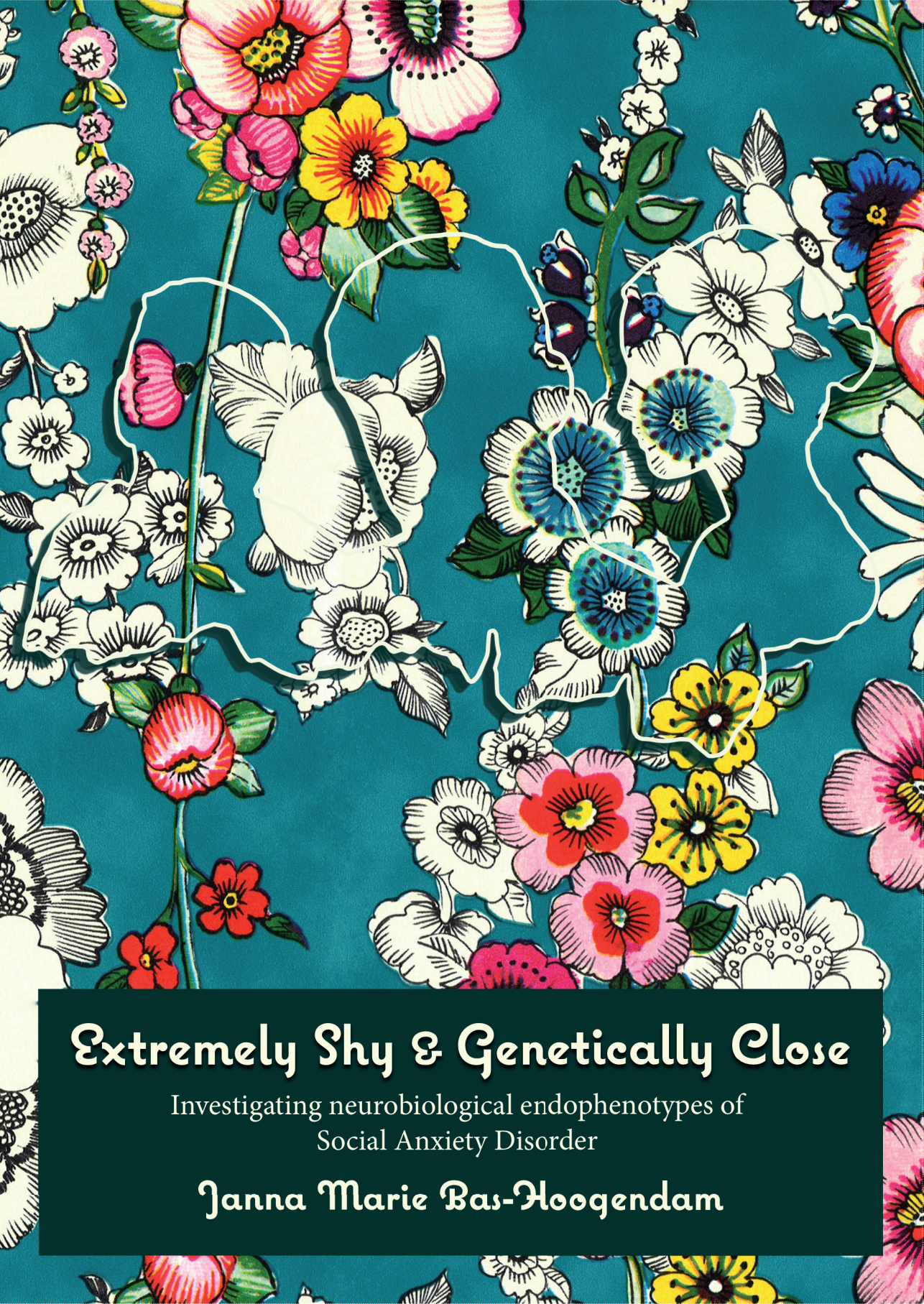
Extremely shy & genetically close : investigating neurobiological endophenotypes of social anxiety disorder

Bas, J.M.



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Janna Marie Bas-Hoogendam

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Chapter 1

Extremely Shy & Genetically Close –
an introduction

Imagine…

… you have to give an important presentation in front of an audience. You are the center of attention and all eyes are on you. How do you feel? Or: you are invited to a party where you don't know anyone. You enter the room and see that the other guests are already seated. What do you experience? Chances are you feel shy and uncomfortable in the beginning, tense maybe, but after a while these feelings will fade and you will have a good time. However, some people remain extremely nervous in social situations and even worry for days or weeks before, and after a social event. These people have an intense fear of being negatively evaluated, and are severely worried about doing something embarrassing in front of others. As a result, they try to avoid social situations as much as possible, and when they actually are in a social situation, they act like wallflowers and don't want to attract attention. This tendency could have a tremendous negative influence on their lives. These individuals suffer from a psychiatric condition: social anxiety disorder (SAD).

We know from previous research that this '*extreme shyness*' develops during childhood and early adolescence. In addition, the disorder often runs in families: being '*genetically close*' to a patient with SAD substantially increases the risk to develop the disorder. But which heritable characteristics make these children and adolescents more susceptible to developing SAD?

Investigating neurobiological endophenotypes of social anxiety disorder

In the novel 'Extremely Loud & Incredibly Close', the nine-year old Oskar Schell wanders through New York City in order to find the lock that belongs to a mysterious key that was owned by his father. His father lost his life in the attack on the World Trade Center on 9/11, and by his search Oskar tries to give meaning to his life (Safran Foer, 2005). This thesis also reflects a search, as the studies described in the present work investigate the behavioral and neurobiological profile of SAD, with a special focus on examining which characteristics are genetically linked to SAD. This is of importance, because these characteristics, the so-called endophenotypes, could provide more insight in the genetic vulnerability to develop SAD.

This chapter offers an introduction to SAD, as well as to the endophenotype concept. Furthermore, previous neuroimaging research on SAD with relevance to this thesis will be discussed. Finally, the studies included in this thesis will be briefly introduced.

Social anxiety disorder: phenotype, prevalence and development

As stated in the Diagnostic and Statistical Manual of Mental Disorders (DSM-5), patients suffering from SAD are characterized by a considerable fear or anxiety in social situations in which the possibility of evaluation by others is present (American Psychiatric Association, 2013). Examples of such situations are performance situations, like speaking, writing or eating in the presence of others, and situations involving social interactions, such as attending parties and meeting unfamiliar people (Furmark, 2002; Neal & Edelmann, 2003). In these social circumstances, patients fear that they will act in a way which will be negatively evaluated by other people, or that they will present themselves with anxiety symptoms like blushing or sweating. They are afraid that their performance in these situations will be humiliating or embarrassing, will lead to rejection by others or the offending of other people. As a result, patients with SAD avoid these social events or endure them with excessive fear or anxiety. Critically, the social situations must almost always elicit fear or anxiety and the fear, anxiety and avoidance should be persistent, lasting at least 6 months (American Psychiatric Association, 2013).

The lifetime prevalence of SAD is estimated between 6 and 13 percent (Bandelow & Michaelis, 2015; Kessler, Petukhova, Sampson, Zaslavsky, & Wittchen, 2012; Ruscio et al., 2008; Stein et al., 2010), and recent data from the World Mental Health survey indicate that the disorder is prevalent across the globe (Stein et al., 2017). SAD often develops during late childhood and adolescence: studies demonstrated that the age of onset is around 10 years of age (Burstein et al., 2011; Ormel et al., 2014). However, the tendency to react to novel persons, experiences, and objects with wariness or avoidant behavior, is already observable in young babies; this characteristic propensity, which is called behavioral inhibition, reflects a stable and innate temperamental trait, and research has shown that behavioral inhibition in children is associated with an increased risk to develop SAD later in life (Clauss, Avery, & Blackford, 2015; Clauss & Blackford, 2012).

SAD is characterized by a persistent course, as shown in adolescents and young adults followed for ten years (Beesdo-Baum et al., 2012), and there is typically a long delay between the onset of the disorder and the first treatment contact (Iza et al., 2013). The effects of the disorder should not be underestimated: patients with SAD often experience problems at school and work, in activities with friends, and in their close relationships (Aderka et al., 2012; Dingemans, van Vliet, Couvée, & Westenberg, 2001; Hendriks et al., 2015; Russell & Topham, 2012). In addition, patients with SAD have an above-average risk of suffering from comorbid psychopathology, like mood disorders such as depression, addiction, and other anxiety disorders (Beesdo et al., 2007; Fehm, Pelissolo, Furmark, & Wittchen, 2005; Ohayon & Schatzberg, 2010), a tendency which is already present in adolescents suffering from SAD (Burstein et al., 2011). All together, these factors make SAD a very disabling condition, with

high costs for society (Acarturk et al., 2009; Dams et al., 2017; Hendriks et al., 2014; Stein & Kean, 2000; Stuhldreher et al., 2014; Wittchen, Fuetsch, Sonntag, Müller, & Liebowitz, 2000).

To avoid individual suffering and societal burden due to SAD as much as possible, effective preventive interventions are important (Craske & Zucker, 2001). To develop them, insight into the factors that make individuals vulnerable for developing SAD is needed. As argued by Beauchaine and colleagues, considering neurobiological processes is therefore essential (Beauchaine, Neuhaus, Brenner, & Gatzke-Kopp, 2008). Indeed, previous research has revealed that various biological, together with psychological and social factors, interact in the development of SAD (Wong & Rapee, 2016). Family- and twin studies have, for example, indicated that SAD has a heritable component (Isomura et al., 2015; Mancini, Van Ameringen, Szatmari, Fugere, & Boyle, 1996; Middeldorp et al., 2005; Scaini, Belotti, & Ogliari, 2014; Stein, Chartier, Hazen, et al., 1998). Still, the genetic variations related to SAD are largely unknown and, for several reasons, not easy to find. First of all, the disorder differs from one patient to another (Hyett & McEvoy, 2018). In addition, it is assumed that multiple interacting genes play a role in the development of SAD (Domschke & Dannlowski, 2010; Meier & Deckert, 2019). As discussed in more detail in *Chapter* 2 of this thesis, this complicates the search for a link between social anxiety and genes (see also the review by Bearden, Jasinska, & Freimer (2009)). However, new approaches and new study designs can help to unravel this connection. An example is the application of the endophenotype concept, which is relatively new in psychiatry (Gottesman & Gould, 2003).

Endophenotype concept

Endophenotypes are measurable characteristics which are located between a phenotype, for instance social anxiety, and specific genetic variations. Examples of endophenotypes are changes in the structure and function of the brain, alterations in cognitive performance, and neurophysiological changes (Glahn, Knowles, et al., 2014). The following criteria are used to determine whether a characteristic is an endophenotype (Glahn, Thompson, & Blangero, 2007; Gottesman & Gould, 2003; Lenzenweger, 2013b; Puls & Gallinat, 2008): 1st the endophenotype should be *associated with the disorder of interest*; 2nd an endophenotype is supposed to be a *stable, state-independent trait, which is already present in a preclinical state*; 3rd an endophenotype should be *heritable*; 4th the endophenotype *co-segregates with the disorder within a family, with nonaffected family members showing altered levels of the endophenotype when compared to the general population*. As more extensively discussed in *Chapter 2*, it is assumed that endophenotypes are easier to detect than the underlying phenotype-related genotype. This way, endophenotypes could help in unraveling the genetic susceptibility to psychiatric disorders. Furthermore, endophenotypes have the potential to increase our un-

derstanding of the pathways leading to pathology (Flint, Timpson, & Munafò, 2014; Miller & Rockstroh, 2013). In addition, as endophenotypes are not necessarily uniquely related to one specific disorder, they could provide insight in the transdiagnostic characteristics of mental disorders (Beauchaine & Constantino, 2017; Miller & Rockstroh, 2013).

In the past decade, the endophenotype approach has been applied to psychiatric disorders like depression (Goldstein & Klein, 2014; Miskowiak et al., 2018), obsessive-compulsive disorder (Bey et al., 2018; de Vries et al., 2013; Vaghi et al., 2017), and schizophrenia (Blakey et al., 2018; Glahn, Williams, et al., 2014; Honea et al., 2008; McCarthy et al., 2018), revealing alterations in brain structure and function in patients as well as in their unaffected relatives. Thereby, these studies provide initial insight in the genetic vulnerability to these disorders, as they show that the changes are not just a manifestation of the disease-state (as the alterations were present in unaffected family members as well), and are likely heritable, because the characteristics were present in both patients and relatives (cf. (Ursu, 2017)). Research on endophenotypes of SAD is, however, still absent, although studies employing case-control designs have already provided valuable insight in the neurobiological changes related to SAD (*endophenotype criterion 1),* as will be summarized later in this chapter. Nevertheless, due to their focus on patients with SAD, these neuroimaging studies were not able to establish the *heritability* of these SAD-related brain characteristics (*endophenotype criterion 3)*, nor could they investigate the *co-segregation within families of probands (first element of endophenotype criterion 4)*. In other words, these studies revealed several *biomarkers* of SAD, being characteristics of brain function and brain structure related to a disorder, but not necessarily causally involved in the mechanistic pathway from genotype to phenotype (Lenzenweger, 2013a); however, whether these characteristics are candidate *endophenotypes* of SAD, and as such reflective of the genetic susceptibility to SAD, is still an open question. Given the heritable background of SAD, investigating whether these biomarkers qualify as endophenotypes could provide important additional knowledge to improve prevention and intervention for children and adolescents who are vulnerable to developing SAD due to their genetic make-up (Dick, 2018).

The Leiden Family Lab study on Social Anxiety Disorder (LFLSAD)

In a first effort to fill this gap in the scientific literature, we performed the Leiden Family Lab study on Social Anxiety Disorder (LFLSAD). As indicated by its name, the LFLSAD involves not only patients with SAD, but also their families, as family studies are particularly suitable to test two important endophenotype criteria and, as such, expand case-control studies (Glahn et al., 2018). First of all, a family design allows for examining whether a candidate endophenotype *co-segregates with the disorder within families* (first element of

endophenotype criterion 4). Furthermore, as multiple family members are investigated, the *heritability* of proposed endophenotypes can be determined (endophenotype criterion 3). In addition, family studies have enhanced statistical power to delineate associations between genotypes and phenotypes, and are cost-efficient (Glahn et al., 2018).

The LFLSAD aims to profile neurobiological endophenotypes of SAD, as measured with magnetic resonance imaging (MRI) and electroencephalography (EEG). The background and design of the study are outlined in *Chapter 3*. This thesis describes the results of several MRI studies which were part of the LFLSAD; the findings of the EEG session are reported in the thesis of Anita Harrewijn (Harrewijn, 2017). The MRI paradigms used within the LFLSAD are depicted in *Figure 1.1*. These particular paradigms were carefully chosen and developed based on the results of previous neuroimaging research on SAD biomarkers, as these studies provided evidence for the first endophenotype criterion of *association with the disorder*. In the following, I will briefly summarize these findings.

Neuroimaging research on SAD

In the last decades, neuroimaging research on biomarkers of SAD has expanded: while early imaging studies focused on key structures in the brain like the amygdala (Birbaumer et al., 1998) and subcortical areas (Potts, Davidson, Krishnan, & Doraiswamy, 1994; Schneider et al., 1999), more recent studies aimed to characterize SAD-related changes in both the structure and function of the whole brain.

To start, structural MRI studies comparing gray matter volumes between patients with SAD and healthy control participants demonstrated widespread alterations in the structure of the brain: changes were found in the frontal, temporal and parietal cortex, as well as in subcortical areas like the amygdala, thalamus and putamen (Brühl, Hänggi, et al., 2014; Irle, Barke, Lange, & Ruhleder, 2014; Meng et al., 2013; Talati, Pantazatos, Schneier, Weissman, & Hirsch, 2013). The studies described in *Chapter 4* and *Chapter 5* build on these insights and investigate whether structural brain characteristics are candidate endophenotypes of SAD (*Figure 1.1A*). We examined evidence for the endophenotype criterion of *co-segregation with the disorder within families* and established *heritability*.

In addition to these structural MRI studies, functional MRI (fMRI) studies yielded important insights in neurobiological characteristics related to SAD. FMRI studies use changes in the blood-oxygen-level dependent (BOLD) signal in order to obtain an estimation of neural activity. Most fMRI studies on SAD use stimuli which are anxiety-provoking for patients. Example of such stimuli are photographs of faces with negative or neutral expressions, stories describing social situations, or sentences involving personal feedback (Brühl, Delsignore, Komossa, & Weidt, 2014). Such stimuli elicited increased brain activation in patients with SAD in several brain areas, including the amygdala, insula and prefrontal

cortex. In addition, enhanced brain responsivity of the parietal cortex has been associated with SAD. The involvement of these areas in the pathophysiology of SAD was confirmed by the results of a meta-analysis on fMRI findings (Brühl, Delsignore, et al., 2014).

Notably, several fMRI studies provided evidence for correlations between these increases in brain activation and the level of social anxiety symptoms; for example, Frick and colleagues reported a positive correlation between amygdala reactivity to emotional faces and social anxiety severity (Frick, Howner, Fischer, Kristiansson, & Furmark, 2013). Such associations support the hypothesis that neurobiological brain alterations, as measured with MRI, underlie the thoughts and behavior associated with SAD and are valuable biomarkers of the disorder. This idea is also substantiated by studies investigating treatment effects in SAD. To illustrate, Phan and colleagues reported that the exaggerated amygdala response to fearful faces, which was present in patients with SAD before treatment, significantly reduced after a twelve-week treatment with the selective serotonin reuptake inhibitor sertraline (Phan et al., 2013). These findings indicate that fMRI studies yield important and relevant insights in the neurobiological brain alterations that are functionally related to SAD. *Chapters 8, 9 and 10* of this thesis extend these biomarker studies, by investigating whether these alterations in brain activity could be considered candidate SAD endophenotypes. We focused on two neurobiological processes which are highly relevant for SAD patients: the processing of neutral faces by the amygdala and the processing of social norm violations, a paradigm which primarily targeted the prefrontal cortex (*Figure 1.1C-D*). Again, we investigated the *co-segregation of brain activation with the disorder within families* and estimated *heritability*.

Another line of neuroimaging research on SAD investigates changes in the connections between brain regions. Such networks can be visualized using fMRI and diffusion tensor imaging (DTI). In this context, fMRI studies estimate functional connections by exploring correlations in brain activation patterns, based on the idea that connected regions show similar reactivity patterns (Damoiseaux et al., 2006). DTI scans map connections between areas by enabling reconstruction of white matter tracts (Chanraud, Zahr, Sullivan, & Pfefferbaum, 2010). Previous work, as summarized by Cremers and Roelofs (2016), suggests that SAD patients are characterized by changes in subcortical networks (Arnold Anteraper et al., 2014), in networks involved in social cognition and self-reflection (Heitmann et al., 2016; Liao, Chen, et al., 2010), as well as in the white matter tract that connects the amygdala and the prefrontal cortex (Baur et al., 2011; Baur, Hänggi, Langer, & Jäncke, 2013). Within the LFLSAD MRI paradigm, we acquired data on functional (resting-state) as well as on structural (DTI) connectivity (*Figure 1.1B, Figure 1.1E*). These data are presently analyzed, and not part of this thesis.

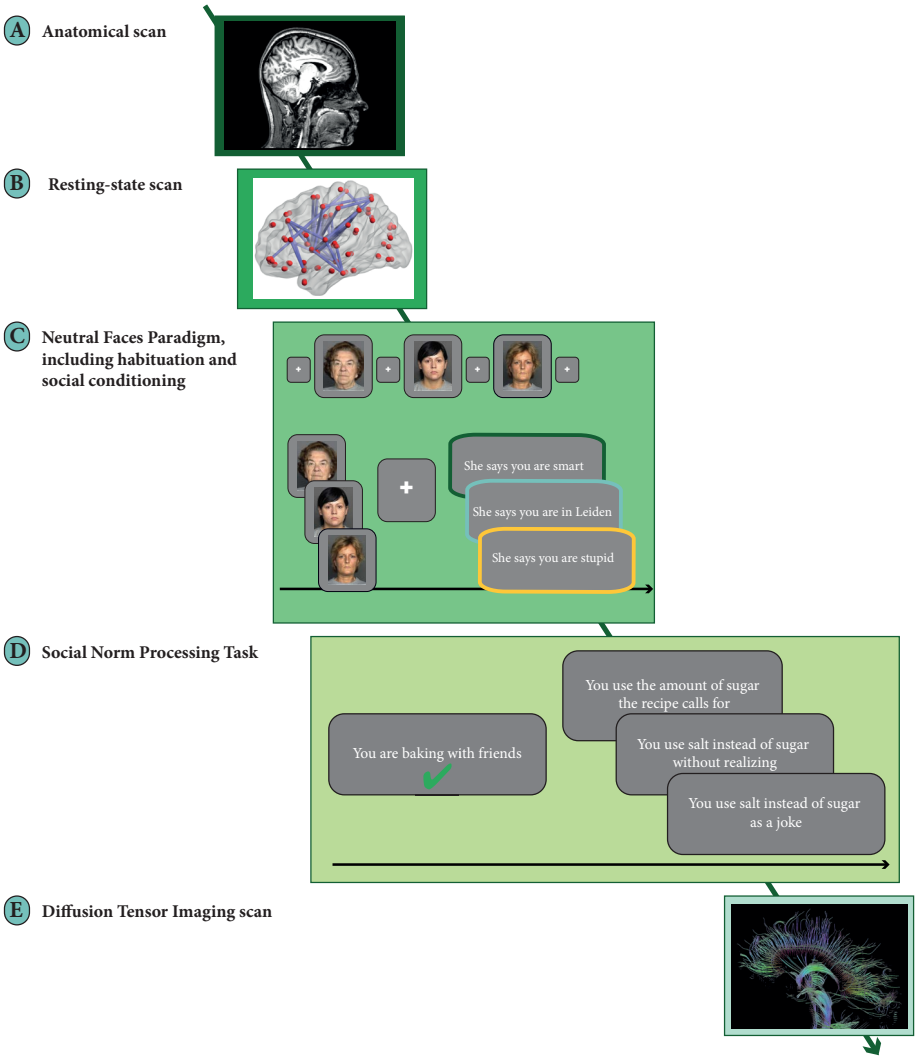

Figure 1.1 Magnetic resonance imaging (MRI) protocol of the Leiden Family Lab study on Social Anxiety Disorder.
A structural MRI scan, aimed to acquire a detailed anatomical scan of the brain (***Figure 1.1A***) and a resting-state scan (in which participants had their eyes closed; ***Figure 1.1B***) were followed by two functional (f)MRI paradigms: the Neutral Faces Paradigm (***Figure 1.1C***) and the Social Norm Processing Task - revised (***Figure 1.1D***). At the end of the scan protocol, diffusion tensor imaging (DTI) scans were acquired to visualize the structural connectivity of the brain (***Figure 1.1E***).

This thesis

The studies enclosed in this thesis aim to gain more insight in several neurobiological endophenotypes of SAD. In *Chapter 2*, I describe the endophenotype approach in detail, and consider existing evidence for neurobiological candidate endophenotypes of SAD, focusing on the function of the amygdala and medial prefrontal cortex, changes in brain structure, and on the connections between brain regions. We review to which extent previous studies provide support for these characteristics meeting the endophenotype criteria. As specific endophenotype studies on SAD are lacking, we used the findings of studies on SAD biomarkers, as well as results from work in healthy participants and animal studies, in order to create a summary of current evidence for neurobiological SAD endophenotypes. This overview substantiated the choice of MRI paradigms used in the LFLSAD.

Chapter 3 describes the design of LFLSAD. This study offers, due to its unique design involving patients with SAD and their family members of two generations, the opportunity to investigate which neurobiological characteristics *co-segregate with the disorder within families* (endophenotype criterion 4, element 1) and are *heritable* (endophenotype criterion 3).

The second part of this thesis addresses changes in brain structure related to SAD. In *Chapter 4*, I describe an international, multi-center mega-analysis on structural MRI scans of 174 patients with SAD and 213 healthy control participants. Within this sample, we investigated changes in gray matter between the groups by using voxel-based morphometry (VBM), establishing structural biomarkers of SAD. In *Chapter 5*, we build upon this work: we used data from the LFLSAD to examine whether gray matter characteristics like cortical thickness, cortical surface area and volumetric indices of subcortical brain structures are not just biomarkers, but also candidate endophenotypes of social anxiety (*Figure 1.1A)*.

In the third part of this thesis, I outline studies investigating neurobiological changes in brain function related to social anxiety, using two paradigms. The first paradigm, the revised Social Norm Processing Task (SNPT-R) concerns the processing of social norm violations and pays special attention to the intention underlying a social norm transgression, because intentional and unintentional social norm violations are contrasted (*Figure 1.1D*). This paradigm is highly relevant in the context of SAD, as it directly relates to the fear of SAD patients to behave in an embarrassing way in front of others. *Chapter 6* outlines the characteristics of the SNPT-R, which we developed in order to investigate the behavioral and neural correlates of processing social norm transgressions in children, adolescents as well as in adults. In *Chapter 7*, I summarize the results of a study on the relation between social anxiety and behavioral ratings on the SNPT-R in a sample from the general population. Subsequently, we used data from the LFLSAD to investigate whether behavioral and neural correlates of processing unintentional social norm violations are candidate endophenotypes of social anxiety. The results of this study are described in *Chapter 8*.

The second fMRI paradigm of the LFLSAD is described in *Chapter 9* and *Chapter 10*. In these chapters, I outline the Neutral Faces Paradigm (NFP), which was designed to investigate the association between social anxiety and brain activation related to the processing of neutral faces (*Figure 1.1C*). In the first part of the paradigm, neutral faces were repeatedly presented, and we used these data to examine whether the predicted decline in brain activation over time (the habituation response) is a candidate endophenotype of social anxiety. This study is summarized in *Chapter 9*. In the second phase of the NFP, the faces were paired with social-evaluative sentences which were either positive, negative or neutral. This enabled us to investigate brain activation in response to faces conditioned with a social-evaluative meaning. These findings are discussed in *Chapter 10*.

The results of the studies included in this thesis are summarized in *Chapter 11*. In a subsequent discussion, I reflect upon what the outcomes of the LFLSAD revealed about the genetic vulnerability to develop SAD. Furthermore, I provide suggestions for future research, and consider several important characteristics of the present work.

Chapter 2

Neurobiological candidate endophenotypes of Social Anxiety Disorder

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Abstract

Social anxiety disorder (SAD) is a disabling psychiatric disorder with a complex pathogenesis. Studies indicated a genetic component in the development of SAD, but the search for genetic mechanisms underlying this vulnerability is complicated. A focus on endophenotypes instead of the disorder itself may provide a fruitful path forward.

Endophenotypes are measurable characteristics related to complex psychiatric disorders and reflective of genetically-based disease mechanisms, and could shed light on the ways by which genes contribute to the development of SAD. We review evidence for candidate MRI endophenotypes of SAD and discuss the extent to which they meet the criteria for an endophenotype, focusing on the amygdala, the medial prefrontal cortex, whole-brain functional connectivity and structural-anatomical changes. Strongest evidence is present for the primary endophenotype criterion of association between the candidate endophenotypes and SAD, while the other criteria, involving trait-stability, heritability and co-segregation of the endophenotype with the disorder within families, warrant further investigation. We highlight the potential of neuroimaging endophenotypes and stress the need for family studies into SAD endophenotypes.

Introduction

Social anxiety disorder (SAD) is a highly disabling disorder with an estimated life-time prevalence of 10 – 15 % (de Graaf, ten Have, van Gool, & van Dorsselaer, 2012; Hendriks et al., 2014; Stein & Kean, 2000; Wittchen et al., 2011). Patients with SAD have an extreme fear of being negatively evaluated in social situations and, as a result, avoid social events or endure them with excessive fear or anxiety (American Psychiatric Association, 2013). SAD usually has its onset during early adolescence (Beesdo-Baum et al., 2015; Haller, Cohen Kadosh, Scerif, & Lau, 2015) and is characterized by a rather chronic, unremitting course (Beesdo-Baum et al., 2012; Blanco et al., 2011; Scholten et al., 2016; Steinert, Hofmann, Leichsenring, & Kruse, 2013), a high association with comorbid psychopathology (Beesdo et al., 2007; Fehm et al., 2005; Kessler, Chiu, Demler, Merikangas, & Walters, 2005), and a reduced quality of life (Acarturk, de Graaf, van Straten, Have, & Cuijpers, 2008; Stein & Kean, 2000). In addition, the direct healthcare costs and indirect economic burdens of SAD, due to lost productivity and early retirement, are high (Acarturk et al., 2009; Fineberg et al., 2013; Gustavsson et al., 2011; Moitra, Beard, Weisberg, & Keller, 2011; Stuhldreher et al., 2014).

The high prevalence, chronic course, and substantial costs of SAD strongly highlight the need for effective preventive interventions and improved treatment options. Yet, our insight into the development of SAD is still rather limited, hindering the possibility to identify early markers for detection and prevention. Previous research points to a complex pathogenesis including genetic vulnerabilities, neurobiological alterations, environmental factors, and psychological mechanisms (Domschke, 2013; Hirshfeld-Becker, Micco, Simoes, & Henin, 2008; Kendler, Gardner, & Lichtenstein, 2008; Wong & Rapee, 2016). Because genetic and neurobiological markers are likely present very early in life, these are potential primary intervention targets. In the present narrative review, we will therefore focus on neurobiological mechanisms involved in the genetic vulnerability to social anxiety.

Genes and SAD

Family- and twin studies show that anxiety disorders are familial and moderately heritable, with heritability estimates for SAD around 50 % (Gottschalk & Domschke, 2016; Isomura et al., 2015; Middeldorp et al., 2005; Scaini et al., 2014; Smoller, 2015; Torvik et al., 2016). These findings are supported by animal studies, which reveal significant heritability of extreme early life anxiety in non-human primates (Fox, Oler, Shackman, et al., 2015; Fox & Kalin, 2014; Oler et al., 2010). Given this heritability, several studies searched for genes associated with SAD. An early genome-wide association (GWA) study, in a sample now considered to be relatively small (17 families, $n = 163$), reported involvement of regions on chromosomes 9, 14, 16 and 18 in SAD (Gelernter, Page, Stein, & Woods, 2004), a linkage-study suggested chromosome 13 as a potential susceptibility locus for 'specific or social phobia' (Fyer et al., 2012), while a recent meta-analysis of GWA-studies on anxiety disorders (the largest

to date, with > 18 000 participants) identified various novel susceptibility loci related to anxiety (Otowa et al., 2016). However, it should be noted that these loci are often very large and span up to hundreds of genes (Domschke, 2013). Recently, a more specific multilevel epigenetic study reported that decreased methylation of the oxytocin receptor (chromosome 3) was related to SAD and SAD-related traits (Ziegler et al., 2015), but research into epigenetic alterations is still in its infancy.

Although these findings for a genetic basis of SAD are promising, they have not yet been replicated. Furthermore, it needs to be investigated whether these genetic findings are specific for SAD, and whether they reflect risk factors for SAD or are rather compensatory changes in response to SAD (Ziegler et al., 2015). Thereby, the genes underlying the vulnerability to SAD are until now largely unknown.

The endophenotype approach

The search for SAD genes is complicated by the heterogeneity of the disorder and the fact that the diagnosis is based on clinical assessments and not on biologically-based measurements (Bearden, Reus, & Freimer, 2004; Glahn et al., 2007; Gottesman & Gould, 2003). In addition, SAD is a polygenic disorder: multiple genetic variants, each with a relatively small effect, interact and lead to disease vulnerability (Binder, 2012; Domschke & Dannlowski, 2010; Fox & Kalin, 2014). These genetic variants are in turn influenced by environmental factors (Gottschalk & Domschke, 2016), further complicating the search for the genetic basis of SAD.

To facilitate the investigation of genetic factors in psychiatric disorders, the endophenotype approach has increasingly received attention (Glahn, Knowles, et al., 2014). Endophenotypes are measurable characteristics that form a causal link between genes and diseases, and are manifestations of underlying disease liability (Lenzenweger, 2013b) (*Figure 2.1*). Criteria used to define endophenotypes are the following (Glahn et al., 2007; Gottesman & Gould, 2003; Lenzenweger, 2013b; Puls & Gallinat, 2008): 1st *association with the disorder*; 2nd being a *stable, state-independent trait, which is already present in a preclinical state*; 3rd being *heritable*; 4th *co-segregation with the disorder within a family, with nonaffected family members showing altered levels of the endophenotype when compared to the general population*. In addition, an endophenotype is ideally more strongly associated with the disorder of interest in comparison to other psychiatric conditions (Lenzenweger, 2013a), but it is also possible that a certain endophenotype affects more than one disorder (Cannon & Keller, 2006) (see the *Discussion* of this Chapter for a more in-depth debate).

Originally, the usefulness of endophenotypes was supposed to lie in discovering the genes predisposing for complex disorders, based on the assumption that endophenotypes have a simpler genetic architecture than the disorders themselves (Glahn et al., 2007; Gottesman & Gould, 2003). This idea was, however, challenged by the results of a meta-analytic review (Flint & Munafò, 2007) which compared the effect sizes of genetic loci contributing

to psychiatric disorders (phenotypes) and loci contributing to endophenotypes. Results showed comparable effect sizes, so the assumption that endophenotypes have a simpler genetic architecture than phenotypes was not supported (Flint & Munafò, 2007). Recently, the findings of this meta-analysis were empirically confirmed by comparing GWA studies investigating the genetic effects related to endophenotypes of schizophrenia (for example, variation in brain structure and measures of cognitive performance) to studies aimed to identify risk genes for the disorder itself (Flint et al., 2014). Again, similar effect sizes were found. So, it is not necessarily true that the genetic architecture of endophenotypes is less complex than that of the disorders themselves (Flint et al., 2014; Glahn, Knowles, et al., 2014; Puls & Gallinat, 2008).

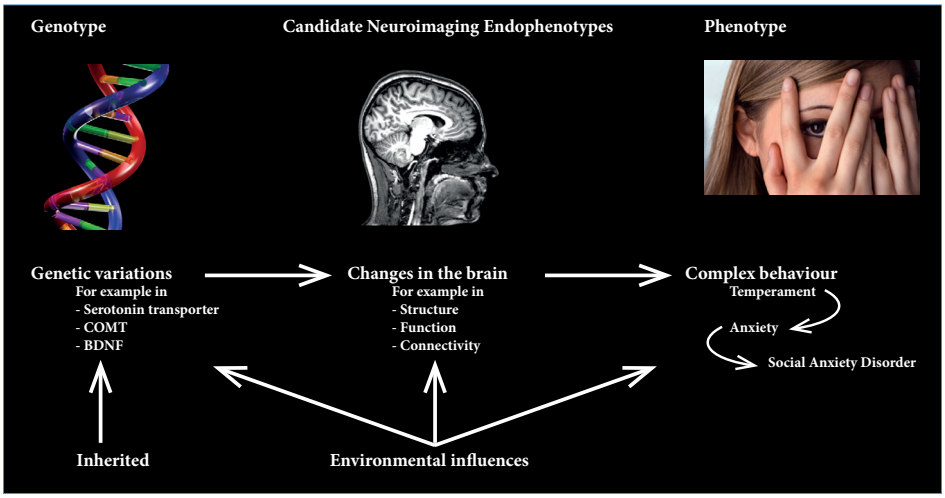

Figure 2.1 The relationship between genetic variation, endophenotype and phenotype.
Inspired by Kendler & Neale (2010). Illustration DNA: Wikimedia Commons, National Human Genome Research Institute, ID 85329. Photograph: *www.smartgirlsgroup.com*

This does not mean, however, that endophenotypes are of limited value. Their usefulness lies in understanding disease mechanisms: based on the assumption that complex disorders could be divided into simpler and more biologically coherent units (endophenotypes), endophenotypes could provide insight into the pathways leading to pathology and could help in discerning the origins of mental disorders (Flint et al., 2014; Miller & Rockstroh, 2013). Furthermore, endophenotypes could support a transdiagnostic perspective on mental disorders, given the fact that endophenotypes could cross traditional diagnostic boundaries (Miller & Rockstroh, 2013). Here, the endophenotype approach fits within the NIMH Research Domain Criteria (RDoC) initiative, a research framework in which not the clinical diagnoses are starting point for investigation, but core features of psychopathology, falling within five research domains (Sanislow et al., 2010). The RDoC initiative explicitly acknowledges that these core features could be present in multiple psychiatric disorders

and promotes the integration of data from several levels, from genes to neural systems to behavior. Goal of this approach is to classify disorders based on 'a deeper understanding of the biological and psychosocial basis' of psychiatric diseases (Insel, 2014). Endophenotypes could be used in this approach, because they provide a bridge between genetic variations at the one hand and psychiatric disorders at the other.

In addition, endophenotypes could aid in the development of improved animal models for psychopathology (Gould & Gottesman, 2006), and, based on the fact that endophenotypes are present prior to disease onset, endophenotypes can be used to identify individuals at risk (Puls & Gallinat, 2008). This is of uttermost importance, given the fact that early detection of psychopathology and subsequent use of preventive interventions can improve long-term prognosis, reduce the substantial burden and cost of SAD, and lower the risk of developing co-morbid psychopathology (Beauchaine et al., 2008). Furthermore, endophenotypes could provide clues for improvement of treatments for psychiatric disorders and guide in the selection of appropriate pharmacological interventions (Garner, Möhler, Stein, Mueggler, & Baldwin, 2009).

The endophenotype approach has been used successfully to investigate the genetic basis of several psychiatric disorders including depression (Goldstein & Klein, 2014; Hasler & Northoff, 2011), schizophrenia (Glahn, Williams, et al., 2014; Sutcliffe, Harneit, Tost, & Meyer-Lindenberg, 2016), bipolar disorder (Fears et al., 2015) and obsessive-compulsive disorder (Menzies et al., 2007), but research on endophenotypes for SAD is still in its infancy.

Review objectives

In this narrative review of empirical research from various sources, we explore potential candidate endophenotypes of social anxiety. We will focus on neurobiological measurements from magnetic resonance imaging (MRI) – a safe, non-invasive, widely applied and relatively accessible method used to investigate the structure and function of the human brain –, based on the assumption that changes in brain structure and function underlie thoughts and behavior associated with anxiety and anxiety disorders. In addition, we will refer to results from positron emission tomography (PET) studies, a method used for in vivo molecular imaging (Vaquero & Kinahan, 2015). Candidate endophenotypes were selected based on recent neuroimaging work on SAD (Brühl, Delsignore, et al., 2014) and include: function and connectivity of the amygdala, function of the medial prefrontal cortex, whole-brain network function and brain structure. We will qualitatively assess the potential of each candidate endophenotype using the four criteria listed in *Table 2.1* and illustrated in *Figure 2.2*.

The *association with disorder* (criterion 1) will be evaluated by discussing neuroimaging research that compares SAD patients with healthy participants. Given the fact that the candidate endophenotypes were selected based on studies on SAD, we expect relatively strong evidence for this criterion. We will briefly outline the results summarized by Brühl,

Table 2.1 Criteria for an endophenotype (EP).

1	EP is associated with the disorder: present in patients at a significantly different level than in general population
2	EP is a trait characteristic and already present in a preclinical state, reflecting the genetically-based vulnerability to the disorder
3	EP is heritable
4	EP co-segregates with the illness within a family, and nonaffected family members show altered EP levels when compared to the general population

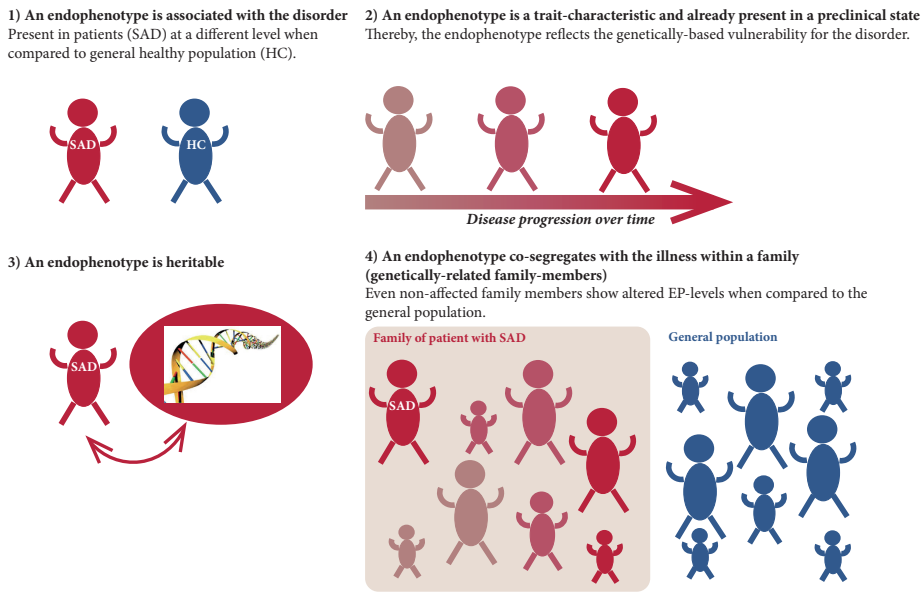

Figure 2.2 Illustration of the four criteria for an endophenotype.

Delsignore, et al. (2014) and extend these findings by summarizing findings of recently published work.

The *trait-stability* of an endophenotype (criterion 2) is ideally examined using longitudinal studies on individuals with SAD, while the *heritability* of an endophenotype (criterion 3) could be estimated from twin-, adoption or family studies. The fourth criterion, the *co-segregation of the endophenotype with illness within families*, is best investigated using studies in families genetically enriched for SAD. However, to the best of our knowledge, there are no longitudinal neuroimaging studies assessing the trait-stability of candidate endophenotypes of SAD, and family studies involving neuroimaging measurements of patients with SAD as well as of their family members, are lacking as well. Direct support for criteria 2, 3 and 4 is therefore mainly absent.

Therefore, we will explore available findings related to these criteria by using a broader perspective, in order to summarize indirect evidence for the candidate endophenotypes. Whether the candidate endophenotypes are *trait characteristics* (criterion 2) will be discussed based on studies investigating the relationship between the candidate endophenotype and several trait characteristics which are assumed to be more or less stable over time. In this light, it is especially useful to look at neuroimaging studies on behavioral inhibition or inhibited temperament. Inhibited temperament is the relatively stable tendency to withdraw from new and unfamiliar objects, situations and people, is already measurable in toddlers, and extreme behavioral inhibition is considered to be a risk factor for SAD (Clauss et al., 2015; Clauss & Blackford, 2012; Essex, Klein, Slattery, Goldsmith, & Kalin, 2010; Henderson, Pine, & Fox, 2015; Rapee, 2014). In addition, we will explore the results of neuroimaging studies on extraversion, a heritable personality trait that is negatively correlated with social anxiety (Bienvenu, Hettema, Neale, Prescott, & Kendler, 2007; Cremers & Roelofs, 2016; Kotov, Gamez, Schmidt, & Watson, 2010; Naragon-Gainey & Watson, 2011). Furthermore, animal studies and longitudinal studies on healthy participants could give insight into the trait-like characteristics and stability of candidate endophenotypes. It is, however, important to realize that measurements of brain activation as assessed by functional (f)MRI are inherently state-dependent, as they reveal the reactivity of brain regions in response to a task or during a certain period of rest. However, we assume that these reactivity patterns, as measured in the MRI scanner, are also reflective of a stable pattern of brain responses in a participant's daily life.

Evidence for *heritability* (criterion 3) will be investigated by describing studies on genetic influences for the candidate endophenotype, for example by summarizing results from studies describing heritability estimates of MRI measurements, and by discussing findings on genetic polymorphisms influencing brain function and structure. In addition, evidence on the *co-segration of the candidate endophenotype with the illness within families* (criterion 4) will be summarized when available.

Amygdala: function and functional connectivity

Neuroimaging research on SAD has generally focused on the amygdala, a key part of a broader circuit, known as the extended amygdala, which includes amygdala sub-nuclei like the central nucleus and basolateral complex, the bed nucleus of the stria terminalis (BNST), and the shell of the nucleus accumbens (Heimer & Van Hoesen, 2006; Janak & Tye, 2015). Although these subregions are functionally heterogeneous (LeDoux, 2007), the majority of past scientific imaging work in humans is based on the whole amygdala region, as imaging of the amygdala is difficult due to magnetic susceptibility differences and most imaging sequences do not have adequete spatial resolution to pinpoint amygdala subnuclei (Robinson,

Windischberger, Rauscher, & Moser, 2004) (but see for recent work with improved imaging parameters (Hrybouski et al., 2016)).

The amygdala is well known for its role in detecting cues that are predictive of potential threats (Fox, Oler, Tromp, Fudge, & Kalin, 2015; Hariri & Whalen, 2011) and individual differences in amygdala functioning are related to the etiology of anxiety (Shackman et al., 2016).

Criterion 1

Heightened amygdala reactivity in response to novel faces is consistently associated with SAD (Birbaumer et al., 1998; Blair, Geraci, Korelitz, et al., 2011; Evans et al., 2008; Fonzo et al., 2015; Hahn et al., 2011; Klumpp, Angstadt, Nathan, & Phan, 2010; Sladky et al., 2012; Stein, Goldin, Sareen, Zorrilla, & Brown, 2002; Straube, Kolassa, Glauer, Mentzel, & Miltner, 2004; Yoon, Fitzgerald, Angstadt, McCarron, & Phan, 2007). This is indicative of an exaggeration of the healthy response to novel and salient stimuli. In addition, increased amygdala reactivity in SAD patients has been reported in studies using SAD-specific symptom-provoking paradigms, like anticipation of giving a speech (Boehme, Ritter, et al., 2014; Lorberbaum et al., 2004; Tillfors, Furmark, Marteinsdottir, & Fredrikson, 2002), giving a speech (Tillfors et al., 2001), reading sentences containing self-referential criticism (Månsson et al., 2016) or receiving peer feedback (Guyer et al., 2008), as well as during unspecific tasks like anticipating (Brühl et al., 2011) or perceiving negative emotional images (Shah, Klumpp, Angstadt, Nathan, & Phan, 2009). Two recent meta-analyses confirmed that increased amygdala reactivity is observed in SAD (Brühl, Delsignore, et al., 2014; Gentili et al., 2016). In addition, Blair and colleagues reported that both adult as well as adolescent SAD patients demonstate amygdala hyperreactivity, supporting the assumption that perturbations in amygdala activation are present over the course of the disorder (Blair, Geraci, Korelitz, et al., 2011).

It is important to note that the amygdala does not function in isolation but is connected with other brain areas (Fox, Oler, Tromp, et al., 2015; Kim et al., 2011; LeDoux, 2007). Amygdala connectivity has been interrogated in SAD patients, using both resting-state methods to assess intrinsic connectivity and various tasks to assess functional task-based connectivity. Several studies showed differences in amygdala connectivity with a variety of brain regions. Resting-state studies demonstrated lower connectivity between the amygdala and the inferior temporal gyrus (Liao, Qiu, et al., 2010), the orbitofrontal cortex (Hahn et al., 2011) and the anterior cingulate cortex (Prater, Hosanagar, Klumpp, Angstadt, & Phan, 2013). Task-based studies using emotional faces show a functional disruption in the negative feedback loop between the amygdala and OFC (Sladky et al., 2015), increased connectivity between amygdala and the fusiform gyrus (Frick et al., 2013a) and increased positive coupling between the amygdala and the dorsal medial prefrontal cortex (Robinson et al., 2014). Furthermore, Cremers and colleagues found a transient decreased negative

functional connectivity between the amygdala and cortical regions involved in emotion regulation during anticipation of giving a public speech (Cremers et al., 2014). The divergent findings of these connectivity studies could be partly explained by the different conditions (rest or task), but in order to use amygdala connectivity as a reliable endophenotype of SAD, more research in larger samples is needed to establish which connectivity changes are consistently associated with SAD.

Criterion 2

There are several lines of evidence suggesting that amygdala functioning is a trait characteristic. First, research on healthy participants shows that inter-subject variability in temperamental traits, which are considered to be more or less stable over time, relates to differences in amygdala function and connectivity. For example, amygdala hyperreactivity was present in young adults with anxiety-related temperamental traits (Stein, Simmons, Feinstein, & Paulus, 2007). The most consistent relation is reported between inhibited temperament and hyperactive amygdala response to stimuli (for a review and meta-analysis see Clauss et al., 2015). To illustrate, adolescents with an inhibited temperament had an exaggerated amygdala response to emotional faces (Pérez-Edgar et al., 2007), and adults who had been characterized as inhibited at the age of two show elevated amygdala reactivity in response to novel faces (Schwartz, Wright, Shin, Kagan, & Rauch, 2003). Furthermore, behavioral inhibition during childhood predicts negative amygdala-frontal connectivity during an attention-bias task involving angry faces in young adulthood (Hardee et al., 2013). Another study demonstrated changes (both increases as well as decreases) in resting-state functional connectivity between subnuclei of the amygdala and the prefrontal cortex, striatum, anterior insula, and cerebellum in young adults with a history of behavioral inhibition (Roy et al., 2014). In addition, a high degree of social inhibition was associated with reduced resting-state connectivity between the superficial amygdala and the rostral cingulate cortex, and between the centromedial amygdala and the dorsal anterior cingulate cortex (Blackford et al., 2014). Furthermore, trait anxiety (and not state anxiety) predicted lower intrinsic functional connectivity between the amygdala and the entire cerebral cortex (He, Xu, Zhang, & Zuo, 2015). Findings on the relation between trait extraversion and amygdala activation are mixed, as both positive (Canli et al., 2002) as well as negative associations (Hooker, Verosky, Miyakawa, Knight, & D'Esposito, 2008) between extraversion and amygdala reactivity are reported (for a comprehensive review, see Kennis, Rademaker, & Geuze (2013). Studies on the functional connectivity of the amygdala revealed increased functional connectivity between the amygdala and brain regions involved in reward processing (Aghajani et al., 2014; Rohr et al., 2015) related to extraversion.

The relation between amygdala function and temperamental traits has been further explored in a specific line of research focusing on amygdala habituation. Studies in healthy participants have indicated that the amygdala response to facial stimuli declines when the

stimuli are presented repeatedly without meaningful consequences. This process, called habituation, is one of the most basic forms of social learning (Blackford, Allen, Cowan, & Avery, 2013; Zald, 2003). Amygdala habituation can be reliably assessed using fMRI (Plichta et al., 2014). Importantly, habituation is an adaptive process, because it enables individuals to focus their attention on novel stimuli with potential meaningful information. A failure to habituate may reflect inefficient processing of novel information. More specifically, a failure to habituate to social stimuli results in a sustained and heightened amygdala response, which may contribute to feelings of anxiety and uncertainty in unfamiliar social situations. Therefore, several research groups have investigated the relationship between amygdala habituation and inhibited temperament (Beaton et al., 2008; Blackford et al., 2013; Blackford, Avery, Cowan, Shelton, & Zald, 2011; Schwartz et al., 2012). These studies demonstrated an increased response to familiarized faces and a failure of the amygdala to habituate in response to repeatedly presented faces, in participants with an inhibited temperament (Blackford et al., 2013, 2011; Schwartz et al., 2012) and shy adults (Beaton et al., 2008). These results strengthen the idea that a more intense and prolonged amygdala response to familiar faces represents a neural substrate underlying the timid and anxious behavior of inhibited people, and, because of the relationship between inhibited temperament and SAD (Clauss & Blackford, 2012), provide evidence for impaired amygdala habituation as an endophenotype for SAD. Additional support comes from a study showing decreased habituation (Schneider et al., 1999) and an increased amygdala response during habituation in SAD patients (Veit et al., 2002), but it should be noted that two other studies did not provide evidence for failed habituation in SAD (Campbell et al., 2007; Sladky et al., 2012). However, these latter studies did not use a passive viewing design which could explain why these studies did neither provide evidence for failed habituation in SAD nor for 'normal' habituation in healthy participants (Campbell et al., 2007; Sladky et al., 2012).

The findings on the relationship between temperamental traits and amygdala functioning in healthy participants are paralleled by results from animal research, demonstrating a trait-like pattern of amygdala activation independent of context: young rhesus monkeys with anxious temperament (AT) show increased amygdala reactivity both in a stressful as well as in a safe context, suggesting that amygdala hyperreactivity is a stable characteristic of AT (Fox, Shelton, Oakes, Davidson, & Kalin, 2008). In addition, amygdala hyperreactivity is associated with multiple dimensions of AT (Shackman et al., 2013), and reduced functional connectivity between the amygdala and prefrontal cortex was reported in anxious monkeys as well as in anxious children (Birn et al., 2014). These findings confirm the evidence from human studies that amygdala hyperreactivity and reduced functional connectivity are trait characteristics of anxious temperament.

There is also evidence for additional state-influences on amygdala reactivity, both in healthy participants as well as in SAD patients. This does, however, not conflict with the trait-stability of endophenotypes: it is acknowledged that a specific challenge (for example,

participating in an experiment in the case of SAD) can reveal an endophenotype (Gould & Gottesman, 2006; Lenzenweger, 2013a). The level of social anxiety in healthy participants influenced amygdala reactivity during social conditioning (Pejic, Hermann, Vaitl, & Stark, 2013), while Brühl and colleagues reported that both the level of trait anxiety as well as the state-dependent level of social anxiety symptoms correlated with reactivity of the amygdala in SAD patients (Brühl et al., 2011). In addition, a significant but weak association between the intensity of social anxiety symptoms and left amygdala reactivity in SAD patients has been demonstrated (Shah et al., 2009).

Findings from studies on the effect of interventions on amygdala reactivity in SAD are mixed. Amygdala activation decreased when patients applied emotion regulation (Brühl, Herwig, Delsignore, Jäncke, & Rufer, 2013), as a result of internet-delivered cognitive behavioral therapy (CBT) (Månsson et al., 2013, 2016) and due to the use of selective serotonin reuptake inhibitors (Faria et al., 2012; Phan et al., 2013) or treatment with oxytocin (Labuschagne et al., 2010). Furthermore, oxytocin modulated functional connectivity of the amygdala (Gorka et al., 2015), and symptom improvement due to treatment with either citalopram or CBT was shown to reduce regional blood flow in the bilateral amygdala (Furmark et al., 2002). However, amygdala responsiveness was not related to treatment outcome in another study using CBT (Klumpp, Fitzgerald, & Phan, 2013).

Criterion 3

As far as we are aware of, there are no studies that directly investigated the heritability of amygdala functioning in healthy participants or patients with SAD, for example using twin- or family studies. However, three lines of evidence point towards genetic influences on amygdala functioning.

To start, various studies in healthy participants have indicated that genes involved in monoaminergic neurotransmission, for example the serotonin transporter gene variation (*5-HTTLPR*), the catechol-o-methyl transferase (*COMT*) gene and the monoamine oxidase A (*MAO-A*) gene influence amygdala functioning (Domschke et al., 2012; Hariri et al., 2002; Kempton et al., 2009; Lonsdorf et al., 2011; Rao et al., 2007). These effects have been confirmed by research on multiple species (Akimova, Lanzenberger, & Kasper, 2009; Caspi, Hariri, Holmes, Uher, & Moffitt, 2010) and by several meta-analyses (Munafò, Brown, & Hariri, 2008; Murphy et al., 2013). However, it should be noted that a recent study was unable to replicate the effect of *5-HTTLPR* variation on amygdala reactivity (Bastiaansen et al., 2014), which could be explained by the rather small contribution of this gene-variant to amygdala reactivity and the fact that the effect may be overestimated due to publication biases (Bastiaansen, de Vries, & Munafò, 2015; Murphy et al., 2013).

In addition, several studies investigated the effect of *5-HTT* genetic variation in SAD patients, confirming the relationship between carrying the short allele of this gene and increased amygdala reactivity (Battaglia et al., 2012; Furmark et al., 2004, 2009) and showing

a link between serotonin-related genotype, amygdala response and the effect of placebo-induced relief of SAD (Furmark et al., 2008). Furthermore, resting-state PET-studies on SAD patients reported reduced binding of the serotonin-1A receptor (Lanzenberger et al., 2007) and increased serotonin synthesis and transporter availability in the amygdala (Frick et al., 2015), a finding recently replicated in an independent sample in which a functional relation between serotonin formation and the tryptophan hydroxylase-2 (*TPH2 G-703T*) polymorphism was reported (Furmark et al., 2016). However, a linkage study in 122 first-degree family members of SAD patients did not yield evidence for a link between SAD and the *5-HTT* gene (Stein, Chartier, Kozak, King, & Kennedy, 1998).

A third line of evidence comes from research on the relation between amygdala activation, genetic variation and AT. Smoller and colleagues demonstrated that variations in the gene encoding the regulator of G protein signaling 2 (*RGS2*), a quantitative trait locus previously linked to anxious behavior in mice, were associated with the level of introversion (a personality trait related to SAD) as well as with the level of amygdala responsiveness during emotion processing, accounting for 15 % of the variance in amygdala activation in humans (Smoller et al., 2008). Converging evidence for a genetic influence on amygdala functioning in relation with AT comes from research on rhesus monkeys (Fox & Kalin, 2014). Studies showed altered expression of genes involved in amygdalar neuroplasticity in anxious young monkeys (Fox et al., 2012), demonstrated AT-related changes in neuropeptide Y gene receptor in the amygdala (Roseboom et al., 2014) and identified several genes with AT-associated methylation changes in the central nucleus of the amygdala (Alisch et al., 2014). In addition, significant heritability of AT-related glucose metabolism in the extended amygdala was demonstrated (Fox, Oler, Shackman, et al., 2015), although another study reported that amygdala functioning predictive of AT was not significantly heritable ((Oler et al., 2010) but see also (Meyer-Lindenberg, 2010) for a commentary). Together, the studies reviewed provide proof for genetic influences on amygdala functioning. However, they also illustrate that many genetic variations are likely to interact in constituting the risk for anxiety.

Criterion 4

To the best of our knowledge, no study has investigated amygdala functioning in SAD patients and their relatives at the same time. However, results from a recent high-risk study support the assumption that the SAD-related alterations in the limbic system are also found in family members of SAD patients. Children who had at least one parent with SAD ($n = 20$) showed hyperreactivity of limbic regions in response to emotional stimuli when compared to normal-risk children (Christensen, Van Ameringen, & Hall, 2015).

Taken together, the studies reviewed suggest that amygdala function and functional connectivity meet the endophenotype criterion of association with SAD. Furthermore, there is support for amygdala functioning and connectivity as relatively stable, trait-like characteristics underlying the vulnerability to SAD. In addition, several lines of evidence

provide evidence for genetic influences on amygdala functioning, while the familial co-segregation warrants more attention in future studies.

Medial prefrontal cortex: function

A core characteristic of SAD is the fear of being negatively evaluated by others (American Psychiatric Association, 2013). It is hypothesized that biases in information processing, such as the tendency to interpret ambiguous social events as negative, distorted self-referential processing, and increased attention to negative responses, play an important role in the development and maintainance of this component of SAD (Clark & McManus, 2002; Spurr & Stopa, 2002). Evidence for disturbed emotional and self-related processing in SAD has been recently reviewed (Jazaieri, Morrison, Goldin, & Gross, 2014; Stein, 2015) and several studies have linked these disturbances to altered functioning of the medial prefrontal cortex (mPFC), a brain area implicated in self-referential processing and social cognition (Amodio & Frith, 2006; Northoff et al., 2006) and the conditioning and extinction of fear (Kim et al., 2011; Quirk, Garcia, & González-Lima, 2006). The mPFC can be roughly divided into two functionally heterogeneous regions: the ventral medial prefrontal cortex (vmPFC) consisting of the subgenual anterior cingulate, ventromedial prefrontal and medial orbitofrontal cortex, mainly involved in the implicit regulation of emotion, and the dorsal medial prefrontal (dmPFC) area, including supragenual anterior cingulate and medial frontal gyrus, important for the appraisal and expression of emotions (Etkin, Büchel, & Gross, 2015; Etkin, Egner, & Kalisch, 2011; Kim et al., 2011). Structural and functional studies have indicated that the mPFC has strong connections with the amygdala (Ghashghaei, Hilgetag, & Barbas, 2007), and anxiety-related changes in both mPFC responsiveness as well as in the connectivity between the mPFC and amygdala have been reported (see review by Kim et al., (2011)). Especially alterations in the function and connectivity of the vmFPC have been associated with anxiety, as this region has a pivotal role in inhibiting conditioned fear and the extincion of a fear response (Blackford & Pine, 2012), but alterations in the dmPFC in SAD have also been reported.

Criterion 1

When we focus on the disturbances in self-related and emotional processing in SAD, studies point towards mPFC hyperreactivity, in both ventral and dorsal areas. SAD patients have increased mPFC activation levels while reading stories describing unintentional social norm transgressions (Blair et al., 2010), in response to self-related comments (Blair et al., 2008; Blair, Geraci, Otero, et al., 2011), and when viewing non-threatening sad faces (Labuschagne et al., 2011). In addition, mPFC hyperreactivity is present in SAD patients during the processing of disorder-related words like 'speech', 'to blush' and 'awkward' (Boehme, Ritter, et

al., 2015). Furthermore, anxious adolescents have increased mPFC responses to faces paired with anxiety-provoking sentences (Peris & Galván, 2013). The hyperreactivity of the mPFC in SAD was confirmed in a meta-analysis (Brühl, Delsignore, et al., 2014). Together, these studies provide evidence for the contribution of the mPFC in the SAD-related interpretation biases and disturbances in self-related processing, and highlight the potential of mPFC hyperresponsiveness as an endophenotype of SAD, although more research is needed to clarify the specific functional roles of the vmPFC and the dmPFC in SAD.

Criterion 2

The trait stability of mPFC functioning has received little attention until now. Several studies investigated the relation between mPFC functioning and temperamental traits (for a review see Kennis et al. (2013), although it should be noted that only one study investigated the relation with self-referential processing and social cognition in healthy participants. Pfeifer and colleagues demonstrated that adolescents, who are generally characterized by increased social concerns, have increased mPFC reactivity during direct self-reflection when compared to adults (Pfeifer et al., 2008). Other studies investigated the relation between inhibited temperament and prefrontal functioning using non-social tasks. Boys who were socially withdrawn during childhood showed increased mPFC responsiveness when anticipating rewards at age 20 (Morgan, Shaw, & Forbes, 2015), while adults with childhood behavioral inhibition had increased activation levels in the mPFC during conflict detection (Jarcho et al., 2013), during attention control in the context of threatening emotional faces (Jarcho et al., 2014), and during anticipation of viewing fearful faces (Clauss, Avery, et al., 2014).

The relation between inhibited temperament and hyperresponsiveness of the mPFC was recently confirmed in a meta-analysis on 13 fMRI studies (Clauss et al., 2015), while research in young rhesus monkeys demonstrated a genetic correlation between orbitofrontal brain metabolism and anxious temperament (Fox, Oler, Shackman, et al., 2015). Furthermore, studies in high socially-anxious participants demonstrated mPFC hyperresponsiveness during a paradigm in which participants were asked to focus their attention on their own bodily states, thoughts, emotions and moods in a simulated social situation (Boehme, Miltner, & Straube, 2015), and while they received social feedback on their performance during a speech task (Heitmann et al., 2014). It should, however, be noted that a study on participants with self-reported subclinical social anxiety (Abraham et al., 2013) was unable to replicate the mPFC hyperresponsivess to self-referential critisism which was previously reported by (Blair et al., 2008). Nevertheless, the majority of these findings provide cautious evidence that hyperresponsiveness of the mPFC is a trait- or vulnerability marker of anxious temperament.

Criterion 3

In comparison to the number of studies investigating genetic influences on amygdala functioning, research on the mPFC is relatively scarce. To the best of our knowledge, no study has investigated the heritability of mPFC functioning in humans. Furthermore, we are not aware of studies investigating genetic influences on mPFC function specifically in SAD. However, research on healthy participants showed an effect of variation in the serotonin transporter polymorphism (*5-HTTLPR*) on the level of mPFC activation while the participants thought about their own negative personality traits, like being lazy or greedy (Ma et al., 2014), and during reflective thinking about the discrepancy between the actual and ideal self (Shi et al., 2015), suggesting that the *5-HTTLPR* polymorphism influences self-referential processing in the mPFC.

To conclude, mPFC hyperreactivity is associated with SAD. Several lines of evidence suggest that mPFC hyperreactivity could be considered a trait characteristic, although more research, for example longitudinal research on participants at high risk for developing social anxiety, is needed to establish this with more certainty. Direct evidence regarding the heritability of this aberrant mPFC functioning in SAD and data on familial co-segregation are, however, missing, although several polymorphisms have been shown to influence mPFC activation levels.

Whole-brain functional connectivity

Over the past several years, researchers have increasingly recognized that brain regions are connected and that disturbances within brain networks could influence the onset, expression and course of diseases (Fornito, Zalesky, & Breakspear, 2015; MacNamara, DiGangi, & Phan, 2016; Sylvester et al., 2012). Thus, the field has shifted from studying specific brain regions to examining brain networks.

Criterion 1

Such network-based studies showed SAD-related changes in functional brain networks, revealing changes in functional connectivity (FC) during rest (Arnold Anteraper et al., 2014; Ding et al., 2011; Geiger et al., 2016; Liao, Chen, et al., 2010; Liao, Qiu, et al., 2010; Liu et al., 2015; Pannekoek et al., 2013) as well as during task-performance (Danti et al., 2010; Gentili et al., 2009; Giménez et al., 2012; Hahn et al., 2011; Klumpp, Angstadt, & Phan, 2012). Although the findings of these studies are mixed, probably due to relatively small sample sizes and the use of different analysis methods (see review by Brühl, Delsignore, et al. (2014)), most prominent FC changes seem to be present in the default-mode network (DMN), which is involved in social cognition and self-referential processes (Gentili et al., 2009; Liao, Chen, et al., 2010); subcortical networks involving the amygdala, caudate,

pallidum and nucleus accumbens (Arnold Anteraper et al., 2014; Manning et al., 2015); and in prefrontal and orbitofrontal networks (Ding et al., 2011). Based on the accumulated evidence to date, alterations in FC in SAD are likely. However, more research in bigger samples and with standardized methods is needed to establish the direction of SAD-related changes in FC.

Criterion 2

Several studies on healthy participants investigated the relation between FC and temperamental traits. Resting-state connectivity between the amygdala and cingulate cortex, as well as intrinsic connectivity in the DMN, the dorsal attention network, the executive control network and salience network, are influenced by trait 'social inhibition' (Blackford et al., 2014), while a recent study showed that changes in intrinsic connectivity of the DMN are already present in children (age 9 - 12 y) who are at temperamental high risk for developing social anxiety (Taber-Thomas, Morales, Hillary, & Pérez-Edgar, 2016). Furthermore, the personality trait 'extraversion' is associated with changes in whole-brain functioning connectivity (Adelstein et al., 2011; Gao et al., 2013; Lei, Zhao, & Chen, 2013), while other studies showed that individual scores of trait 'harm avoidance' (Markett et al., 2013) and trait levels of social anxiety in healthy participants (Gentili et al., 2015) moderate resting-state functional connectivity. There is also evidence for state-influences on FC: state anxiety in healthy participants correlates with resting-state amygdala-insula FC (Baur, Hänggi, et al., 2013), while a recent study on a large, population-based sample (n = 587) showed that FC measures are influenced by both stable, trait-like characteristics, as well as by state-dependent aspects (Geerligs, Rubinov, Cam-Can, & Henson, 2015) – for a review see Dubois (2016).

A couple of studies investigated the relation between the state level of social anxiety symptoms and FC measures, with mixed findings. Pannekoek and colleagues reported differences in resting-state FC in limbic and salience networks between healthy participants and SAD patients, but did not find a relationship between the level of social anxiety symptoms and FC in the patient group (Pannekoek et al., 2013). This supports the idea that FC in SAD is a trait characteristic. However, other studies reported a relationship between social anxiety symptom severity and FC (Dodhia et al., 2014; Liao, Chen, et al., 2010), and an effect of a single dose of oxytocin on resting-state amygdala-frontal connectivity in SAD patients (Dodhia et al., 2014). Future studies using standardized analysis methods should therefore investigate whether changes in FC are a trait characteristic of SAD, for example by investigating whether within-subject changes in social anxiety levels alter FC characteristics, and by examining whether changes in FC are already present in individuals at high risk for developing SAD. Mega-analyses, in which researchers combine resting-state data sets in order to maximize statistical power, could also be beneficial in examining FC changes in SAD.

Criterion 3

There is ample evidence for genetic influences on functional brain networks (Fornito et al., 2011; Glahn et al., 2010; Sinclair et al., 2015; Thompson, Ge, Glahn, Jahanshad, & Nichols, 2013). Recently, a set of 136 genes influencing FC has been identified (Richiardi et al., 2015). Furthermore, variations in the COMT genotype are shown to influence connectivity of the prefrontal cortex (Tunbridge, Farrell, Harrison, & Mackay, 2013) and the DMN (Liu et al., 2010), while variations in the serotonin receptor (*5-HT1A*) modulate activity within the DMN as well (Hahn et al., 2012). Thereby, these studies suggest that FC is at least partly heritable.

To summarize, studies have provided insight in FC changes associated with SAD. In addition, there is evidence that FC networks are generally heritable and related to trait characteristics, although these networks are influenced by state-to-state variations as well. More studies using standardized acquisition- and analysis methods are needed to establish which FC changes are robustly associated with the disorder. In addition, the state-independency and familial co-segregation of these changes call for further investigation.

Structural-anatomical changes

Since more than two decades, neuroimaging data have been used to investigate disorder-related changes in the structure of the brain. Although it is generally believed that the original goal of ascribing psychiatric disorders to specific brain areas is unlikely to be achieved due to the complex nature of such disorders, studies into the structural changes associated with psychopathology are useful to get insight in the neurobiological changes underlying these disorders, especially when a network approach is applied (Menon, 2011) .

Therefore, a handful of studies have investigated anatomical brain changes in SAD, examining alterations in gray matter volumes and differences in the integrity of white matter tracts, which will be reviewed separately in the following subsections.

Gray matter

Criterion 1

Results on gray matter (GM) density changes in SAD point towards alterations in subcortical regions like the amygdala and hippocampus, but it should be noted that these findings often lack consistency (reviewed by Brühl, Delsignore, et al. (2014)). For example, increased amygdala volumes have been found in patients with SAD (Machado-de-Sousa et al., 2014) and in young adults with inhibited temperament (Clauss, Seay, et al., 2014), while a recent treatment study revealed that successful CBT treatment decreased amygdala GM volume in SAD (Månsson et al., 2016). Interestingly, this treatment-related decrease in amygdala GM volume mediated the relationship between decreased neural reactivity of the amygdala and the reduction in social anxiety symptoms after treatment (Månsson et al., 2016), provid-

ing evidence for a link between structural and functional alterations. However, two other studies reported decreases in amygdala volume in SAD (Irle et al., 2010; Meng et al., 2013). These inconsistent results are probably due to the relatively small sample sizes or differences in methodology between studies (see (Montag, Reuter, Jurkiewicz, Markett, & Panksepp, 2013) for a critical review). However, findings from studies on GM changes in other brain regions are more consistent, showing decreases in GM in the orbitofrontal cortex and insula (Syal et al., 2012; Talati, Pantazatos, et al., 2013) and GM-increases in parietal regions (Brühl, Hänggi, et al., 2014; Irle et al., 2014; Talati, Pantazatos, et al., 2013; Tükel et al., 2015) and the temporal cortex (Frick et al., 2013b; Frick et al., 2014; Irle et al., 2014; Talati et al., 2013; Tükel et al., 2015). A recent multi-center mega-analysis on GM volumes in the largest sample to date (174 patients with SAD and 213 healthy control participants) suggests increased GM volume in the right putamen in SAD patients (Bas-Hoogendam, van Steenbergen, Pannekoek, et al., 2017), but more research in bigger samples is needed to establish which GM changes are consistently related to SAD.

Criterion 2

A couple of studies investigated the relation between GM and trait-characteristics, but their results are heterogeneous as well. To illustrate, Cherbuin and colleagues reported a positive relationship between hippocampal volume and inhibited temperament (Cherbuin et al., 2008), while another study demonstrated a negative relationship between hippocampal gray matter and anxiety-like traits (Yamasue et al., 2008). Clauss and co-workers, on the other hand, did not report changes in the hippocampus, but found an association between inhibited temperament and increased volumes of the amygdala and caudate, while further analyses showed that the increase in amygdala volume was positively associated with amygdala reactivity to neutral faces (Clauss, Seay, et al., 2014). This finding of increased amygdala volume is supported by a recent meta-analysis on the GM changes underlying personality traits linked to the vulnerability to anxiety, which revealed increased GM density in the left amygdala in individuals with high negative emotionality-related traits (Mincic, 2015). It should, however, be noted that other studies reported opposite findings, namely an association between increased amygdala volume and extraversion (Cremers et al., 2011) and positive emotionality traits (Lewis et al., 2014), or no relation between amygdala activation and extraversion (Wright et al., 2006). These contradictory findings stress again the need for further research.

Criterion 3

A considerable amount of neuroimaging studies have provided evidence for strong genetic influences on brain anatomy (for reviews see (Blokland, de Zubicaray, McMahon, & Wright, 2012; Peper, Brouwer, Boomsma, Kahn, & Hulshoff Pol, 2007; Thompson et al., 2001)). Of special interest for this review are the results of recent twin studies, indicating that the volumes

of subcortical brain structures, including the amygdala, are highly heritable from childhood on, and that the heritability estimates for these structures are stable over the years (den Braber et al., 2013; Rentería et al., 2014; Swagerman, Brouwer, Geus, Pol, & Boomsma, 2014). A GWA-study of the Enhancing Neuro Imaging Genetics through Meta-Analysis (ENIGMA) consortium on more than 30.000 structural MRI datasets revealed common genetic variants influencing the volumes of several subcortical brain structures (Hibar et al., 2015). Furthermore, an interesting interaction between genotype, gender and amygdala volume has been found: females with two short alleles of the serotonin transporter gene *5-HTTLPR* had the highest anxiety scores and the largest amygdala volume (Cerasa et al., 2014).

Taken together, these results indicate that GM changes are, given their high heritability and their relationship with functional alterations in brain reactivity (Clauss, Seay, et al., 2014; Månsson et al., 2016), potential endophenotypes for SAD, although future research is needed to confirm which trait-like GM alterations are typically associated with SAD.

White Matter

Criterion 1

White matter (WM) density can be investigated using diffusion tensor imaging (DTI) (Thomason & Thompson, 2011). A limited number of DTI studies have investigated global WM volume in SAD: one study found a reduction in global WM, while four other studies reported no differences between SAD patients and healthy controls (see Brühl, Delsignore, and colleagues (2014) for a review). Therefore, the current state of evidence does not support global WM volume as a characteristic of SAD. However, the majority of DTI studies in SAD have focused on the integrity of one specific tract, the uncinate fasciculus (UF). This WM tract connects the amygdala with frontal cortices, including the mPFC and orbitofrontal cortex (Von Der Heide, Skipper, Klobusicky, & Olson, 2013). The mPFC is thought to regulate amygdala output and it is hypothesized that a strong connection between the amygdala and the mPFC leads to lower anxiety levels (Kim et al., 2011). SAD is repeatedly associated with reduced UF integrity (Baur et al., 2011; Baur, Brühl, et al., 2013; Phan et al., 2009). In the case of UF hypoconnectivity, control by the mPFC is likely to fail, leading to the exaggerated amygdala response in SAD (Ayling, Aghajani, Fouche, & van der Wee, 2012). This makes UF hypoconnectivity an etiologically valid candidate endophenotype of SAD. In order to get a complete view of WM changes related to SAD, future studies should examine whole brain WM integrity and investigate systematically whether other WM tracts also show SAD-related differences.

Criterion 2

Several studies investigated the relationship between anxiety-related traits and WM integrity. In line with the results of studies on SAD (Baur et al., 2011; Baur, Brühl, et al., 2013; Phan et al., 2009), two studies on healthy participants demonstrated a negative association

between trait anxiety and UF integrity (Baur, Hänggi, & Jäncke, 2012; Kim & Whalen, 2009), while another study showed a negative relation between the trait harm avoidance and WM integrity in the UF (Westlye, Bjørnebekk, Grydeland, Fjell, & Walhovd, 2011). Furthermore, reduced WM integrity of the UF was found in unmedicated preadolescent children (age 8 - 12) with anxiety disorders, suggesting that WM alterations are not caused by illness chronicity or medication use, but play a role in the pathogenesis (Tromp et al., 2015). Together, these results strengthen the idea that reduced WM integrity of the UF is a state-independent characteristic of (social) anxiety.

Criterion 3

Multiple DTI studies have indicated that WM brain characteristics are heritable (Blokland et al., 2012; Bohlken et al., 2014; Shen et al., 2014), and a meta-analysis showed that the UF has a high heritability estimate of 0.7 (Kochunov et al., 2014). This was confirmed by a recent twin-study, reporting that genetic factors explained 64 - 80 % of variance in UF microstructure (Budisavljevic et al., 2016). More specifically, structural integrity of the UF is influenced by genetic variations in the brain-derived neurotrophic factor (*BDNF*) (Carlson, Cha, Harmon-Jones, Mujica-Parodi, & Hajcak, 2014) and the *5-HTTLPR* genotype (Klucken et al., 2015; Pacheco et al., 2009).

Concluding, reduced integrity of the UF meets the endophenotype criteria of association with SAD, trait-stability and heritability. Whether this structural brain alteration and social anxiety co-segregate within families has not been examined.

Discussion

Evidence for candidate MRI endophenotypes of SAD

Here, we reviewed empirical evidence for several candidate neurobiological endophenotypes of social anxiety. Endophenotypes as measurable characteristics that are related to the disorder and reflective of genetically-based disease mechanisms. We focused on MRI measurements, and candidate endophenotypes included: function and functional connectivity of the amygdala, function of the medial prefrontal cortex (mPFC), changes in whole-brain functional connectivity (FC) and structural-anatomical alterations. Results are summarized in *Table 2.2*. Not surprisingly, given the selection of candidate endophenotypes based on studies on SAD as reviewed by Brühl and colleagues (2014a), we found strongest evidence for all candidate endophenotypes for the first endophenotype criterion, the association with the disorder. Evidence for the other endophenotype criteria (being a trait-characteristic; being heritable; and co-segregation with the illness within families) was, however, more suggestive than definitive, given the fact that direct research on these criteria is still scarce. However, available evidence from other lines of research provides circumstantial evidence

for the potential of these candidate endophenotypes of SAD. For example, studies provided evidence that amygdala-hyperreactivity and FC measures are influenced by stable trait characteristics such as inhibited temperament. Furthermore, studies on healthy participants indicate that reactivity of the amygdala and the mPFC, the strength of FC in several brain networks, changes in GM and the integrity of a specific white matter tract associated with SAD, the UF, are influenced by genetic variations. These findings highlight the potential of these neuroimaging markers as candidate endophenotypes of SAD.

Table 2.2 Evidence for candidate MRI endophenotypes of social anxiety disorder.

	Associated with SAD	Trait characteristic	Heritable	Co-segregation with illness within families
Amygdala				
Hyperreactivity and changes in functional connectivity	***	***, although also evidence for state-influences	Not directly investigated; however, genetic influences have been shown ***	*
Medial prefrontal cortex				
Hyperreactivity	***	***	Not directly investigated; however, genetic influences have been shown *	TBI
Whole-brain functional connectivity (FC)				
Altered FC during rest	***	***, although also evidence for state-influences	Not directly investigated; however, genetic influences have been shown ***	TBI
Altered FC during task performance	**	TBI	TBI	TBI
Structural-anatomical changes				
GM changes	***	**, although also evidence for state-influences	***	TBI
WM changes: integrity UF	**	**	***	TBI

Abbreviations
GM: gray matter; SAD: social anxiety disorder; TBI: to be investigated; UF: uncinate fasciculus; WM: white matter.

Footnotes
*: Evidence from 1-2 independent studies.
**: Evidence from 3-4 independent studies.
***: Evidence from ≥ 5 independent studies or a meta-analysis.

Directions for future research and outstanding questions

The reviewed evidence in favour of considering these characteristics as candidate endophenotypes of SAD is still circumstantial. To directly investigate the *heritability* and familial *co-segregation of candidate endophenotypes,* as well as their *trait-stability* (criterion 2, 3 and 4), longitudinal multiplex family studies involving patients with SAD and their family members, preferably from multiple generations, are the most optimal approach (Cannon & Keller, 2006; DeLisi, 2016). We feel the evidence summarized here provides a solid empirical background to perform such labour- and cost intensive studies, which extend the present studies comparing SAD patients and healthy control participants. To the best of our knowledge, the Leiden Family Lab study on Social Anxiety Disorder is the first comprehensive study aimed to establish neuroimaging endophenotypes of SAD. In this study, patients with SAD, their siblings and children, as well as the partners of each family member, are investigated (total sample size 134 participants of two generations, including 19 SAD patients; MRI sample size 114 participants; age range participants 8.9 – 61.5 y; see pre-registration of this study in (Bas-Hoogendam et al., 2014a)). The data are presently analyzed.

Two other outstanding issues where the endophenotype-field needs to decide upon concern the criteria for defining endophenotypes. First of all, the criterion of trait-stability or state-independency warrants more attention. An open question is, for example, whether endophenotypes could change as a result of a successful treatment. We speculate that the degree of expression of a certain endophenotype could be altered as a result of an intervention, but we hypothesize that, based on the genetic basis of the endophenotype, the endophenotype could still be detected in successfully treated patients when compared to healthy control participants without the endophenotype.

Another open question involves the specificity of endophenotypes for a particular disorder. Although the candidate endophenotypes discussed in this review are in general strongly associated with SAD, several of these characteristics are also related to other anxiety and mood disorders. For example, amygdala hyperreactvity in response to facial expressions has been found in patients with posttraumatic stress disorder (Shin et al., 2005), in participants at high risk for developing anxiety and depression (Wolfensberger, Veltman, Hoogendijk, Boomsma, & de Geus, 2008) and in patients with generalized anxiety disorder (GAD) and panic disorder (Fonzo et al., 2015); FC changes in the default mode network are demonstrated in several neuropsychiatric disorders, including depression (Whitfield-Gabrieli & Ford, 2012); alterations in mPFC functioning related to self-referential processing have been reported in patients with major depressive disorder (Nejad, Fossati, & Lemogne, 2013), while decreased white matter integrity of the UF was also present in GAD patients (Tromp et al., 2012). These findings raise the question whether these changes could serve as endophenotypes for SAD, or are rather reflective of endophenotypes for anxiety and mood disorders in general. We argue, based on the argumentation proposed by Cannon and Keller (2006), that specificity is not a prerequisite for an endophenotype. Given the fact that

several anxiety and mood disorders often run together within families (Hettema, Neale, & Kendler, 2001; Sharma, Powers, Bradley, & Ressler, 2016; Smoller, Block, & Young, 2009), it is possible that certain endophenotypes affect more than one disorder. Discovering these endophenotypes could even be helpful in unraveling the shared genetic background of these disorders (Bearden & Freimer, 2006; Cannon & Keller, 2006; Puls & Gallinat, 2008).

Methodological considerations of the present review

Given the paucity of studies examining directly whether neurobiological characteristics of SAD meet the criteria for endophenotypes (except for multiple studies on the *association with the disorder*, criterion 1), two important methodological considerations with respect to the present review should be made. First, we can not exclude that studies on SAD endophenotypes have been performed, but were not published due to negative results (i.e. a publication bias). However, we think the lack of longitudinal and family studies on SAD is primarily due to the fact that such studies are time- and cost intensive, and are hard to perform given the inherent characteristic of SAD patients to avoid attention to their impairments because they are ashamed of or underestimate their condition (Dingemans et al., 2001; Fehm et al., 2005; Ruscio et al., 2008; Stein & Stein, 2008; Wittchen & Fehm, 2003).

Second, because of the limited number of studies on neurobiological endophenotypes of SAD, the present review is a narrative rather than a systematic review. By describing results from studies which investigated evidence in relation to endophenotype criteria in multiple, related fields of research, we aimed to illustrate the endophenotype criteria using key examples from, for example, research on healthy participants with certain personality traits, and from animal research. This approach was also used in recent reviews on endophenotypes of major depressive disorder (Goldstein & Klein, 2014; Hasler & Northoff, 2011), and suited the aim of the present review, which was to explore the usefulness of endophenotypes in studying the development of SAD and to discuss the way the endophenotype approach can be applied to the field. Although we tried our best to be comprehensive, by including studies who reported results which were not in favour of certain characteristics as being endophenotypes of SAD and null findings as well, the fact that we were not able to systematically review evidence for SAD endophenotypes is a potential limitation of the present work.

Conclusions

Endophenotypes are measurable characteristics that are related to complex psychiatric disorders and reflective of genetically-based disease mechanisms. In this review, we evaluated the usefulness of endophenotypes and summarized evidence in support of neuroimagingendophenotypes of social anxiety disorder (SAD). Results are promising, but they also stress the need for further research, especially using longitudinal family studies, to assess the trait-stability of the candidate endophenotypes and the co-segregation of the endophenotype with the disorder. In addition, we pinpointed outstanding questions for the field.

Based on the circumstantial evidence already available to date, we feel neuroimaging studies have great potential to detect endophenotypes of SAD. These endophenotypes could be especially valuable in giving more insight into the mechanisms leading to this complex psychiatric disorder, which in turn provides clues for better preventive interventions and more effective treatments. Therefore, we strongly urge the need for future research specifically aimed at establishing neuroimaging endophenotypes of SAD.

Chapter 3

The Leiden Family Lab study on Social Anxiety Disorder: a multiplex, multigenerational family study on neurocognitive endophenotypes

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Abstract

Objectives

Social anxiety disorder (SAD) is a serious and prevalent psychiatric condition, with a heritable component. However, little is known about the characteristics that are associated with the genetic component of SAD, the so-called 'endophenotypes'. These endophenotypes could advance our insight in the genetic susceptibility to SAD, as they are on the pathway from genotype to phenotype. The Leiden Family Lab study on Social Anxiety Disorder (LFLSAD) is the first multiplex, multigenerational study aimed to identify neurocognitive endophenotypes of social anxiety.

Methods

The LFLSAD is characterized by a multidisciplinary approach and encompasses a variety of measurements, including a clinical interview, functional and structural magnetic resonance imaging (MRI) and an electroencephalography (EEG) experiment. Participants are family members from two generations, from families genetically enriched for SAD.

Results

The sample (n = 132 participants, from nine families) was characterized by a high prevalence of SAD, in both generations (prevalence (sub)clinical SAD: 38.3 %). Furthermore, (sub)clinical SAD was positively related to self-reported social anxiety, fear of negative evaluation, trait anxiety, behavioral inhibition, negative affect and the level of depressive symptoms.

Conclusions

By the multidimensional character of the measurements and thorough characterization of the sample, the LFLSAD offers unique opportunities to investigate candidate neurocognitive endophenotypes of SAD.

Introduction

Social anxiety disorder (SAD) is a prevalent mental disorder, with an estimated lifetime prevalence around 13 % (Kessler et al., 2012). Patients with SAD have an extreme fear of being negatively evaluated by others in social situations (American Psychiatric Association, 2013). SAD has a considerable impact on the life of patients, as the disorder has a typical onset during late childhood or early adolescence, and is characterized by a chronic course (Beard, Moitra, Weisberg, & Keller, 2010; Beesdo-Baum et al., 2012; Haller, Cohen Kadosh, Scerif, & Lau, 2015; Miers, Blöte, de Rooij, Bokhorst, & Westenberg, 2013; Miers, Blöte, Heyne, & Westenberg, 2014; Steinert, Hofmann, Leichsenring, & Kruse, 2013; Westenberg, Gullone, Bokhorst, Heyne, & King, 2007; Wittchen & Fehm, 2003). SAD patients experience impairments in multiple domains, including education, work, and social life; they report a lower quality of life, and suffer often from comorbid psychopathology, like other anxiety disorders, depression and substance abuse (Acarturk, de Graaf, van Straten, Have, & Cuijpers, 2008; Dingemans, van Vliet, Couvée, & Westenberg, 2001; Fehm, Pelissolo, Furmark, & Wittchen, 2005; Mack et al., 2015; Meier et al., 2015; Stein & Stein, 2008). Insight in the factors that play a role in the development of SAD is therefore of uttermost importance, in order to be able to reduce long-term effects of SAD by developing effective preventive interventions and early treatment programs (Beauchaine et al., 2008).

Several studies have indicated that genetic predispositions, as well as environmental, biological, and temperamental factors interact in the pathogenesis of SAD, as reviewed by Wong & Rapee (2016), Spence & Rapee (2016) and Fox & Kalin (2014). Family- and twin studies pointed to a heritability of SAD of around 50 % (Bandelow et al., 2016; Gottschalk & Domschke, 2016; Isomura et al., 2015; Smoller, 2015); however, the search for specific genes underlying the susceptibility to SAD has been proven difficult. To start, SAD is a heterogeneous disorder and the diagnosis is based on clinical assessments and not on biologically-based measurements (Bearden et al., 2004; Glahn et al., 2007; Gottesman & Gould, 2003). In addition, it is assumed that multiple interacting genetic variants, with relatively small individual effects, contribute to the vulnerability to SAD, complicating their detection (Binder, 2012; Munafò & Flint, 2014b). Furthermore, epigenetic mechanisms, reflecting the interaction between genetic background and environmental influences, are of importance, requiring multi-level studies integrating data on psychopathology, (epi)genetics and environment (Gottschalk & Domschke, 2016; Schiele & Domschke, 2017). Given these complexities, studies into the genes that contribute to the pathophysiology may be facilitated by defining endophenotypes related to SAD (Bas-Hoogendam et al., 2016).

Endophenotypes are measurable characteristics on the pathway from genotype to phenotype (Gottesman & Gould, 2003; Lenzenweger, 2013b) and offer several possibilities to advance our understanding of the genetic susceptibility to SAD (Bas-Hoogendam et al., 2016). Endophenotypes could shed light on the pathways leading to disorder phenotypes (Flint et

al., 2014; Miller & Rockstroh, 2013), can be used to identify individuals at risk (Puls & Gallinat, 2008), and could aid in the development of animal models for psychopathology (Gould & Gottesman, 2006). Furthermore, they offer starting points for therapeutic interventions (Garner et al., 2009) and can be useful in trans-diagnostic research as proposed by the NIMH Research Domain Criteria (RDoC) (Sanislow et al., 2010). For a conceptual framework on neurobiological endophenotypes of SAD, we refer to Bas-Hoogendam et al. (2016).

Endophenotypes are defined as meeting the following criteria (Glahn et al., 2007; Gottesman & Gould, 2003; Lenzenweger, 2013b; Puls & Gallinat, 2008): 1st they are *associated with the disorder*; 2nd they are *state-independent traits, already present in a preclinical state*; 3rd they are *heritable*; 4th they *co-segregate with the disorder within families of probands, with non-affected family members showing altered levels of the endophenotype in comparison to the general population*. Furthermore, endophenotypes are ideally more strongly related to the disorder of interest in comparison to other psychiatric conditions (Lenzenweger, 2013a), but given the shared genetic influences between psychiatric disorders, certain endophenotypes are likely related to more than one disorder (Cannon and Keller, 2006).

Objective of the Leiden Family Lab study on Social Anxiety Disorder

To determine which disease-related characteristics may serve as endophenotypes, participants with SAD as well as their relatives need to be extensively phenotyped. Families are essential to allow investigating the *heritability* of the feature (criterion 3) and the *co-segregation of the candidate endophenotype with the disorder within the family* (criterion 4, first element), while case-control studies and longitudinal studies are needed to examine the other endophenotype criteria (criterion 1 and criterion 2, respectively) (Bas-Hoogendam et al., 2016). In addition, adequately matched control families are needed to investigate the second element of criterion 4, namely whether *non-affected family members show altered levels of the endophenotype when compared to the general population*. To the best of our knowledge, the Leiden Family Lab study on Social Anxiety Disorder (LFLSAD) is the first multiplex (i.e., multiple cases of the disorder within one family), multigenerational family study aimed to determine neurocognitive endophenotypes of SAD, as measured with magnetic resonance imaging (MRI) and electroencephalography (EEG), investigating the *heritability* of candidate endophenotypes and the *co-segregation of the candidate endophenotypes with the disorder within the family*. Two important aspects of the study deserve to be highlighted.

First, the multiplex, multigenerational design was chosen to maximize statistical power to detect genetic and environmental influences on SAD-related characteristics. Having multiple cases within a family instead of sporadic cases enriches the sample for a heritable basis of the disease and the detection of genetic factors. Furthermore, a sample consisting of large families, composed of several related nuclear families (parents with their children), is likely to share more heritable factors than a same-sized sample of unrelated nuclear families,

hence more statistical power to distinguish shared environmental effects from genetic effects (Williams & Blangero, 1999), cf. Gur et al. (2007).

Second, the LFLSAD focuses on neurocognitive SAD endophenotypes as measured with MRI and EEG, as these are both non-invasive, widely applied, and safe methods to investigate structural and functional properties of the human brain. Importantly, these methods are complementary: EEG has good temporal precision to capture electrocortical activity associated with attentional SAD-related biases and can be used to study candidate endophenotypes related to processing social judgments (Harrewijn, van der Molen, van Vliet, Tissier, & Westenberg, 2018; Van der Molen et al., 2014) and to performing a public speaking task (Harrewijn, van der Molen, van Vliet, Houwing-Duistermaat, & Westenberg, 2017; Harrewijn, Van der Molen, & Westenberg, 2016). MRI enables precise spatial localization of the brain regions implicated in SAD, and provides valuable insights in the structure and connectivity of the brain, and the functioning of brain regions like the amygdala and the prefrontal cortex during viewing neutral faces in a habituation and conditioning task (cf. (Bas-Hoogendam, van Steenbergen, Westenberg, & van der Wee, 2015; Blackford et al., 2013, 2011; Davis, Johnstone, Mazzulla, Oler, & Whalen, 2010)) and processing social norm violations (Bas-Hoogendam, van Steenbergen, Kreuk, van der Wee, & Westenberg, 2017a; Bas-Hoogendam, van Steenbergen, van der Wee, & Westenberg, 2018; Blair et al., 2010). Typically, neurocognitive endophenotypes are assumed to be closer to the genotype than, for example psychological constructs (Cannon & Keller, 2006). However, data collection in the LFLSAD was not limited to these measures: in order to achieve comprehensive phenotyping of the participants, a variety of additional measurements was included, as described in detail below. To this aim, the LFLSAD was performed by a multidisciplinary team of clinicians, neuroscientists, and statisticians.

In the current paper, the design and methods of the LFLSAD are presented. Furthermore, characteristics of the LFLSAD sample are described, including analyses on its psychological features. Hypotheses with respect to the candidate neurocognitive endophenotypes have been pre-registered in 2014 on the Open Science Framework website (osf.io) and are available online (links provided in the *Supplemental Methods*) (Bas-Hoogendam et al., 2014a). Results of the analyses into candidate neurocognitive endophenotypes of SAD are reported in other papers and conference abstracts (Bas-Hoogendam, van Steenbergen, van der Wee, & Westenberg, 2017c, 2017b; Bas-Hoogendam, van Steenbergen, et al., 2015; Harrewijn, van der Molen, et al., 2017; Harrewijn et al., 2018) and in preparation.

Methods

Study design and setting

The Leiden Family Lab study on Social Anxiety Disorder (LFLSAD) is a cross-sectional, two-generation multiplex family study on the neurocognitive characteristics that are genetically linked to SAD. The study is a collaboration between Leiden University (Institute of Psychology) and the Leiden University Medical Center (LUMC; Departments of (Child) Psychiatry and Department of Medical Statistics and Bioinformatics) and is embedded within the Leiden University research profile area 'Health, prevention and the human life cycle'. Data collection took place at Leiden University and the LUMC between October 2013 and July 2015.

Sample

Families were considered eligible for inclusion when they contained at least one adult, aged 25 - 55 years, with a primary diagnosis of SAD (from now on referred to as the 'proband'), of whom at least one child, aged 8 - 21 years and living at home with the proband, showed SAD symptoms at a clinical or subclinical level (referred to as the 'proband's SA-child'). For these participants, comorbidity with other internalizing disorders was allowed; however, families were excluded when the proband or the proband's SA-child suffered of other psychiatric diagnoses, especially developmental disorders (e.g. autism).

In addition to the proband and its SA-child, the proband's spouse, other children (age ≥ 8 years) as well as the proband's sibling(s) and their spouse(s) with their child(ren) (age ≥ 8 years) were invited to participate. In *Figure 3.1* we depict a pedigree starting with the grandparental generation (0) on which no data was collected for reasons of feasibility; probands and siblings belonging to generation 1; and proband's and siblings' offspring (generation 2). We aimed to include families with at least 8 family members, to enable reliable estimations of the relation between endophenotype and SAD.

Family members of the proband and proband's SA-child were included independent of the presence of psychopathology. All participants were required to have sufficient comprehension of the Dutch language.

Sample size & power calculation

The aim of the LFLSAD was twofold. First, the study aimed to estimate the association between SAD and neurocognitive putative endophenotypes (Bas-Hoogendam et al., 2014a, 2016; Harrewijn, Schmidt, Westenberg, Tang, & van der Molen, 2017; Harrewijn, van der Molen, et al., 2017; Harrewijn et al., 2018); second, the significance of clustering of these endophenotypes within families (i.e., genetic effects) was addressed. To estimate this heritability, a joint mixed model taking the ascertainment process and familiar relationships into account, will be used (Tissier, Tsonaka, Mooijaart, Slagboom, & Houwing-Duistermaat, 2017). Power calculations, performed by co-author JHD, revealed that 12 families with 8 - 12 family members (average:

10 members per family) were required for sufficient power (i.e., minimally 80 %) to investigate these two questions (details provided in *Supplemental Methods*).

Procedure

Recruitment

Families were recruited through media exposure, such as interviews in Dutch newspapers, on television and radio; furthermore, the study was brought to the attention of patient organizations like the 'Anxiety, Compulsion and Phobia association' (in Dutch: 'Angst, Dwang en Fobie stichting') and the 'Association of Shy People' ('Vereniging van Verlegen Mensen'), to clinical psychologists, general practitioners, and mental health care organizations. In the media items, we asked families in which multiple family members experienced 'extreme shyness' to contact us.

Screening-procedure and inclusion of families

Potential probands were screened for eligibility by a telephone call or an email, depending on their preference. This screening contained questions with respect to the presence of social anxiety in the proband and the proband's SA-child, the age of the proband and his/her child(ren), and the potential number of family members that could be invited for the study. In addition, probands were further informed about the study. When they passed the screening and showed interest in participation, an information letter was sent to the proband and his/her nuclear family members, containing detailed information about the study. Two weeks later, participants were contacted by telephone and any questions about the study were answered. Next, the proband, the proband's spouse and the proband's SA-child were invited to come to the LUMC for an introductory meeting and structured clinical interview, in order to confirm the presence of a primary diagnosis of SAD (proband) and (sub)clinical social anxiety (proband's SA-child). Furthermore, a screening was performed to exclude the presence of autism in the proband and the proband's SA-child.

When the inclusion criteria were met, the proband and his/her nuclear family were included in the study and invited for the remaining measurements (*Table 3.1*). In addition, we asked the proband to contact his/her sibling(s), in order to confirm that they were interested to be informed about participation in the study. Given a positive response, these siblings, together with their partner and/or children, were invited to participate by the investigators. Given the inherent characteristic of socially anxious people to avoid new situations and their tendency to stay out of the spotlights, we encouraged participants to visit the lab together with their family members, in order to make them feel more comfortable. Although we emphasized the importance of including as many family members as possible within the study, we also indicated that each individual was free to decide whether or not to participate (*Figure 3.1*).

Table 3.1 Measurements included in the LFLSAD.

	Measurements	Instrument	Age group (years)
Clinical interview	Diagnoses of mental (axis-1) disorders according to DSM criteria	M.I.N.I.-Plus	18+
		M.I.N.I.-Kid	8-17
Questionnaires	Social anxiety symptoms	LSAS-SR	18+
		SAS-A	8-17
	Fear of negative evaluation	BFNE-II-R	8+
	General anxiety	STAI-trait	8+
		STAI-state (before and after MRI scan)	8+
	Depressive symptoms	BDI-II	18+
		CDI	8-17
	Affect	PANAS	8+
	Temperament	BIS/BAS	13+
		BIS/BAS-C	8-12
	Autism screening	AQ	18+
		SRS, completed by both parents about their child(ren)	8-17
	Handedness	EHI	8+
Estimation of intelligence	IQ	WAIS-IV subtests (similarities & block design)	17+
		WISC subtests (similarities & block design)	8-16
MRI scan	Structural and functional MRI		8+
EEG experiment	EEG measurement, including collection of saliva for cortisol measurements		8+
Genotyping	Collection of saliva	Oragene•DNA OG-500 kit	8+

Abbreviations

M.I.N.I.-Plus: Mini-Plus International Neuropsychiatric Interview (MINI Plus version 5.0.0) (Sheehan et al., 1998; van Vliet & de Beurs, 2007); M.I.N.I.-Kid: MINI Kid interview version 6.0 (Bauhuis, Jonker, Verdellen, Reynders, & Verbraak, 2013; Sheehan et al., 2010); LSAS-SR: Liebowitz Social Anxiety Scale – self report (Fresco et al., 2001; Mennin et al., 2002); SAS-A : Social Anxiety Scale – adolescents (La Greca & Lopez, 1998); BFNE-II-R: revised Brief Fear of Negative Evaluation-II scale (Carleton, McCreary, Norton, & Asmundson, 2006; Leary, 1983); STAI: State-Trait Anxiety Inventory (Spielberger et al., 1970); BDI-II= Beck Depression Inventory-II (Beck, Steer, & Brown, 1996; Van der Does, 2002); CDI: Children's Depression Inventory (Kovacs, 1983, 1985; Timbremont & Braet, 2002); PANAS: Positive and Negative Affect Schedule (Peeters, Ponds, & Vermeer, 1996; Watson, Clark, & Tellegen, 1988); BIS/BAS: Behavioral Inhibition and Behavioral Activation Scales (Carver & White, 1994); BIS/BAS-C: Behavioral Inhibition and Behavioral Activation Scales for children (Muris, Meesters, de Kanter, & Timmerman, 2005); AQ: Autism-spectrum Quotient questionnaire (Baron-Cohen, Wheelwright, Skinner, Martin, & Clubley, 2001); SRS: Social Responsiveness Scale (Constantino et al., 2003; Roeyers, Thys, Druart, De Schryver, & Schittekatte, 2011); EHI: Edinburgh Handedness Inventory (Oldfield, 1971); WAIS: Wechsler Adult Intelligence Scale IV (Wechsler, Coalson, & Raiford, 2008); WISC: Wechsler Intelligence Scale for Children III (Wechsler, 1991); MRI: magnetic resonance imaging; EEG: electroencephalography.

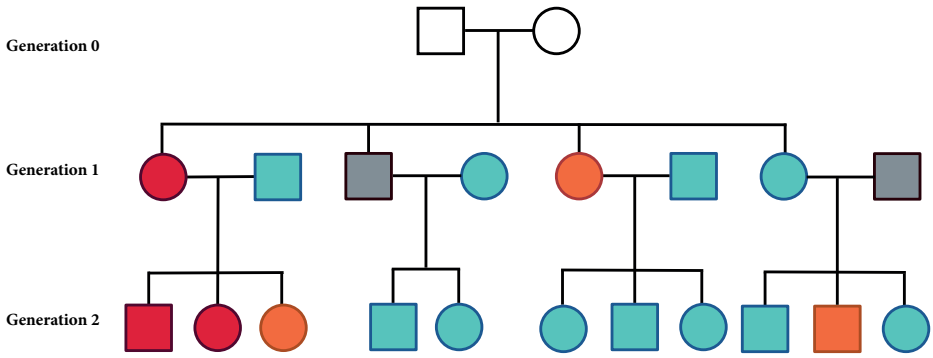

Figure 3.1 Example of a family within the Leiden Family Lab study on Social Anxiety Disorder.
Families were included based on the combination of a parent with SAD ('proband'; depicted in red) and a proband's child with SAD (red) or subclinical SA (orange). In addition, family members of two generations were invited, independent from the presence of SAD within these family members (no SAD: light blue; did not participate: gray). Grandparents (generation 0; white) were not invited for participation. This family is slightly modified to guarantee anonymity; however, the number of family members and the frequency of (sub)clinical SAD are depicted truthfully. Squares and circles represent men and women, respectively.

Ethics

The study (P12.061) was approved by the Medical Ethical Committee of the LUMC in June 2012. All participants received written and verbal information with respect to the objectives and procedure of the study; information letters were age-adjusted, to make them understandable for children and adolescents as well. Participants provided informed consent prior to participation, according to the Declaration of Helsinki. Both parents signed the informed consent form for their children, while children between 12 and 18 years of age signed the form themselves as well. Every participant received €75 for participation (duration whole test procedure, including breaks: 8 hours) and travel expenses were reimbursed. Furthermore, participants were provided with lunch / diner, snacks, and drinks during their visit to the lab. Confidentiality of the research data was maintained by the use of a unique research ID number for each participant.

Measurements

All participants took part in the same measurements; the order of the measurements differed between participants depending on their availability and lab resources. However, as described above, for the proband, the proband's spouse, and the proband's SA-child, the clinical interview always preceded the other measurements. Age-appropriate instruments were used to evaluate certain constructs. Measurements are listed in *Table 3.1* and explained below.

Diagnosis of mental disorders

Structured clinical interviews using the Mini-International Neuropsychiatric Interview (M.I.N.I.)-Plus (version 5.0.0) (Sheehan et al., 1998; van Vliet & de Beurs, 2007) or the M.I.N.I.-Kid (version 6.0) (Bauhuis, Jonker, Verdellen, Reynders, & Verbraak, 2013; Sheehan et al., 2010) were used to determine the presence of psychiatric diagnoses according to DSM-IV-TR criteria (axis-1). Interviews were conducted by trained clinicians, and were recorded. These recordings were used to determine the presence of (sub)clinical SAD. Clinical SAD was diagnosed using the DSM-IV-TR criteria for the generalized subtype of SAD, but the clinician verified whether the DSM-5 criteria for SAD were also met in order to establish the diagnosis. Participants were classified as having subclinical SAD when they met the criteria for SAD as described in the DSM-5, but without showing obvious impairments in social, occupational, or other important areas of functioning (criterion G) (American Psychiatric Association, 2013).

Self-report assessments of anxiety and associated constructs

Social anxiety was assessed on a dimensional scale using the self-report version of the Liebowitz Social Anxiety Scale (LSAS-SR) (Fresco et al., 2001; Mennin et al., 2002) or the Social Anxiety Scale for Adolescents (SAS-A) (La Greca & Lopez, 1998). The LSAS-SR measures fear in and avoidance of situations that are likely to elicit social anxiety, with good internal consistency (Heimberg et al., 1999). The SAS-A determines social anxiety in children and adolescents, with satisfactory levels of internal consistency (Miers et al., 2013).

Fear of negative evaluation was assessed with the revised Brief Fear of Negative Evaluation (BFNE)-II-R scale (Carleton, McCreary, Norton, & Asmundson, 2006), which is a revision of the BFNE questionnaire (Leary, 1983). The BFNE-II-R is a self-report questionnaire with excellent internal consistency and good convergent validity (Carleton, Collimore, & Asmundson, 2007).

The State-Trait Anxiety Inventory (STAI) ((Spielberger, Gorsuch, & Lushene (1970); see Spielberger & Vagg (1984) for psychometric properties) was used to determine self-reported trait anxiety, as well as state anxiety before and after the MRI scan.

Severity of self-reported depressive symptoms was assessed using the Beck Depression Inventory-II (BDI-II) (Beck, Steer, & Brown, 1996; Van der Does, 2002) or the Children's Depression Inventory (CDI) (Kovacs, 1983, 1985; Timbremont & Braet, 2002). Due to ethical reasons, an item asking about suicide was removed from the CDI (cf. Miers, Blöte, & Westenberg (2010)).

The general mood of the participant, experienced in the last couple of weeks, was assessed by the self-report Positive and Negative Affect Schedule (PANAS) (Peeters, Ponds, & Vermeer, 1996; Watson, Clark, & Tellegen, 1988), which is a reliable and valid instrument to measure affect (Crawford & Henry, 2004).

The sensitivity for the temperamental traits 'behavioral inhibition' and 'behavioral activation' was assessed using the self-report BIS/BAS (Carver & White, 1994; Franken, Muris, & Rassin, 2005) or the BIS/BAS scales for children (BIS/BAS-C) (Muris, Meesters, de Kanter, & Timmerman, 2005).

Autism screening

Adult participants were screened for autism using the self-report Autism-spectrum Quotient (AQ) questionnaire (Baron-Cohen, Wheelwright, Skinner, Martin, & Clubley, 2001); parents completed the Dutch version of Social Responsiveness Scale about their child(ren) (Constantino et al., 2003; Roeyers, Thys, Druart, De Schryver, & Schittekatte, 2011).

Handedness

Handedness was assessed with the Edinburgh Handedness Inventory (EHI) (Oldfield, 1971).

Estimation of intelligence

Two subscales of the Wechsler Adult Intelligence Scale-IV (WAIS-IV) (Wechsler, Coalson, & Raiford, 2008) or Wechsler Intelligence Scale for Children-III (WISC) (Wechsler, 1991), the similarities (verbal comprehension) and block design (perceptual reasoning) subtests, were administered to obtain an estimate of cognitive functioning.

Structural and functional MRI measurements

A detailed description of the MRI session is included in the *Supplemental Methods*. The session consisted of a high-resolution T1 scan, two diffusion tensor imaging scans and a magnetization transfer ratio scan. In addition, a high-resolution EPI scan and a B0 field map were acquired. Functional MRI data were collected during resting-state and during two functional paradigms: an amygdala paradigm investigating amygdala habituation (based on the work of Blackford, Allen, Cowan, & Avery, 2013; Blackford, Avery, Cowan, Shelton, & Zald, 2011; Schwartz, Wright, Shin, Kagan, Whalen, et al., 2003; Schwartz, Wright, Shin, Kagan, & Rauch, 2003) and conditioning (Davis et al., 2010), and the revised Social Norm Processing Task (Bas-Hoogendam, van Steenbergen, Kreuk, et al., 2017a).

EEG measurements

A detailed description of the EEG session is included in the *Supplemental Methods*. The session consisted of multiple resting-state measurements, as well as two task paradigms: a social judgment paradigm (Harrewijn et al., 2018; Van der Molen et al., 2014) and a social performance task (Harrewijn, van der Molen, et al., 2017; Harrewijn et al., 2016). At several time points before and during this task, task-induced mood was measured and saliva samples were collected to measure cortisol.

Biosampling for DNA isolation

Saliva samples were collected for future genotyping, using the Oragene•DNA OG-500 self-collection kits (Genotek, Ottawa, Ontario, Canada).

Data analysis for the current paper

Sample characterization

We investigated socio-demographic differences between the generations using chi-square tests (male / female ratio, native country, and education level) and linear regression models (age and estimated IQ). These regression models were fitted in R (R Core Team, 2016), with generation as independent variable. Because of the relationships between the participants, genetic correlations between family members were modeled by including random effects (lmekin function).

Next, in order to verify that the LFLSAD sample is genetically enriched for SAD, several analyses were performed. First, the presence of clinical and subclinical SAD was determined. Furthermore, the heritability of (sub)clinical SAD within the sample was estimated using the software package SOLAR (Sequential Oligogenic Linkage Analysis Routines; Almasy & Blangero, 1998). Heritability indicates how strong genetic effects influence a certain trait, and is defined as the proportion of the variation in a phenotype that can be attributed to additive genetic effects (Almasy & Blangero, 2010; Wray & Visscher, 2008). SOLAR uses maximum likelihood techniques to attribute variance in the phenotype to either genetic or environmental effects, based on a kinship matrix for the genetic component and an identity matrix for the unique environmental component. Here, we did not include a shared environmental component, to keep the model as simple as possible. We corrected for ascertainment (de Andrade & Amos, 2000) by indicating that families were selected based on the proband and the proband's SA-child. Age and gender were included as covariates, and were removed from the model when their effect was not significant ($p > 0.05$).

Characterization of participants with and without (sub)clinical SAD

To further characterize the sample, we investigated differences between participants with and without (sub)clinical SAD with respect to male / female ratio, generation, presence of (comorbid) psychopathology (chi-square tests; Bonferroni-corrected p-value for psychopathology: $p = 0.003$ (15 tests)), age and estimated IQ (regression models with genetic correlations as random effects). Furthermore, we examined the relationships between (sub) clinical SAD and self-reported levels of anxiety and anxiety-related constructs. When different questionnaires were used for adults and children/adolescents, z-scores were used (see *Supplemental Methods* for reference values). The following constructs were investigated: level of social anxiety (z-score), level of fear of negative evaluation, level of depressive symptoms (z-score), level of negative affect, level of trait anxiety and the level of inhibited temperament (z-score). Regression models were fitted in R, with (sub)clinical SAD as the

independent variable; the outcomes of the questionnaires were the dependent variables of interest. Age and gender were included as covariates, and the genetic correlations between family members were modeled by including random effects. A Bonferroni-corrected *p*-value of 0.008 was used (six tests).

Results

Recruitment and inclusion

Given the nature of SAD, recruitment of families meeting the inclusion criteria was a time-consuming process, taking place between Summer 2013 and Summer 2015. Nine families were included in the LFLSAD, including 133 family members (*Figure 3.2*). All probands were recruited by media exposure and contacts with patient associations, and none of the probands had been treated for SAD before entering the study. Due to insufficient proficiency of the Dutch language, data of one participant (partner of a proband's sibling) were excluded. Socio-demographic characteristics of the remaining sample (n = 132) are summarized in *Table 3.2*.

Table 3.2 Socio-demographic characteristics of the LFLSAD sample, per generation.

	Generation 1 (n = 62)	**Generation 2 (n = 70)**	**Statistical analysis**
Gender (n)			$\chi^2(1) = 1.05$, $p = 0.38$
Male / Female	29 / 33	39 / 31	
Age (in years, mean ± SD)	46.2 ± 6.6	17.9 ± 6.2	$\beta = -30.4$, $p < 0.001$
Range	31.0 - 61.5	8.2 - 32.2	
Native country (n)			$\chi^2(1) = 0.40$, $p = 0.84$
The Netherlands	57	65	
Other	5	5	
Education level (n)†			$\chi^2(1) = 3.28$, $p = 0.19$
Low	11	22	
Intermediate	25	26	
High	25	22	
Estimated IQ (mean ± SD)‡	104.0 ± 11.8	107.2 ± 10.6	$\beta = 2.5$, $p = 0.13$

Footnotes

†: Generation 1 (education completed): data from 61 participants; generation 2 (education completed or currently following): data from 70 participants.

‡: Generation 1: data from 58 participants; generation 2: data from 66 participants.

Education level was classified as follows: low: primary education (elementary school) and pre-vocational education; intermediate: higher secondary education (higher general continued education, pre-university secondary education) and post-secondary education (intermediate vocational education); high: tertiary education (higher professional education, university).

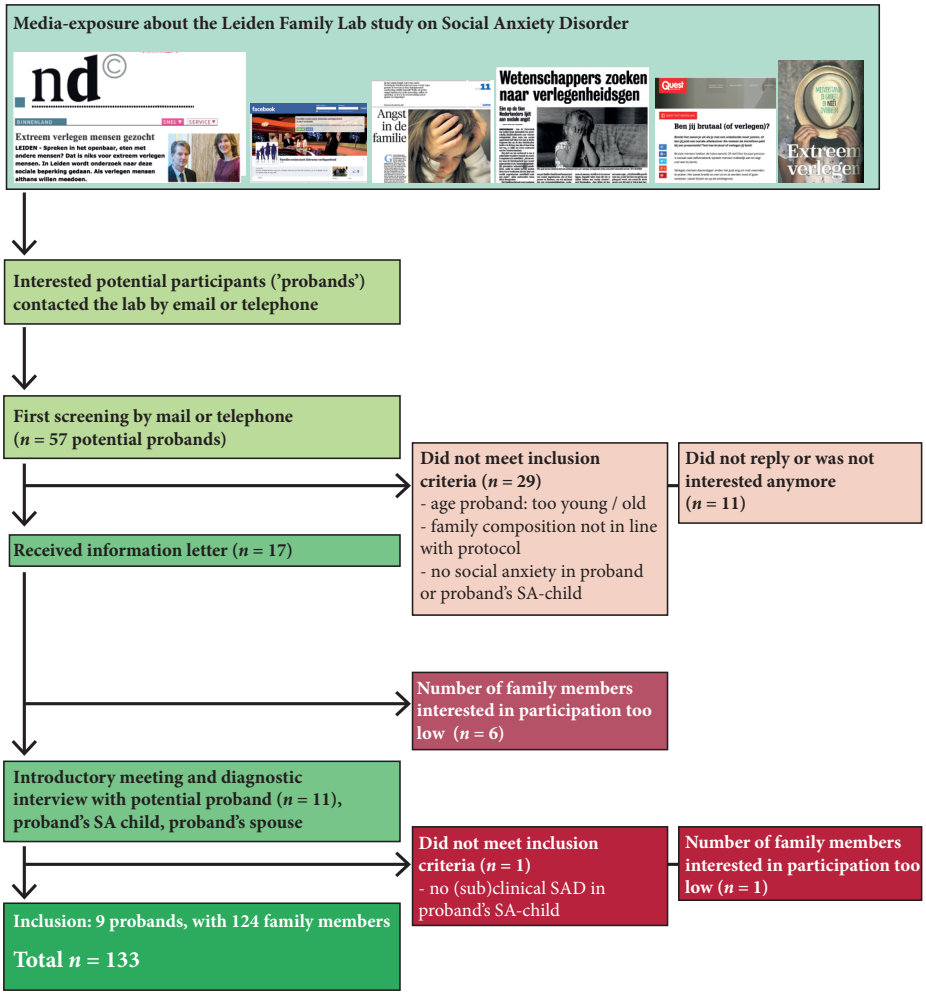

Figure 3.2 Flowchart of the Leiden Family Lab study on Social Anxiety Disorder.

On average, each family contained 14.7 participating family members (range: 4 - 35). The sample included 68 males and 64 females, who were equally divided over the generations. As expected based on the design, the generations differed significantly in age, but not in estimated IQ (*Table 3.2*). Availability of data is illustrated in *Figure 3.3*.

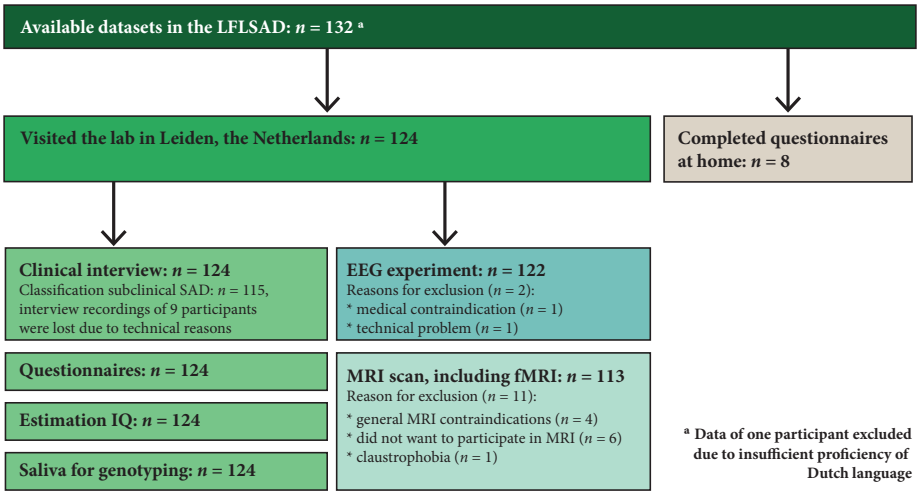

Figure 3.3 Overview of available data within the Leiden Family Lab study on Social Anxiety Disorder.

Characterization of the LFLSAD sample

An overview of clinical diagnoses within the sample is presented in *Table 3.3*, whereas scores on the dimensional self-assessments of anxiety and anxiety-related constructs are displayed in *Table 3.4.* Diagnostic interviews showed that social anxiety was highly prevalent within the sample, in both generations: in addition to the nine probands, who were selected based on a primary diagnosis of SAD, ten of their family members (generation 1: $n = 6$; generation 2: $n = 4$, of whom three proband's SA-children) met the criteria for clinical SAD. Furthermore, 25 family members (six of them proband's SA-children) were classified as having subclinical SAD. Total percentage of (sub)clinical SAD cases within the sample was 38.3 % (generation 1: 40.4 %; generation 2: 36.5 %). The validity of the diagnoses as established by the clinical interviews was confirmed by the self-report questionnaires: participants meeting the DSM-criteria for generalized SAD ($n = 19$) also met literature-based cutoff scores for generalized social anxiety (score ≥ 60 on LSAS (Mennin et al., 2002) or a score ≥ 50 on SAS-A (Storch, Masia-Warner, Dent, Roberti, & Fisher, 2004), with an average score (± SD) of 68.1 ± 24.2 on the LSAS ($n = 17$) and a score of 55.5 ± 0.7 ($n = 2$) on the SAS-A, whereas participants with subclinical SAD reported scores of 38.2 ± 23.7 (LSAS; $n = 12$) and 37.5 ± 9.7 (SAS-A; $n = 13$) respectively.

A heritability analysis using SOLAR indicated that (sub)clinical SAD had a moderately high heritability, which was significant at trend-level ($h^2 = 0.43$, $p = 0.09$). Age and gender did not significantly influence the model and were therefore removed (age: $p = 0.78$; gender: $p = 0.62$).

Comorbid diagnoses in the nine probands included depression (past, $n = 3$), panic disorder ($n = 2$), agoraphobia (current, $n = 2$), specific phobia ($n = 1$) and obsessive-compulsive

disorder (n = 1). Assessment of other psychopathology in their family members indicated that depression (past and current, n = 24), agoraphobia (past and current, n = 7) and panic disorder (n = 5) were most common diagnoses in the LFLSAD sample. Furthermore, several participants met criteria for alcohol dependence (current and lifetime, n = 6), dysthymia (current and past n = 5), specific phobia (n = 4), generalized anxiety disorder (n = 3), separation anxiety (n = 1), drug dependence (n = 1) and bulimia nervosa (n = 1) (*Table 3.3*).

Table 3.3 Clinical diagnoses of DSM-axis 1 diagnoses within the LFLSAD sample, per generation.

	Generation 1	**Generation 2**
SAD (number of cases; %)†	15; 25.9 %	4; 6.1 %
Subclinical SAD (number of cases; %)‡	6; 11.5 %	19; 30.2 %
(Sub)clinical SAD - cumulative (number of cases; %)‡	21; 40.4 %	23; 36.5 %
Other psychopathology§		
Depressive episode - current	1	1
Depressive episode - past	16	9
Dysthymia - current	1	2
Dysthymia - past	1	1
Panic disorder - lifetime	6	1
Agoraphobia - current	5	2
Agoraphobia - lifetime	1	1
Separation anxiety - present	n.a	1
Specific phobia - present	2	3
Generalized anxiety disorder - present	3	0
Obsessive-compulsive disorder - present	1	0
Alcohol dependence - present	1	1
Alcohol dependence - lifetime	1	3
Drug dependence - lifetime	1	0
Bulimia nervosa - present	1	0

Abbreviation

n.a: not assessed.

Footnotes

† Generation 1: data from 58 participants; generation 2: data from 66 participants (30 participants: M.I.N.I.-Plus; 36 participants: M.I.N.I.-Kid).

‡ Generation 1: data from 52 participants; generation 2: data from 63 participants.

§ Generation 1: data from 58 participants; generation 2: data from 60 participants (30 participants: M.I.N.I.-Plus; 30 participants M.I.N.I.-Kid).

Table 3.4 Self-report assessments of anxiety and associated constructs within the LFLSAD sample, per generation.

		Generation 1	Generation 2
LSAS-SR†	*Total*	31.4 ± 25.0 (2 – 95)	33.7 ± 23.3 (7 – 98)
	Fear	16.1 ± 13.0 (0 – 52)	17.0 ± 13.2 (0 – 58)
	Avoidance	15.3 ± 12.8 (0 – 50)	16.7 ± 11.1 (2 – 42)
SAS-A‡	*Total*		35.8 ± 9.2 (20 – 56)
	Fear of negative evaluation		14.9 ± 5.2 (8 – 26)
	Social avoidance and distress – new		13.9 ± 4.6 (6 – 26)
	Social avoidance and distress - general		6.9 ± 2.3 (4 -14)
BFNE-II-R¶	*Total*	16.3 ± 11.6 (0 – 48)	15.0 ± 10.5 (0 – 47)
STAI – trait§	*Total*	36.0 ± 10.4 (20 – 64)	35.0 ± 8.1 (21 – 57)
BDI†	*Total*	7.3 ± 8.1 (0 – 32)	7.6 ± 7.0 (1 – 30)
CDI‡	*Total*		6.6 ± 4.5 (0 – 23)
Positive affect§	*Total*	32.3 ± 7.3 (15 – 47)	32.7 ± 5.7 (21 – 45)
Negative affect§	*Total*	17.5 ± 6.9 (10 – 40)	16.9 ± 5.0 (10 – 31)
BIS-BAS$^{\Delta}$	*BIS – Total*	19.8 ± 4.5 (7 – 28)	18.5 ± 3.9 (9 – 28)
	BAS – Total	37.2 ± 5.0 (26 – 50)	39.1 ± 4.3 (31 – 48)
BIS-BAS C●	*BIS – Total*		7.2 ± 4.2 (1 – 17)
	BAS – Total		17.6 ± 5.2 (9 – 27)

Abbreviations
LSAS-SR: Liebowitz Social Anxiety Scale – self report (Fresco et al., 2001; Mennin et al., 2002); SAS-A: Social Anxiety Scale – adolescents (La Greca & Lopez, 1998); BFNE-II-R: revised Brief Fear of Negative Evaluation-II scale (Carleton et al., 2006; Leary, 1983); STAI: State-Trait Anxiety Inventory (Spielberger et al., 1970); BDI-II: Beck Depression Inventory-II (Beck et al., 1996; Van der Does, 2002); CDI: Children's Depression Inventory (Kovacs, 1983, 1985; Timbremont & Braet, 2002); BIS/BAS: Behavioral Inhibition and Behavioral Activation Scales (Carver & White, 1994); BIS/BAS-C: Behavioral Inhibition and Behavioral Activation Scales for children (Muris et al., 2005).

Footnotes
† Generation 1: data from 62 participants; generation 2: data from 33 participants.
‡ Generation 2: data from 37 participants.
¶ Generation 1: data from 60 participants; generation 2: data from 70 participants.
§ Generation 1: data from 62 participants; generation 2: data from 70 participants.
$^{\Delta}$ Generation 1: data from 62 participants; generation 2: data from 52 participants.
● Generation 2: data from 18 participants.

Values represent mean ± standard deviation (range).

Characterization of participants with and without (sub)clinical SAD

A characterization of the participants with and without (sub)clinical SAD is presented in *Table 3.5*. There were no differences between family members with and without (sub) clinical SAD with respect to the presence of other DSM-diagnoses (at Bonferonni-corrected

Table 3.5 Characteristics of participants with and without (sub)clinical SAD.

	(Sub)clinical SAD (*n* = 44)	No SAD (*n* = 71)	Statistical analysis
Demographics			
Male / Female (n)	22/ 22	35 / 36	$\chi^2(1) = 0.005$, $p = 1.00$
Generation 1 / Generation 2 (n)	21/ 23	31 / 40	$\chi^2(1) = 0.18$, $p = 0.70$
Age in years	30.0 ± 15.5	30.8 ± 15.8	$\beta = 0.82$, $p = 0.78$
Estimated IQ	104.6 ± 11.8	105.7 ± 10.8	$\beta = 1.39$, $p = 0.50$
Other psychopathology (*n*) †			
Depressive episode – current	1	1	$\chi^2(1) = 0.16$, $p = 1.00$
Depressive episode - past	12	11	$\chi^2(1) = 3.00$, $p = 0.09$
Dysthymia - current	3	0	$\chi^2(1) = 5.32$, $p = 0.047$*
Dysthymia - past	1	1	$\chi^2(1) = 0.17$, $p = 1.00$
Panic disorder – lifetime	5	2	$\chi^2(1) = 3.88$, $p = 0.10$
Agoraphobia – current	5	2	$\chi^2(1) = 3.88$, $p = 0.10$
Agoraphobia - lifetime	0	2	$\chi^2(1) = 1.18$, $p = 0.53$
Separation anxiety - present	0	1	$\chi^2(1) = 0.63$, $p = 1.00$
Specific phobia - present	2	3	$\chi^2(1) = 0.02$, $p = 1.00$
Generalized anxiety disorder - present	2	1	$\chi^2(1) = 1.19$, $p = 0.55$
Obsessive-compulsive disorder - present	1	0	$\chi^2(1) = 1.74$, $p = 0.37$
Alcohol dependence - present	1	1	$\chi^2(1) = 0.16$, $p = 1.00$
Alcohol dependence - lifetime	1	3	$\chi^2(1) = 0.25$, $p = 1.00$
Drug dependence - lifetime	1	0	$\chi^2(1) = 1.78$, $p = 0.36$
Bulimia nervosa - present	1	0	$\chi^2(1) = 1.74$, $p = 0.37$
Self-report measurements			
Social anxiety symptoms (z-score)	3.0 ± 3.3	0.2 ± 1.8	*See Table 3.6*
Fear of negative evaluation	23.4 ± 12.5	12.5 ± 8.0	*See Table 3.6*
Trait anxiety	39.1 ± 9.6	32.9 ± 8.5	*See Table 3.6*
Behavioral inhibition (z-score)	0.4 ± 1.2	-0.4 ± 1.0	*See Table 3.6*
Depressive symptoms (z-score)	0.0 ± 0.8	-0.5 ± 0.7	*See Table 3.6*
Negative affect	20.6 ± 6.9	15.3 ± 4.7	*See Table 3.6*

Footnotes

†: Generation 1: data from 52 participants; generation 2: data from 57 participants (28 participants: M.I.N.I.-Plus; 29 participants M.I.N.I.-Kid).

Values represent mean ± standard deviation, unless otherwise specified.

Statistical significance

* Significant at uncorrected *p*-value of 0.05.

p-value < 0.003). However, all self-reported measures of interest were significantly related to (sub)clinical SAD (*Table 3.6*). Age was not a significant predictor in the models; gender was, however, significantly related to the level of the level of behavioral inhibition (at Bonferroni-corrected *p*-value < 0.008), the level of fear of negative evaluation and the level of negative affect (at uncorrected *p*-value < 0.05), with higher levels in females compared to males.

Table 3.6 Associations with (sub)clinical SAD.

		Relation with (sub) clinical SAD		Relation with age		Relation with gender	
Constructs	***n***	**β (SE)**	***p***	**β (SE)**	***p***	**β (SE)**	***p***
Social anxiety (*z*-score)	115	2.76 (0.45)	$1.3 * 10^{-9}$ **	0.02 (0.01)	0.10	0.40 (0.44)	0.36
Fear of negative evaluation	113	10.83 (1.85)	$5.0 * 10^{-9}$ **	0.08 (0.06)	0.18	4.10 (1.80)	0.02 *
Trait anxiety	115	5.97 (1.67)	$3.5* 10^{-4}$ **	0.02 (0.05)	0.69	3.09 (1.63)	0.06
Behavioral inhibition (*z*-score)	115	0.82 (0.19)	$1.7 * 10^{-5}$ **	0.00 (0.01)	0.49	0.71 (0.19)	$1.2 * 10^{-4}$ **
Depressive symptoms (*z*-score)	115	0.53 (0.14)	$1.4* 10^{-4}$ **	0.00 (0.00)	0.37	0.17 (0.14)	0.2
Negative affect	115	5.32 (1.04)	$3.1 * 10^{-7}$ **	0.02 (0.03)	0.64	2.54 (1.02)	0.01 *

Statistical significance

* Significant at uncorrected *p*-value of 0.05.

** Significant at Bonferroni-corrected *p*-value of 0.008.

Discussion

Here, we describe the background, objective, design and methods of the Leiden Family Lab study on Social Anxiety Disorder (LFLSAD), and present data characterizing the sample. The study is unique in several aspects.

To start, the LFLSAD is the first multiplex, multigenerational family study on SAD, including 132 participants from nine families. The composition of the sample (families were selected based on at least two SAD cases within one nuclear family, multiplex, and multiple nuclear families involving two generations from the same family were included, multigenerational; see *Figure 3.1*) boosts statistical power to observe genetic and environmental effects on SAD-related traits (Williams & Blangero, 1999).

In addition, families were recruited from the general population (*Figure 3.2*) and none of the participants with SAD within the sample ($n = 19$) was treated for the disorder before entering the study. This is in line with several reports on social anxiety, indicating that SAD is frequently underdiagnosed because of the low help-seeking behavior of patients; furthermore, SAD is often not adequately recognized by clinicians (Alonso et al., 2018; Dingemans et al., 2001; Fehm et al., 2005; Ruscio et al., 2008). Thereby, the sample of the LFLSAD represents socially-anxious families from the community (Dingemans et al., 2001), including participants who are on a daily basis limited by their SAD symptoms (following

criterion G of the DSM-5 definition, stating that 'the fear, anxiety, or avoidance causes clinically significant distress or impairment in social, occupational, or other important areas of functioning') (American Psychiatric Association, 2013), but those SAD cases are not a selection of cases who have received treatment for SAD in the past.

Next, following our criteria which were aimed to include families who were enriched for genetic susceptibility to SAD, the disorder was highly prevalent within the sample: while the lifetime prevalence of SAD is estimated to be around 13 % in the general population (Kessler et al., 2012), the prevalence of (sub)clinical SA in the sample was 38.3 %, with a heritability of 0.43. In addition, the scores on the dimensional self-assessments of social anxiety were indicative of elevated levels of social anxiety. It's interesting to note that, although SAD is often comorbid with major depressive disorder (MDD) (Meier et al., 2015), the prevalence of depressive episodes within the sample was in the range of the general population: the lifetime prevalence of past and/or present depressive episodes within the LFLSAD was 22.9 % (27 cases in 118 participants), while population studies indicated that the lifetime prevalence of MDD within the community ranges between 17.1 % (Jacobi et al., 2004) and 28.2 % (Vandeleur et al., 2017). These results suggest that the sample is specifically enriched for SAD and not for depression.

Furthermore, as the majority of the participants (n = 124) visited the lab in Leiden and completed a variety of measurements including, among others, a structured clinical interview, self-report questionnaires, and collection of saliva for future genotyping (*Table 3.1; Figure 3.3*), the LFLSAD sample is an extensively characterized sample. This enables detailed (future) analyses on the relationship between the social anxiety phenotype on the one hand and neurocognitive candidate endophenotypes of SAD on the other.

Here, we presented data on the relationship between (sub)clinical SAD and anxiety-related constructs, showing that (sub)clinical SAD is positively related to increased levels of self-reported social anxiety, fear of negative evaluation and depressive symptoms, to higher trait anxiety, to the temperamental tendency to be behaviorally inhibited, and to higher levels of negative affect (*Table 3.6*). These findings are in line with previous reports indicating a relationship between (sub)clinical social anxiety and these self-reported traits (Bas-Hoogendam, van Steenbergen, Pannekoek, et al., 2017; Campbell et al., 2009; Carleton et al., 2007; Clauss & Blackford, 2012; Goldin, Manber, Hakimi, Canli, & Gross, 2009; Harrewijn et al., 2016; Rytwinski et al., 2009; Stein, Chartier, Lizak, & Jang, 2001) and underscore the validity of the LFLSAD sample.

Looking back at the power analyses performed before the start of the study, which showed that including twelve families of on average ten family members would result in sufficient statistical power, the actual LFLSAD sample contains less families (i.e. nine), but with, on average, more family members per family (14.7 family members). In comparison to the original sample composition, this actual sample contains comparable statistical power to investigate candidate endophenotypes of SAD. The first results on neurocognitive

endophenotypes emerging from the LFLSAD (Bas-Hoogendam, van Steenbergen, van der Wee, et al., 2017c, 2017b; Bas-Hoogendam, van Steenbergen, et al., 2015; Harrewijn, van der Molen, et al., 2017; Harrewijn et al., 2018) underscore the potential of such a study design.

Some limitations of the LFLSAD design should be mentioned. First of all, the LFLSAD has a relatively small sample size, which is due to the novelty and complexity of performing a family study in this population. Furthermore, given the cross-sectional nature of the study, the LFLSAD data do not allow for testing the *state-independency of the candidate neurocognitive endophenotypes* (endophenotype criterion 2). In addition, as no control families were included, comparing the *levels of the candidate endophenotypes between non-affected family members and participants from the general population* (second part of endophenotype criterion 4) is not possible. Finally, we did not acquire data with respect to potential environmental influences like traumatic life events and aversive social experiences, which could play an important role in the etiology and maintenance of SAD (Brook & Schmidt, 2008; Norton & Abbott, 2017; Wong & Rapee, 2016).

Conclusion

To conclude, the LFLSAD provides a unique opportunity to examine candidate neurocognitive endophenotypes of this serious disorder. It is our hope that the results of this study will provide clues for future directed gene-linkage studies, to gain more insight in the genetic vulnerability to SAD.

Supplemental Methods

Pre-registration LFLSAD

Following a pilot phase of the study and upon approval of the Medical Ethical Committee of the LUMC, the basic concepts and hypotheses of the LFLSAD were pre-registered on the Open Science Framework (osf.io) website (https://osf.io/erums/register/564d31db8c5e4a7c9694b2c0).

The components of this pre-registration are publicly available and are listed below.

- Wiki of the project 'Profiling Endophenotypes in Social Anxiety Disorder: a neurocognitive approach': osf.io/q4wx2/
- Hypothesized Endophenotype: Amygdala (MRI): osf.io/erums
- Hypothesized Endophenotype: Prefrontal Cortex (MRI): osf.io/y5m8q
- Hypothesized Endophenotype: Structure and Connectivity (MRI): osf.io/5dgki
- Hypothesized Endophenotype: Resting-state (EEG): osf.io/gqnit
- Hypothesized Endophenotype: Social Evaluation (EEG): osf.io/gncf6/
- Hypothesized Endophenotype: Social Performance (EEG): osf.io/ru958

Power analyses

Power was computed by simulation, based on an endophenotype with a heritability of 60 % and a correlation of 70 % with SAD; prevalence of SAD was set at 10 %. Families were generated using linear mixed models and correlations between family members were modeled via normally distributed random effects with a correlation structure of two times the kinship matrix. Only families with at least two affected members in one nuclear family were used for estimation of the power.

Detailed procedure structural and functional MRI measurements

Participants were screened for MRI eligibility and invited for participation in the MRI experiment when they had no contraindications (like, for example, metal implants or pregnancy) for undergoing an MRI scan. Preceding the experimental session, children and adolescents were familiarized with the MRI procedure using a mock scanner (Galván, 2010). All participants received explanation of the MRI paradigms and practiced short versions of the MRI tasks on a laptop before the experiment. They were informed about the safety procedures and they were told that they could refrain from continuing the experiment at any time. State anxiety was assessed before and after the MRI scan by a Dutch-translation of the STAI (Spielberger et al., 1970). Scanning was performed using a 3.0 T Philips Achieva MRI scanner (Philips Medical Systems, Best, The Netherlands), equipped with a 32-channel Sensitivity Encoding (SENSE) head coil.

The MRI session consisted of a high-resolution T1 scan, two diffusion tensor imaging (DTI) scans (anterior-to-posterior and posterior-to-anterior direction) and a magnetization transfer ratio (MTR)-scan. In addition, a high-resolution EPI scan and a B0 field map were acquired for registration purposes. Furthermore, fMRI data were collected during resting-state (eyes closed condition) and during two functional paradigms: an amygdala paradigm investigating amygdala habituation, (based on the work of Blackford, Allen, Cowan, & Avery, 2013; Blackford, Avery, Cowan, Shelton, & Zald, 2011; Schwartz, Wright, Shin, Kagan, Whalen, et al., 2003; Schwartz, Wright, Shin, Kagan, & Rauch, 2003; Wedig, Rauch, Albert, & Wright, 2005) and conditioning (Davis et al., 2010) and the revised social norm processing task (SNPT-R) (Bas-Hoogendam, van Steenbergen, Kreuk, van der Wee, & Westenberg, 2017).

Total duration of the MRI protocol was 55 minutes. After the MRI scan, participants completed the second phase of the SNPT-R on a laptop, and they were debriefed about the amygdala paradigm. Furthermore, they were instructed not to share the details of the MRI session with their family members. Total duration of the MRI session was 2.5 hours.

Detailed procedure EEG measurements

Two weeks before the EEG session, participants were asked to send in a portrait photograph of themselves for a task about first impressions. Participants were informed that a panel of peers would evaluate their photograph. This was a cover story to elicit feelings of social evaluation (Harrewijn et al., 2018; Van der Molen et al., 2014). Few days before the EEG session, participants were reminded of the EEG session and were asked to come in with clean hair. When participants arrived in the lab, we explained the EEG procedure and attached the electrodes. EEG was recorded using the BioSemi Active Two system (Biosemi, Amsterdam, The Netherlands) with 64 Ag-AgCl electrodes mounted in an electrode cap (10/20 placement) and 8 external electrodes (to measure horizontal/vertical eye movements, heart rate and for offline re-referencing).

The EEG session consisted of several elements. After a resting-state measurement (eyes closed), participants performed a social judgment paradigm (Harrewijn et al., 2018; Van der Molen et al., 2014) followed by another resting-state measurement (eyes closed). Subsequently, participants were informed about the second EEG task, as they did not know beforehand about this task. EEG data were acquired during a social performance task (Harrewijn, van der Molen, et al., 2017; Harrewijn et al., 2016), while participants watched a neutral nature movie for 20 minutes (to allow for cortisol measures), and during resting-state (eyes closed). Then, the electrodes were detached and participants filled out a questionnaire about their health. Finally, we debriefed all participants and asked them not to tell their family members about the EEG tasks. Total duration of the EEG session was 2.5 hours.

Calculation *z*-scores

We characterized the LFLSAD sample by comparing the level of social anxiety symptoms (assessed by the LSAS-SR or the SAS-A), the level of fear of negative evaluation (assessed by the BFNE-II-R), the level of behavioral inhibition (BIS; assessed by the BIS/BAS and BIS/BAS-C) and the level of depressive symptoms (assessed with the BDI or CDI) with those of community samples, by computing z-scores. We used the following reference values (mean ± SD):

— LSAS-SR: 13.5 ± 12.7 (Fresco et al., 2001);
— SAS-A: 34.7 ± 2.3 (Miers, Blöte, Bögels, & Westenberg, 2008);
— behavioral inhibition BIS/BAS: 20.0 ± 3.8 (Carver & White, 1994);
— behavioral inhibition BIS/BAS-C: 6.9 ± 3.9 (Muris et al., 2005);
— BDI-II: 10.6 ± 10.9 (Roelofs et al., 2013);
— CDI: 8.9 ± 5.4, unpublished data from the study by (Miers et al., 2008).

Part 2

Structural brain characteristics as putative SAD endophenotypes

Chapter 4

Voxel-based morphometry multi-center mega-analysis of brain structure in Social Anxiety Disorder

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Abstract

Social anxiety disorder (SAD) is a prevalent and disabling mental disorder, associated with significant psychiatric co-morbidity. Previous research on structural brain alterations associated with SAD has yielded inconsistent results concerning the direction of the changes in gray matter (GM) in various brain regions, as well as on the relationship between brain structure and SAD-symptomatology. These heterogeneous findings are possibly due to limited sample sizes. Multi-site imaging offers new opportunities to investigate SAD-related alterations in brain structure in larger samples.

An international multi-center mega-analysis on the largest database of SAD structural T1-weighted 3T MRI scans to date was performed to compare GM volume of SAD patients (n = 174) and healthy control (HC) participants (n = 213) using voxel-based morphometry. A hypothesis-driven region of interest (ROI) approach was used, focusing on the basal ganglia, the amygdala-hippocampal complex, the prefrontal cortex, and the parietal cortex. SAD patients had larger GM volume in the dorsal striatum when compared to HC participants. This increase correlated positively with the severity of self-reported social anxiety symptoms. No SAD-related differences in GM volume were present in the other ROIs.

Thereby, the results of this mega-analysis suggest a role for the dorsal striatum in SAD, but previously reported SAD-related changes in GM in the amygdala, hippocampus, precuneus, prefrontal cortex and parietal regions were not replicated. Our findings emphasize the importance of large sample imaging studies and the need for meta-analyses like those performed by the Enhancing NeuroImaging Genetics through Meta-Analysis (ENIGMA) Consortium.

Introduction

Social anxiety disorder (SAD) is one of the most common anxiety disorders (Stein & Stein, 2008), with an estimated lifetime prevalence between 6 and 13 % (Kessler et al., 2012; Stein et al., 2010). Patients with SAD are characterized by intense fear of, distress in, and avoidance of situations in which they may be scrutinized (American Psychiatric Association, 2013). The disorder is highly disabling, as impairments in social life and work situations are frequently reported (Mack et al., 2015). In addition, the disorder is associated with significant psychiatric co-morbidity, such as depressive disorders and substance abuse (Stein & Stein, 2008). These findings stress the need for improvements in the treatment of SAD. Understanding the neurobiological mechanisms that underlie this disorder has the potential to advance treatment.

Previous magnetic resonance imaging (MRI) studies on brain anatomy differences in SAD have reported heterogeneous findings, implicating regions such as the frontal cortex, the parietal cortex, occipital cortex, temporal regions and subcortical limbic areas, as reviewed by Brühl, Delsignore, et al. (2014); see also Goodkind et al. (2015) reporting on a transdiagnostic meta-analysis of structural neuroimaging studies. Several of these changes were correlated with clinical characteristics, such as the severity of social anxiety symptoms (Brühl, Hänggi, et al., 2014; Frick, Engman, et al., 2014; Irle et al., 2010, 2014; Liao et al., 2011; Syal et al., 2012; Talati, Pantazatos, et al., 2013) or disease duration (Meng et al., 2013). In addition, recent treatment studies in SAD patients have identified structural changes in bilateral caudate and putamen, right thalamus and cerebellum after eight weeks of paroxetine treatment (Talati, Pantazatos, Hirsch, & Schneier, 2015), and alterations in parieto-occipital and prefrontal GM volumes after cognitive behavioral group therapy (Steiger et al., 2017), while a classification study using multi-voxel pattern analysis was able to discriminate SAD patients from healthy control participants based on the pattern of regional gray matter (GM) volume over the whole brain (Frick, Gingnell, et al., 2014). Together, these studies provide evidence for the idea that certain brain regions are clinically associated with SAD.

Functional MRI (fMRI) studies have also identified important candidate brain regions that may be related to structural changes associated with SAD-related psychopathology. These fMRI studies, typically examining brain activity in response to emotional stimuli or in response to cognitive tasks (Brühl, Delsignore, et al., 2014), most consistently point towards an increase of brain activation in SAD in the bilateral amygdala and hippocampus, prefrontal brain regions, bilateral insula, bilateral parietal cortex and bilateral precuneus, while findings on the direction of changes in the basal ganglia are mixed (Brühl, Delsignore, et al., 2014; Cremers & Roelofs, 2016). In addition, studies on functional connectivity, during rest as well as during cognitive tasks (Brühl, Delsignore, et al., 2014), revealed changes in connectivity of, among others, the putamen (Cremers, Veer, Spinhoven, Rombouts, & Roelofs, 2015) and the amygdala (Hahn et al., 2011; Pannekoek et al., 2013; Sladky et al.,

2015), while recent positron emission tomography (PET) studies showed decreased serotonin receptor binding (Lanzenberger et al., 2007) and increased serotonin synthesis and transporter availability in the hippocampus, amygdala, anterior cingulate cortex (ACC) and striatal regions like the putamen and globus pallidus (Frick et al., 2015; Furmark et al., 2016). These results, together with the findings of a treatment study revealing a relationship between changes in amygdala structure and amygdala function in SAD (Månsson et al., 2016), suggest that the brain regions showing functional changes in SAD overlap to a large extent with the regions that have showed differences in brain structure.

However, the available evidence with respect to structural brain alterations in SAD is inconclusive, as both increases as well as decreases in GM volumes in various brain regions have been reported (Brühl, Delsignore, et al., 2014). Furthermore, findings concerning the relationship between brain structure and SAD-symptoms are inconsistent (Brühl, Hänggi, et al., 2014; Frick, Engman, et al., 2014; Irle et al., 2014; Tükel et al., 2015). These heterogeneous results are possibly due to differences in the employed methods, as well as the relatively small sample sizes employed in studies on SAD-related changes in brain structure (ranging from 12 to 67 SAD patients), and variability in clinical parameters between the samples. Recent advances in multi-site imaging offer new opportunities to investigate the structural brain alterations associated with SAD.

In this international multi-center mega-analysis, which is part of the European and South African Research Network in Anxiety Disorders (EURSANAD) program initiated by the Anxiety Disorders Research Network (Baldwin & Stein, 2012), we investigated GM volume in a priori defined regions of interest (ROIs) in a sample of 174 SAD patients and 213 healthy control participants, using an optimized voxel-based morphometry (VBM) protocol (Ashburner & Friston, 2000; Lerch et al., 2017). VBM analyses have the advantage of using unbiased, standardized methods to investigate brain structure, and have been extensively used to investigate alterations in brain morphology across numerous major psychiatric conditions (Ashburner & Friston, 2000; Goodkind et al., 2015). The large sample of the present work provides the best statistical power to date to investigate GM alterations associated with SAD. Data were collected in multiple scan centers located in five countries (Germany, South Africa, Sweden, the Netherlands and the United States of America; *Figure 4.1*). Based on the available evidence reviewed above, our analysis focused on changes in GM volume in four a priori defined ROIs that seem to be most prominently involved in SAD: the basal ganglia, the amygdala-hippocampal complex, the prefrontal cortex and the parietal cortex including the precuneus. Given the mixed findings on SAD-related increases versus decreases in GM in the previous structural MRI studies (Brühl, Delsignore, et al., 2014), we did not make specific predictions about the direction of the changes within these ROIs. Significant results within the ROIs were followed up by regression analyses to investigate the relationship between GM volumes and the severity of social anxiety symptoms within the patient group.

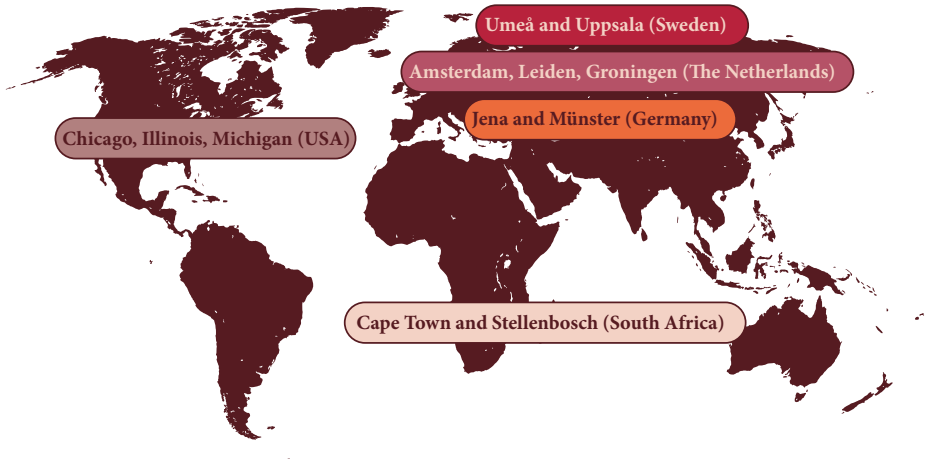

Figure 4.1 Research sites represented in mega-analysis.

Material and methods

Participants

Structural T1-weighted 3T MRI scans were collected at research centers located in Europe, Africa and North-America, and brought together for quality control and initial analysis in Cape Town, South-Africa. Final analyses took place in Leiden, the Netherlands. The initial sample consisted of 251 SAD patients and 230 healthy control (HC) participants (*Table 4.1*), and results on these datasets have been published previously (Boehme, Ritter, et al., 2014, 2015; Boehme, Mohr, Becker, Miltner, & Straube, 2014; Cremers et al., 2014; Geiger et al., 2016; Howells et al., 2015; Klumpp et al., 2016; Månsson et al., 2013, 2015; Pannekoek et al., 2013; Phan et al., 2013; Syal et al., 2012; van Tol et al., 2010) – see *Supplemental Methods* for more details on the in- and exclusion criteria and recruitment of participants for each sample. At each site, the local ethical committee approved data collection and all participants provided written informed consent after the procedure had been fully explained.

Participants (18 years or older) were recruited through public announcements (online and within the community), consumer advocacy groups, general practitioners and clinical centers, and screened using structured clinical interviews in their native language: the Mini-International Neuropsychiatric Interview (Sheehan et al., 1997), the Composite Interview Diagnostic Instrument version (Kessler & Ustün, 2004) or the Structured Clinical Interview for DSM-IV disorders (First, Spitzer, Gibbon, Williams, & Benjamin, 1998). SAD patients had to meet criteria for a primary diagnosis of SAD, while HC participants had to be free of any psychopathology. General MRI contraindications (ferromagnetic implants, claustrophobia, pregnancy) were a reason for exclusion in both groups.

Table 4.1 Sample composition and number of scans.

		Initial # scans		Excluded # scans				Included # scans		
Country	**Research center**	**SAD**	**HC**	**Comorbidity[a]**	**Insufficient quality[b]**	**Brain pathology**	**Other reason[c]**	**SAD**	**HC**	**Total**
Germany	University of Jena; University of Münster	53	22	31	3	0	0	19	22	41
The Netherlands	VU Medical Center Amsterdam, NESDA Study	10	27	1	3	0	0	6	27	33
	University of Groningen, NESDA Study	9	12	1	1	0	0	8	11	19
	Leiden University Medical Center, NESDA Study	9	26	1	0	0	0	8	26	34
	Leiden University Medical Center, Social Anxiety Study	20	20	0	0	0	0	20	20	40
South-Africa	University of Cape Town; Stellenbosch University	18	17	2	10	0	0	12	11	23
Sweden	Umeå University	26	26	0	3	0	0	26	23	49
	Uppsala University	24	0	0	0	0	24	0	0	0
United States of America	University of Chicago	27	25	3	4	0	0	24	21	45
	University of Illinois	12	12	1	0	0	0	12	11	23
	University of Michigan	43	43	2	3	1	0	39	41	80
Total		251	230	42	27	1	24	174	213	387

Abbreviations and symbols

SAD: social anxiety disorder patients; HC: healthy control participants. #: number of scans.

Footnotes

[a] Other than depression or anxiety (SAD patients only).

[b] Insufficient scan-quality: scans with motion artefacts, scans being unsegmentable or scans for which brain extraction failed after multiple attempts.

[c] No data from HC participants to balance design.

In addition to the T1-weighted 3T MRI scans, demographic (age, gender, handedness) and clinical data were collected at each research center. Furthermore, information about education level, comorbidity, medication use and the scores on several questionnaires (Liebowitz Social Anxiety Scale (LSAS) (Heimberg et al., 1999), Beck Depression Inventory (BDI) (Beck, Steer, & Carbin, 1988) and State-Trait Anxiety Inventory (STAI) (Spielberger et al., 1970)) were available for a subset of participants.

Data acquisition, quality checks and final sample

Parameters of the T1-weighted MRI scans are presented in *Table 4.2*. Scans from SAD patients with comorbid psychopathology other than any other anxiety disorder or major depressive disorder (MDD) were excluded from the analysis (n = 42, see *Supplemental Table S4.1*). Next, scans were extensively checked for pathology and quality, leading to the exclusion of an additional 28 scans (*Table 4.1*). Furthermore, all scans from the research center in Uppsala (n = 24 SAD patients) were excluded due to the lack of scans from HC participants from this center, necessary for our analytic approach. This resulted in a final sample of 174 SAD patients and 213 HC participants. Characteristics of the final sample are presented in *Table 4.3*. Statistical analyses on differences between groups were performed using IBM SPSS Statistics (Version 23), with a significance level of $p < 0.05$.

Voxel-based morphometry analysis

Voxel-wise GM volumes were investigated using an optimized voxel-based morphometry (VBM) protocol, using the default pipeline as implemented in FSL (version 5.0.7) (Good et al., 2001; Smith et al., 2004). Structural T1-weighted images were first brain-extracted using FSL and FreeSurfer software. Each brain was closely visually inspected and brain-extraction was repeated until all non-brain tissue was properly removed from the image. Subsequently, images were segmented into GM, white matter (WM) and cerebrospinal fluid (CSF) (Zhang, Brady, & Smith, 2001). Next, a study-specific GM template was created, in order to avoid biases during registration that could favour either the SAD or HC-group (Good et al., 2001), by randomly selecting GM images from an equal number of SAD patients and HC participants from each research center (n = 166 SAD patients and n = 166 HC participants). These GM images were non-linearly registered to the Montreal Neurological Institute (MNI) T1-template brain, averaged and flipped along the x-axis to create a left-right symmetric study-specific GM template with a resolution of 2 x 2 x 2 mm. Subsequently, the original GM images from all participants were non-linearly registered to this template (Andersson, Jenkinson, & Smith, 2007), modulated to correct for local expansion or contraction and smoothed using a kernel with an isotropic Gaussian kernel (σ = 3 mm).

Table 4.2 Characteristics of T1-weighted MRI scans.

Country	Research Site / Sample	Scanner	Voxels	Dimensions	Reference
Germany	University of Jena; University of Münster	Siemens/ TrioTim 3T	192 x 256 x 256	1 x 1 x 1 mm	(Boehme, Ritter, et al., 2014, 2015; Boehme, Mohr, et al., 2014)
The Netherlands	VU Medical Center Amsterdam, NESDA study	Philips 3 T	170 x 256 x 256	1 x 1 x 1 mm	(Pannekoek et al., 2013, 2015; Penninx et al., 2008; van Tol et al., 2010)
	University of Groningen - NESDA study	Philips 3 T	170 x 256 x 256	1 x 1 x 1 mm	
	Leiden University Medical Center - NESDA study	Philips 3 T	170 x 256 x 256	1 x 1 x 1 mm	
	Leiden University Medical Center - Social Anxiety Study	Philips 3 T	256 x 256 x 140	0.875 x 0.875 x 1.2 mm	(Cremers et al., 2014)
South-Africa	University of Cape Town; Stellenbosch University	Siemens Magnetom Allegra 3T	128 x 256 x 256	1.33 x 1 x 1 mm	(Geiger et al., 2016; Howells et al., 2015; Syal et al., 2012)
Sweden	Umeå University	General Electric 3T	512 x 512 x 176	0.48 x 0.48 x 1 mm	(Månsson et al., 2013, 2015)
	Uppsala University	Philips Achieva 3T	480 x 480 x 170	0.5 x 0.5 x 1 mm	
United States of America	University of Chicago	GE Signa System 3T	256 x 256 x 120	0.94 x 0.94 x 1.5 mm	(Klumpp et al., 2016; Phan et al., 2013)
	University of Illinois	GE Signa System 3T	256 x 256 x 182	0.86 x 0.86 x 1 mm	
	University of Michigan	GE Signa System 3T	256 x 256 x 124	1 x 1 x 1.2 mm	

Region of interest (ROI) analysis: differences between groups

In order to maximize the statistical power to detect GM differences between SAD patients and HC participants, we used a region of interest (ROI) approach (Poldrack, 2007), focusing on brain areas in which functional and structural brain changes related to SAD have been reported previously (see *Introduction*). Four ROIs were created in standard space (resolution 2 x 2 x 2 mm) using the Harvard-Oxford Cortical Structural Atlas and Harvard-Oxford Subcortical Structural Atlas implemented in FSLView (version 3.2.0). The *basal ganglia* ROI consisted of voxels with a probability of at least 50 % of belonging to the bilateral accumbens, caudate, pallidum or putamen (total size of ROI: 3224 voxels, 25792 mm^3). The second ROI, the *amygdala-hippocampus* ROI, consisted of voxels with a probability of at least 50 % of belonging to the bilateral amygdala, hippocampus and the anterior and posterior para-

hippocampal gyrus (total size of ROI: 3066 voxels, 24528 mm^3). The *prefrontal cortex* ROI included voxels with a probability of at least 50 % of belonging to the middle frontal gyrus, the subcallosal cortex, the anterior cingulate gyrus, paracingulate gyrus, frontal medial cortex and frontal orbital cortex (total size of ROI: 20601 voxels, 164808 mm^3). Finally, the *parietal* ROI encompassed voxels with a probability of at least 50 % of belonging to the superior parietal lobule, the precuneus cortex and the posterior cingulate gyrus (total size of ROI: 5478 voxels, 43824 mm^3).

Within these ROIs, we examined differences in GM volume between SAD patients and HC participants using a general linear model (GLM). In this model, scan center (coded by dummy variables) and gender were added as nuisance regressors, and age and total GM volume were included as covariates. Before we analyzed this GLM, we tested the homogeneity of regression slopes assumption that applies to covariate analysis, by building a separate GLM that included a diagnosis-by-age and a diagnosis-by-total GM regressor in addition to the other regressors. No significant interactions at the whole-brain level were observed, thus justifying the use of the abovementioned GLM that investigated the effect of diagnosis while correcting for the covariates.

Voxelwise statistics were applied using permutation-based non-parametric testing (5000 permutations), correcting for multiple comparisons across space. FSL's default threshold-free cluster enhancement (TFCE) was used to detect significant clusters (Smith & Nichols, 2009) and we used a familywise error (FWE)-corrected threshold of $p < 0.05$ within each ROI. Given the fact that ROIs were a priori defined and are part of a network of brain areas involved in SAD (Brühl, Delsignore, et al., 2014), we report *p*-values uncorrected for the number of ROIs. Significant results within the ROIs were followed up by a multiple regression analysis using IBM SPSS Statistics, in order to examine the relationship between average individual GM volume in the extracted cluster and the severity of total social anxiety symptoms (measured with the LSAS), while controlling for scan center, gender, age and total GM volume. In line with previous work (Frick, Engman, et al., 2014; Irle et al., 2014; Meng et al., 2013; Syal et al., 2012), this analysis was performed in SAD patients only.

For reasons of completeness, we also performed an exploratory whole-brain VBM analysis to examine a main effect of diagnosis and interactions with age and scan center outside the predefined ROIs using the same GLM. Again, we used TFCE-results based on an FWE-corrected threshold of $p < 0.05$.

Results

Sample characteristics

Characteristics of SAD patients (n = 174) and HC participants (n = 213) are presented in *Table 4.3*. SAD patients did not differ from HC participants in terms of age, gender distribu-

Table 4.3 Demographic and clinical characteristics of social anxiety disorder (SAD) patients and healthy control (HC) participants.

	SAD (*n* = 174)	HC (*n* = 213)	Statistical analysis
	Mean ± SD	**Mean ± SD**	***p***
Age (years)	30.6 ± 10.0	32.4 ± 10.5	0.13 [g]
Age of onset (years)[a]	14.8 ± 7.1		
	***n* (%)**	***n* (%)**	***p***
Males	72 (41.4)	97 (45.5)	0.41 [h]
Education level[b]			0.10 [h]
Low	1 (0.7)	6 (3.2)	
Intermediate	56 (36.8)	54 (29.0)	
High	95 (62.5)	126 (67.7)	
Right-handed	172 (98.9)	206 (96.7)	0.17 [h]
Comorbidity			
SAD only	114 (65.5)		
SAD + MDD	8 (4.6)		
SAD + MDD + PD	2 (1.1)		
SAD + GAD	10 (5.7)		
SAD + GAD + SP	3 (1.7)		
SAD + GAD + PD	2 (1.1)		
SAD + PD	3 (1.7)		
SAD + SP	6 (3.4)		
Unknown	26 (14.9)		
Medication use at time of scan[c]	24 (14.2)		
SSRI	17		
Betablocker	2		
Antidepressivum NOS	4		
Unknown medication	1		
	Mean ± SD	**Mean ± SD**	***p***
LSAS[d]	77.9 ± 17.9	14.3 ± 12.6	<0.001 [g]
BDI[e]	13.8 ± 8.8	2.3 ± 3.2	<0.001 [g]
STAI– State [f]	43.2 ± 10.1	20.9 ± 11.0	<0.001 [i]
STAI -Trait [f]	50.1 ±10.2	22.6 ± 11.5	<0.001 [i]
Total GMV (mL)	519.3 ± 49.9	522.3 ± 58.7	0.47 [g]

Abbreviations

BDI: Beck Depression Inventory; GAD: generalized anxiety disorder; GMV: Gray Matter Volume; LSAS: Liebowitz Social Anxiety Scale; MDD: Major Depressive Disorder; NOS: not otherwise specified; PD: panic disorder; SD: standard deviation; SP: specific phobia; SSRI: selective serotonin reuptake inhibitor; STAI: State-Trait Anxiety Inventory.

Footnotes

[a] Data from 65 SAD patients.
[b] Data from 152 SAD patients and 186 HC participants.
[c] Data from 169 SAD patients.
[d] Data from 148 SAD patients and 140 HC participants.
[e] Data from 113 SAD patients and 111 HC participants.
[f] Data from 75 SAD patients and 73 HC participants.
[g] Independent Samples Mann-Whitney U test.
[h] χ^2 test.
[i] Independent Samples T-Test.

tion, level of education, handedness and total GM volume, but they reported significantly more social anxiety symptoms (measured with the LSAS) and anxiety symptoms (measured with the STAI) in comparison to HC participants. In addition, SAD patients reported significantly more depressive symptoms than HC participants, as measured with the BDI. It should, however, be noted that the degree of reported depression symptoms in the SAD patients indicates only minimal depression (mean ± standard deviation: 13.8 ± 8.8) (Beck et al., 1988), whereas the mean scores on the LSAS for the SAD patients (mean ± standard deviation: 77.9 ± 17.9) are in line with a clinical diagnosis of SAD (Mennin et al., 2002).

ROI analyses: differences between SAD patients and HC participants

There was an effect of diagnosis in the basal ganglia ROI: SAD patients had larger GM volume in the right putamen, extending into the pallidum (*Figure 4.2A-B*; extent = 78 voxels, peak coordinate in MNI space (x, y, z): 26, -8, 0; $p = 0.022$, small-volume corrected; result did not survive correction when all ROIs were taken together), with a small effect size ($\beta = 0.14$, Cohen's d = 0.20). A subsequent analysis, that regressed social anxiety symptoms within the SAD patients on individual extracted GM volume in this region, revealed a significant positive correlation with a small effect size (zero-order correlation: Spearman's rho = 0.21, $p = 0.010$; multiple regression analysis while controlling for scan center, gender, age and total GM volume: $\beta = 0.13$, $p = 0.048$; see also *Figure 4.2C*).

Given the fact that SAD often co-occurs with major depressive disorder (MDD) (Stein & Stein, 2008), we investigated whether the GM difference in the putamen was influenced by comorbid depression, by performing three subsequent analyses. Firstly, we excluded SAD patients with a diagnosis of comorbid MDD (excluded: $n = 10$ SAD patients; *Table 4.3*) and performed a multiple regression analysis with individual GM volume of the right putamen cluster as dependent variable, and diagnosis as independent variable while controlling for scan center, age, gender and total GM volume (remaining sample: $n = 164$ SAD patients and 213 HC participants). This analysis still showed a significant effect of diagnosis ($\beta = 0.14$, $p = 0.002$). Secondly, we excluded participants with a BDI score ≥ 30, indicating severe depression (Beck et al., 1988), (excluded: $n = 7$ SAD patients; remaining sample: $n = 106$ SAD patients and 111 HC participants). Again, the effect of diagnosis was significant ($\beta = 0.14$, $p = 0.017$). In the third analysis, we examined the relationship between BDI score and GM volume in the SAD group ($n = 113$ SAD patients; regression analysis, controlling for scan center, age, gender, and total GM volume). This analysis revealed a significant effect of BDIscore on GM volume ($\beta = 0.17$, $p = 0.034$). Importantly, when LSAS score and BDI score were both entered in the regression model, the effect of BDI was not significant anymore ($\beta = 0.13$, $p = 0.13$), while LSAS score was still a significant predictor of GM volume ($\beta = 0.16$, $p = 0.049$). These results indicate that variation in BDI scores in the SAD sample did not significantly account for GM variance in the putamen-pallidum over and above effects of LSAS.

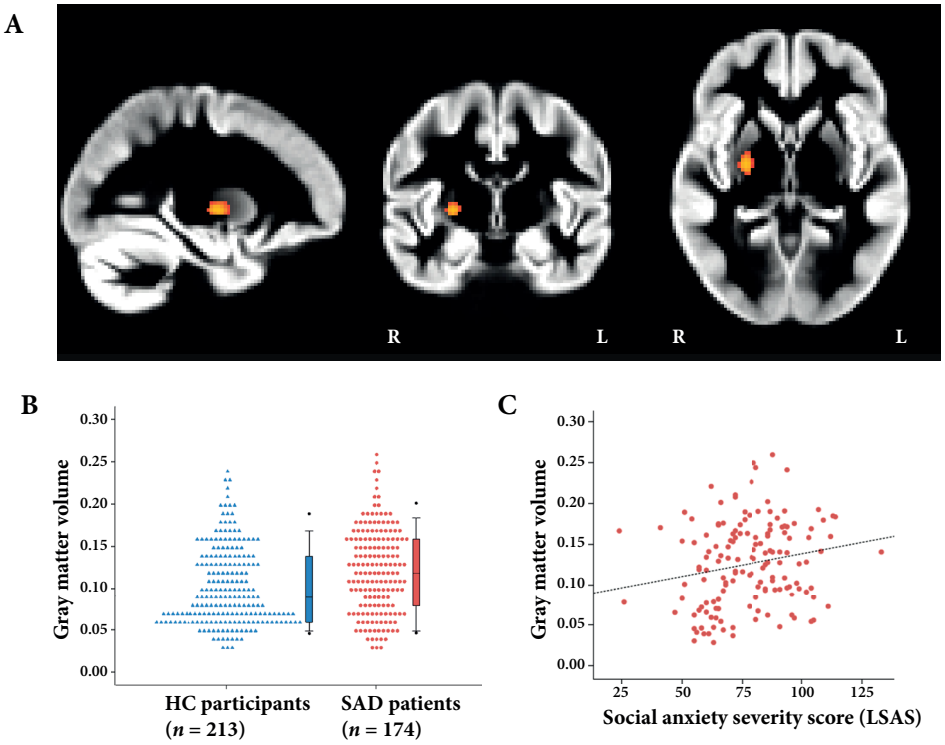

Figure 4.2 Larger GM volume in social anxiety disorder (SAD) patients relative to healthy control (HC) participants.
Figure 4.2A Larger gray matter (GM) volume in SAD patients relative to HC participants in the right dorsal striatum ($p < 0.05$, small-volume corrected).
Figure 4.2B Dot density plot illustrating the group difference in GM volume in the dorsal striatum.
Figure 4.2C Scatterplot illustrating the relationship between social anxiety symptoms in a subset of SAD patients ($n = 148$; measured with the Liebowitz Social Anxiety Scale, LSAS) and GM volume in the dorsal striatum (Spearman's rho = 0.21, $p < 0.05$).

However, when we performed two additional sensitivity analyses to investigate the effect of 1st general comorbidity and 2nd medication use on the GM difference in the putamen, using multiple regression analyses with individual GM volume of the right putamen cluster as dependent variable, and diagnosis as independent variable while controlling for scan center, age, gender and total GM volume, the effect of diagnosis lacked significance (sensitivity analysis 1, including only patients without comorbidity: remaining sample: $n = 114$ SAD patients and 213 HC participants; $\beta = 0.06$, $p = 0.28$; sensitivity analysis 2, including only patients without present medication use: remaining sample: $n = 59$ SAD patients and 117 HC participants; $\beta = 0.13$, $p = 0.13$).

There were no clusters in the basal ganglia ROI where HC participants had larger GM volume relative to SAD patients. In addition, we did not find significant group differences in

the other ROIs using the VBM approach. To explore these null findings, we extracted the individual GM volumes from the regions within each of the larger ROIs tested and examined the presence of between-group differences using multiple regression analyses controlled for scan center, age, gender and total GM volume. Because of the exploratory nature of these analyses, we corrected for the number of tests using Bonferroni-correction (13 regions, $p \leq 0.004$). There were no regions in which the effect of diagnosis was significant at this Bonferroni-corrected significance level (*Supplemental Table S4.2*), although two effects were significant at the uncorrected level. Furthermore, we explored the possibility that these null findings were present due to gender differences between patients, by investigating gender-by-diagnosis interactions. Again, no significant interactions were found at the Bonferroni-corrected significance level ($p \leq 0.004$) (*Supplemental Table S4.2*).

Whole-brain analysis: no group differences

The exploratory whole-brain VBM analysis did not reveal a significant main effect of diagnosis. Significant diagnosis-by-age or diagnosis-by-scan center interactions were also not observed at whole-brain level.

Discussion

In this study we investigated differences in GM volume between SAD patients and HC participants, in the largest sample of 3T structural MRI scans available for analysis to date (n = 174 SAD patients and 213 HC participants). We used a hypothesis-driven ROI approach and focused on differences in GM volume in the amygdala-hippocampal complex, the basal ganglia, the prefrontal cortex and parietal areas. The results showed larger GM volume in the right putamen in SAD patients in comparison to HC participants (*Figure 4.2A-B*), and this increase in GM was positively correlated with the total score on the Liebowitz Social Anxiety Scale (LSAS) within the patient group (*Figure 4.2C*). This effect remained significant when we performed several sensitivity analyses examining the effect of comorbid depression; however, the effect did not survive in two other sensitivity analyses in which patients with any type of comorbidity and medication use were excluded, possibly due to the fact that the remaining sample size was relatively small.

We did, however, not find diagnosis-related alterations in GM volumes in the amygdala-hippocampal, prefrontal or parietal ROIs. Furthermore, there were no group differences in an exploratory whole-brain analysis. To examine these results, we performed post-hoc analyses to examine group differences in individual structures of these ROIs, but again, no SAD-related GM differences were present (*Supplemental Table S4.2*). Furthermore, we checked whether GM differences between male and female SAD patients might have

confounded the results, but we did not find significant gender-by-diagnosis interactions (*Supplemental Table S4.2*).

No SAD-related changes in amygdala-hippocampal, prefrontal and parietal ROIs

The null findings in the amygdala-hippocampal, prefrontal, and parietal ROIs were unexpected, because previous studies have reported SAD-related changes in GM in, among others, the amygdala, hippocampus, precuneus, prefrontal cortex, and parietal regions (Brühl, Hänggi, et al., 2014; Irle et al., 2010, 2014; Liao et al., 2011; Machado-de-Sousa et al., 2014; Meng et al., 2013; Syal et al., 2012; Talati, Pantazatos, et al., 2013; Tükel et al., 2015). Although applying the usual caveat when interpreting null effects, our results based on the largest SAD sample to date suggest that GM volume in regions outside the basal ganglia is likely not systematically related to SAD and thus might not underlie the alterations in brain functioning consistently reported and replicated in these regions (Brühl, Delsignore, et al., 2014). This idea is in line with the findings of a recent voxel-wise machine learning study, which suggested that SAD is easier to detect using multivariate analyses that take into account the global relationships between GM volume alterations in different regions, than by applying analyses that only focus on local changes in specific brain regions (Frick, Gingnell, et al., 2014).

With respect to the previous studies reporting SAD-related GM differences, it should be noted that the findings of these studies were often inconsistent, with increases as well as decreases in the same regions having been reported. E.g. for the amygdala, see (Irle et al., 2010; Machado-de-Sousa et al., 2014; Meng et al., 2013); see also (Brühl, Hänggi, et al., 2014; Syal et al., 2012) reporting no volumetric differences between SAD patients and HC participants, and the work of Shang et al. (2014), who did not observe changes in amygdalar GM volumes in a meta-analysis on structural neuroimaging findings across several anxiety disorders. These inconsistencies are most likely due to small sample sizes, which may have increased the probability of obtaining false-positive findings (Blackford, 2017; Button et al., 2013) – see also Cremers & Roelofs (2016) for a critical overview of neuroimaging research findings in SAD. Furthermore, the inconsistencies are likely due to differences in methodology, for example the use of manual vs. automatic segmentation, the choice and size of ROIs, and to differences in clinical characteristics. Thus, the results of this study stress the need for studies with sufficient sample sizes and meta-analyses such as those performed by the Enhancing NeuroImaging Genetics through Meta-Analysis (ENIGMA) Consortium and its working groups (Bearden & Thompson, 2017; Thompson et al., 2014).

Larger GM volume in right putamen

We did find GM differences in the right putamen, which, together with the caudate, forms the dorsal striatum (Marchand, 2010). The striatum is the major input structure of the basal ganglia, receiving information from the cortex, amygdala and hippocampus. The dorsal

striatum is part of a network that is important for learning actions based on their predicted outcomes (i.e. reward-related behavior), as well as for regulating cognitive and emotional behavior (Marchand, 2010; Shohamy, 2011; Stathis, Panourias, & Themistocleous, Sakas, 2007); for a recent review on the role of the striatum in anxiety we refer to Lago, Davis, Grillon, & Ernst (2017). Interestingly, our findings converge with earlier research on the structural and functional basis of inhibited temperament, a characteristic that refers to the innate tendency to be shy, quiet and extremely cautious in novel social and non-social situations (Miskovic & Schmidt, 2012). Inhibited temperament substantially increases the risk for developing SAD (Clauss & Blackford, 2012; Fox & Kalin, 2014) and is correlated with larger volumes of both the amygdala and the caudate in young adults, and hyperactivation in, among other areas, putamen, globus pallidus and caudate (Clauss, Seay, et al., 2014; Clauss et al., 2015) – see *Supplemental Table S4.3* for coordinates of these and other findings discussed in this section. Moreover, Clauss and colleagues showed that the GM increase in the caudate was positively related to the level of activation in this area in response to neutral faces (Clauss, Seay, et al., 2014). Because larger GM volume of the caudate was also associated with increased functional connectivity to regions that respond to social stimuli, the authors have proposed that larger caudate volume might facilitate the saliency of social and novel stimuli for individuals with an inhibited temperament, which could predispose them for developing SAD (Clauss, Seay, et al., 2014). Combined with our observation that SAD is associated with larger GM volume in the putamen, it may be hypothesized that structural changes in the dorsal striatum, as an integral part of limbic circuitry (Stathis et al., 2007), might underlie the biased processing of stimuli typically observed in SAD (Miskovic & Schmidt, 2012).

Evidence consistent with this idea comes from recent fMRI studies on SAD-related threat processing (Cremers et al., 2015; Heitmann et al., 2016). Anticipation of social punishment versus reward was associated with increased local activity in the putamen in SAD patients compared to healthy controls. In addition, SAD patients showed increased negative connectivity between the putamen and the ACC during social punishment and reward compared to HC participants (Cremers et al., 2015). Another study indicated that viewing ecologically valid, disorder-related complex visual scenes evoked increased activation in SAD patients in, among others, the putamen and globus pallidus. Here, hyperactivation in the dorsal striatum was accompanied by increased connectivity with the amygdala, medial prefrontal cortex and ACC, regions playing an important role in emotion processing (Heitmann et al., 2016). These findings are supported by another resting-state study indicating hyperconnectivity of the putamen and the globus pallidus in SAD (Arnold Anteraper et al., 2014) and two meta-analyses on task-related activity in SAD, reporting increased activation of the globus pallidus (Gentili et al., 2016; Hattingh et al., 2013).

Additional support for our hypothesis comes from a within-subject longitudinal study on the neuro-anatomical effects of paroxetine in a small sample of fourteen patients with

SAD, showing treatment-related decreases in symptom severity and concomitant reductions in GM in bilateral caudate and putamen (Talati et al., 2015). Furthermore, a 1H-magnetic resonance spectroscopy study demonstrated a relationship between social anxiety symptoms and the concentration of choline metabolites in the left caudate and right putamen (Howells et al., 2015), while single-photon emission computed tomography (SPECT) studies reported on alterations in the striatal dopaminergic system in patients with SAD (Schneier et al., 2000; Tiihonen et al., 1997; van der Wee et al., 2008), which are possibly related to striatal dysfunction (Sareen et al., 2007a). In addition, two recent PET studies indicated enhanced serotonin synthesis capacity in the striatum (Frick et al., 2015; Furmark et al., 2016). Given the role of serotonin in neuroplasticity and brain circuit development (Lesch & Waider, 2012), concomitant brain structure alterations may be expected in this region. Combined with these previous findings, our results support the idea stated before (Brühl, Delsignore, et al., 2014; Gentili et al., 2016), that SAD-related changes in brain function and structure may be found outside the traditional fear circuitry, consisting of the amygdala, insula, prefrontal cortex and anterior cingulate cortex (Etkin & Wager, 2007).

Notwithstanding the results of the present study, it should be noted that, despite the use of the largest database of structural MRI scans of SAD patients available to date, the effect sizes obtained in our study were small (see *Figure 4.3* for an illustration of the relationship between effect size and the power to detect an effect, given the sample size of our study). However, small effect sizes are not uncommon for studies on structural brain abnormalities in mental disorders (Ioannidis, 2011); we refer the reader to the recent viewpoint articles by Blackford (2017) and Reddan and colleagues (Reddan, Lindquist, & Wager, 2017) for important insights on improving the validity and reproducibility of neuroimaging studies in psychiatry. Furthermore, because of the hypothesis-driven ROI approach, we did not correct the *p*-value for the number of ROIs tested. In addition, it should be mentioned that the GM increase was present in a region with a low GM density (mean GM volume ± SD in significant cluster: SAD patients: 0.12 ± 0.05; HC participants 0.10 ± 0.05; see also *Figure 4.2B*). Together with the fact that it is hard to link neuroimaging results showing changes in brain structure directly to underlying cellular and molecular mechanisms like synaptogenesis, neurogenesis and changes in neuronal morphology (Lerch et al., 2017; Zatorre, Fields, & Johansen-Berg, 2012), this finding underscores that more research is needed to understand how the macroscopic SAD-related GM increase relates to effects at the microscopic level. It is also unclear, given the correlational nature of this study, whether and how structural differences in the dorsal striatum might play a causal or compensatory role in the pathogenesis of SAD. This underscores the need for future longitudinal studies on SAD, as well as for experiments that incorporate the dorsal striatum in animal models of social anxiety (compare (Fox & Kalin, 2014)).

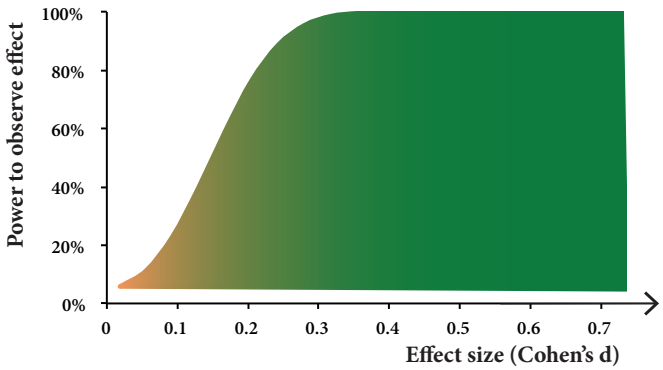

Figure 4.3 Illustration of relation between effect size and power to detect effect.
Power to observe an effect as a function of the real effect size given the current sample size (n = 174 SAD patients and n = 213 HC participants), calculated using *https://www.ai-therapy.com/psychology-statistics/power-calculator*.

Study limitations and future studies

The present study has several limitations. First, data on medication use and comorbidity were not available for all participants (*Table 4.3*). Furthermore, only the current use of medication and present comorbidity were known, so we could not exclude heterogeneity within the sample due to past medication use or past comorbidity. Another possible source of heterogeneity within the sample arises from the fact that we pooled data from multiple research centers located in various countries, which could add confounding effects of, for example, ethnicity and differences in scanner settings. However, we do not believe that these potential confounds have substantially influenced our results, as we corrected for scan center within our statistical model and since we did not find any diagnosis-by-scan center effects.

In the present study, we have exclusively investigated SAD-related differences in GM volumes. Future studies on structural brain alterations should examine changes in other parameters of brain anatomy, like cortical thickness, white matter integrity, and the shape of brain structures. The latter is especially interesting, given the recent insight that the shape of the putamen exhibits moderate-to-high heritability (Ge et al., 2016; Roshchupkin et al., 2016). This, together with the understanding that SAD is familial and moderately heritable (Isomura et al., 2015; Middeldorp et al., 2005; Scaini et al., 2014; Torvik et al., 2016), raises the question whether putamen shape could be considered a candidate endophenotype of SAD (compare (Bas-Hoogendam et al., 2016)) and it will be interesting to investigate this in future studies. In addition, it would be worthwhile to perform multivariate pattern analyses (Adluru et al., 2013; Pereira, Mitchell, & Botvinick, 2009) to examine whether it is possible to discriminate SAD patients from HC participants based on GM volumes – see for example (Frick, Gingnell, et al., 2014). Together with ongoing work on the functional brain altera-

tions, as well as with the results of PET studies on brain metabolism in SAD, these findings may aid in unraveling the neurobiological basis of this serious and disabling disorder.

Conclusions

In summary, the results of the present mega-analysis of the largest database of SAD brain scans to date showed larger GM volume in the dorsal striatum in SAD, which correlated positively with the severity of self-reported social anxiety symptoms. Combined with previous work on inhibited temperament and imaging studies on SAD, our results suggest that the dorsal striatum may play a role in the biased processing of social stimuli that is characteristic of SAD psychopathology. Importantly, we could not replicate GM alterations in the amygdala, hippocampus, prefrontal cortex and precuneus, regions previously implicated in SAD in imaging studies with smaller sample sizes. We take these null findings as an indication that large sample sizes and investigations such as the meta-analyses performed by the ENIGMA Consortium are necessary for the reliable detection of neuro-anatomical changes in SAD.

Supplemental Methods

Details on in- and exclusion criteria for each site/study, as described in original research protocols and/or publications.

Germany

Full name of research centers Biological Psychology, University of Jena, Germany; Institute of Medical Psychology and Systems Neurosciences, University of Münster, Germany.

Data published in Boehme, Ritter, et al. (2014, 2015); Boehme, Mohr, et al. (2014).

Exclusion criteria both groups General fMRI contraindications (e.g. ferromagnetic implants, pregnancy, claustrophobia, …).

Inclusion criteria SAD Primary diagnosis of SAD – determined by extensive semi-structured interviews with individual subjects. For SAD, diagnoses were confirmed (and comorbidities were assessed) by clinical psychologists administering the Structured Clinical Interview for DSM-IV Axis I and II disorders (SCID I and II; (Fydrich, Renneberg, Schmitz, & Wittchen, 1997; Wittchen, 1997).

Exclusion criteria SAD (i) a diagnosis of obsessive–compulsive disorder, current alcohol or substance abuse, any psychotic disorder or dementia and current primary or secondary major depression; (ii) a history of seizures or head injury with loss of consciousness; (iii) a severe uncontrollable medical condition; or (iv) the use of any psychotropic medication within the preceding 6 months.

Inclusion criteria HC Healthy adults, age-, gender-, and education-matched to patients.

Exclusion criteria HC Presence of any psychopathology.

Recruitment of participants Both groups were recruited via public announcement (flyers distributed at university, in the community, and by online advertisement on department website).

The Netherlands - The Netherlands Study of Depression and Anxiety (NESDA)

Full name of research centers Leiden University Medical Center, Leiden, the Netherlands; VU University Medical Center, Amsterdam, the Netherlands; University Medical Center Groningen, Groningen, the Netherlands.

Design of NESDA published in Penninx et al. (2008); *data published in* Pannekoek et al. (2013, 2015; van Tol et al. (2010).

Exclusion criteria both groups Presence or history of major internal or neurological disorder, dependence on or recent abuse (past year) of alcohol and/or drugs, hypertension, and general magnetic resonance imaging contraindications.

Inclusion criteria SAD Half-year diagnosis of SAD, established using the structured Composite International Diagnostic Interview (lifetime version 2.1) given by a trained interviewer (Robins et al., 1988).

Exclusion criteria SAD Known personality disorders; presence of axis-I disorders other than MDD, PD, SAD, or GAD and any use of psychotropic medication other than stable use of SSRIs or infrequent benzodiazepine use (ie, equivalent to 2 doses of 10 mg of oxazepam 3 times per week or use within 48 hours prior to scanning).

Inclusion criteria HC Controls were currently free of, and had never met criteria for, depressive or anxiety disorders or any other axis-I disorder and were not taking any psychotropic drugs.

Exclusion criteria HC Lifetime DSM-IV diagnosis, established using the structured Composite International Diagnostic Interview (lifetime version 2.1) given by a trained interviewer (Robins et al., 1988).

Recruitment of participants (cited from (Penninx et al., 2008)): "NESDA has been designed to be representative of those with depressive and anxiety disorders in different health care settings and stages of the developmental history. Therefore, the sample is stratified for setting (community, primary care and specialized mental health) and set up to include a range of psychopathology: those with no symptoms or disorders ('controls'), those with earlier episodes or at risk because of subthreshold symptoms or family history, and those with a current first or recurrent depressive or anxiety disorder. The focus is on Dysthymia, Major Depressive Disorder, General Anxiety Disorder, Panic Disorder, Social Phobia and Agoraphobia. A general inclusion criterion was an age of 18 through 65 years."

"In order to maintain representativity, only two exclusion criteria existed: (1) a primary clinical diagnosis of a psychiatric disorder not subject of NESDA which will largely affect course trajectory: psychotic disorder, obsessive-compulsive disorder, bipolar disorder, or severe addiction disorder, and (2) not being fluent in Dutch since language problems would harm the validity and reliability of collected data."

"The NESDA community sample builds on two cohorts that were already available through prior studies. The first cohort is from the Netherlands Mental Health Survey and Incidence Study (NEMESIS), a community-based study described in detail elsewhere (Bijl et al., 1998)."

"The second cohort exists of participants of the Adolescents at Risk for Anxiety and Depression (ARIADNE) study (Landman-Peeters et al., 2005), a prospective cohort study among 528 biological children (aged 13– 25 years) of parents who were treated for depressive or anxiety disorder as outpatient at a mental health organization."

"Recruitment from primary care practices: Primary care patients were recruited from 65 general practitioners (GPs) in the vicinity of the field sites (Amsterdam, Groningen, Leiden). In selecting these GPs, attention was paid to the use of an appropriate electronic patient record databases which allows uniform data extraction for research purposes."

"Recruitment from mental health organizations: The specialized mental health patients were recruited from outpatient clinics of regional facilities for mental health care around the three research sites."

The Netherlands - Social Anxiety Study

Full name of research center Leiden University Medical Center, Leiden, the Netherlands.

Data published in Cremers et al. (2014, 2015).

Inclusion criteria SAD SAD participants had to meet criteria for general SAD according to DSM-IV as a primary diagnosis (1994) based on the Mini-International Neuropsychiatric Interview (MINI; (Sheehan et al., 1997)).

Exclusion criteria SAD Other co-morbid anxiety, psychotic or substance abuse disorders.

Inclusion criteria HC Matched to SAD with respect to age, gender and years of education; no history of psychiatric diseases or psychotropic medication use.

Recruitment of participants SAD participants were recruited through an advertisement (n = 7), local participating treatment centers (n = 8) and, social anxiety web forums (n = 5).

South Africa

Full name of research centers University of Cape Town, Cape Town, South Africa; US/UCT MRC Unit on Anxiety & Stress Disorders, Department of Psychiatry, University of Stellenbosch, Cape Town, South Africa.

Data published in Geiger et al.(2016) ; Hattingh et al. (2013); Howells et al. (2015); Syal et al. (2012).

Inclusion criteria SAD Primary diagnosis of SAD, established by SCID-I; SCID-OCSD done by clinical psychologist / psychiatrist; right-handed.

Exclusion criteria SAD Clinically significant comorbidity; Psychotropic medication; Psychotic disorder.

Inclusion criteria HC No psychiatric disorders; Righthanded.

Exclusion criteria HC Medication use. Psychotic disorder.

Recruitment of participants Advertisements in media; radio talks; Letters to clinicians. Consumer advocacy groups (e.g. SADAG).

Sweden

Full name of research center Uppsala University, Department of Psychology, Uppsala, Sweden

Data published in Frick, Engman, et al. (2014).

Inclusion criteria SAD Social anxiety disorder (social phobia), according to DSM-IV, must be the main diagnosis as assessed with the structured clinical interview for DSM disorders. Otherwise somatically healthy; age 18 or older but not postmenopausal; willingness to participate in a symptom provocation brain imaging trial.

Exclusion criteria SAD Treatment of social anxiety within the three months preceding the study; Current serious or dominant psychiatric disorder other than social anxiety disorder (e.g., psychosis, major depressive disorder, bipolar disorder); Suicidal ideation; Chronic use of prescribed medication that could influence the results; Abuse of alcohol or narcotics; Pregnancy or planned pregnancy during the study period; Menopause; Previous PET examination; Contra-indications for MRI investigations (e.g. implants or other metal objects in the body, brain and heart operations).

Inclusion criteria HC Somatically healthy; Age 18 or older but not postmenopausal; Willingness to participate in a symptom provocation brain imaging trial.

Exclusion criteria HC History of or current psychiatric disorder. To exclude the presence of psychopathology, Mini International Neuropsychiatric Interview was administered by psychology grad students, (during their final phase of education) trained in the administration of the interviews and under supervision. Suicidal ideation; Chronic use of prescribed medication that could influence the results; Abuse of alcohol or narcotics; Pregnancy or planned pregnancy during the study period; Menopause; Previous PET examination; Contrainidications for MRI investigations (e.g. implants or other metal objects in the body, brain and heart operations).

Recruitment of participants SAD patients were recruited through newspaper advertisements and volunteered to participate by signing up at a dedicated website. HC participants were recruited from public bulletin boards at Uppsala University.

United States of America - University of Michigan, University of Chicago

Full name of research centers UIC: University of Illinois at Chicago Mood and Anxiety Disorders Research Program; UofM: University of Michigan; UofC: University of Chicago Brain Imaging and Emotions Laboratory.

Data published in Phan et al. (2013).

Inclusion criteria both groups Able to give informed consent; Physically healthy; Age 18-55.

Exclusion criteria both groups Clinically significant medical or neurologic condition; Life history of bipolar disorder, schizophrenia, presence of an organic mental syndrome, mental retardation, or pervasive developmental disorder; Positive drug screen results; Pregnant or lactating; Left-handed; Presence of ferrous-containing metal in the body; Inability to tolerate small, enclosed spaces; Unwilling/unable to sign informed consent.

Inclusion criteria SAD Current social anxiety disorder, generalized subtype; Master's level clinician determined diagnoses by SCID for DSM-IV; LSAS > 60 at screening visit.

Exclusion criteria SAD Primary comorbid anxiety disorder; Current Major Depressive Disorder or Major Depression within the past 6 months; HAM-D > 18; Current alcohol/drug abuse or dependence or within the past year; Current suicidal ideation; Diagnosis of any of the following Axis II personality disorders: paranoid, schizoid, schizotypal, antisocial, borderline, histrionic, narcissistic. Concomitant treatments with psychotropic/psychoactive medications within the last 2 weeks (8 weeks for fluoxetine, 4 weeks for MAOIs) before screening, including beta-adrenergic blockers, SSRIs, benzodiazepines, tricyclic/mono-amine oxidase inhibitor antidepressants, lithium, antiepileptic/anticonvulsants, neuroleptic/antipsychotics; Clinically significant medical condition which interferes with the metabolism of sertraline (e.g. severe hepatic or renal insufficiency); Ongoing psychotherapy treatment; History of known or suspected hypersensitivity to sertraline or another SSRI; Prior failure of response to sertraline or another SSRI for social anxiety.

Inclusion criteria HC Free of a lifetime diagnosis of any Axis I or Axis II disorder

Recruitment of participants Community, internet, clinic.

United States of America - University of Illinois

Full name of research centers University of Illinois at Chicago Mood and Anxiety Disorders Research Program.

Data published in Klumpp et al. (2015).

Inclusion criteria both groups Age 18-55; Subject is able to give informed consent; Physically healthy.

Exclusion criteria both groups Clinically significant and active medical or neurological condition; Primary comorbid anxiety disorder (defined by which disorder was the more debilitating and clinically salient than SAD or MDD); Life history of bipolar disorder, schizophrenia, or presence of an organic mental syndrome, mental retardation, or pervasive developmental disorder; Life history of or current psychotic symptoms; Current alcohol/drug abuse or dependence or in the past 6 months based on the SCI; Current suicidal/homicidal ideation (i.e., an active suicidal plan or history of serious suicide attempt in the last six months); Evidence of chronic self-injurious behavior in the past six months (i.e., cutting, burning, etc.) as determined by self-report and the Primary Investigator; Prior treatment of a clinical dose of cognitive behavioral therapy as determined by the Primary Investigator; Ongoing active psychotherapy (e.g. CBT) treatment of any kind; Current treatment with any psychotropic medication (anti-depressants, anti-obsessionals, anxiolytics, anti-psychotics, mood stabilizers); prior

treatment with a psychotropic medication is not an exclusion criteria as long as the treatment was discontinued at least 2 weeks prior to study entry (4 weeks if potential participant was taking fluoxetine); Presence of ferrous-containing metal in the body; Inability to tolerate small, enclosed spaces.

Inclusion criteria SAD Current SAD based on SCID diagnosis; Master's level clinician determined diagnoses by SCID for DSM-IV; LSAS score of ≥ 55 for SAD.

Inclusion criteria HC Free of a lifetime diagnosis of Axis I or Axis II disorder.

Recruitment of participants Community, internet, clinic.

Supplemental Tables

Supplemental Table S4.1 Scans excluded based on comorbidity other than anxiety and/or MDD.

Exclusion comorbidity	Number of scans
SAD + OCD	3
SAD + OCD + panic disorder	1
SAD + personality disorder	8
SAD + dysthymia	5
SAD + dysthymia + personality disorder	3
SAD + dysthymia + personality disorder + panic disorder	1
SAD + dysthymia + personality disorder + OCD	1
SAD + MDD + alcohol dependency	1
SAD + MDD + personality disorder	8
SAD + MDD + alcohol dependency + personality disorder	2
SAD + MDD + eating disorder + personality disorder	1
SAD + MDD + panic disorder + personality disorder	1
SAD + self-injury disorder	1
SAD + cannabis dependency	2
SAD + dissociative disorder NOS	1
SAD + sexual arousal disorder	1
SAD + panic disorder + eating disorder	1
SAD + somatoform disorder + intermittent explosive disorder	1
Total	42

Abbreviations
MDD: major depressive disorder; NOS: not otherwise specified; OCD: obsessive-compulsive disorder.

Supplemental Table S4.2 Overview results multiple regression analyses individual regions.

		Mean GM[a]		Effect of diagnosis		Interaction diagnosis x gender	
Original ROI	**Individual regions**	**SAD**	**HC**	**β**	***p* value uncorrected[b]**	**β**	***p* value uncorrected[b]**
Prefrontal cortex	Anterior cingulate gyrus	0.585	0.591	-0.040	0.417	-0.075	0.098
	Frontal medial cortex	0.566	0.584	-1.020	0.015	0.012	0.763
	Frontal orbital cortex	0.580	0.578	0.023	0.614	0.013	0.750
	Middle frontal gyrus	0.529	0.529	0.006	0.905	-0.082	0.065
	Paracingulate gyrus	0.614	0.632	-0.102	0.029	-0.016	0.710
	Subcallosal cortex	0.591	0.587	0.021	0.583	2.053	0.041
Amygdala-hippocampus	Amygdala	0.649	0.639	0.033	0.318	0.330	0.275
	Hippocampus	0.613	0.605	0.039	0.258	0.033	0.302
	Anterior parahippocampal gyrus	0.661	0.663	-0.013	0.774	0.062	0.159
	Posterior parahippocampal gyrus	0.506	0.510	-0.030	0.537	0.120	0.008
Parietal	Precuneus	0.576	0.586	-0.050	0.245	-0.028	0.486
	Superior parietal lobule	0.473	0.481	-0.050	0.304	0.010	0.832
	Posterior cingulate gyrus	0.573	0.585	-0.072	0.130	0.010	0.823

Footnotes

[a] Estimated marginal mean;

[b]: None of these effects survived correction for multiple comparisons (13 regions, Bonferroni-corrected *p*-value: $p \leq 0.004$).

Supplemental Table S4.3 Coordinates of findings summarized in *Discussion*.

		Coordinates peak voxel		
Publication	**Region**	**x**	**y**	**z**
Clauss et al., 2014	Left caudate	-19	6	17
Clauss et al., 2015	Left caudate	-14	10	18
	Right amygdala, parahippocampal gyrus, globus pallidus, putamen	22	-6	-16
Cremers et al., 2015	Left putamen	-20	12	4
Heitmann et al., 2016	Left globus pallidus / putamen	-22	-1	2
Arnold Anteraper et al., 2014	Caudate seed	*Seeds for functional connectivity analysis defined according to the Wake Forest University Pickatlas*		
	Left and right putamen seeds			
	Globus pallidus seeds			
Gentili et al., 2016	Right globus pallidus	18	-2	-8
Hattingh et al., 2013	Right globus pallidus	20	-2	-8
Talati et al., 2015	Right caudate, putamen	21	14	-3
	Left caudate, putamen	-12	14	-5
Howells et al., 2015	Left caudate	*Not applicable (MRS voxels)*		
	Right putamen			
Schneier et al., 2000	Striatum	*Not further specified*		
Tiihonen et al., 1997	Striatum	*Not further specified*		
Van der Wee et al., 2008	Striatum – right putamen (post-hoc)	*Manually drawn VOIs for SPECT analyses*		
Frick et al., 2015	Caudate nucleus	*ROIs defined according to the Wake Forest University Pickatlas*		
	Putamen			
Furmark et al., 2016	Globus pallidus	24	2	0
	Putamen	-26	-14	18

Abbreviations

MRS: magnetic resonance spectroscopy; ROI: region of interest; VOI: volume of interest.

Chapter 5

Subcortical brain volumes, cortical thickness and cortical surface area in families genetically enriched for Social Anxiety Disorder – a multiplex multigenerational neuroimaging study

Published as:

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Abstract

Background

Social anxiety disorder (SAD) is a disabling psychiatric condition with a genetic background. Brain alterations in gray matter (GM) related to SAD have been previously reported, but it remains to be elucidated whether GM measures are candidate endophenotypes of SAD. Endophenotypes are measurable characteristics on the causal pathway from genotype to phenotype, providing insight in genetically-based disease mechanisms. Based on a review of existing evidence, we examined whether GM characteristics meet two endophenotype criteria, using data from a unique sample of SAD patients and their family members of two generations. First, we investigated whether GM characteristics co-segregate with social anxiety within families genetically enriched for SAD. Secondly, heritability of the GM characteristics was estimated.

Methods

Families with a genetic predisposition for SAD participated in the Leiden Family Lab study on SAD; T1-weighted MRI brain scans were acquired (n = 110, eight families). Subcortical volumes, cortical thickness and cortical surface area were determined for a priori determined regions of interest (ROIs). Next, associations with social anxiety and heritabilities were estimated.

Findings

Several subcortical and cortical GM characteristics, derived from frontal, parietal and temporal ROIs, co-segregated with social anxiety within families (uncorrected p-level) and showed moderate to high heritability.

Interpretation

These findings provide preliminary evidence that GM characteristics of multiple ROIs, which are distributed over the brain, are candidate endophenotypes of SAD. Thereby, they shed light on the genetic vulnerability to SAD. Future research is needed to confirm these results and to link them to functional brain alterations and to genetic variations underlying these GM changes.

Funding

Leiden University Research Profile 'Health, Prevention and the Human Life Cycle'.

Research in context

Evidence before this study

Social anxiety disorder (SAD) is a prevalent psychiatric condition characterized by intense fear of negative evaluation in social situations. SAD typically develops during late childhood or adolescence and has a strong negative impact on patients' lives. Previous studies showed that SAD has a familial background. However, it's unknown which heritable characteristics make children and adolescents vulnerable for developing SAD. The endophenotype approach could be helpful to shed more light on the genetic susceptibility to SAD. Endophenotypes are measurable characteristics which are associated with the disorder, heritable, and co-segregate with the disorder within families of patients. Alterations in brain structure are candidate endophenotypes of SAD, as gray matter (GM) characteristics have been shown to be highly heritable. Furthermore, several studies have shown abnormalities of brain structure in SAD.

Added value of this study

To investigate whether specific GM characteristics could serve as endophenotypes for SAD, family studies are needed. The Leiden Family Lab study on Social Anxiety Disorder (LFLSAD) is a unique neuroimaging study, in which patients with SAD as well as their family members of two generations were investigated. Selected families were genetically enriched for SAD and due to the family design of the LFLSAD, we were able to investigate two endophenotype criteria. First, we examined whether GM characteristics co-segregated with social anxiety within the families. Second, we estimated the heritability of the GM characteristics. Our results show that several GM characteristics meet both endophenotype criteria, making them promising candidate endophenotypes of social anxiety.

Implications of all available evidence

The findings provide preliminary evidence that several GM characteristics are genetically linked to social anxiety. Thereby, the results of this study shed light on the genetic vulnerability to SAD.

Introduction

Patients who suffer from social anxiety disorder (SAD) are characterized by an intense fear of negative evaluation by others in social situations (American Psychiatric Association, 2013; Stein & Stein, 2008). As a result, SAD patients try to avoid social situations as much as possible, which leads to disability and serious impairments in important areas of life such as education, work, and social activities (Acarturk et al., 2008; Aderka et al., 2012; Craske et al., 2017; Fehm et al., 2005; Hendriks et al., 2014; Stein & Kean, 2000; Vos et al., 2016; Wittchen et al., 2011). The disorder has a high prevalence (de Graaf et al., 2012; Kessler et al., 2012), is often chronic (Blanco et al., 2011; Wittchen & Fehm, 2003), and has a typical onset during late childhood and early adolescence (Beesdo-Baum et al., 2015; Haller et al., 2015; Leigh & Clark, 2018; Merikangas et al., 2010; Miers et al., 2013, 2014). Furthermore, SAD is associated with high psychiatric comorbidity (Erwin, Heimberg, Juster, & Mindlin, 2002; Meier et al., 2015; Ruscio et al., 2008), adding to its burden on patients. Insight in the development of and vulnerability to SAD is therefore of great importance, as this might aid in developing preventive interventions and effective treatments.

Previous studies indicate that the pathogenesis of SAD is complex: environmental, biological, temperamental, and genetic factors are shown to play a interacting role (Fox & Kalin, 2014; Hirshfeld-Becker, 2010; Wong & Rapee, 2016). With respect to the latter, the heritability of SAD is estimated to be between 39 - 56% (Bandelow et al., 2016; Isomura et al., 2015; Scaini et al., 2014; Smoller, 2015). However, despite the promising results of a handful of studies investigating the genetic background of SAD (Fyer et al., 2012; Gelernter et al., 2004; Otowa et al., 2016; Scaini et al., 2014; Stein et al., 2017, 2001; Stein, Jang, & Livesley, 2002), the genetic variants underlying the vulnerability to SAD are at present still largely unidentified. Detecting such 'SAD genes' is difficult due to several factors. First of all, SAD is a polygenic disorder, and it is widely assumed that various genetic variants, influenced by environmental factors, are involved in its development (Binder, 2012; Gottschalk & Domschke, 2016; Munafò & Flint, 2014a). Furthermore, SAD is a heterogeneous disorder, and the diagnosis is based on clinical interviews and not on biologically-based parameters (Bearden et al., 2004; Hyett & McEvoy, 2018). Thus, investigating endophenotypes might facilitate in unravelling the genetic vulnerability to complex psychiatric disorders like SAD (Iacono, 2018).

Endophenotypes are measurable traits located on the causal pathway from genotype to phenotype (Gottesman & Gould, 2003; Lenzenweger, 2013b), and include, for example, neurobiological changes in brain structure and function. Criteria for endophenotypes are the following (Glahn et al., 2007; Lenzenweger, 2013a; Puls & Gallinat, 2008): 1st they are *associated with the disorder*; 2nd they are *state-independent traits, already present in a preclinical state*; 3rd they are *heritable*; 4th they *co-segregate with the disorder within families of probands, with non-affected family members showing altered levels of the endophenotype in comparison*

to the general population. As reviewed in our earlier work (Bas-Hoogendam et al., 2016), endophenotypes have the potential to shed more light on the mechanisms involved in the etiology of SAD.

In the present work, we provide a comprehensive overview of existing evidence and investigate whether gray matter (GM) structural brain characteristics, as measured with magnetic resonance imaging (MRI), are candidate endophenotypes of SAD. Based on previous findings, and as summarized in Bas-Hoogendam et al. (2016), there are two important reasons to do so.

To start, differences in GM between SAD patients and healthy controls have been reported for a number of subcortical, frontal, temporal and parietal regions (Bas-Hoogendam, van Steenbergen, Pannekoek, et al., 2017; Brühl, Hänggi, et al., 2014; Frick, Engman, et al., 2014; Frick, Howner, Fischer, Eskildsen, et al., 2013; Irle et al., 2014; Liao et al., 2011; Machado-de-Sousa et al., 2014; Meng et al., 2013; Syal et al., 2012; Talati, Pantazatos, et al., 2013; Tükel et al., 2015; Zhao et al., 2017) – see *Table 5.1* for an overview of MRI studies on GM in SAD. Furthermore, changes in brain structure were shown to be associated with clinical characteristics (Bas-Hoogendam, van Steenbergen, Pannekoek, et al., 2017; Brühl, Hänggi, et al., 2014; Frick, Engman, et al., 2014; Irle et al., 2010, 2014; Liao et al., 2011; Syal et al., 2012; Talati, Pantazatos, et al., 2013; Tükel et al., 2015), while treatment-related changes in brain structure in SAD patients have also been described (Cassimjee et al., 2010; Steiger et al., 2017; Talati et al., 2015). Although it should be noted that the findings reported in these studies are heterogeneous (see *Table 5.1* and review by Brühl and colleagues (2014)), and have small effect sizes (Bas-Hoogendam, van Steenbergen, Pannekoek, et al., 2017), a machine learning study was able to discriminate SAD patients from healthy controls based on GM changes over the whole brain (Frick, Gingnell, et al., 2014). Furthermore, higher levels of social anxiety in healthy women were related to increased volumes of the amygdala, nucleus accumbens, and striatal regions like the putamen and caudate nucleus (Günther et al., 2018), while structural brain alterations have also been reported in anxious children and adolescents (Gold et al., 2016, 2017; Milham et al., 2005; Mueller et al., 2013; Strawn et al., 2015). In addition, changes in brain structure have been reported in participants who were classified as being 'behaviorally inhibited' (Barrós-Loscertales et al., 2006; Cherbuin et al., 2008; Clauss, Seay, et al., 2014; Fuentes et al., 2012; Levita et al., 2014; Schwartz et al., 2010; Sylvester et al., 2015), which refers to the innate, temperamental trait associated with an increased vulnerability to develop SAD (Clauss & Blackford, 2012). Together, these results suggest that structural brain alterations in GM might be related to SAD.

A second reason to consider GM brain characteristics as candidate endophenotypes is the fact that numerous studies, both in healthy controls as well as in several patient groups, have indicated that brain structure is to a great extent determined by genetic influences. For example, studies revealed that genetic variants affect the thickness and surface area of cortical GM (Chen et al., 2015; Eyler et al., 2011; Joshi et al., 2011; Strike et al., 2018; Thompson

et al., 2001; Wen et al., 2016), as well as intracranial volume (ICV) (Adams et al., 2016) and subcortical brain volumes (den Braber et al., 2013; Hibar et al., 2015; Rentería et al., 2014; Stein et al., 2012; Whelan et al., 2015); the findings with respect to subcortical volumetric measures have recently been replicated and extended in a genome-wide association analysis in over 40,000 individuals (Satizabal et al., 2017). In addition, the neuroanatomical shape of subcortical structures has been shown to be significantly heritable (Ge et al., 2016; Roshchupkin et al., 2016). Furthermore, the results of studies in various patient populations, for example in twins (dis)concordant for bipolar disorder (Bootsman et al., 2015) and in families with multiple cases of schizophrenia (Roalf et al., 2015) corroborate with these findings, showing that both the volume as well as the shape of subcortical structures are heritable. A meta-analysis of twin studies confirmed that global brain volumes, volumes of subcortical brain areas, as well as measures of cortical thickness, are all highly or moderately-to-highly heritable (Blokland et al., 2012); see also the review by Peper and colleagues (2007).

The present work used MRI data from the Leiden Family Lab study on Social Anxiety Disorder (LFLSAD) (Bas-Hoogendam, Harrewijn, et al., 2018) to explore whether GM brain characteristics (volumes of subcortical structures; estimations of cortical thickness (CT), and measures of cortical surface area (CSA)) are endophenotypes of SAD. The LFLSAD is a multiplex (i.e., families were selected based on a minimum of two (sub)clinical SAD cases within one nuclear family), multigenerational (i.e., multiple nuclear families encompassing two generations from the same family took part) family study on SAD, in which nine families who were genetically enriched for SAD were included (total $n = 132$). Such a family design is particularly powerful to investigate genetic and environmental influences on SAD-related characteristics (Williams & Blangero, 1999).

We examined two endophenotype criteria. First, we investigated whether alterations in GM brain characteristics *co-segregate with social anxiety within the families* (first element of endophenotype criterion 4); second, we estimated the *heritability* of these measures (endophenotype criterion 3). The structural brain phenotypes were established using the FreeSurfer software package (version 5.3) and we employed a hypothesis-driven region-of-interest (ROI) approach based on the results of previous studies.

With respect to the subcortical volumes, we focused on the putamen and pallidum, based on the findings of a recent mega-analysis on SAD reporting increased GM related to SAD in these regions (Bas-Hoogendam, van Steenbergen, Pannekoek, et al., 2017), which were recently replicated (Günther et al., 2018). In addition, we investigated the association between social anxiety and volumes of the amygdala and hippocampus, given the fact that volumetric changes in these areas in SAD have been reported (Irle et al., 2010; Machado-de-Sousa et al., 2014; Meng et al., 2013), although it should be noted that other studies were not able to replicate these effects (see for example (Bas-Hoogendam, van Steenbergen, Pannekoek, et al., 2017; Brühl, Hänggi, et al., 2014) and *Table 5.1*). These subcortical ROIs are displayed in *Figure 5.1A*.

With respect to the estimates of CT, it should be noted that only a handful of studies have investigated SAD-related alterations in CT, with mixed results (*Table 5.1*). To determine cortical ROIs for the present study, we used the findings from previous work, starting with the work by Brühl and colleagues (2014), who investigated CT in a sample of 46 SAD patients and 46 matched healthy controls; they reported SAD-related increases in CT in the anterior cingulate cortex (ACC), the insula, the dorsolateral prefrontal cortex (DLPFC) including the middle frontal gyrus and the superior frontal lobule, the temporal pole and the parietal cortex (Brühl, Hänggi, et al., 2014). Most of these findings were recently replicated by Zhao and colleagues (2017), who described significant cortical thickening in the ACC, the insula, the superior frontal cortex, as well as in the temporal pole and parietal areas in SAD; in addition, this study mentioned cortical thinning in the orbitofrontal cortex, precentral cortex and the rostral medial frontal cortex. Other work, by Syal and colleagues (2012), reported on cortical thinning in 13 SAD patients, in several temporal, frontal and parietal regions, as well as in the insula and cingulate areas. The selected ROIs based on the results of these three studies are illustrated in *Figure 5.1B* (cortical parcellations as defined in the Desikan-Killiany atlas (Desikan et al., 2006)).

As there are, to the best of our knowledge, no studies on measures of CSA in SAD, the same cortical ROIs were used to investigate alterations in CSA related to SAD. It is of importance to investigate the measures of CT and CSA separately, as it has been shown that these neuroimaging phenotypes reflect different features of cerebral cortical structure. That is, neurons in the cortex are organized in columns running perpendicular to the surface of the brain; CT represents the number of cells within these columns, whereas the size of the CSA is determined by the number of columns in a certain area (Geschwind & Rakic, 2013; Rakic, 1988). Previous research indicated that brain size is primarily determined by the size of CSA (and not by CT) (Im et al., 2008); in addition, CT and CSA are genetically independent and follow different developmental trajectories (Chen et al., 2013; Gilmore, Knickmeyer, & Gao, 2018; Hogstrom, Westlye, Walhovd, & Fjell, 2013; Panizzon et al., 2009; Tamnes et al., 2017; Wierenga, Langen, Oranje, & Durston, 2014; Winkler et al., 2010, 2018). Furthermore, CT and CSA have different predictive values with respect to the development of psychopathology (Bois et al., 2015; Prasad et al., 2010).

Other, non ROI (sub)cortical areas were investigated on an exploratory basis only; results are reported in the *Supplemental Tables* and only briefly mentioned in the Results section. Analyses were corrected for multiple comparisons at a false discovery rate (FDR) of 5 % (Benjamini & Hochberg, 1995), but given the divergent findings of previous studies (*Table 5.1 A-C*), the innovative nature of the present study (to the best of our knowledge, this is the first comprehensive family study on social anxiety) and the fact that brain regions are likely biologically not independent but constitute structural and functional networks (cf. the work of Brühl et al. (2014)), uncorrected *p*-values are reported and discussed as well.

Table 5.1A Overview results of studies on GM in SAD; subcortical areas.

			Subcortical areas				
Publication	**Method**	**Group**	**Amy**	**HiC**	**Thal**	**Putamen**	**Caudate**
(Potts et al., 1994)	Manual segmentation caudate, thalamus, putamen	22 SAD vs 22 HC	n.a.	n.a.	=	=	=
(Cassimjee et al., 2010)	Whole brain VBM (SPM)	11 SAD - treatment effect	=	=	=	=	=
(Irle et al., 2010)	Manual segmentation amygdala & hippocampus	24 SAD vs 24 HC	-	-	n.a.	n.a.	n.a.
(Liao et al., 2011)	Whole brain VBM (SPM)	18 SAD vs 18 HC	=	-	=	=	=
(Syal et al., 2012)	Whole brain CT FreeSurfer; volumes amygdala & hippocampus	13 SAD vs 13 HC	=	=	n.a.	n.a.	n.a.
(Frick, Howner, Fischer, Eskildsen, et al., 2013)	Whole brain CT using FACE	14 male SAD vs 12 HC	=	=	=	=	=
(Meng et al., 2013)	Whole brain VBM (SPM)	20 SAD vs 19 HC	- and negative correlation with disease duration	=	- and positive correlation with age of onset	=	=
(Talati, Pantazatos, et al., 2013) - sample 1	Whole brain VBM (SPM)	16 SAD vs 20 HC (16 PD)	=	+	=	=	=
(Talati, Pantazatos, et al., 2013) - sample 2	Whole brain VBM (SPM)	17 SAD vs 17 HC	=	=	=	=	=
(Brühl, Hänggi, et al., 2014)	Whole brain & ROIs CT FreeSurfer; volumes subcortical ROIs	46 SAD vs 46 HC	=	=	=	=	=
(Frick, Gingnell, et al., 2014)	Whole brain VBM (SPM) + ROI approach; SVM study	14 SAD vs 12 HC	=	=	=	=	=
(Frick, Engman, et al., 2014)	Whole brain VBM (SPM)	48 SAD vs 29 HC	=	=	=	=	=

Table 5.1A Overview results of studies on GM in SAD; subcortical areas. (*continued*)

			Subcortical areas				
Publication	**Method**	**Group**	**Amy**	**HiC**	**Thal**	**Putamen**	**Caudate**
(Irle et al., 2014)	Whole brain VBM (SPM); manual segmentation parietal ROIs	67 SAD vs 64 HC	=	=	=	=	=
(Machado-de-Sousa et al., 2014b)	Manual segmentation amygdala & hippocampus	12 SAD, 12 SA, 14 HC	+	+	n.a.	n.a.	n.a.
(Talati et al., 2015)	Whole brain VBM (SPM)	14 SAD - treatment effect	=	=	- after treatment	- after treatment	- after treatment
(Tükel et al., 2015)	Whole brain VBM (SPM)	27 SAD vs 27 HC	=	=	=	=	=
(Månsson et al., 2016, 2017)	ROIs (amygdala, ACC, insula, hippocampus) as well as whole brain VBM (SPM)	13 SAD - treatment effect	- after treatment	=	=	=	=
(Steiger et al., 2017)	Whole brain cortical volume & CT using FreeSurfer	33 SAD -treatment effect	=	=	=	=	=
(Bas-Hoogendam, van Steenbergen, Pannekoek, et al., 2017)	Whole brain VBM (FSL)	178 SAD vs 213 HC	=	=	=	+	=
(Zhao et al., 2017)	Whole brain VBM (SPM) & whole brain CT using FreeSurfer	24 SAD vs 41 HC (and 37 MDD)	=	=	-	-	=

Abbreviations and symbols

=: no difference; +: increase; -: decrease; n.a.: not data available.

Amy: amygdala; CT: cortical thickness; GM: gray matter; HC: healthy control participants; HiC: hippocampus; MDD: patients with major depressive disorder; PD: patients with panic disorder; ROI: region of interest; SA: social anxiety; SAD: patients with social anxiety disorder; SVM: support vector machine; Thal: thalamus; VBM: voxel-based morphometry.

Table 5.1B Overview results of studies on GM in SAD; frontal and parietal regions.

	Frontal regions						Parietal regions		
Publication	**MPFC**	**DLPFC**	**VLPFC**	**OFC**	**PMC**	**ACC**	**PCC**	**Par**	**PC**
(Potts et al., 1994)	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a	n.a.	n.a.
(Cassimjee et al., 2010)	=	=	=	=	=	=	=	=	=
(Irle et al., 2010)	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
(Liao et al., 2011)	+	=	=	=	=	=	=	=	=
(Syal et al., 2012)	=	-	=	-	-	=	-	-	-
(Frick, Howner, Fischer, Eskildsen, et al., 2013)	=	=	=	=	=	pos. relation symptoms	=	=	=
(Meng et al., 2013)	=	=	=	=	=	=	=	=	-
(Talati, Pantazatos, et al., 2013) – sample 1	-	=	=	=	-	-	-	-	-
(Talati, Pantazatos, et al., 2013) – sample 2	=	-	=	-	+	=	=	+	=
(Brühl, Hänggi, et al., 2014)	=	+	=	=	=	+ ROI approach	=	+	+
(Frick, Gingnell, et al., 2014)	=	=	=	=	=	=	=	=	=
(Frick, Engman, et al., 2014)	=	=	=	=	=	=	pos. relation symptoms	=	=
(Irle et al., 2014)	=	=	=	=	+	=	=	both + and – (neg. relation LSAS avoidance)	both + and – (neg. relation LSAS avoidance)
(Machado-de-Sousa et al., 2014b)	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
(Talati et al., 2015)	=	=	=	=	=	=	=	=	=
(Tükel et al., 2015)	=	=	=	=	=	=	=	+	+
(Månsson et al., 2016, 2017)	- after treatment	=	=	=	=	=	=	=	- after treatment

Table 5.1B Overview results of studies on GM in SAD; frontal and parietal regions. (*continued*)

	Frontal regions						Parietal regions		
Publication	**MPFC**	**DLPFC**	**VLPFC**	**OFC**	**PMC**	**ACC**	**PCC**	**Par**	**PC**
(Steiger et al., 2017)	=	relation with treatment success	=	=	=	=	=	- after treatment	=
(Bas-Hoogendam, van Steenbergen, Pannekoek, et al., 2017)	=	=	=	=	=	=	=	=	=
(Zhao et al., 2017)	-	=	=	-	-	+	=	+	=

Abbreviations and symbols

=: no difference; +: increase; -: decrease; n.a.: not data available.

ACC: anterior cingulate cortex; CT: cortical thickness; DLPFC: dorsolateral prefrontal cortex; GM: gray matter; HC: healthy control participants; MPFC: medial prefrontal cortex; OFC: orbitofrontal cortex; Par: Parietal cortex; PC: (pre)cuneus; PCC: posterior cingulate cortex; PMC: premotor cortex; SAD: patients with social anxiety disorder; VLPFC: ventrolateral prefrontal cortex.

Table 5.1C Overview results of studies on GM in SAD; temporal and occipital regions and cerebellum.

	Temporal regions		Occipital regions		Cerebellum
Publication	**Ins**	**TC**	**Occ**	**FFG**	
(Potts et al., 1994)	n.a.	n.a.	n.a.	n.a.	n.a.
(Cassimjee et al., 2010)	=	- after treatment	=	=	- after treatment
(Irle et al., 2010)	n.a.	n.a.	n.a.	n.a.	n.a.
(Liao et al., 2011)	=	-	=	=	n.a.
(Syal et al., 2012)	-	-	=	-	n.a.
(Frick, Howner, Fischer, Eskildsen, et al., 2013)	=	+	=	+	n.a.
(Meng et al., 2013)	=	=	=	=	n.a.
(Talati, Pantazatos, et al., 2013) sample 1	=	+	+	+	n.a.
(Talati, Pantazatos, et al., 2013) – sample 2	=	both – and +	=	=	n.a.
(Brühl, Hänggi, et al., 2014)	+ (ROI approach, uncorrected)	+ (ROI approach, uncorrected)	=	=	n.a.
(Frick, Gingnell, et al., 2014)	=	=	=	=	n.a.
(Frick, Engman, et al., 2014)	=	=	+	+	n.a.
(Irle et al., 2014)	=	=	=	=	n.a.
(Machado-de-Sousa et al., 2014b)	n.a.	n.a.	n.a.	n.a.	n.a.
(Talati et al., 2015)	=	=	=	=	+ after treatment
(Tükel et al., 2015)	=	+	=	+	n.a.
(Månsson et al., 2016, 2017)	=	=	=	=	n.a.
(Steiger et al., 2017)	=	=	- after treatment	=	n.a.
(Bas-Hoogendam, van Steenbergen, Pannekoek, et al., 2017)	=	=	=	=	n.a.
(Zhao et al., 2017)	+	+	=	=	n.a.

Abbreviations and symbols

=: no difference; +: increase; -: decrease; n.a.: not data available. FFG: fusiform gyrus; Ins: insula; Occ: occipital cortex; TC: temporal cortex.

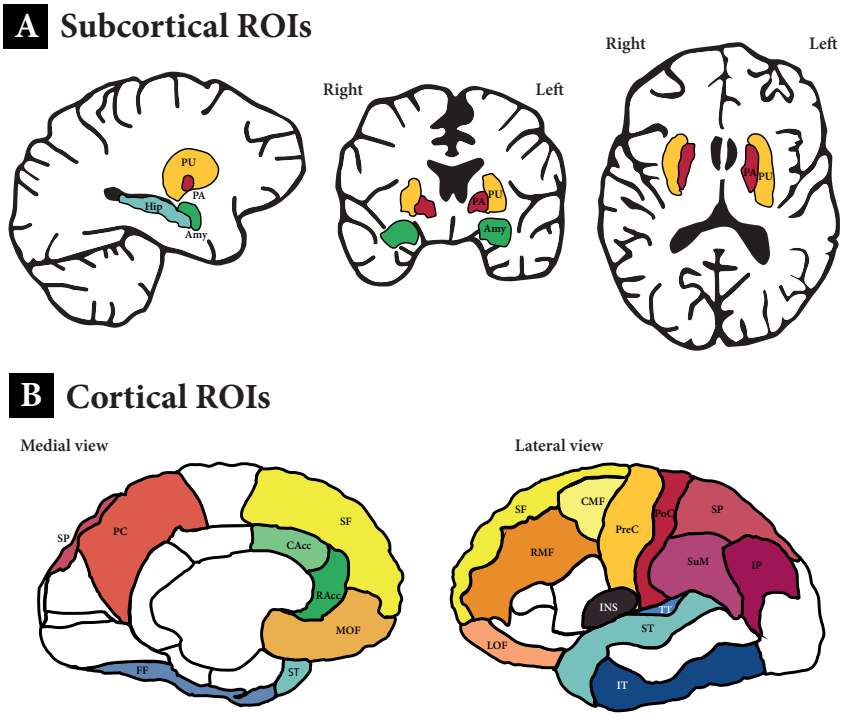


Figure 5.1 Subcortical (A) and cortical (B) regions of interest (ROIs).
Figure 5.1A Amy: amygdala; Hip: hippocampus; PA: pallidum; PU: putamen.
Figure 5.1B **Frontal regions (yellow)**: CMF: caudal middle frontal; LOF: lateral orbitofrontal; MOF: medial orbitofrontal; PreC: precentral; RMF: rostral middle frontal; SF: superior frontal.
Anterior cingulate (green): CAcc: caudal anterior cingulate; RAcc: rostral anterior cingulate.
Insula (purple): INS: insula.
Parietal regions (red): IP: inferior parietal; PC: precuneus; PoC: postcentral; SuM: supramarginal; SP: superior parietal.
Temporal regions (blue): FF: fusiform gyrus; IT: inferior temporal; ST: superior temporal; TT: transverse temporal.

Materials and methods

Participants

Participants included families genetically enriched for SAD, who were part of the LFLSAD (total sample: n = 132, from nine families). The background, objectives and methods of this multiplex, multigenerational family study, as well as the clinical characteristics of the LFLSAD sample and an a priori power analysis are extensively described elsewhere (Bas-Hoogendam, Harrewijn, et al., 2018); in addition, a pre-registration of the study is available online at https://osf.io/e368h/ (Bas-Hoogendam et al., 2014a).

In brief, the LFLSAD sample consists of families who were selected based on the presence of a primary diagnosis of SAD in a parent (aged 25-55 years old; the so-called 'proband') with a child, living at home and aged 8-21 years of age ('proband's SA-child') who met criteria for clinical or subclinical SAD. The age-criterion was based on the fact that adolescence appears to be a critical period for the development of clinical levels of SAD (Haller et al., 2015; Miers et al., 2014), while we used the 'living at home' criterion to minimize the impact of environmental influences, other than the family environment, on the child's phenotype and on the gene-environment interaction, in order to optimize the ability to detect the genotype-endophenotype-phenotype connection.

In addition to the proband and proband's SA-child, the proband's partner and other children from this nuclear family (aged 8 years or older), as well as the proband's sibling(s), with their partners and children (aged 8 years or older) were invited to participate. This way, the sample consisted of family members of two generations (generation 1: generation proband; generation 2: generation proband's SA-child), as depicted in *Figure 5.2*.

Exclusion criteria for the LFLSAD were comorbidity other than internalizing disorders in the proband or proband's SA-child, especially developmental disorders like autism; other family members were included independent from the presence of psychopathology. Furthermore, general MRI contraindications, like metal implants, pregnancy or dental braces, were exclusion criteria for the MRI experiment.

Although we collected MRI data from nine families (n = 113) (Bas-Hoogendam, Harrewijn, et al., 2018), data from one family were excluded from the present analysis, as the proband from this family was not able to participate in the MRI experiment due to an MRI

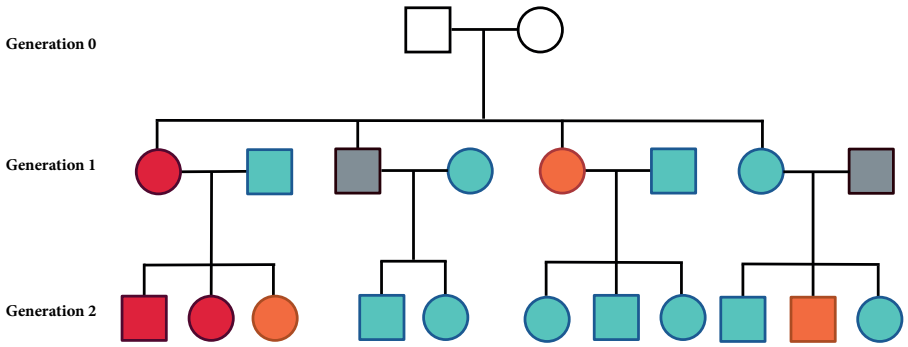


Figure 5.2 Example of a family within the Leiden Family Lab study on Social Anxiety Disorder.
Families were included based on the combination of a parent with SAD ('proband'; depicted in red) and a proband's child with SAD (red) or subclinical SA (orange). In addition, family members of two generations were invited, independent from the presence of SAD within these family members (no SAD: light blue; did not participate: gray). Grandparents (generation 0; white) were not invited for participation. This family is slightly modified to guarantee anonymity; however, the number of family members and the frequency of (sub)clinical SAD are depicted truthfully. Squares and circles represent men and women, respectively.

contraindication, which limited the analyses on the data of this proband's family members (n = 3). Therefore, the remaining sample consisted of 110 family members (56 males) from eight families (mean number of participating family members per family: 13.8; range 5 – 28). These family members were, according to the design, divided over two generations (generation 1: n = 51, 24 males; age (mean ± SD, range) 46.5 ± 6.7 years, 34.3 – 61.5 years; generation 2: n = 59, 32 males, age 18.1 ± 6.0 years, 9.0 – 32.2 years) who differed significantly in age (β = -30.3, $p < 0.001$), but not in male / female ratio ($\chi^2(1) = 0.56$, $p = 0.57$).

Ethics

The LFLSAD was approved by the Medical Ethical Committee of the Leiden University Medical Center (P12.061). Prior to entering the study, interested family members received verbal and written information on the objectives and procedure of the study; information letters were age-adjusted, to make them understandable for participants of all ages. All participants provided informed consent according to the Declaration of Helsinki; both parents signed the informed consent form for their children, while children between 12 and 18 years of age signed the form themselves as well. Every participant received €75 for participation in the LFLSAD (duration whole test procedure, including breaks: 8 hours) and travel expenses were reimbursed. Furthermore, participants were provided with lunch/dinner, snacks and drinks during their visit to the lab. Confidentiality of the research data was maintained by the use of a unique research ID number for each participant.

Data collection LFLSAD: extensive phenotyping

All included family members participated in a range of measurements, as described in Bas-Hoogendam et al. (2018). The presence of DSM-IV diagnoses, with special attention to (sub)clinical SAD, was determined using the Mini-International Neuropsychiatric Interview (M.I.N.I.)-Plus (version 5.0.0) (Sheehan et al., 1998; van Vliet & de Beurs, 2007) or the M.I.N.I.-Kid interview (version 6.0) (Bauhuis et al., 2013; Sheehan et al., 2010); these interviews were conducted by experienced clinicians and were recorded. The diagnosis of clinical SAD was established using the DSM-IV-TR criteria for the generalized subtype of SAD, but the clinician verified whether the DSM-5 criteria for SAD were also met. A diagnosis of subclinical SAD was established when participants met the criteria for SAD as described in the DSM-5, but did not show impairing limitations in important areas of functioning (criterion G) (American Psychiatric Association, 2013).

Furthermore, participants completed age-appropriate questionnaires on several anxiety-related constructs, including, among others, the *level of self-reported social anxiety symptoms* (Liebowitz Social Anxiety Scale (LSAS-SR) (Fresco et al., 2001; Mennin et al., 2002) or the Social Anxiety Scale for adolescents (SAS-A) (La Greca & Lopez, 1998)), the *intensity of fear of negative evaluation* (revised Brief Fear of Negative Evaluation (BFNE – II

scale) (Carleton et al., 2006; Leary, 1983)) and the *level of trait anxiety* (State-Trait Anxiety Inventory (STAI) (Spielberger et al., 1970)).

The severity of *self-reported depressive symptoms* was evaluated using the Beck Depression Inventory (BDI – II) (Beck et al., 1996; Van der Does, 2002) or the Children's Depression Inventory (CDI) (Kovacs, 1985; Timbremont & Braet, 2002). In order to enable analysing the scores of the age-specific questionnaires, *z*-scores were computed as described previously (Bas-Hoogendam, Harrewijn, et al., 2018). In addition, an estimate of *cognitive functioning* was obtained using two subtests of the Wechsler Adult Intelligence Scale IV (WAIS-IV) (Wechsler et al., 2008) or Wechsler Intelligence Scale for Children III (WISC) (Wechsler, 1991), consisting of the similarities (verbal comprehension) and block design (perceptual reasoning) subtests.

MRI procedure and data acquisition

Prior to the MRI scan, all participants were informed about the MRI safety procedures and they were told that they could refrain from continuing the experiment at any time. Children and adolescents were familiarized with the MRI scanner using a mock scanner (Galván, 2010). State anxiety was assessed before and after the MRI scan by a Dutch-translation of the STAI (Spielberger et al., 1970). Scanning was performed using a 3.0 T Philips Achieva MRI scanner (Philips Medical Systems, Best, The Netherlands), equipped with a 32-channel Sensitivity Encoding (SENSE) head coil.

The MRI session (total duration of the MRI protocol: 54 min 47 s) consisted of several structural and functional scans, as described in the design paper on this project (Bas-Hoogendam, Harrewijn, et al., 2018). Of interest for the present work is a high-resolution T1-weighted scan, with the following characteristics: 140 slices, resolution 0.875 mm × 0.875 mm × 1.2 mm, FOV = 224 mm × 168 mm × 177.333 mm, TR = 9.8 ms, TE = 4.59 ms, flip angle = 8∘. All structural MRI scans were inspected by a neuroradiologist; no clinically relevant abnormalities were reported in any of the participants.

MRI processing

Reconstruction of cortical surface, cortical parcellation and cortical thickness estimation, as well as segmentation of subcortical brain structures, was performed using standard procedures in the FreeSurfer software (version 5.3). This software is documented and freely available for download online (http://surfer.nmr.mgh.harvard.edu/) and the technical details of these procedures are described elsewhere (Dale, Fischl, & Sereno, 1999; Fischl et al., 2002; Fischl, Liu, & Dale, 2001; Fischl, Salat, et al., 2004; Fischl, van der Kouwe, et al., 2004; Fischl & Dale, 2000; Fischl, Sereno, & Dale, 1999; Fischl, Sereno, Tootell, & Dale, 1999). These procedures resulted in the extraction of volumes for seven bilateral subcortical GM regions (amygdala, caudate, hippocampus, nucleus accumbens, pallidum, putamen and thalamus) and the lateral ventricles, as well as in the segmentation of the cortex into 68 (34 left and

34 right) GM regions based on the Desikan-Killiany atlas (Desikan et al., 2006). For these regions, mean CT, defined as the closest distance from the gray/white boundary to the gray/cerebral spinal fluid boundary at each location of each participant's reconstructed cortical surface, as well as mean CSA, was determined. The method for the measurement of CT have been validated against both histological analysis (Rosas et al., 2002) and manual measurements (Kuperberg et al., 2003; Salat et al., 2004), and FreeSurfer morphometric procedures have been demonstrated to show good test-retest reliability across scanner manufacturers and across field strengths (Han et al., 2006; Reuter, Schmansky, Rosas, & Fischl, 2012). Subcortical ROIs in the current study were the amygdala, hippocampus, pallidum and putamen; cortical ROIs were the superior frontal gyrus, the caudal middle frontal gyrus, the rostral middle frontal gyrus, the lateral orbitofrontal gyrus, the medial orbitofrontal gyrus, the precentral gyrus, the caudal anterior cingulate, the rostral anterior cingulate, the insula, the superior parietal gyrus, the inferior parietal cortex, the precuneus, the supramarginal gyrus, the postcentral gyrus, the temporal pole, the inferior temporal gyrus, the superior temporal gyrus, the fusiform gyrus and the transverse temporal gyrus (*Figure 5.1*).

Both the subcortical segmentations as well as the segmentations of the cortical GM regions were visually inspected for accuracy and statistically evaluated for outliers according to standardized protocols designed to facilitate harmonized image analysis across multiple sites (http://enigma.ini.usc.edu/protocols/imaging-protocols/). This quality control resulted in the exclusion of, on average, 2.0 % (SD: 4.0 %) of the segmentations per participant for the subcortical measures (absolute number: 0.3 segmentations, range: 0 – 3; SD: 0.6) and 3.4 % (SD: 3.2 %) of the segmentations per participant for the cortical measures (absolute number: 2.3 segmentations, range: 0 - 8; SD: 2.2). In addition, data of one participant (age 9.0 y, generation 2) had to be excluded completely from the analyses because FreeSurfer was not able to reliably reconstruct the brain from the T1-weighted scan. This was due to excessive movement during data acquisition, which was present during both the structural as well as the functional MRI scans of this participant (relative motion parameters exceeded 2.5 mm) (Savalia et al., 2016).

Data of the FreeSurfer segmentations are available at https://osf.io/m8q2z (Bas-Hoogendam, van Steenbergen, Tissier, et al., 2018a).

Statistical analysis

Incidental missing values on the self-report questionnaires were replaced by the mean value of the completed items. We investigated differences between participants with and without (sub)clinical SAD by fitting regression models in R (R Core Team, 2016), with (sub)clinical SAD as the independent variable and the outcomes of the self-report questionnaires (self-reported social anxiety (*z*-score), fear of negative evaluation, level of trait anxiety and level of state anxiety before and after the MRI scan) as dependent variables of interest. Gender and age were included as covariates, and genetic correlations between family members were

modeled by including random effects. *P*-values were corrected for multiple comparisons (seven tests, Bonferroni corrected *p*-value = 0.007). In addition, we compared the presence of (comorbid) psychopathology between participants in the (sub)clinical SAD and no SAD group by performing chi-square tests using IBM SPSS Statistics for Windows (Version 23.0. Armonk, NY: IBM Corp.), while applying a Bonferroni-corrected *p*-value ($p = 0.005$ [10 tests]).

Next, we investigated whether GM brain characteristics are candidate endophenotypes of SAD by focusing on two endophenotype criteria. First, the *co-segregation of the candidate endophenotype with the disorder within families* (first element of endophenotype criterion 4) was examined by performing multiple regression using a linear mixed model in R (R Core Team, 2016). (Sub)clinical SAD was used as the independent variable, as we considered the clinical and subclinical SAD cases to reflect the same phenotype; the GM brain characteristics (subcortical volumes; CT; CSA) were dependent variables. Again, correlations between family members were modeled by including random effects; age (centered) and gender were included as covariates of no interest. In addition, total ICV (centered), mean global cortical thickness (GCT) (centered), or total global cortical surface area (GCSA) (centered) were added as covariates for the analyses on subcortical volumes, CT, and CSA, respectively. Furthermore, in order to obtain a reliable estimate of the main effect of (sub)clinical SAD, a (sub)clinical SAD-by-age interaction term as well as an analysis-dependent interaction term ((sub)clinical SAD-by-total ICV; (sub)clinical SAD-by-mean GCT; (sub)clinical SAD-by-total GCSA) were included in the model. As data on the presence of subclinical SAD were, due to technical reasons, lost for eight family members, data from these participants could not be used for this analysis (remaining sample: $n = 101$). For reasons of completeness, we also investigated the relationship between GM brain characteristics and two continuous measures of social anxiety: self-reported levels of social anxiety (*z*-scores, based on the LSAS and SAS-A) and levels of fear of negative evaluation (FNE) (sample: $n = 109$).

Because of the non-normal distribution of most of the dependent variables, we confirmed the robustness of the used linear mixed model by checking the distribution of the residuals of the phenotypes showing significant results using Shapiro-Wilk normality tests in R; results showed that these residuals followed a normal distribution. Analyses were corrected for multiple comparisons at a false discovery rate (FDR) of 5 % (Benjamini & Hochberg, 1995). In addition to these analyses of interest, we performed two sensitivity analyses to examine whether the results of the association analyses were driven by (comorbid) psychopathology other than SAD or by the severity of depressive symptoms as measured by the BDI-II or the CDI. Therefore, we excluded all participants with past and / or present (comorbid) psychopathology other than SAD (sensitivity analysis 1; note however, that the results may be biased, as the majority of the probands, on which the selection of the families was based, were excluded as well) or added the *z*-score of the level of depressive symptoms as a covariate in the analyses (sensitivity analysis 2).

Second, the *heritability* of the GM brain characteristics (h^2) was estimated (endophenotype criterion 3), by jointly modelling the GM brain characteristics and SAD (on which the selection of the families was based). Random effects were included to model the familial relationships (Tissier et al., 2017). Age (centered and standardized), gender and total ICV (centered and standardized; analyses on subcortical volume), mean GCT (centered and standardized; analyses on CT) or total GCSA (centered and standardized; analyses on surface area) were included as covariates. This approach takes the ascertainment process into account. We tested whether the genetic variance was significantly different from zero (cf. (Ganjgahi et al., 2015)) by using likelihood ratio tests. Significance levels are reported for heritability estimates > 0.10. Again, a FDR of 5 % was applied.

Results

Sample characteristics

Characteristics of the sample are summarized in *Table 5.2*. Seventeen participants were diagnosed with clinical SAD, while an additional 22 were classified as having subclinical SAD (total group (sub)clinical SAD n = 39); the validity of these diagnoses was substantiated by the scores on the self-report questionnaires as described previously (Bas-Hoogendam, Harrewijn, et al., 2018). The family members with (sub)clinical SAD did not differ from family members without SAD (n = 62) with respect to male / female ratio, age and estimated IQ. However, family members in the (sub)clinical SAD group were more often diagnosed with depression (past) and dysthymia (present), although these differences were not significant at a Bonferroni-corrected p-value. In addition, the prevalence of depressive episodes within the sample as a whole was in the range of the general population (Jacobi et al., 2004; Vandeleur et al., 2017), as reported in the design paper on the LFLSAD (Bas-Hoogendam, Harrewijn, et al., 2018). Furthermore, participants with (sub)clinical SAD self-reported significantly higher levels of social anxiety, FNE, trait anxiety, and increased levels of depressive symptoms. Groups did not differ with respect to state anxiety related to the MRI scan. None of the participants with SAD received treatment for the disorder before entering the study (Bas-Hoogendam, Harrewijn, et al., 2018).

General imaging phenotypes

Values of general imaging phenotypes are presented in *Table 5.3*. Participants with and without (sub)clinical SAD did not differ with respect to total ICV, mean GCT and total GCSA, but there were effects of age and gender on these phenotypes, in line with previous findings (Gennatas et al., 2017; Mutlu et al., 2013).

Table 5.2 Characteristics of participants with and without (sub)clinical SAD.

	(Sub)clinical SAD (*n* = 39)	No SAD (*n* = 62)	Statistical analysis
Demographics			
Male / Female (n)	20 / 19	31 /31	$\chi^2(1) = 0.02$, $p = 1.00$
Generation 1 / Generation 2 (n)	19 / 20	27 / 35	$\chi^2(1) = 0.26$, $p = 0.68$
Age in years (mean ± SD); range	30.3 ± 15.5; 9.2 – 59.6	31.3 ± 15.2; 9.4 – 61.5	β (± SE) = -1.0 ± 3.1, $p = 0.76$
Estimated IQ (mean ± SD)	104.3 ± 12.2	105.6 ± 10.5	β (± SE) = -2.1 ± 2.2, $p = 0.33$
Diagnostic information (*n*)			
Clinical SAD	17	0	$\chi^2(1) = 32.5$, $p < 0.001$ **
Depressive episode - present	1	1	$\chi^2(1) = 0.15$, $p = 1.00$
Depressive episode - past	12	9	$\chi^2(1) = 4.8$, $p = 0.04$ *
Dysthymia - present	3	0	$\chi^2(1) = 5.3$, $p = 0.05$ *
Dysthymia - past	1	1	$\chi^2(1) = 0.2$, $p = 1.00$
Panic disorder lifetime	5	2	$\chi^2(1) = 3.9$, $p = 0.10$
Agoraphobia - present	3	2	$\chi^2(1) = 1.2$, $p = 0.35$
Agoraphobia - past	0	2	$\chi^2(1) = 1.2$, $p = 0.53$
Separation anxiety	0	1	$\chi^2(1) = 0.8$, $p = 1.00$
Specific phobia	2	3	$\chi^2(1) = 0.02$, $p = 1.00$
Generalized anxiety disorder - present	1	0	$\chi^2(1) = 1.7$, $p = 0.37$
Self-report measures (mean ± SD)			
Social anxiety symptoms (z-score)	3.0 ± 3.3	0.6 ± 1.5	β (± SE) = 2.6 ± 0.5, $p < 0.001$ **
FNE	23.3 ± 12.3	12.8 ± 8.0	β (± SE) = 10.6 ± 1.9, $p < 0.001$ **
Depressive symptoms (z-score)	0.0 ± 0.9	-0.5 ± 0.7	β (± SE) = 0.5 ± 0.2, $p < 0.001$ **
STAI - trait	38.8 ± 9.4	33.1 ± 8.5	β (± SE) = 5.5 ± 1.8, $p = 0.002$ **
STAI - state pre scan	35.2 ± 7.5	32.2 ± 8.8	β (± SE) = 2.8 ± 1.6, $p = 0.08$
STAI - state post scan	30.8 ± 6.4	28.5 ± 6.4	β (± SE) = 2.2 ± 1.3, $p = 0.09$

Abbreviations

FNE: fear of negative evaluation; SAD: social anxiety disorder; SD: standard deviation; SE: standard error; STAI: state-trait anxiety inventory.

Statistical significance

* Significant at uncorrected *p*-value of 0.05.

** Significant at Bonferroni corrected *p*-value.

Volumes of subcortical brain structures

Using three different models, we investigated whether indices of social anxiety ((sub)clinical SAD, *z*-score of SA, and FNE) were associated with volumes of the subcortical ROIs. Results of the analyses are displayed in *Table 5.4* and *Supplemental Table S5.1*. There were

Table 5.3 General imaging characteristics participants with and without (sub)clinical SAD.

			Effect of (sub)clinical SAD[b]			Effect of social anxiety (*z*-score)[b]			Effect of FNE[b]			Effect of age[b,c]			Effect of gender[b,c]		
	(Sub)clinical SAD[a]	No SAD[a]	β	SE	*p*	β	SE	*p*	β	SE	*p*	β	SE	*p*	β	SE	*p*
Total ICV	1599832.3 ± 161567.6	1628908.4 ± 163820.3	-0.06	0.07	0.41	0.05	0.07	0.49	-0.07	0.07	0.27	-0.13	0.06	0.04*	-0.70	0.07	<0.001 **
Mean GCT	2.55 ± 0.13	2.54 ± 0.14	0.05	0.06	0.45	0.01	0.06	0.88	-0.03	0.06	0.66	-0.69	0.05	<0.001 **	0.07	0.06	0.28
Total GCSA	174163.3 ± 16561.2	176417.4 ± 17792.7	0.00	0.07	0.99	0.05	0.06	0.38	0.02	0.06	0.71	-0.38	0.05	<0.001 **	-0.59	0.07	<0.001 **

Abbreviations

FNE: fear of negative evaluation; GCSA: global cortical surface area (mm^2); GCT: global cortical thickness (mm); ICV: intracranial volume (mm^3); SAD: social anxiety disorder; SE: standard error.

Footnotes

Main effects of (sub)clinical SAD, social anxiety (*z*-score) and FNE are corrected for age (centered), gender and family structure. Reported *p*-values are uncorrected for multiple comparisons.

[a] Uncorrected mean ± standard deviation.

[b] Coefficients represent standardized values .

[c] Effects of age and gender are reported for the models including (sub)clinical SAD, but are comparable to the effects of these covariates in the models including social anxiety (*z*-score) and FNE. Values of the covariates are reported in *Supplemental Table S5.1*.

Statistical significance

* Significant at uncorrected *p*-value of 0.05.

** Significant at Bonferroni-corrected *p*-value.

Table 5.4 Effects of social anxiety on volumes of subcortical ROIs; heritability estimates.

				Effect of (sub) clinical SAD[b]			Effect of social anxiety (*z*-score)[b]			Effect of FNE[b]			Heritability estimate	
		(Sub)clinical SAD[a]	No SAD[a]	β	SE	*p*	β	SE	*p*	β	SE	*p*	h^2	*p*
Amygdala	L	1511.0 ± 150.4	1552.9 ± 190.7	-0.11	0.08	0.19	0.04	0.08	0.58	-0.01	0.08	0.94	0.34	0.009 **
	R	1515.1 ± 192.3	1541.4 ± 196.8	-0.04	0.08	0.62	0.07	0.08	0.40	0.05	0.08	0.55	< 0.10	n.a.
Hippocampus	L	5009.3 ± 611.3	5150.4 ± 544.7	-0.09	0.08	0.26	0.01	0.08	0.89	0.07	0.08	0.39	0.37	0.001 **
	R	4782.6 ± 547.2	4782.3 ± 494.5	0.04	0.08	0.65	0.00	0.08	0.98	0.08	0.08	0.33	0.29	$1.1*10^{-5}$**
Pallidum	L	1777.5 ± 283.8	1711.3 ± 256.1	0.08	0.08	0.35	0.21	0.08	0.01 *	0.21	0.08	0.01 *	0.28	0.038 *
	R	1516.4 ± 220.0	1497.8 ± 203.5	0.00	0.08	0.96	0.08	0.08	0.33	0.12	0.08	0.13	0.45	$1.7*10^{-5}$ **
Putamen	L	6741.4 ± 1028.2	6480.6 ± 931.6	0.15	0.09	0.09	0.07	0.08	0.40	0.08	0.09	0.35	< 0.10	n.a.
	R	5103.5 ± 688.4	5153.7 ± 568.2	-0.04	0.07	0.57	0.07	15.9	0.54	0.06	0.06	0.39	0.61	$5.5*10^{-6}$ **

Abbreviations

FNE: fear of negative evaluation; L: left; n.a.: not applicable; R: right; SAD: social anxiety disorder; SE: standard error.

Footnotes

Main effects of (sub)clinical SAD, social anxiety (*z*-score) and FNE are corrected for age (centered), gender, total intracranial volume (centered) and family-structure. Furthermore, the models including (sub)clinical SAD contained the interaction terms (sub)clinical SAD-by- age (centered) and (sub)clinical SAD-by-total intracranial volume (centered). Values of the covariates are reported in *Supplemental Table S5.1*. Reported *p*-values are uncorrected for multiple comparisons.

[a] Uncorrected mean ± standard deviation.

[b] Coefficients represent standardized values.

Statistical significance

* Significant at uncorrected *p*-value of 0.05.

** Significant at FDR-corrected *p*-value.

no significant associations between the indices of social anxiety and subcortical volumes at the FDR-corrected significance level, but there were positive relationships between the level of self-reported SA and FNE on the one hand and volume of the left pallidum at the other at an uncorrected significance level of $p < 0.05$ (*Figure 5.3A*). Furthermore, volume of the left pallidum was moderately heritable ($h^2 = 0.28$). Heritability estimates of the volumes of other subcortical ROIs are depicted in *Figure 5.4A* and listed in *Table 5.4*.

Cortical thickness of ROIs

Results of the analyses with respect to the thickness of cortical ROIs are displayed in *Table 5.5* and *Supplemental Table S5.1*. Again, we used three different models to test for associations between cortical thickness and, respectively, (sub)clinical SAD, self-reported levels of SA (*z*-score), and FNE. None of the associations was significant at the FDR-corrected significance level; at the uncorrected level ($p < 0.05$), indices of social anxiety were negatively correlated with CT of the right rostral middle frontal gyrus (effect of (sub)clinical SAD and effect of self-reported social anxiety), the left medial orbitofrontal cortex (effect of self-reported social anxiety), the right rostral ACC (effect of (sub)clinical SAD), the left and right superior temporal gyrus (effect of (sub)clinical SAD and effect of FNE, respectively) and the left fusiform gyrus (effect of self-reported social anxiety). Furthermore, there were positive relationships between social anxiety and CT of the left rostral ACC (effect of FNE), the right inferior parietal cortex (effect of (sub)clinical SAD), the left and right supramarginal gyrus (effect of (sub)clinical SAD and effect of FNE, respectively), the left temporal pole (effect of (sub)clinical SAD) and the left transverse temporal gyrus (effect of (sub)clinical SAD) (*Figure 5.3B*). It should be noted that there were significant interactions between (sub)clinical SAD and age with respect to the thickness of the right rostral middle frontal gyrus and the left supramarginal gyrus. These interactions are illustrated in *Supplemental Figure S5.1*.

Considering the regions showing an association between CT and social anxiety in the first place, heritability analyses revealed that CT of the left medial orbitofrontal cortex, the bilateral rostral ACC, the left superior temporal gyrus and the left transverse temporal gyrus displayed moderately high ($h^2 = 0.4 – 0.6$) or even high ($h^2 > 0.6$) heritability. Furthermore, CT of the left supramarginal gyrus and the right superior temporal gyrus had moderate heritability (h^2 between 0.2 and 0.4). These heritability estimates, as well as the estimates for ROIs in which there was no association with social anxiety, are illustrated in *Figure 5.4B* and summarized in *Table 5.5*.

Cortical surface area of ROIs

Results of the analyses with respect to the average CSA of the cortical ROIs are displayed in *Table 5.6* and *Supplemental Table S5.1*. There were no significant relationships between the measures of social anxiety at the corrected significance level, but self-reported social

Table 5.5 Effects of social anxiety on thickness of cortical ROIs; heritability estimates.

				Effect of (sub)clinical SAD[b]			Effect of social anxiety (*z*-score)[b]			Effect of FNE[b]			Heritability estimate	
		(Sub)clinical SAD[a]	No SAD[a]	β	SE	*p*	β	SE	*p*	β	SE	*p*	h^2	*p*
Superior frontal	L	2.93 ± 0.17	2.93 ± 0.20	-0.07	0.04	0.07	-0.07	0.04	0.10	0.01	0.04	0.85	0.11	n.s.
	R	2.92 ± 0.17	2.93 ± 0.20	-0.06	0.04	0.15	0.00	0.04	1.00	0.02	0.04	0.63	< 0.10	n.a.
Caudal middle frontal	L	2.68 ± 0.16	2.65 ± 0.17	0.03	0.06	0.56	0.04	0.06	0.53	0.07	0.06	0.25	< 0.10	n.a.
	R	2.66 ± 0.16	2.63 ± 0.17	0.05	0.06	0.44	0.03	0.06	0.60	-0.02	0.06	0.73	< 0.10	n.a.
Rostral middle frontal	L	2.51 ± 0.17	2.51 ± 0.18	0.00	0.05	0.93	-0.04	0.05	0.41	0.06	0.05	0.26	< 0.10	n.a.
	R	2.44 ± 0.17	2.48 ± 0.18	-0.13	0.05	0.02 *	-0.12	0.05	0.03 *	-0.08	0.05	0.15	< 0.10	n.a.
Lateral orbitofrontal	L	2.80 ± 0.25	2.81 ± 0.20	-0.08	0.06	0.18	-0.06	0.05	0.28	-0.05	0.06	0.34	< 0.10	n.a.
	R	2.71 ± 0.23	2.72 ± 0.22	-0.04	0.06	0.51	-0.02	0.05	0.71	-0.01	0.05	0.86	0.20	n.s.
Medial orbitofrontal	L	2.60 ± 0.20	2.62 ± 0.21	-0.08	0.06	0.18	-0.12	0.06	0.04 *	0.06	0.06	0.32	0.48	0.035 *
	R	2.71 ± 0.28	2.67 ± 0.21	0.05	0.06	0.41	0.03	0.06	0.60	0.09	0.06	0.13	0.19	n.s.
Precentral	L	2.61 ± 0.17	2.59 ± 0.15	-0.01	0.06	0.90	0.02	0.06	0.75	0.03	0.06	0.62	0.22	n.s.
	R	2.59 ± 0.15	2.58 ± 0.16	-0.01	0.06	0.83	0.02	0.06	0.76	0.04	0.06	0.55	< 0.10	n.a.
Caudal anterior cingulate	L	2.96 ± 0.25	2.91 ± 0.26	0.05	0.09	0.58	0.12	0.09	0.16	0.02	0.09	0.80	< 0.10	n.a.
	R	2.72 ± 0.21	2.76 ± 0.25	-0.08	0.08	0.29	-0.15	0.08	0.06	-0.08	0.08	0.33	< 0.10	n.a.
Rostral anterior cingulate	L	3.13 ± 0.26	3.08 ± 0.25	0.05	0.07	0.51	-0.04	0.07	0.60	0.14	0.07	0.05 *	0.48	0.024 *
	R	3.05 ± 0.25	3.15 ± 0.23	-0.18	0.08	0.02 *	-0.07	0.08	0.36	0.09	0.08	0.27	0.48	0.016 **
Insula	L	3.16 ± 0.20	3.17 ± 0.20	-0.05	0.06	0.44	-0.07	0.06	0.23	0.02	0.06	0.77	0.43	0.001 **
	R	3.16 ± 0.21	3.15 ± 0.20	-0.01	0.07	0.93	-0.04	0.07	0.53	-0.06	0.07	0.38	0.29	0.046 *
Superior parietal	L	2.20 ± 0.14	2.19 ± 0.14	0.02	0.05	0.76	0.08	0.05	0.14	0.01	0.05	0.81	0.35	n.s.
	R	2.17 ± 0.16	2.15 ± 0.15	0.07	0.05	0.19	0.02	0.05	0.68	-0.02	0.05	0.63	0.53	0.002 **
Inferior parietal	L	2.53 ± 0.16	2.50 ± 0.16	0.08	0.05	0.11	-0.03	0.05	0.55	-0.03	0.05	0.52	0.35	n.s.
	R	2.56 ± 0.14	2.52 ± 0.15	0.11	0.05	0.03 *	0.07	0.05	0.12	0.01	0.05	0.86	< 0.10	n.a.
Precuneus	L	2.46 ± 0.21	2.44 ± 0.19	0.01	0.05	0.86	0.09	0.05	0.06	0.03	0.05	0.45	0.30	0.045 *
	R	2.45 ± 0.19	2.44 ± 0.20	-0.01	0.06	0.84	0.06	0.05	0.29	-0.06	0.05	0.28	< 0.10	n.a.

Table 5.5 Effects of social anxiety on thickness of cortical ROIs; heritability estimates. (*continued*)

				Effect of (sub)clinical SAD[b]			Effect of social anxiety (*z*-score)[b]			Effect of FNE[b]			Heritability estimate	
		(Sub)clinical SAD[a]	No SAD[a]	β	SE	*p*	β	SE	*p*	β	SE	*p*	h^2	*p*
Supramarginal	L	2.64 ± 0.17	2.58 ± 0.16	0.13	0.06	0.03 *	0.04	0.06	0.53	0.02	0.06	0.73	0.23	n.s.
	R	2.62 ± 0.17	2.58 ± 0.17	0.06	0.06	0.32	0.08	0.06	0.14	0.12	0.06	0.04 *	< 0.10	n.a.
Postcentral	L	2.11 ± 0.18	2.09 ± 0.12	0.02	0.07	0.77	0.08	0.07	0.26	0.02	0.07	0.78	0.19	n.s.
	R	2.03 ± 0.16	2.06 ± 0.13	-0.13	0.07	0.07	-0.03	0.07	0.66	-0.05	0.07	0.50	0.34	n.s.
Temporal pole	L	3.63 ± 0.27	3.49 ± 0.28	0.25	0.09	0.01 *	0.07	0.09	0.48	0.14	0.10	0.15	0.11	n.s.
	R	3.53 ± 0.36	3.50 ± 0.38	0.05	0.10	0.59	-0.02	0.09	0.85	-0.06	0.10	0.51	< 0.10	n.a.
Inferior temporal	L	2.80 ± 0.14	2.77 ± 0.19	0.06	0.07	0.38	0.02	0.06	0.74	-0.06	0.07	0.33	< 0.10	n.a.
	R	2.77 ± 0.15	2.78 ± 0.17	-0.05	0.07	0.47	0.01	0.07	0.89	-0.06	0.07	0.41	< 0.10	n.a.
Superior temporal	L	2.84 ± 0.17	2.87 ± 0.19	-0.21	0.07	0.002 *	-0.09	0.07	0.15	-0.08	0.06	0.21	0.74	7.5*10-5 **
	R	2.89 ± 0.15	2.89 ± 0.17	-0.07	0.06	0.28	-0.05	0.06	0.39	-0.17	0.06	0.01 *	0.23	n.s.
Fusiform	L	2.70 ± 0.16	2.71 ± 0.16	-0.06	0.06	0.34	-0.14	0.06	0.02 *	-0.09	0.06	0.15	< 0.10	n.a.
	R	2.69 ± 0.14	2.69 ± 0.17	0.00	0.06	0.97	0.02	0.06	0.69	0.07	0.06	0.26	< 0.10	n.a.
Transverse temporal	L	2.46 ± 0.30	2.34 ± 0.27	0.15	0.07	0.04 *	0.09	0.07	0.22	0.03	0.07	0.66	0.64	1.7*10-6 **
	R	2.49 ± 0.28	2.45 ± 0.33	0.02	0.08	0.83	0.01	0.08	0.89	0.03	0.08	0.72	0.47	0.001 **

Abbreviations

FNE: fear of negative evaluation; L: left; n.a.: not applicable; n.s.: not significant; R: right; SAD: social anxiety disorder; SE: standard error.

Footnotes

Main effects of (sub)clinical SAD, social anxiety (*z*-score) and FNE are corrected for age (centered), gender, mean global cortical thickness (centered) and family-structure. Furthermore, the models including (sub)clinical SAD contained the interaction terms (sub)clinical SAD* age (centered) and (sub)clinical SAD* mean global cortical thickness (centered). Values of the covariates are reported in *Supplemental Table S5.1*. Reported *p*-values are uncorrected for multiple comparisons.

[a] Uncorrected mean ± standard deviation.

[b] Coefficients represent standardized values.

Statistical significance

* Significant at uncorrected *p*-value of 0.05.

** Significant at FDR-corrected *p*-value.

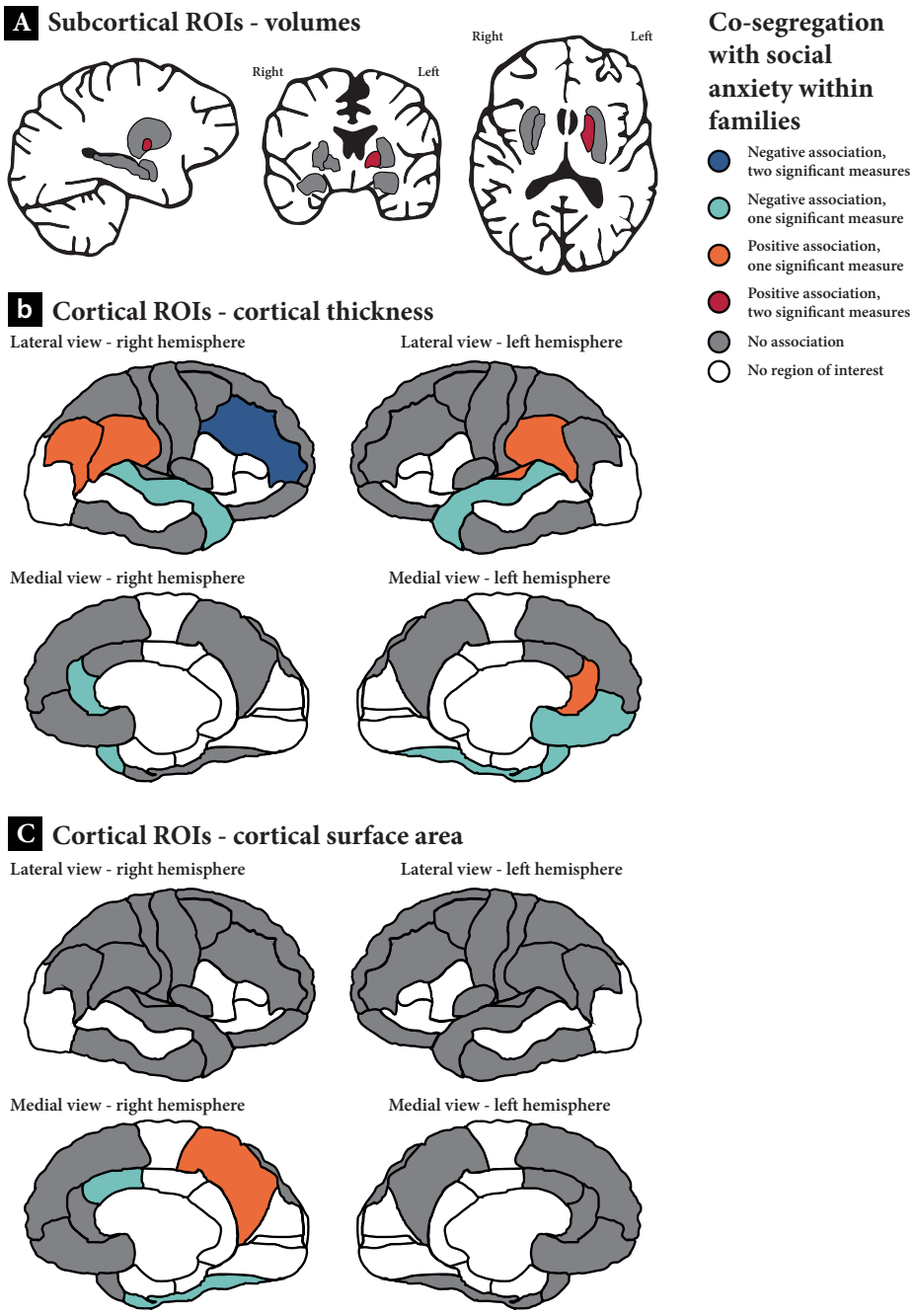


Figure 5.3 Relationship between indices of social anxiety and gray matter characteristics.

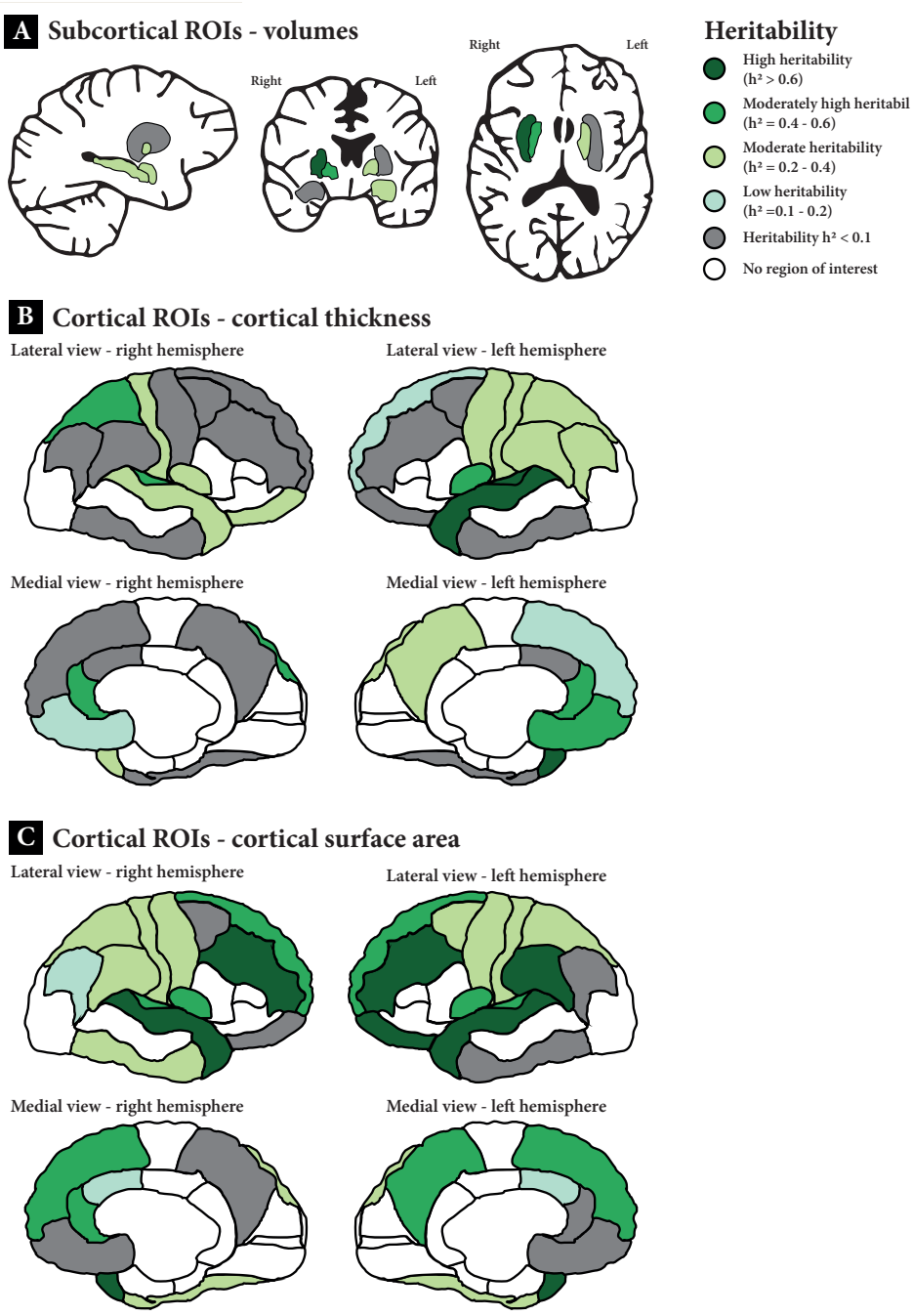


Figure 5.4 Heritability estimates of gray matter characteristics.

Table 5.6 Effects of social anxiety on surface area of cortical ROIs; heritability estimates.

				Effect of (sub)clinical SAD[b]			Effect of social anxiety (z-score)[b]			Effect of FNE[b]			Heritability estimate	
		(Sub)clinical SAD[a]	No SAD[a]	β	SE	*p*	β	SE	*p*	β	SE	*p*	h^2	*p*
Superior frontal	L	7412.1 ± 884.9	7448.6 ± 809.4	0.09	0.05	0.07	0.01	0.05	0.91	-0.01	0.05	0.89	0.59	0.003 **
	R	7112.2 ± 809.1	7268.8 ± 845.4	-0.04	0.05	0.44	0.00	0.05	0.94	0.07	0.05	0.15	0.48	0.001 **
Caudal middle frontal	L	2416.5 ± 360.0	2398.2 ± 400.2	0.05	0.07	0.46	0.08	0.07	0.24	0.10	0.07	0.13	0.31	0.036 *
	R	2241.5 ± 371.9	2206.5 ± 355.9	0.07	0.07	0.36	0.02	0.07	0.83	0.01	0.07	0.90	0.54	0.057
Rostral middle frontal	L	5947.1 ± 805.4	6080.3 ± 862.2	0.00	0.05	0.97	-0.02	0.05	0.59	0.00	0.04	0.97	0.66	2.7*10-4 **
	R	6277.7 ± 924.3	6347.6 ± 934.5	0.04	0.04	0.33	0.00	0.04	0.92	-0.01	0.04	0.83	0.79	1.6*10-5 **
Lateral orbitofrontal	L	2566.5 ± 215.2	2644.7 ± 287.5	0.03	0.06	0.56	0.08	0.06	0.16	-0.02	0.05	0.75	0.76	9.3*10-6 **
	R	2569.0 ± 260.1	2596.0 ± 282.4	0.01	0.08	0.94	0.01	0.07	0.90	-0.12	0.07	0.09	< 0.10	n.a.
Medial orbitofrontal	L	1913.4 ± 239.6	1942.0 ± 253.0	0.00	0.07	0.97	0.06	0.07	0.41	0.03	0.07	0.73	< 0.10	n.a.
	R	1872.9 ± 205.4	1872.6 ± 195.2	0.04	0.08	0.61	0.06	0.07	0.45	0.06	0.07	0.41	< 0.10	n.a.
Precentral	L	4821.7 ± 450.3	4887.3 ± 491.4	-0.04	0.06	0.54	0.02	0.06	0.77	0.05	0.06	0.45	0.26	0.035 *
	R	4924.3 ± 502.2	4925.0 ± 461.3	0.04	0.06	0.48	0.10	0.06	0.09	0.03	0.06	0.57	0.24	0.049 *
Caudal anterior cingulate	L	641.3 ± 120.6	678.0 ± 172.2	-0.05	0.08	0.54	0.06	0.08	0.43	-0.01	0.08	0.89	0.17	n.s.
	R	819.9 ± 178.4	834.2 ± 150.3	-0.02	0.09	0.79	-0.05	0.08	0.51	-0.16	0.08	0.05*	0.16	n.s.
Rostral anterior cingulate	L	828.2 ± 161.3	850.7 ± 155.6	-0.01	0.07	0.93	0.05	0.07	0.50	-0.01	0.07	0.94	< 0.10	n.a.
	R	673.7 ± 158.3	711.7 ± 117.4	-0.07	0.08	0.36	0.02	0.08	0.79	-0.06	0.08	0.42	0.44	0.002 **
Insula	L	2277.4 ± 188.0	2275.6 ± 225.0	0.04	0.06	0.56	0.00	0.06	0.98	-0.04	0.06	0.47	0.49	0.004 **
	R	2272.2 ± 271.2	2327.7 ± 262.0	-0.03	0.08	0.69	-0.08	0.07	0.30	0.06	0.07	0.39	0.45	0.002 **
Superior parietal	L	5611.9 ± 584.8	5609.0 ± 693.8	0.00	0.06	0.99	0.04	0.06	0.54	0.00	0.06	0.99	0.39	0.029 *
	R	5620.2 ± 734.3	5631.8 ± 664.2	0.00	0.07	0.98	0.06	0.06	0.35	0.09	0.07	0.19	0.35	n.s.
Inferior parietal	L	4751.4 ± 749.4	4880.4 ± 663.4	-0.02	0.06	0.78	-0.02	0.06	0.67	-0.01	0.05	0.78	< 0.10	n.a.
	R	5618.7 ± 811.9	5843.9 ± 918.8	-0.10	0.06	0.06	-0.09	0.05	0.10	-0.04	0.06	0.43	0.17	n.s.
Precuneus	L	3853.2 ± 504.3	3922.2 ± 504.2	0.00	0.05	0.94	0.02	0.05	0.73	0.02	0.05	0.66	0.47	2.4*10-5 **
	R	4063.1 ± 527.7	4095.5 ± 534.8	0.04	0.06	0.48	0.03	0.06	0.65	0.13	0.06	0.02*	< 0.10	n.a.

Table 5.6 Effects of social anxiety on surface area of cortical ROIs; heritability estimates. (*continued*)

				Effect of (sub)clinical SAD[b]			Effect of social anxiety (z-score)[b]			Effect of FNE[b]			Heritability estimate	
		(Sub)clinical SAD[a]	No SAD[a]	β	SE	*p*	β	SE	*p*	β	SE	*p*	h^2	*p*
Supramarginal	L	3966.7 ± 608.7	4110.0 ± 664.4	-0.09	0.06	0.16	-0.01	0.06	0.85	-0.02	0.06	0.67	0.75	1.1*10-6 **
	R	3895.4 ± 616.5	3922.6 ± 652.1	0.06	0.06	0.30	-0.03	0.06	0.62	-0.05	0.06	0.36	0.32	0.005 **
Postcentral	L	4249.8 ± 634.9	4323.8 ± 511.6	-0.02	0.06	0.70	-0.03	0.06	0.60	-0.02	0.06	0.79	0.29	0.034 *
	R	4086.7 ± 597.0	4147.1 ± 536.8	-0.01	0.06	0.89	0.03	0.06	0.63	-0.06	0.06	0.35	0.22	0.049 *
Temporal pole	L	474.7 ± 59.4	484.3 ± 64.4	-0.05	0.09	0.56	0.03	0.08	0.74	-0.02	0.09	0.82	0.15	0.028 *
	R	403.2 ± 64.7	407.1 ± 52.8	0.01	0.09	0.93	0.02	0.09	0.80	0.02	0.09	0.82	0.34	0.005 **
Inferior temporal	L	3544.0 ± 452.0	3583.9 ± 591.1	0.02	0.06	0.70	-0.02	0.06	0.75	-0.04	0.06	0.51	< 0.10	n.a.
	R	3314.4 ± 508.7	3367.0 ± 490.0	0.01	0.05	0.91	-0.02	0.06	0.69	-0.02	0.05	0.70	0.38	0.004 **
Superior temporal	L	3947.8 ± 457.5	3921.2 ± 476.2	0.06	0.06	0.30	0.04	0.06	0.49	0.07	0.05	0.20	0.92	5.8*10-6 **
	R	3773.6 ± 407.2	3675.4 ± 315.8	0.08	0.05	0.15	0.00	0.05	0.96	0.06	0.05	0.26	0.75	3.8*10-4 **
Fusiform	L	3405.1 ± 446.8	3371.7 ± 474.6	0.09	0.07	0.18	-0.08	0.07	0.22	0.01	0.06	0.92	0.34	0.004 **
	R	3162.8 ± 408.3	3274.9 ± 451.4	-0.09	0.06	0.13	-0.12	0.05	0.02*	-0.04	0.05	0.47	0.33	3.6*10-6 **
Transverse temporal	L	482.6 ± 75.0	495.4 ± 77.4	-0.03	0.08	0.71	-0.01	0.08	0.92	0.01	0.08	0.93	0.55	0.004 **
	R	361.1 ± 57.5	368.8 ± 67.7	-0.02	0.08	0.79	0.02	0.08	0.84	0.03	0.08	0.72	0.52	0.002 **

Abbreviations

FNE: fear of negative evaluation; L: left; n.a.: not applicable; n.s.: not significant; R: right; SAD: social anxiety disorder; SE: standard error.

Footnotes

Main effects of (sub)clinical SAD, social anxiety (*z*-score) and FNE are corrected for age (centered), gender, total global cortical surface area (centered) and family-structure. Furthermore, the models including (sub)clinical SAD contained the interaction terms (sub)clinical SAD* age (centered) and (sub)clinical SAD* total global cortical surface area (centered). Values of the covariates are reported in *Supplemental Table S5.1*. Reported *p*-values are uncorrected for multiple comparisons.

[a] Uncorrected mean ± standard deviation.

[b] Coefficients represent standardized values.

Statistical significance

* Significant at uncorrected *p*-value of 0.05.

** Significant at FDR-corrected *p*-value.

anxiety was negatively related to the CSA of the right fusiform gyrus at the uncorrected level. In addition, the level of FNE was negatively related to the CSA of the right caudal ACC and positively associated with CSA of the right precuneus (*Figure 5.3C*). Analyses on the heritability of CSA of these ROIs indicated that CSA of the right fusiform gyrus was moderately high ($h^2 = 0.33$). Heritability estimates of other ROIs are depicted in *Figure 5.4C* and listed in *Table 5.6*.

Sensitivity analyses

Results of the sensitivity analyses showed comparable associations between the indices of social anxiety and the GM characteristics as the main analyses of interest. That is, in both sensitivity analyses (sensitivity analysis 1: participants with past and/or present (comorbid) psychopathology other than SAD were excluded; remaining $n = 70$; sensitivity analysis 2: the level of depressive symptoms was added as a covariate), we found a positive association with volume of the left pallidum, changes in cortical thickness in frontal, parietal and temporal areas, as well as alterations in cortical surface area of the precuneus and fusiform gyrus (all at $p < 0.05$, uncorrected). These findings are illustrated in *Supplemental Figure S5.2* and *Supplemental Figure S5.3*; detailed statistics are available in *Supplemental Table S5.2* and *Supplemental Table S5.3*.

Other subcortical and cortical brain regions (non ROIs)

For reasons of completeness, results of the association analyses on subcortical and cortical regions that were not a priori selected (non ROIs) are reported in *Supplemental Table S5.4*. In brief, none of the subcortical non ROIs showed an association with any of the indices of social anxiety. With respect to the cortical measurements: cortical thickness was positively related to indices of social anxiety in some regions (right banks of the superior temporal sulcus, bilateral lingual gyrus, right lateral occipital gyrus and left pars triangularis), while indices of social anxiety were related to cortical surface area of the left parahippocampal gyrus, the right pars opercularis and the right banks of the superior temporal sulcus. However, none of these results survived multiple comparisons correction.

Discussion

Aim of the present study was to investigate whether structural gray matter (GM) brain characteristics could serve as candidate endophenotypes of social anxiety disorder (SAD) (Bas-Hoogendam et al., 2016). Data from the Leiden Family Lab study on Social Anxiety Disorder (LFLSAD) were used, as the multiplex, multigenerational family design of this study enables investigating two important endophenotype criteria (Bas-Hoogendam, Harrewijn, et al., 2018). First of all, we investigated whether the candidate endophenotypes

co-segregated with social anxiety within the families, by studying the association between GM characteristics and three indices of social anxiety in families genetically enriched for SAD: the diagnosis of (sub)clinical SAD, self-reported levels of social anxiety, and self-reported levels of fear of negative evaluation (FNE). Secondly, we examined the *heritability* of the GM phenotypes. We investigated subcortical brain volumes, cortical thickness (CT) measures and estimates of cortical surface area (CSA) and used a hypothesis-driven region of interest (ROI) approach, focusing on regions in which SAD-related alterations have been reported previously (*Figure 5.1*), although it should be noted that the results of these studies, as summarized in *Table 5.1*, often lack consistency. Findings of these analyses will be considered in the following. We start with reviewing GM characteristics meeting both criteria for being a candidate endophenotype of social anxiety, as they 1st *co-segregated with social anxiety within families*, and 2nd were at least moderately *heritable*. Next, we discuss the results of the association and heritability analyses in more detail, and consider them in the light of previous work.

Candidate endophenotypes of SAD

When combining the results of the association analyses with those of the heritability analyses, several GM characteristics turn out to be promising candidate endophenotypes of social anxiety, although it should be noted that the results of the association analyses did not survive correction for multiple comparisons. We summarized these findings in *Figure 5.5*. This figure illustrates that the structural changes in GM which are genetically related to SAD are widespread over the brain, as they involve subcortical (pallidum) as well as cortical areas, including frontal, parietal and temporal regions. Interestingly, several of these cortical areas, namely the medial orbitofrontal cortex, the ACC, the supramarginal gyrus and the fusiform gyrus, are part of the extended neurobiological model of SAD as proposed by Brühl and colleagues (Brühl, Delsignore, et al., 2014). This model of SAD, which is mainly based on data from functional MRI and the results of resting-state and functional connectivity studies, describes a hyperactive fear and anxiety circuit (Etkin, 2012; Etkin & Wager, 2007), consisting of the amygdala, insula, ACC, and prefrontal cortex, as well as hyperactive but less connected parieto-occipital regions. Furthermore, recent studies on connectivity showed widespread changes in functional networks in SAD (Cui et al., 2017; Yang et al., 2019; Yuan et al., 2018). Together, these findings converge with the results of the present study, as they indicate that the neurobiological brain changes related to SAD are not limited to the regions traditionally implicated in fear and anxiety, but are distributed in larger networks in the brain (cf. the recent commentary by Frick (2017)).

Although it is difficult to relate the structural alterations described here to functional brain changes, the results of functional MRI studies on SAD offer an interesting starting point. Most fMRI studies on SAD employ paradigms involving faces, as these are anxiety-provoking stimuli, and the results often point to increased brain responses in several brain

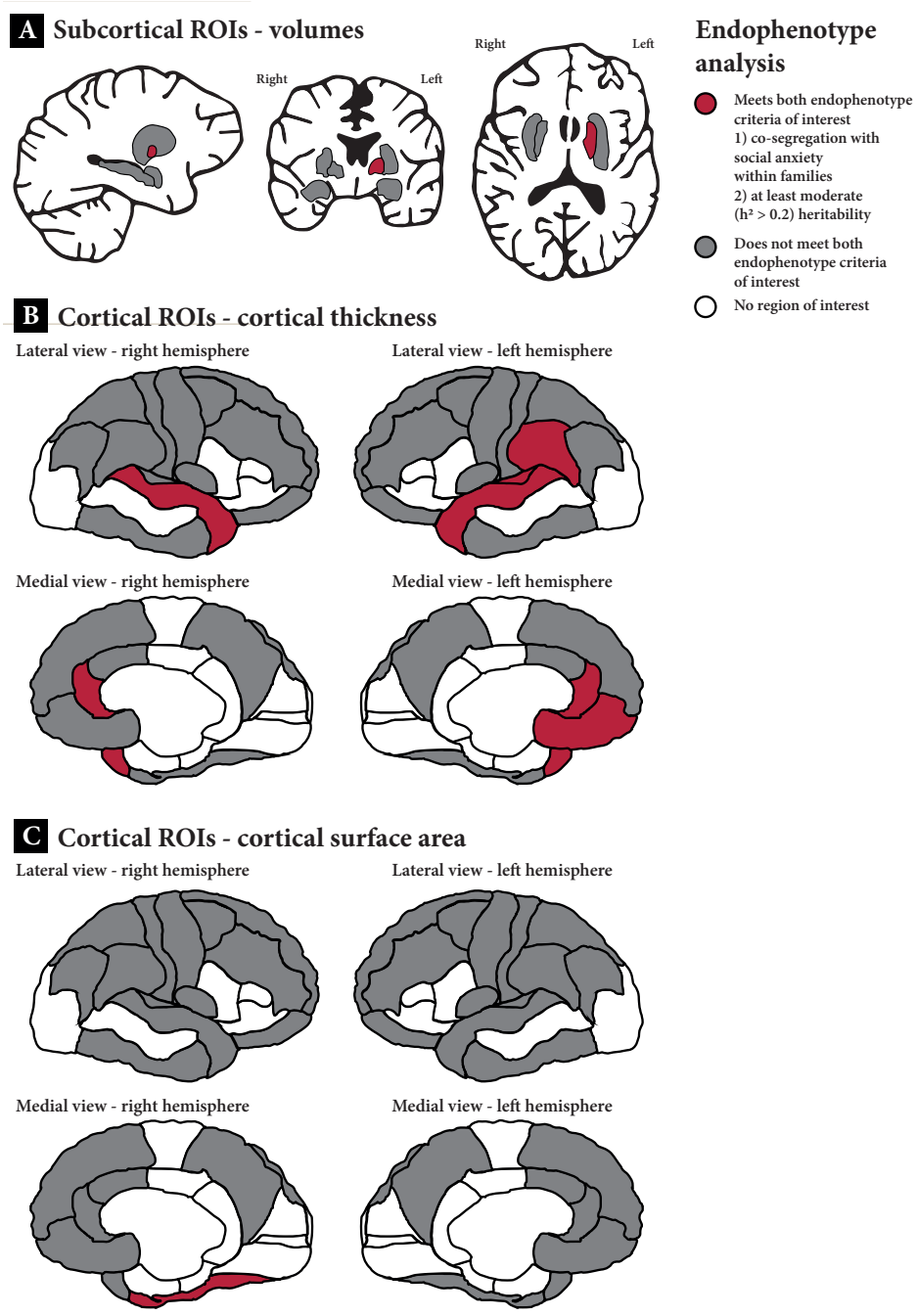


Figure 5.5 Overview of gray matter candidate endophenotypes of social anxiety.

areas, including the candidate endophenotype regions of the present study (*Figure 5.5*). The rostral ACC, for example, a region involved in emotional processing, resolving emotional conflicts and guiding socially-driven human interactions (Etkin et al., 2011; Etkin, Egner, Peraza, Kandel, & Hirsch, 2006; Lavin et al., 2013), showed increased activation in patients with SAD in response to angry (Blair, Geraci, Korelitz, et al., 2011), disgust (Amir et al., 2005) and sad faces (Labuschagne et al., 2011). Furthermore, several studies reported increased responsiveness of the superior temporal gyrus and the fusiform gyrus related to facial emotion processing in SAD (Binelli et al., 2014; Evans et al., 2008; Straube, Mentzel, & Miltner, 2005; Ziv, Goldin, Jazaieri, Hahn, & Gross, 2013), while increased activation of the medial orbitofrontal cortex was found when patients with SAD looked at harsh faces (Goldin, Manber, et al., 2009). However, as these results provide only indirect indications of the psychological alterations which might be related to the structural GM changes, future studies, for example using advanced MR sequences, are needed to gain more insight in the cellular bases of structural brain alterations (Lerch et al., 2017) and to link them more directly to functional brain changes related to SAD. In addition, animal studies, in particular in non-human primates, enabling a translational approach, should further advance our understanding of the molecular and genetic underpinnings of anxiety-related brain changes (cf. (Fox & Kalin, 2014; McGregor et al., 2018)).

Co-segregation of GM characteristics with social anxiety within families

When we consider the results of the association analyses, no significant associations between social anxiety and the GM characteristics were present at an FDR-corrected significance level (*Table 5.4, Table 5.5*, and *Table 5.6*). At an uncorrected significance level ($p < 0.05$), several interesting patterns with respect to the association with social anxiety emerged (*Figure 5.3*), which deserve to be discussed in detail.

To start with the *subcortical* ROIs, we found a positive association between both the level of self-reported social anxiety as well as with the level of self-reported FNE on the one hand and the volume of the left pallidum on the other (*Figure 5.3, Table 5.4*). This result is in line with findings of a mega-analysis on 174 patients with SAD and 213 healthy control participants, showing larger GM volume in the dorsal striatum, including the pallidum and the putamen; in this study, the increase in GM was positively related to the level of self-reported social anxiety (Bas-Hoogendam, van Steenbergen, Pannekoek, et al., 2017). Recently, the positive relationship between social anxiety and volume of the dorsal striatum was replicated in a sample of healthy young women with a broad range of social anxiety levels (Günther et al., 2018), while a study on the structural correlates of 'intolerance of uncertainty', a psychological construct that is related to anxiety, indicated a positive relationship between intolerance of uncertainty and bilateral striatal volume, in particular the putamen and pallidum (Kim et al., 2017). Interestingly, these findings and the increase in pallidum volume reported in the present work fit within the recent focus on the striatum

as being an important structure in the anxiety circuitry of the brain (Lago et al., 2017) and are potentially reflective of the role of the pallidum and putamen in processing emotions and reward (Arsalidou, Duerden, & Taylor, 2012), as both processes have been shown to be associated with altered brain activation levels in these regions in patients with SAD (Binelli et al., 2014; Cremers et al., 2015; Heitmann et al., 2016; Shah et al., 2009).

Next, we investigated *cortical* GM characteristics. We examined estimates of CT as well as of CSA separately, as these measures show different developmental courses, are genetically independent and have distinct associations with the risk of developing psychopathology (Bois et al., 2015; Chen et al., 2013; Gilmore et al., 2018; Hogstrom et al., 2013; Panizzon et al., 2009; Prasad et al., 2010; Tamnes et al., 2017; Wierenga et al., 2014; Winkler et al., 2010). Our results converge with these findings, as there were no cortical ROIs in which both the estimates of CT and CSA were associated with social anxiety (cf. *Figure 5.3B* and *Figure 5.3C*).

The analyses on CT (*Table 5.5*) revealed that social anxiety was related to cortical thickening of the left rostral ACC, the right inferior parietal cortex, the left and right supramarginal gyrus, the left temporal pole, and the left transverse temporal gyrus; furthermore, there were associations between social anxiety and cortical thinning of the right rostral middle frontal gyrus, the left medial orbitofrontal gyrus, the right rostral ACC, the bilateral superior temporal gyrus, and the left fusiform gyrus (*Figure 5.3B*). To facilitate the discussion, we summarized these findings together with the results of previous studies on the association between social anxiety and CT (Brühl, Hänggi, et al., 2014; Syal et al., 2012; Zhao et al., 2017) in *Table 5.7*. This summary shows the divergence of the results with respect to the relation between social anxiety and CT. That is, our results showing decreases in CT in frontal ROIs coincide with those of Syal et al. (2012) and Zhao et al. (2017), while Brühl and colleagues (2014) reported increased CT in frontal areas. The increases in CT in the left rostral ACC and several parietal regions found in the present study are in line with the results described by Brühl, Hänggi, et al. (2014) and Zhao et al. (2017), but it should be noted that Syal et al. (2012) outlined decreased CT in parietal regions; furthermore, the cortical thinning of the right rostral ACC of the present work has not been described previously. In addition, we found both increases as well as decreases in CT in the temporal ROIs; the increase in CT of the temporal pole corresponded to the results of Brühl, Hänggi, et al. (2014) and Zhao et al. (2017) (but note that Syal et al. reported a decrease in CT in this area), while the decreases in CT (superior temporal gyrus and fusiform gyrus) were in line with the data of Syal and colleagues (2012) and with the results of a voxel-based morphometry study involving 68 anxiety patients without comorbidity (van Tol et al., 2010). Furthermore, it should be mentioned that we could not replicate previous findings on SAD-related changes in CT in frontal areas like the superior frontal gyrus, the caudal middle frontal cortex, the lateral orbitofrontal gyrus, and the precentral gyrus, nor did we found changes in CT in the

Table 5.7 Summary of results with respect to the association between SAD and cortical thickness.

		Present work	Previous work		
		LFLSAD 39 (sub)clinical SAD with their family members (*n* = 62)	**Syal et al. (2012) 13 SAD vs 13 HC**	**Brühl et al. (2014) 46 SAD vs 46 HC**	**Zhao et al. (2017) 24 SAD vs 41 HC**
Frontal	*Superior frontal*	*n.s.*	-	+	+
	Caudal middle frontal	*n.s.*	-	+	+
	Rostral middle frontal	-	-	+	-
	Lateral orbitofrontal	*n.s.*	-	*n.s.*	-
	Medial orbitofrontal	-	-	*n.s.*	*n.s.*
	Precentral	*n.s.*	-	*n.s.*	-
ACC	*Caudal anterior cingulate*	*n.s.*	*n.s.*	+	+
	Rostral anterior cingulate	+ (left) and - (right)	*n.s.*	+	+
Insula	*Insula*	*n.s.*	-	+	+
Parietal	*Superior parietal*	*n.s.*	-	+	+
	Inferior parietal	+	*n.s.*	+	*n.s.*
	Precuneus	*n.s.*	-	+	*n.s.*
	Supramarginal	+	-	*n.s.*	+
	Postcentral	*n.s.*	-	*n.s.*	*n.s.*
Temporal	*Temporal pole*	+	-	+	+
	Inferior temporal	*n.s.*	-	*n.s.*	+
	Superior temporal	-	-	*n.s.*	*n.s.*
	Fusiform gyrus	-	-	*n.s.*	*n.s.*
	Transverse temporal	+	-	*n.s.*	*n.s.*

Abbreviations and symbols
+: increase; -: decrease; HC: healthy control; n.s.: not significant; SAD: patient with social anxiety disorder.

caudal ACC, the insula, the superior parietal gyrus, the precuneus, the postcentral gyrus, and the inferior temporal gyrus.

Taken together, the inconsistency of the results, as well as the small effect sizes (Brühl, Hänggi, et al., 2014), and the fact that *p*-values often don't survive comparison for multiple comparisons (this study and (Brühl, Hänggi, et al., 2014; Syal et al., 2012)) indicate that studies with large sample sizes and meta-analyses such as those performed by the Enhancing NeuroImaging Genetics through Meta-Analysis (ENIGMA) Consortium (Bearden & Thompson, 2017; Groenewold et al., 2018; Thompson et al., 2014) are needed to increase the reproducibility and validity of results of studies on the relation between social anxiety and cortical thickness (Blackford, 2017). The results of the present study could serve as a starting point for such future studies.

To the best of our knowledge, this study was the first to explore the relationship between social anxiety and CSA, although Steiger and colleagues investigated changes in cortical

volume, which is the product of CT and CSA, in a treatment study on SAD patients (Steiger et al., 2017). Results showed decreases in CSA in the right caudal ACC and right fusiform gyrus, as well as an increase in **CSA in the right precuneus (Table 5.6 and Figure 5.3C).**

Heritability of GM characteristics

We used a newly developed model to estimate the heritability of the GM brain characteristics, which is, to the best of our knowledge, the only available analysis model taking the specific ascertainment process of the present study and the familial relationships between the participants into account (Tissier et al., 2017). As expected based on the results of previous studies (Blokland et al., 2012; Chen et al., 2015; den Braber et al., 2013; Eyler et al., 2011; Hibar et al., 2015; Strike et al., 2018; Thompson et al., 2001), the majority of the GM measures of interest were (at least) moderately heritable (*Figure 5.4; Table 5.4, Table 5.5,* and *Table 5.6)*. It should be noted that we could not replicate the significant heritability estimates of some of the GM measures as reported in other work, but estimates of heritability are often highly variable across studies (Blokland et al., 2012) and across brain regions (Strike et al., 2018); we refer to the recent work of Patel and colleagues reporting on the effects of different estimation approaches on heritability estimates (Patel et al., 2018). Furthermore, these divergent results could also be due to the relatively small sample size and specific data structure of the present study, in which a limited number of families (n = 8) was included, with a broad range in the size of the families (range in number of participating family members per family 5 – 28).

Limitations and suggestions for future studies

The present study is unique as it is the first neuroimaging family study on SAD involving two generations, enabling the investigation of the potential of structural GM characteristics as candidate endophenotypes of SAD. Several limitations of the present study should be mentioned. First of all, the sample size of the MRI sample of the LFLSAD was relatively small, which was partly caused by the loss of data points due to technical reasons and as a result of thorough quality control. Secondly, as this was a cross-sectional study, the *trait stability* of the GM characteristics (endophenotype criterion 2) could not be investigated. Third, we should mention the issue of psychiatric comorbidity, which was present in the LFLSAD sample as could be expected based on the comorbidity associated with SAD (Erwin, Heimberg, Juster, & Mindlin, 2002; Hyett & McEvoy, 2018; Meier et al., 2015; Ruscio et al., 2008). We performed two sensitivity analyses to address this issue; in the first analysis, we excluded participants with past and/or present (comorbid) psychopathology other than SAD, in the second we added the level of depressive symptoms as a covariate. The results of these sensitivity analyses were in line with those of the main analyses, but these analyses were limited by a small sample size (sensitivity analysis 1) and the fact that we only controlled for the level of depressive symptoms (sensitivity analysis 2), and not

for other comorbidity. Furthermore, as the regression models tested were already complex and computationally demanding due to the family structure of the sample, we could not investigate the potentially moderating or mediating effects of factors like trait anxiety, education level, IQ, and socioeconomic status (Brito & Noble, 2014; Noble et al., 2015), nor did we examine the non-linear effects of age on the GM characteristics (Wierenga et al., 2014). As technical advances are constantly being made, future studies will most likely be able to perform more advanced analyses taking these factors into account. In addition, as the LFLSAD did not include control families from the general population, we were not able to assess the second part of endophenotype criterion 4, namely, *whether the levels of the candidate endophenotypes differed between nonaffected family members and participants from the general population*. Furthermore, as most of the results presented here did not survive corrections for multiple comparisons, future studies, preferably with a longitudinal design and larger sample sizes, are needed to confirm these findings. In addition, as we have not yet analyzed the genetic data that was acquired in the LFLSAD (Bas-Hoogendam, Harrewijn, et al., 2018), we could not link the GM changes to genetic variations. Moreover, future studies should investigate to which extent the GM alterations are specific to social anxiety (cf. (Zhao et al., 2017)). Finally, we employed a ROI approach in this study, as this enabled implementing the complex family structure of the sample in the analyses. However, as vertex-based and voxel-based morphometry studies have the potential to detect more subtle alterations in brain structure (Ashburner & Friston, 2001; Clarkson et al., 2011; Lerch et al., 2017), we recommend these techniques for future studies when they become available for family studies with complex (family) designs.

To conclude, the results of this study suggest that several structural GM alterations are heritable and co-segregate with social anxiety within families genetically enriched for SAD. Thereby, these GM characteristics are promising candidate endophenotypes of SAD and have the potential to offer novel insights in the genetic neurobiological vulnerability to this disabling psychiatric condition. Future replication studies are important to confirm these preliminary findings.

Declaration of interests

Janna Marie Bas-Hoogendam received a travel grant to present preliminary results of this study at the WASAD-SFB-TRR58 2017 meeting, organized by the World Association for Stress Related and Anxiety Disorders (from 14-16 September 2017, Würzburg, Germany) (Bas-Hoogendam, van Steenbergen, van der Wee, & Westenberg, 2017d).

Supplemental Tables

The supplemental tables belonging to this chapter are, due to their size, publicly available online at the Open Science Framework Database *(Bas-Hoogendam, van Steenbergen, Tissier, et al., 2018a)*. https://osf.io/m8q2z/

Supplemental Table S5.1

Detailed statistics of effects of social anxiety on, and heritability estimates of parameters of interest: general imaging phenotypes (tab 1); subcortical volumes (tab 2); cortical thickness (tab 3); cortical surface area (tab 4).

Supplemental Table S5.2

Detailed statistics of effects of social anxiety on parameters of interest in sample without comorbidity (sensitivity analysis 1): subcortical volumes (tab 1); cortical thickness (tab 2); cortical surface area (tab 3).

Supplemental Table S5.3

Detailed statistics of effects of social anxiety on parameters of interest, corrected for level of depressive symptoms (sensitivity analysis 2): subcortical volumes (tab 1); cortical thickness (tab 2); cortical surface area (tab 3).

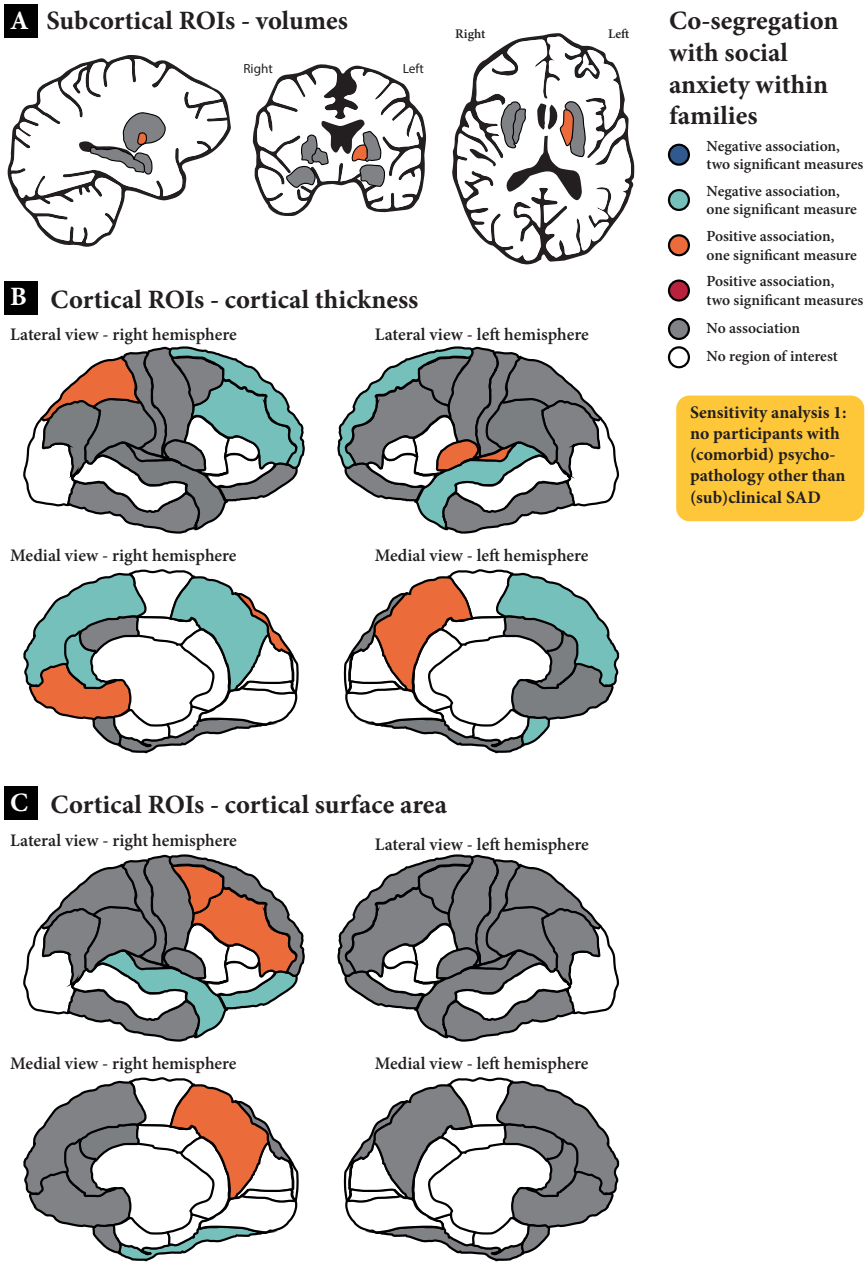
Supplemental Table S5.4

Detailed statistics of effects of social anxiety on, and heritability estimates of non-ROIs: subcortical volumes (tab 1); cortical thickness (tab 2); cortical surface area (tab 3).

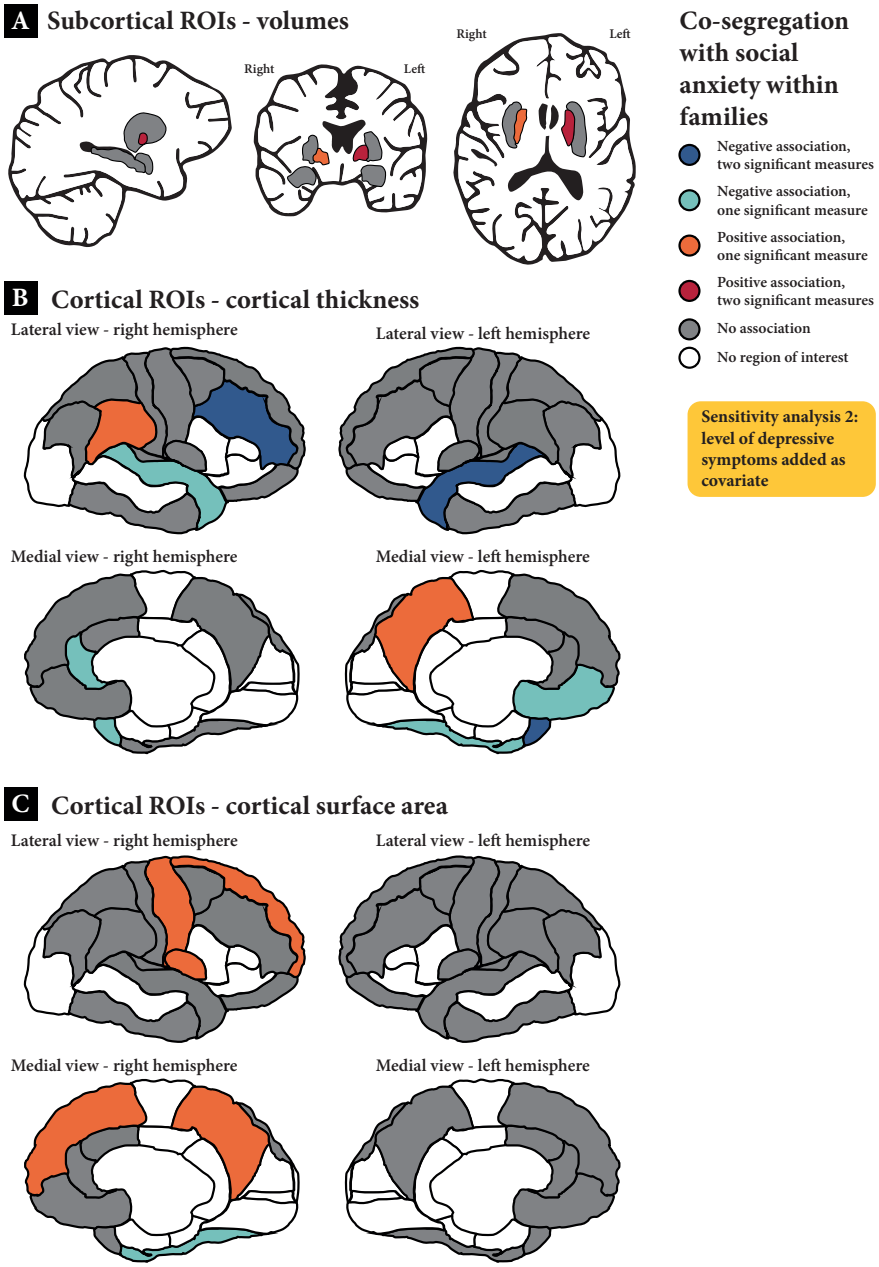
Supplemental Figure S5.4 QR code for easy access to Supplemental Tables belonging to Chapter 5.

Supplemental Figures

Supplemental Figure S5.1 Illustration of interaction (sub)clinical SAD-by-age.



Supplemental Figure S5.2 Relationship between indices of social anxiety and gray matter characteristics in selection of LFLSAD sample: participants with (comorbid) psychopathology other than (sub)clinical SAD were excluded (sensitivity analysis 1).



Supplemental Figure S5.3 Relationship between indices of social anxiety and gray matter characteristics, corrected for level of depressive symptoms (sensitivity analysis 2).

Part 3

Functional brain characteristics as putative SAD endophenotypes

Chapter 6

How embarrassing! The behavioral and neural correlates of processing social norm violations

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Abstract

Social norms are important for human social interactions, and violations of these norms are evaluated partly on the intention of the actor. Here, we describe the revised Social Norm Processing Task (SNPT-R), a paradigm enabling the study of behavioral and neural responses to intended and unintended social norm violations among both adults and adolescents. We investigated how participants (adolescents and adults, n = 87) rate intentional and unintentional social norm violations with respect to inappropriateness and embarrassment, and we examined the brain activation patterns underlying the processing of these transgressions in an independent sample of 21 adults using functional magnetic resonance imaging (fMRI). We hypothesized to find activation within the medial prefrontal cortex, temporo-parietal cortex and orbitofrontal cortex in response to both intentional and unintentional social norm violations, with more pronounced activation for the intentional social norm violations in these regions (Berthoz, Armony, Blair, & Dolan, 2002).

Participants' ratings confirmed the hypothesis that the three types of stories are evaluated differently with respect to intentionality: intentional social norm violations were rated as the most inappropriate and most embarrassing. Furthermore, fMRI results showed that reading stories on intentional and unintentional social norm violations evoked activation within the frontal pole, the paracingulate gyrus and the superior frontal gyrus. In addition, processing unintentional social norm violations was associated with activation in, among others, the orbitofrontal cortex, middle frontal gyrus and superior parietal lobule, while reading intentional social norm violations was related to activation in the left amygdala. These regions have been previously implicated in thinking about one's self, thinking about others and moral reasoning.

Together, these findings indicate that the SNPT-R could serve as a useful paradigm for examining social norm processing, both at the behavioral and the neural level.

Introduction

In the present work, we describe the revised Social Norm Processing Task (SNPT-R), a paradigm enabling the study of behavioral and neural responses to intended and unintended social norm violations among both adults and adolescents. More specifically, we investigated how participants rate intentional and unintentional social norm violations with respect to inappropriateness and embarrassment, and we examined the brain activation patterns underlying the processing of these transgressions.

Social norms are crucial in creating and maintaining social relationships, because they specify what is acceptable in a certain social group (Bicchieri, 2006; Cialdini & Goldstein, 2004). Transgressions of these norms induce self-conscious emotions like embarrassment and guilt (Jankowski & Takahashi, 2014; Robins & Schriber, 2009; Tangney, Stuewig, & Mashek, 2006). These emotions are prosocial, because they lead to action tendencies which are important to restore the social order (Eisenberg, 2000; Feinberg, Willer, & Keltner, 2012; Haidt, 2003).

Several studies have investigated the behavioral and neural responses to violations of norms and the associated prosocial emotions, for example while focusing on making moral judgments (Chakroff et al., 2016; Knutson et al., 2010; Schaich Borg, Hynes, Van Horn, Grafton, & Sinnott-Armstrong, 2006), the emergence of human social values (Zahn et al., 2009), the effect of the presence or absence of an audience on processing moral and social transgressions (Finger, Marsh, Kamel, Mitchell, & Blair, 2006), and the experience of self-conscious moral emotions like shame and guilt (Mclatchie, Giner-Sorolla, & Derbyshire, 2016; Michl et al., 2014; Takahashi et al., 2004). While these paradigms investigated several aspects of norm processing, the focus of the Social Norm Processing Task (SNPT), originally developed and described by Berthoz et al. (Berthoz et al., 2002; Berthoz, Grèzes, Armony, Passingham, & Dolan, 2006) and used in a subsequent study (Blair et al., 2010), is on the effect of intention on the judgment of social norm violations. The concept of 'intentionality' has been extensively studied (Cova, Dupoux, & Jacob, 2012; Knobe, 2003; Malle & Knobe, 1997) and the effect of the actor's intention on the evaluation of an action has been shown previously (cf. (Chakroff et al., 2016; Knutson et al., 2010; Schaich Borg et al., 2006)). For example, intentional harmful acts were judged worse (Chakroff et al., 2016) and more 'wrong' (Schaich Borg et al., 2006) than accidental harmful acts.

In the SNPT, participants read and evaluate stories describing neutral social situations and situations in which social norms are intentionally or unintentionally transgressed (Berthoz et al., 2002). Social norms, in this task, are widely shared beliefs on appropriate behavior in a social situation, i.e. in a situation where others are present. It should, however, be noted that several other definitions of 'social norms' exist, for example in the context of economic decision games (O'Callaghan et al., 2016; Ruff, Ugazio, & Fehr, 2013; Sanfey, Stallen, & Chang, 2014; Spitzer, Fischbacher, Herrnberger, Grön, & Fehr, 2007; Yuan Zhang,

Yu, Yin, & Zhou, 2016). Furthermore, there is a debate about how social norms are different from moral norms and decency norms, a discussion which is outside the scope of this paper (Bicchieri, 2006; Brennan, Eriksson, Goodin, & Southwoord, 2013; Colombo, 2014; Lisciandra, Postma-Nilsenová, & Colombo, 2013).

Results on the SNPT (Berthoz et al., 2002) revealed that participants evaluated the stories differently with respect to inappropriateness and embarrassment: healthy male participants (n = 12) rated intentional social norm violations as more inappropriate when compared to unintentional norm violations, while they considered the unintentional norm violations as the most embarrassing (Berthoz et al., 2002, 2006). These findings indicate that the evaluation of social norm violations is determined to a great extent by the intention of the actor (Berthoz et al., 2006), given the fact that the consequences of the intentional and the unintentional social norm violations are in general the same (Berthoz et al., 2002). Furthermore, neuroimaging data on the SNPT indicated that reading stories describing intentional and unintentional social norm violations evoked activation within the medial and superior prefrontal cortex, the left temporo-parietal junction and left orbito-frontal cortex, while the intentional condition (compared to unintentional condition) was associated with stronger activation within the medial and superior frontal cortex, anterior cingulate gyrus, parietal regions including the precuneus, left superior occipital gyrus and, as shown in a re-analysis of the data, the left amygdala (Berthoz et al., 2006).

In addition to the study by Berthoz et al. on healthy male participants (Berthoz et al., 2002), the SNPT was used in an imaging study on social anxiety disorder (SAD) (Blair et al., 2010). Patients with SAD are characterized by an intense fear of negative evaluation (Stein & Stein, 2008), which was reflected by aberrant behavioral and neural responses to the SNPT. At the behavioral level, SAD patients (n = 16) reported higher levels of inappropriateness and embarrassment across all conditions (intentional, unintentional and neutral), when compared to healthy control participants (n = 16). Furthermore, increased activation in the medial prefrontal cortex in response to unintentional norm violations was present in SAD (Blair et al., 2010), suggesting altered self-referential processing. These findings indicate that the SNPT is a useful paradigm to investigate the neurobehavioral correlates of social anxiety, but we suggest, in line with Berthoz and colleagues (Berthoz et al., 2002), that the SNPT can also be utilized in future research on the vulnerability to other psychiatric and neurological conditions in which social behavior is typically affected.

However, previous work on the SNPT (Berthoz et al., 2002; Blair et al., 2010) has several limitations, hindering its future use. Both studies had small sample sizes (n = 12 (Berthoz et al., 2002) and n = 16 healthy participants (Blair et al., 2010)), and included only adult participants, while Berthoz and colleagues (2002) examined solely males. Furthermore, given the focus of these studies on the neural correlates of social norm processing, behavioral responses were not described in detail. In addition, different versions of the SNPT were used: while the SNPT employed by Blair and colleagues (2010) only comprised impersonal

stories (i.e. the story protagonist is a character like 'Joanna'), Berthoz et al. (2002, 2006) used a combination of personal and impersonal stories (i.e. the story protagonist is 'you' or the story protagonist is a character like 'Joanna', respectively), as well as 'nonsense' stories composed of unrelated words, which were not further analyzed. Furthermore, the imaging parameters of the paradigms vary to a great extent: the paradigm by Berthoz and colleagues (Berthoz et al., 2002, 2006) has a duration of more than 50 minutes, while the task used by Blair et al. lasts around 15 minutes. Finally, the stories of these SNPT-versions are not publicly available. Taken together, these differences make it hard to compare the results of these studies and to obtain a clear picture of social norm processing in healthy participants, which could serve as a reference for future studies in patients.

Here, we describe, building upon the work of Berthoz et al. (2002, 2006) and Blair et al. (2010), an adapted version of the SNPT: the revised Social Norm Processing Task (SNPT-R). In line with previous versions of the SNPT, the SNPT-R contains stories describing neutral social situations, stories on unintentional social norm violations, and stories depicting intentional social norm violations. However, in contrast to earlier versions of the SNPT (Berthoz et al., 2002, 2006; Blair et al., 2010), the SNPT-R uses only personal stories in order to maximize personal involvement of the participants while reading the stories (cf. (Finger et al., 2006)). In line with this, we developed four age- and gender specific versions of SNPT-R, making the paradigm appropriate for participants of different ages. Other changes relative to previous versions of the SNPT involve a shortening of the duration of the paradigm relative to the paradigm by Berthoz et al. (2002), mainly by omitting the 'nonsense' stories, and improvements in the fMRI design like the use of a jittered presentation of a fixation cross between the stories.

Main aim of the present study was to validate the SNPT-R, by replicating the findings of previous versions of the SNPT. First, we examined the behavioral ratings of inappropriateness and embarrassment for the three types of stories in a sample of adolescents and adults (n = 87). We hypothesized to find an effect of intention on the evaluation of the stories, both on the ratings of inappropriateness and embarrassment, as reported previously (Berthoz et al., 2002). Secondly, we investigated neural responses to the stories using fMRI, in an independent sample of 21 adults, aiming to replicate the results described by Berthoz et al. (2002, 2006). More specifically, we expected to find activation within the medial prefrontal cortex, temporo-parietal cortex and orbitofrontal cortex in response to both intentional and unintentional social norm violations, with more pronounced activation for the intentional social norm violations in these regions (Berthoz et al., 2002). Furthermore, we hypothesized that intentional social norm violations would be associated with left amygdala activation as reported by (Berthoz et al., 2006).

The present study extends previous work on the SNPT in two ways. First, we use a larger sample of participants, including both genders and with a broader age range. Secondly, by publishing the stories used in the SNPT-R (*Supplemental Table S6.1* and osf.io/

pt4qt (Bas-Hoogendam, van Steenbergen, Kreuk, van der Wee, & Westenberg, 2017b)), as well as the code for stimulus presentation (available on osf.io/pt4qt (Bas-Hoogendam, van Steenbergen, Kreuk, et al., 2017b)), and the data acquired in the present study (osf.io/pt4qt (Bas-Hoogendam, van Steenbergen, Kreuk, et al., 2017b) and http://neurovault.org/collections/QCZKVNWZ/), we aim to encourage the use of the SNPT-R in future studies.

Materials and methods

Participants

One hundred eight participants were included in the study, divided into two independent samples. Sample size was determined by availability of subjects and resources. The first sample (from now on referred to as 'behavioral sample') consisted of adolescents and adults who performed the SNPT-R on a laptop or personal computer, while the second sample was comprised of adults who were scanned using fMRI while reading the stories ('imaging sample'). All participants were required to have Dutch as their first language, to be in good health and to be free of past and present psychopathology as assessed by a self-report questionnaire. General contraindications for undergoing an MRI scan and left-handedness, evaluated by a self-report questionnaire, were exclusion criteria for the imaging sample.

Ninety-four participants signed up for the behavioral experiment; four participants were excluded from participation because they did not meet the selection criteria (present medication use: $n = 3$; present phyisical disorder: $n = 1$). Furthermore, data from three participants were excluded from analysis because they performed a version of the SNPT-R that did not match their age, leaving a total of 87 participants in the behavioral sample.

Twenty-three participants were screened for participation in the imaging study; one participant was excluded due to past psychopathology, one MRI session was aborted due to participant claustrophobia, resulting in a sample of 21 participants. A neuroradiologist examined all structural MRI scans; no clinically relevant abnormalities were present in any of the participants included in the imaging sample.

All participants (and in case of minors below 18 years of age, both parents) signed informed consent prior to participation. The study was approved by the Psychology Research Ethics Committee of Leiden University (behavioral sample; study numbers 2282269557 and 8070826266) and the Medical Ethical Committee of the Leiden University Medical Center (imaging sample; protocol number P12.061). Participants were recruited via flyers, in-class announcements and by word of mouth and tested between July 2013 and December 2015 (imaging sample: July - August 2013; behavioral sample adults: November - December 2014; behavioral sample adolescents: June 2015 - December 2015). After performing the experiment, participants were debriefed about the aim of the study and received a compensation

for partaking in the experiment (imaging sample: monetary reward; behavioral sample adults: study credits; behavioral sample adolescents: chocolate bar).

Sample characteristics are summarized in *Table 6.1*. Participants of the behavioral sample were divided into four groups (group 1: boys < 18 years of age; group 2: girls < 18 years of age; group 3: men ≥ 18 years of age; group 4: women ≥ 18 years of age), based on the four age- and gender specific versions of the SNPT-R. As a consequence, groups differed significantly with respect to age (oneway ANOVA: $F(3,86) = 59.0$, $p < 0.001$): boys and girls did not differ in age (independent-samples t-test: $t(27) = -0.38$, *ns*), but the men were significantly older when compared to the women ($t(35.8) = 3.1$, $p = 0.004$). In the imaging sample, there were no age differences between men and women ($t(19) = 0.41$, *ns*).

Table 6.1 Characteristics participants.

Behavioral sample	**Boys (*n* = 13)**	**Girls (*n* = 16)**	**Men (*n* = 29)**	**Women (*n* = 29)**
Age in years *mean ± SD (range)*	14.0 ± 1.2 (12.7 - 16.5)	14.2 ± 1.4 (12.5 - 17.0)	21.1 ± 3.1 (18.5 - 32.6)	19.2 ± 1.2 (18.1 - 24.1)
Imaging sample			**Men (*n* = 6)**	**Women (*n* = 15)**
Age in years *mean ± SD (range)*			25.8 ± 9.3 (18.7 - 44.1)	24.0 ± 9.7 (18.1 - 57.1)

Abbreviation
SD: standard deviation.

Social Norm Processing Task (SNPT-R)

Participants performed the revised Social Norm Processing Task (SNPT-R), an adaptation of the Social Norm Processing Task described by (Berthoz et al., 2002, 2006; Blair et al., 2010). The SNPT-R consists of two phases: a story-reading phase and a rating phase (*Figure 6.1*). In the story-reading phase, participants read short stories written in second person. Each story consisted of two sentences, a stem sentence (duration: 3 s) and an ending sentence (duration: 6 s). The stories described either a situation in which no social norm was transgressed ('neutral condition'), a situation in which a social norm was unintentionally transgressed ('unintentional condition') or a situation in which a social norm was intentionally transgressed ('intentional condition'). It is important to note that the unintentional ('You are baking with friends. You use salt instead of sugar without realizing.') and intentional ('You are baking with friends. You use salt instead of sugar as a joke.') condition differ only in the intention of the actor, while we aimed to keep the actual outcome of the action (for example, a distasteful cake) in general the same (although the outcome of some intentional stories could be considered to be more severe in comparison to the outcome of the matching unintentional story, inherent to the verb used to describe intentionality; we refer the reader to the *Supplemental Analysis* and *Supplemental Table S6.2* for a sensitivity analysis).

The stories in the SNPT-R were developed in collaboration with Karina S. Blair, author of previous work on the SNPT (Blair et al., 2010). All stories described everyday social

situations, in which the protagonist was accompanied by at least one other person, and the stories outlined relative innocuous violations of conventional social norms, in which no severe harm was done to others. The stories were heterogeneous with respect to the context (for example, in the presence of one friend or in public space like an airport) and the nature of the social norm transgression (for example, breaking decency rules versus hurting somebody), in order to increase the external validity of the paradigm. Stories were developed to be suitable for a broad audience and age range (for children from age 8). However, given the fact that the stories of the SNPT-R were all personal (written in 'you' form) in order to maximize personal involvement of participants, some small changes were necessary in stories describing age- or genderspecific elements. Therefore, four age- and gender specific versions of the task were developed: for boys < 18 years of age (version 1), girls < 18 years of age (version 2), men ≥ 18 years of age (version 3) and women ≥ 18 years of age (version 4). For example, the school environment (< 18 years) was replaced for a work environment (≥ 18 years of age), and 'bikini bottoms' (females) for 'swimming trunks' (males). However, these changes were only minimal (see *Supplemental Table S6.1* and osf.io/pt4qt for a full list of stories included in the SNPT-R (Bas-Hoogendam, van Steenbergen, Kreuk, et al., 2017b)).

In line with the SNPT described by Blair et al. (2010), twenty-six stem sentences were developed, with three different types of ending. Therefore, the SNPT-R consisted of 78 short stories in total. These stories were presented in a pseudo-random order using E-Prime software (version 2.0.10, Psychology Software Tools; script available at osf.io/pt4qt (Bas-Hoogendam, van Steenbergen, Kreuk, et al., 2017b)), separated by a fixation cross (jittered duration between 2 - 7 s, determined using Optseq software (https://surfer.nmr.mgh.harvard.edu/optseq/), mean duration fixation: 3.5 s) and divided into two consecutive blocks of 39 stories (duration each block: 8 min 44 s). Participants were instructed to imagine themselves in the social situations described and to press a button with their right index finger after reading the stem sentence of each story. A button press within 3 s resulted in visual feedback to the participant (a green checkmark presented beneath the sentence). This element was added to the paradigm in order to be able to check whether participants engaged with the task. Prior to the start of the experiment, all participants were familiarized with the story-reading phase by performing a short version of the task (using five stories).

In the (unannounced) rating phase of the task (*Figure 6.1*), participants read all stories again and were asked to rate them on a five-point Likert scale on embarrassment (ranging from 1, not embarrassing at all, to 5, extremely embarrassing) and inappropriateness (ranging from 1, not inappropriate at all, to 5, extremely inappropriate), similar as in the SNPT described by Blair and colleagues (2010). These tasks were also presented using E-Prime software (version 2.0.10, Psychology Software Tools; scripts available at osf.io/pt4qt (Bas-Hoogendam, van Steenbergen, Kreuk, et al., 2017b)).

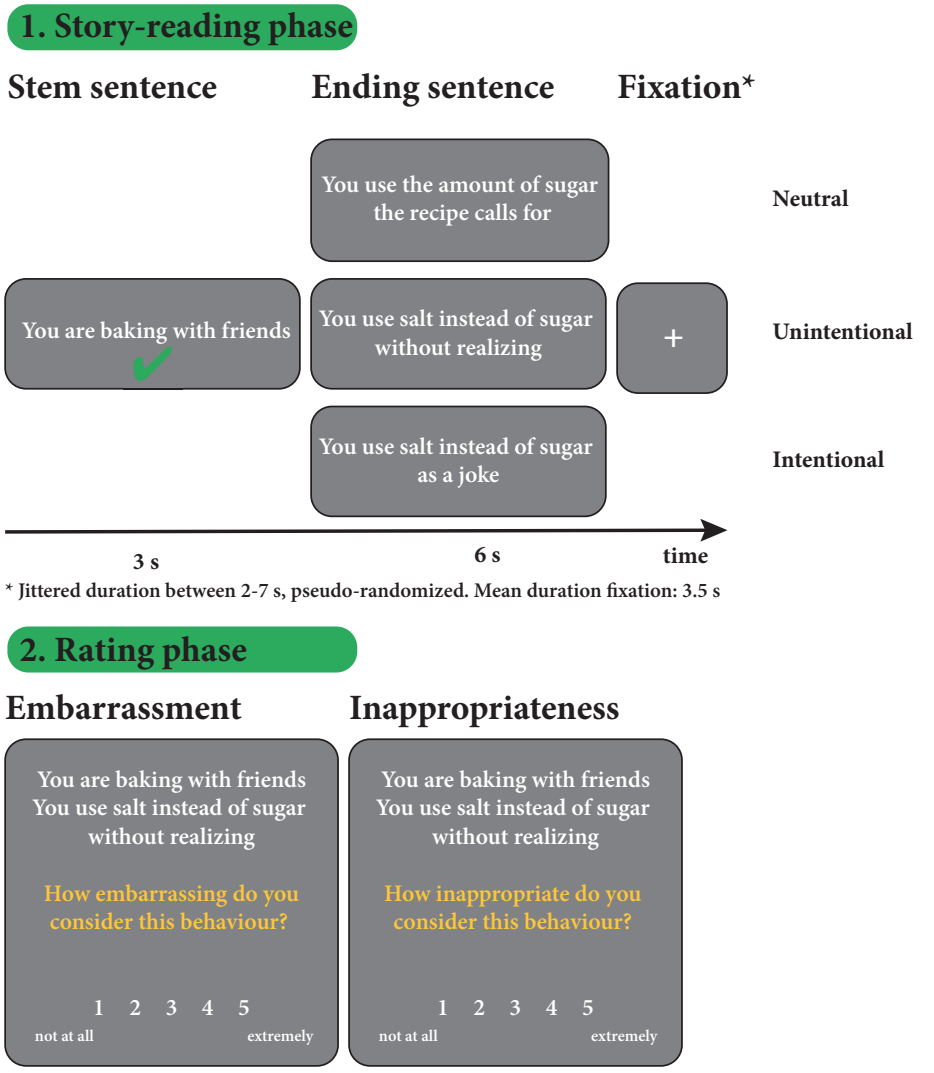

Figure 6.1 Overview of the revised Social Norm Processing Task (SNPT-R).
During the story-reading phase (1), participants read stories consisting of a stem sentence and an ending sentence, describing either a neutral social situation, a situation in which a social norm was unintentionally transgressed or situation in which a social norm was violated intentionally. Participants were instructed to imagine themselves in the situation described. In the rating phase (2), participants rated all stories on embarrassment and inappropriateness.

Procedure

Participants of the behavioral sample completed both the story-reading phase as well as the rating phase of the SNPT-R on a laptop or personal computer in a quiet environment, at

the Faculty of Social and Behavioral Sciences, Leiden University (adult participants) or at a secondary school in the Netherlands (adolescent participants). After performing the SNPT-R, participants completed, depending on their age, the self-report version of the Liebowitz Social Anxiety Scale (Heimberg et al., 1999) or the Social Anxiety Scale for adolescents (La Greca & Lopez, 1998), and the Brief Fear of Negative Evaluation-R scale (Carleton et al., 2006). These results are not discussed in the present paper.

Participants of the imaging sample performed the story-reading phase of the SNPT-R in the MRI scanner, located at the Leiden University Medical Center (LUMC). Imaging data were collected during the story-reading phase using a Philips 3.0 T Achieva MRI scanner (Philips Medical Systems, Best, The Netherlands), equipped with a 32-channel SENSE (Sensitivity Encoding) head coil. During the two blocks of the story-reading phase, functional scans were acquired using T2* weighted echo-planar imaging (repetition time (TR) = 2200 ms, echo time (TE) = 30 ms, 38 axial slices, descending acquisition, 2.75 mm × 2.75 mm × 2.75 mm + 10 % interslice gap, field of view (FOV) = 220 mm × 115 mm × 220 mm, 230 volumes/block). The first six volumes of these scans were dummy volumes and removed to allow for equilibration of T1 saturation effects. A 3D T1-weighted anatomical scan was acquired for within-subject registration purposes before the SNPT-R (TR = 9.8 ms, TE = 4.59 ms, flip angle = 8◦, 140 slices, 0.875 mm × 0.875 mm × 1.2 mm, FOV = 224 mm × 168 mm × 177.333 mm). The task was part of a larger scanning session including other fMRI tasks, a resting-state scan, and a diffusion tensor imaging (DTI) scan.

Following the scan-session, participants performed the rating phase of the SNPT-R on a laptop in a quiet room next to the MRI scanner.

Data analysis

Behavioral ratings

Statistical analyses of the ratings of embarrassment and inappropriateness for the stories of the SNPT-R were performed using IBM SPSS Statistics for Windows (Version 23.0. Armonk, NY: IBM Corp.). Internal consistency of the task conditions (intentional, unintentional and neutral) was determined by calculating Cronbach's α for the ratings of inappropriateness and embarrassment, and for the difference score (again both for inappropriateness and embarrassment) between the intentional and unintentional condition for each set of stories.

Repeated measures ANOVAs with condition (intentional, unintentional, neutral) as a within-subjects factor were used to investigate differences between task conditions. Furthermore, group (based on the version of the SNPT-R; group 1: boys < 18 years of age; group 2: girls < 18 years of age; group 3: men ≥ 18 years of age; group 4: women ≥ 18 years of age) was added as a between-subjects factor. The SPSS code for analysis of the behavioral data is available at osf.io/pt4qt (Bas-Hoogendam, van Steenbergen, Kreuk, et al., 2017b). For all analyses, significance level was set at $p \leq 0.05$ and Greenhouse–Geisser correction was used when the assumption of sphericity was violated.

Imaging data

Analysis of fMRI data was performed using FEAT (FMRI Expert Analysis Tool; version 6.00) (Jenkinson, Beckmann, Behrens, Woolrich, & Smith, 2012; Smith et al., 2004), (FSL, RRID:SCR_002823; scripts available at osf.io/pt4qt (Bas-Hoogendam, van Steenbergen, Kreuk, et al., 2017b)). Prestatistics processing consisted of motion correction using MCFLIRT (Jenkinson, Bannister, Brady, & Smith, 2002), non-brain removal using BET (Smith, 2002), spatial smoothing using a Gaussian kernel of full-width half-maximum (FWHM) 6.0 mm, grand-mean intensity normalization of the entire 4D dataset by a single scaling factor in order to enable higher-level analyses, and high-pass temporal filtering (Gaussian-weighted least-squares straight line fitting, with $\sigma = 30.0$ s). Functional scans of each participant were registered to the individual 3D T1-weighted anatomical scan using FLIRT (Jenkinson et al., 2002; Jenkinson & Smith, 2001) and subsequently registered to the Montreal Neurological Institute (MNI) T1-template brain (resolution 2 mm) using FNIRT nonlinear registration (Andersson et al., 2007). Next, event-related statistical analysis of the time-series was carried out in native space using FILM with local autocorrelation correction (Woolrich, Ripley, Brady, & Smith, 2001). For each participant, four explanatory variables (EVs) with their temporal derivatives were included in the general linear model, representing the presentation of 1st a stem sentence, 2nd a neutral ending sentence, 3rd an unintentional norm violation ending and 4th an intentional norm violation ending. Onset of the EVs was determined using custom-written scripts in Matlab (Mathworks; code available at osf.io/pt4qt (Bas-Hoogendam, van Steenbergen, Kreuk, et al., 2017b)). The stem EV had a duration of 3 s, the ending EVs had a duration of 6 s. EVs were convolved with a double gamma hemodynamic response function. In addition, nuisance regressors were included for time-points corresponding to motion outliers using the FSL motion outliers program (http://fsl.fmrib.ox.ac.uk/fsl/fslwiki/FSLMotionOutliers), which defined outlier time-points using the 75th percentile plus 1.5 times the InterQuartileRange criterion. The mean number of excluded time-points for block 1 and 2 of the story-reading phase of the SNPT-R was 12.00 (range: 1 - 24 volumes) and 13.24 (range: 3 - 26 volumes), corresponding to respectively 5.2% and 5.8% of the volumes for each block.

Subsequently, four contrasts of interest were defined, following the contrasts described by Berthoz et al. (2002): 1st intentional norm violation endings > neutral endings; 2nd unintentional norm violation endings > neutral endings; 3rd intentional norm violation endings > unintentional norm violation endings; 4th unintentional norm violation endings > intentional norm violation endings. We verified whether the individual scans were registered correctly and confirmed that relative motion parameters did not exceed 2.5 mm. Subsequently, the individual contrast images of the two story-reading blocks of the SNPT-R were combined using a within-subject multi-session fixed-effects analysis and the resulting contrast images were submitted to higher-level mixed-effects group analyses using FMRIB's Local Analysis of Mixed Effects (FLAME-1) (Beckmann, Jenkinson, & Smith, 2003;

Woolrich, 2008; Woolrich, Behrens, Beckmann, Jenkinson, & Smith, 2004). We performed whole-brain analyses to investigate clusters related to the four contrasts of interest and tested clusters for significance using a height threshold of $z > 2.3$ and a cluster-corrected significance threshold of $p < 0.05$, using Gaussian random field theory (Worsley, 2001). In addition, we determined, in line with the analysis described by Berthoz et al. (2002), common areas activated by the intentional and unintentional norm violations by applying a binary mask of the areas significantly activated by contrast 2 (unintentional norm violation endings > neutral endings) to contrast 1 (intentional norm violation endings > neutral endings), again using the above-mentioned statistical thresholds.

Furthermore, we investigated, following Berthoz and colleagues (2006) who re-analyzed the data of Berthoz et al. (2002) to test the hypothesis that the amygdala is pivotal in processing one's own intentional social norm transgressions, a hypothesis which was confirmed, activation within the left amygdala for the contrasts involving intentional norm violations. Therefore, we used a mask that was created in standard space (resolution 2 x 2 x 2 mm) using the Harvard-Oxford Subcortical Structural Atlas implemented in FSLView (version 3.2.0), which included voxels with a probability of at least 50 % of belonging to the left amygdala. Again, a height threshold of $z > 2.3$ and a cluster-corrected significance threshold of $p < 0.05$ was used.

Unthresholded statistical maps have been uploaded to NeuroVault.org (Gorgolewski et al., 2015) and are available at http://neurovault.org/collections/QCZKVNWZ/ as well as at osf.io/pt4qt (Bas-Hoogendam, van Steenbergen, Kreuk, et al., 2017b).

Results

Behavioral ratings

Task characteristics

The items of the SNPT-R for each condition were shown to have good internal consistency with respect to the ratings of both embarrassment (intentional: Cronbach's α = 0.94; unintentional: Cronbach's α = 0.90; neutral: Cronbach's α = 0.73) and inappropriateness (intentional: Cronbach's α = 0.85; unintentional: Cronbach's α = 0.88; neutral: Cronbach's α = 0.66). Furthermore, Cronbach's α on the difference scores (intentional vs. unintentional) was high (embarrassment: Cronbach's α = 0.90; inappropriateness: Cronbach's α = 0.84), indicating that the stories were internally consistent with respect to the difference between the intentional and unintentional condition.

Differences between task conditions and effects of group (behavioral sample)

Ratings for the three task conditions of the SNPT-R (behavioral sample) are presented in *Table 6.2* and *Figure 6.2* (for ratings at individual and story level, we refer the reader to

Table 6.2 Ratings of inappropriateness and embarrassment for the *SNPT-R* stories – behavioral sample.

	Inappropriateness			**Embarrassment**		
	Intentional	***Unintentional***	***Neutral***	***Intentional***	***Unintentional***	***Neutral***
Total sample	4.43 ± 0.36	2.93 ± 0.51	1.29 ± 0.20	3.83 ± 0.67	3.50 ± 0.56	1.25 ± 0.21
Boys (n = 13)	4.01 ± 0.40	2.71 ± 0.48	1.25 ± 0.21	3.49 ± 0.59	3.19 ± 0.63	1.27 ± 0.22
Girls (n = 16)	4.43 ± 0.34	2.94 ± 0.41	1.24 ± 0.17	3.75 ± 0.62	3.36 ± 0.45	1.23 ± 0.12
Men (n = 29)	4.44 ± 0.33	3.03 ± 0.48	1.29 ± 0.19	3.76 ± 0.62	3.35 ± 0.52	1.20 ± 0.20
Women (n = 29)	4.60 ± 0.23	2.92 ± 0.58	1.33 ± 0.21	4.09 ± 0.72	3.87 ± 0.44	1.31 ± 0.23

Footnote
Values represent mean ± standard deviation.

Supplemental Table S6.3; original E-Prime output files and csv files are also available at osf.io/pt4qt (Bas-Hoogendam, van Steenbergen, Kreuk, et al., 2017b)). Given the unequal sample sizes, we checked whether the variances were significantly different between the groups. This was not the case: for both the embarrassment and inappropriateness data, Box's Test of Equality of Covariance Matrices was not significant (inappropriateness: Box's M = 20.7, F(18, 10272.5) = 1.1, p = 0.38; embarrassment: Box's M = 17.4, F(18, 10272.5) = 0.89, p = 0.59). Furthermore, Levene's Test of Equality of Error Variance was not significant (inappropriateness intentional: F(3,83) = 2.14, p = 0.10; inappropriateness unintentional: F(3,83) = 1.01, p = 0.39; inappropriateness neutral: F(3,83) = 0.49, p = 0.69; embarrassment intentional: F(3,83) = 0.50, p = 0.69; embarrassment unintentional F(3,83) = 1.40, p = 0.25; embarrassment neutral: F(3,83) = 1.13, p = 0.34), indicating that the assumptions for interpreting the results of the repeated measures ANOVA are met.

Repeated measures ANOVAs (condition x group) showed significant effects of condition on both the ratings of embarrassment (F(1.7,144.4) = 790.8, $p < 0.001$, $\eta^2 = 0.90$) and inappropriateness (F(1.7,137.1) = 2018.5, $p < 0.001$, $\eta^2 = 0.96$). In addition, there were significant effects of group on the ratings of embarrassment (F(3,83) = 7.02, $p < 0.001$, $\eta^2 = 0.20$) and ratings of inappropriateness (F(3,83) = 3.9, p = 0.011, $\eta^2 = 0.12$), as well as interaction effects between group and condition (embarrassment: F(5.2,144.4) = 2.5, p = 0.03, $\eta^2 = 0.009$; inappropriateness: F(5.0,137.1) = 3.0, p = 0.01, $\eta^2 = 0.004$) (*Figure 6.2*).

Post-hoc paired-samples t-tests showed that the mean ratings of inappropriateness were significantly higher for the intentional stories relative to the unintentional stories (t(86) = 27.7, $p < 0.001$, r = 0.95), while the unintentional stories were rated as more inappropriate compared to the neutral stories (t(86) = 34.0, $p < 0.001$, r = 0.96). A similar pattern was found for the ratings of embarrassment: participants rated the intentional stories as the most embarrassing (intentional > unintentional: t(86) = 4.6, $p < 0.001$, r = 0.44), and the unintentional stories as more embarrassing when compared to the neutral stories (t(86) = 40.3, $p < 0.001$, r = 0.97). Separate repeated measures ANOVAs for each group confirmed that the effect of condition was significant for all age- and gender specific versions of the task, both for inappropriateness and embarrassment (effect of condition on inappropriateness:

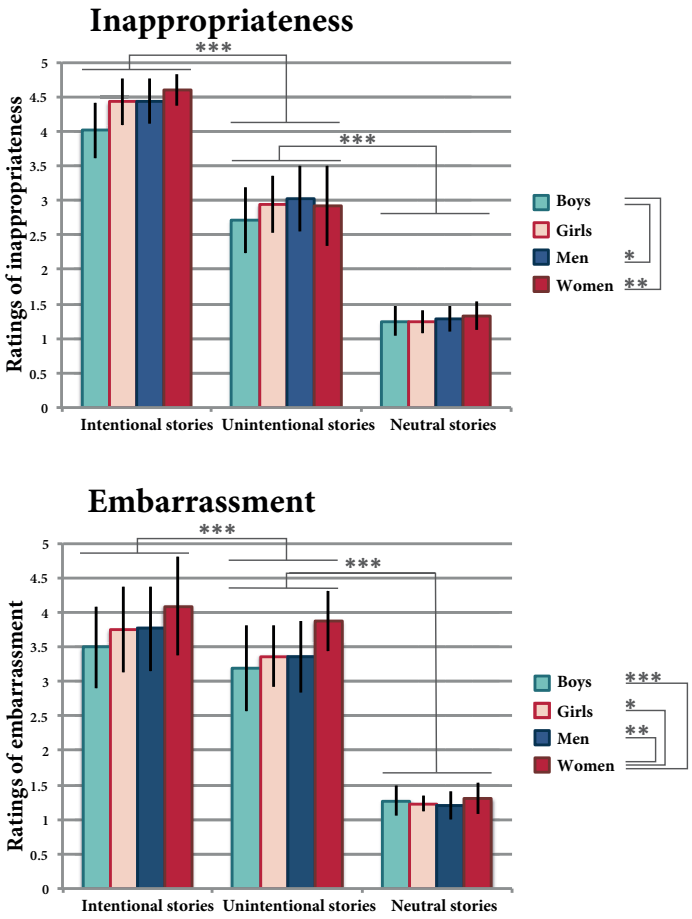

Figure 6.2 Behavioral ratings on the SNPT-R (*n* = 87, behavioral sample).
Stories describing intentional social norm violations were rated as more inappropriate and more embarrassing when compared to stories on unintentional social norm violations, while unintentional stories were considered more inappropriate and more embarrassing in comparison to neutral stories. Boys rated the stories as less inappropriate when compared to men and women; women rated the stories as more embarrassing in comparison to the other groups. Data are presented as means ± SD.

*: $p \leq 0.05$; **: $p \leq 0.01$; ***: $p \leq 0.001$.

boys: $F(2,24) = 255.0$, $p < 0.001$, $\eta^2 = 0.96$; girls: $F(2,30) = 627.8$, $p < 0.001$, $\eta^2 = 0.98$; men: $F(2,56) = 709.8$, $p < 0.001$, $\eta^2 = 0.96$; women: $F(1.4,39.3) = 845.8$, $p < 0.001$, $\eta^2 = 0.97$; effect of condition on embarrassment: boys: $F(2,24) = 99.2$, $p < 0.001$, $\eta^2 = 0.89$; girls: $F(2,30) = 146.9$, $p < 0.001$, $\eta^2 = 0.91$; men: $F(2,56) = 356.0$, $p < 0.001$, $\eta^2 = 0.93$; women: $F(1.5,42.3) = 351.8$, $p < 0.001$, $\eta^2 = 0.93$).

Post-hoc tests (corrected for multiple comparisons using Bonferroni correction) indicated that boys rated the stories as less inappropriate when compared to men ($p = 0.03$) and

women ($p = 0.01$), while a follow-up oneway ANOVA showed that this effect was specific for the intentional condition ($F(3,86) = 10.6$, $p < 0.001$, $\eta^2 = 0.28$), with significant differences between boys and the other groups (Bonferroni-corrected comparisons: boys < girls, $p = 0.003$; boys < men: $p = 0.001$; boys < women: $p < 0.001$). There were no group differences with respect to the ratings of inappropriateness for the unintentional ($F(3,86) = 1.2$, *ns*) and neutral stories ($F(3,86) = 0.9$, *ns*).

Women rated the stories overall as more embarrassing in comparison to boys ($p = 0.001$), girls ($p = 0.03$) and men ($p = 0.003$), and a follow-up oneway ANOVA indicated that this effect was present in the intentional ($F(3,86) = 2.9$, $p = 0.04$, $\eta^2 = 0.10$; women > boys, $p = 0.04$) and the unintentional condition ($F(3,86) = 8.2$, $p < 0.001$, $\eta^2 = 0.23$; women > boys: $p = 0.001$; women > girls: $p = 0.009$; women > men: $p = 0.001$; all comparisons Bonferroni-corrected for multiple comparisons). There were no differences between the groups with respect to the embarrassment-ratings of the neutral condition ($F(3,86) = 1.7$, *ns*).

Differences between task conditions and effects of group (imaging sample)

Ratings for the three task conditions of the SNPT-R (imaging sample) are presented in Table 6.3 (for ratings at individual and story level, we refer the reader to *Supplemental Table 6.4*; original E-Prime output files and csv files are also available at osf.io/pt4qt (Bas-Hoogendam, van Steenbergen, Kreuk, et al., 2017b)). Repeated measures ANOVAs replicated all significant effects found in the behavioral sample. That is, there was a significant effect of condition for both the ratings of embarrassment ($F(2,38) = 216.1$, $p < 0.001$, $\eta^2 = 0.91$) and inappropriateness ($F(1.5,28.2) = 271.0$, $p < 0.001$, $\eta^2 = 0.92$), with the highest ratings of embarrassment and inappropriateness for the intentional stories (embarrassment: intentional > unintentional: $t(20) = 3.9$, $p = 0.001$, $r = 0.66$; unintentional > neutral: $t(20) = 17.3$, $p = 0.001$, $r = 0.97$; inappropriateness: intentional > unintentional: $t(20) = 17.9$, $p < 0.001$, $r = 0.97$; unintentional > neutral: $t(20) = 12.0$, $p < 0.001$, $r = 0.94$). Furthermore, there were significant effects of group on the ratings of embarrassment and inappropriateness (embarrassment: $F(1,19) = 5.8$, $p = 0.03$, $\eta^2 = 0.23$; inappropriateness: $F(1,19) = 4.7$, $p = 0.04$, $\eta^2 = 0.20$), with higher ratings for women compared to men. In addition, results showed a significant interaction between condition and group on the ratings of inappropriateness ($F(1.5, 28.2) = 4.4$, $p = 0.03$, $\eta^2 = 0.01$), while this interaction was significant at trend level for the ratings of embarrassment ($F(2,38) = 3.0$, $p = 0.06$, $\eta^2 = 0.01$). Oneway ANOVAs indicated that women rated intentional social norm violations as more inappropriate relative to men ($F(1,20) = 5.4$, $p = 0.03$, $\eta^2 = 0.22$), and unintentional social norm violations as both more inappropriate ($F(1,20) = 5.7$, $p = 0.03$, $\eta^2 = 0.23$) and more embarrassing ($F(1,20) = 7.6$, $p = 0.01$, $\eta^2 = 0.29$). The other comparisons were not significant (embarrassment intentional: $F(1,20) = 3.3$, *ns*; embarrassment neutral: $F(1,20) = 0.25$, *ns*; inappropriateness neutral: $F(1,20) = 0.14$, *ns*).

Table 6.3 Ratings of inappropriateness and embarrassment for the *SNPT-R* – imaging sample.

	Inappropriateness			**Embarrassment**		
	Intentional	***Unintentional***	***Neutral***	***Intentional***	***Unintentional***	***Neutral***
Total sample	4.37 ± 0.49	3.11 ± 0.60	1.40 ± 0.32	4.00 ± 0.62	3.53 ± 0.60	1.33 ± 0.27
Men (n = 6)	4.01 ± 0.71	2.66 ± 0.77	1.44 ± 0.57	3.64 ± 0.60	3.03 ± 0.72	1.28 ± 0.34
Women (n = 15)	4.51 ± 0.30	3.28 ± 0.44	1.38 ± 0.18	4.16 ± 0.59	3.73 ± 0.43	1.35 ± 0.24

Footnote
Values represent mean ± standard deviation.

Imaging data

Behavioral responses during story-reading phase

We verified whether participants engaged with the task during the story-reading phase by examining the behavioral responses of the participants (i.e. a button press during the presentation of the stem sentence). On average, participants responded to 96 % of trials (number of missed responses / block of 39 trials (mean ± SD): 1.6 ± 1.8, range 0 - 8), indicating good task compliance.

Intentional norm violations versus neutral stories

Reading stories describing intentional social norm violations evoked activation in a cluster encompassing the paracingulate gyrus, superior frontal gyrus and frontal pole, extending into the left inferior frontal gyrus, frontal operculum cortex and left caudate (p = 0.01; cluster-size 748 voxels; peak coordinate in MNI space (x, y, z): -10, 28, 36; peak z-value = 3.39), when compared to reading neutral stories (*Table 6.4; Figure 6.3A*). Furthermore, significant activation was present in the left amygdala, revealed by a post-hoc analysis using a mask of the left amygdala (p = 0.033; cluster-size 21 voxels; peak coordinate in MNI space (x, y, z): -18, -10, -12; peak z-value = 3.15).

Unintentional norm violations versus neutral stories

Reading stories describing unintentional social norm violations evoked activation in a cluster including the left superior frontal gyrus, left middle frontal gyrus, left frontal pole, left paracingulate gyrus and right superior frontal gyrus ($p < 0.001$; cluster-size 1604 voxels; peak coordinate in MNI space (x, y, z): -14, 52, 14; peak z-value = 3.99), when compared to reading neutral stories (*Table 6.4; Figure 6.3B*).

Intentional versus unintentional norm violations

There were no clusters where reading the intentional norm violations evoked more activation when compared to reading the unintentional norm violations (using a height threshold of $z > 2.3$ and a cluster-corrected significance threshold of $p < 0.05$). Even when we restricted the analysis to the regions reported in (Berthoz et al., 2002), using a region

of interest approach (spheres with a radius of 5 mm around the coordinates reported for the contrast intentional > unintentional transgressions) and applied a liberal threshold ($p < 0.05$, uncorrected), no significant activation was found. Furthermore, no activation was present in the left amygdala.

Unintentional versus intentional norm violations

Comparison of brain activation related to reading the unintentional norm violations versus intentional norm violations revealed three clusters (*Table 6.4; Figure 6.3C*). The first cluster contained the left orbitofrontal cortex, left paracingulate gyrus and subcallosal cortex, and extended into the right frontal medial cortex ($p < 0.001$; cluster-size 2179 voxels; peak coordinate in MNI space (x, y, z): -26, 36, -14; peak *z*-value = 4.39). The second cluster encompassed the right postcentral gyrus and right middle frontal gyrus ($p = 0.002$; cluster-size 982 voxels; peak coordinate in MNI space (x, y, z): 38, -36, 66; peak *z*-value = 3.42), the third cluster was located in the left lateral occipital cortex and the left superior parietal lobule ($p = 0.003$; cluster-size 926 voxels; peak coordinate in MNI space (x, y, z): -34, -64, 58; peak *z*-value = 3.80).

Overlap between intentional and unintentional norm violations

In line with the work of Berthoz and colleagues (2002), we also examined common areas activated by the intentional and unintentional norm violations. We created a binary mask of the significant activation cluster of contrast 2 (unintentional norm violation endings > neutral endings) and investigated activation related to contrast 1 (intentional norm violation endings > neutral endings) within this mask. We found three clusters of common activation (*Table 6.4; Figure 6.3D*): a cluster encompassing left and right superior frontal gyrus ($p = 0.02$; cluster-size 167 voxels; peak coordinate in MNI space (x, y, z): 4, 56, 34; peak *z*-value = 3.43), a cluster in the left paracingulate gyrus extending into the left superior frontal gyrus ($p = 0.02$; cluster-size 150 voxels; peak coordinate in MNI space(x, y, z): -12, 52, 16; peak *z*-value = 3.23) and a cluster in the left frontal pole ($p = 0.05$; cluster-size 98 voxels; peak coordinate in MNI space (x, y, z): -26, 40, 40; peak *z*-value = 2.99).

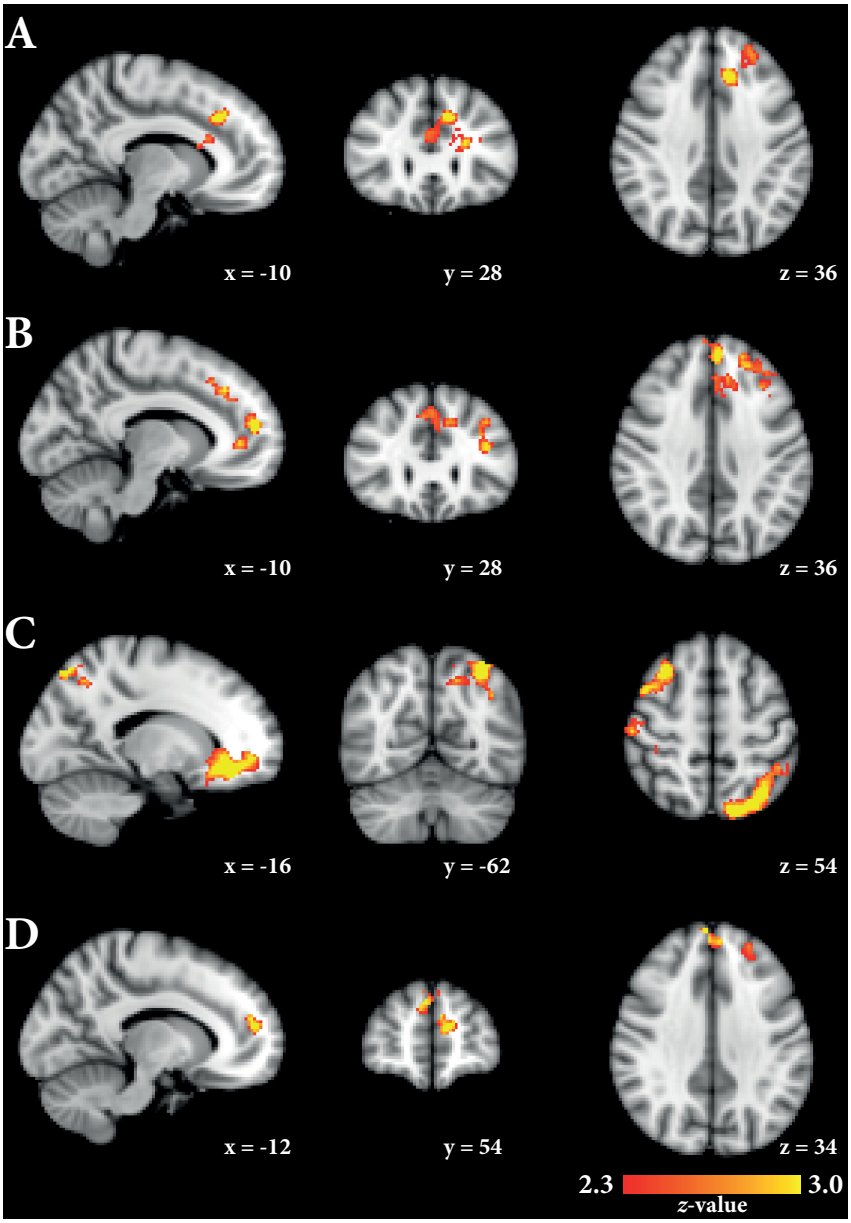

Figure 6.3 Significant activation clusters related to processing stories describing social norm violations.
Figure 6.3A Contrast intentional > neutral;
Figure 6.3B Contrast unintentional > neutral;
Figure 6.3C Contrast unintentional > intentional;
Figure 6.3D Overlap intentional & unintentional.
Clusters are superimposed on the template MNI_T1_152_2mm_brain (partial brain coverage; inferior parts of the frontal medial cortex and superior parts of the postcentral gyrus are not included). All images are displayed according to radiological convention: right in image is left in brain.

Table 6.4. Brain activity related to reading social stories describing intentional and unintentional norm violations versus neutral situations.

Cluster	Region	*Z*-score	Peak coordinates (MNI space)			Cluster size
			x	y	z	
Intentional norm violations vs neutral stories						
1	Left paracingulate gyrus / superior frontal gyrus	3.39	-10	28	36	748
	Left frontal pole	2.99	-26	40	40	
	Left frontal operculum cortex	2.87	-44	12	6	
	Left amygdala*	3.15	-18	-10	-12	21
Unintentional norm violations vs neutral stories						
1	Left paracingulate gyrus / superior frontal gyrus	3.99	-14	52	14	1604
	Left superior frontal gyrus	3.72	-4	46	38	
	Left middle frontal gyrus	3.46	-36	30	20	
	Right superior frontal gyrus	3.46	8	52	28	
	Left frontal pole	3.39	-20	42	32	
Intentional versus unintentional norm violations						
	No significant clusters					
Unintentional versus intentional norm violations						
1	Left orbitofrontal cortex	4.39	-26	36	-14	2179
	Left paracingulate gyrus	3.52	-10	48	-6	
	Right frontal medial cortex	3.52	2	52	-8	
	Subcallosal cortex	3.46	2	26	-8	
2	Right postcentral gyrus	3.42	38	-36	66	982
	Right middle frontal gyrus	3.30	32	20	54	
3	Left lateral occipital cortex	3.80	-34	-64	58	926
	Left superior parietal lobule	3.36	-36	-58	48	
Overlap unintentional and intentional norm violations						
1	Right superior frontal gyrus	3.43	4	56	34	167
	Left superior frontal gyrus	3.15	-6	50	36	
2	Left paracingulate gyrus	3.23	-12	52	16	150
	Left superior frontal gyrus	2.98	-6	54	22	
3	Left frontal pole	2.99	-26	40	40	98

Footnote

* : post-hoc analysis using mask of left amygdala.

Discussion

In the present study, we investigated the behavioral and neural correlates of social norm processing in two independent samples, using a new instrument: the revised Social Norm Processing Task (SNPT-R). The SNPT-R, based on a task originally developed by Berthoz and colleagues (2002, 2006) and used by Blair et al. (2010), entails three conditions, allowing the investigation of the neural responses and behavioral ratings related to processing 1st stories describing intentional violations of social norms, 2nd stories on unintentional violations of social norms, and 3rd neutral social stories (*Figure 6.1*), in both adolescents and adults. We examined the behavioral ratings of the stories (concerning inappropriateness and embarrassment) in a sample of adolescents and adults (n = 87), and examined both the behavioral as well as the neural correlates of social norm processing using functional magnetic resonance imaging (fMRI) in an independent sample of 21 adults. Our overall aim was to replicate the results from previous versions of the SNPT (Berthoz et al., 2002, 2006; Blair et al., 2010) and to describe the characteristics of the SNPT-R in detail, in order to enable the use of this paradigm in future studies involving both healthy participants and patient populations. Findings are discussed below.

Ratings of embarrassment and inappropriateness: dependent on type of story

In a large sample of adolescents and adults, we examined the ratings of inappropriateness and embarrassment concerning the three types of stories. Because all stories were written in second person ('you') and participants were asked to imagine themselves in the situation described, the ratings reflect how participants evaluate their own social norm transgressions. Results indicated a consistent effect of condition: participants rated the stories describing intentional social norm violations as the most inappropriate and the most embarrassing, while the unintentional social norm transgressions were rated more inappropriate and more embarrassing than the neutral stories (*Table 6.2; Figure 6.*2). These effects of condition were confirmed in the behavioral ratings by another, independent sample (n = 21) of adults (*Table 6.3*). Again, intentional social norm violations were rated as more inappropriate and more embarrassing than unintentional social norm violations. It is important to mention that we aimed to keep the actual outcomes of the intentional and unintentional social norm transgressions as far as possible the same. Thereby, these results indicate that participants consider their intention of importance for the evaluation of the transgression. The higher levels of inappropriateness for the intentional social norm violations indicate that participants are familiar with social conventions, while we hypothesize that the higher levels of embarrassment for the intentional social norm violations indicate that participants 1st realize that intentional actions decrease their personal reputation to a greater extent than unintentional actions (Moll & Schulkin, 2009), and 2nd that they are aware that intentional social norm violations require more prosocial behavior (i.e. by communicating to others

that they recognize and regret their misbehavior and that they will do better in the future, as defined by Miller (2007)) than unintentional social norm violations.

Our finding with respect to the pattern of inappropriateness ratings is in line with the results of Berthoz and colleagues, demonstrating that healthy males (n = 12) rated intentional norm violations as more inappropriate than unintentional norm violations (Berthoz et al., 2002). However, the ratings of embarrassment reported here do not coincide with those described in Berthoz et al. (2002), who found that mean embarrassment ratings were significantly higher for the unintentional social norm violations than for the intentional social norm violations. Nevertheless, our results seem to be in line with the behavioral ratings on embarrassment by healthy participants (n = 16) in the study by Blair et al. (2010), showing slightly higher ratings of embarrassment for intentional than for unintentional social norm violations - although this study did not statistically test within-group differences between the task conditions. These discrepancies stress the need for replication studies.

It is important to note that the SNPT-R differs from previous versions of the paradigm (Berthoz et al., 2002, 2006; Blair et al., 2010) in the sense that the SNPT-R includes four age- and gender specific versions: for boys < 18 years of age, girls < 18 years of age, men ≥ 18 years of age and women ≥ 18 years of age. These versions were created in order to maximize the personal involvement of participants with the task, which was important because we aimed to investigate the behavioral and neural responses involved in evaluating one's own actions (cf. (Finger et al., 2006)). We investigated whether the effect of condition on inappropriateness and embarrassment was present in all participant groups. Results showed that this was indeed the case, indicating that all four versions of the SNPT-R enable distinguishing intentional and unintentional social norm violations based on behavioral ratings of inappropriateness and embarrassment. We did find, however, differences between the groups: boys considered the stories as less inappropriate when compared to the adult groups (both men and women), while women reported higher levels of embarrassment when rating the stories (in comparison to all other groups; *Figure 6.2*). We hypothesize that these effects are due to gender differences and developmental changes in moral sensitivity (Jennings, Mitchell, & Hannah, 2015; Krettenauer, Colasante, Buchmann, & Malti, 2014; You, Maeda, & Bebeau, 2011), but future research is needed to examine this in detail.

Processing stories on social norm violations: overlapping and differential activation patterns for intentional and unintentional violations

Imaging results showed that reading stories describing social norm violations (both intentional and unintentional) evoked overlapping activation within the frontal pole, the paracingulate gyrus, and the superior frontal gyrus, relative to reading neutral social stories (*Table 6.4; Figure 6.3*). Furthermore, we observed activation within the middle frontal gyrus related to reading unintentional social norm violations when compared to reading neutral stories, while reading intentional social norm violations (in comparison to reading neutral

stories) evoked activation within the frontal pole, paracingulate gyrus and frontal operculum cortex. In addition, reading stories on intentional social norm transgressions was related to activation in the left amygdala. When contrasting unintentional and intentional norm violations, differential activation was found within three clusters: a cluster encompassing the left orbitofrontal cortex, frontal medial cortex and subcallosal cortex, a cluster involving the right postcentral gyrus and right middle frontal gyrus, and an occipital-parietal cluster (*Table 6.4; Figure 6.3*). There were no clusters where reading the intentional norm violations evoked more activation in comparison to the unintentional norm violations.

These results are largely in line with the findings of Berthoz and colleagues (2002), who investigated the neural systems underlying the processing of social norm transgressions in a sample of twelve healthy male participants; they reported activation in several regions in the medial prefrontal cortex in response to social norm violations, as well as in the orbitofrontal cortex, temporo-parietal regions and the basal temporal cortex. Furthermore, a re-analysis of the same dataset revealed enhanced activation in the left amygdala in response to intentional social norm violations, a finding that was replicated in the present study. In addition, our findings coincide with the results of neuroimaging studies considering brain activation related to thinking about the self and thinking about others, and of studies on moral reasoning – processes which are important in evaluating social norm violations (Denny, Kober, Wager, & Ochsner, 2012; Finger et al., 2006; Gallagher & Frith, 2003; Michl et al., 2014; Moll, de Oliveira-Souza, Bramati, & Grafman, 2002; Northoff et al., 2006; Schaich Borg et al., 2006; Takahashi et al., 2004). More specifically, the paracingulate gyrus and superior frontal gyrus, activated by both intentional and unintentional social norm violations, have been implicated previously in mentalizing (Gallagher & Frith, 2003) and the experience of shame (Michl et al., 2014), embarrassment (Takahashi et al., 2004) and guilt (Morey et al., 2012), while activation within the frontal pole is associated with moral reasoning (Schaich Borg et al., 2006). Furthermore, the ventral medial frontal cortex and orbitofrontal cortex, in this study activated by unintentional social norm violations, were found to be involved in self-related judgements (Denny et al., 2012), self-referential processing (Northoff et al., 2006), moral emotions (Moll, de Oliveira-Souza, Eslinger, et al., 2002; Moll, de Oliveira-Souza, Bramati, et al., 2002) and in evaluative processes of embarrassment (Takahashi et al., 2004). Our results build upon these findings and provide more insight in the neural processes underlying dealing with one's own social norm transgressions.

It should, however, be noted that we did not find significant clusters when contrasting intentional versus unintentional social norm violations, while Berthoz et al. (2002) reported more pronounced activation in several prefrontal, temporal and parietal regions when investigating this contrast. This discrepancy is possibly due to differences in task parameters (the task employed by Berthoz and colleagues involved both personal and impersonal stories, as well as stories comprised of 'unrelated words' (Berthoz et al., 2002, 2006), while the SNPT-R only involved personal stories written in second-person), and the use of a

more stringent statistical threshold in the present study. In addition, we cannot exclude the possibility that the participants' initial reaction to the stories, while reading them in the MRI scanner, differs from the reaction as reflected in the ratings after the scan session. These ratings indicated higher levels of embarrassment and inappropriateness for the stories on intentional social norm violations, but it is possible that unintentional transgressions evoked more arousal on the first time reading, which is reflected in increased activation levels in the brain. However, data to test this hypothesis are not available.

Limitations and suggestions for future research

In line with previous work on the SNPT (Berthoz et al., 2002; Blair et al., 2010), we focused on the experience of embarrassment in relation to social norm violations. However, given the fact that social norm violations could also evoke other reactions, future studies could investigate how participants rate the stories with respect to the experience of other prosocial emotions like shame and guilt (Jankowski & Takahashi, 2014; Robins & Schriber, 2009; Tangney et al., 2006), as well as look into the potential positive outcomes of social norm transgressions for the transgressor (van Kleef, Wanders, Stamkou, & Homan, 2015).

A limitation of the present study is the relatively small sample size of the adolescent sample (13 boys and 16 girls). However, the distribution of the variances was not significantly different between the groups and the effect of condition on behavioral ratings was robustly present in all samples (all $p < 0.001$, both for ratings of inappropriateness and embarrassment), so we feel our data provide substantial support for the usefulness of the SNPT-R in these populations.

Another shortcoming is the fact that we did not acquire imaging data in the adolescent sample. As a result, we were not able to investigate developmental changes in brain activation related to social norm processing. Given the fact that adolescence is a time period characterized by influential changes in social-affective and social cognitive abilities (Crone & Dahl, 2012; Haller et al., 2015), it could be hypothesized that reading one's own social norm violations evokes differential activation patterns in adolescents in comparison to adults. Future studies, in line with the behavioral study by Lahat and colleagues (Lahat, Helwig, & Zelazo, 2012), could investigate this topic.

Furthermore, based on the results of Blair et al. (2010), showing aberrant behavioral and neural responses to social norm violations in patients with SAD, and given the fact that social anxiety symptoms are present at a continuum, ranging from a total lack of symptoms to normal levels of social anxiety or even mild social fears, in the normal population (Rapee & Spence, 2004), future studies could investigate the relation between self-report measures of social anxiety and behavioral ratings of social norm violations in healthy participants. In addition, we suggest that the SNPT-R could be used to investigate the behavioral and neural correlates of social norm processing in other patient populations in which disturbances of social behavior are present, for example in patients with frontal brain lesions, patients with

frontotemporal dementia and patients with personality disorders. Using the SNPT-R across disorders is in line with the Research Domain Criteria project (RDoC), which proposes a framework for conducting research in which core symptoms (in this case: disturbances in social behavior) are studied at different levels and across diagnostic classifications of disorders, in order to gain more insight in the mechanisms underlying normal and abnormal behavior (Insel, 2014).

Conclusions

To conclude, the data presented here provide support for the use of the SNPT-R to investigate the behavioral and neural substrates of social norm processing. Intentional social norm violations were rated as more inappropriate and more embarrassing when compared to unintentional social norm violations, while reading stories describing these violations evoked activation within the frontal pole, the paracingulate gyrus and the superior frontal gyrus. Furthermore, processing unintentional social norm violations was associated with activation in, among others, the orbitofrontal cortex, middle frontal gyrus and superior parietal lobule, while reading intentional social norm violations was related to activation in the left amygdala. These regions have been previously implicated in thinking about one's self, thinking about others and moral reasoning. These findings indicate that the SNPT-R could serve as a useful paradigm for examining social norm processing, both at the behavioral and neural level.

Supplemental Analysis

Sensitivity analysis on outcome stories

We aimed to keep the actual outcome of the action described in the intentional condition the same as the outcome of the action described in the unintentional condition. However, as the editor pointed out in his comments on a previous version of this manuscript, the phrasing of the stories differs between the intentional and unintentional condition. In some stories, we only varied words like 'purposefully' and 'by accident', in other stories we used a different verb that in itself explained whether the action was intentional or unintentional. We chose to do so to make the paradigm more lively (we thought that only varying the words 'purposefully' and 'by accident' would make the task monotonous), and more realistic. However, these differences in phrasing could have induced differences in how participants considered the outcomes of the actions, which could have subsequently influenced their ratings of inappropriateness and embarrassment.

We performed a sensitivity analysis to investigate whether these phrasing differences between the stories could have systematically influenced our results. First, we determined for each story whether the outcome of the intentional action could be considered different (more severe) relative to the effect of the action described in the unintentional condition. This seems to be the case for ten stories (*Supplemental Table S6.2*); for these stories, it is uncertain whether research participants consider the effects of the intentional and unintentional condition the same.

Second, to investigate the extent to which these stories might have influenced the embarrassment and inappropriateness ratings, we performed a sensitivity-analysis by excluding these stories. Repeated measures ANOVAs (condition-by-group) on the remaining 16 stories showed the same effects as reported for the full set of stories: we found significant effects of condition on both the ratings of embarrassment ($F(1.7,143.8) = 643.44$, $p < 0.001$) and inappropriateness ($F(1.8,150.8) = 1740.5$, $p < 0.001$). Post-hoc paired-samples t-tests confirmed that the mean ratings of inappropriateness were significantly higher for the intentional stories relative to the unintentional stories ($t(86) = 25.5$, $p < 0.001$), while the unintentional stories were rated as more inappropriate compared to the neutral stories ($t(86) = 33.0$, $p < 0.001$). Furthermore, intentional stories were rated as more embarrassing than the unintentional stories ($t(86)= 2.7$, $p = 0.007$), while unintentional stories were more embarrassing relative to neutral stories ($t(86) = 38.4$, $p < 0.001$).

In our opinion, these findings indicate that the overall effect that we have reported in the manuscript does not depend on the stories in which the outcome of the intentional and the unintentional action could be considered to be different.

Supplemental Tables

Supplemental Figure S6.1 QR code for easy access to Supplemental Table S6.1.

Supplemental Table S6.1 Full list of *SNPT-R* stories.
Stories are available in Dutch, as used in the LFLSAD, and in English.
Furthermore, stories are available for download at the Open Science Framework Database (Bas-Hoogendam, van Steenbergen, Kreuk, et al., 2017b). https://osf.io/pt4qt/files/ - Methods – SNPT-R stories

	Dutch	English
1	Je wilt een ritje in de achtbaan maken	You want to take a ride on the roller coaster
Intentional	Je ziet de lange rij en dringt voor	You see the long queue, and push in at the front of the line
Unintentional	Je ziet de lange rij niet en gaat vooraan staan	You don't see the long queue, and go and stand at the front
Neutral	Je ziet de lange rij en sluit achteraan	You see the long queue, and go and stand at the end
2	Je loopt op het strand	You are walking on the beach
Intentional	Je ziet een zonnende vrouw liggen en schopt tegen haar been	You see a woman sunbathing and kick her leg
Unintentional	Je ziet een zonnende vrouw niet liggen en struikelt over haar been	You don't notice a woman sunbathing and accidentally trip over her leg
Neutral	Je ziet een zonnende vrouw liggen en loopt om haar heen	You see a woman sunbathing and step around her
3	Je hebt een glas cola in je hand (jongens/meisjes); Je hebt een glas wijn in je hand (mannen/vrouwen)	You are holding a glass of coke (boys/girls); You are holding a glass of wine (men/women)
Intentional	Je loopt naar een vriend en gooit cola (wijn) over hem heen	You walk over to a friend and throw the coke (wine) on him
Unintentional	Je struikelt en morst cola (wijn) over een vriend	You stumble and spill coke (wine) on a friend
Neutral	Je drinkt de cola (wijn) samen met een vriend op	You and your friend finish the glass of coke (wine)
4	Je vouwt in de pauze een papieren vliegtuigje (jongens/meisjes) Je vouwt een papieren vliegtuigje terwijl je op je werk bent (mannen/vrouwen)	During break, you make a paper airplane (boys/girls) While you are at work, you make a paper airplane (men/women)
Intentional	Je gooit het vliegtuigje naar je leraar (collega) en het raakt zijn hoofd	You throw the airplane at your teacher (colleague) and the airplane hits his head
Unintentional	Je gooit het vliegtuigje naar een open raam maar het raakt het hoofd van je leraar (collega)	You throw the airplane at the open window, but the airplane hits your teacher (colleague) on the head
Neutral	Je gooit het vliegtuigje door het open raam naar buiten	You aim the airplane at the open window, and the airplane flies through the window
5	Je gaat naar de wc	You go to the toilet
Intentional	Je ziet dat er iemand op zit, maar trekt de deur open	You see that it's occupied but you open the door
Unintentional	Je ziet niet dat er iemand op zit en doet de deur open	You don't see that it's occupied and you open the door

	Dutch	English
Neutral	Je ziet dat er iemand op zit en wacht voor de deur	You see that it's occupied and wait in front of the door
6	Je krijgt een zelfgebakken koekje van een vriendin	You receive a cookie your friend baked
Intentional	Je vindt het koekje vies en spuugt het uit	You dislike it and spit it out
Unintentional	Je verslikt je in het koekje en spuugt het uit	You take a bite of the cookie, choke, and spit it out
Neutral	Je neemt een hap en eet het koekje op	You take a bite of the cookie, chew, and swallow it
7	Je bent op een feestje	You are at a party
Intentional	Je gooit je drankje over een andere gast	You throw your drink over another guest
Unintentional	Je struikelt en morst je drankje over een andere gast	You trip, and spill your drink over another guest
Neutral	Je geeft je drankje aan een andere gast	You give your drink to another guest
8	Je staat op een balkon boven een drukke stoep	You are standing on a balcony above a busy street
Intentional	Je ziet een vrouw en spuugt op haar hoofd	You see a woman below and spit on her head
Unintentional	Je spuugt en raakt per ongeluk een vrouw op haar hoofd	You spit and hit a woman by accident
Neutral	Je spuugt naar beneden en raakt een lege plek op de stoep	You see an empty patch below, spit and hit the ground
9	Je gaat bij je oma eten (jongens/meisjes); Je gaat bij je schoonmoeder eten (mannen/vrouwen)	You go to your grandmother's house for dinner (boys/girls); You go to your mother-in-law's for dinner (men/women)
Intentional	Je vindt het eten niet lekker en spuugt het uit	You do not like the food and spit it out
Unintentional	Je voelt je helemaal niet lekker en moet overgeven	You feel unwell and vomit over the table
Neutral	Je vindt het eten lekker en eet het op	You like the food and eat it all
10	Je zit in de klas en moet heel nodig naar de wc (jongens/meisjes); Je zit in een vergadering en moet heel nodig naar de wc (mannen/vrouwen)	You are in the classroom and really need to pee (boys/girls); You are in a meeting and really need to pee (men/women)
Intentional	Je wilt het niet ophouden en plast op de grond	You don't want to hold it in and pee on the floor in front of everyone
Unintentional	Je kan het niet ophouden en plast in je broek	You can't hold it in and wet yourself in front of everyone
Neutral	Je kan het ophouden en gaat na de les naar de wc	You hang on until the end of the meeting and go to the toilet afterwards
11	Je komt door de regen kletsnat aan op school (jongens/meisjes); Je komt door de regen kletsnat aan op je werk (mannen/vrouwen)	You get soaked on the way to school (boys/girls); You get soaked on the way to the office (men/women)

	Dutch	English
Intentional	Je kleedt je uit waar iedereen bij is om droge kleren aan te trekken	You undress in front of everyone in order to change clothes
Unintentional	Je trekt je natte kleren uit en dan komen opeens klasgenoten (collega's) aanlopen	You undress, but suddenly classmates (colleagues) walk in on you
Neutral	Je gaat naar je werkplek en laat je kleren opdrogen	You sit down at your desk and wait to get dry
12	Je drinkt een glas cola bij de lunch op school (jongens/meisjes); Je drinkt een glas cola bij de lunch op je werk	You drink a glass of coke for lunch at school (boys/girls); You drink a glass of coke for lunch at the office (men/women)
Intentional	Je ziet je leraar (baas) en laat expres een harde boer	You see your teacher (boss) and burp in front of him
Unintentional	Je ziet je leraar (baas) niet en laat per ongeluk een harde boer	You don't see your teacher (boss), and burp in front of him
Neutral	Je ziet je leraar (baas) en kan een boer nog net inhouden	You see your teacher (boss) and are able to swallow the burp
13	Je bent op het vliegveld en moet naar de wc	You need to go to the toilet at the airport
Intentional	Je ziet een damestoilet maar je gaat toch naar binnen (jongens/mannen); Je ziet een herentoilet maar je gaat toch naar binnen (meisjes/vrouwen)	You notice the sign on the wall but go into the women's toilet anyway (boys/men); You notice the sign on the wall but go into the men's toilet anyway (girls/women)
Unintentional	Je ziet niet dat het een damestoilet is en je gaat naar binnen (jongens/mannen); Je ziet niet dat het een herentoilet is en je gaat naar binnen (jongens/mannen)	You don't see the sign on the wall and go into the women's toilet (boys/men); You don't see the sign on the wall and go into the men's toilet (girls/women)
Neutral	Je ziet het damestoilet en zoekt dan naar het herentoilet (jongens/mannen); Je ziet het herentoilet en zoekt dan naar het damestoilet (meisjes/vrouwen)	You see the sign of the women's toilet and look for the men's toilet (boys/men); You see the sign of the women's toilet and look for the women's toilet (girls/women)
14	Je bent in het zwembad	You are at the swimming pool
Intentional	Je duikt in het water en trekt je zwembroek uit (jongens/mannen); Je duikt in het water en trekt je bikinibroekje uit (meisjes/vrouwen)	You dive in the water and take off your swimming trunks (boys/men); You dive in the water and take off your bikini bottoms (girls/women)
Unintentional	Je duikt in het water en je zwembroek (bikinibroekje) zakt af	You dive in the water and your swimming trunks (bikini bottoms) fall off
Neutral	Je duikt in het water en trekt je zwembroek (bikinibroekje) weer goed	You dive in the water and adjust your swimming trunks (bikini bottoms)
15	Je bent in de klas aan het lezen (jongens/meisjes); Je bent op je kantoor met collega's (mannen/vrouwen)	You and your class are quietly reading (boys/girls); You are at the office with your colleagues (men/women)
Intentional	Je laat expres een harde wind	You decide to fart loudly
Unintentional	Je laat per ongeluk een harde wind	You accidentally fart loudly

	Dutch	English
Neutral	Je moet een wind laten maar houdt het tegen	You have to fart but you can hold it in
16	Je speelt voetbal	You are playing football
Intentional	Je schiet expres de bal in je eigen doel	You purposefully kick the ball in your own team's goal
Unintentional	Je schiet per ongeluk de bal in je eigen doel	You accidentally kick the ball in your own team's goal
Neutral	Je schiet de bal in het doel van de tegenstander	You purposefully kick the ball in the other team's goal
17	Op bezoek bij familie begint je neus te lopen	While visiting family, your nose starts to run
Intentional	Je veegt je neus af aan het tafellaken	You wipe your nose on the table cloth
Unintentional	Je neus drupt per ongeluk op het tafellaken	Your nose drips by accident on the table cloth
Neutral	Je snuit je neus in een zakdoekje	You wipe your nose in a tissue
18	Je bakt een appeltaart samen met vrienden	You are baking an apple pie with your friends
Intentional	Voor de grap gebruik je zout in plaats van suiker	You use salt instead of sugar as a joke
Unintentional	Je vergist je en gebruikt zout in plaats van suiker	You use salt instead of sugar without realizing
Neutral	Je gebruikt de juiste hoeveelheid suiker	You use the amount of sugar the recipe calls for
19	Je hebt met een vriend afgesproken om een computerspelletje te spelen (jongens/meisjes); Je hebt met een vriend afgesproken om koffie te gaan drinken (mannen/vrouwen)	You've arranged to meet a friend to play a computer game (boys/girls); You've arranged to meet a friend for coffee (men/women)
Intentional	Je besluit om weg te blijven	You decide not to turn up
Unintentional	Je vergeet de afspraak	You forget to go
Neutral	Je gaat naar de afspraak	You go and meet your friend
20	Je past op de hamster van de buren	You are petsitting for your neighbour
Intentional	Je besluit de hamster niet te voeren en de hamster gaat dood	You decide not to feed the hamster, and it dies
Unintentional	Je vergeet de hamster te voeren en de hamster gaat dood	You forget to feed the hamster, and it dies
Neutral	Je voert de hamster totdat de buren weer terug zijn	You feed the hamster until the neighbour's return
21	Je verft het haar van een vriend (jongens/mannen); Je verft het haar van een vriendin (meisjes/vrouwen)	You are dyeing your friend's hair
Intentional	Je verft het haar expres blauw	You purposefully dye it blue

		Dutch	English
	Unintentional	Er gaat iets mis en het haar wordt blauw	You mess up and her hair becomes blue
	Neutral	Zoals afgesproken verf je het haar blauw	You dye it blue as planned
22		Je bent aan de telefoon met een vriend (jongens/mannen); Je bent aan de telefoon met een vriendin (meisjes/vrouwen)	You are on the phone with a friend
	Intentional	Je vindt het gesprek niet interessant en je hangt op zonder gedag te zeggen	You feel bored with the conversation and hang up without saying goodbye
	Unintentional	De telefoon glijdt uit je hand en de verbinding wordt verbroken	The telephone slips out of your hand and the connection is lost
	Neutral	Na gedag te zeggen zet je de telefoon uit	After saying goodbye you hang up
23		Je zit in de bus naast een onbekende man	You are on the bus next to a stranger
	Intentional	Je leunt tegen de schouder van je buurman en gaat slapen	You decide to sleep and lean on your neighbour's shoulder
	Unintentional	Je valt in slaap tegen de schouder van je buurman	You fall asleep, slumped against your neighbour's shoulder
	Neutral	Je valt in slaap tegen het raam	You fall asleep leaning against the window
24		Je bent in de klas (jongens/meisjes); Je bent aan het werk (mannen/vrouwen)	You are in your classroom (boys/girls); You are at the office (men/women)
	Intentional	Je ziet de leraar (je baas) aankomen en gooit de deur voor zijn neus dicht	You see your teacher (boss) coming and you slam the door in his face
	Unintentional	Je ziet de leraar (je baas) niet aankomen en doet de deur vlak voor hem dicht	You don't see your teacher (boss) coming and you shut the door right in front of him
	Neutral	Je ziet de leraar (je baas) aankomen en houdt de deur voor hem open	You see your teacher (boss) coming and you hold the door open for him
25		Je bent erg verkouden	You have a cold
	Intentional	Voor de lol nies je in het gezicht van een klasgenoot (jongens/meisjes); Voor de lol nies je in het gezicht van een collega (mannen/vrouwen)	You sneeze in your classmate's face for a laugh (boys/girls); You sneeze in your colleague's face for a laugh (men/women)
	Unintentional	Je moet ineens niezen en niest in het gezicht van een klasgenoot (collega)	All of a sudden you have to sneeze and you sneeze in a classmate's (colleague's) face
	Neutral	Je moet niezen en houdt je hand voor je mond	You have to sneeze and you cover your mouth with your hand
26		Je laat de hond uit	You are walking the dog
	Intentional	Je ziet dat de hond op straat poept maar je loopt door	You see the dog defecating on the street but you keep walking
	Unintentional	Je ziet niet dat de hond op straat poept en je loopt door	You don't see the dog defecating on the street and you keep walking
	Neutral	Je ziet dat de hond op straat poept en je ruimt het op	You see the dog defecating on the street and you clean it up

Supplemental Table S6.2 Comparison of outcome *SNPT-R* stories (sensitivity analysis).

	Intentional	Unintentional	Outcome same severity?
1	You see the long queue, and push in at the front of the line	You don't see the long queue, and go and stand at the front	Questionable
2	You see a woman sunbathing and kick her leg	You don't notice a woman sunbathing and accidentally trip over her leg	Questionable
3	You walk over to a friend and throw the coke on him	You stumble and spill coke on a friend	Questionable
4	You throw the airplane at your teacher and the airplane hits his head	You throw the airplane at the open window, but the airplane hits your teacher on the head	Yes
5	You see that it's occupied but you open the door	You don't see that it's occupied and you open the door	Questionable
6	You dislike it and spit it out	You take a bite of the cookie, choke, and spit it out	Yes
7	You throw your drink over another guest	You trip, and spill your drink over another guest	Questionable
8	You see a woman below and spit on her head	You spit and hit a woman by accident	Yes
9	You do not like the food and spit it out	You feel unwell and vomit over the table	Questionable
10	You don't want to hold it in and pee on the floor in front of everyone	You can't hold it in and wet yourself in front of everyone	Questionable
11	You undress in front of everyone in order to change clothes	You undress, but suddenly classmates walk in on you	Yes
12	You see your teacher and burp in front of him	You don't see your teacher, and burp in front of him	Yes
13	You notice the sign on the wall but go into the women's toilet anyway	You don't see the sign on the wall and go into the women's toilet	Yes
14	You dive in the water and take off your swimming trunks	You dive in the water and your swimming trunks fall off	Yes
15	You decide to fart loudly	You accidentally fart loudly	Yes
16	You purposefully kick the ball in your own team's goal	You accidentally kick the ball in your own team's goal	Yes
17	You wipe your nose on the table cloth	Your nose drips by accident on the table cloth	Questionable
18	You use salt instead of sugar as a joke	You use salt instead of sugar without realizing	Yes
19	You decide not to turn up	You forget to go	Yes
20	You decide not to feed the hamster, and it dies	You forget to feed the hamster, and it dies	Yes
21	You purposefully dye it blue	You mess up and his hair becomes blue	Yes
22	You feel bored with the conversation and hang up without saying goodbye	The telephone slips out of your hand and the connection is lost	Yes

Supplemental Table S6.2 Comparison of outcome *SNPT-R* stories (sensitivity analysis). (*continued*)

	Intentional	Unintentional	Outcome same severity?
23	You decide to sleep and lean on your neighbour's shoulder	You fall asleep, slumped against your neighbour's shoulder	Questionable
24	You see your teacher coming and you slam the door in his face	You don't see your teacher coming and you shut the door right in front of him	Questionable
25	You sneeze in your classmate's face for a laugh	All of a sudden you have to sneeze and you sneeze in a classmate's face	Yes
26	You see the dog defecating on the street but you keep walking	You don't see the dog defecating on the street and you keep walking	Yes

Supplemental Figure S6.2 QR code for easy access to Supplemental Tables S6.3-4.

Supplemental Table S6.3 and Supplemental Table S6.4

These supplemental tables are, due to their size, publicly available online at the Open Science Framework Database (Bas-Hoogendam, van Steenbergen, Kreuk, et al., 2017b). https://osf.io/pt4qt/files/ - Results – Behavioral Data – S2 Dataset and S3 Dataset

Chapter 7

Not intended, still embarrassed: social anxiety is related to increased levels of embarrassment in response to unintentional social norm violations

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Abstract

Background

Social anxiety disorder (SAD) is associated with altered social norm (SN) processing: SAD patients rate stories on SN violations as more inappropriate and more embarrassing than healthy participants, with the most prominent effect for stories on unintentional SN violations (i.e. committing a blunder). Until now it's unknown how levels of social anxiety (SA) are related to ratings of SN violations in the general population, in which SA-symptoms are present at a continuum. More insight in this relationship could improve our understanding of the symptom profile of SAD. Therefore, we investigated the relation between ratings of SN violations and SA-levels in the general population.

Methods

Adults and adolescents (n = 87) performed the revised Social Norm Processing Task (SNPT-R) and completed self-report questionnaires on social anxiety. Repeated measures ANCOVAs were used to investigate the effect of SA on the ratings of inappropriateness and embarrassment.

Results

As hypothesized, participants with higher SA-levels rated SN violations as more inappropriate and more embarrassing. Whereas participants with low-to-intermediate SA-levels rated unintentional SN violations as less embarrassing than intentional SN violations, participants with high SA-levels (z-score SA $\geq$ 1.6) rated unintentional SN violations as equally embarrassing as intentional SN-violations.

Conclusions

These findings indicate that increased embarrassment for unintentional SN violations is an important characteristic of social anxiety. These high levels of embarrassment are likely related to the debilitating concern of socially-anxious people that their skills and behavior do not meet expectations of others, and to their fear of blundering. This concern might be an important target for future therapeutic interventions.

Introduction

Social anxiety (SA) is an emotion that is experienced by most people with some regularity. Typically, people want to make a good impression when they are in a social situation, and when committing a blunder in the presence of others, people tend to feel embarrassed or ashamed. However, the experience of social anxiety varies between people, ranging from discomfort in specific social situations for some individuals to an intense fear in almost all social situations for others (Miskovic & Schmidt, 2012). At the upper end of this 'continuum of social anxiety' (Rapee & Spence, 2004) lies social anxiety disorder (SAD), a psychiatric condition which is, according to the Diagnostic and Statistical Manual of Mental Disorders (DSM-5), characterized by an intense fear of being negatively evaluated in social situations (American Psychiatric Association, 2013). This fear of social-evaluative stimuli (Wong & Rapee, 2016), which is out of proportion to the actual threat and to the sociocultural context (Heimberg et al., 2014; Leichsenring & Leweke, 2017) leads to the avoidance of social situations and results in significant disturbances in a person's everyday life (American Psychiatric Association, 2013; Stein & Stein, 2008). The typical onset of SAD is during childhood or adolescence and several environmental as well as intrinsic factors like genetic influences, biological factors as well as cognitive biases interact in the development of the disorder (Spence & Rapee, 2016).

Previous studies have indicated that SAD patients experience disturbances in self-referential processing and have biases concerning the opinion of others about them: they have increased self-portrayal concerns (Moscovitch et al., 2013), for example when it concerns their social rank (Berger, Keshet, & Gilboa-Schechtman, 2017; Gilboa-Schechtman et al., 2017) or their own social performance (Gavric, Moscovitch, Rowa, & McCabe, 2017; Glazier & Alden, 2017), they overestimate the negative consequences of their own social blunders (Moscovitch, Waechter, Bielak, Rowa, & McCabe, 2015), and are characterized by negatively biased learning about themselves from social feedback (Koban et al., 2017). Furthermore, clinical SAD is associated with an increased belief in negative interpretations of social situations (Loscalzo, Giannini, & Miers, 2017), and SAD patients focus predominantly on potentially embarrassing events when they evaluate themselves in a social context (Blair & Blair, 2012). Such negative self-beliefs, which are already present in adolescents with SAD (Blöte, Miers, Heyne, Clark, & Westenberg, 2014; Schreiber & Steil, 2013), are related to increased negative emotions like fear and anxiety, and induce maladaptive behavioral responses like safety behaviors, which, consecutively, lead to the maintenance of social anxiety (Goldin, Manber-Ball, Werner, Heimberg, & Gross, 2009; Piccirillo, Dryman, & Heimberg, 2016). It has been argued that SAD patients are 'uniquely and primarily concerned about characteristics of self that they perceive as being deficient or contrary to perceived societal expectations or norms' (Moscovitch, 2009). According to this view, one of the main concerns of SAD patients is the fear that they will unintentionally

commit an embarrassing behavioral blunder in a social situation (Moscovitch, 2009), which let us to hypothesize that social anxiety is specifically related to the experience of increased embarrassment in reaction to unintentional social norm violations.

This idea was previously examined by investigating the behavioral data of a functional magnetic resonance imaging (fMRI) study using the social norm processing task (SNPT) (Blair et al., 2010). In this task, participants read three types of short stories: stories describing neutral social situations, stories on unintentional social norm (SN) transgressions (i.e. committing a blunder) and stories describing intentional SN transgressions (i.e. breaking conventional rules) and they are asked to imagine themselves in the situation described. Subsequently, participants rate the stories on inappropriateness and embarrassment. Thereby, the SNPT enables investigating the effect of intention on these ratings. Blair and colleagues (2010) showed that, while SAD patients had higher self-reported levels of inappropriateness and embarrassment across all conditions, the effect of SAD was most pronounced for unintentional SN violations: adult patients with generalized SAD (n = 16) rated these unintentional transgressions as significantly more embarrassing when compared to healthy participants (n = 16). Furthermore, the fMRI analyses revealed that reading the unintentional stories evoked increased activation in the ventromedial prefrontal cortex in SAD. This activation was considered to represent increased self-referential processing and was taken to indicate that SAD patients judge unintentional SN violations as more self-relevant than healthy participants (Blair et al., 2010).

The results of this study (Blair et al., 2010), which was the first, and, to the best of our knowledge, the only study to date investigating the difference between processing intentional and unintentional SN violations in SAD, provide important initial evidence that the intention underlying a SN violation is a determining factor in the experience of embarrassment in social anxiety: although SAD patients reported higher embarrassment for all social situations, they differed most from control participants when they considered unintentional transgressions (Blair et al., 2010). However, the sample size of the study was relatively small. In addition, participants performed an 'impersonal' version of the SNPT, in which the stories described behavior of an unknown character like 'Joanna' (cf. (Berthoz et al., 2002)), as a result of which it could be questioned whether the ratings reflect the participants' opinion about their own SN violations. Furthermore, it is unknown if the effect of intention on the level of embarrassment also holds for participants with higher SA-levels in the general population.

Here, we investigated the relation between self-reported SA and behavioral ratings of SN violations in a sample of adults and adolescents from the general population (n = 87), using the revised Social Norm Processing Task (SNPT-R) (Bas-Hoogendam, van Steenbergen, Kreuk, et al., 2017a). In the SNPT-R, the three types of stories were written in second-person, in order to let the ratings reflect how participants think about their own SN viola-

tions. Data of this sample on the SNPT-R have been published previously (Bas-Hoogendam, van Steenbergen, Kreuk, et al., 2017a).

Based on previous work (Blair et al., 2010), we hypothesized that higher SA-levels within the general population would be predictive of a general effect of SA, reflected by higher ratings of inappropriateness and embarrassment for all stories and of an intention-specific effect of SA, namely an even more pronounced increase in embarrassment ratings for stories on unintentional SN violations. More insight in this relationship could help further unravel mechanisms involved in the etiology and maintenance of social anxiety and may identify potential novel targets for prevention and intervention.

Methods

Participants

Participants were adults and adolescents from the general population (n = 87; age range 12.5 – 32.6 y), the same as those described previously (Bas-Hoogendam, van Steenbergen, Kreuk, et al., 2017a); details of the inclusion procedure are described in the *Supplemental Methods*. They had Dutch as their first language and were free of past and present psychopathology as assessed by a self-report questionnaire. After explanation of the procedure, all participants (and in case of minors below 18 years of age, both parents) signed informed consent according to the Declaration of Helsinki. The Psychology Research Ethics Committee of Leiden University approved the experiment.

Social Norm Processing Task

Participants performed the revised Social Norm Processing Task (SNPT-R), described in detail previously (Bas-Hoogendam, van Steenbergen, Kreuk, et al., 2017a). The SNPT-R consists of two phases (*Figure 6.1*).

In the first phase, participants read three types of short stories: stories on situations in which no social norm (SN) was violated (neutral condition; for example: 'You are baking an apple pie with your friends. You use the amount of sugar the recipe calls for'), stories describing unintentional SN violations (unintentional condition; 'You are baking an apple pie with your friends. You use salt instead of sugar without realizing') and stories outlining intentional SN violations (intentional condition; 'You are baking an apple pie with your friends. You use salt instead of sugar as a joke'). Stories in the unintentional and intentional condition described relatively innocent violations of conventional social norms, in situations where at least one other person was present. The intentional and unintentional stories differed only in the intention of the actor, while the actual result of the violation (for example: a distasteful cake) was kept as much as possible the same. Stories were written in second-person and participants were instructed to imagine themselves in the situations,

in order to maximize their personal involvement (cf. (Finger et al., 2006)). Therefore, four age- and gender specific versions of the task were used: for boys < 18 years, girls < 18 years, men ≥ 18 years and women ≥ 18 years. The task consisted of 78 stories and a full list of stories is provided in *Supplemental Table S6.1* (Bas-Hoogendam, van Steenbergen, Kreuk, et al., 2017a). We refer the reader to this work and to the Open Science Framework (OSF) project (Bas-Hoogendam, van Steenbergen, Kreuk, et al., 2017b) for more details on task parameters and scripts for task presentation.

Secondly, there was an unannounced rating-phase, in which participants were asked to rate all stories on a five-point Likert scale on embarrassment (from 1, not embarrassing at all, to 5, extremely embarrassing) and inappropriateness (from 1, not inappropriate at all, to 5, extremely inappropriate). These ratings were the output measures used in this study.

Both phases of the SNPT-R were presented using E-Prime software (version 2.0.10, Psychology Software Tools; available at osf.io/pt4qt (Bas-Hoogendam, van Steenbergen, Kreuk, et al., 2017b)).

Self-report questionnaires

As there exists, to the best of our knowledge, no instrument which is suitable to reliably assess the level of social anxiety in both adults and adolescents, two questionnaires were used to determine social anxiety: depending on their age, participants completed the self-report version of the Liebowitz Social Anxiety Scale (LSAS) (Heimberg et al., 1999) or the Social Anxiety Scale for Adolescents (SAS-A) (La Greca & Lopez, 1998). The LSAS is a questionnaire for adults measuring fear in and avoidance of situations that are likely to elicit social anxiety (Fresco et al., 2001; Heimberg et al., 1999). The SAS-A (La Greca & Lopez, 1998) measures social anxiety in adolescents, with satisfactory levels of internal consistency (Miers et al., 2013).

Boys and girls did not differ in self-reported SA as measured with the SAS-A (independent-samples t-test: $t(27) = -0.41$, $p = 0.69$), while women reported significantly more SA-symptoms compared to men, as measured with the LSAS ($t(56) = -3.24$, $p = 0.002$) (cf. (Asher, Asnaani, & Aderka, 2017; Carleton et al., 2007; Duke, Krishnan, Faith, & Storch, 2006; Ingles, La Greca, Marzo, Garcia-Lopez, & Garcia-Fernandez, 2010; Turk et al., 1998)) (*Table 7.1*). Because we aimed to investigate the relation between self-reported SA and ratings on the SNPT-R within each group of participants (boys < 18 years; girls < 18 years; men ≥ 18 years; women ≥ 18 years), rather than over the whole sample (an analysis which could be influenced by age- and gender differences), we normalized the scores on the LSAS and SAS-A within each group and used the *z*-scores (SA-*z*) for further analyses. The validity of this measure was established by additional analyses, separate for the adolescent and adult sample, using the original LSAS and SAS-A scores; in these analyses, we observed in general the same pattern of results as described in the Results section. Furthermore,

exploratory analyses indicated no significant interactions between SA-*z*, age group (adult vs. adolescents) and behavioral ratings.

After *z*-standardizing the scores on the LSAS and SAS-A within each group, one participant (male) was considered an outlier (SA-*z* = 3.27) and removed from subsequent analyses (remaining sample: $n = 86$).

Procedure

The experiment took place at the Faculty of Social and Behavioral Sciences, Leiden University, the Netherlands (adult participants) and at a secondary school in the Netherlands (adolescent participants). Participants performed both phases of the SNPT-R and the self-report questionnaires on a laptop in a quiet environment.

Data analysis

Statistical analyses of the ratings of embarrassment and inappropriateness for the SNPT-R stories were performed using IBM SPSS Statistics for Mac (Version 24.0). The relationships between behavioral ratings of inappropriateness and embarrassment and social anxiety were investigated using repeated measures ANCOVAs with condition (intentional; unintentional; neutral) as within-subjects factor and SA-*z* as covariate. Significant effects of condition were further investigated using paired-samples t-tests; significant effects of SA-*z* were examined using separate regression analyses for each condition (independent variable: SA-*z*; dependent variables: ratings), while significant interactions between condition and SA-*z* were explored using regression analyses with the difference scores of the ratings as dependent variables (e.g., ΔIntentional_unintentional = intentional score minus unintentional score). For reasons of completeness and in line with the analyses reported previously (Bas-Hoogendam, van Steenbergen, Kreuk, et al., 2017a), we repeated the above described ANCOVAs with group (based on the four versions of the task; group 1: boys < 18 years; group 2: girls < 18 years; group 3: men ≥ 18 years; group 4; women ≥ 18 years) as additional between-subjects factor. Results of these analyses are summarized in *Supplemental Table S7.1* and *Supplemental Table S7.2* and discussed in the *Supplemental Results*. For all analyses, significance level was set at $p \leq 0.05$; Greenhouse–Geisser correction was used when the assumption of sphericity was violated.

Results

Participants

Characteristics of the participants, divided into four groups based on the age- and gender-specific versions of the SNPT-R, are summarized in *Table 7.1*. Data are also available at osf.io/j58yc/(Bas-Hoogendam, van Steenbergen, van der Wee, & Westenberg, 2017a). Using

literature-based cutoff scores, 8 adults (14% of the adult sample; LSAS score ≥ 60 (Mennin et al., 2002)) and 2 adolescents (7 % of the adolescent sample; SAS-A score ≥ 50 (Storch et al., 2004)) met the criteria for generalized SAD.

Table 7.1 Characteristics participants.

	Boys (*n* = 13)	**Girls (*n* = 16)**	**Men (*n* = 29)**	**Women (*n* = 29)**
Age (years)	14.0 ± 1.2 (12.7 - 16.5)	14.2 ± 1.4 (12.5 - 17.0)	21.1 ± 3.1 (18.5 - 32.6)	19.2 ± 1.2 (18.1 - 24.1)
Social anxiety				
SAS-A	36.3 ± 9.2 (20 - 54)	37.6 ± 8.2 (26 - 56)	n.a	n.a
LSAS	n.a	n.a	31.0 ± 17.1 (2 - 87)	47.3 ± 21.0 (16 - 89)

Abbreviations
LSAS: Liebowitz Social Anxiety Scale (Heimberg et al., 1999); SAS-A: Social Anxiety Scale for Adolescents (La Greca & Lopez, 1998); n.a: not applicable.

Footnote
Values represent mean ± standard deviation (range).

Relationship between ratings and self-reported social anxiety

Ratings of inappropriateness and embarrassment on the SNPT-R are summarized in *Table 7.2*; for group-specific ratings, we refer the reader to (Bas-Hoogendam, van Steenbergen, Kreuk, et al., 2017a).

Table 7.2 Ratings of embarrassment and inappropriateness on the *SNPT- R*.

	Inappropriateness			**Embarrassment**		
	Intentional	***Unintentional***	***Neutral***	***Intentional***	***Unintentional***	***Neutral***
Total sample	4.42 ± 0.36	2.93 ± 0.51	1.29 ± 0.20	3.83 ± 0.67	3.50 ± 0.56	1.25 ± 0.21

Footnote
Values represent mean ± standard deviation.

Inappropriateness

A repeated measures ANCOVA (condition-by-SA-*z*) on inappropriateness ratings indicated a main effect of condition ($F(1.8,149.5) = 2230.9$, $p < 0.001$, partial $\eta^2 = 0.97$), a main effect of SA-*z* ($F(1,84) = 7.1$, $p = 0.009$, partial $\eta^2 = 0.08$), and an interaction between condition and SA-*z* ($F(1.8,149.5) = 3.9$, $p = 0.026$, partial $\eta^2 = 0.05$). Paired-samples t-tests revealed that intentional stories were rated more inappropriate than unintentional stories ($t(85) = 27.4$, $p < 0.001$) and unintentional stories as more inappropriate than neutral stories ($t(85) = 33.6$, $p < 0.001$), as reported previously (Bas-Hoogendam, van Steenbergen, Kreuk, et al., 2017a).

As illustrated in *Figure 7.1* (left panel), subsequent regression analyses revealed positive relationships between SA-*z* and ratings of inappropriateness in the unintentional (β = 0.30, p = 0.005; 95 % confidence interval (CI): 0.10 – 0.55) and neutral condition (β = 0.25, p = 0.020; 95 % CI: 0.04 – 0.50), but not in the intentional condition (β = 0.085, *ns*; 95 % CI: -0.14 – 0.33). The interaction between SA-*z* and condition was further investigated using regression analyses on difference scores. Results showed that SA-*z* was positively related to ΔUnintentional_intentional (β = 0.24, p = 0.024; 95 % CI: 0.04 - 0.49) and to ΔUnintentional_neutral (β = 0.23, p = 0.033; 95 % CI: 0.20 – 0.48), but not related to ΔIntentional_neutral (β = -0.05, *ns*; 95 % CI: -0.29–0.18). These findings indicate that the slope of the regression line for the relationship between SA-*z* and inappropriateness in the unintentional condition is significantly steeper when compared to the slopes of the

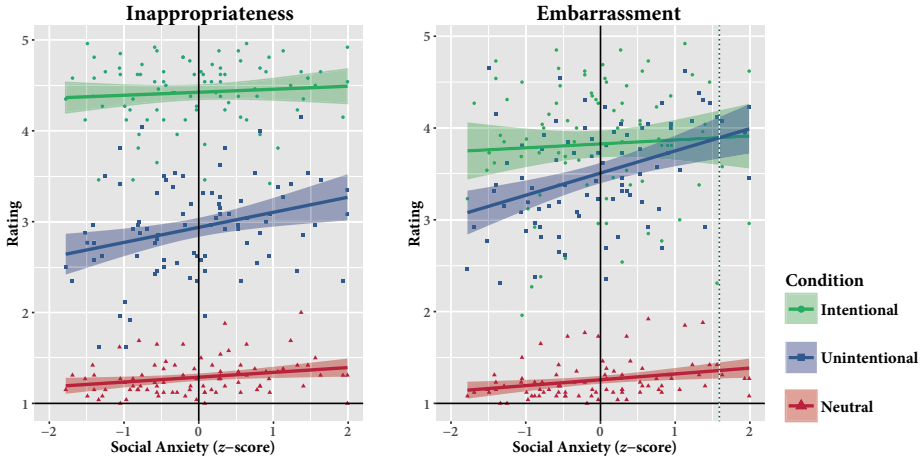

Figure 7.1 Relationships between social anxiety (*z*-standardized) and ratings on the SNPT-R.
Shaded areas represent 95% confidence intervals.

regression lines for the relationships between SA-*z* and inappropriateness in the intentional and neutral condition.

Embarrassment

A repeated measures ANCOVA on the ratings of embarrassment showed a main effect of condition ($F(1.8,151.0) = 909.9$, $p < 0.001$, partial $\eta^2 = 0.92$), a main effect of SA-*z* ($F(1,84) = 7.4$, $p = 0.008$, partial $\eta^2 = 0.08$), and an interaction between condition and SA-*z* ($F(1.8,151.0) = 4.7$, $p = 0.013$, partial $\eta^2 = 0.05$). Intentional stories were rated as more embarrassing than unintentional stories ($t(85) = 4.5$, $p < 0.001$) and unintentional stories as more embarrassing than neutral stories ($t(85) = 39.9$, $p < 0.001$) (Bas-Hoogendam, van Steenbergen, Kreuk, et al., 2017a).

Regression analyses revealed significant positive relationships between SA-*z* and embarrassment in the unintentional condition (β = 0.40, $p < 0.001$; 95 % CI: 0.22 – 0.65) and the neutral condition (β = 0.28, $p = 0.008$; 95 % CI: 0.08 – 0.53), but not between SA-*z* and embarrassment in the intentional condition (β = 0.06, ns; 95 % CI: -0.17 – 0.30) (*Figure 7.1*, right panel). The interaction between SA-*z* and condition was further investigated using regression analyses on difference scores. Results showed a significant positive relationship between SA-*z* and ΔUnintentional_intentional (β = 0.28, $p = 0.01$; 95 % CI: 0.07 - 0.53), a positive relationship with ΔUnintentional_neutral (β = 0.32, $p = 0.003$; 95 % CI: 0.12 – 0.57), but no relationship between SA-*z* and ΔIntentional_neutral (β = -0.03, *ns*; 95 % CI: -0.27 – 0.20). Again, these findings indicate that the effect of SA-*z* is most pronounced (i.e., steepest slope) on the embarrassment ratings in the unintentional condition. Interestingly, the regression lines depicting the relationships between SA-*z* and embarrassment on the intentional and unintentional condition intersect at intermediate-to-high SA-levels (SA-*z* = 1.6; *Figure 7.1*, right panel): while individuals with low SA-levels give lower embarrassment scores for unintentional versus intentional SN transgressions, this effect disappears with increasing levels of social anxiety and is no longer significant at high SA-levels in our sample.

Discussion

We investigated the relationship between self-reported levels of social anxiety (SA) and behavioral ratings on the revised Social Norm Processing Task (SNPT-R) in adults and adolescents from the general population. Previous research showed that patients with social anxiety disorder (SAD) rated all SNPT-stories as significantly more inappropriate and more embarrassing compared to healthy participants, with the most noticeable effect for the unintentional condition (Blair et al., 2010). These findings support the hypothesis proposed by Moscovitch (2009) that one of the main concerns of SAD patients is the fear that they will unintentionally commit an embarrassing behavioral blunder in a social situation. Building upon this work, we predicted a general effect of SA, namely, that higher SA-levels in the general population would be associated with higher ratings of inappropriateness and embarrassment. Furthermore, we expected to find an intention-specific effect of SA, reflected by a more pronounced effect of SA on embarrassment ratings for stories on unintentional SN violations.

These hypotheses were confirmed: overall, participants with higher SA-levels rated the stories as more inappropriate and more embarrassing (*Figure 7.1*), while subsequent regression analyses revealed how this general effect of SA was related to the different conditions of the inappropriateness and embarrassment ratings. In the intentional condition, inappropriateness and embarrassment ratings were unrelated to SA, suggesting that SA did not influence people's basic judgment of SN violations: breaking conventional rules is

simply considered 'not done' and evokes embarrassment independent of the level of SA. However, SA-levels were positively related to inappropriateness and embarrassment in the neutral condition, which might reflect the general tendency of socially-anxious individuals to feel uncomfortable in social situations (Miskovic & Schmidt, 2012). The strongest positive relationships were present between SA-levels and ratings of inappropriateness and embarrassment in the unintentional condition. Importantly, we found an intention-specific effect of SA for embarrassment when comparing the unintentional and intentional conditions. While participants with low-to-intermediate SA-levels rated stories on unintentional SN violations as less embarrassing than stories describing intentional SN violations, participants with high SA-levels (z-score SA $\geq$ 1.6) rated unintentional SN violations as equally embarrassing as intentional SN violations (*Figure 7.1*, right panel). In other words, participants with lower SA-levels distinguish between breaking conventional rules and committing a blunder in their embarrassment ratings: they take the intention underlying the transgression into account and report less embarrassment when the action was unintentional. However, participants with higher SA-levels do not make this distinction. Note that this intention-specific effect of SA was not found for inappropriateness: individuals with higher SA-levels did still distinguish between intentional and unintentional SN violations with respect to inappropriateness.

These findings hint at a dissimilarity in the cognitive and affective evaluation of SN violations: at the cognitive level (evaluation of inappropriateness), individuals with high SA-levels are not all that different from those with low SA-levels; at the affective level (evaluation of embarrassment), however, they fail to make the distinction between intentional and unintentional SN violations. This increased experience of embarrassment could contribute to the development and maintenance of SAD, as embarrassment is a self-conscious emotion with two sides: although it is a prosocial emotion signaling the recognition of misbehavior and holding the promise that the mistake will not happen again, it also represents negative self-evaluations (Feinberg et al., 2012; Jankowski & Takahashi, 2014; Miller, 2014). When embarrassment occurs too often and too intensely, these negative self-evaluations can lead to an overestimation of the extent to which a misstep is important to others, to misplaced and needless concerns about other people's judgment, and to timid, passive behavior (Miller, 2007) – a tendency that characterizes socially-anxious people.

The results reported here extend those of Blair and colleagues (Blair et al., 2010), by showing that the aberrant behavioral response to unintentional SN violations observed in SAD patients is also present in participants from the general population with high SA-levels. Furthermore, our finding of increased embarrassment for unintentional SN violations is in line with previous work, which indicated that both SAD patients as well as participants with high SA-levels overestimate the negative consequences of unintentional social blunders (Moscovitch, Rodebaugh, & Hesch, 2012; Moscovitch et al., 2015). In addition, our results link to the idea that both negative interpretation biases as well as disordered self-referential

processing, at the cognitive and neural level, are important characteristics of SA (Abraham et al., 2013; Blair, Geraci, Otero, et al., 2011; Blair & Blair, 2012; Boehme, Miltner, et al., 2015; Clark & McManus, 2002; Giménez et al., 2012; Hirsch & Clark, 2004; Kreifelts et al., 2014; Miers et al., 2008; Morrison & Heimberg, 2013; Müller-Pinzler et al., 2015; Ziv et al., 2013). Furthermore, studies have indicated that SA is associated with increased levels of perfectionism, especially with heightened concerns over making mistakes (Antony, Purdon, Huta, & Swinson, 1998; Ashbaugh et al., 2007; Cox & Chen, 2015; Newby et al., 2017) and with high levels of self-criticism (Cox, Fleet, & Stein, 2004). Together with these observations, our results support the idea that participants with high SA-levels are characterized by a fear of blundering in a social situation and by a strong concern that their skills and behavior do not meet perceived societal expectations (Moscovitch, 2009). Thereby, our findings contribute to understanding the symptom profile in at-risk populations and in SAD patients, and could aid in improving preventive and therapeutic interventions for this disorder. Cognitive behavioral therapy could, for example, challenge the concern of patients that their self-characteristics are deficient and do not satisfy societal norms, and help patients to realize that the consequences of unintentional blunders are probably not as bad as they consider them to be (Moscovitch, 2009). This is of importance, given that the increased experience of embarrassment leads to maladaptive coping strategies like avoidance and safety behaviors, which are maintaining factors of SAD (Hofmann, 2007; Piccirillo et al., 2016; Wong & Rapee, 2016).

A limitation of the present study is the relatively small sample size, especially given the fact that participants were divided into four groups based on the versions of the SNPT-R (versions for respectively boys, girls, men and women). Especially the number of included adolescents is limited, as a result of which we were unable to investigate whether age influences the relationship between SA and embarrassment. Given that adolescence is a critical time period for the onset of SAD (Haller et al., 2015), a longitudinal study on a large sample of adolescents could give more insight in the role of embarrassment in the development of SAD. In addition, the presence of past and present psychopathology in the sample was only assessed by self-report, which could lead to an underestimation of psychopathology. For example, SAD patients are often underestimating their condition and refrain from consulting their general practitioner, which may lead to underdiagnosis (Dingemans et al., 2001). Actually, ten participants of the current sample met the criteria for generalized SAD as based on the cutoff scores for the LSAS and SAS-A (Mennin et al., 2002; Storch et al., 2004), but due to the lack of structured clinical interview, these diagnoses could not be confirmed by a clinician.

Furthermore, we did not acquire neuroimaging data, thus we could not relate our data to neural activity (cf. (Blair et al., 2010)). Future imaging studies could investigate whether SA-levels alter activation in brain regions involved in SN processing. Because increased activation in the ventromedial prefrontal cortex in SAD patients in response to unintentional

SN violations has been reported (Blair et al., 2010), we hypothesize that higher SA-levels are related to differential activation within this region. Such an experiment could provide more insight in the neural basis of the altered SN processing associated with high SA-levels. Furthermore, family studies involving SAD patients as well as their relatives could investigate whether altered SN processing and the associated neural pattern are heritable characteristics, representing an endophenotype of SAD (Bas-Hoogendam et al., 2014a, 2016). This could enhance our understanding of the familial component of SAD.

Conclusions

To conclude, the data presented here show that high levels of social anxiety in the general population are associated with increased embarrassment for unintentional social norm violations. Although the generalizability of our results might be limited by the relatively small sample size, these findings provide more insight in the core fear of socially-anxious individuals and offer clues for therapeutic interventions.

Supplemental Methods

Participants

Participants were recruited via flyers, in-class announcements and by word of mouth and tested between November 2014 and December 2015 (adults: November - December 2014; adolescents: June 2015 - December 2015). Adolescents were recruited at a secondary school in the Netherlands; adults were students from Leiden University, Faculty of Social and Behavioral Sciences. After performing the experiment, participants were debriefed about the aim of the study and they received a compensation for partaking in the experiment (adults: study credits; adolescents: chocolate bar).

Ninety-four participants signed up for the current experiment; four participants were excluded from participation because they did not meet the selection criteria (n = 3: present medication use; n = 1: present physical disorder). Furthermore, data from three participants were excluded from the analyses because they performed a version of the SNPT-R that did not match their age. Therefore, the total sample size of the present study was 87 participants.

Supplemental Results

ANCOVAs including group as between-subjects factor

The results of the ANCOVAs (summarized in *Supplemental Table S7.1* and *Supplemental Table S7.2*), including group as a between-subject factor, show that the effects of SA-*z* on the ratings of inappropriateness and embarrassment, as well as the interactions between SA-*z* and condition, are comparable to the effects reported in the main text of the paper.

Furthermore, the significant effects of group (both on the ratings of inappropriateness as well as on the ratings of embarrassment) and the interactions between group and condition, are in line with the results reported previously (Bas-Hoogendam, van Steenbergen, Kreuk, et al., 2017a). However, neither the SA-*z*-by-group-by-condition interactions, nor the SA-*z*-by-group interactions, reached significance. We take these findings as an indication that the effects of SA-*z*, as described in the paper, are not influenced by group.

Supplemental Tables

Supplemental Table S7.1 ANCOVA Condition x group, with SA-*z* as covariate – inappropriateness ratings.

Within-subjects effects	F	*p*	Partial η^2
Main effect condition	F(1.6, 127.9) = 2065.3	$p < 0.001$	0.96
Interaction condition x *SA*-z	F(1.6, 127.9) = 4.3	$p = 0.022$	0.05
Interaction condition x group	F(4.9, 127.9) = 3.3	$p = 0.008$	0.11
Interaction condition x group x *SA*-z	F(4.9, 127.9) = 0.8	$p = 0.53$	0.03
Between-subjects effects			
Main effect *SA*-z	F(1, 78) = 7.5	$p = 0.008$	0.09
Main effect group	F(3, 78) = 4.2	$p = 0.008$	0.14
Interaction *SA*-z x group	F(3, 78) = 0.1	$p = 0.94$	0.005

Footnote
In line with Bas-Hoogendam et al. (2017), group is based on the version of the *SNPT-R*: group 1: boys < 18 years of age; group 2: girls < 18 years of age; group 3: men ≥ 18 years of age; group 4: women ≥ 18 years of age.

Supplemental Table S7.2 ANCOVA Condition x group, with SA-*z* as covariate – embarrassment ratings.

Within-subjects effects	F	*p*	Partial η^2
Main effect condition	F(1.6, 127.7) = 837.6	$p < 0.001$	0.92
Interaction condition x SA-*z*	F(1.6, 127.7) = 5.9	$p = 0.006$	0.07
Interaction condition x group	F(4.9, 127.7) = 2.7	$p = 0.03$	0.09
Interaction condition x group x SA-*z*	F(4.9, 127.7) = 1.8	$p = 0.11$	0.07
Between-subjects effects			
Main effect SA-*z*	F(1, 78) = 10.7	$p = 0.002$	0.12
Main effect group	F(3, 78) = 7.5	$p < 0.001$	0.22
Interaction SA-*z* x group	F(3, 78) = 0.9	$p = 0.42$	0.04

Footnote
In line with Bas-Hoogendam et al. (2017), group is based on the version of the *SNPT-R*: group 1: boys < 18 years of age; group 2: girls < 18 years of age; group 3: men ≥ 18 years of age; group 4: women ≥ 18 years of age.

Chapter 8

Altered neurobiological processing of unintentional social norm violations: a multiplex, multigenerational fMRI study on social anxiety endophenotypes

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Abstract

Background

Patients with social anxiety disorder (SAD) fear negative evaluation in social situations. Specifically, previous work indicated that social anxiety is associated with increased medial prefrontal cortex (mPFC) activation in response to unintentional social norm (SN) transgressions, accompanied by increased embarrassment ratings for such SN violations. Here, we used data from the multiplex, multigenerational Leiden Family Lab study on SAD, which involved two generations of families genetically enriched for SAD, and investigated whether these neurobiological and behavioral correlates of unintentional SN processing are SAD endophenotypes. Of four endophenotype criteria, we examined two: the *co-segregation of these characteristics with social anxiety (SA) within families of SAD-probands* and the *heritability* of the candidate endophenotypes.

Methods

Participants (n = 110, age-range 9.0 - 61.5 years, eight families) performed the revised Social Norm Processing Task; functional magnetic resonance imaging (fMRI) data and behavioral ratings related to this paradigm were used to examine whether brain activation in response to processing unintentional SN violations and ratings of embarrassment were associated with SA-levels. Next, heritability of these measurements was estimated.

Results

As expected, voxelwise fMRI analyses revealed positive associations between SA-levels and brain activation in the mPFC and a cluster encompassing the medial temporal gyrus, superior temporal gyrus and superior temporal sulcus, and these brain activation levels displayed moderate to moderately-high heritability. Furthermore, although SA-levels correlated positively with behavioral ratings of embarrassment for SN transgressions, these behavioral characteristics were not heritable.

Conclusions

These results show, for the first time, that brain responses in the mPFC and medial temporal gyrus, superior temporal gyrus and superior temporal sulcus, related to processing unintentional SN violations, provide a neurobiological candidate endophenotype of SAD.

Introduction

Social anxiety disorder (SAD), a prevalent anxiety disorder, is characterized by an onset during early adolescence, a chronic course and a high risk of comorbid psychopathology (Beesdo-Baum et al., 2015; Blanco et al., 2011; Haller et al., 2015; Kessler et al., 2012; Merikangas et al., 2010; Stein et al., 2017). Furthermore, treatment for SAD is at present often suboptimal (Weisberg, Beard, Moitra, Dyck, & Keller, 2014). Thereby, this psychiatric condition has a large negative impact on the patients' lives (McKnight, Monfort, Kashdan, Blalock, & Calton, 2016; Wittchen et al., 2000) as well as on society (Baxter, Vos, Scott, Ferrari, & Whiteford, 2014). It is therefore essential to gain a better understanding of the vulnerability to develop SAD, in order to improve preventive and therapeutic interventions (Marín, 2016).

A defining feature of SAD-psychopathology is the fear to act in a way that will be embarrassing and humiliating (American Psychiatric Association, 2013). More specifically, it has been postulated that an important fear of SAD patients concerns that they will 'unintentionally generate an embarrassing behavioral blunder in a social situation' (Moscovitch, 2009). The neurobiological and behavioral correlates of this fear of negative evaluation, which is out of proportion to the context and actual threat (Heimberg et al., 2014), can be assessed using the Social Norm Processing Task (SNPT) (Berthoz et al., 2002). In this paradigm, participants read and evaluate three types of stories: stories describing unintentional social norm (SN) violations, stories on intentional SN violations and stories on neutral social situations. This enables examining the effect of intention on processing SN transgressions.

Two previous studies have used the SNPT to investigate SN processing related to social anxiety (SA), indicating that socially-anxious people show increased sensitivity to unintentional SN violations. The first study, an imaging study comparing 16 SAD patients with 16 healthy participants (Blair et al., 2010), revealed increased activation related to unintentional SN violations in the medial prefrontal cortex (mPFC) in SAD patients. Furthermore, patients rated all stories as more inappropriate and more embarrassing, with the most prominent effect for the unintentional SN violations, which SAD patients considered significantly more embarrassing than control subjects did (Blair et al., 2010). This effect of SA on the embarrassment ratings for unintentional SN violations was recently replicated in a community sample (Bas-Hoogendam, van Steenbergen, van der Wee, et al., 2018). Using a revised version of the SNPT (SNPT-R), which enabled investigating SN processing in children, adolescents and adults (Bas-Hoogendam, van Steenbergen, Kreuk, et al., 2017a), we reproduced the general effect of SA on ratings of inappropriateness and embarrassment, and the specific effect of SA on embarrassment ratings for unintentional SN violations: while participants with low-to-intermediate SA-levels rated unintentional SN transgressions as less embarrassing compared to intentional SN transgressions, participants with higher SA-levels rated the unintentional SN violations as equally embarrassing as the

intentional SN violations (Bas-Hoogendam, van Steenbergen, van der Wee, et al., 2018). This distinct experience of embarrassment is a critical factor underlying the development and maintenance of SA, as it could lead to negative self-evaluations and to the increased concerns about the judgments of others which are typical to SAD (Stein, 2015; Wong & Rapee, 2016).

These results suggest that aberrant processing of unintentional SN transgressions, at both the neurobiological and behavioral level, reflects an important component of the SAD phenotype. No study has, however, investigated whether these correlates of SN processing are potential endophenotypes of SAD. Endophenotypes are measurable and heritable traits located on the causal pathway from genotype to phenotype and reflect genetically-based disease mechanisms (Gottesman & Gould, 2003); this definition distinguishes endophenotypes from 'biomarkers', which do not necessarily have a genetic basis, and from the 'intermediate/extended phenotype concept', which is usually used to describe a subclinical form of a serious psychiatric disorder (Lenzenweger, 2013a). As described in more detail elsewhere (Bas-Hoogendam et al., 2016; Miller & Rockstroh, 2013; Puls & Gallinat, 2008), endophenotypes could advance our insight in the pathways leading to serious psychopathology, have potential to identify individuals at risk and can be valuable for improvement of therapeutic interventions. An endophenotype is supposed to be *associated with the disor*der (criterion 1), *state-independent and already present in a preclinical state* (criterion 2), and *heritabl*e (criterion 3). Furthermore, an endophenotype should *co-segregate with the disorder within families of probands, with non-affected family members showing altered levels of the endophenotype in comparison to the general population* (criterion 4) (Glahn et al., 2007; Lenzenweger, 2013a; Puls & Gallinat, 2008). Given that twin- and family studies suggest that genetic factors are involved in the pathogenesis of SAD, by reporting heritability estimates for SAD between 39 - 56% (Bandelow et al., 2016; Isomura et al., 2015; Scaini et al., 2014) as well as a significantly increased risk to develop the disorder in first-degree relatives of SAD patients (Merikangas, Lieb, Wittchen, & Avenevoli, 2003; Stein, Chartier, Hazen, et al., 1998), exploring whether the neurobiological and behavioral correlates of SN processing are candidate endophenotypes will provide more insight into the genetic vulnerability to this impairing disorder (Bas-Hoogendam et al., 2016).

Here, we tested the hypothesis that brain activation related to processing unintentional SN violations, as well as behavioral ratings related to such SN transgressions, are candidate SAD endophenotypes (pre-registration of hypotheses publicly available at osf.io/y5m8q (Bas-Hoogendam et al., 2014c)). We used data from the Leiden Family Lab study on Social Anxiety Disorder (LFLSAD), a unique multiplex, multigenerational family study (Bas-Hoogendam, Harrewijn, et al., 2018). This design is especially suitable to investigate candidate endophenotypes of SAD, as it allows for testing two endophenotype criteria in the same sample: *co-segregation of the candidate endophenotype with social anxiety within families of probands* and the *heritability* of the candidate endophenotype. Based on pre-

vious findings (Bas-Hoogendam, van Steenbergen, van der Wee, et al., 2018; Blair et al., 2010), we predicted a positive correlation with brain activation in the mPFC, specifically related to processing unintentional SN violations; furthermore, we hypothesized to find a positive association between SA-levels and embarrassment ratings on the unintentional SN violations. Next, as genetic influences on brain activation (Blokland et al., 2012; Mattay & Goldberg, 2004; Shan et al., 2016), as well as on personality, temperamental and emotional traits (Nivard et al., 2014; Sallis, Davey Smith, & Munafò, 2018; Stein, Jang, et al., 2002) have been demonstrated, we expected these candidate endophenotypes to be at least moderately ($h^2 \geq 0.20$) heritable.

Methods and materials

Participants

Participants were part of the LFLSAD, including two generations of families genetically enriched for SAD (total sample: $n = 132$, nine families; MRI sample: $n = 110$, eight families; we refer the reader to the *Supplemental Methods* for details about ethics, recruitment and exclusion criteria, as well as an a priori power calculation). More information with respect to the background, aims and methodology of the LFLSAD is provided elsewhere (Bas-Hoogendam, Harrewijn, et al., 2018); a pre-registration is available online (Bas-Hoogendam et al., 2014b). The sample consists of nuclear families who were invited for participation based on the combination of parent with a primary diagnosis of SAD (age 25 - 55 years; 'proband') and a child who met criteria for (sub)clinical SAD (age 8 - 21 years; 'proband's SA-child'). In addition to these two SAD cases, the proband's partner and other children from this nuclear family (age ≥ 8 years), as well as the proband's sibling(s), with their partners and children (age ≥ 8 years), were invited. Thereby, the LFLSAD sample consists of family members of two generations (*Figure 3.1*). Participants took part in several measurements, including a diagnostic interview, self-report questionnaires and an MRI scan (Bas-Hoogendam, Harrewijn, et al., 2018). The LFLSAD was approved by the Medical Ethical Committee of the Leiden University Medical Center and all participants provided informed consent.

Phenotyping

Family members participated in various measurements in order to enable extensive phenotyping (Bas-Hoogendam, Harrewijn, et al., 2018). Here, we focus on the measures of SA (see *Supplemental Methods* and *Supplemental Results* for an extended characterization of the LFLSAD sample). The presence of DSM-IV diagnoses was determined using the Mini-International Neuropsychiatric Interview (M.I.N.I.)-Plus (v5.0.0) (Sheehan et al., 1998; van Vliet & de Beurs, 2007) or the M.I.N.I.-Kid interview (v6.0) (Bauhuis et al., 2013; Sheehan

et al., 2010). Clinical SAD was established using the DSM-IV-TR criteria for the generalized subtype of SAD, but a clinician verified whether the DSM-5 criteria for SAD were also met. A diagnosis of subclinical SAD was established when participants met the DSM-5 criteria for SAD, but did not show impairing limitations in important areas of functioning (American Psychiatric Association, 2013). Furthermore, participants completed age-appropriate questionnaires on the level of SA: the Liebowitz Social Anxiety Scale (Fresco et al., 2001) or the Social Anxiety Scale for Adolescents (La Greca & Lopez, 1998). *Z*-scores were computed (Bas-Hoogendam, Harrewijn, et al., 2018) in order to use these scores over the whole sample,.

MRI experiment

Prior to the MRI scan, participants were informed about the safety procedures, and they were told that they could refrain from continuing the experiment at any time. Children and adolescents were familiarized with the MRI scanner using a mock scanner (Galván, 2010) and all participants received instructions about the task paradigms presented during the scan session. Scanning was performed using a 3.0 T Philips Achieva MRI scanner (Philips Medical Systems, Best, The Netherlands), equipped with a 32-channel Sensitivity Encoding head coil. The MRI experiment (total duration MRI protocol: 54 min 47 s) consisted of several structural scans (Bas-Hoogendam, van Steenbergen, Tissier, et al., 2018b) and functional task paradigms (Bas-Hoogendam, Harrewijn, et al., 2018), including the SNPT-R (Bas-Hoogendam, van Steenbergen, Kreuk, et al., 2017a). Scan parameters are reported in the *Supplemental Methods*.

Revised Social Norm processing Task (SNPT-R)

The SNPT-R (Bas-Hoogendam, van Steenbergen, Kreuk, et al., 2017a) is composed of a story-reading phase, taking place in the MRI scanner, and an unannounced rating phase on completion of the MRI scan (*Figure 8.1*). During the story-reading phase, short stories written in second person are presented. Stories consisted of two sentences: a stem sentence (for example: 'You are baking an apple pie with friends.') followed by an ending sentence which described either a neutral social situation ('…You use the amount of sugar the recipe calls for.'), a situation in which a social norm was unintentionally transgressed ('….You use salt instead of sugar without realizing.') or a situation in which a social norm was intentionally transgressed ('…You use salt instead of sugar as a joke.'). Stories were suitable for a broad audience and age range. However, because of the second-person form of the stories, small adaptations were made in stories describing age- or gender specific elements. Therefore, the SNPT-R has four age- and gender specific versions (boys / girls / men / women). We refer to *Supplemental Table S6.1* for all SNPT-R stories; stories are also available online at the website of the Open Science Framework (osf.io/pt4qt) (Bas-Hoogendam, van Steenbergen, Kreuk, et al., 2017b).

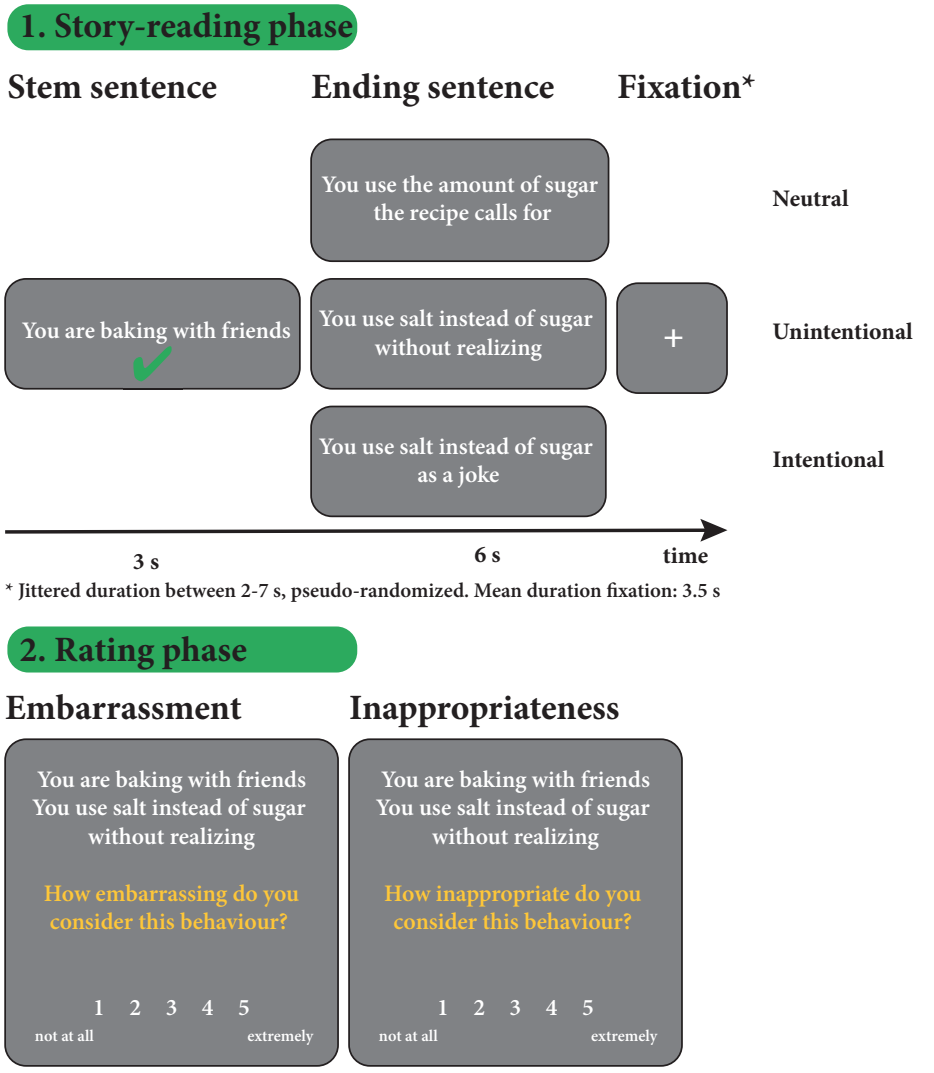


Figure 8.1 Overview of the revised Social Norm Processing Task (SNPT-R).
During the story-reading phase (1), participants read stories consisting of a stem sentence and an ending sentence, describing either a neutral social situation, a situation in which a social norm was unintentionally transgressed or situation in which a social norm was violated intentionally. Participants were instructed to imagine themselves in the situation described. In the rating phase (2), participants rated all stories on embarrassment and inappropriateness.

The SNPT-R consists of 26 stem sentences, each combined with the three different types of endings. These 78 stories were divided into two consecutive blocks of 39 stories. Participants were instructed to imagine themselves in the social situations and to press a

button after reading the stem sentence of each story, in order to verify participants' task engagement.

After the scan, participants rated all stories on a five-point Likert scale on embarrassment and inappropriateness (*Figure 8.1*). Presentation parameters are described in the *Supplemental Methods*.

Data analysis

Sample characteristics

We replaced incidental missing values on the self-report questionnaires by the average value of the completed items. Differences between participants with and without (sub)clinical SAD were examined by fitting regression models in R (R Core Team, 2016),with (sub)clinical SAD as the independent variable and the level of self-reported social anxiety (*z*-score) as dependent variable. Gender and age were included as covariates; genetic correlations between family members were modeled by including random effects.

Imaging data

General processing

Functional (f)MRI data were pre-processed using FMRIB Software Library, version 5.0.9 (Jenkinson et al., 2012); next, event-related statistical analysis was performed (details in *Supplemental Methods*). Briefly, the general linear model included four explanatory variables with their temporal derivatives, representing the presentation of a stem sentence, a neutral ending (EN), an unintentional SN violation ending (EU) and an intentional SN violation ending (EI). Three contrasts were defined: EI > EN, EU > EN and EU > EI. We verified the main effects of the SNPT-R on brain activation by using contrasts EI > EN and EU > EN (*Supplemental Results; Supplemental Table S8.3; Supplemental Figure S8.1*), while the contrast EU > EI was used for the endophenotype analysis, following previous results (Blair et al., 2010).

Neuroimaging candidate endophenotypes

The *co-segregation between SA and brain activation related to processing unintentional SN violations within the families* was investigated using regression models in R (R Core Team, 2016), with self-reported SA (*z*-score, centered) as independent variable and individual activation level related to the contrast EU > EI as dependent variable. Analyses with (sub) clinical SAD as a discrete predictor are included in the *Supplemental Methods* and *Supplemental Results*. Correlations between family members were modeled by including random effects; age (centered) and gender (centered) were included as covariates. Models were ran for each voxel separately, in order to determine the effect of SA on a whole-brain voxelwise basis. Results (*z*-scores) were transformed into a nifti-image with the same dimensions of the MNI T1-template brain. Clusters within this nifti-image, representing the association

between SA and brain activation, were corrected for multiple comparisons at the whole-brain level using the FSL tool easythresh (cluster threshold: $z > 3.1$, cluster extent threshold $p < 0.01$) (Worsley, 2001). Subsequent sensitivity analyses were performed to investigate whether the results of the association analyses were driven by the severity of depressive symptoms or by (comorbid) psychopathology other than SAD (*Supplemental Methods* and *Supplemental Results; Supplemental Tables S8.5-8.7; Supplemental Figures S8.2-8.3*).

Next, we determined the *heritability* of brain activation for voxels in the significant clusters. Voxelwise heritability estimates were obtained with a method which takes the ascertainment process into account and incorporates familial relationships (Tissier et al., 2017). Age and gender (both centered) were included as covariates.

Behavioral Data

Responses during story-reading phase

Analysis of the behavioral responses during the story-reading phase confirmed that participants paid attention to the stories (*Supplemental Results*).

Behavioral candidate endophenotypes

The *co-segregation between SA and the post-MRI SNPT-R ratings within the families* was investigated using linear mixed models in R (package: *coxme*), with self-reported SA (*z*-score, centered) as predictor of interest. Analyses with (sub)clinical SAD (discrete predictor) are described in *Supplemental Methods* and *Supplemental Results*. Separate models were used to investigate the ratings of embarrassment and inappropriateness. Task condition (intentional / unintentional / neutral), age- and gender specific task version (modeled using the dummy variables gender and age group (boys / girls vs men / women)), as well as three interaction terms (condition-by-gender, condition-by-age group, condition-by-SA-level) were added as independent variables and tested for significance. Random effects were included to account for genetic correlations between family members and within-subject correlations between task conditions. Interaction terms lacking significance were removed from the final models. Significance level was set a $p < 0.05$.

Next, we investigated whether the behavioral outcomes were *heritable*, focusing on the ratings displaying a significant association with SA. We estimated heritability by applying an approach that takes the ascertainment process into account and incorporates familial relationships, by jointly modelling the ratings and phenotype on which the family selection was based and by including random effects (Tissier et al., 2017). Age group and gender were included as possible confounders.

Results

Sample characteristics

Characteristics of the sample after quality control (n = 109 for the behavioral analyses; n = 99 for the fMRI analyses; see *Supplemental Results* for a detailed description of data availability) are summarized in *Table 8.1*. Family members with (sub)clinical SAD (n = 22 subclinical SAD; n = 17 clinical SAD) did not differ from family members without SAD (n = 62) with respect to male / female ratio and age. However, as expected, family members with (sub)clinical SAD reported higher levels of social anxiety. A detailed characterization of the sample, including clinical diagnoses other than SAD, is provided in the *Supplemental Results (Supplemental Tables S8.1-S8.2)*.

Table 8.1 Characteristics of participants with and without (sub)clinical SAD.

	Behavioral sample[a]			**fMRI sample[a]**		
	(Sub)clinical SAD (n = 39)	**No SAD (n = 62)**	**Statistical analysis**	**(Sub)clinical SAD (n = 33)**	**No SAD (n = 58)**	**Statistical analysis**
Demographics						
Male / Female (n)	20 / 19	30 /32	$\chi^2(1) = 0.08$, $p = 0.84$	16 / 17	29 / 29	$\chi^2(1) = 0.02$, $p = 1.00$
Generation 1 / Generation 2 (n)	19 / 20	27 / 35	$\chi^2(1) = 0.26$, $p = 0.68$	19 / 14	27 / 31	$\chi^2(1) = 1.02$, $p = 0.39$
Age in years (mean ± SD; range)	30.3 ± 15.5; 9.2 – 59.6	31.3 ± 15.2; 9.0 – 61.5	β (± SE) = -0.9 ± 3.1, $p = 0.76$	33.4 ± 14.9; 13.3 – 59.6	32.7 ± 14.8; 9.6 – 61.5	β (± SE) = 0.7 ± 3.2, $p = 0.83$
Diagnostic information						
Clinical SAD (n)	17	0		15	0	
Self-report measures						
Social anxiety symptoms (z-score; mean ± SD)	3.0 ± 3.3	0.6 ± 1.5	β (± SE) = 2.4 ± 0.5, $p < 0.001$	2.9 ± 3.0	0.7 ± 1.3	β (± SE) = 2.4 ± 0.4, $p < 0.001$

Abbreviations

SAD: social anxiety disorder; SD: standard deviation.

Footnote

[a]: Due to technical reasons, data on the presence of (sub)clinical SAD were lost for eight family members. Data from these participants were, however, included in the endophenotype analyses using SA-level (z-score) as a predictor (behavioral sample: n = 109; fMRI sample: n = 99).

Neuroimaging candidate endophenotypes

Whole-brain voxelwise regression analyses revealed two clusters in which self-reported SA-level was positively associated with brain activation related to the contrast EU > EI (*Table 8.2, Figure 8.2*). The first cluster (6647 voxels, $p = 1.8*10^{-7}$; corrected for multiple comparisons at the whole-brain level) was located in the occipital pole and encompassed the temporal occipital fusiform cortex, lateral occipital cortex, right superior temporal gyrus (STG), right medial temporal gyrus (MTG), superior temporal sulcus (STS) and cuneal cortex. The second cluster (1589 voxels, $p = 0.003$; corrected for multiple comparisons at the whole-brain level) comprised the frontal pole, extending to the paracingulate gyrus and mPFC. There were no clusters displaying negative relationships with SA, while visual inspection of the data confirmed the absence of outliers. Follow-up analyses confirmed the specificity of this positive association for processing unintentional SN violations, while sensitivity analyses, taking the effect of depressive symptoms and (comorbid) psychopathology other than SAD into account, further supported our results *(Supplemental Results)*.

Table 8.2 Effect of self-reported social anxiety on processing unintentional norm violations.

			Peak coordinates (MNI space)			Cluster
Cluster	**Region**	***Z*-score**	**x**	**y**	**z**	**size**
Unintentional norm violations vs intentional norm violations						
1	Temporal occipital fusiform cortex	5.45	32	-60	-18	6647
	Occipital pole	5.29	10	-94	26	
	Superior temporal gyrus, posterior division	4.31	62	-6	-8	
	Medial temporal gyrus, posterior division	3.66	60	-22	-10	
	Cuneal cortex	3.54	20	-76	32	
2	Frontal pole	5.75	-10	56	32	1589
	Frontal pole / frontal medial cortex	3.71	0	58	-4	

Subsequent voxelwise heritability analyses within the two clusters indicated that activation within the right MTG/STG/STS and mPFC, paracingulate cortex and frontal pole was heritable, with 91 voxels (cluster MTG/STG/STS) and 188 voxels (cluster mPFC) showing at least moderate ($h^2 \geq 0.20$) heritability, with some voxels displaying moderately-high ($h^2 \geq 0.40$) heritability (*Figure 8.3*).

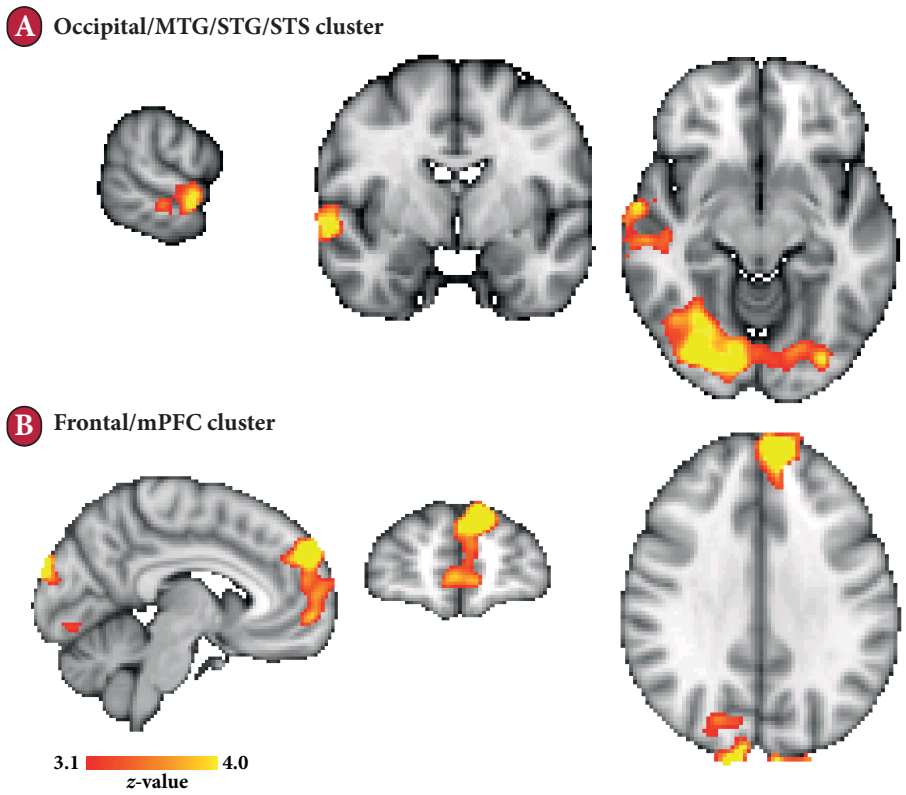


Figure 8.2 Brain activation (related to processing unintentional social norm violations) co-segregates with social anxiety within families.
Significant positive associations between social anxiety (*z*-scores) and activation related to processing stories on unintentional social norm violations versus intentional social norm violations (EU > EI). Coordinates of displayed slices (MNI, xyz): 64, -4, -10 (occipital/MTG/STG/STS cluster) and -6, 56, 32 (frontal/mPFC cluster). Clusters are displayed on the template MNI_T1_152_2mm_brain (partial brain coverage: inferior parts of the frontal medial cortex, superior parts of the postcentral gyrus as well as parts of the cerebellum are not included). Images are displayed according to radiological convention: right in the image is left in the brain.
MTG/STG/STS: medial temporal gyrus/ superior temporal gyrus/ superior temporal sulcus. mPFC: medial prefrontal cortex.

Behavioral candidate endophenotypes

Post-MRI SNPT-R ratings are summarized in *Table 8.3*; detailed results for each task version (boys / girls / men / women) are included in *Supplemental Table S8.8* and illustrated in *Supplemental Figure S8.4*. Analyses revealed significant associations between SA and embarrassment, but no relation with inappropriateness. Follow-up analyses indicated positive relationships between SA and embarrassment in all three conditions (*Figure 8.4*), while sensitivity analyses indicated that these effects were not driven by the clinical SAD cases within the sample (*Supplemental Results*).

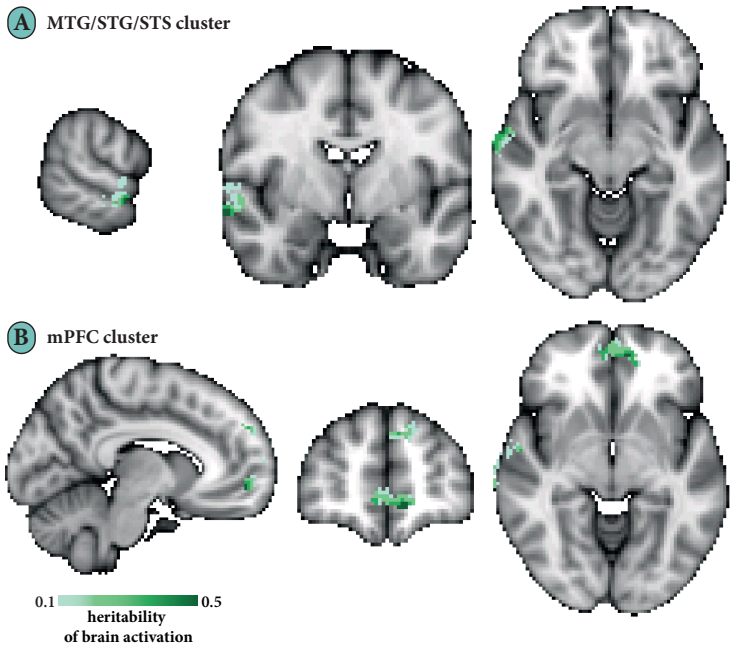


Figure 8.3 Voxelwise heritability estimates.
Coordinates of displayes slices (MNI, xyz): 64, -4, -10 (MTG/STG/STS cluster; 91 voxels with $h^2 \geq 0.20$) and -10, 52, -6 (mPFC cluster; 188 voxels with $h^2 \geq 0.20$).

Table 8.3 Ratings of inappropriateness and embarrassment.

			Effect of social anxiety (*z*-score)		Heritability
	(Sub)clinical SAD	No SAD	β ± SE	*p*	h^2
Inappropriateness			0.002 ± 0.009	0.84	
Intentional stories	4.36 ± 0.40	4.36 ± 0.43	n.i.	n.i.	n.i.
Unintentional stories	2.98 ± 0.73	2.98 ± 0.64	n.i.	n.i.	n.i.
Neutral stories	1.39 ± 0.34	1.31 ± 0.29	n.i.	n.i.	n.i
Embarrassment			0.03 ± 0.01	0.003 *	
Intentional stories	3.92 ± 0.72	3.89 ± 0.58	0.06 ± 0.02	0.010 *	0.17
Unintentional stories	3.45 ± 0.54	3.23 ± 0.51	0.06 ± 0.02	0.003 *	0.01
Neutral stories	1.38 ± 0.38	1.25 ± 0.24	0.03 ± 0.01	0.024 *	0.02

Abbreviations
h^2: heritability estimate; n.i.: not investigated; SAD: social anxiety disorder. SE: standard error.

Footnore
Values represent mean ± standard deviation.

Statistical significance
* : significant at $p < 0.05$.

Heritability analyses demonstrated that embarrassment ratings on the intentional stories had low heritability ($h^2 = 0.17$), while embarrassment scores for unintentional and neutral stories were not heritable (*Table 8.3*).

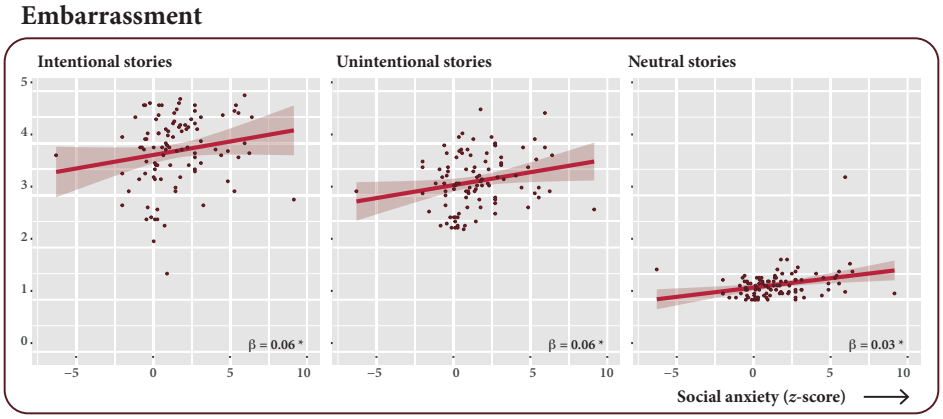


Figure 8.4 Embarrassment ratings co-segregate with social anxiety within families.
Correlation between level of self-reported social anxiety (*z*-score) and ratings of embarrassment. Shaded area represents 95% confidence interval. Asterisks indicate effects of social anxiety at $p < 0.05$.

Discussion

Here, we provide evidence that brain activation related to processing unintentional social norm (SN) violations is a neurobiological candidate endophenotype of social anxiety disorder (SAD), by using data from the Leiden Family Lab study on SAD (LFLSAD) (Bas-Hoogendam, Harrewijn, et al., 2018). This study, with its unique multiplex and multigenerational design, was especially designed to explore SAD endophenotypes (Bas-Hoogendam et al., 2014a), and our data revealed that SAD-related neurobiological alterations in processing unintentional SN violations *co-segregated with social anxiety (SA) within families of probands* ($n = 99$). Next, our data indicated that these aberrant brain activation patterns displayed moderate to moderately-high heritability, providing support for the endophenotype criterion of *heritability*. Thereby, we replicate and extend previous work on the processing of SN violations in SAD, which provided support for the endophenotype criterion of *association with the disorder,* by reporting increased brain activation in the medial prefrontal cortex (mPFC) related to processing unintentional SN violations in SAD patients (Blair et al., 2010). In addition to these neurobiological alterations, we found positive relationships between SA and ratings of embarrassment within the families, but as these behavioral measures were not heritable, our data do not provide support for these ratings as candidate endophenotypes of SAD.

Level of mPFC and MTG/STG/STS activation as a candidate SAD endophenotype

fMRI data revealed a positive relationship between SA-level within the families and brain activation in the frontal cortex, including the mPFC, in response to unintentional SN violations (versus intentional SN violations), as well as an association with activation within the occipital cortex and medial/superior temporal gyrus (MTG/STG), including the superior temporal sulcus (STS) between them (*Table 8.2, Figure 8.2*). Furthermore, activation clusters within the mPFC and MTG/STG/STS displayed moderate to moderately high heritability (maximum $h^2 = 0.47$) (*Figure 8.3*). Thereby, activation within the mPFC and MTG/STG/STS is a promising neurobiological endophenotype of SAD.

The heightened mPFC reactivity in response to unintentional SN violations confirmed our hypothesis, as this finding is in line with previous work reporting on 16 patients with generalized SAD (Blair et al., 2010). The mPFC is engaged during social cognitive processing, including self-referential processing (Amodio & Frith, 2006; Jenkins & Mitchell, 2011) and as such, the exaggerated mPFC activation during processing unintentional SN violations supports the idea that SAD patients consider these transgressions as extremely self-relevant, probably because these unintentional transgressions relate to their strong fear of unintentionally generating an embarrassing behavioral blunder in a social situation (Blair & Blair, 2012; Moscovitch, 2009). The importance of the mPFC in the neurobiological characterization of SAD is further supported by studies indicating increased mPFC activation related to self-referential statements and criticism (Blair et al., 2008; Blair, Geraci, Otero, et al., 2011), as well as in response to performance feedback (Heitmann et al., 2014); see reviews by (Brühl, Delsignore, et al., 2014; Miskovic & Schmidt, 2012).

Although not a priori hypothesized, the increased activation in the posterior STG/MTG/STS could concur with the role of this area in social cognition (Beauchamp, 2015; Deen, Koldewyn, Kanwisher, & Saxe, 2015; Schirmer, Meck, & Penney, 2016), including, but not limited to, understanding intentions from other people's actions (Frith & Frith, 2007; Pelphrey, Morris, & McCarthy, 2004; Saxe, Xiao, Kovacs, Perrett, & Kanwisher, 2004). Interestingly, recent work demonstrated that the posterior STS is involved in experiencing embarrassment with another person's mishaps (Paulus, Müller-Pinzler, Jansen, Gazzola, & Krach, 2015), while work on SAD demonstrated increased bilateral STS activation in response to emotional faces (Gentili et al., 2008; Straube et al., 2004). Furthermore, the STS is functionally connected to the amygdala (Gorka, Torrisi, Shackman, Grillon, & Ernst, 2018; Pitcher, Japee, Rauth, & Ungerleider, 2017). Based on these findings, we cautiously hypothesize that the heightened posterior temporal activation in response to unintentional SN violations could represent the increased affective value that socially-anxious people attribute to making an unintentional slip. Furthermore, as these temporal regions are involved in visual processing and visual imagery (Ganis, Thompson, & Kosslyn, 2004), enhanced activation

within these areas could also represent the increased saliency of the social situations for socially-anxious participants when they imagine themselves in the hypothetical scenarios.

Embarrassment co-segregates with SA within families

Within the LFLSAD sample, family members with higher levels of self-reported SA rated all types of stories as more embarrassing (*Figure 8.4*). These findings are in line with previous work (Bas-Hoogendam, van Steenbergen, van der Wee, et al., 2018; Blair et al., 2010), and confirm the notion that feeling embarrassed is an important characteristic of social anxiety. Our results did not, however, support the specific effect of SA on embarrassment in the unintentional condition, which was reported previously, nor did we replicate the effect of SA on the ratings of inappropriateness (Bas-Hoogendam, van Steenbergen, van der Wee, et al., 2018; Blair et al., 2010), indicating the need for future studies to unravel this complex pattern. Furthermore, heritability of these behavioral endophenotypes was low (intentional condition) or absent (unintentional and neutral condition). So, the present findings reinforce the view that increased reports of embarrassment are associated with SA, but as these embarrassment levels have heritability estimates below our predefined threshold, they do not meet criteria for being a candidate endophenotype.

Limitations and directions for future research

Although the results presented here, from a unique two-generation neuroimaging family study on SAD which was especially designed to explore two endophenotype criteria (Bas-Hoogendam, Harrewijn, et al., 2018), provide support for the *co-segregation of the candidate endophenotypes with social anxiety within families of probands* (criterion 4, first part) and the endophenotype criterion of *heritability* (criterion 3), the cross-sectional nature of the LFLSAD and the lack of control families do not allow for investigation of the *state-independency* (criterion 2) of the candidate endophenotypes, nor do the data enable assessing whether *non-affected family members show altered levels of the endophenotype in comparison to the general population* (criterion 4, second part). Future studies, employing a longitudinal design and including control families from the general population, are needed to investigate these criteria and to replicate the current findings. In addition, given the heterogeneity in the SAD phenotype (Heimberg et al., 2014), future studies could consider using individually-tailored stimuli (cf. (Simon, Kaufmann, Müsch, Kischkel, & Kathmann, 2010)). We hypothesize that such stimuli, representing social situations that are most anxiety-provoking for participants with SAD, might yield even stronger neurobiological and behavioral responses compared to those of the present study. Furthermore, given the fact that the SNPT-R has age- and gender-adjusted task versions, the present design does not allow for determining effects of age and gender on the candidate endophenotypes.

Another interesting avenue for future research would be to link the altered brain activation observed here to changes in brain structure. In a previous study on the same sample, we

found a negative correlation between SA-levels and cortical thickness of the left mPFC and bilateral STG (Bas-Hoogendam, van Steenbergen, Tissier, et al., 2018b). In addition, cortical thickness of the left mPFC and left STG displayed moderately-high and high heritability (Bas-Hoogendam, van Steenbergen, Tissier, et al., 2018b). However, due to the complexity of the present association analyses, in which we had to account for the family structure of the data, we were not able to consider the connection between brain structure and brain function on a voxelwise basis. Moreover, it should be noted that a voxel-based morphometry mega-analysis on the largest sample of SAD patients to date did not reveal gray matter differences in frontal and temporal areas (Bas-Hoogendam, van Steenbergen, Pannekoek, et al., 2017). Furthermore, the alterations in function are specific to processing unintentional SN violations, while the structural changes are independent of any task condition. Therefore, more research is needed to unravel the complex relationship between brain structure and function (Bas-Hoogendam, 2019; Lerch et al., 2017). Besides, longitudinal MRI studies (cf. (Steiger et al., 2017)) could explore the potential of cognitive behavioral therapy enriched with neurofeedback (Haller et al., 2015), to specifically target the altered brain activation patterns in the mPFC. Finally, as we have not yet considered the genetic data collected within the LFLSAD (Bas-Hoogendam, Harrewijn, et al., 2018), we are at present not able to relate the alterations in brain activation to genetic variations, which would be a next step in order to further unravel the genetic susceptibility to SAD.

Conclusions

The findings of this study provide considerable support for increased brain activation in the mPFC and MTG/STG/STS, related to the processing of unintentional SN violations, as a neurobiological candidate SAD endophenotype. Thereby, these results offer novel insights in the neurobiological pathways leading to SAD.

Supplemental Methods

Participants

Exclusion criteria

There was one important exclusion criterion in the LFLSAD, being comorbidity other than internalizing disorders or substance abuse in the proband or proband's SA-child; other family members were included independent from the presence of psychopathology. Insufficient comprehension of the Dutch language was an exclusion criteria for the whole sample, and general MRI contraindications, for example pregnancy, metal implants or dental braces, led to exclusion of the MRI experiment (Bas-Hoogendam, Harrewijn, et al., 2018).

Ethical procedure

Both parents signed the informed consent form for their children, and children between 12 and 18 years of age signed the form themselves as well. Family members received €75 for participation. Confidentiality of the data was maintained by the use of a unique research ID number for each participant.

Recruitment

Families were recruited through media exposure, like interviews in Dutch newspapers, on television and radio; furthermore, the study was brought to the attention of patient organizations, to clinical psychologists, general practitioners and mental health care organizations. Recruitment was targeted at families in which multiple family members experienced 'extreme shyness' and took place between Summer 2013 and Summer 2015. Details about the screening and inclusion flow of the LFLSAD are provided in Bas-Hoogendam et al. (2018).

A priori power calculation and sample size

A priori power calculations were performed to estimate the required sample size of the LFLSAD, as described previously in Bas-Hoogendam, Harrewijn, et al. (2018). Power was computed by simulation, based on an endophenotype with a heritability of 60 % and a correlation of 70 % with SAD; the prevalence of SAD was set at 10 %. Families were generated using linear mixed models and we modeled correlations between family members via normally distributed random effects with a correlation structure of two times the kinship matrix. Only families with at least two affected members in one nuclear family were used for estimation of the power. These power calculations revealed that 12 families with 8 - 12 family members (average: 10 members per family) were required for sufficient power (i.e., minimally 80%) to 1st estimate the association between SAD and neurocognitive putative endophenotypes and 2nd to determine the significance of clustering of these endophenotypes within families (i.e., genetic effects).

Phenotyping

The presence of DSM-IV diagnoses was determined using the Mini-International Neuropsychiatric Interview (M.I.N.I.)-Plus (version 5.0.0) (Sheehan et al., 1998; van Vliet & de Beurs, 2007) or the M.I.N.I.-Kid interview (version 6.0) (Bauhuis et al., 2013; Sheehan et al., 2010); these interviews were administered by experienced clinicians and recorded. Special attention was paid to the presence of (sub)clinical SAD: clinical SAD was established using the DSM-IV-TR criteria for the generalized subtype of SAD, but the clinician verified whether the DSM-5 criteria for SAD were also met. We chose a priori to include patients with generalized SAD, as this is the most prevalent subtype, with a strong familiar pattern and an early age of onset (D'Avanzato & Dalrymple, 2016). A diagnosis of subclinical SAD was established when participants met the criteria for SAD as described in the DSM-5, but did not show impairing limitations in important areas of functioning (criterion G) (American Psychiatric Association, 2013).

In addition to the clinical interviews and the self-report questionnaires on social anxiety (the Liebowitz Social Anxiety Scale (LSAS-SR) (Fresco et al., 2001; Mennin et al., 2002) or the Social Anxiety Scale for adolescents (SAS-A) (La Greca & Lopez, 1998)), participants completed several questionnaires on anxiety-related constructs.

The intensity of fear of negative evaluation was assessed using the revised Brief Fear of Negative Evaluation (BFNE) – II scale (Carleton et al., 2006; Leary, 1983).

Furthermore, the level of self-reported depressive symptoms was evaluated using the Beck Depression Inventory (BDI– II) (Beck et al., 1996; Van der Does, 2002) or the Children's Depression Inventory (CDI) (Kovacs, 1985; Timbremont & Braet, 2002).

The State-Trait Anxiety Inventory (STAI) (Spielberger et al., 1970) (see (Spielberger & Vagg, 1984) for psychometric properties) was used to determine self-reported trait anxiety, as well as state anxiety before and after the MRI scan.

The sensitivity for the temperamental traits 'behavioral inhibition' and 'behavioral activation' was assessed using the self-report BIS/BAS (Carver & White, 1994; Franken et al., 2005) or the BIS/BAS scales for children (BIS/BAS-C) (Muris et al., 2005).

Two subscales of the Wechsler Adult Intelligence Scale-IV (WAIS-IV) (Wechsler et al., 2008) or Wechsler Intelligence Scale for Children-III (WISC) (Wechsler, 1991), the similarities (verbal comprehension) and block design (perceptual reasoning) subtests, were administered to obtain an estimate of cognitive functioning.

MRI parameters

During the SNPT-R, fMRI scans were acquired using T2*-weighted echo-planar imaging (EPI). Characteristics of these scans with the following characteristics: 38 axial slices, 2.75 mm x 2.75 mm x 2.75 mm + 10 % interslice gap, field of view (FOV) = 220 mm x 115 mm x 220 mm, repetition time (TR) = 2200 ms, echo time (TE) = 30 ms. The first six volumes of

each fMRI scan were dummy volumes; these volumes were removed to allow for equilibration of T1 saturation effects.

In addition, a high-resolution EPI-scan (84 axial slices, 1.964 mm x 1.964 mm x 2 mm, FOV = 220 mm x 168 mm x 220 mm, TR = 2200 ms, TE = 30 ms) and a high-resolution T1-weighted scan (140 slices, resolution 0.875 mm × 0.875 mm × 1.2 mm, FOV = 224 mm × 168 mm × 177.333 mm, TR = 9.8 ms, TE = 4.59 ms, flip angle = 8◦) were acquired. These scans were used for within-subject registration purposes; furthermore, the structural T1-scans were inspected by a neuroradiologist, but no clinically relevant abnormalities were present in any of the participants.

Revised Social Norm Processing Task (SNPT-R)

Story-reading phase

The SNPT-R has 78 stories which were presented in a pseudo-random order using E-Prime software (version 2.0.10, Psychology Software Tools; script available at osf.io/pt4qt (Bas-Hoogendam, van Steenbergen, Kreuk, et al., 2017b)). Stem sentences were presented for 3 s, while ending sentences had a duration of 6 s. Stories were separated by a fixation cross (jittered duration between 2 - 7 s, determined using Optseq software (https://surfer.nmr.mgh.harvard.edu/optseq/), mean duration fixation: 3.5 s) and the 78 stories were divided into two consecutive blocks of 39 stories (duration each block: 8 min 44 s). Importantly, the stories in the unintentional and intentional condition differed only in the intention of the actor, while the actual outcome of the action (for example, a distasteful pie) was in general the same (see (Bas-Hoogendam, van Steenbergen, Kreuk, et al., 2017a) for a sensitivity analysis).

Rating phase

During the post MRI scan rating phase, participants rated the stories on a 5-point Likert scale on embarrassment (ranging from 1, not embarrassing at all, to 5, extremely embarrassing) and inappropriateness (ranging from 1, not inappropriate at all, to 5, extremely inappropriate), using a laptop. These tasks were presented using E-Prime software (version 2.0.10, Psychology Software Tools) and the scripts are available at osf.io/pt4qt (Bas-Hoogendam, van Steenbergen, Kreuk, et al., 2017b). Note that the behavioral ratings were performed outside the MRI scanner, for two main reasons. First of all, we aimed to measure brain activation of the participants while they just 'imagined themselves' as being in a certain social situation, without 'priming' or directing participants to think about embarrassment or inappropriateness specifically. Secondly, we had to take the duration of the MRI session into account: the whole MRI session lasted around one hour; having participants rate the stories during the MRI scan would make the session too long and too demanding. As a result, however, we are not able to disentangle which brain areas are activated during

thinking about the inappropriateness of the stories or while considering the amount of embarrassment related to the stories.

fMRI data

General processing steps

FMRI data were denoised using FIX (FMRIB's ICA-based X-noiseifier), a publicly available plugin for FSL (FMRIB Software Library, version 5.0.9) (Jenkinson et al., 2012), which provides an automatic solution for denoising fMRI data via accurate classification of ICA components (Griffanti et al., 2014; Salimi-Khorshidi et al., 2014). Next, data underwent several preprocessing steps using FEAT (FMRI Expert Analysis Tool; version 6.00) (Jenkinson et al., 2012; Smith et al., 2004), including motion correction using MCFLIRT (Jenkinson et al., 2002), spatial smoothing using a Gaussian kernel of full-width half-maximum (FWHM) 6.0 mm and grand-mean intensity normalization of the entire 4D dataset by a single scaling factor in order to enable higher-level analyses and registration. Scans were first registered to high-resolution EPI images, which were registered to T1 images, which in turn were registered to the Montreal Neurological Institute (MNI) T1-template brain (resolution 2 mm) using FNIRT nonlinear registration (warp resolution 10 mm) (Andersson et al., 2007; Jenkinson et al., 2002; Jenkinson & Smith, 2001). Next, ICA-AROMA (ICA-based Automatic Removal of Motion Artifacts) was used to remove motion-related artefacts (Pruim, Mennes, van Rooij, et al., 2015; Pruim, Mennes, Buitelaar, & Beckmann, 2015). Data were then submitted to FEAT to perform non-brain removal using BET (Smith, 2002), high-pass temporal filtering (Gaussian-weighted least-squares straight line fitting, with sigma = 30.0 s) and registration. Functional scans of each participant were registered to the individual 3D T1-weighted anatomical scan using FLIRT (Jenkinson et al., 2002; Jenkinson & Smith, 2001) and subsequently registered to the MNI T1-template brain (resolution 2 mm) using FNIRT nonlinear registration (warp resolution 10 mm) (Andersson et al., 2007).

Event-related statistical analysis of the time-series was carried out in native space following the method described in (Bas-Hoogendam, van Steenbergen, Kreuk, et al., 2017a). We used FILM with local autocorrelation correction (Woolrich et al., 2001) and included four explanatory variables (EVs) with their temporal derivatives in the general linear model. These EVs were convolved with a canonical double gamma hemodynamic response function and represented the presentation of 1st a stem sentence, 2nd a neutral ending (EN), 3rd an unintentional SN violation ending (EU) and 4th an intentional SN violation ending (EI). The stem EV had a duration of 3 s, ending EVs had a duration of 6 s; onset of the EVs for each individual was determined using custom-written scripts in MATLAB (Mathworks; code available at osf.io/pt4qt (Bas-Hoogendam, van Steenbergen, Kreuk, et al., 2017b)). Subsequently, three contrasts were defined: 1st EI > EN; 2nd EU > EN; 3rd EU > EI. Contrasts 1 and 2 were used to validate the main effect of the SNPT-R on brain activation (Bas-Hoogendam,

van Steenbergen, Kreuk, et al., 2017a), while contrast 3 (EU > EI) was the contrast of interest for the endophenotype analysis, following previous results (Blair et al., 2010).

We checked whether the individual scans were registered correctly and confirmed that relative motion parameters did not exceed 2.5 mm. The individual contrast images of the two story-reading blocks were combined using a within-subject multi-session fixed-effects analysis. The resulting contrast images were submitted to higher-level mixed-effects group analyses using FMRIB's Local Analysis of Mixed Effects (Beckmann et al., 2003; Woolrich, 2008; Woolrich et al., 2004).

Validation of whole-brain activation patterns

To compare the task-related brain activation patterns to previous findings (Bas-Hoogendam, van Steenbergen, Kreuk, et al., 2017a), whole-brain analyses were used to investigate clusters related to the contrasts EI > EN and EU > EN. To keep the analyses comparable with our previous work, we used a cluster threshold of $z > 2.3$ and a cluster extent threshold $p < 0.05$, but we also used a more stringent threshold (cluster-threshold $z > 3.1$, cluster extent threshold $p < 0.01$).

Endophenotype analyses with (sub)clinical SAD as predictor

For reasons of completeness, we performed voxelwise analyses using (sub)clinical SAD as a discrete predictor in addition to the main analyses using self-reported SA-level (continuous variable) as a predictor. In these analyses, individual activation level related to the contrast EU > EI was used as dependent variable. Correlations between family members were modeled by including random effects; age (centered) and gender (centered) were included as covariates. Models were run for each voxel separately, in order to determine the effect of (sub) clinical SAD on a whole-brain voxelwise basis. Results (z-scores) were transformed into a nifti-image with the same dimensions of the MNI T1-template brain. Clusters within this nifti-image, representing the association between SA and brain activation, were corrected for multiple comparisons at the whole-brain level using the FSL tool easythresh (cluster threshold: $z > 3.1$, cluster extent threshold $p < 0.01$) (Worsley, 2001).

Sensitivity analyses

We performed two sensitivity analyses to examine whether the results of the association analysis (effect of self-reported social anxiety (z-score) on brain activation related to EU > EI) were driven by the severity of depressive symptoms as measured by the BDI-II or the CDI or by (comorbid) psychopathology other than SAD (cf. (Bas-Hoogendam, van Steenbergen, Tissier, et al., 2018b)). To this aim, we added the z-score of the level of depressive symptoms as a covariate in the voxelwise analysis (sensitivity analysis 1) or excluded all family members with past and/ or present psychopathology other than SAD and repeated the association analysis (sensitivity analysis 2). Note however, that this latter analysis may

yield biased and weaker results, as the majority of the probands, on which the selection of the families was based, had comorbid psychopathology and were thus excluded. We used the same statistical threshold as for the main analyses ($z > 3.1$, cluster-threshold $p < 0.01$).

Behavioral data

Endophenotype analyses with (sub)clinical SAD as predictor

For reasons of completeness, we performed analyses using (sub)clinical SAD as a discrete predictor in addition to the main analyses using self-reported SA-level (continuous variable) as a predictor. Separate models were used to investigate the ratings of embarrassment and inappropriateness. Task condition (intentional / unintentional / neutral), age- and gender specific task version (modeled using the dummy variables gender and age group (boys and girls vs men and women)), as well as three interaction terms (condition-by-gender, condition-by-age group, and condition-by-(sub)clinical SAD) were added as independent variables and tested for significance. Random effects were included to account for the genetic correlations between family members and the within-subject correlations between the task conditions. Interaction terms lacking significance were removed from the final models. Significance level was set at $p < 0.05$.

Supplemental Results

Data availability

We acquired MRI data from nine families ($n = 113$) (Bas-Hoogendam, Harrewijn, et al., 2018), but data from one family ($n = 3$ family members) had to be excluded as the proband from this family was not able to participate in the MRI experiment due to an MRI contra-indication. Furthermore, two young participants (aged 18.8 y and 9.4 y) quitted the MRI session before they had completed the two blocks of the SNPT-R, although one of them did perform the rating phase of the SNPT-R after the scan session. As a result, 109 datasets were available for the analyses with respect to the ratings, while 108 fMRI datasets were available for further fMRI pre-processing and quality control. Upon inspection of the relative motion parameters, fMRI data of nine participants had to be excluded, as their motion parameters for at least one of the two blocks of the SNPT-R exceeded 2.5 mm. Thus, the sample available for the fMRI analyses consisted of the data of 99 family members.

Due to technical reasons, data on the presence of subclinical SAD were lost for eight family members and data from these participants were therefore not included in the analysis with respect to the co-segregation of the candidate endophenotypes with (sub)clinical SAD within families.

Sample characteristics

We refer to *Supplemental Table S8.1* and *Supplemental Table S8.2* for detailed information about the sample. Following the design of the study, family members originated from two generations, which differed significantly in age (behavioral sample: $\beta \pm SE = -30.2 \pm 0.7$, $p < 0.001$; fMRI sample: $\beta \pm SE = -29.6 \pm 0.7$, $p < 0.001$), but not in male / female ratio (behavioral sample: $\chi^2(1) = 0.44$, $p = 0.57$; fMRI sample: $\chi^2(1) = 0.50$, $p = 0.55$). Family members with and without (sub)clinical SAD did not differ with respect to male / female ratio, age and estimated IQ. Groups did differ, however, in comorbidity rates: family members with (sub)clinical SAD were more often diagnosed with depression (past), dysthymia (present) and panic disorder. These differences were, however, only significant at an uncorrected significance level. Furthermore, family members with (sub)clinical SAD reported higher levels of fear of negative evaluation, more depressive symptoms, higher levels of trait anxiety and behavioral inhibition (BIS), as well as lower levels of behavioral activation (BAS).

FMRI data

Validation of whole-brain activation patterns

Results of the whole-brain analyses investigating activation related to the two task contrasts EI > EN and EU > EN are summarized in *Supplemental Table S8.3* and *Supplemental Figure S8.1.* In short, the analyses replicated the results reported for a sample of 21 healthy adults (Bas-Hoogendam, van Steenbergen, Kreuk, et al., 2017a), although the current activation clusters were more extended, probably due to the larger sample size of the present study.

***Contrast EI > EN** (Supplemental Figure S8.1A)*

Reading stories on intentional social norm violations (contrasted with reading neutral social stories) was associated with activation in three clusters. The first cluster encompassed the bilateral orbital frontal cortex, the bilateral inferior frontal gyrus, bilateral frontal operculum cortex and bilateral precentral gyrus, extended into subcortical structures like the bilateral amygdala, caudate, putamen, pallidum and thalamus, as well as into occipital areas such as the bilateral lateral occipital cortex, occipital fusiform gyrus and the occipital pole. The second cluster was comprised of the frontal pole, the superior frontal gyrus, the anterior cingulate gyrus and the paracingulate gyrus, while the third cluster included the right precentral and postcentral gyrus.

***Contrast EU > EN** (Supplemental Figure S8.1B)*

Activation related to reading unintentional social norm violations was found in two clusters, again when compared to reading neutral social stories. The first cluster contained the left frontal operculum cortex and inferior frontal gyrus, the left thalamus, the left amygdala, the left superior temporal gyrus and occipital areas like the lingual gyrus, the intracalcarine cortex and the bilateral occipital pole. The second cluster encompassed the superior frontal

gyrus and frontal pole as well as the anterior and posterior cingulate gyrus and the paracingulate gyrus.

Findings were confirmed by analyses using a more stringent threshold (cluster threshold $z > 3.1$, cluster extent threshold $p < 0.01$) – those are reported in *Supplemental Table S8.4*.

Endophenotype analyses with (sub)clinical SAD as predictor

We investigated whether brain activation related to the contrast EU > EI *co-segregated with SA* by performing whole-brain voxelwise regression analyses. The regression analysis using discrete (sub)clinical SAD as a predictor did not yield clusters surviving the predefined threshold. So, it should be noted that we did find a positive association between brain activation and self-reported SA (continuous predictor), but not with (sub)clinical SAD (discrete predictor). We speculate that this lack of a correlation is power-related, as the fMRI sample only contained 33 (sub)clinical SAD cases. This indicates the need for replication of the present findings in a larger sample.

Follow-up analyses

We explored whether the results of the difference contrast EU > EI were driven by a positive relationship between SA-levels and the processing of unintentional SN violations or by a negative association of SA-levels with processing intentional SN violations. We extracted the individual activation levels for the contrasts 'EU > baseline' and 'EI > baseline' within the significant clusters and performed two regression analyses in R (predictor: self-reported SA; dependent variables: activation related to 'EU > baseline' and 'EI > baseline', respectively; models corrected for age and gender; genetic correlations between family members were taken into account). These analyses showed a positive relationship between SA-levels and activation related to processing unintentional SN violations (contrast EU > baseline: $\beta \pm SE = 2.18 \pm 0.80$, $p = 0.006$), but no association between SA-level and activation related to processing intentional SN violations (contrast EI > baseline: $\beta \pm SE = 0.67 \pm 0.76$, $p = 0.36$). We further confirmed the specificity of the main finding for processing unintentional SN violations by repeating the whole-brain voxelwise analyses on the contrasts EI>EN and 'all endings (EU + EI + EN) > baseline'. These analyses did not yield significant clusters at the predefined significance level.

Sensitivity analyses

Results of the first sensitivity analysis, with the level of depressive symptoms as an additional covariate, confirmed the clusters found in the main analysis and revealed even a third cluster showing a significant association between self-reported SA and brain activation related to processing unintentional SN violations in the left temporal pole (*Supplemental Table S8.5, Supplemental Figure S8.2*).

In the second sensitivity analysis, we excluded all participants with past and/or present comorbid psychopathology other than SAD; this resulted in a sample of 64 participants, of which 15 in the (sub)clinical SAD group. Next, we repeated the association analysis with self-reported social anxiety as predictor. The analysis with the standard (stringent) statistical threshold ($z > 3.1$, $p < 0.01$), confirmed the positive association between self-reported social anxiety and brain activation in the occipital pole (*Supplemental Table S8.6; Supplemental Figure S8.3A*), in line with the main analysis. The association between social anxiety and activation in the frontal/mPFC cluster was, however, not significant at this significance level; when we applied a less stringent threshold ($z > 2.3$, $p < 0.05$), we did find a significant positive association (*Supplemental Table S8.7; Supplemental Figure S8.3B*).

Behavioral data

Behavioral responses during story-reading phase

Examination of the behavioral responses during the story-reading phase showed that two participants (female, aged 41 y; male, aged 20 y) forgot to press the button during the first block of the SNPT-R; in between the two blocks, these participants indicated upon request that they had read the stories and after additional instructions, they responded well to the sentences presented in the second block of the task. The other participants ($n = 97$) responded to 95.3 % of the trials (number of missed responses / block of 39 trials (mean ± SD): 1.8 ± 2.5, range 0 - 15), indicating good task compliance.

Effects of gender and age group

Behavioral ratings for each task version (based on age and gender; versions for boys / girls / men/ women) are summarized in *Supplemental Table S8.8* and *Supplemental Figure S8.4*. Detailed statistics are presented in *Supplemental Table S8.9* and *Supplemental Table S8.10*. Females rated the stories are more inappropriate and more embarrassing, while adults (men / women) rated the stories are more inappropriate than the children and adolescents (aged 8 - 18 years) did.

Endophenotype analyses with (sub)clinical SAD as predictor

Analyses examining the *co-segregation of (sub)clinical SAD (discrete variable) with the behavioral candidate endophenotypes within the families* revealed a main effect of (sub) clinical SAD on the ratings of embarrassment, with higher ratings for family members with (sub)clinical SAD, but not on the ratings of inappropriateness (*Supplemental Table S8.9; Supplemental Figure S8.5*). The interaction between (sub)clinical SAD and condition was not significant, while exploratory follow-up analyses on the embarrassment ratings separately for each condition (with age group and gender as covariates) indicated that (sub) clinical SAD was associated with higher embarrassment ratings on the unintentional ($\beta \pm SE = 0.23 \pm 0.10$, $p = 0.025$) and neutral stories ($\beta \pm SE = 0.14 \pm 0.06$, $p = 0.03$) (*Supplemental*

Figure S8.5), but not on the intentional stories (β ± SE = 0.06 ± 0.10, $p = 0.62$). Furthermore, there were main effects of condition and gender, as well as interaction effects for both types of ratings, comparable to previous findings on the SNPT-R (Bas-Hoogendam, van Steenbergen, Kreuk, et al., 2017a; Bas-Hoogendam, van Steenbergen, van der Wee, et al., 2018) (*Supplemental Table S8.9*).

Sensitivity analyses

We performed two additional analyses to investigate whether the effects of social anxiety on the embarrassment ratings were driven by the participants with a diagnosis of clinical SAD.

In the first analysis, we compared the embarrassment ratings for all three task conditions between participants with clinical SAD ($n = 17$) and participants with subclinical SAD ($n = 22$); as in the main analyses, gender and age group were added as covariates and we included random effects to account for the genetic correlations between family members. Results showed no significant differences between the groups in embarrassment ratings for the intentional (β ± SE = 0.03 ± 0.24, $p = 0.89$), unintentional (β ± SE = -0.05 ± 0.16, $p = 0.76$) or neutral condition (β ± SE = -0.01 ± 0.06, $p = 0.80$).

Secondly, we excluded all participants with clinical SAD (remaining $n = 92$) and repeated the analyses with self-reported social anxiety as predictor for the embarrassment ratings. In line with the results of the main analysis, we found a significant main effect of self-reported social anxiety (β ± SE = 0.03 ± 0.01, $p = 0.02$), a main effect of condition (β ± SE = -1.16 ± 0.12, $p < 0.001$), a main effect of gender (β ± SE = 0.56 ± 0.19, $p = 0.003$) and an interaction between condition and gender (β ± SE = -0.17 ± 0.07, $p = 0.01$) (*Supplemental Table S8.11*). Subsequent analyses for the separate conditions revealed a significant positive effect of self-reported social anxiety on the embarrassment ratings for the unintentional condition (β ± SE = 0.05 ± 0.02, $p = 0.03$), while the associations for the other conditions were significant at trend level (intentional: β ± SE = 0.05 ± 0.03, $p = 0.09$; neutral: β ± SE = 0.03 ± 0.02, $p = 0.07$); these non-significant results are most likely due to the loss of statistical power.

Supplemental Tables

Supplemental Table S8.1 Detailed characteristics of participants with and without (sub)clinical SAD: demographics and clinical information.

	Behavioral sample[a]			fMRI sample[a]		
	(Sub)clinical SAD (*n* = 39)	No SAD (*n* = 62)	Statistical analysis	(Sub)clinical SAD (*n* = 33)	No SAD (*n* = 58)	Statistical analysis
Demographics						
Male / Female (n)	20 / 19	30 /32	$\chi^2(1) = 0.08$, $p = 0.84$	16 / 17	29 / 29	$\chi^2(1) = 0.02$, $p = 1.00$
Generation 1/ Generation 2 (n)	19 / 20	27 / 35	$\chi^2(1) = 0.26$, $p = 0.68$	19 / 14	27 / 31	$\chi^2(1) = 1.02$, $p = 0.39$
Age in years (mean ± SD)	30.3 ± 15.5	31.3 ± 15.2	β (± SE)=-0.9 ± 3.1, p= 0.76	33.4 ± 14.9	32.7 ± 14.8	β (± SE) = 0.7 ± 3.2, $p = 0.83$
Estimated IQ (mean ± SD)	104.3 ± 12.2	105.9 ± 10.5	β (± SE)=-2.7±2.2, p= 0.22	103.6 ± 12.6	106.0 ± 10.8	β (± SE) = -2.8 ± 2.4, $p = 0.23$
Diagnostic information (n)						
Clinical SAD	17	0	$\chi^2(1) = 32.5$, $p < 0.001$	15	0	$\chi^2(1) = 31.6$, $p < 0.001$
Depressive episode present	1	1	$\chi^2(1) = 0.15$, $p = 0.70$	1	1	$\chi^2(1) = 0.2$, $p = 0.65$
Depressive episode past	12	9	$\chi^2(1) = 4.8$, $p = 0.03$	12	9	$\chi^2(1) = 6.1$, $p = 0.01$
Dysthymia present	3	0	$\chi^2(1) = 5.3$, $p = 0.02$	3	0	$\chi^2(1) = 5.8$, $p = 0.02$
Dysthymia past	1	1	$\chi^2(1) = 0.2$, $p = 0.65$	0	1	$\chi^2(1) = 0.5$, $p = 0.47$
Panic disorder lifetime	5	2	$\chi^2(1) = 3.9$, $p = 0.05$	5	2	$\chi^2(1) = 4.5$, $p = 0.03$
Agoraphobia present	3	2	$\chi^2(1) = 1.2$, $p = 0.27$	2	2	$\chi^2(1) = 0.4$, $p = 0.52$
Agoraphobia past	0	2	$\chi^2(1) = 1.2$, $p = 0.28$	0	1	$\chi^2(1) = 0.5$, $p = 0.46$
Separation anxiety	0	1	$\chi^2(1) = 0.8$, $p = 0.37$	0	0	n.a.
Specific phobia	2	3	$\chi^2(1) = 0.02$, $p = 0.90$	2	2	$\chi^2(1) = 0.40$, $p = 0.53$
Generalized anxiety disorder	1	0	$\chi^2(1) = 1.7$, $p = 0.19$	1	0	$\chi^2(1) = 1.9$, $p = 0.17$
Obsessive-compulsive disorder	1	0	$\chi^2(1) = 1.7$, $p = 0.19$	1	0	$\chi^2(1) = 1.9$, $p = 0.17$
Attention deficit hyperactivity disorder (ADHD)	3	1	$\chi^2(1) = 2.3$, $p = 0.13$	2	1	$\chi^2(1) = 1.2$, $p = 0.27$
Alcohol dependency present	1	1	$\chi^2(1) = 0.2$, $p = 0.70$	1	1	$\chi^2(1) = 0.2$, $p = 0.65$
Alcohol dependency lifetime	1	3	$\chi^2(1) = 0.3$, $p = 0.62$	1	3	$\chi^2(1) = 0.2$, $p = 0.67$

Supplemental Table S8.1 Detailed characteristics of participants with and without (sub)clinical SAD: demographics and clinical information. (*continued*)

	Behavioral sample[a]			fMRI sample[a]		
	(Sub)clinical SAD (*n* = 39)	No SAD (*n* = 62)	Statistical analysis	(Sub)clinical SAD (*n* = 33)	No SAD (*n* = 58)	Statistical analysis
Present psychotropic medication (n)	4	3	$\chi^2(1) = 1.1$, $p = 0.30$	2	3	$\chi^2(1) = 0.3$, $p = 0.86$
Antidepressants not otherwise specified	3	0		2	0	
ADHD medication not otherwise specified	1	3		0	3	

Footnote

[a]: Due to technical reasons, data on the presence of (sub)clinical SAD were lost for eight family members. Data from these participants were, however, included in the endophenotype analyses using SA-level (*z*-score) as a predictor (behavioral sample: *n* = 109; fMRI sample: *n* = 99).

Supplemental Table S8.2 Detailed characteristics of participants with and without (sub)clinical SAD: scores on self-report questionnaires.

	Behavioral sample			fMRI sample		
	(Sub)clinical SAD (*n* = 39)	No SAD (*n* = 62)	Statistical analysis	(Sub)clinical SAD (*n* = 33)	No SAD (*n* = 58)	Statistical analysis
Self-report measures						
Social anxiety symptoms (z-score; mean ± SD)	3.0 ± 3.3	0.6 ± 1.5	β ± SE = 2.4 ± 0.5, $p < 0.001$	2.9 ± 3.0	0.7 ± 1.3	β ± SE = 2.4 ± 0.4, $p < 0.001$
Fear of negative evaluation (mean ± SD)	23.3 ± 12.3	13.0 ± 8.0	β ± SE = 10.3 ± 2.0, $p < 0.001$	23.4 ± 11.7	13.1 ± 8.0	β ± SE = 10.3 ± 2.1, $p < 0.001$
Depressive symptoms (z-score; mean ± SD)	0.0 ± 0.9	-0.5 ± 0.7	β ± SE = 0.5 ± 0.2, $p < 0.001$	0.1 ± 0.9	-0.5 ± 0.7	β ± SE = 0.6 ± 0.2, $p < 0.001$
STAI – state pre scan (mean ± SD)	n.a	n.a		34.4 ± 7.4	31.5 ± 8.2	β ± SE = 3.1 ± 1.6, $p = 0.06$
STAI – state post scan (mean ± SD)	n.a	n.a		30.5 ± 6.4	28.1 ± 6.2	β ± SE = 2.4 ± 1.4, $p = 0.07$
STAI – trait (mean ± SD)	38.8 ± 9.4	33.0 ± 8.5	β ± SE = 5.5 ± 1.8, $p = 0.002$	39.1 ± 9.4	33.1 ± 8.6	β ± SE = 5.8 ± 1.9, $p = 0.003$
BIS (z-score; mean ± SD)	0.4 ± 1.3	-0.4 ± 0.9	β ± SE = 0.8 ± 0.2, $p < 0.001$	0.3 ± 1.1	-0.4 ± 0.9	β ± SE = 0.7 ± 0.2, $p < 0.001$
BAS (z-score; mean ± SD)	-0.9 ± 1.0	-0.6 ± 1.0	β ± SE = -0.5 ± 0.2, $p = 0.02$	-1.0 ± 0.9	-0.6 ± 1.0	β ± SE = -0.5 ± 0.2, $p = 0.008$

Abbreviations

n.a.: not applicable; SD: standard deviation; SE: standard error.

Supplemental Table S8.3 Brain activity related to reading social stories describing intentional and unintentional social norm violations versus neutral situations ($z > 2.3$, $p < 0.05$).

Cluster	Region	*Z*-score	Peak coordinates (MNI space)			Cluster size
			x	y	z	
Intentional norm violations vs neutral stories (EI > EN)						
1	Left orbital frontal cortex	8.53	-36	22	-14	41548
	Right orbital frontal cortex	6.91	30	20	-16	
	Right occipital pole	5.95	10	-88	40	
	Right operculum cortex	5.62	46	10	0	
	Right operculum cortex / inferior frontal gyrus	5.51	46	26	2	
	Left supramarginal gyrus	5.47	-62	-42	30	
2	Superior frontal gyrus	7.75	-2	54	28	13572
	Paracingulate gyrus	7.31	-6	54	16	
	Anterior cingulate gyrus	7.28	-2	22	22	
	Superior frontal gyrus	6.56	8	12	62	
	Left frontal pole	6.46	-28	46	28	
	Anterior cingulate gyrus	5.95	-2	16	38	
3	Right precentral gyrus	6.25	52	0	48	1429
	Right postcentral gyrus	3.85	64	-8	46	
Unintentional norm violations vs neutral stories (EU > EN)						
1	Lingual gyrus	5.64	-8	-82	-2	27196
	Intracalcarine cortex	5.62	12	-82	2	
	Intracalcarine cortex	5.49	18	-68	2	
	Left orbitofrontal cortex	5.29	-36	22	-14	
	Left frontal operculum cortex	5.09	-44	14	4	
	Right occipital pole	5.06	28	-92	36	
2	Superior frontal gyrus	5.92	0	56	30	6250
	Anterior cingulate gyrus	4.99	-2	18	28	
	Left frontal pole	4.95	-26	48	30	
	Superior frontal gyrus	4.75	0	10	62	
	Posterior cingulate gyrus	4.48	-4	-22	42	
	Paracingulate gyrus	3.90	-6	36	28	

Supplemental Table S8.4 Brain activity related to reading social stories describing intentional and unintentional social norm violations versus neutral situations ($z > 3.1$, $p < 0.01$).

Cluster	Region	*Z*-score	Peak coordinates (MNI space)			Cluster size
			x	y	z	
Intentional norm violations vs neutral stories (EI > EN)						
1	Occipital pole	5.95	10	-88	40	9845
2	Superior frontal gyrus	7.75	-2	54	28	9590
3	Left orbitofrontal cortex	8.53	-36	22	-14	7549
4	Right orbitofrontal cortex	6.91	30	20	-16	3243
5	Right precentral gyrus	6.25	52	0	48	911
6	Left supramarginal gyrus	5.47	-62	-42	30	696
7	Left precentral gyrus	5.14	-44	-14	42	602
8	Right superior temporal gyrus, posterior part	5.12	48	-32	2	601
Unintentional norm violations vs neutral stories (EU > EN)						
1	Lingual gyrus	5.64	-8	-82	-2	9735
2	Superior frontal gyrus	5.92	0	56	30	2572
3	Left orbitofrontal cortex	5.29	-36	22	-14	1766
4	Left thalamus	4.02	-6	-10	6	550

Supplemental Table S8.5 Effect of self-reported social anxiety on processing unintentional social norm violation – with level of depressive symptoms as additional covariate ($z > 3.1$, $p < 0.01$).

Cluster	Region	*Z*-score	Peak coordinates (MNI space)			Cluster size
			x	y	z	
Unintentional norm violations vs intentional norm violations (EU > EI)						
1	Temporal occipital fusiform cortex	6.19	30	-60	-18	12436
	Occipital pole	5.18	10	-94	26	
	Superior temporal gyrus, posterior division	4.33	62	-6	-8	
	Cuneal cortex	4.01	20	-76	32	
	Medial temporal gyrus, posterior division	3.75	60	-22	-10	
2	Frontal pole	6.10	-10	56	32	3520
	Frontal pole / frontal medial cortex	3.30	0	58	-4	
3	Inferior Temporal gyrus	4.79	-54	-18	-22	1464

Supplemental Table S8.6 Effect of self-reported social anxiety on processing unintentional social norm violation – in sample without (comorbid) psychopathology except for SAD ($z > 3.1$, $p < 0.01$).

Cluster	Region	*Z*-score	Peak coordinates (MNI space)			Cluster size
			x	y	z	
Unintentional norm violations vs intentional norm violations (EU > EI)						
1	Occipital pole	5.28	8	-92	26	4850
	Temporal occipital fusiform cortex	5.24	34	-58	-20	
	Occipital fusiform gyrus	4.02	32	-82	-12	

Supplemental Table S8.7 Effect of self-reported social anxiety on processing unintentional social norm violation – in sample without (comorbid) psychopathology except for SAD ($z > 2.3$, $p < 0.05$).

Cluster	Region	*Z*-score	Peak coordinates (MNI space)			Cluster size
			x	y	z	
Unintentional norm violations vs intentional norm violations (EU > EI)						
1	Lateral occipital cortex	5.38	50	-82	8	40324
	Occipital pole	5.28	8	-92	26	
	Frontal pole	4.45	-10	56	30	
	Posterior cingulate gyrus	4.35	-10	-46	30	

Supplemental Table S8.8 Ratings of inappropriateness and embarrassment – summarized for each task version (based on age and gender).

	(Sub)clinical SAD	No SAD		(Sub)clinical SAD	No SAD
Inappropriateness			Embarrassment		
Intentional	4.36 ± 0.40	4.36 ± 0.43	*Intentional*	3.92 ± 0.72	3.89 ± 0.58
Boys	3.99 ± 0.45	4.17 ± 0.69	Boys	3.59 ± 0.67	3.95 ± 0.63
Girls	4.40 ± 0.28	4.29 ± 0.52	Girls	3.84 ± 0.73	3.85 ± 0.48
Men	4.32 ± 0.36	4.35 ± 0.41	Men	3.75 ± 0.62	3.63 ± 0.61
Women	4.63 ± 0.23	4.46 ± 0.32	Women	4.30 ± 0.72	4.13 ± 0.49
Unintentional	2.98 ± 0.73	2.98 ± 0.64	*Unintentional*	3.45 ± 0.54	3.23 ± 0.51
Boys	3.14 ± 0.68	3.19 ± 0.66	Boys	3.14 ± 0.51	3.33 ± 0.49
Girls	2.84 ± 0.78	3.15 ± 0.52	Girls	3.56 ± 0.44	3.43 ± 0.36
Men	3.00 ± 0.62	2.99 ± 0.65	Men	3.32 ± 0.50	3.12 ± 0.52
Women	2.92 ± 0.87	2.83 ± 0.65	Women	3.73 ± 0.52	3.23 ± 0.55
Neutral	1.39 ± 0.34	1.31 ± 0.29	*Neutral*	1.38 ± 0.38	1.25 ± 0.24
Boys	1.57 ± 0.52	1.19 ± 0.15	Boys	1.29 ± 0.27	1.19 ± 0.16
Girls	1.18 ± 0.13	1.44 ± 0.63	Girls	1.28 ± 0.18	1.40 ± 0.52
Men	1.40 ± 0.26	1.30 ± 0.22	Men	1.51 ± 0.64	1.22 ± 0.19
Women	1.33 ± 0.28	1.32 ± 0.19	Women	1.38 ± 0.20	1.25 ± 0.16

Footnote

Values represent mean ± standard deviation.

Supplemental Table S8.9 Effect of (sub)clinical SAD on ratings – detailed statistics.

	Inappropriateness			Embarrassment		
	β	SE	*p*	β	SE	*p*
(Sub)clinical SAD	0.06	0.05	0.24	0.13	0.06	0.02
Condition *Intentional, unintentional, neutral*	-1.15	0.12	< 0.001	-1.07	0.11	< 0.001
Gender *male, female*	0.30	0.12	0.01	0.62	0.17	< 0.001
Age group *boys / girls vs men / women*	0.36	0.13	0.007	0.03	0.06	0.65
Condition-by-gender	-0.09	0.05	0.04	-0.20	0.06	0.001
Condition-by- age group	-0.12	0.05	0.02	*not significant and not included in final model*		
Condition-by-(sub)clinical SAD	*not significant and not included in final model*			*not significant and not included in final model*		

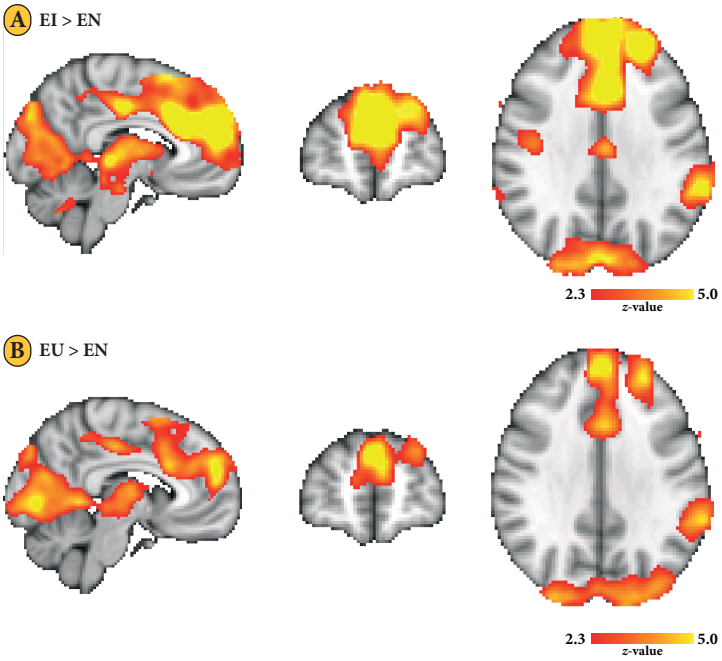
Supplemental Table S8.10 Effect of social anxiety (*z*-score) on ratings – detailed statistics.

	Inappropriateness			Embarrassment		
	β	**SE**	***p***	**β**	**SE**	***p***
Z-score SA	0.002	0.009	0.84	0.03	0.01	0.003
Condition *intentional, unintentional, neutral*	-1.14	0.11	< 0.001	-1.07	0.11	< 0.001
Gender *male, female*	0.33	0.11	0.003	0.69	0.17	< 0.001
Age group *boys / girls vs men / women*	0.32	0.13	0.01	-0.003	0.06	0.96
Condition-by-gender	-0.11	0.05	0.01	-0.22	0.06	p < 0.001
Condition-by- age group	-0.11	0.05	0.02	*not significant and not included in final model*		
Condition-by-*z*-score SA	*not significant and not included in final model*			*not significant and not included in final model*		

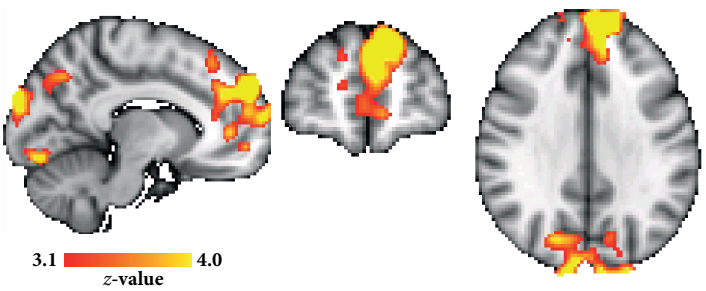
Supplemental Table S8.11 Effect of social anxiety (*z*-score) on embarrassment ratings – detailed statistics for sample without clinical SAD cases.

	Embarrassment		
	β	**SE**	***p***
Z-score SA	0.03	0.01	0.019
Condition *intentional, unintentional, neutral*	-1.16	0.12	< 0.001
Gender *male, female*	0.56	0.19	0.003
Age group *boys / girls vs men / women*	-0.007	0.07	0.92
Condition-by-gender	-0.17	0.07	0.01

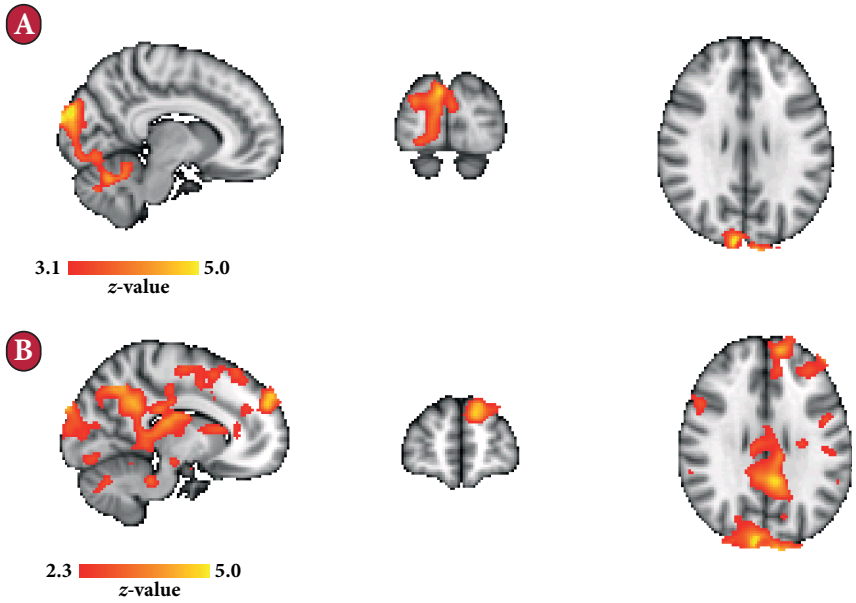
Supplemental Figures



Supplemental Figure S8.1 Significant activation patterns related to processing stories on social norm violations.
Clusters are displayed on the temple MNI_T1_152_2mm_brain (partial brain coverage: inferior parts of the frontal medial cortex, superior parts of the postcentral gyrus as well as parts of the cerebellum are not included). Images are displayed according to radiological convention: right in the image is left in the brain. Coordinates of displayed slices (MNI, xyz): -6, 56, 32. Cluster-threshold $z > 2.3$, cluster extent threshold $p < 0.05$.



Supplemental Figure S8.2 Significant positive associations between social anxiety (*z*-scores) and activation related to processing stories on unintentional social norm violations, corrected for level of depressive symptoms (sensitivity analysis 1).
Clusters are displayed on the temple MNI_T1_152_2mm_brain (partial brain coverage: inferior parts of the frontal medial cortex, superior parts of the postcentral gyrus as well as parts of the cerebellum are not included). Images are displayed according to radiological convention: right in the image is left in the brain. Coordinates of displayed slices (MNI, x, y, z): -10, 56, 32.

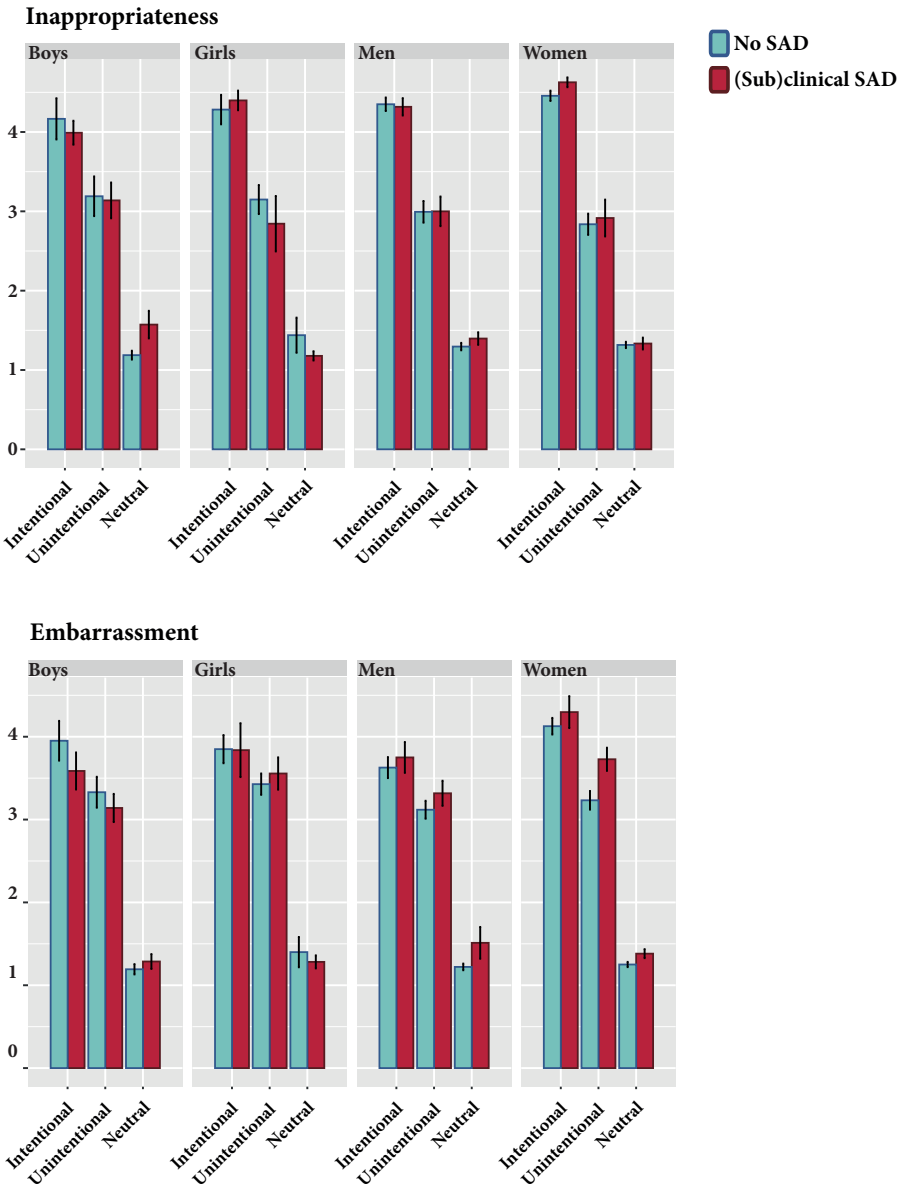


Supplemental Figure S8.3 Significant positive associations between social anxiety (*z*-scores) and activation related to processing stories on unintentional social norm violations, sample without (comorbid) psychopathology other than SAD (sensitivity analysis 2).

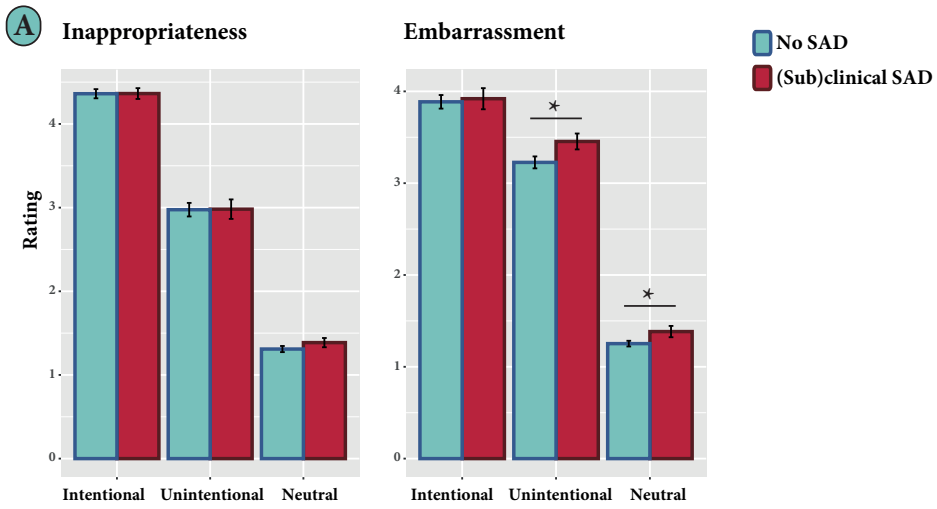
Clusters are displayed on the temple MNI_T1_152_2mm_brain (partial brain coverage: inferior parts of the frontal medial cortex, superior parts of the postcentral gyrus as well as parts of the cerebellum are not included). Images are displayed according to radiological convention: right in the image is left in the brain.

Figure S8.3A Threshold $z > 3.1$, $p < 0.01$. Coordinates of displayed slices (MNI, x, y, z): 10, -90, 28.

Figure S8.3B Threshold $z > 2.3$, $p < 0.05$. Coordinates of displayed slices (MNI, x, y, z): -12, 56, 28.



Supplemental Figure S8.4 Behavioral ratings on the SNPT-R, summarized for each task version (based on age and gender).
Bars represent means ± standard errors of the mean.



Supplemental Figure S8.5 Behavioral ratings on the SNPT-R – effect of (sub)clinical SAD.
Bars represent means ± standard errors of the mean.

Chapter 9

Impaired neural habituation to neutral faces in families genetically enriched for Social Anxiety Disorder

A revised version of this chapter is accepted for publication as:

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Abstract

Background

Social anxiety disorder (SAD) is an incapacitating disorder, running in families. Previous work associated social fearfulness with a failure to habituate, but the habituation response to neutral faces has, as of yet, not been investigated in patients with SAD and their family members concurrently. Here, we examined whether impaired habituation to neutral faces is a putative neurobiological endophenotype of SAD, by using data from the multiplex and multigenerational Leiden Family Lab study on SAD.

Methods

Participants (n = 110, age-range 9.2 - 61.5 y) performed a habituation paradigm involving neutral faces, as these are strong social stimuli with an ambiguous meaning. We used fMRI data to investigate whether brain activation related to habituation was associated with the level of social anxiety within the families. Furthermore, heritability of the neural habituation response was estimated.

Results

Our data revealed a relationship between impaired habituation to neutral faces and social anxiety in the right hippocampus and right amygdala. In addition, our data indicated that this habituation response displayed moderate to moderately-high heritability in the right hippocampus.

Conclusions

The present results provide support for altered habituation as a candidate SAD endophenotype: impaired neural habitation co-segregrated with the disorder within families, and was heritable. These findings shed light on the genetic susceptibility to SAD.

Introduction

Social anxiety disorder (SAD) is a highly prevalent and incapacitating disorder with a genetic background (Isomura et al., 2015; Stein et al., 2017; Stein & Stein, 2008). The underlying neurobiology is still not fully elucidated, hampering progress in prevention and therapies. A potential neurobiological marker for SAD is the reactivity of the brain to novel stimuli, and, more specific, the change in this response over time, called habituation.

Habituation, which can be reliably established using functional (f)MRI (Plichta et al., 2014), is the adaptive decrease in the automatic response to a novel stimulus presented multiple times without meaningful consequences (Ramaswami, 2014; Rankin et al., 2009). Several lines of evidence implicate impaired habituation in social anxiety: a prolonged habituation response, for example in the amygdala, has been linked to inhibited temperament (Blackford et al., 2013, 2011; Schwartz et al., 2012; Schwartz, Wright, Shin, Kagan, & Rauch, 2003), a stable trait which is considered to be a risk factor for SAD (Clauss et al., 2015; Clauss & Blackford, 2012); furthermore, a study in a community sample of young adults revealed slower neural habituation of neutral faces in individuals with higher levels of social fearfulness (Avery & Blackford, 2016). These findings are further supported by work in nonhuman primates with an anxious temperament (cf. (Fox & Kalin, 2014)) and a recent study demonstrating that a sustained amygdala response to neutral stimuli predicts a worse response to attention bias modification treatment in transdiagnostic clinical anxiety (Woody et al., 2019). Together, these observations support the link between impaired habituation and the vulnerability to social anxiety. Furthermore, they provide initial evidence for the neural habituation response to neutral faces, which could be considered as strong social stimuli with an ambiguous meaning in social situations and as such as ecologically relevant in the context of social anxiety, as a social anxiety endophenotype.

Endophenotypes are measurable, heritable characteristics, that constitute a causal connection between a certain genotype and a phenotype, and shed light on genetically-based disease mechanisms (Bas-Hoogendam et al., 2016; Gottesman & Gould, 2003). Importantly, not all disease-related traits are endophenotypes; by definition, endophenotypes should be *associated with the disorder* (criterion 1), *state-independent and already present in a preclinical state* (criterion 2), and *heritable* (criterion 3). Furthermore, an endophenotype should *co-segregate with the disorder within families of probands, with non-affected family members showing altered levels of the endophenotype in comparison to the general population* (criterion 4) (Glahn et al., 2007; Lenzenweger, 2013a; Puls & Gallinat, 2008). Nevertheless, as the neural habituation response has, as of yet, not been investigated in patients with SAD and their family members simultaneously, evidence with respect to the endophenotype criteria of *co-segregation within families* and *heritability* is currently lacking. Investigating these criteria is, however, of importance, given the genetic susceptibility to SAD and the typical onset of SAD during adolescence (Knappe, Beesdo-Baum, & Wittchen, 2010).

In the present work, we investigated neural habituation in two generations of families genetically enriched for SAD; these families were part of the Leiden Family Lab study on SAD (LFLSAD), a unique neuroimaging study with a multiplex and multigenerational design which was especially designed to delineate putative endophenotype of social anxiety (Bas-Hoogendam, Harrewijn, et al., 2018). Here, we examined whether impaired habituation *co-segregated with social anxiety (SA) within families* (first element of criterion 4); furthermore, the family-data enabled establishing the *heritability* of the neural habituation response (criterion 3). Based on the evidence summarized above, we predicted an association between SA and impaired neural habituation; furthermore, as genetic influences on the neural habituation response have been demonstrated (Lonsdorf et al., 2011; Perez-Rodriguez et al., 2017; Piel et al., 2018; Wiggins, Swartz, Martin, Lord, & Monk, 2014), we expected the habituation response to be at least moderately ($h^2 \geq 0.20$) heritable.

Materials and methods

Participants

Participants (n = 110; eight families) originated from the LFLSAD (*Figure 9.1A*); families within the LFLSAD were invited based on the combination of a primary diagnosis of SAD in a parent (aged 25 - 55 years; 'proband') and a child who met criteria for clinical or subclinical SAD (aged 8 - 21 years and living at home with the proband; 'proband's SA-child'). Together with these two SAD-cases, first- and second-degree family members of two generations were invited to participate, being the proband's partner and other children of the nuclear family (age ≥ 8 years), as well as the proband's sibling(s), with their partners and children (age ≥ 8 years). A detailed description of the study design, the exclusion criteria, recruitment procedure and an a priori power calculation are provided elsewhere (Bas-Hoogendam, Harrewijn, et al., 2018) and described in the *Supplemental Methods*; furthermore, a pre-registration is publicly available (Bas-Hoogendam et al., 2014a, 2014b). The study was approved by the Medical Ethical Committee of the Leiden University Medical Center and participants provided informed consent according to the Declaration of Helsinki. All participants completed a number of measurements, such as a diagnostic interview, self-report questionnaires and an MRI scan (Bas-Hoogendam, Harrewijn, et al., 2018).

Table 9.1 Characteristics of participants within the LFLSAD.

	(Sub)clinical SAD (n = 37)[†]	No SAD (n = 61)	Statistical analysis
Demographics			
Male / Female (n)	18 / 19	31 / 30	$\chi^2(1) = 0.04$, $p = 0.84$[‡]
Generation 1 / Generation 2 (n)	19 / 18	27 / 34	$\chi^2(1) = 0.47$, $p = 0.50$[‡]
Age in years (mean ± SD, range)	31.3 ± 15.2, 9.2 – 59.6	31.6 ± 15.2, 9.4 – 61.5	β ± SE = -0.3 ± 3.1, $p = 0.93$[$]
Estimated IQ (mean ± SD)	103.8 ± 12.0	105.5 ± 10.5	β ± SE = -2.0 ± 2.2, $p = 0.36$[$]
Diagnostic information (n)			
Clinical SAD	17	0	$\chi^2(1) = 33.9$, $p < 0.001$[‡]
Depressive episode present	1	1	$\chi^2(1) = 0.2$, $p = 0.69$ [‡]
Depressive episode past	12	9	$\chi^2(1) = 4.9$, $p = 0.03$ [‡]
Dysthymia present	3	0	$\chi^2(1) = 5.4$, $p = 0.02$ [‡]
Dysthymia past	1	1	$\chi^2(1) = 0.2$, $p = 0.65$ [‡]
Panic disorder lifetime	5	2	$\chi^2(1) = 4.0$, $p = 0.05$ [‡]
Agoraphobia present	3	2	$\chi^2(1) = 1.3$, $p = 0.26$ [‡]
Agoraphobia past	0	2	$\chi^2(1) = 1.2$, $p = 0.28$ [‡]
Separation anxiety	0	1	$\chi^2(1) = 0.8$, $p = 0.38$ [‡]
Specific phobia	2	3	$\chi^2(1) = 0.02$, $p = 0.89$ [‡]
Generalized anxiety disorder	1	0	$\chi^2(1) = 1.8$, $p = 0.19$ [‡]
Obsessive-compulsive disorder	1	0	$\chi^2(1) = 1.8$, $p = 0.19$ [‡]
Attention deficit hyperactivity disorder	3	1	$\chi^2(1) = 2.5$, $p = 0.11$ [‡]
Alcohol dependency present	1	1	$\chi^2(1) = 0.2$, $p = 0.70$ [‡]
Alcohol dependency lifetime	1	3	$\chi^2(1) = 0.2$, $p = 0.62$ [‡]
Present psychotropic medication	4	3	$\chi^2(1) = 1.1$, $p = 0.30$ [‡]
Self-report measures			
Social anxiety symptoms (z-score; mean ± SD)	2.9 ± 3.3	0.6 ± 1.5	β ± SE = 2.5 ± 0.5, $p < 0.001$[$]

Abbreviations
SA, social anxiety; SAD, social anxiety disorder; SD, standard deviation; SE, standard error.

Footnotes
[†] Due to technical reasons, data on the presence of subclinical SAD were lost for seven family members. Data from these participants were, however, included in the endophenotype analyses using SA-level (z-score) as a predictor (n = 105).
[‡] Chi-square tests in SPSS (version 25).
[$] Regression models in R (https://www.r-project.org), in which genetic correlations between family members were modelled by including random effects.

Data acquisition and analyses

Phenotyping

Experienced clinicians confirmed the presence of clinical SAD, subclinical SAD (hereafter, the term '(sub)clinical SAD' will be used to refer to both clinical and subclinical SAD) and other DSM-IV diagnoses using the Mini-International Neuropsychiatric Interview (M.I.N.I.)-Plus or the M.I.N.I.-Kid interview. Clinical SAD was established using the DSM-IV-TR criteria for the generalized subtype of SAD, while the interviewer verified whether the DSM-5 criteria for SAD were also met. A diagnosis of subclinical SAD was established when participants met the criteria for SAD as described in the DSM-5, but did not show impairing limitations in important areas of functioning.

Furthermore, participants filled out age-matched questionnaires on SA symptoms, being the Liebowitz Social Anxiety Scale (participants ≥ 18 years of age) or the Social Anxiety Scale for adolescents (participants < 18 years of age) (Fresco et al., 2001; La Greca & Lopez, 1998). In order to use these scores over the whole sample, *z*-scores were computed as described elsewhere (Bas-Hoogendam, Harrewijn, et al., 2018). We refer the reader to *Supplemental Table S9.1* for an extended characterization of the LFLSAD sample.

Demographics and clinical characteristics

Incidental missing values on the questionnaires were replaced by the average value of the completed items. Participants with and without (sub)clinical SAD were compared by fitting regression models in R (R Core Team, 2016), with (sub)clinical SAD as the independent variable and the level of self-reported social anxiety (*z*-score) as dependent variable. Gender and age were included as covariates; genetic correlations between family members were modelled by including random effects.

Habituation paradigm during functional (f)MRI

The habituation paradigm was part of a larger scan protocol (total duration MRI protocol: 54 min 47 s), consisting of structural scans (Bas-Hoogendam, van Steenbergen, Tissier, et al., 2018b) and functional task paradigms (Bas-Hoogendam, van Steenbergen, Kreuk, et al., 2017a; Bas-Hoogendam, van Steenbergen, Tissier, van der Wee, & Westenberg, 2019). Details on the MRI experiment (3.0 T Philips MRI scanner) are provided in the *Supplemental Methods*.

During the habituation paradigm, three neutral faces from the FACES database (Ebner, Riediger, & Lindenberger, 2010) were repeatedly presented (*Figure 9.1B*); see *Supplemental Methods* for the selected faces. We chose neutral faces, as they have an ambiguous meaning in a social context, leading to amygdala activation in both people with and without social fear (Whalen, 2007); thereby, these faces offer the best starting point for studying differential habituation patterns. The habituation paradigm started with the presentation of a fixation cross (24 s), followed by the presentation of the neutral faces. The faces were presented in

blocks of 24 s; within each block a neutral face was repeatedly presented (48 times) for 200 ms with a 300 ms interstimulus interval. There were 6 face blocks (2 blocks for each face), in order to resemble the design described previously (Wedig et al., 2005), and face blocks were separated by the presentation of a fixation cross (duration 12 s). An additional 12 s fixation cross was presented at the end of the paradigm. Gendermatched faces were presented in pseudo-random order, and participants were instructed to keep looking at the faces and the fixation crosses.

fMRI data: habituation response

fMRI data were pre-preprocessed following standard procedures using FSL (RRID:SCR_002823), described in the *Supplemental Methods*. Event-related statistical analyses were performed in native space, using FILM with local autocorrelation correction (Woolrich et al., 2001); in the general linear model, we included regressors modelling the presentation of the faces during the *first half* and *last (second) half* of the blocks (*Figure 9.1B*). Regressors were convolved with a canonical double gamma hemodynamic response function; furthermore, their temporal derivatives were included. We investigated habituation by using the contrast 'first half > last half' and applied a hypothesis-driven region of interest (ROI) approach focusing on the regions described by Avery & Blackford (Avery & Blackford, 2016), being the amygdala, hippocampus, ventromedial prefrontal cortex (vmPFC), orbitofrontal cortex, fusiform face area (FFA), primary visual cortex (V1) and extrastriate visual cortex; we added the bed nucleus of stria terminalis (BNST), given its role in anxiety (Avery, Clauss, & Blackford, 2015; Clauss, Avery, Benningfield, & Blackford, 2019; Figel et al., 2019). Specifics on these ROIs are available in the *Supplemental Methods*. We established in which ROIs habituation was present at the group level (cluster threshold $z > 2.3$, extent threshold $p < 0.05$) and used these ROIs for the subsequent endophenotype analysis.

fMRI data: endophenotype analysis

We examined the *co-segregation of the habituation response with the disorder within families*within the ROIs showing significant habituation-related activation. We used voxelwise multivariable regression models (predictor: SA-level; corrected for family structure, age and gender). Results (*z*-scores) were transformed into a nifti-image with the same dimensions of the MNI T1-template brain. Clusters within these images, mirroring the relation between SA and brain activation, were corrected for multiple comparisons within each bilateral ROI mask using the FSL tool easythresh (cluster threshold $z > 2.3$, extent threshold $p < 0.05$) (Worsley, 2001).

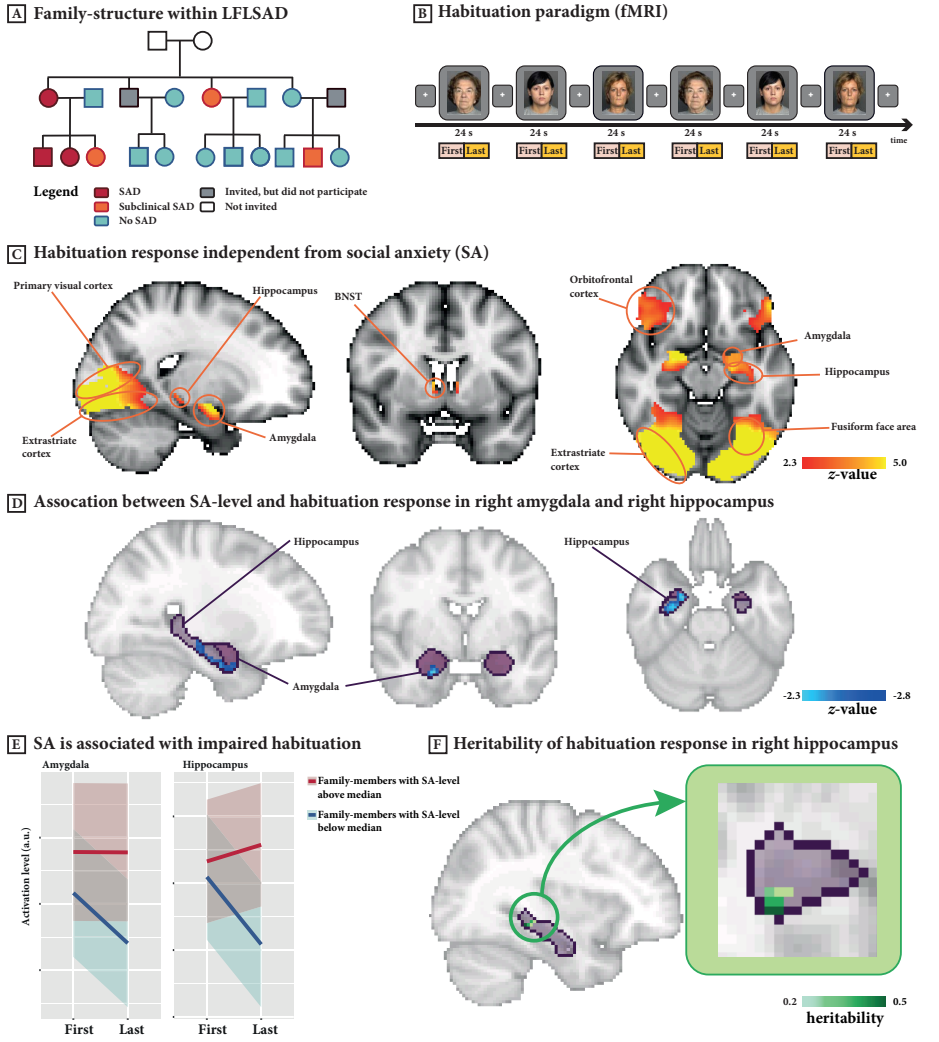

Figure 9.1 Failure to habituate within families genetically enriched for social anxiety disorder.

Figure 9.1A The LFLSAD sample comprises families who were invited to participate based on the combination of a primary diagnosis of SAD in a parent (aged 25 - 55 years old; 'proband'; depicted in red) and a proband's child with SAD (red) or subclinical SAD (orange). Furthermore, family members of two generations were invited (age ≥ 8 years), independent from the presence of SAD within these family members (no SAD: light blue; did not participate: gray). Grandparents (white) were not invited to participate. Squares and circles represent men and women, respectively. This figure is a modified reprint of Figure 1 of Bas-Hoogendam, Harrewijn, et al. (2018).

Figure 9.1B The habituation paradigm during functional (f)MRI scanning.

Figure 9.1C Significant habituation responses (brain activation 'first half > last half') in the bilateral amygdala, BNST, hippocampus, primary visual cortex, fusiform face area, extrastriate cortex and orbitofrontal cortex (n = 105). Coordinates of displayed slices (MNI, x, y, z): 26, 2, -26 (left and right image); 24, -2, -26 (middle image). Images are displayed according to radiological convention: right in the image is left in the brain.

Figure 9.1D Negative association between SA-level and habituation in the right amygdala and right hippocampus. Coordinates of displayed slices (MNI, x, y, z): 26, -10, -26 (left and right image); 26, -2, -20 (middle image).
Figure 9.1E SA-level was positively related with brain activation levels during the presentation of the faces in the last half of the blocks, while there was no correlation between SA and activation during the first half of the presentation blocks.
Figure 9.1F Heritability of brain activation in the right hippocampus. Coordinates of displayed slices (MNI, x, y, z): 34, -34, -8.

Next, we determined the *heritability* of brain activation for voxels in the significant clusters. Voxelwise heritability estimates were obtained with a method which takes the ascertainment process into account and incorporates familial relationships (Tissier et al., 2017). Age and gender (both centered) were included as covariates. For reasons of completeness, we also performed analyses with (sub)clinical SAD as a discrete predictor, as well as sensitivity analyses on the effect of (comorbid) psychopathology other than SAD, and the influence of depressive symptoms (*Supplemental Methods* and *Supplemental Results*).

Results

Demographics and clinical characteristics

Sample characteristics (n = 105 after quality control; see *Supplemental Results*) are summarized in *Table 9.1*. Family members with (sub)clinical SAD were more often diagnosed with depression (past) and dysthymia (present), but these differences were only significant at an uncorrected significance level (cf. (Bas-Hoogendam, van Steenbergen, Tissier, et al., 2019)). For a detailed phenotyping of the LFLSAD sample we refer the reader to the *Supplemental Results*.

fMRI analyses

Habituation response

Analyses over the whole sample revealed significant habituation responses (brain activation 'first > last') within most of the ROIs including the bilateral amygdala, BNST, hippocampus, V1, FFA, extrastriate cortex and orbitofrontal cortex (*Figure 9.1C; Table 9.2*), confirming the effectiveness of the paradigm for studying the neural correlates of the habituation response. No significant habituation was present in the vmPFC.

Endophenotype analyses

Voxelwise regression analyses revealed that SA-level was associated with reduced neural habituation in the right amygdala (cluster characteristics: 27 voxels, p = 0.013; max z-value: 2.82) and right hippocampus (cluster characteristics: 136 voxels, p = 0.04; max z-value: 3.13) (*Figure 9.1D*). Follow-up analyses on the individual activation levels within the significant

clusters indicated that family members with high SA-levels showed a failed habituation response in the late part of the task and not a heightened novelty response in the early part of the task (*Figure 9.1E*). To specify, SA-level was positively related with brain activation levels during the presentation of the faces in the *last half* of the blocks (amygdala: β ± SE = 1.23 ± 0.67, $p = 0.07$; hippocampus: β ± SE = 1.53 ± 0.57, $p = 0.007$), while there was no relation between SA and activation during the *early* presentation of the faces in the *first half* of the blocks (amygdala: β ± SE = -0.39 ± 0.72, $p = 0.59$; hippocampus: β ± SE = -0.25 ± 0.72, $p = 0.73$; regression analyses corrected for age, gender and family structure).

Voxelwise heritability analyses within the clusters showing an association with SA-level revealed that the neural habituation response within the right hippocampus was heritable, with 13 voxels showing at least moderate heritability ($h^2 > 0.20$)(*Figure 9.1F*). In the other ROIs, no association with SA was present at the pregnificance level.

Table 9.2 Neural habituation in regions of interest (ROIs) at group level.

			Peak coordinates (MNI space)			
ROI	**Left / right**	***Z*-score**	**x**	**y**	**z**	**Cluster size**
Amygdala	Left	5.26	-20	-12	-12	191
	Right	5.88	20	-4	-12	225
BNST	Left	3.53	-8	4	6	5
	Right	4.50	8	4	6	9
Extrastriate cortex	Left/right	9.49	-30	-86	-14	8736
FFA	Left	8.99	-26	-84	-18	964
	Right	8.67	30	-78	-2	1200
Hippocampus	Left	5.26	-20	-12	-12	244
	Right	5.88	20	-4	-12	233
Orbitofrontal cortex	Left	4.76	-52	36	-12	952
	Right	5.44	34	26	-26	1205
V1	Left/right	9.42	6	-90	0	2880
vmPFC	*No significant clusters*					

Abbreviations
BNST: bed nucleus of stria terminalis; FFA: fusiform face area; V1: primary visual cortex; vmPFC: ventromedial prefrontal cortex.

Discussion

The present findings provide evidence that altered habituation is an endophenotype of social anxiety disorder (SAD). First, we showed that impaired habituation to neutral faces in neural structures supporting threat (amygdala) and memory-related processes (hippocampus) is associated with SA within families genetically enriched for SAD, supporting

the endophenotype criterion of *co-segregration within families* (criterion 4, first element). Next, our data indicated that the habituation response to neutral faces in the hippocampus is partly *heritable* (endophenotype criterion 3). Thereby, these results from the multiplex, multigenerational Leiden Family Lab study on Social Anxiety Disorder add substantially to prior work indicating an *association* between impaired habituation and SA (endophenotype criterion 1) and studies on the *trait-stability* of the habituation response (endophenotype criterion 2) (Avery & Blackford, 2016; Blackford et al., 2013, 2011) (cf. (Bas-Hoogendam et al., 2016)), and shed light on the genetic pathways leading to SAD.

Impaired habituation in families genetically enriched for SAD

As habituation is an adaptive process, reflecting a basic, non-associative learning mechanism that acts like 'an intelligent firewall' that filters out irrelevant sensory information (Poon & Young, 2006), the failure to habituate likely contributes to the feelings of uncertainty that characterize individuals with high SA levels: at the neurobiological level, these individuals keep considering neutral social stimuli as being alarming, which makes them feel uncomfortable in social situations and contributes to aberrant social behavior. Although previous neuroimaging studies on habituation in patients with SAD yielded divergent results (Campbell et al., 2007; Sladky et al., 2012), potentially due to differences in task characteristics (cf. the extended discussion in (Bas-Hoogendam et al., 2016)), our findings are in line with work on participants with high levels of behavioral inhibition (Blackford et al., 2013; Schwartz et al., 2012; Schwartz, Wright, Shin, Kagan, & Rauch, 2003), as well as with the results of a study in individuals with high levels of social fearfulness (Avery & Blackford, 2016). Interestingly, impairments in neural habituation have also been reported in other neuropsychiatric disorders in which social behavior is altered, like autism and schizophrenia (Blackford, Williams, & Heckers, 2015; Kleinhans et al., 2009; Williams, Blackford, Luksik, Gauthier, & Heckers, 2013, and review by McDiarmid, Bernardos, & Rankin, 2017), indicating that impaired habituation is not specifically related to SA. However, as argued more extensively in Bas-Hoogendam et al. (2016), specificity is not a prerequisite for an endophenotype, as endophenotypes that are related to more than one disorder could advance transdiagnostic research on the shared genetic background of these disorders (Bearden & Freimer, 2006).

Habituation response in hippocampus, but not the amygdala, is heritable

The dissociation in heritability of the habituation response between the amygdala and hippocampus was unexpected, as previous studies indicated genetic influences on both hippocampus activation (Kauppi, Nilsson, Persson, & Nyberg, 2014) and amygdala reactivity (Lonsdorf et al., 2011; Munafò et al., 2008; Murphy et al., 2013). However, the results presented here are in line with findings from a multigenerational family study in rhesus monkeys, revealing significant heritability of metabolic activity predictive of anxious temperament in hippocampal regions, but not in the amygdala (Oler et al., 2010). Together, these findings

suggest that the impaired habituation response in the amygdala, although associated with SA, does not meet the endophenotype criterion of *heritability*, illustrating the distinction between disease-related neurobiological traits (biomarkers) and endophenotypes, with the latter having a genetic link with the disorder (cf. (Lenzenweger, 2013a)), and underscoring the value of studies using a family-design (Glahn et al., 2018). Furthermore, these findings support the notion that both genes and environment play a role in the development of SAD (Bas-Hoogendam, Roelofs, Westenberg, & van der Wee, 2019), and indicate that research on the interaction between these factors is important.

Habituation in other ROIs

Although the brain response to neutral faces habituated in several ROIs besides the amygdala and hippocampus, namely in the BNST, extrastriate cortex, FFA, orbitofrontal cortex and V1, we did not find an association with SA within these regions. Thereby, we could not replicate previous work demonstrating a relationship between social fearfulness and a slower habituation response to neutral faces in the orbitofrontal cortex, FFA, extrastriate cortex and V1 (Avery & Blackford, 2016). It should, however, be noted that this study employed a task paradigm in which the neutral faces were presented 1, 3, 5 or 7 times; as a result, their design allowed for investigation of habituation within specific repetition windows, for example from first to third presentation, third to fifth presentation and fifth to seventh presentation (Avery & Blackford, 2016). Interestingly, the effect of social fearfulness on habituation in the hippocampus was present over the whole paradigm, in line with our findings. The effects in the other ROIs were, however, only present in specific time windows (first to third and third to fifth presentation) which, arguably, could explain why we did not find associations with SA within these regions in the present study. Future studies, using the same task parameters and analysis methods as described by Avery & Blackford (2016), are needed to explore whether the associations between social fearfulness and neural habituation at specific timing intervals are also present in families genetically enriched for SAD.

Clinical implications

In addition to providing insight into the genetic susceptibility to SAD, our results might have potential clinical relevance, for example when considering the effect of exposure therapy. Exposure therapy, targeted at diminishing anxiety levels by repeated confrontations with the feared stimulus (i.e. a social situation), is often applied in SAD as part of cognitive behavioral therapy, with typically only small to moderate effects (Carpenter et al., 2018; Klumpp & Fitzgerald, 2018). Importantly, the effect of exposure therapy is thought to rely (at least partly) on habituation responses. Indeed, a recent meta-analysis showed a positive association between both within-session as well as between-session habituation on the one hand, and treatment outcome on the other (Rupp, Doebler, Ehring, & Vossbeck-Elsebusch, 2017). Furthermore, a research paper on adults with speaking anxiety indicated

that less amygdala activation during extinction learning predicted greater reduction in SA symptoms two weeks after a session of exposure (Ball, Knapp, Paulus, & Stein, 2017), while another study in SAD patients indicated that a decrease in regional cerebral blood flow in the amygdala was associated with anxiety reduction following repeated stress exposure (Åhs, Gingnell, Furmark, & Fredrikson, 2017). These results suggest that impaired habituation might have a negative consequence on the outcome of exposure therapy, but more research is needed to test this hypothesis. In this light, the role of inhibitory learning is also relevant: inhibitory learning, involving the amygdala, hippocampus, as well as the prefrontal cortex, and aimed at inhibiting the original feared association by a newly formed association representing safety, has been proposed as an alternative mechanism underlying exposure therapy (Craske, Liao, Brown, & Vervliet, 2012; Craske, Treanor, Conway, Zbozinek, & Vervliet, 2014). We hypothesize that a focus on inhibitory learning in exposure therapy might yield better outcomes in anxiety patients with impaired habituation responses.

Limitations and future studies

As the LFLSAD had a cross-sectional design and was intended to investigate the endophenotype criteria with respect to *co-segregation* and *heritability* (Bas-Hoogendam, Harrewijn, et al., 2018), we were not able to establish the *trait stability* of the candidate endophenotype (endophenotype criterion 2), nor could we examine the *difference in neural habituation between nonaffected family members within the sample and participants from the general population* (endophenotype criterion 4, second element). To investigate whether neural habituation meets these criteria, longitudinal studies, including families enriched for SAD as well as control families from the general population, are necessary. Furthermore, the present promising results paved the way for future analyses on the genetics underlying neural habituation: we did collect genetic data on the LFLSAD sample (Bas-Hoogendam, Harrewijn, et al., 2018), but we have not yet investigated whether specific genetic variations or epigenetic changes (cf. (Alisch et al., 2014; Dannlowski et al., 2011; Domschke et al., 2012; Ziegler et al., 2015)) are associated with the impaired neural habituation response. Such an investigation would be the following stage in disentangling the genetic vulnerability to SAD.

Finally, given work reporting changes in functional and structural connectivity of the amygdala in SAD (Brühl, Delsignore, et al., 2014), future studies should explore whether these aberrant connectivity patterns meet criteria for being candidate SAD endophenotypes.

Conclusion

The findings reported here support the hypothesis that impaired neural habituation to neutral faces is a promising neural candidate endophenotype of SAD, as our data revealed that

impaired habituation to neutral faces, expressed as a prolonged response to these faces in the right hippocampus and right amygdala, *co-segregated with social anxiety within families of probands*. Next, our data indicated that brain activation related to habituation displayed moderate to moderately-high heritability in the right hippocampus, providing support for the endophenotype criterion of *heritability*. Thereby, the present results offer novel insights in the neurobiological pathways leading to SAD.

Supplemental Methods

Participants

Exclusion criteria

There was one important exclusion criterion in the LFLSAD, being comorbidity other than internalizing disorders or substance abuse in the proband or proband's SA-child; other family members were included independent from the presence of psychopathology. Insufficient comprehension of the Dutch language was an exclusion criteria for the whole sample, and general MRI contraindications, for example pregnancy, metal implants or dental braces, led to exclusion of the MRI experiment (Bas-Hoogendam, Harrewijn, et al., 2018).

Recruitment

Families were recruited through media exposure, like interviews in Dutch newspapers, on television and radio; furthermore, the study was brought to the attention of patient organizations, to clinical psychologists, general practitioners and mental health care organizations. Recruitment was targeted at families in which multiple family members experienced 'extreme shyness' and took place between Summer 2013 and Summer 2015. Details about the screening and inclusion flow of the LFLSAD are provided in (Bas-Hoogendam, Harrewijn, et al., 2018).

Ethics

Both parents signed the informed consent form for their children, and children between 12 and 18 years of age signed the form themselves as well. Participants received a financial compensation of €75. Confidentiality of the data was maintained by the use of a unique research ID number for each family member.

A priori power calculation and sample size

A priori power calculations were performed to estimate the required sample size of the LFLSAD, as described previously in (Bas-Hoogendam, Harrewijn, et al., 2018). Power was computed by simulation, based on an endophenotype with a heritability of 60 % and a correlation of 70 % with SAD; the prevalence of SAD was set at 10 %. Families were generated using linear mixed models and we modeled correlations between family members via normally distributed random effects with a correlation structure of two times the kinship matrix. Only families with at least two affected members in one nuclear family were used for estimation of the power. These power calculations revealed that 12 families with 8 - 12 family members (average: 10 members per family) were required for sufficient power (i.e., minimally 80 %) to 1st estimate the association between SAD and neurocognitive putative endophenotypes and 2nd to determine the significance of clustering of these endophenotypes within families (i.e., genetic effects).

Phenotyping

The presence of DSM-IV diagnoses was determined using the Mini-International Neuropsychiatric Interview (M.I.N.I.)-Plus (version 5.0.0) (Sheehan et al., 1998; van Vliet & de Beurs, 2007) or the M.I.N.I.-Kid interview (version 6.0) (Bauhuis et al., 2013; Sheehan et al., 2010); these interviews were administered by experienced clinicians and recorded. Special attention was paid to the presence of (sub)clinical SAD: clinical SAD was established using the DSM-IV-TR criteria for the generalized subtype of SAD, but the clinician verified whether the DSM-5 criteria for SAD were also met. We chose a priori to include patients with generalized SAD, as this is the most prevalent subtype, with a strong familiar pattern and an early age of onset (D'Avanzato & Dalrymple, 2016). A diagnosis of subclinical SAD was established when participants met the criteria for SAD as described in the DSM-5, but did not show impairing limitations in important areas of functioning (criterion G) (American Psychiatric Association, 2013).

In addition to the clinical interviews and the self-report questionnaires on social anxiety (the Liebowitz Social Anxiety Scale (LSAS-SR) (Fresco et al., 2001; Mennin et al., 2002) or the Social Anxiety Scale for adolescents (SAS-A) (La Greca & Lopez, 1998)), participants completed several questionnaires on anxiety-related constructs.

The intensity of fear of negative evaluation was assessed using the revised Brief Fear of Negative Evaluation (BFNE) – II scale (Carleton et al., 2006; Leary, 1983).

Furthermore, the level of self-reported depressive symptoms was evaluated using the Beck Depression Inventory (BDI– II) (Beck et al., 1996; Van der Does, 2002) or the Children's Depression Inventory (CDI) (Kovacs, 1985; Timbremont & Braet, 2002).

The State-Trait Anxiety Inventory (STAI) (Spielberger et al., 1970) (see (Spielberger & Vagg, 1984) for psychometric properties) was used to determine self-reported trait anxiety, as well as state anxiety before and after the MRI scan.

The sensitivity for the temperamental traits 'behavioral inhibition' and 'behavioral activation' was assessed using the self-report BIS/BAS (Carver & White, 1994; Franken et al., 2005) or the BIS/BAS scales for children (BIS/BAS-C) (Muris et al., 2005).

Two subscales of the Wechsler Adult Intelligence Scale-IV (WAIS-IV) (Wechsler et al., 2008) or Wechsler Intelligence Scale for Children-III (WISC) (Wechsler, 1991), the similarities (verbal comprehension) and block design (perceptual reasoning) subtests, were administered to obtain an estimate of cognitive functioning.

MRI experiment

Prior to the MRI scan, participants were informed about the safety procedures and they were told that they could refrain from continuing the experiment at any time. Children and adolescents were familiarized with the MRI scanner using a mock scanner (Galván, 2010) and all participants received instructions about the task paradigms presented during the scan session. Scanning was performed using a 3.0 T Philips Achieva MRI scanner (Philips

Medical Systems, Best, The Netherlands), equipped with a 32-channel Sensitivity Encoding head coil. During the habituation paradigm, fMRI scans were acquired using T2*-weighted echo-planar imaging (EPI). These scans had the following characteristics: 38 axial slices, 2.75 mm x 2.75 mm x 2.75 mm + 10 % interslice gap, field of view (FOV) = 220 mm x 115 mm x 220 mm, repetition time (TR) = 2200 ms, echo time (TE) = 30 ms. The first six volumes of each fMRI scan were dummy volumes; these volumes were removed to allow for equilibration of T1 saturation effects.

In addition, a high-resolution EPI scan (84 axial slices, 1.964 mm x 1.964 mm x 2 mm, FOV=220 mm x 168 mm x 220 mm, TR = 2200 ms, TE = 30 ms) and a high-resolution T1-weighted scan (140 slices, resolution 0.875 mm × 0.875 mm × 1.2 mm, FOV = 224 mm × 168 mm × 177.333 mm, TR = 9.8 ms, TE = 4.59 ms, flip angle = 8◦) were acquired. These scans were used for within-subject registration purposes; furthermore, the structural T1-scans were inspected by a neuroradiologist, but no clinically relevant abnormalities were present in any of the participants.

Habituation paradigm

Faces

We selected the following faces from the FACES database (Ebner et al., 2010): M049, M072 and M089 (faces of men; mean age: 24 y) and F069, F152 and F171 (faces of women; mean age: 25.7 y).

fMRI data

General processing steps

FMRI data were denoised using FIX (FMRIB's ICA-based X-noiseifier), a publicly available plugin for FSL (FMRIB Software Library, version 5.0.9) (Jenkinson et al., 2012), which provides an automatic solution for denoising fMRI data via accurate classification of ICA components (Griffanti et al., 2014; Salimi-Khorshidi et al., 2014). Next, data underwent several preprocessing steps using FEAT (FMRI Expert Analysis Tool; version 6.00) (Jenkinson et al., 2012; Smith et al., 2004), including motion correction using MCFLIRT (Jenkinson et al., 2002), spatial smoothing using a Gaussian kernel of full-width half-maximum (FWHM) 6.0 mm and grand-mean intensity normalization of the entire 4D dataset by a single scaling factor in order to enable higher-level analyses, and registration. Scans were first registered to high-resolution EPI images, which were registered to T1 images, which in turn were registered to the Montreal Neurological Institute (MNI) T1-template brain (resolution 2 mm) using FNIRT nonlinear registration (warp resolution 10 mm) (Andersson et al., 2007; Jenkinson et al., 2002; Jenkinson & Smith, 2001). Next, ICA-AROMA (ICA-based Automatic Removal of Motion Artifacts) was used to remove motion-related artefacts (Pruim, Mennes, van Rooij, et al., 2015; Pruim, Mennes, Buitelaar, et al., 2015). Data were then submitted to FEAT to perform non-brain removal using BET (Smith, 2002), high-pass

temporal filtering (Gaussian-weighted least-squares straight line fitting, with sigma = 30.0 s) and registration. Functional scans of each participant were registered to the individual 3D T1-weighted anatomical scan using FLIRT (Jenkinson et al., 2002; Jenkinson & Smith, 2001) and subsequently registered to the MNI T1-template brain (resolution 2 mm) using FNIRT nonlinear registration (warp resolution 10 mm) (Andersson et al., 2007).

We checked whether the individual scans were registered correctly and confirmed that relative motion parameters did not exceed 2.5 mm.

Definition of the regions of interest (ROI)

The amygdala ROI was defined using the Harvard-Oxford atlas implemented in FSLview, using a threshold of 50 %. Replicating the methods by (Avery & Blackford, 2016), the hippocampus, primary visual cortex (V1) and extrastriate cortex ROI were defined using the AAL (automated anatomical labeling) standard masks (Tzourio-Mazoyer et al., 2002); for V1, we selected the calcarine fissure mask, while the extrastriate cortex ROI consisted of the lingual gyrus, the inferior occipital cortex and the middle occipital cortex. Because we did not perform a standard fusiform face area (FFA) localizer task as described by (Avery & Blackford, 2016), the FFA ROI was based on the AAL atlas as well. The ventromedial prefrontal cortex and orbitofrontal cortex ROI were defined following the population masks described by (Mackey & Petrides, 2010) – see Avery & Blackford (2016).

For the BNST ROI we used a mask that was previously created using an ultra-high field (7T) MRI and a specialized GRASE sequence to trace the BNST (Avery et al., 2014).

Endophenotype analyses with (sub)clinical SAD as predictor

For reasons of completeness, we performed voxelwise analyses using (sub)clinical SAD as a discrete predictor, in addition to the main analyses using self-reported SA-level (continuous variable) as a predictor. In these analyses, individual activation level related to the contrast 'first > last' was used as dependent variable. Correlations between family members were modeled by including random effects; age and gender (both centered) were included as covariates. Models were run for each voxel separately. Results (z-scores) were transformed into a nifti-image with the same dimensions of the MNI T1-template brain. Clusters within the ROIs were corrected for multiple comparisons using the FSL tool easythresh (cluster threshold: $z > 2.3$, cluster extent threshold $p < 0.05$) (Worsley, 2001).

Sensitivity analyses

We performed two sensitivity analyses to examine whether the results of the association analysis (effect of self-reported social anxiety on brain activation related to 'first half > last half') were driven by the severity of depressive symptoms as measured by the BDI-II or the CDI, or by (comorbid) psychopathology other than SAD (cf. (Bas-Hoogendam, van Steenbergen, Tissier, et al., 2018b, 2019)). To this aim, we added the z-score of the level

of depressive symptoms as a covariate in the voxelwise analysis (sensitivity analysis 1) or excluded all family members with past and / or present psychopathology other than SAD and repeated the association analysis (sensitivity analysis 2). Note however, that this latter analysis may yield biased and weaker results, as the majority of the probands, on which the selection of the families was based, had comorbid psychopathology and were thus excluded. We used the same statistical threshold as for the main analyses, within the bilateral ROIs ($z > 2.3$, cluster-threshold $p < 0.05$).

Supplemental Results

Data availability

We acquired MRI data from nine families ($n = 113$) (Bas-Hoogendam, Harrewijn, et al., 2018), but data from one family ($n = 3$ family members) had to be excluded as the proband from this family was not able to participate in the MRI experiment due to an MRI contra-indication. As a result, 110 datasets were available for further fMRI pre-processing and quality control. Two datasets could not be used due to an imaging artefact, while the relative motion parameters of three other participants exceeded 2.5 mm. So, 105 fMRI datasets were available for further analysis of brain activation related to habituation. Furthermore, data on the presence of (sub)clinical SAD were lost for several family members due to technical reasons.

Sample characteristics

In line with the design of the study, participants originated from two generations, which differed significantly in age ($\beta \pm SE = -30.1 \pm 0.7$, $p < 0.001$), but not in male / female ratio ($\chi^2(1) = 0.75$, $p = 0.38$). In line with previous reports on this sample (Bas-Hoogendam, van Steenbergen, Tissier, et al., 2018b, 2019), family members with and without (sub)clinical SAD did not differ with respect to male / female ratio, age and estimated IQ. Groups did differ, however, in comorbidity rates: family members with (sub)clinical SAD were more often diagnosed with depression (past) and dysthymia (present). These differences were, however, only significant at an uncorrected significance level. Furthermore, family members with (sub)clinical SAD reported higher levels of fear of negative evaluation, more depressive symptoms, higher levels of trait anxiety and behavioral inhibition (BIS), as well as lower levels of behavioral activation (BAS) (*Supplemental Table S9.1*).

fMRI data

Endophenotype analyses with (sub)clinical SAD as predictor

The regression analysis using discrete (sub)clinical SAD as a predictor did not yield clusters within the ROIs surviving the predefined threshold. So, although we did find an association

between the habituation response and self-reported SA (continuous predictor), there was no relation with (sub)clinical SAD (discrete predictor). We speculate that this lack of a correlation is power-related, as the fMRI sample only contained 37 (sub)clinical SAD cases. This indicates the need for replication of the present findings in a larger sample.

Sensitivity analyses

Results of the first sensitivity analysis, with the level of depressive symptoms as an additional covariate, confirmed the relationship between SA and reduced neural habituation in the right amygdala (cluster characteristics: 29 voxels, $p = 0.014$; max z-value: 2.83), while the relationship between SA and habituation in the right hippocampus was not significant.

In the second sensitivity analysis, we excluded all participants with past and/or present comorbid psychopathology other than SAD; this resulted in a sample of 58 participants, of which 12 in the (sub)clinical SAD group. Next, we repeated the association analysis with self-reported social anxiety as predictor; this analysis confirmed the relation between SA level and impaired habituation in the right hippocampus (cluster characteristics: 89 voxels, $p = 0.047$; max z-value: 3.86); no significant clusters were present in the right amygdala.

Supplemental Table

Supplemental Table S9.1 Detailed characteristics of participants with and without (sub)clinical SAD: scores on self-report questionnaires.

	(Sub)clinical SAD (*n* = 37)[a]	No SAD (*n* = 61)	Statistical analysis
Self-report measures			
Social anxiety symptoms (z-score; mean ± SD)	2.9 ± 3.3	0.6 ± 1.5	β ± SE = 2.5 ± 0.5, $p < 0.001$
Fear of negative evaluation (mean ± SD)	23.6 ± 12.4	12.7 ± 8.0	β ± SE = 10.9 ± 2.0, $p < 0.001$
Depressive symptoms (z-score; mean ± SD)	0.1 ± 0.9	-0.5 ± 0.7	β ± SE = 0.5 ± 0.2, $p < 0.001$
STAI – trait (mean ± SD)	38.9 ± 9.6	33.0 ± 8.6	β ± SE = 5.6 ± 1.9, $p = 0.003$
BIS (z-score; mean ± SD)	0.3 ± 1.3	-0.4 ± 0.9	β ± SE = 0.7 ± 0.2, $p < 0.001$
BAS (z-score; mean ± SD)	-1.0 ± 0.8	-0.6 ± 1.0	β ± SE = -0.5 ± 0.2, $p = 0.007$

Footnote

[a] Due to technical reasons, data on the presence of subclinical SAD were lost for seven family members. Data from these participants were, however, included in the endophenotype analyses using SA-level (*z*-score) as a predictor (*n* = 105).

Chapter 10

Amygdala hyperreactivity to faces conditioned with a social-evaluative meaning - a multiplex, multigenerational fMRI study on social anxiety endophenotypes

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Abstract

Social anxiety disorder (SAD) runs in families, but the neurobiological pathways underlying the genetic susceptibility towards SAD are largely unknown. Here, we employed an endophenotype approach, and tested the hypothesis that amygdala hyperreactivity to faces conditioned with a social-evaluative meaning is a candidate SAD endophenotype. We used data from the multiplex, multigenerational Leiden Family Lab study on Social Anxiety Disorder (eight families, n = 105) and investigated amygdala activation during a social-evaluative conditioning paradigm with high ecological validity in the context of SAD. Three neutral faces were repeatedly presented in combination with socially negative, positive or neutral sentences. We focused on two endophenotype criteria: *co-segregation of the candidate endophenotype with the disorder within families*, and *heritability*.

Analyses of the fMRI data were restricted to the amygdala as a region of interest, and revealed that bilateral amygdala hyperreactivity in response to the conditioned faces co-segregated with social anxiety within the families. Furthermore, multiple voxels within these amygdala clusters were at least moderately heritable. Taken together, these findings show that amygdala engagement in response to conditioned faces with a social-evaluative meaning qualifies as a neurobiological candidate endophenotype of social anxiety. Thereby, these data shed light on the genetic vulnerability to develop SAD.

Introduction

Social anxiety disorder (SAD), one of the most prevalent anxiety disorders, has a typical onset during adolescence and runs in families (Haller et al., 2015; Isomura et al., 2015). Patients with the disorder have an extreme fear of evaluation by others and avoid social situations as much as possible (Stein & Stein, 2008). Furthermore, SAD is associated with a chronic course, high rates of comorbid psychopathology, reduced quality of life and far-reaching impairments in school, work and relations (Dams et al., 2017; Fehm et al., 2005). Given the severe consequences of the disorder, for patients and their families as well as for society, insight in the neurobiological functional brain alterations underlying the genetic vulnerability to develop SAD is essential.

One of the key structures in the socially-anxious brain is the amygdala (cf. reviews by (Brühl, Delsignore, et al., 2014; Etkin & Wager, 2007; Garner et al., 2009)). The amygdala is essential for processing environmental stimuli and learning their predictive value, as demonstrated in both humans and animals (Hariri & Whalen, 2011; Janak & Tye, 2015; Olsson & Phelps, 2007; Paton, Belova, Morrison, & Salzman, 2006). More specifically, an elegantly designed neuroimaging study by Davis and colleagues (2010) has provided strong evidence for the role of the amygdala in learning the social value of biologically-relevant cues. The authors employed a conditioning paradigm, in which three neutral faces (conditioned stimuli, CS) were consistently paired with either a positive endorsement, a negative comment, or a socially-neutral statement (unconditioned stimuli, US; Davis et al., 2010); importantly, as these sentences were directly addressing the participant, the presentation of these face-sentence combinations created a social-evaluative learning context. Behavioral ratings of likeability indicated that healthy participants learned the social value of the faces, and functional magnetic resonance imaging (fMRI) data revealed the involvement of various amygdala subregions during social-evaluative learning (Davis et al., 2010).

At present, and to the best of our knowledge, this social-evaluative conditioning paradigm has not been used in SAD. Nevertheless, given the heightened fear of negative as well as positive evaluation that characterizes socially-anxious individuals (Reichenberger et al., 2019; Teale Sapach, Carleton, Mulvogue, Weeks, & Heimberg, 2014), investigating the neurobiological underpinnings of social-evaluative learning is of uttermost relevance in SAD (cf. (Pittig, Treanor, LeBeau, & Craske, 2018)). An electromyography (EMG) study in patients with SAD, using a differential fear conditioning paradigm in which neutral faces (CS) were paired with positive, neutral or negative facial expressions and verbal feedback (US) addressing the participant, reported an elevated fear-potentiated startle reflex in response to faces conditioned with critical facial expressions and insults in SAD patients, while no group differences were present with respect to subjective ratings of the conditioned stimuli, nor during extinction learning (Lissek et al., 2008).

Subsequent studies used slightly adapted versions of this differential fear conditioning paradigm. The first, an EMG study on individuals with clinical SAD and participants with high levels of social anxiety (SA) could, however, not replicate fear conditioning in the physiological data, and did not find SA-related differences with respect to self-report measures of anxiety, unpleasantness and arousal due to conditioning (Tinoco-González et al., 2015). The second study (Ahrens, Mühlberger, Pauli, & Wieser, 2015), using electroencephalography (EEG), paired neutral faces (CS) with three types of verbal feedback (positive, neutral or negative; US), and demonstrated impaired electrocortical differentiation in students with high levels of SA: while low socially-anxious individuals showed differential visuocortical processing in relation to the three conditions, with highest cortical activity to faces paired with insults and lowest activity to faces paired with compliments, this distinction was absent in high socially-anxious participants. Again, no group differences were found with respect to ratings of valence (Ahrens et al., 2015).

Due to the methodology used, these studies were, however, not able to investigate amygdala reactivity during social conditioning. To the best of our knowledge, only one fMRI study has explored the relation between SA and amygdala activation during social conditioning using disorder relevant stimuli. In that study, Pejic et al. (2013) paired neutral faces (CS) with film-clips of critical comments (US), and showed positive correlations between SA and amygdala activation during social conditioning; at the behavioral level, participants with higher SA-levels reported stronger increases in unpleasantness and fear following social conditioning (Pejic et al., 2013).

Together, these findings suggest that SA is associated with altered physiological and neural responses during social conditioning, although it should be noted that only one study so far explored amygdala activation. Furthermore, results on the relation between SA and behavioral indices of social conditioning are mixed. In addition, as Pejic and colleagues (2013) used a sample of healthy students with varying levels of SA and only employed negative unconditioned stimuli, the relation between SA and amygdala function related to social conditioning has until now not been investigated in patients with SAD, and the effect of positive and neutral comments as unconditioned stimuli is at present still unknown. Moreover, it has not been examined whether amygdala activation during social-evaluative learning is a candidate endophenotype of SAD. Such research is however, important, as endophenotypes, which are located on the causal pathway from genotype to phenotype, could shed light on the mechanisms by which genetic risk unfolds (Dick, 2018), and as such, could aid in unravelling the genetic susceptibility to SAD and offer new insights in targets for prevention and intervention (Bas-Hoogendam et al., 2016).

By definition, endophenotypes are quantitative characteristics which are *associated with the disorder* (criterion 1), *state-independent and already present in a preclinical state* (criterion 2), *heritable* (criterion 3), and display *co-segregation with the disorder within families of probands, with non-affected family members showing altered levels of the endophenotype in*

comparison to the general population (criterion 4) (Glahn et al., 2007; Lenzenweger, 2013a). The endophenotype approach has yielded promising results in other psychiatric disorders, for example in depression (Goldstein & Klein, 2014), schizophrenia and psychosis (Blakey et al., 2018; Glahn, Williams, et al., 2014; Sutcliffe et al., 2016) and obsessive–compulsive disorder (Taylor, 2012) but research on neurobiological endophenotypes of SAD is still scarce.

Here, we present data from the Leiden Family Lab study on Social Anxiety Disorder (LFLSAD), comprising a unique sample of families genetically enriched for SAD (Bas-Hoogendam, Harrewijn, et al., 2018). This multiplex (i.e. multiple cases of SAD), multigenerational family design is eminently suitable to test two important endophenotype criteria within the same sample, being the heritability and co-segregation of a certain candidate endophenotype within families. Using the social conditioning paradigm developed by Davis and colleagues (2010) for the first time in the context of SAD, we investigated whether amygdala reactivity during social-evaluative learning could serve as a candidate neurobiological endophenotype of SAD. First, we examined evidence for the endophenotype criterion of *co-segregation of the candidate endophenotype with SA within the families* (first element of criterion 4); in case of affirmative results, we established *heritability* (criterion 3). Based on previous research summarized above, we predicted a positive relationship between SA-level and amygdala activation in response to the conditioned stimuli. Furthermore, on a more exploratory basis as research on this subject is still scarce, we examined the relation between SA-level and amygdala activation over time, as well as in response to the three particular conditions. Behavioral ratings were used to validate the paradigm; in addition, their relation with SA-level was explored.

Experimental procedures

Participants

The sample consisted of participants from the LFLSAD, in which families genetically enriched for SAD are included. Families were invited for participation based on the combination of a primary diagnosis of SAD in a parent (aged 25 - 55 years old; 'proband') and a child who met criteria for clinical or subclinical SAD ('proband's SA-child'). The proband's SA-child (age 8 - 21 years) should live at home with the proband; comorbidity other than internalizing disorders or substance abuse was an exclusion criterion for the proband and proband's SA-child. Besides these two SAD-cases, first- and second-degree family members of two generations were invited to participate, being the proband's partner and other children of the nuclear family (age ≥ 8 years), as well as the proband's sibling(s), with their partners and children (age ≥ 8 years). These family members were included independent from the presence of psychopathology. Insufficient comprehension of the Dutch language

was an exclusion criterion for all participants, and general MRI contraindications led to exclusion of the MRI experiment.

Following this inclusion strategy, the LFLSAD sample (total sample: n = 132, nine families; MRI sample: n = 110, eight families; more information about recruitment is included in the *Supplemental Methods*) consists of family members of two generations (*Figure 3.1*). Participants completed a number of measurements, such as a diagnostic interview, self-report questionnaires and an MRI scan (Bas-Hoogendam, Harrewijn, et al., 2018). The study was approved by the Medical Ethical Committee of the Leiden University Medical Center and all participants provided informed consent according to the Declaration of Helsinki. Detailed information about the LFLSAD and an a priori power calculation for the study are outlined in a designpaper (Bas-Hoogendam, Harrewijn, et al., 2018); furthermore, the study was pre-registered online (Bas-Hoogendam et al., 2014a, 2014b).

Phenotyping

In order to facilitate extensive phenotyping, the LFLSAD protocol consisted of several measurements (Bas-Hoogendam, Harrewijn, et al., 2018) (cf. *Supplemental Methods*). The following assessments are relevant for the present work.

Experienced clinicians determined the presence of DSM-IV diagnoses using the Mini-International Neuropsychiatric Interview (M.I.N.I.)-Plus (Sheehan et al., 1998) or the M.I.N.I.-Kid interview (Sheehan et al., 2010). Given the nature of the LFLSAD sample, special attention was paid to the presence of (sub)clinical SAD. Clinical SAD was established using the DSM-IV-TR criteria for the generalized subtype of SAD, but the clinician verified whether the DSM-5 criteria for SAD were also met. A diagnosis of subclinical SAD was established when participants met the criteria for SAD as described in the DSM-5, but did not show impairing limitations in important areas of functioning (criterion G) (American Psychiatric Association, 2013). The interviews were recorded.

Furthermore, participants completed age-appropriate questionnaires on the level of SA-symptoms, being the Liebowitz Social Anxiety Scale for adults (Fresco et al., 2001) and the Social Anxiety Scale for adolescents (La Greca & Lopez, 1998), as well as on the level of depressive symptoms (Beck Depression Inventory (Beck et al., 1996) or the Children's Depression Inventory (Kovacs, 1985). To enable interpreting the scores of the age-appropriate questionnaires over the whole sample, z-scores were computed (Bas-Hoogendam, Harrewijn, et al., 2018). Incidental missing values were replaced by the average value of the completed items.

MRI experiment

Scanning was performed using a 3.0T Philips Achieva MRI scanner. The MRI experiment consisted of several structural scans (Bas-Hoogendam, van Steenbergen, Tissier, et al., 2018b) and functional task paradigms (Bas-Hoogendam, van Steenbergen, Blackford, et

al., 2019; Bas-Hoogendam, van Steenbergen, Kreuk, et al., 2017a; Bas-Hoogendam, van Steenbergen, Tissier, et al., 2019); details are provided in the *Supplemental Methods*.

Social-evaluative conditioning paradigm

The social-evaluative conditioning paradigm was part of the neutral faces paradigm (NFP), in which we investigated both the initial habituation response to neutral faces as well as brain activation associated with learning their social-evaluative value (*Figure 10.1*). Neutral faces were selected from the FACES database, a set of well-validated images of naturalistic faces of women and men (Ebner et al., 2010). Participants were presented with faces matching their own sex, so we selected photographs of three young males and three young females (see *Supplemental Methods* for details on these faces). Stimuli were presented using E-Prime software version (2.0.10, Psychology Software Tools).

The NFP consists of two phases, a habituation phase (HP) and the social-evaluative conditioning phase (SCP). Findings on the HP are reported elsewhere (Bas-Hoogendam, van Steenbergen, Blackford, et al., 2019); for reasons of completeness, a description of the HP is included in the *Supplemental Methods*. During the SCP, which was based on the paradigm by (Davis et al., 2010), three neutral faces, which had been shown to the participants already during the HP, were again presented, but now each face was consistently combined with one type of social-evaluative sentence: positive, negative or neutral. That is, after presentation of the face (duration: 1 s; CS) a social-evaluative sentence was presented (duration: 2 s; US) (*Figure 10.1*). One face was always followed by a positive endorsement (for example: 'he/she says you are smart'), the second face was accompanied by a negative comment ('he/she says you are stupid'), while the last face was combined with a socially-neutral statement ('he/she says you are in Leiden'). There were four different sentences within each category (see *Supplemental Table S10.1* for a list of all sentences), and each face-sentence combination was shown three times. This resulted in 12 trials per condition and a total of 36 trials. The order of the face-sentence combinations was pseudorandomized and the combinations of the faces with the type of self-relevant sentences were counterbalanced across the participants.

Participants were instructed to look at the faces and to read the accompanying sentences. As the face presentation always preceded the sentence presentation, participants learned what type of social-evaluative sentence would follow upon presentation of a certain face. The intervals between the presentation of the face and the presentation of the sentence, as well as the intertrial intervals, were jittered in order to optimize the estimation of the blood-oxygen-level-dependent response related to the presentation of the faces and the presentation of the sentences (jitter face-sentence: range 1.5 s – 2.5 s, mean 2.0 s; intertrial interval: range 2.0 s – 3.5 s, mean 2.7 s; cf. (Davis et al., 2010)). Total duration of the SCP was 5 min 41 s.

At three times during the NFP, participants were asked to rate the faces on likeability and arousal in line with the paradigm described by (Davis et al., 2010); the first measurement

was before the HP (T1), the second between the HP and SCP (T2), while the last measurement followed the end of the SCP (T3; *Figure 10.1*). These ratings were used to investigate the initial rating of the faces (T1); furthermore, the ratings were used to assess whether participants learned the social-evaluative value of the faces (i.e. to validate the SCP), and to examine the association between this learning process and social anxiety. The three faces were presented sequentially on the screen, accompanied by the question 'How much do you like this person?' (range from -4, 'not at all' to 4, 'very much'), and, on a second screen, the question 'How much emotion do you experience when seeing this person?' (ranging from 1, 'none' to 9 ,'a lot'). Prior to the start of the MRI scan, participants were familiarized with these ratings by performing a short version of the task (with faces not used in the fMRI task) on a laptop.

Data analysis

Sample characteristics

We compared participants with and without (sub)clinical SAD on demographic variables and on the level of self-reported SA, by performing chi-square tests in SPSS (version 25) and by fitting multi-level regression models in R (R Core Team, 2016). Within these regression models, we modelled genetic correlations between family members by including random effects.

Behavioral data

We examined whether participants learned the social-evaluative value of the faces by performing a repeated measures ANOVA in SPSS, with condition (3 levels: positive, negative, neutral) and time (3 levels: T1, T2 and T3) as within-subjects factors. Significance level was set at $p \leq 0.05$; we applied Greenhouse–Geisser correction when the assumption of sphericity was violated.

Furthermore, we investigated whether the initial behavioral response to the faces, as well as the likeability ratings related to learning their value in the social-evaluative context, were associated with SA. To examine the initial response, we used the average of likeability ratings over the three faces provided at T1 (Likeability_T1); to examine the effect of the social-evaluative context, we calculated the difference in likeability scores between T2 and T3 over the three conditions (ΔLikeability_T3_T2), and for the three conditions separately; furthermore, we calculated difference scores to explore whether SA-level was differentially associated with learning the value of the negative, neutral and positive conditioned faces (ΔLikeability_T3_T2_Neg_vs_Neu; ΔLikeability_T3_T2_Neg_vs_Pos; ΔLikeability_T3_T2_Pos_vs_Neu).

We investigated the association between SA and these likeability ratings using linear mixed models in R (package: *coxme*), with self-reported SA (continuous variable; *z*-score, centered) as predictor of interest. Separate models were used to investigate the initial re-

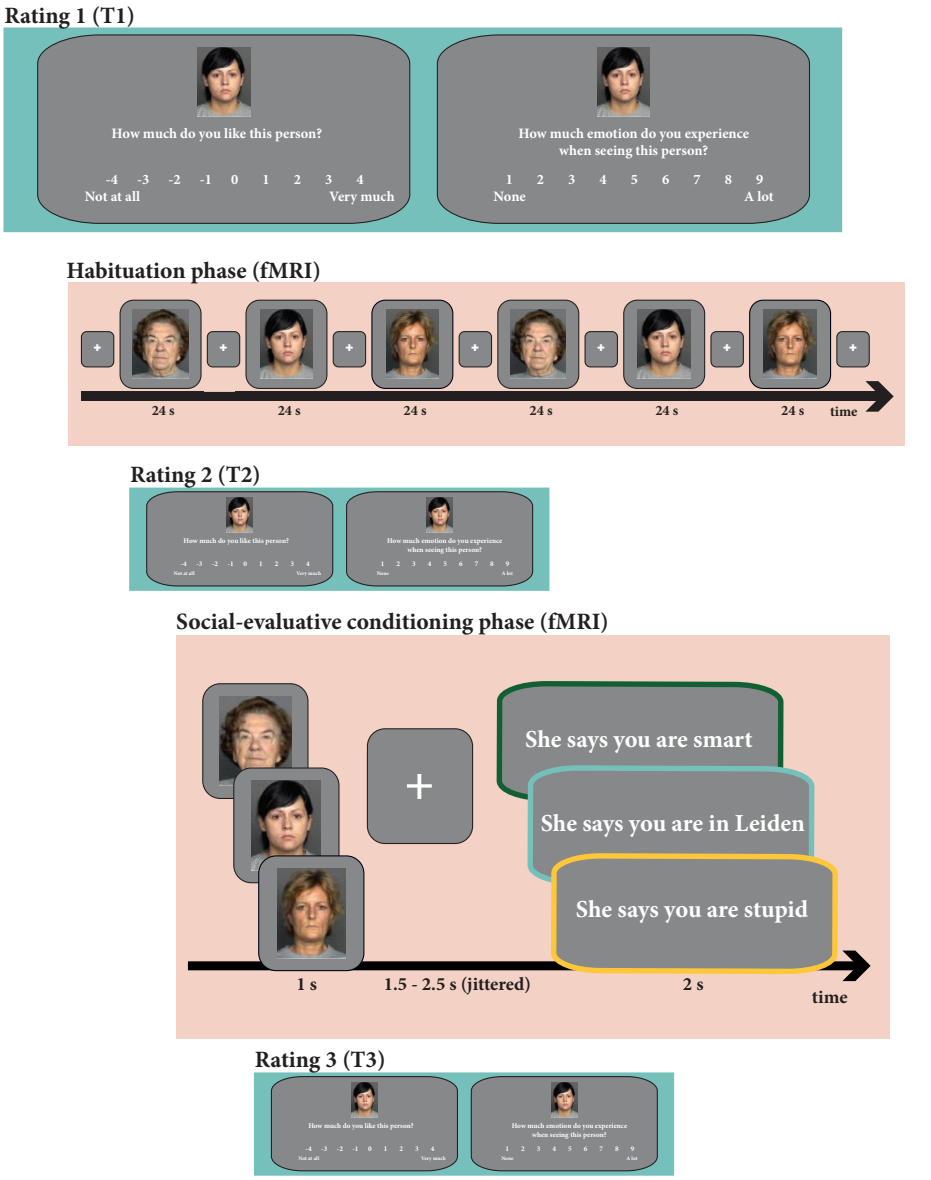


Figure 10.1 Overview of the neutral faces paradigm (NFP).
Stimuli were neutral faces selected from the FACES database (Ebner et al., 2010); the paradigm consists of two fMRI phases, being a habituation phase (described in more detail in *Supplemental Methods* as well as in (Bas-Hoogendam, van Steenbergen, Blackford, et al., 2019) and the social-evaluative conditioning phase (SCP) which is discussed in the present work. During the SCP, three neutral faces were consistently paired with either a positive endorsement, a negative comment or a socially-neutral statement, enabling participants to learn the social value of each face. At three time-points during the NFP, participants rated the faces on likeability and arousal.

sponse to the faces and the difference scores representing learning the value of the faces in the social-evaluative context. Random effects were included to account for the genetic correlations between family members; age (centered) and gender (centered) were added as covariates of no interest. Significance level was set at $p < 0.05$.

fMRI data

General processing steps and statistical analysis

Functional MRI data were pre-preprocessed using standardized procedures in FSL (Jenkinson et al., 2012) – see a detailed description of the processing steps in the *Supplemental Methods* and (Bas-Hoogendam, van Steenbergen, Blackford, et al., 2019; Bas-Hoogendam, van Steenbergen, Tissier, et al., 2019). Event-related statistical analysis was performed in native space, using FILM with local autocorrelation correction (Woolrich et al., 2001). Following previous analyses (Davis et al., 2010), we included twelve explanatory variables (EVs) as well as their temporal derivatives in the general linear model. The EVs represented the presentation of the faces belonging to the three conditions (negative, neutral, positive) and the presentation of the negative, neutral and positive social-evaluative sentences; separate EVs were created for the stimuli presented during the first half and the last half of the SCP, in order to enable investigating social-evaluative learning over time (cf. (Davis et al., 2010)). As the present work focuses on the processing of the conditioned stimuli (the faces), brain responses to the sentences will not be further analyzed. EVs were convolved with a double gamma hemodynamic response function and onset of the EVs for each individual was determined using custom-written scripts in Matlab. The fixation cross between the face and sentence stimuli and the fixation cross between the trials were not modeled and therefore served as the implicit baseline to which EVs could be compared.

We defined several contrasts of interest. First of all, we examined the contrast 'all faces > baseline', in order to investigate brain activation related to viewing the conditioned stimuli (i.e. faces with a social-evaluative meaning). Furthermore, we examined habituation (cf. (Davis et al., 2010)) by contrasting the faces presented during the first half of the SCP with the faces presented during the last half of the SCP; we refer to this contrast as 'all faces early > all faces late'. Next, we investigated valence-effects by contrasting the conditioned stimuli in the three different conditions ('negative > neutral'; 'negative > positive'; 'positive > neutral').

Brain activation at group level

For all contrasts of interest, we determined brain activation over the whole sample in the amygdala, by using masks of the left and right amygdala (mask description included in the *Supplemental Methods* and illustrated in *Supplemental Figure S10.1*; cluster threshold $z > 2.3$, cluster extent threshold $p < 0.05$ within the unilateral regions of interest (ROIs)).

Furthermore, for reasons of completeness, we also report explorative whole-brain analyses (cluster threshold $z > 3.1$, extent threshold $p < 0.05$).

Neurobiological candidate endophenotypes

We tested whether altered amygdala activation in response to conditioned faces could serve as a candidate SAD endophenotype, and investigated the *co-segregation of the candidate endophenotype with the disorder within families* using regression models in R, with self-reported SA-level (*z*-score; centered) as independent variable and individual activation level related to the contrasts of interest as dependent variables. Correlations between family members were modeled by including random effects; age and gender (both centered) were included as covariates of no interest. Furthermore, analyses were corrected for the level of depressive symptoms (*z*-score; centered). Models were ran for each voxel separately and results (*z*-scores) were transformed into a nifti-image with the dimensions of the MNI T1-template brain.

We examined the relation with SA within the clusters representing significant amygdala activation at group level; results were corrected for multiple comparisons using the FSL-tool easythresh (cluster threshold $z > 2.3$, cluster extent threshold $p < 0.05$, minimum of 10 voxels) (Worsley, 2001). For reasons of completeness, we also investigated the association with SA at the level of whole brain activation (*Supplemental Results; Supplemental Figure S10.2*). A subsequent sensitivity analysis was performed to investigate whether the results of the association analyses were driven by (comorbid) psychopathology other than SAD (*Supplemental Methods*).

Next, we determined the *heritability* of brain activation for voxels in the significant clusters. Heritability estimates were obtained with a method which takes the ascertainment process into account and incorporates familial relationships (Tissier et al., 2017). Age and gender (both centered) were included as covariates.

Results

Sample characteristics

Details on quality checking and data availability are provided in the *Supplemental Results*. Characteristics of the samples ($n = 108$ for the behavioral analyses, data on subclinical SAD available for 102 participants; $n = 105$ for the fMRI analyse, data on subclinical SAD available for 98 participants) are presented in *Table 10.1*. Family members with (sub)clinical SAD did not differ from family members without SAD with respect to male / female ratio and age, but they reported higher levels of social anxiety and more depressive symptoms. We refer the reader to *Supplemental Tables S10.2-3* for a detailed characterization of the sample.

Table 10.1 Characteristics of participants with and without (sub)clinical SAD.

	Behavioral samplea			fMRI samplea		
	(Sub)clinical SAD (*n* = 39)	No SAD (*n* = 63)	Statistical analysis	(Sub)clinical SAD (*n* = 38)	No SAD (*n* = 60)	Statistical analysis
Demographics						
Male / Female (n)	20 /19	31 /32	$\chi^2(1) = 0.04$, $p = 0.84$	19 /19	30 /30	$\chi^2(1) = 0.0$, $p = 1.00$
Generation 1 / Generation 2 (n)	19 /20	27 /36	$\chi^2(1) = 0.33$, $p = 0.56$	19 /19	27 /33	$\chi^2(1) = 0.23$, $p = 0.63$
Age in years, (mean ± SD, (range))	30.3 ± 15.5 (9.2 – 59.6)	30.9 ± 15.4 (9.0 – 61.5)	β ± SE = -0.6 ± 3.1, $p = 0.85$	30.9 ± 15.3 (9.2 – 59.6)	31.9 ± 15.0 (9.4 – 61.5)	β ± SE =-1.0 ± 3.1, $p = 0.73$
Diagnostic information						
Clinical SAD (n)	17	0		17	0	
Self-report measures						
Social anxiety (z-score; mean ± SD)	3.0 ± 3.3	0.5 ± 1.6	β ± SE = 2.6 ± 0.5, $p < 0.001$	3.0 ± 3.1	0.6 ± 1.5	β ± SE = 2.6 ± 0.5, $p < 0.001$
Depression (z-score; mean ± SD)	0.0 ± 0.9	-0.5 ± 0.7	β ± SE = 0.5 ± 0.2, $p < 0.001$	0.01 ± 0.8	-0.5 ± 0.7	β ± SE = 0.5 ± 0.2, $p < 0.001$

Abbreviations

SD, standard deviation; SE, standard error.

Footnote

[a]: Due to technical reasons, data on the presence of subclinical SAD were lost for several family members. Data from these participants were, however, included in the endophenotype analyses using SA-level (*z*-score) as a predictor (behavioral sample: *n*=108; fMRI sample: *n*=105).

Behavioral data

Validation of the SCP

Likeability ratings for the faces, provided at three timepoints during the NFP, are provided in *Table 10.2* and illustrated in *Figure 10.2A*. As expected, a repeated measures ANOVA with condition and time as within-subject factors indicated a significant interaction between time and condition ($F(3.4,362.8) = 37.2$, $p < 0.001$, $\eta^2 = 0.18$). Subsequent repeated measures ANOVAs separately for each timepoint, with condition as within-subjects factor, indicated that the faces did not differ with respect to likeability at T1 ($F(2,214) = 1.0$, $p = 0.38$, $\eta^2 = 0.009$) and T2 ($F(2,214) = 0.9$, $p = 0.40$, $\eta^2 = 0.009$), which validated the use of these faces for the subsequent SCP. Indeed, after the SCP (T3), a significant effect of condition was present ($F(1.8,194.5) = 34.5$, $p < 0.001$, $\eta^2 = 0.24$), indicating that participants learned the social-evaluative value of the faces; this finding is in line with the original report on this paradigm (Davis et al., 2010).

Table 10.2 Behavioral ratings on the neutral faces paradigm.

Likeability ratings (mean ± SD)	**T1**	**T2**	**T3**
Average	0.7 ± 1.1	0.8 ± 1.1	0.7 ± 1.1
Negative	0.8 ± 1.6	0.8 ± 1.6	-0.3 ± 1.8
Neutral	0.7 ± 1.6	0.9 ± 1.6	1.0 ± 1.5
Positive	0.6 ± 1.6	0.7 ± 1.6	1.3 ± 1.6
Effect of social anxiety (*z*-score)[a]	β ± SE	*p*	
Likeability_T1	0.07 ± 0.04	0.07	
ΔLikeability_T3_T2	-0.08 ± 0.03	0.003	
ΔLikeability _T3_T2_positive	-0.06 ± 0.06	0.27	
ΔLikeability_T3_T2_ negative	-0.11 ± 0.06	0.07	
ΔLikeability-T3_T2_ neutral	-0.07 ± 0.05	0.15	

Abbreviations
SD, standard deviation; SE, standard error.

Footnote
[a] Corrected for age, gender and family structure.

Association analyses showed that the initial response to the neutral faces (Likeability_T1) was not significantly related to SA-level within the families (*Table 10.2*). SA-level was, however, associated with the change in likeability ratings due to social-evaluative conditioning: there was a significant negative relation between SA-level and ΔLikeability_T3_T2, indicating that the addition of the social-evaluative sentences (US) was aversive for family members with higher SA-levels (*Table 10.2; Figure 10.2B upper half*). This effect was present regardless of the valence of the comments: follow-up analyses indicated that the negative association between SA and ΔLikeability_T3_T2 was present in all three conditions (*Table 10.2; Figure 10.2B lower half*), while subsequent regression analyses on the difference scores

between the conditions confirmed that the relationship between SA and ΔLikeability_T3_T2 was not different between the conditions (ΔLikeability_T3_T2_Neg_vs_Neu: β ± SE = -0.04 ± 0.07, p = 0.57; ΔLikeability_T3_T2_Neg_vs_Pos: β ± SE = -0.05 ± 0.09, p = 0.60; ΔLikeability_T3_T2_Pos_vs_Neu: β ± SE = 0.007 ± 0.08, p = 0.93). A sensitivity analysis on the difference in likeability between T1 and T2 confirmed that the effect of SA was specific for the SCP of the NFP (*Supplemental Results*).

In addition to these likeability ratings, we included ratings of arousal in the NFP in line with the task description by Davis et al. (2010). However, it was hard to find a good transcription of the term 'arousal' when translating the question from English to Dutch. Indeed, participants indicated during debriefing that they struggled to interpret the question with

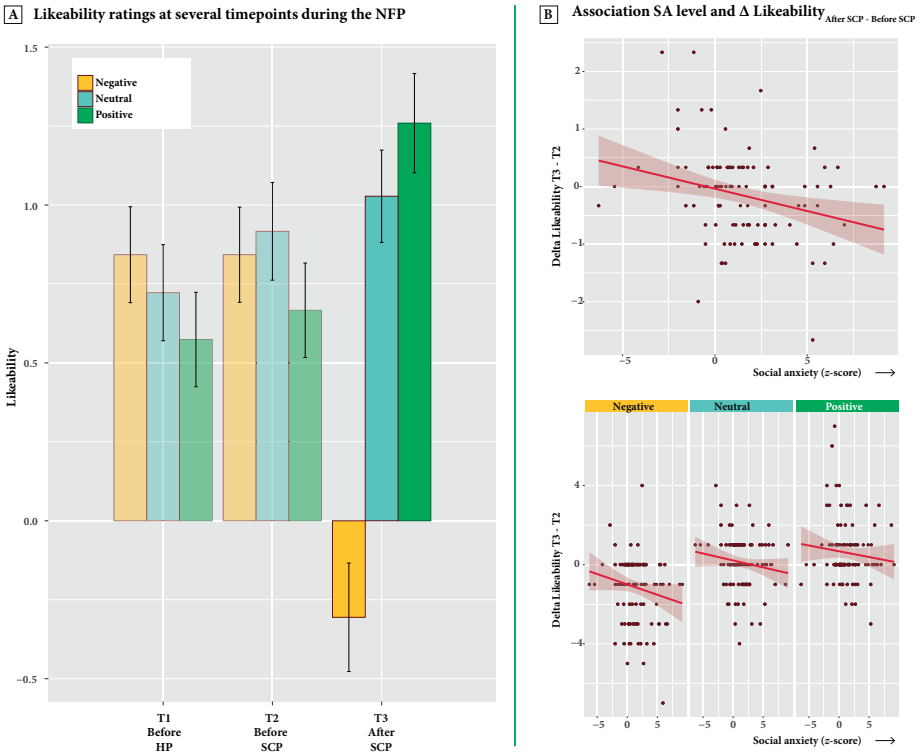


Figure 10.2 Behavioral ratings on the NFP.

Figure 10.2A Ratings of likeability for the three conditions at the three timepoints. Faded colors at T1 and T2 indicate that the faces were not conditioned yet; at T3, participants had learned the social-evaluative value of the faces, as indicated by a significant interaction between time and condition, as well as an effect of condition at T3. Errorbars represent standard errors of the mean.

Figure 10.2B Association between the level of social anxiety (SA) and learning the social-evaluative value of the faces (ΔLikeability_T3_T2), depicted over all conditions (upper half) and separate for the three conditions (lower half).

respect to arousal. Data showed that the changes in the arousal ratings due to conditioning resembled the pattern of the likeability ratings (i.e. increase for the positive condition and decrease for the negative condition), and did not, as expected based on the findings by Davis et al. (2010), reflect increased levels of arousal for the faces conditioned with the positive and negative social-evaluative sentences when compared to the neutrally-conditioned faces. Therefore, the arousal ratings will not be further considered; for reasons of completeness, they are available in *Supplemental Table S10.4*.

fMRI data

Brain activation at group level

Significant activation related to the contrasts of interest is summarized in Table 10.3 and illustrated in *Figure 10.3* (amygdala ROIs). These results confirmed the role of the amygdala during social-evaluative learning, previously described by Davis et al., (2010). In short, the ROI-analyses on the contrast 'all faces > baseline', 'negative > neutral', and 'negative > positive' revealed bilateral amygdala activation, while the contrast 'all faces early > all faces late' showed activation in the right amygdala. No amygdala activation was present for the contrast 'positive > neutral'. The latter contrast was therefore not further investigated in the endophenotype analysis.

Neurobiological candidate endophenotypes

Voxelwise regression analyses on the association between self-reported SA and amygdala activation related to viewing the conditioned stimuli (faces with a social-evaluative meaning) revealed significant positive associations within both the left and right amygdala (*Table 10.4; Figure 10.4*). The amygdala findings were replicated in a sensitivity analysis, in which data from participants with (comorbid) psychopathology other than SAD were excluded (*Supplemental Table S10.5; Supplemental Figure S10.3*). Within the right amygdala cluster, 22 voxels had at least moderate heritability (range: $h^2 = 0.20$ (moderate heritability) – 0.63 (high heritability)); in the left amygdala, only one voxel survived the threshold of $h^2 \geq 0.20$ (*Table 10.4*).

Analyses on the association with SA for the other contrasts of interest('all faces early >all faces late'; 'negative > neutral'; negative > positive') did not yield significant results within the amygdala.

Table 10.3 Brain activation independent from level of social anxiety.

Cluster	Region	*Z*-score	Peak coordinates (MNI space)			Cluster size
			x	y	z	
All faces > baseline						
Whole brain						
1	Occipital pole, fusiform gyrus	12.1	14	-94	2	81562
	Middle temporal gyrus	8.30	-62	-46	6	
	Middle frontal gyrus	9.90	-40	0	48	
	Orbitofrontal cortex	7.60	-46	28	-8	
	Amygdala, left	7.47	-20	-6	-14	
	Amygdala, right	6.27	22	-4	-18	
2	Caudate, right	4.93	16	8	8	397
Amygdala ROI						
1	Amygdala, left	7.47	-20	-6	-14	738
2	Amygdala, right	6.27	22	-4	-18	884
All faces early > all faces late						
Whole brain						
1	Occipital pole	4.77	16	-92	-4	2335
Amygdala ROI						
1	Amygdala, right	3.02	16	-4	-12	44
Negative > neutral						
Whole brain						
1	Anterior cingulate gyrus	4.35	8	44	8	963
2	Supramarginal gyrus, right	4.33	64	-40	8	513
3	Precentral gyrus, right	4.80	44	8	26	377
4	Cerebellum, left	3.81	-32	-86	-32	367
5	Superior temporal gyrus, right	5.04	44	-26	-2	347
Amygdala ROI						
1	Amygdala, left	3.87	-16	-8	-10	182
2	Amygdala, right	3.36	16	-12	-10	64
Negative > positive						
Whole brain						
1	Inferior frontal gyrus, right	4.36	50	16	20	572
Amygdala ROI						
1	Amygdala, left	3.37	-16	-10	-12	109
2	Amygdala, right	2.75	18	-4	-14	48
Positive > neutral						
Whole brain analysis						
1	Lingual gyrus, right	5.16	20	-66	-12	3876
	Lingual gyrus, left	4.63	-18	-74	-2	
	Lateral occipital cortex	4.59	30	-80	12	
Amygdala ROI		*No significant clusters*				

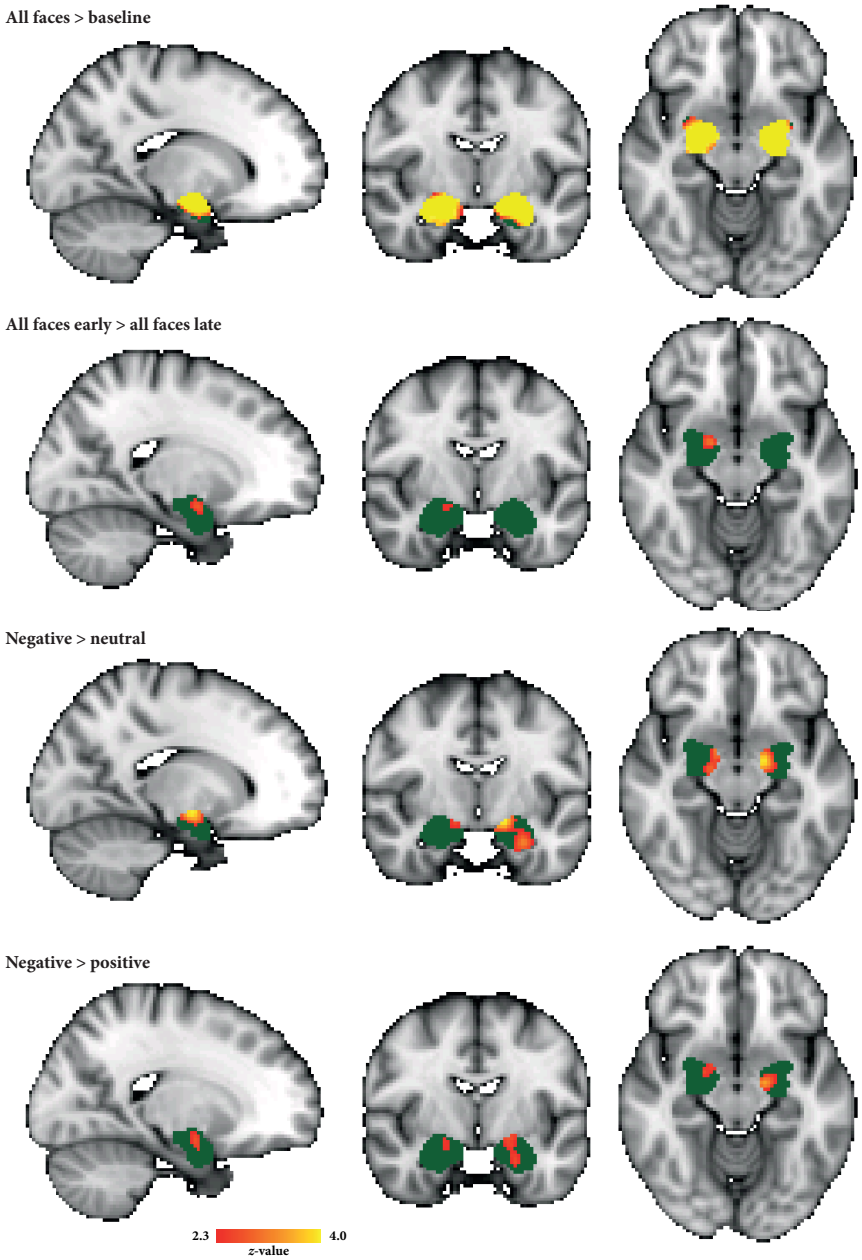


Figure 10.3 Amygdala activation (group level).
Activation related to contrasts of interest within the amygdala regions of interest (depicted in green), over the whole sample (n = 105). The contrast 'positive > neutral' did not yield significant amygdala activation. Coordinates displayed slices (MNI space, x,y,z): -16,-8,-12 (contrasts 'all faces > baseline' and 'negative > neutral') and 20,-6,-12 (contrasts 'all faces early > all faces late' and 'negative > positive'). Images are displayed according to radiological convention: right in the image is left in the brain.

Table 10.4 Effect of self-reported social anxiety on neutral face processing.

Region		*Z*-score	Peak coordinates (MNI space) x	y	z	Cluster size	Number of voxels with $h^2 > 0.20$	Mean h^2, range
All_faces > baseline								
Amygdala	Left	2.65	-28	-6	-14	36	1	0.27, n.a.
	Right	3.01	28	-10	-14	164	22	0.31, 0.20–0.63

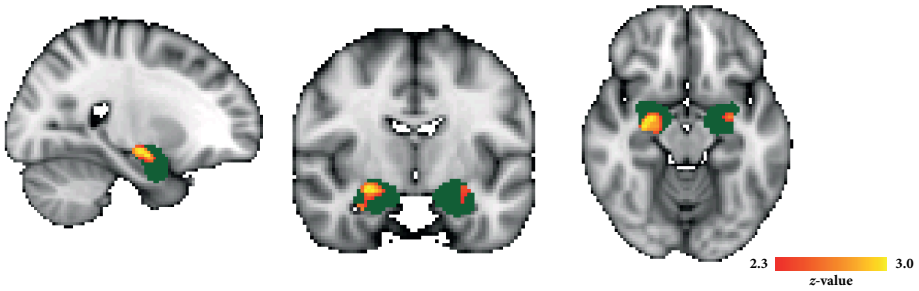


Figure 10.4 Association between social anxiety and brain activation in the amygdala.
Amygdala activation related to viewing faces conditioned with a social-evaluative meaning (versus baseline) co-segregates with social anxiety within families. Significant positive associations between social anxiety and activation were present in both the left (36 voxels) and right (164 voxels) amygdala.
Coordinates displayed slices (MNI space, x,y,z): 24,-8,-14. Images are displayed according to radiological convention: right in the image is left in the brain.

Discussion

Here, we demonstrated substantial evidence for amygdala hyperactivation, in response to faces conditioned with a social-evaluative meaning, as a putative neurobiological social anxiety disorder (SAD) endophenotype. Using a conditioning paradigm with high ecological validity in the context of SAD, in a unique sample of families genetically enriched for SAD (n = 105) (Bas-Hoogendam, Harrewijn, et al., 2018), we showed that amygdala reactivity *co-segregated with social anxiety within families of probands* (endophenotype criterion 4, first element); furthermore, multiple voxels within these amygdala clusters displayed at least moderate ($h^2 \geq 0.20$) *heritability* (endophenotype criterion 3). Thereby, we extend previous work on the role of the amygdala in SAD (see summary by (Bas-Hoogendam et al., 2016)), and offer novel insights into the genetic vulnerability to SAD.

Amygdala hyperreactivity during social-evaluative learning

The positive association between SA-level and amygdala activation to social-evaluative conditioned faces (conditioned stimuli, CS) confirmed our a priori prediction, which

was based on a previous neuroimaging study reporting increased SA-related amygdala activation during conditioning of socially threatening stimuli (Pejic et al., 2013). Here, we extend these findings, by using a paradigm which included three types of social evaluation (negative, neutral and positive; unconditioned stimuli, US), and demonstrated amygdala hyperreactivity within SAD patients as well as their family members.

Interestingly, although the analyses using other contrasts of interest, defined to determine amygdala activation during the course of the social-evaluative conditioning phase (SCP; contrast 'all faces early > all faces late') and related to the three different US conditions ('negative > neutral'; 'negative > positive'), revealed overall amygdala engagement at the group level, in line with the results of Davis and colleagues (2010), they did not yield significant associations with SA. These results suggest that the SA-related amygdala hyperreactivity seems not to differ between the first and last half of the social-evaluative conditioning phase (SCP), nor was this amygdala hyperreactivity specific for faces conditioned with negative, neutral or positive sentences, although we obviously cannot exclude that the lower statistical power inherent to these difference contrasts (i.e. containing less trials) limited us to detect significant effects of SA. We argue that these results reflect that the amygdala hyperreactivity in family members with high SA-levels is related to the social-evaluative context of the SCP, in which participants were directly addressed ('He says you are …'), rather than to the valence of the sentences (for example, 'He says you are boring' (negative), 'He says you are smart' (positive) or 'He says you are in Leiden'(neutral)). This idea is supported by the behavioral data, as these showed that family members with higher SA-levels rated all faces as less likeable after conditioning, independent from the value of the conditioning sentences (US). Together, these findings underscore the increased saliency of social information, being it negative, positive, or neutrally loaded, in social anxiety, which was present even without a cover story (note that we did not pretend that the faces belonged to 'real people' who did judge the participants in real-life; cf. (Harrewijn et al., 2018)).

The present results concur with contemporary models of social anxiety, acknowledging the multidimensional nature of the disorder (Reichenberger & Blechert, 2018). For example, as illustrated by a recent study, SAD patients displayed elevated scores on fear of negative evaluation as well as on fear of positive evaluation, combined with altered psychophysiological responses to negative as well as to positive social-evaluative videos (Reichenberger et al., 2019). Our findings support the view that social anxiety involves fear and avoidance of all potential social-evaluative interpersonal interactions (Miskovic & Schmidt, 2012), and emphasize that, although the fear of negative evaluation is especially prominent in SAD, the central fear in socially anxious individuals concerns the view that their self-characteristics are deficient or contrary to perceived societal expectations (Moscovitch, 2009). It is of importance to acknowledge this comprehensive fear in cognitive-behavioral therapy for SAD.

Furthermore, our results broaden the knowledge with respect to amygdala overreactivity in SAD. A recent meta-analysis indicated that SA is associated with increased amygdala

responsiveness related to face perception processing (Gentili et al., 2016), and it is commonly hypothesized that amygdala hyperreactivity is reflective of the heightened threat processing that characterizes SAD (Brühl, Delsignore, et al., 2014). Indeed, hyperactivation of the amygdala in response to socially-relevant stimuli has been repeatedly reported in SAD patients, as well as in children and adolescents with anxiety disorders (Blair, Geraci, Korelitz, et al., 2011; Ferri, Bress, Eaton, & Proudfit, 2014; Figel et al., 2019; Kraus et al., 2018;. Williams et al., 2015). However, to the best of our knowledge, the present results are the first demonstrating amygdala hyperreactivity in response to conditioned faces with a social-evaluative meaning, and the first to detect amygdala overreactivity within a sample of patients with SAD as well as their family members of two generations.

Co-segregation within families

The unique multiplex and multigenerational family-design of the LFLSAD enabled us to investigate two endophenotype criteria within the same sample, namely the *co-segregation within families* and the *heritability* of the candidate endophenotype. In addition to the association between amygdala hyperreactivity and the level of SA within the families, our data revealed that amygdala hyperactivation displayed moderate to even high heritability. Thereby, our results extend previous work reporting genetic influences on amygdala activation (cf. (Bas-Hoogendam et al., 2016)) and indicate that amygdala hyperreactivity is not just a biomarker of SAD (a characteristic associated with the disorder, which is not necessarily positioned on the pathway from genotype to phenotype; cf. (Beauchaine & Constantino, 2017; Lenzenweger, 2013a)), but reflective of the genetic vulnerability to SAD, thus providing a starting point for the development of preventive and therapeutic interventions (Beauchaine et al., 2008).

Amygdala function, structure and connectivity

In the present study, we used a mask of the extended amygdala, based on previous work using this paradigm (Davis et al., 2010), and in line with theories on the role of the extended amygdala in conditioning and treat processing (Fox, Oler, Tromp, et al., 2015; Shin & Liberzon, 2010). The amygdala consists of several subnuclei, being the laterobasal, centromedial, and superficial nucleus, with distinct connectivity patterns with other brain regions (Kerestes, Chase, Phillips, Ladouceur, & Eickhoff, 2017; Roy et al., 2009); furthermore, these connectivity patterns display different relationships with anxiety-related temperamental traits (Blackford et al., 2014; Roy et al., 2014).

According to a probabilistic atlas (Amunts et al., 2005), the hyperreactivity of the amygdala in the present study maps to the bilateral laterobasal nuclei. These nuclei receive information from sensory cortical regions, frontal brain areas and subcortical regions, and play a role in associative processing of environmental cues and the integration of this information with self-relevant cognition (Bzdok, Laird, Zilles, Fox, & Eickhoff, 2013). Future studies

could explore if there are SA-related changes in connectivity of these nuclei (cf. (Pannekoek et al., 2013; Prater et al., 2013)), and whether such alterations are heritable.

Furthermore, it is interesting to note that, in contrast to the consistent findings with respect to amygdala hyperactivation in SAD, findings on SAD-related alterations in amygdala structure are inconclusive (Brühl, Delsignore, et al., 2014). However, both a recent mega-analysis (Bas-Hoogendam, van Steenbergen, Pannekoek, et al., 2017) as well as a recent meta-analysis ((Wang et al., 2018) cf. the commentary by (Bas-Hoogendam, 2019)) did not report structural alterations in the amygdala in SAD patients, while we, in a previous study on the LFLSAD sample, did not detect SA-related differences in amygdala volume in socially-anxious families (Bas-Hoogendam, van Steenbergen, Tissier, et al., 2018b). Together, these findings suggest that alterations in amygdala function, rather than in its structure, are associated with SAD.

Limitations and future research

The LFLSAD was especially designed to investigate the endophenotype criteria of *co-segregation* and *heritability*. Longitudinal studies involving control families from the general population are essential to assess other endophenotype criteria, like the *trait-stability of the candidate endophenotype* (criterion 2) and the *difference between non-affected family members and participants from the general population* (criterion 4, second element). Furthermore, as the present work focused on the amygdala as an a priori defined, hypothesis-based region of interest, and we only performed an exploratory whole-brain analysis on the association with SA with a stringent statistical threshold, we might have missed functional SA-related alterations in other brain areas. For example, a recent study on reversal learning indicated that trait SA influenced learning rate-related activation of the dorsal anterior cingulate cortex (Piray, Ly, Roelofs, Cools, & Toni, 2018), while Blair et al. (2016) reported, besides amygdala hyperactivation, increased responsiveness of frontal and parietal cortices during social reference learning in SAD patients. Future studies could explore whether these regions display SA-related functional alterations during social conditioning as well.

In conclusion, the results of the present study provide evidence for bilateral amygdala hyperactivation in response to conditioned faces with a social-evaluative meaning as a candidate neurobiological SAD endophenotype. As such, these findings shed novel light on the genetic susceptibility to SAD.

Supplemental Methods

Participants

Recruitment and ethics

Families were recruited through media exposure, like interviews in Dutch newspapers, on television and radio; furthermore, the study was brought to the attention of patient organizations, to clinical psychologists, general practitioners and mental health care organizations. Recruitment was targeted at families in which multiple family members experienced 'extreme shyness' and took place between Summer 2013 and Summer 2015. Details about the screening and inclusion flow of the LFLSAD are provided in Bas-Hoogendam et al. (2018).

Both parents signed the informed consent form for their children, and children between 12 and 18 years of age signed the form themselves as well. Participants received a financial compensation. Confidentiality of the data was maintained by the use of a unique research ID number for each family member.

Phenotyping

The presence of DSM-IV diagnoses was determined using the Mini-International Neuropsychiatric Interview (M.I.N.I.)-Plus (version 5.0.0) (Sheehan et al., 1998; van Vliet & de Beurs, 2007) or the M.I.N.I.-Kid interview (version 6.0) (Bauhuis et al., 2013; Sheehan et al., 2010); these interviews were administered by experienced clinicians and recorded.

In addition to the clinical interviews and the self-report questionnaires on social anxiety (the Liebowitz Social Anxiety Scale (LSAS-SR) (Fresco et al., 2001; Mennin et al., 2002) or the Social Anxiety Scale for adolescents (SAS-A) (La Greca & Lopez, 1998)), participants completed several questionnaires on anxiety-related constructs.

The intensity of fear of negative evaluation was assessed using the revised Brief Fear of Negative Evaluation (BFNE) – II scale (Carleton et al., 2006; Leary, 1983).

Furthermore, the level of self-reported depressive symptoms was evaluated using the Beck Depression Inventory (BDI– II) (Beck et al., 1996; Van der Does, 2002) or the Children's Depression Inventory (CDI) (Kovacs, 1985; Timbremont & Braet, 2002).

The State-Trait Anxiety Inventory (STAI) (Spielberger et al., 1970) (see (Spielberger & Vagg, 1984) for psychometric properties) was used to determine self-reported trait anxiety, as well as state anxiety before and after the MRI scan.

The sensitivity for the temperamental traits 'behavioral inhibition' and 'behavioral activation' was assessed using the self-report BIS/BAS (Carver & White, 1994; Franken et al., 2005) or the BIS/BAS scales for children (BIS/BAS-C) (Muris et al., 2005).

Two subscales of the Wechsler Adult Intelligence Scale-IV (WAIS-IV) (Wechsler et al., 2008) or Wechsler Intelligence Scale for Children-III (WISC) (Wechsler, 1991), the

similarities (verbal comprehension) and block design (perceptual reasoning) subtests, were administered to obtain an estimate of cognitive functioning.

MRI experiment: detailed description

Prior to the MRI scan, participants were informed about the safety procedures and they were told that they could refrain from continuing the experiment at any time. Children and adolescents were familiarized with the MRI scanner using a mock scanner (Galván, 2010) and all participants received instructions about the task paradigms presented during the scan session. Scanning was performed using a 3.0 T Philips Achieva MRI scanner (Philips Medical Systems, Best, The Netherlands), equipped with a 32-channel Sensitivity Encoding head coil. The total duration of the MRI scan protocol was 54 min 47 s.

During the neutral faces paradigm, fMRI scans were acquired using T2*-weighted echo-planar imaging (EPI). Characteristics of these scans with the following characteristics: 38 axial slices, 2.75 mm x 2.75 mm x 2.75 mm + 10 % interslice gap, field of view (FOV) = 220 mm x 115 mm x 220 mm, repetition time (TR) = 2200 ms, echo time (TE) = 30 ms. The first six volumes of each fMRI scan were dummy volumes; these volumes were removed to allow for equilibration of T1 saturation effects.

In addition, a high-resolution EPI scan (84 axial slices, 1.964 mm x 1.964 mm x 2 mm, FOV = 220 mm x 168 mm x 220 mm, TR = 2200 ms, TE = 30 ms) and a high-resolution T1-weighted scan (140 slices, resolution 0.875 mm × 0.875 mm × 1.2 mm, FOV = 224 mm × 168 mm × 177.333 mm, TR = 9.8 ms, TE = 4.59 ms, flip angle = 8◦) were acquired. These scans were used for within-subject registration purposes; furthermore, the structural T1-scans were inspected by a neuroradiologist, but no clinically relevant abnormalities were present in any of the participants.

Neutral faces paradigm

Habituation phase

The first phase of the NFP paradigm, the habituation phase (HP), was inspired by the paradigm described by Wedig and colleagues (Wedig et al., 2005) and by several other paradigms on habituation (Blackford et al., 2013; Schwartz, Wright, Shin, Kagan, Whalen, et al., 2003; Schwartz, Wright, Shin, Kagan, & Rauch, 2003). This habituation phase started with the presentation of a fixation cross (24 s), followed by the presentation of three neutral faces. The faces were presented in blocks of 24 s, and within each block a neutral face was repeatedly presented (48 times) for 200 ms with a 300 ms interstimulus interval. There were six face blocks (two blocks for each face), in order to resemble the design described previously (Wedig et al., 2005), and face blocks were separated by the presentation of a fixation cross (duration 12 s). An additional 12 s fixation cross was presented at the end of the habituation phase. Faces were presented in pseudo-random order and participants were instructed to keep looking at the faces and the fixation crosses. Total duration of the habitu-

ation phase was 2 min 42 s. Results of the HP are reported elsewhere (Bas-Hoogendam, van Steenbergen, Blackford, et al., 2019).

Faces

We selected the following faces from the FACES database (Ebner et al., 2010): M049, M072 and M089 (faces of men; mean age: 24 y) and F069, F152 and F171 (faces of women; mean age: 25.7 y).

fMRI data

General processing steps

FMRI data were denoised using FIX (FMRIB's ICA-based X-noiseifier), a publicly available plugin for FSL (FMRIB Software Library, version 5.0.9) (Jenkinson et al., 2012), which provides an automatic solution for denoising fMRI data via accurate classification of ICA components (Griffanti et al., 2014; Salimi-Khorshidi et al., 2014). Next, data underwent several preprocessing steps using FEAT (FMRI Expert Analysis Tool; version 6.00) (Jenkinson et al., 2012; Smith et al., 2004), including motion correction using MCFLIRT (Jenkinson et al., 2002), spatial smoothing using a Gaussian kernel of full-width half-maximum (FWHM) 6.0 mm and grand-mean intensity normalization of the entire 4D dataset by a single scaling factor in order to enable higher-level analyses and registration. Scans were first registered to high-resolution EPI images, which were registered to T1 images, which in turn were registered to the Montreal Neurological Institute (MNI) T1-template brain (resolution 2 mm) using FNIRT nonlinear registration (warp resolution 10 mm) (Andersson et al., 2007; Jenkinson et al., 2002; Jenkinson & Smith, 2001). Next, ICA-AROMA (ICA-based Automatic Removal of Motion Artifacts) was used to remove motion-related artefacts (Pruim, Mennes, van Rooij, et al., 2015; Pruim, Mennes, Buitelaar, et al., 2015). Data were then submitted to FEAT to perform non-brain removal using BET (Smith, 2002), high-pass temporal filtering (Gaussian-weighted least-squares straight line fitting, with sigma = 30.0 s) and registration. Functional scans of each participant were registered to the individual 3D T1-weighted anatomical scan using FLIRT (Jenkinson et al., 2002; Jenkinson & Smith, 2001) and subsequently registered to the MNI T1-template brain (resolution 2 mm) using FNIRT nonlinear registration (warp resolution 10 mm) (Andersson et al., 2007). We checked whether the individual scans were registered correctly and confirmed that relative motion parameters did not exceed 2.5 mm.

Region of interest – amygdala mask

Masks for the amygdala were based on the Harvard-Oxford Subcortical Structural Atlas implemented in FSLview. We used a liberal threshold of 5 %, based on the findings by Davis et al. on the social conditioning paradigm (Davis et al., 2010): we transformed the coordinates of their results (reported in Talairach-space) to MNI space and chose the threshold of

our mask in such a way that the coordinates of their findings (in medial ventral amygdala, dorsal amygdala/substantia innominate and lateral ventral amygdala) were included in our ROIs. Furthermore, because of the laterality of the results reported by Davis and colleagues (2010), we used unilateral masks and investigated effects within the left and right amygdala separately. Masks are depicted in *Supplemental Figure S10.1.*

Sensitivity analysis

We performed a sensitivity analysis to examine whether the results of the association analyses (effect of self-reported social anxiety (*z*-score) on brain activation related to the contrast 'all faces > baseline') were driven by (comorbid) psychopathology other than SAD (cf. (Bas-Hoogendam, van Steenbergen, Tissier, et al., 2018b)). To this aim, we excluded all family members with past and / or present psychopathology other than SAD and repeated the association analysis. Note however, that this analysis may yield biased results, as the majority of the probands, on which the selection of the families was based, had comorbid psychopathology and were thus excluded. We used the same statistical threshold as for the main analyses (within the amygdala ROIs: $z > 2.3$, cluster-threshold $p < 0.05$).

Supplemental Results

Data availability

We collected MRI data from nine families ($n = 113$) (Bas-Hoogendam, Harrewijn, et al., 2018), but we had to exclude data from one family ($n = 3$ family members) as this family's proband was not able to participate in the MRI experiment due to an MRI contraindication. Due to technical problems, behavioral data were lost for two participants (behavioral sample: $n = 108$), while 110 imaging data sets were available for fMRI pre-processing and quality control. Two datasets could not be used due to an imaging artefact, while the relative motion parameters of three other participants exceeded 2.5 mm. As a result, 105 fMRI datasets were available for further analysis of brain activation related to the social conditioning phase. Furthermore, data on the presence of (sub)clinical SAD were lost for several family members.

Sample characteristics

We refer to *Supplemental Table S10.2* and *Supplemental Table S10.3* for detailed information about the sample. In line with the design of the study, participants originated from two generations, which differed significantly in age (behavioral sample: $\beta \pm SE = -30.3 \pm 0.7$, $p < 0.001$; fMRI sample: $\beta \pm SE = -30.1 \pm 0.7$, $p < 0.001$), but not in male / female ratio (behavioral sample: $\chi^2(1) = 0.57$, $p = 0.56$; fMRI sample: $\chi^2(1) = 0.76$, $p = 0.44$). In line with previous reports on this sample (Bas-Hoogendam, van Steenbergen, Tissier, et al.,

2019), family members with and without (sub)clinical SAD did not differ with respect to male / female ratio, age and estimated IQ. Groups did differ, however, in comorbidity rates: family members with (sub)clinical SAD were more often diagnosed with depression (past), dysthymia (present) and panic disorder. These differences were, however, only significant at an uncorrected significance level. Furthermore, family members with (sub)clinical SAD reported higher levels of fear of negative evaluation, more depressive symptoms, higher levels of trait anxiety and behavioral inhibition (BIS), as well as lower levels of behavioral activation (BAS).

Behavioral data

Ratings of arousal

Arousal ratings are summarized in *Supplemental Table S10.4*. As several participants indicated during debriefing that they struggled to interpret the arousal question correctly, results of these ratings will therefore not be further considered.

Behavioral candidate endophenotypes: sensitivity analysis

A sensitivity analyses, investigating the effect of SA-level on the difference in likeability between T1 and T2 (ΔLikeability_T2_T1) showed no significant association between these scores and social anxiety ($\beta \pm SE = -0.02 \pm 0.03$, $p = 0.51$), confirming that the effect of SA was specific for the SCP of the NFP.

fMRI data

Whole brain analyses on association with SA

For reasons of completeness, we investigated the association between SA-level and brain activation at the whole-brain level (cluster threshold $z > 3.1$, extent threshold $p < 0.05$), in addition to the ROI analyses within the amygdala. There were no significant clusters for the contrasts 'all faces > baseline', 'all faces early > all faces late', and 'negative > neutral'. For the contrast 'negative > positive', we found a positive relation between SA-level and brain activation in the right frontal pole (cluster size: 392 voxels, $p = 0.008$, max z-value 5.05, MNI coordinates (x,y,z) peak voxel: 24, 52, -14) (*Supplemental Figure S10.2*).

Sensitivity analyses

In the sensitivity analysis, we excluded all participants with past and/or present comorbid psychopathology other than SAD; this resulted in a sample of 58 participants, of which 14 in the (sub)clinical SAD group. Next, we repeated the association analysis with self-reported social anxiety as predictor (corrected for age, gender and level of depressive symptoms). These analyses confirmed the amygdala findings for the contrast 'all faces > baseline' (*Supplemental Table S10.5; Supplemental Figure S10.3).*

Supplemental Tables

Supplemental Table S10.1 Sentences included in the SCP.

Negative comments	Positive endorsements	Socially-neutral statements
He / she thinks you are lazy	He / she thinks you are active	He / she thinks you are in the MRI scanner
He / she thinks you are boring	He / she thinks you are nice	He / she thinks you speak Dutch
He / she says you are stupid	He / she says you are smart	He / she says you are in Leiden
He / she says you are greedy	He / she says you are generous	He / she says you are righthanded / lefthanded*

Footnote

* This sentence was adapted based on the scores on the Edinburgh Handedness Inventory (Oldfield, 1971).

Supplemental Table S10.2 Detailed characteristics of participants with and without (sub)clinical SAD: demographics and clinical information.

	Behavioral samplea		
	(Sub)clinical SAD (*n* = 39)	**No SAD (*n* = 63)**	**Statistical analysis**
Demographics			
Male / Female (n)	20 / 19	31 /32	$\chi^2(1) = 0.04$, $p = 0.84$
Generation 1 / Generation 2 (n)	19 / 20	27 / 36	$\chi^2(1) = 0.33$, $p = 0.56$
Age in years (mean ± SD)	30.3 ± 15.5	30.9 ± 15.4	β ± SE = -0.6 ± 3.1, $p = 0.85$
Estimated IQ (mean ± SD)	104.3 ± 12.2	105.7 ± 10.4	β ± SE=-2.2 ± 2.2, $p = 0.32$
Diagnostic information (*n*)			
Clinical SAD	17	0	$\chi^2(1) = 32.9$, $p < 0.001$
Depressive episode present	1	1	$\chi^2(1) = 0.2$, $p = 0.69$
Depressive episode past	12	9	$\chi^2(1) = 4.9$, $p = 0.03$
Dysthymia present	3	0	$\chi^2(1) = 5.4$, $p = 0.02$
Dysthymia past	1	1	$\chi^2(1) = 0.2$, $p = 0.65$
Panic disorder lifetime	5	2	$\chi^2(1) = 4.0$, $p = 0.05$
Agoraphobia present	3	2	$\chi^2(1) = 1.3$, $p = 0.26$
Agoraphobia past	0	2	$\chi^2(1) = 1.2$, $p = 0.28$
Separation anxiety	0	1	$\chi^2(1) = 0.8$, $p = 0.39$
Specific phobia	2	3	$\chi^2(1) = 0.02$, $p = 0.89$
Generalized anxiety disorder	1	0	$\chi^2(1) = 1.8$, $p = 0.18$
Obsessive-compulsive disorder	1	0	$\chi^2(1) = 1.8$, $p = 0.18$
Attention deficit hyperactivity disorder (ADHD)	3	1	$\chi^2(1) = 2.5$, $p = 0.11$
Alcohol dependency present	1	1	$\chi^2(1) = 0.2$, $p = 0.70$
Alcohol dependency lifetime	1	3	$\chi^2(1) = 0.2$, $p = 0.63$
Present psychotropic medication (*n*)	4	3	$\chi^2(1) = 1.1$, $p = 0.30$
Antidepressants, not otherwise specified	3	0	
ADHD medication, not otherwise specified	1	3	

Abbreviations

SD, standard deviation; SE, standard error.

Footnote

[a]: Due to technical reasons, data on the presence of subclinical SAD were lost for six family members. Data from these participants were, however, included in the endophenotype analyses using SA-level (*z*-score) as a predictor (behavioral sample: $n = 108$).

Supplemental Table S10.3 Detailed characteristics of participants with and without (sub)clinical SAD: scores on self-report questionnaires.

	Behavioral sample[a]		
	(Sub)clinical SAD (*n* = 39)	No SAD (*n* = 63)	Statistical analysis
Self-report measures			
Social anxiety symptoms (z-score; mean ± SD)	3.0 ± 3.3	0.5 ± 1.6	β ± SE = 2.6 ± 0.5, $p < 0.001$
Fear of negative evaluation (mean ± SD)	23.3 ± 12.3	12.9 ± 8.0	β ± SE = 10.4 ± 2.0, $p < 0.001$
Depressive symptoms (z-score; mean ± SD)	0.0 ± 0.9	-0.5 ± 0.7	β ± SE = 0.5 ± 0.2, $p < 0.001$
STAI – trait (mean ± SD)	38.8 ± 9.4	33.2 ± 8.5	β ± SE = 5.3 ± 1.8, $p = 0.003$
BIS (z-score; mean ± SD)	0.4 ± 1.3	-0.4 ± 0.9	β ± SE = 0.8 ± 0.2, $p < 0.001$
BAS (z-score; mean ± SD)	-0.9 ± 1.0	-0.6 ± 1.0	β ± SE = -0.5 ± 0.2, $p = 0.02$

Abbreviations
SD: standard deviation; SE: standard error.

Footnote
[a]: Due to technical reasons, data on the presence of (sub)clinical SAD were lost for six family members. Data from these participants were, however, included in the endophenotype analyses using SA-level (*z*-score) as a predictor (behavioral sample: $n = 108$).

Supplemental Table S10.4 Arousal ratings (*n* = 108).

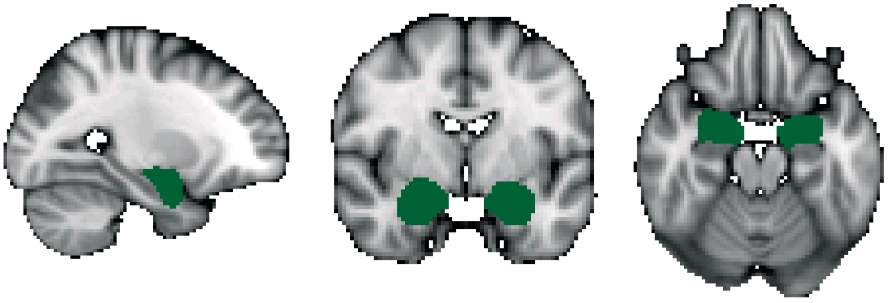
Condition	T1	T2	T3
Negative	3.1 ± 1.6	3.2 ± 1.6	2.9 ± 1.6
Neutral	2.9 ± 1.7	3.0 ± 1.7	3.1 ± 1.8
Positive	2.9 ± 1.5	2.9 ± 1.6	3.4 ± 1.8

Footnote
Values represent mean ± standard deviation.

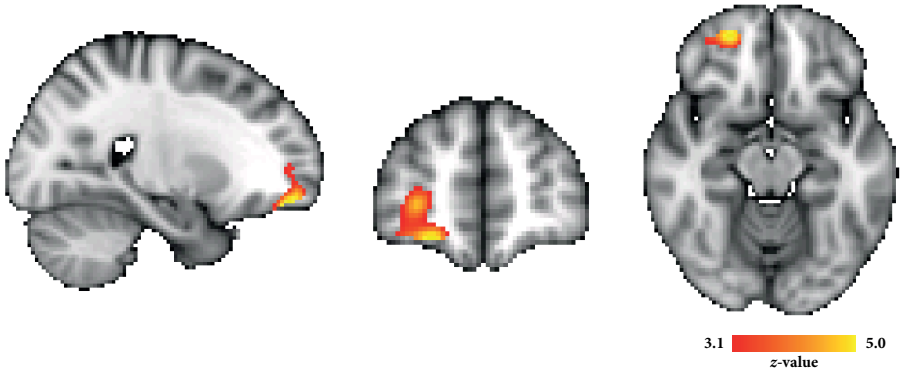
Supplemental Table S10.5 Sensitivity analyses in sample without (comorbid) psychopathology other than SAD.

			Peak coordinates (MNI space)			
Region		***Z*-score**	**x**	**y**	**z**	**Cluster size**
All faces > baseline						
Amygdala	Left	2.98	-24	-12	-18	38
	Right	4.61	26	-8	-14	161

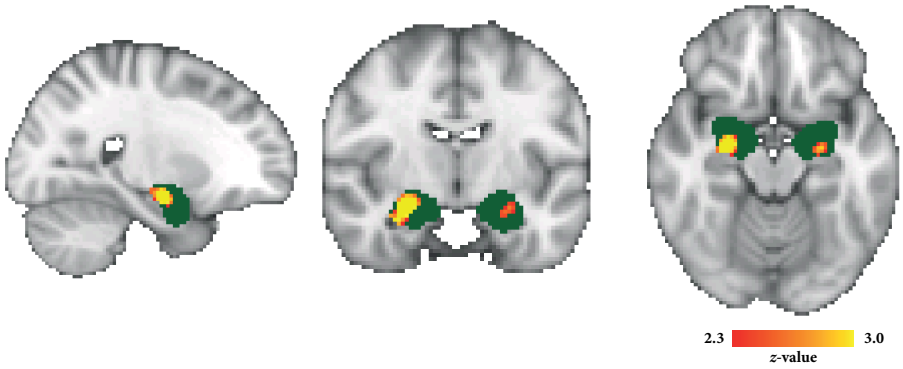
Supplemental Figures



Supplemental Figure S10.1 Mask of the amygdala regions of interest.
Coordinates displayed slices (MNI space, x,y,z): -26, -4, -20. Masks are displayed on the template MNI_T1_152_2mm_brain and images are displayed according to radiological convention: right in the image is left in the brain.



Supplemental Figure S10.2 Results whole-brain analysis on the relation between SA-level and brain activation (contrast 'negative > positive').
Positive association between SA-level and brain activation related to the contrast 'negative > positive' in the left frontal pole. Coordinates displayed slices (MNI space, x,y,z): 24, -52, -14.
Clusters are displayed on the template MNI_T1_152_2mm_brain and images are displayed according to radiological convention: right in the image is left in the brain.



Supplemental Figure S10.3 Results sensitivity analyses in sample without (comorbid) psychopathology other than SAD (*n* = 58).

Positive association between SA-level and brain activation related to the contrast ' all faces > baseline' in both the left and right amygdala. Coordinates displayed slices (MNI space, x,y,z): 24, -8, -16. Clusters are displayed on the temple MNI_T1_152_2mm_brain and images are displayed according to radiological convention: right in the image is left in the brain.

Part 4

Neurobiological SAD endophenotypes: summary and discussion

Chapter 11

Extremely Shy & Genetically Close -
what have we learned and how to proceed?

Extremely Shy & Genetically Close: the scope of this thesis in short

Social anxiety disorder (SAD) is a serious psychiatric condition, which typically evolves during late childhood and early adolescence (Beesdo-Baum et al., 2012; Haller et al., 2015; Miers et al., 2013, 2014; Wittchen & Fehm, 2003). Patients are '*extremely shy*': they are afraid of a negative evaluation by others and avoid social situations as much as possible, leading to significant adverse effects on important areas of functioning (American Psychiatric Association, 2013; Leichsenring & Leweke, 2017; Stein & Stein, 2008). As SAD is characterized by a chronic course, insight in the factors that make children and adolescents vulnerable to develop SAD is pivotal to get grip on the disorder and to prevent its lifelong negative consequences (Craske & Zucker, 2001; Knappe et al., 2010).

Previous work on SAD has identified several biological, psychological, and social factors that play a role in the development and the maintenance of SAD (Bas-Hoogendam, Roelofs, et al., 2019; Wong & Rapee, 2016). This thesis builds upon the results of family- and twin studies, which demonstrated that the genetic makeup of individuals is one of the contributing factors to the development of SAD: being '*genetically close*' to a patient with SAD leads to an enhanced risk to develop the disorder (Isomura et al., 2015; Merikangas et al., 2003; Stein, Chartier, Hazen, et al., 1998). Previous studies reported heritability estimates of SAD around 50 % (Bandelow et al., 2016), but little is known about the genetic variations underlying the susceptibility to SAD.

The work presented in this thesis aims to deepen our knowledge of the genetic vulnerability to SAD, by focusing on neurobiological endophenotypes as measured with structural and functional magnetic resonance imaging (MRI). Endophenotypes are measurable characteristics on the pathway from genotype to phenotype, and because of their intermediate position, they provide, once identified, a stepping stone for further investigation of the underlying genetic variations (Gottesman & Gould, 2003; Lenzenweger, 2013b). Here, we describe the main findings of the studies outlined in this thesis. Next, we integrate them into a graphical summary which reflects the genetic vulnerability to SAD, and highlight emerging patterns. Furthermore, we use this summary as a starting point to outline directions for future research. In addition, methodological and ethical characteristics of the Leiden Family Lab study on Social Anxiety Disorder are discussed.

Main findings

The endophenotype concept and the identification of endophenotypes in the Leiden Family Lab study on Social Anxiety Disorder

In order to begin the search for SAD endophenotypes with a clear picture of the concept, I started this thesis with a literature review on candidate endophenotypes of SAD, as described in *Chapter 2*. We summarized previous work on endophenotypes, which mentioned the following criteria for endophenotypes: 1st endophenotypes should be *associated with the disorder*; 2nd they are supposed to represent *stable, state-independent traits, which are already present in a preclinical state*; 3rd endophenotypes are *heritable*; 4th endophenotypes *co-segregate with the disorder within families of probands, with nonaffected family members showing altered levels of the endophenotype when compared to the general population*. Following these criteria, endophenotypes are more than just 'biomarkers' of a certain disorder: while a biomarker could be any measurable indicator that is associated with a particular disease, it does not necessarily have a genetic basis; endophenotypes, on the other hand, are by definition heritable and supposed to be reflective of genetically-based disease mechanisms (Lenzenweger, 2013a). So, as stated by Lenzenweger, '*all endophenotypes are biomarkers, but not all biomarkers are endophenotypes*' (2013a, page 187). Next, we outlined the value of applying the endophenotype approach to SAD, and explained how endophenotypes could aid in understanding disease mechanisms. Furthermore, we discussed that endophenotypes are useful to identify individuals at risk.

Following these considerations, we investigated which neurobiological measurements from MRI are potential candidate endophenotypes of SAD, by summarizing results of empirical research from various research fields. We described evidence supporting the potential of several neurobiological characteristics as SAD endophenotypes, namely the function and functional connectivity of the amygdala, the function of the medial prefrontal cortex, whole-brain functional connectivity and structural-anatomical brain changes.

These candidate endophenotypes were topic of investigation within the Leiden Family Lab study on Social Anxiety Disorder (LFLSAD). The background, design and methodology of this study are outlined in *Chapter 3*. The multigenerational (i.e. family members of two generations participated in the LFLSAD) and multiplex (i.e. families contained at least two (sub)clinical SAD cases) design of the LFLSAD was especially chosen to examine what case-control studies cannot determine, namely the *co-segregation of the candidate endophenotypes within families of probands* (first element of criterion 4) and the *heritability* of the candidate endophenotypes (criterion 3).

Structural brain characteristics as putative SAD endophenotypes

In the second part of this thesis, I focused on SAD-related changes in brain structure. We investigated gray matter characteristics in two different samples. In *Chapter 4*, the findings

of an international mega-analysis on the largest database of SAD structural T1-weighted 3T MRI scans to date are described. In this study, we examined whether gray matter volume was *associated with the disorder* (endophenotype criterion 1). We used voxel-based morphometry (VBM), a standardized method which estimates gray matter volume on a voxelwise basis. Results indicated that patients with SAD (n = 174) had increased gray matter volume in the putamen and pallidum in comparison to healthy participants (n = 213). Interestingly, this increase in putamen volume was positively related to the level of social anxiety symptoms in the patient group, which provides additional support for the endophenotype criterion of *association with the illness*. Taken together, these findings indicate that gray matter volume in the dorsal striatum is a biomarker of SAD.

Building upon this work, we next explored whether gray matter characteristics could also be considered candidate endophenotypes of SAD, by using data from the LFLSAD sample (*Chapter 5*). In this sample, we tested two other endophenotype criteria: the *co-segregation of social anxiety with the gray matter characteristics within the families,* and the *heritability* of the candidate endophenotypes. At the time of the analysis, the complex family design of the LFLSAD precluded performing a whole-brain VBM analysis, like we did in the mega-analysis. Therefore, we used a different approach to investigate gray matter characteristics, and estimated 1st the *volumes* of subcortical structures, 2nd the *thickness* of cortical brain areas, and 3rd the *surface area* of cortical regions, by using the automated software pipeline of the FreeSurfer program. We restricted our analyses to regions on which effects of SAD had been previously reported. Results confirmed the positive association between volume of the pallidum and social anxiety, this time within families genetically enriched for SAD, and revealed that pallidum volume was moderately heritable. Furthermore, several cortical gray matter characteristics, extracted from frontal, parietal and temporal regions, *co-segregated with social anxiety within the families* and had moderate to high *heritability*. So, although it should be noted that the association results did not survive correction for the number of statistical tests, the findings of this study provide preliminary evidence that gray matter characteristics of various brain regions are candidate SAD endophenotypes.

Functional brain characteristics as putative SAD endophenotypes

Part three of the present work addressed functional brain alterations associated with SAD. Previous work, as reviewed by Brühl and colleagues (Brühl, Delsignore, et al., 2014) and Cremers & Roelofs (2016), indicated an association between SAD and hyperactivation of subcortical, frontal, parietal and occipital brain areas. In the majority of the studies, this overreactivity was evoked by functional paradigms addressing specific SAD-related fears. In the LFLSAD, we employed two functional paradigms, each targeted at specific neurocognitive components of SAD, and examined evidence for brain activation, as measured with fMRI, as candidate endophenotypes of SAD.

Processing unintentional social norm violations

The first paradigm, the Social Norm Processing Task (SNPT), taps into the fear of socially-anxious individuals that they will unintentionally break a social norm in the presence of others, and focuses on the function of the medial prefrontal cortex. In this paradigm, three different types of stories on social situations are presented, which enables investigating the behavioral and neurobiological correlates of processing intentional and unintentional social norm violations (Berthoz et al., 2002). Building upon previous versions of the SNPT (Berthoz et al., 2002, 2006; Blair et al., 2010), we created a revised version of the paradigm (SNPT-R) which allows for using the paradigm in participants of different ages (from age 8); furthermore, we incorporated some methodological improvements. In *Chapter 6,* we described the results of a validation study of the SNPT-R, which we performed in two samples of healthy adolescents and adults. Participants rated the stories differently, depending on the intention underlying the social norm violation: intentional social norm violations were considered as more inappropriate and more embarrassing when compared to unintentional social norm violations. Furthermore, fMRI data revealed both overlapping as well as differential brain activation patterns for reading intentional and unintentional social norm violations.

In a follow-up study on this sample, we explored the relationship between self-reported social anxiety and ratings of inappropriateness and embarrassment related to the different types of stories (*Chapter 7*). In line with our hypotheses, which were based on previous work on the SNPT in patients with SAD (Blair et al., 2010), we found a positive relationship between social anxiety and the ratings, with the most pronounced effect for the embarrassment ratings of the unintentional social norm violations: while individuals with low-to-intermediate social anxiety levels rated the unintentional social norm transgressions as less embarrassing when compared to the intentional social norm transgressions (i.e., these individuals make a distinction between breaking conventional rules, *by intention*, and committing a blunder, *unintentionally*, when they rate the stories on embarrassment), individuals with high social anxiety levels consider unintentional social norm violations as equally embarrassing as intentional social norm transgressions. We suggest that this increased experience of embarrassment, which often represents a negative self-evaluation, plays a role in the development and maintenance of SAD.

In the next chapter, we described the results of the SNPT-R within the LFLSAD (*Chapter 8*). Based on our own study and previous work on the SNPT in SAD patients, which demonstrated increased embarrassment accompanied by increased activation in the medial prefrontal cortex, in response to unintentional social norm violations (Blair et al., 2010), thus supporting the *association with the disorder* (endophenotype criterion 1), we tested the hypothesis that the neurobiological and behavioral correlates of processing unintentional social norm transgressions could serve as endophenotypes of SAD. In line with the approach described in *Chapter 5*, we investigated the *co-segregation of the candidate endo-*

phenotypes with social anxiety within the families and estimated their *heritability.* Indeed, the fMRI data revealed that brain responses to unintentional social norm violations, in the medial prefrontal cortex and in a cluster encompassing the medial temporal gyrus, superior temporal gyrus and superior temporal sulcus, were *positively related with levels of social anxiety within the families* of the LFLSAD; furthermore, these brain activation levels were at least moderately *heritable*. Our hypothesis with respect to the ratings of embarrassment was partly supported: while we found a positive correlation between social anxiety and embarrassment, this effect was not specific for the unintentional condition of the SNPT-R, and heritability estimates of these ratings were low or even absent. In sum, the results of this study provided evidence for hyperactivation in the medial prefrontal cortex and temporal brain regions, in response to unintentional social norm violations, as putative SAD endophenotypes.

Processing neutral faces

The second fMRI paradigm within the LFLSAD concerned the processing of faces with a neutral expression, as these are strong social stimuli with an ambiguous meaning; this paradigm focused on the function of the amygdala, a key structure in emotional processing. We created the Neutral Faces Paradigm (NFP) to explore brain activation related to two different aspects of processing neutral faces. In the first phase of the NFP, the habituation phase (HP), we tested whether impaired habituation to neutral faces (i.e. the adaptive decline in brain activation to a stimulus which is presented multiple times without meaningful consequences) could be considered a candidate endophenotype of SAD. This hypothesis was based on previous research on individuals with inhibited temperament, which is an important risk factor for the development of social anxiety, and in participants with high levels of social fearfulness (Avery & Blackford, 2016; Blackford et al., 2013), reporting failed habituation within these groups. Results of our study, described in *Chapter 9*, revealed that the neural habituation response, in the right hippocampus and amygdala, was impaired in family members with high levels of social anxiety, providing support for the endophenotype criterion of *co-segregation within the families*. Subsequent *heritability* analyses revealed that the neural habituation response within the right hippocampus was at least moderately heritable. Taken together, these findings indicate that altered neural habituation in the hippocampus is a putative SAD endophenotype.

The second phase of the NFP concerned the social-evaluative conditioning of the faces. By consistently pairing three neutral faces with social-evaluative sentences with a positive ('He says you are smart'), negative ('He says you are stupid') or neutral ('He says you are in Leiden') content, participants learned the social-evaluative value of each face. Previous work on this paradigm indicated amygdala engagement during this learning process (Davis et al., 2010), but the relation between amygdala activation related to this social-evaluative conditioning paradigm (SCP) and social anxiety has not been investigated, let alone within

families genetically enriched for SAD. In *Chapter 10*, I outline a study in which we investigated amygdala functioning related to social-evaluative conditioning in the families of the LFLSAD. Our data indicated bilateral amygdala hyperactivation to faces conditioned with a social-evaluative meaning, which *co-segregated with social anxiety within the families*, and displayed at least moderate *heritability*. Interestingly, this amygdala hyperreactivity was present for all conditions of the SCP, indicating that being directly addressed ('He says you are…') strongly activates the amygdala in socially-anxious family members, independent from the context of the evaluation. In sum, these results provide evidence for amygdala activation in response to faces with a learned social-evaluative meaning as a neurobiological candidate endophenotype of SAD.

Integrative graphical summary of the neurobiological genetic susceptibility to SAD

Based on the findings described above, I created a graphical summary of the neurobiological genetic susceptibility to SAD. This summary, depicted in *Figure 11.1,* outlines structural and functional brain alterations which, based on data of the LFLSAD, meet the criterion of *co-segregation with social anxiety within families of probands* and display at least moderate *heritability* ($h^2 \geq 0.20$), and could therefore be considered as candidate endophenotypes of SAD (brain regions with bright colors). Furthermore, null findings with respect to the analyses with respect to brain structure (cf. *Chapter 5*) are depicted (areas in gray); regions that were not specifically investigated in the present work are shown in white. Although it is important to stress that this summary reflects 'work in progress', as will be discussed more extensively later in this section, I want to highlight several interesting patterns.

Multiple brain regions are implicated in the genetic vulnerability to SAD

First of all, this summary illustrates that the brain characteristics related to the genetic vulnerability to SAD are spread over the brain, as they involve subcortical, frontal, parietal and temporal regions (*Figure 11.1*). It is interesting to note that these regions, whose function and / or structure qualifies as candidate SAD endophenotype, are to a great extent in line with the regions summarized in the neurobiological model of SAD, which was proposed by Brühl and colleagues a couple of years ago (Brühl, Delsignore, et al., 2014). This model, based on a qualitative review and meta-analysis of 76 neuroimaging studies on adult patients with SAD, described SAD-related changes in brain function in subcortical, frontal, parietal and occipital areas, as well as alterations in the connections between these regions. Interestingly, while Brühl et al. (2014) extended an older neurobiological model outlined by Etkin and Wager, describing functional alterations in the so-called 'fear circuit' (amygdala, parahippocampal gyrus, globus pallidus, insula, inferior frontal gyrus), as well as in the

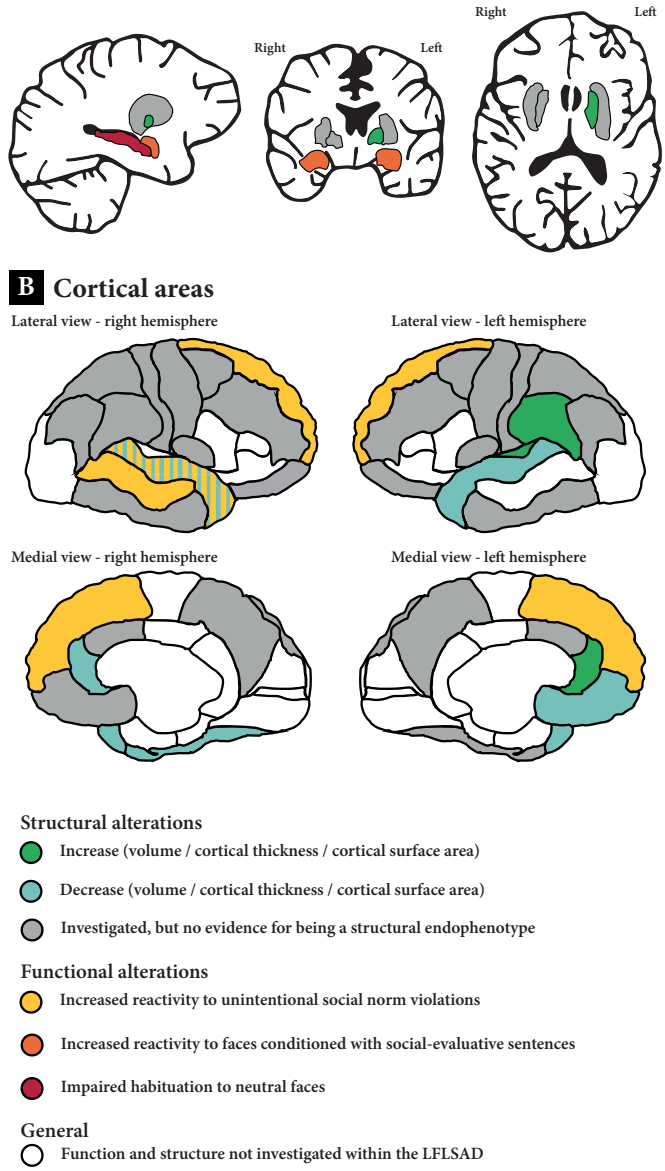


Figure 11.1 Graphical summary of neurobiological endophenotypes of SAD, as revealed by data from the LFLSAD.

Regions in *bright colors* indicate areas in which brain function and / or structure co-segregates with social anxiety within families of probands, and which display at least moderate heritability. Regions in *gray* represent null findings with respect to structural endophenotypes, while regions depicted in *white* were not specifically investigated.

fusiform gyrus and superior temporal gyrus (Etkin & Wager, 2007), we now extend the model by Brühl et al. by providing insight in the SAD-related brain characteristics which are not just biomarkers (i.e. associated with the disorder, but not necessarily located on the causal pathway from genotype to phenotype), but qualify, based on the results of the LFLSAD, as candidate endophenotypes, and are as such thought to be part of the neural mechanisms that translate genetic effects into disorder phenotypes (Meyer-Lindenberg & Weinberger, 2006). This distinction is important, as it implies that the brain alterations summarized in *Figure 11.1* reflect the genetic vulnerability to *develop* SAD and are not the *result* of the (often chronic) course of the disorder, nor could they be attributed to the effects of psychological treatment, psychotropic medication, or comorbid psychopathology (Beauchaine & Constantino, 2017; Lenzenweger, 2013a). As such, our findings indicate that SAD is a multi-circuit brain disorder already at the level of the endophenotype.

The dorsal striatum: a new player in anxiety research

Second, special attention needs to be paid to the dorsal striatum, including the pallidum and putamen. This subcortical brain area has received increasingly more attention in the field of anxiety research, but was not yet part of the neurobiological model by Brühl et al. (2014). In two separate studies, being a mega-analysis on a large international dataset of patients with SAD as well as healthy control participants (described in *Chapter 4),* and an endophenotype study within families genetically enriched for SAD (*Chapter 5),* we found positive associations between social anxiety and gray matter volume of this region; furthermore, these alterations *co-segregated* with social anxiety within families and were moderately *heritable*. Recently, these findings were replicated in two other samples with relevance for SAD. First of all, a neuroimaging study on healthy participants demonstrated a robust positive correlation between the concept 'intolerance of uncertainty' and striatal volume (Kim et al., 2017), while a study on healthy women demonstrated that socially anxious tendencies were associated with an enlarged striatum (Günther et al., 2018). Interestingly, a recent study on the common underlying structural brain alterations across four psychiatric disorders, including hundreds of patients with depression, post-traumatic stress disorder, obsessive-compulsive disorder and schizophrenia, as well as a small number of unaffected first-degree relatives, reported strong evidence for putamen enlargement as a transdiagnostic marker of the familial vulnerability to psychopathology (Gong et al., 2019). Our findings concur with this result, not only with respect to the involvement of the dorsal striatum in psychopathology, but also in light of the genetic susceptibility to develop psychopathology; however, these observations also question the specificity of striatal enlargement as an endophenotype for SAD. Nevertheless, as outlined by Cannon and Keller (2006), specificity is not a prerequisite for an endophenotype, as particular endophenotypes could predispose for multiple anxiety and mood disorders. In line with this reasoning, I propose that the findings with respect to striatal enlargement are reflective of the shared genetic background of anxiety disorders,

depression and related phenotypes (Cannon & Keller, 2006; Ohi, Otowa, Shimada, Sasaki, & Tanii, 2019; Shimada-Sugimoto, Otowa, & Hettema, 2015); cf. a recent analysis showing a high degree of genetic correlation among psychiatric disorders (Anttila et al., 2018).

The idea of striatal enlargement as a transdiagnostic feature is corroborrated by a recent review, highlighting the important role of the striatum in three behavioral processes that are very relevant in psychopathology, as these processes include 1st attention, 2nd conditioning, and 3rd motivation (Lago et al., 2017). Furthermore, a large (> 30 000 MRI scans) genome-wide association study revealed several genetic variants influencing variation in putamen volume; intriguingly, these genetic variants were thought to affect developmental pathways such as apoptosis, axon guidance and vesicle transport, and, as suggested by the authors, could therefore aid in determining mechanisms of neuropsychiatric disorders (Hibar et al., 2015). Taken together, I feel the role of the dorsal striatum in anxiety, both with respect to its structure as well as its function, deserves attention in future research on the genetic vulnerability to psychopathology in general, and social anxiety in particular.

A hyperactive emotional brain

Another striking point, as well as a similarity between our summary of the neurobiological genetic susceptibility to SAD (*Figure 11.1*) and the model by Brühl et al. (2014), is the fact that both models only describe *increases* in brain reactivity. We found increased amygdala activation in response to faces conditioned with social-evaluative sentences (*Chapter 10),* increased brain responses in the medial prefrontal cortex and medial and superior temporal gyrus to unintentional social norm transgressions (*Chapter 8*), as well as a prolonged reactivity of the hippocampus and amygdala in response to neutral faces *Chapter 9*); the meta-analysis underlying the neurobiological model of Brühl et al. (2014) showed increased activation in the regions of the fear circuit (including, among others, the amygdala and prefrontal cortex), as well as in parietal and medial occipital brain regions.

In the discussion of their neurobiological model of SAD, Brühl and colleagues attribute the hyperactivation in the fear circuit to the increased levels of arousal and negative valence, and an overall exaggerated response of the emotional system in SAD, while they propose three hypotheses with respect to the increased responsiveness of prefrontal areas, based on previous work (Brühl, Delsignore, et al., 2014). As prefrontal areas are generally implicated in emotion regulative functions (Buhle et al., 2013), the hyperactivation in these regions could reflect either attempts of these areas to down-regulate the hyperactive limbic system, or indicate activity related to reinterpretation emotion regulation strategies (cf. (Phan et al., 2005; Picó-Pérez, Radua, Steward, Menchón, & Soriano-Mas, 2017)). A third hypothesis, originally suggested by Robinson and colleagues (Robinson, Charney, Overstreet, Vytal, & Grillon, 2012) and highlighted by Brühl et al. (2014), states that the increased prefrontal responsiveness is driven by the increased activity in the amygdala and is actual the result of increased functional coupling in the prefrontal - amygdala-circuit during aversive process-

ing in pathological anxiety (cf. (Robinson et al., 2014)). At present, convincing support for one of these hypotheses is lacking, as studies on this matter in SAD are scarce and inconsistent (Brühl, Delsignore, et al., 2014).

With respect to the heightened responsiveness of the parieto-occipital regions, Brühl et al. hypothesize that this activity is reflective of the increased attempts of the regulatory parietal areas to decrease activation within the fear circuit, as the parietal hyperactivation is predominantly found in studies on emotion regulation (Brühl, Delsignore, et al., 2014). In the LFLSAD, we did not find alterations in parietal function, which could possibly be attributed to the fact that our functional paradigms did not involve specific emotion regulation tasks. Future work, involving emotion regulation paradigms in families genetically enriched for SAD, is essential to determine whether parietal hyperactivation qualifies as a candidate endophenotype of the disorder, or is merely a biomarker associated with disease state.

Despite the attention for these SAD-related increases, it is important to mention that several previous neuroimaging studies reported decreases in brain response in SAD patients, related to various tasks. For example, Sareen and colleagues demonstrated reduced activation related to implicit learning in the caudate, insula and inferior parietal lobe (Sareen et al., 2007), while another study found decreased responsiveness of the left orbitofrontal cortex in SAD patients during the anticipation of emotional stimuli (Brühl et al., 2011). However, these changes did not survive the threshold for statistical significance in the meta-analysis underlying the neurobiological model by Brühl et al (2014), nor did we find SAD-related decreases in brain activation within the LFLSAD, although it should be noted that we did not employ the specific task paradigms used in the studies by Brühl et al. (2011) and Sareen et al. (2007).

Work in progress and directions for future research

This brings us to an important general remark with respect to the summary presented in *Figure 11.1*. As stated earlier, I want to emphasize that this overview of the neurobiological genetic susceptibility to SAD is not yet complete and needs to be complemented by further work. In the following, I will outline four lines of future research which are essential in this respect, and focus consecutively on the potential of using other neurocognitive paradigms, the investigation of brain connectivity, and outstanding questions with respect to the stability of the candidate endophenotypes. Furthermore, I will consider how the endophenotype data of the LFLSAD could be used as a starting point in subsequent studies into (epi)genetic risk variants for SAD.

The use of new neurocognitive paradigms

To start, several important neurobiological alterations which have previously been *associated with SAD* (endophenotype criterion 1) still need to be investigated using an endophenotype approach. That is, while time-constraints within the LFLSAD MRI protocol only allowed for the inclusion of two functional MRI paradigms, which were carefully chosen based on their relevance for the social anxiety phenotype and the promising results of earlier work as summarized in *Chapter 2* (cf. *Table 2.2*), work by other researchers in the field has provided evidence for multiple other neurocognitive SAD-related alterations in brain function.

For example, recent fMRI studies using novel paradigms in the context of SAD have revealed altered functional responses in specialized sensory brain areas underpinning the general processing of human voices and faces (Kreifelts et al., 2019), increased phasic activation in the bed nucleus of the stria terminalis and central amygdala during the anticipation of aversive events (Figel et al., 2019), continued increased reactivity of the amygdala, temporo-parietal junction and insula in response to task-irrelevant social distractors during a performance task (Kim et al., 2018), and differential activation in prefrontal areas in response to social and negative feedback (Peterburs, Sandrock, Miltner, & Straube, 2016). In addition, the already mentioned study by Sareen et al. (2007) revealed reduced striatal activation in implicit sequence learning in SAD, while Brühl and colleagues (2011) reported on altered brain activation in the orbitofrontal cortex (decreased activation), thalamus, amygdala and temporo-occipital and parietal areas (increased activation), during the anticipation of non-specific, general emotional stimuli.

This selection of recent work on SAD biomarkers indicates that SAD is associated with functional brain alterations in regions which were not specifically investigated within the LFLSAD; furthermore, these findings pinpoint that different neurocognitive paradigms could evoke different functional changes within the same regions. So, the functional alterations depicted in Figure *11.1* are *specific* for the paradigms employed in the LFLSAD, and do not automatically reflect the responsiveness of these regions in SAD in *general*, independent from the context (cf. a recent study indicating that the enhanced amygdala response in SAD seems to be specific to socially-relevant stimuli rather than to aversive stimuli in general (Kraus et al., 2018)). In other words, the summary in *Figure 11.1* is not complete with respect to the neurocognitive functions involved, and the regions implicated; future studies are needed to explore whether the functional alterations, demonstrated by other research groups and mentioned above, are not only biomarkers of SAD, but meet the additional criteria for being endophenotypes as well.

Examination of brain connectivity

Another topic of future investigation is whether changes in the connectivity of the socially-anxious brain meet the criteria for being candidate endophenotypes. As described in *Chapter 1* and *Chapter 2,* brain connectivity can be determined by outlining the density

of white matter tracts between brain regions using diffusion tensor imaging (structural connectivity), or by detecting correlations in brain activation patterns across regions using fMRI (functional connectivity) (Fornito & Bullmore, 2015). Within the LFLSAD, data to establish both types of connectivity were collected (*Figure 1.1*); these data are currently analyzed and not part of this thesis.

Investigating brain connectivity is important, as regions in the brain do not function in isolation, but are tightly connected and part of large-scale networks; moreover, changes in connectivity could play a role in the development, expression and course of psychopathology (Bassett & Sporns, 2017; Bassett, Xia, & Satterthwaite, 2018; Buckholtz & Meyer-Lindenberg, 2012; Morgan, White, Bullmore, & Vértes, 2018; Sylvester et al., 2012). Furthermore, genetic influences on connectivity are repeatedly established (Thompson et al., 2013) and microscale alterations, for example in gene expression, are thought to underlie macroscale networks (Scholtens & van den Heuvel, 2018). Moreover, a recent study indicated that functional brain networks have unique characteristics for each individual, which are stable over months to years (Horien, Shen, Scheinost, & Constable, 2019). Together, these observations provide support for the endophenotype criteria of *association with the disorder, trait-stability over time* and *heritability,* suggesting that indices of connectivity have good potential to qualify as candidate endophenotypes.

To the best of our knowledge, no study to date has explored brain connectivity as a candidate endophenotype of SAD, although several studies revealed alterations in structural and functional connectivity associated with the disorder (cf. the discussion on this topic in *Chapter 2*). Most consistent findings concern reduced white matter integrity of the uncinate fasciculus, the white matter tract between the amygdala and frontal cortices (Baur et al., 2011; Baur, Brühl, et al., 2013; Phan et al., 2009), as well as alterations in functional connectivity within the default-mode network (Gentili et al., 2009; Liao, Chen, et al., 2010) and in prefrontal, limbic and subcortical networks (Arnold Anteraper et al., 2014; Manning et al., 2015; Pannekoek et al., 2013; Yang et al., 2019). With respect to the amygdala, task-dependent changes in amygdala connectivity have also been reported (Minkova et al., 2017), as well as changes in connectivity due to treatment (Brown et al., 2019; Young et al., 2017). Furthermore, a meta-analysis on > 800 individuals with different levels of anxiety or anxiety disorders investigated intra- and inter-network functional connectivity, and revealed hypo-connectivity between the executive control network (consisting of the dorsolateral prefrontal cortex, inferior parietal lobe and dorsomedial prefrontal cortex) on the one hand and the affective network (including, among others, the amygdala) and default mode network on the other hand; in addition, hypo-connectivity within the salience network (including the anterior insula and dorsal anterior cingulate cortex) was associated with anxiety and anxiety disorders (Xu et al., 2019).

Again, in line with the discussion with respect to striatal volume earlier in this chapter, it should be noted that these findings are probably not specific for (social) anxiety. A

meta-analysis on white matter integrity in SAD, depression, bipolar disorder, obsessive-compulsive disorder and post-traumatic stress disorder revealed transdiagnostic reductions in white matter integrity (Jenkins et al., 2016), while a study on a large sample of twins oversampled for psychopathology showed nonspecific changes in white matter related to a general transdiagnostic psychopathology factor, as well as changes which were associated with internalizing and externalizing factors (Hinton et al., 2019). Furthermore, two recently published papers provided evidence for transdiagnostic alterations in functional connectivity in networks underlying cognitive performance (Sha, Wager, Mechelli, & He, 2019) and networks supporting executive control and self-referential processes (Elliott, Romer, Knodt, & Hariri, 2018). Therefore, endophenotype studies dedicated to SAD, as well as large-scale transdiagnostic studies on the connectivity of the human brain are needed to explore which alterations in brain connectivity increase the genetic vulnerability to social anxiety and internalizing psychopathology in general.

Candidate endophenotypes: subject to change?

The LFLSAD was designed as a cross-sectional study, in which participants were measured only once. As a result, we were not able to investigate whether the candidate endophenotypes depicted in *Figure 11.1* remain *stable* over time (endophenotype criterion 2; *Figure 2.2*). Furthermore, although we corrected our analyses for the effect of age, we were not able to investigate specific age-related trajectories of change with respect to brain structure and brain function, nor were we, given the relatively small number of adolescents (the MRI sample contained 41 participants in the age-range 8 - 21 years) able to focus on the complex changes taking place during adolescence. These are however, important issues. Therefore, I argue that longitudinal studies, in which participants are repeatedly investigated, could provide valuable knowledge on outstanding questions concerning the stability of the endophenotype, and on the interaction between developmental changes and neurobiological alterations underlying the risk for developing SAD (cf. the recent statement paper by Haller, Mills, Hartwright, David, & Cohen Kadosh (2018)). In the following, I will briefly reflect upon these matters.

As discussed in more detail in *Chapter 2,* endophenotypes are, given their genetically-based origin, supposed to be *trait-characteristics which are already present in a preclinical state*. This does, however, not necessarily imply that endophenotypes could not change over time; previously, we have argued that endophenotypes can become more prominent in case of clinical SAD, and that their expression can be lower in patients with SAD who are successfully treated (*Chapter 2;* Bas-Hoogendam et al., 2016). Future longitudinal studies, involving patients with SAD as well as studies in participants with varying levels of social anxiety, are needed to investigate the within-subject correlation between the level of social anxiety symptoms and the expression of the candidate endophenotypes. In addition, treatment studies are essential to determine whether the candidate SAD endophenotypes are

useful targets for therapeutic or even preventive interventions, for example using cutting-edge techniques which enable altering the function of specific brain regions, like real-time fMRI-based neurofeedback (Brühl, Scherpiet, et al., 2014; Cohen Kadosh & Staunton, 2019; Herwig et al., 2019; Sitaram et al., 2016) and non-invasive brain stimulation (Hallett, 2000; Hoogendam, Ramakers, & Di Lazzaro, 2010; Nitsche et al., 2008; Vicario, Salehinejad, Felmingham, Martino, & Nitsche, 2019).

Another relevant question which could be addressed using a longitudinal design concerns the influence of development on the candidate endophenotypes. The typical age of onset of SAD is during late childhood and early adolescence, a period of time in which major dynamic changes in the brain take place (Casey, Jones, & Hare, 2008; Paus, Keshavan, & Giedd, 2008). Such changes include, among others, structural and functional alterations in regions that were investigated in this thesis. That is, previous work has provided ample evidence that essential parts of the social-affective brain, for instance perceptual brain areas (i.e. temporal regions involved in the processing of social stimuli), executive systems (including prefrontal areas) and regions involved in affect and motivation (amygdala, striatum) undergo major changes during the transition from childhood to adulthood (Blakemore, 2008; Crone & Dahl, 2012; Mills, Lalonde, Clasen, Giedd, & Blakemore, 2012; Nelson, Jarcho, & Guyer, 2015). Furthermore, changes in brain connectivity, with regional differences in maturation, have been demonstrated in typically developing individuals aged between 7 and 23 years (Wierenga et al., 2016). It is argued that these brain changes, in interaction with changes in the social environment during adolescence, contribute to the increased vulnerability to develop SAD during this period of life (Caouette & Guyer, 2014; Haller et al., 2015). However, how these maturational and environmental changes interact with the neurobiological brain characteristics that reflect the genetic predisposition to develop SAD, is at present unexplored terrain.

To investigate these questions, I plead for longitudinal studies in large samples of adolescents, both with and without familial risk for SAD. Such studies enable a better understanding of the interaction between brain maturation and genetic risk factors during adolescence, and provide the opportunity to explore how individual characteristics impact the expression of the candidate endophenotypes and the risk for developing SAD (cf. (Crone & Elzinga, 2015)).

Investigation of (epi)genetic variations

Within the LFLSAD, we collected saliva for genotyping purposes, using the Oragene•DNA OG-500 self-collection kits (Genotek, Ottawa, Ontario, Canada). These data are, however, not yet analyzed. In the near future, we aim to examine whether specific (epi)genetic variations underlie the neurobiological candidate endophenotypes summarized in *Figure 11.1*. As the genetic architecture of endophenotypes is not necessarily less complex when compared to the genetic background of the phenotypes (cf. the extended discussion on this

topic in *Chapter 2* and the work by (Flint & Munafò, 2007; Flint et al., 2014)), we will use a hypothesis-driven approach based on previous work in the field. For example, as previous studies indicated that variations in the serotonin transporter (*5-HTTLPR*) gene, the catechol-o-methyl transferase (*COMT*) gene and the monoamine oxidase A (*MAO-A*) gene influenced reactivity of the amygdala (Domschke et al., 2012; Hariri et al., 2002; Kempton et al., 2009; Lonsdorf et al., 2011), we will investigate whether specific variants of these genes display a relationship with the hyperreactivity of the amygdala as reported in *Chapter 10*. Furthermore, the effect of epigenetic changes on the functional amygdala response is worthy of investigation (Nikolova et al., 2014; Nikolova & Hariri, 2015; Puglia, Lillard, Morris, & Connelly, 2015; Schiele & Domschke, 2017; Ziegler et al., 2015). Such studies are the next step in unraveling the genetic vulnerability to SAD.

Methodological considerations

In addition to the strengths and limitations of the individual studies, which are discussed in the preceding chapters of this thesis, I want to highlight the most prominent characteristics of the LFLSAD.

The unique character of a multiplex, multigenerational, neuroimaging family study

To start, the LFLSAD is the first and, to the best of our current knowledge, the only two-generation family study on SAD which includes neuroimaging measurements. As mentioned previously, this family design was chosen to facilitate testing of two endophenotype criteria, namely the *co-segregation of the candidate endophenotypes with social anxiety within the families* and the *heritability of the candidate endophenotypes,* in the same sample, with highest possible statistical power to detect genetic and environmental influences on SAD-related characteristics (Williams & Blangero, 1999). Recently, Glahn and colleagues (2018) outlined several advantages of studying families when examining genetic risk variants for psychopathology, in comparison to testing unrelated individuals. First of all, the environmental variation among family members is smaller, leading to less noise in the data and increased statistical power to detect genotype-phenotype associations. Furthermore, family-based designs are more cost-effective, for example when it comes to whole genome-sequencing (Glahn et al., 2018).

Nevertheless, family studies involving multiple generations and including extended families, which investigate neurobiological underpinnings of the genetic risk to develop psychopathology, are scarce. Previous family studies in the field of SAD were epidemiologic controlled family studies of probands with anxiety disorders, or high-risk studies in children of parents with anxiety (Knappe, Beesdo, Fehm, Lieb, & Wittchen, 2009; Mancini

et al., 1996; Merikangas et al., 2003). These studies revealed convincing evidence for the familial aggregation of SAD, a finding which was recently confirmed by a meta-analysis (Lawrence, Murayama, & Creswell, 2019), while a large longitudinal study in twins between ages 3 and 63 years indicated that the stability in symptoms of depression and anxiety over the lifespan was largely due to genetic effects (Nivard et al., 2015). These studies support the genetic background of SAD but did, however, not provide insight in the underlying neurobiological mechanisms, due to the absence of neuroimaging measurements. On the other hand, I am aware of several neuroimaging studies on high-risk offspring of parents with (social) anxiety (Christensen et al., 2015; Suffren, Chauret, Nassim, Lepore, & Maheu, 2019), but data-collection (diagnostic interviews) in these studies was limited to the nuclear family, while MRI data were collected only in the high-risk offspring.

When considering research on other psychiatric disorders, I have knowledge of several studies which have, to a certain extent, a family design comparable to that of the LFLSAD. To start, the 3G parenting study on the intergenerational transmission of parenting styles, stress and emotion regulation, which is, like the LFLSAD, part of the Leiden University Research Profile 'Health, Prevention and the Human Life Cycle', also uses a multi-generational family design in combination with neuroimaging methods (van den Berg et al., 2018; van den Berg, Tollenaar, Compier-de Block, Bakermans-Kranenburg, & Elzinga, 2019). Furthermore, I know of a longitudinal three-generation family study on major depressive disorder, which was initiated in 1982 and includes EEG and MRI measurements. In this study, probands with a diagnosis and probands without psychopathology, with their offspring (children and grandchildren) are being followed for over 25 years (Talati, Weissman, & Hamilton, 2013). In addition, there are several family studies on schizophrenia, bipolar disorder, depression and obsessive-compulsive disorder which included neuroimaging measurements, but these studies typically only involved patients, unaffected relatives and unrelated control subjects, and did not invite entire families for participation (for some recent examples, see (Blakey et al., 2018; Goghari, MacDonald, & Sponheim, 2014; Miskowiak et al., 2018; Vaghi et al., 2017; Yalin et al., 2019)). Thus, to the best of our knowledge, the LFLSAD is unique, not only within the field of research on social anxiety, but even broader within the field of neurobiological research on the genetic vulnerability to develop psychopathology.

Next, it is important to note that patients recruited as part of a family study may differ from patients who are recruited 'on their own'. It has previously been shown that patients with schizophrenia ascertained through a family-based design (and thus requiring intact family relationships) were younger, with higher levels of education and better performance on some neurocognitive domains when compared to patients in a case-control study (Gur et al., 2015). Based on this report, Glahn et al. state that 'studies that use case-control ascertainment may tap into populations with more severe forms of illness that are exposed to less favorable factors compared to those ascertained through designs that require family participation' (Glahn et al., 2018, page 8). This bias could also apply to the LFLSAD, as selec-

tion of the families was based on the combination of a parent with clinical SAD ('proband') and a child with clinical or subclinical SAD (*Chapter 3);* furthermore, we aimed to include families with at least eight family members, implicating, in most cases, that the proband needed to have at least one sibling with a partner and / or children, who the proband had to contact, in order to ask whether they were open to receive information about the study (see the detailed description of the inclusion procedure in *Chapter 3*). Thus, given the finding that SAD patients are less likely to be married (Wells, Tien, Garrison, & Eaton, 1994), the observation that a lifetime diagnosis of social phobia is associated with a significantly greater likelihood of reporting dissatisfaction with one's family life (Stein & Kean, 2000), and a study showing that social anxiety is associated with deficits in relationship maintenance behavior (Wenzel, Graff-Dolezal, Macho, & Brendle, 2005), it is possible that patients included in the LFLSAD had less severe forms of the disorder.

However, according to Glahn et al., 'designs that require multiple affected individuals in a family may result in a *more severe* phenotypic profile (..) as compared to simplex families' (Glahn et al., 2018). This also applies to the LFLSAD, as we selected families with at least two (sub)clinical SAD cases, leading to a sample which was indeed enriched for SAD (cf. the results described in *Chapter 3*). Furthermore, it is important to note that all SAD patients included in the LFLSAD met the DSM-IV-TR criteria for the generalized subtype of SAD, while a clinician verified whether the DSM-5 criteria for SAD were also met (American Psychiatric Association, 2013; Heimberg et al., 2014). Therefore, we feel the patients included in the LFLSAD are on a daily basis limited by their SAD symptoms (following criterion G of the DSM-5 definition, stating that 'the fear, anxiety, or avoidance causes clinically significant distress or impairment in social, occupational, or other important areas of functioning') and do not represent cases with less severe social anxiety. Taken together, we feel the sample of the LFLSAD does not consist of a particular selection of less severe SAD cases (cf. *Chapter 3*).

Limitations of the LFLSAD

Some limitations of the LFLSAD need to be mentioned. As already discussed in several chapters of this thesis, the lack of control families precluded examining whether *non-affected family members showed altered levels of the endophenotype in comparison to the general population* (second element of criterion 4). Furthermore, the *stability of the endophenotype* (endophenotype criterion 2) could not be investigated due to the cross-sectional design. With respect to the neuroimaging analyses, the complex family-structure of the data, which we took into account in the structural analyses (*Chapter 5)* as well as in the voxelwise analyses of brain function (*Chapters 8, 9 and 10)* using multivariable regression models, impeded adding additional elements to the analyses. For example, at present it is statistically and computationally too demanding to examine whether factors like IQ, education level, or socioeconomic status had a moderating or mediating effect on the expression of the candidate endophenotypes, nor could we perform psychophysiological interaction (PPI)

analyses to investigate whether SA-related alterations in brain activation were accompanied by differences in functional connectivity specific to the task (cf. Bas-Hoogendam, Andela, et al. (2015)). It is our hope that future studies will be able to perform such analyses, due to technical developments and improvements.

Another limitation concerns the sample size of the LFLSAD. Although the overall size of the MRI sample (in most analyses, the remaining dataset after extensive quality checking exceeded 100 participants) is not unusual in the field (cf. recent neuroimaging case-control studies on SAD involving respectively *12 vs. 14*, *23 vs. 23* , *28 vs. 27*, and *58 vs. 16* (SAD vs. healthy control) participants (Davies et al., 2017; Heeren et al., 2017; Kreifelts et al., 2017; Yun et al., 2017)), the sample size of the LFLSAD was too small to test the effects of additional parameters like, for example, temperamental characteristics, trait anxiety and negative affect, in a reliable manner (Blackford, 2017).

Finally, it is vital to realize that, while the studies included in this thesis focused on the neurobiological alterations underlying the genetic vulnerability to develop SAD, and data-collection was not designed for analyses on environmental influences, such factors are also relevant in the development of the disorder. Importantly, these factors do not exert their effects independently, but rather interact with the inherited vulnerability to develop SAD (Bas-Hoogendam, Roelofs, et al., 2019; Wong & Rapee, 2016). Therefore, I will briefly highlight some interesting findings from other researchers in this area. I will focus on parental influences, as the children included in the LFLSAD did not only inherit a genetic risk to develop SAD, but also grew up in a possibly altered family environment due to their parent's SAD.

A striking example of the genotype - environment interaction in the development of social anxiety was provided by a study with a prospective adoption design, in which 275 adoption-linked families, each including an adopted child, adoptive parents, and a birth mother were investigated (Natsuaki et al., 2013). Anxious behavior in the children was assessed when they were between 18 and 27 months of age, and results indicated that toddlers whose birth mothers met criteria for SAD showed elevated levels of anxious behavior in a social situation at 27 months of age, but only when their adoptive mothers were less emotionally and verbally responsive at 18 months of age. Interestingly, children at high genetic risk to develop SAD, who experienced higher levels of their adoption mothers' responsiveness, did not show an elevation in social anxiety (Natsuaki et al., 2013). Other studies also demonstrated effects of parental anxiety, general parental psychopathology and parenting style on the development of anxiety in their children (Aktar, Majdandžić, de Vente, & Bögels, 2013; Lieb et al., 2000; Pahl, Barrett, & Gullo, 2012); furthermore, specific maternal and paternal effects are reported (Aktar, Bockstaele, Perez-Edgar, Wiers, & Bögels, 2018; Bögels & Perotti, 2011; Bynion, Blumenthal, Bilsky, Cloutier, & Leen-Feldner, 2017; Knappe, Beesdo-Baum, Fehm, Lieb, & Wittchen, 2012).

These findings suggest a complex interplay between the innate temperament of the child and parental factors in the development of SAD (Knappe et al., 2010; Ollendick & Benoit, 2012), and indicate that investigating their interaction with neurobiological alterations is of importance to unravel the complex pathways leading to SAD. In this light, it is essential to mention that, within family studies, genetic and environmental factors are likely entangled with each other; that is to say, common traits may not only be the result of genetic influences, but could also be transmitted via shared environmental factors and model learning (Bandelow et al., 2016; Talati, Weissman, et al., 2013) As expressed in a statement paper on the importance of translational epidemiology in psychiatry, 'disorders that are highly familial are likely genetic, but nongenetic risks can also run in families' (Weissman, Brown, & Talati, 2011, page 605). In line with Bandelow and colleagues (2016), I propose that twin- and adoption studies are essential to separate genetics, shared environmental and other influences.

Ethical considerations

As described in the preceding chapters, the research protocol of the LFLSAD was approved by the Medical Ethical Committee of the Leiden University Medical Center. Furthermore, all participants were extensively informed about the objectives and procedure of the study, and provided informed consent prior to participation (*Chapter 3)*. However, studies with a multigenerational family design could bring forward specific ethical questions, inherent to their unique character. Here, I want to highlight some ethical considerations, inspired by the discussion on this topic with respect to high-risk studies as provided by Mesman (2015).

To start with a critical notion, one could argue that inviting family members to participate in a study on familial extreme shyness may lead to distress, caused by increased awareness of their 'at risk status'. Furthermore, the fact that whole families are invited could potentially lead to group pressure, which could limit family members in their subjective feeling of free choice with respect to their decision to participate in the study.

However, although we did not systematically ask family members about their experience of participating in the LFLSAD, we predominantly received positive feedback. First of all, participants told us that the study made them aware that their 'extreme shyness', which in many cases limited them in their daily lives, was not a personal shortcoming, but a psychiatric disorder, which was associated with alterations in their brain and subject of investigation. This recognition was important for many participants within the LFLSAD, and also opened up the conversation about their personal struggles. Several participants disclosed that the study made them realize that they 'were not the only ones' who experienced social anxiety. In some cases, family members did not know about their sibling's social anxiety, and participating in the study helped them to share their experiences.

In addition, as most participants with SAD indicated that they were motivated to take part in the study because they 'wanted to know how they could prevent suffering in their

children', I don't believe that inviting family members put them in an 'at risk status'; after all, their motivation implicated that they were already aware of socially-anxious tendencies in their offspring, and that they were worried about the development of more severe social anxiety.

Importantly, I think that participation in the LFLSAD could have contributed to a lower threshold for help-seeking in both parents and offspring. Although none of the participants with SAD within the sample was treated for the disorder before entering the study, several participants indicated that they wanted to receive treatment to reduce their social anxiety, following their participation. This is important, as patients with SAD do not easily seek treatment, most likely due to embarrassment or an underestimation of their condition. As a result, there is a striking delay between the age of onset of SAD and the age of first therapy, even up to 15 years (Alonso et al., 2018; Dingemans et al., 2001; Iza et al., 2013). In addition, it was recently demonstrated that children with anxiety disorders often face barriers to treatment access, with 'parents not knowing where or from whom to seek help' as the most common access barrier (Salloum, Johnco, Lewin, McBride, & Storch, 2016). To illustrate, a report described that less than 1 % of all children of patients with severe depressive and / or anxiety disorders in the Netherlands participated in preventive intervention programs (as discussed in (Potijk, Drost, Havinga, Hartman, & Schoevers, 2019)). This is a concern, as treatment during sensitive periods in brain development, preferably before the onset of clear psychiatric symptoms, might prevent the development of full-blown anxiety disorder later in life (Hirshfeld-Becker & Biederman, 2002; Marín, 2016; Sylvester, 2018; Talati, Weissman, et al., 2013). The promising results of a recent randomized controlled trial in offspring of anxious parents underscore this idea: findings indicated that a brief prevention program significantly reduced the incidence of anxiety disorders and the severity of anxiety symptoms over a 1-year period in high-risk offspring (Ginsburg, Drake, Tein, Teetsel, & Riddle, 2015). Thus, preventive interventions using a family-focused approach, preferably embedded within routine adult psychiatric care, are important to reduce future suffering in offspring at risk for anxiety disorders (Knappe et al., 2010; Potijk et al., 2019).

Taken together, it is my hope that, in addition to its scientific results which could lead to improvements in treatment at the long term, the LFLSAD also resulted in direct positive personal benefits for the participants. Continued translational research, putting socially-anxious participants 'into the spotlight', is of uttermost relevance to reduce the everyday, often unnoticed, suffering of these patients.

Concluding remarks

In conclusion, the studies summarized in this thesis provide new insights in the neurobiological vulnerability to SAD. Using data from the unique multiplex, multigenerational LFLSAD,

we identified several structural and functional brain alterations which *co-segregated with social anxiety within families of probands* and were at least moderately *heritable*, making them promising candidate endophenotypes of SAD. Future studies are needed to investigate additional neurobiological endophenotypes, and to establish the stability and development of the candidate endophenotypes over time. Furthermore, whether the neurobiological candidate endophenotypes are useful targets for intervention needs to be examined. Moreover, which (epi)genetic variations give rise to the neurobiological alterations is still an open question. The promising results of the present work offer a starting point for follow-up studies on the genetic susceptibility to SAD.

Hoofdstuk 12

Extreem Verlegen & Genetisch Verwant –
samenvatting in het Nederlands

Stel je voor …

… dat je een presentatie moet geven voor een groep mensen. Je staat in het middelpunt van de belangstelling en alle ogen zijn op jou gericht. Hoe zou je je voelen? Of denk je eens in: je bent uitgenodigd voor een feestje waar je verder niemand kent. Je komt de feestruimte binnen en ziet dat alle andere gasten al een plekje hebben gevonden. Wat gaat er dan door je heen? Waarschijnlijk voel je je in het begin verlegen en niet zo op je gemak, maar na verloop van tijd zal deze spanning verdwijnen en heb je het naar je zin.

Er zijn echter mensen die zich in sociale situaties voortdurend extreem ongemakkelijk en gespannen voelen. Sterker nog, ze maken zich al zorgen vóórdat ze zich in een sociale setting bevinden, en ook na afloop piekeren ze over de indruk die ze hebben achtergelaten. Deze mensen hebben een extreme angst om negatief beoordeeld te worden, en zijn erg bezorgd dat ze iets doms of beschamends doen in het bijzijn van anderen. Door deze angst vermijden ze sociale situaties voor zover dat mogelijk is, en als ze er niet onder uit kunnen komen om zich in het gezelschap van anderen te begeven zijn ze als muurbloempjes die het liefst niet op willen vallen. Dit gedrag heeft vaak ernstige negatieve effecten op hun welzijn. Wanneer deze angst hun leven daadwerkelijk beperkt is er sprake van een psychiatrische aandoening: sociale angststoornis (SAS).

Eerder onderzoek heeft laten zien dat deze '*extreme verlegenheid*' al tijdens de kindertijd en vroege adolescentie tot uiting komt. Ook is bekend dat sociale angst vaak binnen families voor komt; iemand die '*genetisch verwant*' is aan een patiënt met SAS heeft een substantieel hogere kans om zelf ook de ziekte te ontwikkelen. Maar welke erfelijke karakteristieken maken deze kinderen en jongeren kwetsbaar om SAS te ontwikkelen?

Onderzoek naar de neurobiologische endofenotypes van sociale angststoornis

In de roman 'Extreem Luid & Ongelooflijk Dichtbij' dwaalt de negenjarige Oskar Schell door New York om het slot te vinden waarin een mysterieuze sleutel past. Deze sleutel was van zijn vader, die om het leven kwam bij de aanslag op het World Trade Center op 9/11. De zoektocht door New York helpt Oskar om het verlies van zijn vader een plek te geven.

Dit proefschrift beschrijft ook een zoektocht: de studies die in dit boek zijn opgenomen richten zich op het vaststellen van veranderingen in gedrag en neurobiologische breinkarakteristieken die gerelateerd zijn aan sociale angst. Ik onderzocht met name welke karakteristieken genetisch gekoppeld zijn aan SAS. Dit is van belang, omdat deze karakteristieken, die 'endofenotypes' worden genoemd, ons meer kunnen leren over de genetische kwetsbaarheid voor het ontwikkelen van SAS.

In dit hoofdstuk vat ik kort samen wat SAS is en waarom het van belang is om onderzoek te doen naar de factoren die een rol spelen bij de ontwikkeling van deze aandoening. Ik introduceer het endofenotype concept en leg uit wat de Leidse Familiestudie naar Sociale Angststoornis uniek maakt. Vervolgens beschrijf ik kort de inhoud van de afzonderlijke

hoofdstukken van dit proefschrift, en zet per hoofdstuk de belangrijkste resultaten uiteen. Na een samenvatting van deze bevindingen reflecteer ik op deze uitkomsten, en beschrijf wat deze resultaten ons leren over de genetische kwetsbaarheid voor SAS. Ook geef ik aan welk vervolgonderzoek van belang is.

Sociale angststoornis

In de vijfde editie van de 'Diagnostic and Statistical Manual of Mental Disorders (DSM-5)', het handboek waarin criteria voor psychiatrische aandoeningen uiteengezet worden, wordt beschreven dat patiënten met SAS gekarakteriseerd worden door een 'duidelijke angst of vrees voor één of meer sociale situaties waarin de betrokkene wordt blootgesteld aan mogelijke kritische beoordeling door anderen' (American Psychiatric Association, 2013). Voorbeelden van zulke sociale situaties zijn gelegenheden waarin men een bepaalde taak moet uitvoeren, bijvoorbeeld het geven van een presentatie, en situaties met sociale interacties, zoals het bezoeken van feestjes of het ontmoeten van nieuwe mensen (Furmark, 2002; Neal & Edelmann, 2003). In deze sociale situaties zijn patiënten met SAS bang dat hun gedrag negatief beoordeeld zal worden door anderen, en ze vrezen dat ze op zullen vallen door zichtbare uitingen van hun sociale angst, zoals door blozen of zweten. SAS patiënten zijn altijd bang voor het begaan van een blunder in het bijzijn van anderen, en daarom vermijden ze sociale situaties zoveel als mogelijk is. Wanneer vermijden geen optie is, ervaren ze veel spanning en angst tijdens deze sociale aangelegenheden. Volgens de DSM-5 criteria voor een sociale angststoornis moeten deze angsten in alle sociale situaties en gedurende lange tijd, tenminste 6 maanden, aanwezig zijn.

Meerdere onderzoeken hebben laten zien dat SAS bij 6 tot 13 % van de bevolking voor komt (Bandelow & Michaelis, 2015; Kessler, Petukhova, Sampson, Zaslavsky, & Wittchen, 2012; Ruscio et al., 2008; Stein et al., 2010); bovendien maken recente gegevens van een 'World Mental Health' onderzoek duidelijk dat SAS niet cultuurgebonden is, maar over de hele wereld voor komt (Stein et al., 2017). SAS komt over het algemeen gedurende de late kinderjaren en vroege adolescentie tot ontwikkeling: de diagnose SAS kan vaak al op tienjarige leeftijd gesteld worden (Burstein et al., 2011; Ormel et al., 2014). De neiging om terughoudend en vermijdend te reageren op nieuwe personen, ervaringen en objecten is echter al veel eerder, namelijk al in jonge baby's, op te merken. Deze karakteristieke reactie, die 'gedragsvermijdend' wordt genoemd, weerspiegelt een stabiel en aangeboren temperamentskenmerk, en uit eerder onderzoek is gebleken dat kinderen met een gedragsvermijdend temperament een verhoogd risico hebben om later in hun leven SAS te ontwikkelen (Clauss et al., 2015; Clauss & Blackford, 2012).

Typerend voor SAS is het hardnekkige en chronische beloop van de aandoening (Beesdo-Baum et al., 2012); bovendien wachten mensen die aan SAS lijden vaak jaren voordat ze met hun klachten naar een arts of psycholoog gaan om voor hun angsten behandeld te worden (Iza et al., 2013). De gevolgen van de ziekte zijn echter niet gering: patiënten met SAS

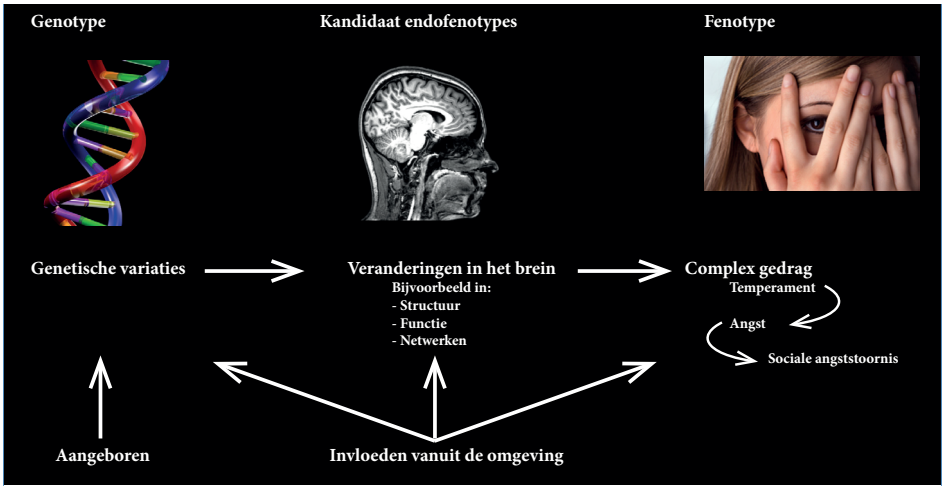
ervaren vaak problemen tijdens hun studie en werk, in activiteiten met vrienden, en in hun intieme relaties (Aderka et al., 2012; Dingemans et al., 2001; Hendriks et al., 2015; Russell & Topham, 2012). Verder lijden patiënten met SAS bovengemiddeld vaak aan andere psychiatrische aandoeningen, zoals stemmingsstoornissen, verslaving, en andere angststoornissen (Beesdo et al., 2007; Fehm et al., 2005; Ohayon & Schatzberg, 2010). Samengenomen leiden deze factoren ertoe dat SAS een aandoening is die het leven van patiënten ernstig belemmert, en die tot hoge kosten leidt voor de maatschappij, bijvoorbeeld door ziekteverzuim en verminderde productiviteit (Acarturk et al., 2009; Dams et al., 2017; Hendriks et al., 2014; Stein & Kean, 2000; Stuhldreher et al., 2014; Wittchen, Fuetsch, Sonntag, Müller, & Liebowitz, 2000).

Om deze ernstige gevolgen van SAS zoveel mogelijk te voorkomen zijn effectieve preventieve interventies, die erop gericht zijn om de ontwikkeling van de ziekte tegen te gaan, van groot belang (Craske & Zucker, 2001). Om zulke therapieën te kunnen ontwikkelen is het nodig om te weten welke factoren ervoor zorgen dat bepaalde kinderen en jongeren extra gevoelig zijn om SAS te ontwikkelen: wat maakt hen kwetsbaar?

Het is essentieel om daarbij oog te hebben voor neurobiologische processen (Beauchaine et al., 2008), aangezien eerder onderzoek aangetoond heeft dat biologische factoren, samen met psychologische en sociale factoren, een rol spelen in de ontwikkeling van SAS (Wong & Rapee, 2016). Onderzoeken in families en tweelingen hebben bijvoorbeeld laten zien dat SAS deels erfelijk is (Isomura et al., 2015; Mancini, Van Ameringen, Szatmari, Fugere, & Boyle, 1996; Middeldorp et al., 2005; Scaini, Belotti, & Ogliari, 2014; Stein, Chartier, Kozak, King, & Kennedy, 1998), maar de genetische variaties die hieraan ten grondslag liggen zijn tot op heden nog grotendeels onbekend. Bovendien zijn deze genetische variaties moeilijk op een betrouwbare manier vast te stellen. Dat wordt deels veroorzaakt door het feit dat SAS op verschillende manieren tot uiting komt (Hyett & McEvoy, 2018): sommige patiënten worden vooral belemmerd tijdens het geven van presentaties, terwijl anderen het niet aandurven om een artikel terug te brengen naar een winkel, of ervoor terugdeinzen om iets te eten in het bijzijn van anderen. Bovendien wordt SAS niet door één enkele genetische variatie bepaald: aangenomen wordt dat meerdere kleine veranderingen in het DNA van belang zijn in de ontwikkeling van de stoornis (Domschke & Dannlowski, 2010; Meier & Deckert, 2019). Dit zorgt ervoor dat het moeilijk is om een link te leggen tussen variaties in het DNA en sociale angst (Bas-Hoogendam et al., 2016; Bearden et al., 2009). Nieuwe onderzoeksparadigma's en studie-opzetten kunnen echter helpen om meer inzicht te krijgen in de genetische kwetsbaarheid voor het ontwikkelen van SAS. Een voorbeeld van zo'n nieuwe benadering betreft onderzoek naar *endofenotypes* van psychiatrische aandoeningen (Gottesman & Gould, 2003).

Het endofenotype concept

Endofenotypes zijn meetbare karakteristieken die de schakel vormen tussen individuele waarneembare eigenschappen aan de ene kant (‘*fenotype*’), en de onderliggende genetische variaties aan de andere kant (‘*genotype*’). In dit proefschrift onderzoek ik endofenotypes van het fenotype ‘sociale angst’ (*Figuur 12.1*). Voorbeelden van endofenotypes zijn veranderingen in de structuur en functie van de hersenen, afwijkingen in cognitief functioneren, en neurofysiologische veranderingen (Glahn, Knowles, et al., 2014).



Figuur 12.1 De relatie tussen genotype, endofenotype, en fenotype.
Figuur geïnspireerd door het werk van Kendler & Neale (2010). Illustratie DNA: Wikimedia Commons, National Human Genome Research Institute, ID 85329. Foto: *www.smartgirlsgroup.com*. Deze figuur is gebaseerd op Figure 1, gepubliceerd in Bas-Hoogendam et al. (2016, *Neuroscience & Biobehavioral Reviews*).

Het is van belang om te realiseren dat niet elk meetbaar kenmerk een endofenotype is; in de wetenschappelijke literatuur worden vier belangrijke criteria gebruikt om dit vast te stellen (Glahn et al., 2007; Gottesman & Gould, 2003; Lenzenweger, 2013b; Puls & Gallinat, 2008). Ten eerste moet een endofenotype *geassocieerd zijn met* een bepaalde aandoening. Volgens het tweede criterium moet een endofenotype *stabiel zijn* over de tijd, terwijl het derde criterium beschrijft dat een endofenotype *erfelijk* moet zijn. Ten slotte moet een endofenotype *samen met de aandoening vóórkomen binnen families, waarbij het van belang is dat het endofenotype ook meetbaar is in familieleden zonder de aandoening, wanneer deze familieleden vergeleken worden met individuen uit de gemiddelde bevolking* (endofenotype criterium 4). Door deze kenmerken kan een endofenotype meer inzicht geven in de genetische kwetsbaarheid voor het ontwikkelen van psychiatrische stoornissen, en in de manier waarop genetische variaties leiden tot deze aandoeningen (Flint et al., 2014; Miller & Rockstroh, 2013). Ook kunnen endofenotypes, omdat ze niet noodzakelijkerwijs uniek gerelateerd zijn aan één specifieke aandoening, kennis opleveren over de overlappende,

zogenaamde *transdiagnostische,* karakteristieken van mentale aandoeningen (Beauchaine & Constantino, 2017; Miller & Rockstroh, 2013).

In de afgelopen tien jaar is de endofenotype benadering gebruikt om meer inzicht te krijgen in verschillende psychiatrische aandoeningen, zoals depressie (Goldstein & Klein, 2014), obsessieve-compulsieve dwangstoornis (Bey et al., 2018; de Vries et al., 2013; Vaghi et al., 2017), en schizofrenie (Blakey et al., 2018; Glahn, Williams, et al., 2014; Honea et al., 2008; McCarthy et al., 2018). Deze onderzoeken lieten veranderingen in de structuur en functie van het brein zien die niet alleen aanwezig waren in patiënten, maar ook in gezonde familieleden van deze patiënten. Daarmee ondersteunen deze bevindingen het idee dat genetische variaties een rol spelen in de kwetsbaarheid voor deze aandoeningen, aangezien de hersenveranderingen niet alleen gerelateerd zijn aan het hebben van een psychiatrische aandoening, maar ook aanwezig zijn in verwanten van patiënten die *niet* aan deze stoornissen lijden. Ook maken deze resultaten duidelijk dat deze hersenveranderingen *erfelijk* zijn.

Onderzoek naar endofenotypes van SAS is tot op heden echter niet uitgevoerd. Er zijn wel onderzoeken bekend waarin patiënten met SAS vergeleken zijn met gezonde deelnemers. Deze studies, die bijvoorbeeld gebruik maakten van functionele magnetische resonantie imaging (fMRI), hebben waardevolle inzichten opgeleverd met betrekking tot neurobiologische hersenveranderingen die *geassocieerd zijn met SAS* (endofenotype criterium 1). De familieleden van SAS patiënten werden echter niet onderzocht, waardoor deze studies niet in staat waren om te bepalen of deze hersenveranderingen ook *erfelijk* zijn (endofenotype criterium 3); ook kon niet worden vastgesteld of de hersenveranderingen *samen met de aandoening vóórkomen binnen families* (eerste deel van endofenotype criterium 4). In andere woorden, deze onderzoeken brachten diverse *biomarkers* van SAS aan het licht: karakteristieken in de structuur en functie van het brein die gerelateerd zijn aan een bepaalde aandoening, maar die niet noodzakelijkerwijs op een functionele manier betrokken bij de ontwikkeling van de ziekte. Biomarkers kunnen bijvoorbeeld ook het gevolg zijn van het jarenlange ziekteproces of van het gebruik van medicatie (Lenzenweger, 2013a). Het is echter onbekend of deze eigenschappen ook als *endofenotypes* beschouwd kunnen worden, die een uiting zijn van de genetische gevoeligheid om SAS te ontwikkelen. Omdat SAS vaak binnen families voorkomt en deels erfelijk is, kan onderzoek naar endofenotypes van SAS belangrijke kennis opleveren over de genetische kwetsbaarheid voor het ontwikkelen van de aandoening. Deze inzichten kunnen vervolgens gebruikt worden voor het ontwikkelen van nieuwe behandelmogelijkheden, en het verbeteren van bestaande therapieën (Dick, 2018).

De Leidse Familiestudie naar Sociale Angststoornis

De Leidse Familiestudie naar Sociale Angststoornis (in het Engels: *Leiden Family Lab study on Social Anxiety Disorder*, LFLSAD) is opgezet als een eerste stap om het hierboven beschreven hiaat in de wetenschappelijke literatuur met betrekking tot endofenotypes van SAS te vullen. Zoals de naam aangeeft werden in de LFLSAD niet alleen patiënten met

SAS onderzocht: ook hun familieleden werden uitgenodigd voor deelname. We kozen voor deze aanpak omdat familiestudies bij uitstek geschikt zijn om twee belangrijke criteria voor endofenotypes te testen (Glahn et al., 2018). Ten eerste maakt een familiestudie het mogelijk om te onderzoeken of de endofenotypes *samen met de aandoening in de families vóórkomen* (eerste deel van endofenotype criterium 4). Ten tweede is het, doordat meerdere familieleden onderzocht worden, mogelijk om een schatting van de *erfelijkheid* van de endofenotypes te verkrijgen (endofenotype criterium 3). Verder hebben familiestudies goed statistisch onderscheidingsvermogen om betekenisvolle relaties tussen genotypes en fenotypes te detecteren, en zijn familiestudies kostenefficiënt (Glahn et al., 2018). Het doel van de LFLSAD was om neurobiologische endofenotypes van SAS vast te stellen, met behulp van magnetische resonantie imaging (MRI) en elektro-encefalografie (EEG). In *Hoofdstuk 3* beschrijf ik de achtergrond en opzet van het onderzoek in meer detail. In de rest van dit proefschrift vat ik de resultaten van een aantal MRI studies samen; de bevindingen van het EEG onderzoek zijn beschreven in het al eerder verschenen proefschrift van Anita Harrewijn (Harrewijn, 2017).

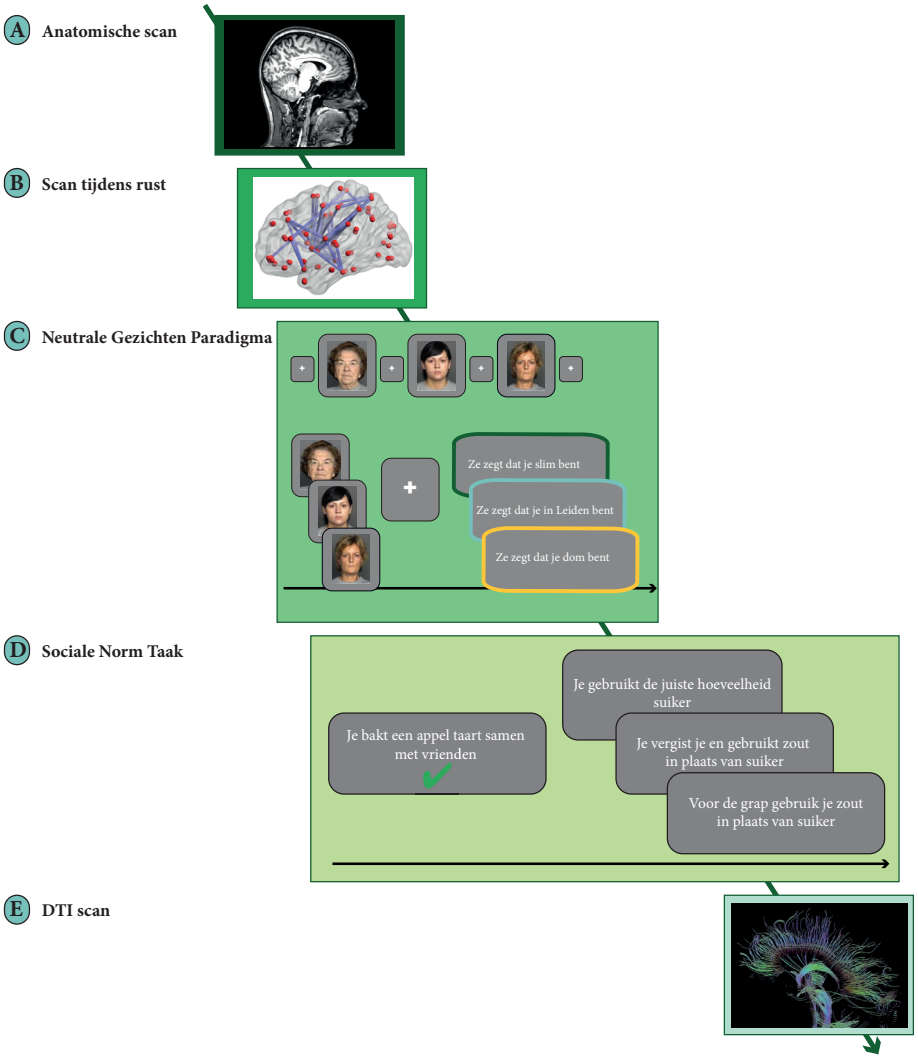
De verschillende MRI scans die ik binnen het LFLSAD onderzoek heb gebruikt zijn weergegeven in *Figuur 12.2*. We kozen deze MRI paradigma's op basis van eerder beeldvormend onderzoek naar biomarkers van SAS, omdat deze studies al bewijs leverden voor het eerste endofenotype criterium, namelijk de *associatie van het endofenotype met SAS*. In onze keuze namen we ook mee dat de MRI paradigma's gericht waren op verschillende regio's in het brein, en dat we vermoedden dat veranderingen deels erfelijk bepaald zouden zijn. In de volgende paragraaf vat ik de belangrijkste resultaten van dit eerdere onderzoek kort samen.

Beeldvormend onderzoek naar SAS

Het neuroimaging onderzoek naar sociale angst is in de afgelopen jaren steeds verder uitgebreid: terwijl de focus van de eerste beeldvormende onderzoeken naar SAS vooral lag op specifieke hersenstructuren zoals de amandelkern (amygdala) (Birbaumer et al., 1998) en diepgelegen delen van de hersenen (ook wel 'subcorticale gebieden' genoemd) (Potts et al., 1994; Schneider et al., 1999), is recent onderzoek erop gericht om breder in kaart te brengen welke veranderingen in de *structuur* en *functie* van het brein geassocieerd zijn met SAS.

MRI kan allereerst gebruikt worden om onderzoek te doen naar de structuur, oftewel de anatomie van het brein. Zulke studies meten bijvoorbeeld de grootte van bepaalde hersenkernen, schatten de dikte van het laagje grijze stof aan de buitenkant van het brein, en brengen in kaart wat de oppervlakte van hersengebieden is. Een recent samenvattend artikel van Brühl en collega's liet zien dat er bij patiënten met SAS, in vergelijking met gezonde deelnemers, door het hele brein kleine anatomische verschillen meetbaar zijn (Brühl, Delsignore, et al., 2014). In *Hoofdstuk 4* en *Hoofdstuk 5* van dit proefschrift bouw ik op deze resultaten verder; ik onderzoek daar veranderingen in de structuur van het brein in een groot, internationaal onderzoek, en analyseer vervolgens of deze anatomische hersenveran-

deringen endofenotypes van SAS zijn, door te bepalen of deze hersenveranderingen *samen met SAS binnen families vóórkomen* en of deze *erfelijk* zijn (*Figuur 12.2A*).



Figuur 12.2 Magnetische resonantie imaging (MRI) protocol van de Leidse Familiestudie naar Sociale Angststoornis (LFLSAD).
Een structurele MRI scan, bedoeld om een gedetailleerd beeld te krijgen van de anatomie van het brein (***Figuur 12.2A***) en een rust scan (waarin deelnemers hun ogen gesloten hadden; ***Figuur 12.2B***) werden gevolgd door twee functionele (f)MRI paradigma's: het Neutrale Gezichten Paradigma ***Figuur 12.2C***) en de Sociale Norm Taak (***Figuur 12.2D***). Aan het eind van het scanprotocol werden diffusie tensor imaging (DTI) scans gemaakt om de wittestofverbindingen in het brein in kaart te brengen (***Figuur 12.2E***). Het hele protocol duurde ongeveer een uur.

Met behulp van functioneel (f)MRI onderzoek is het mogelijk om inzicht te krijgen in de manier waarop de hersenen *functioneren*. Daarbij wordt gebruik gemaakt van veranderingen in het zuurstofgehalte in het bloed; deze veranderingen zijn een indicatie van de mate van activatie in een bepaald hersengebied. fMRI studies naar SAS maken vaak gebruik van stimuli die bij patiënten met SAS angst opwekken. Voorbeelden van zulke stimuli zijn foto's van gezichten met een negatieve (bijvoorbeeld boos of angstig) of neutrale gezichtsuitdrukking, verhaaltjes die sociale situaties beschrijven, of zinnen waarin persoonlijke feedback gegeven wordt (Brühl, Delsignore, et al., 2014). Eerder onderzoek heeft laten zien dat zulke stimuli leiden tot meer hersenactiviteit in patiënten met SAS, waarbij verschillende hersengebieden betrokken zijn. Verder is er meermaals een verband gevonden tussen de ernst van SAS (de hoeveelheid symptomen) en de mate waarin deze verhoogde hersenactiviteit gemeten wordt (Frick, Howner, Fischer, Kristiansson, et al., 2013), en hebben onderzoeken aan kunnen tonen dat een succesvolle behandeling met angstremmende medicijnen geassocieerd is met een verlaging van deze intensere hersenreactie op sociale stimuli (Phan et al., 2013). Deze bevindingen maken duidelijk dat functionele beeldvormende onderzoeken belangrijke en relevante kennis opleveren over de neurobiologische veranderingen in de functie van de hersenen bij patiënten met SAS.

In de *Hoofdstukken 8, 9* en *10* beschrijf ik onderzoek waarin ik gebruik heb gemaakt van fMRI om te onderzoeken of deze functionele hersenveranderingen niet alleen biomarkers van SAS zijn, maar ook als endofenotypes van SAS beschouwd kunnen worden. Ik richtte me daarbij op twee hersenfuncties die bijzonder relevant zijn in de context van SAS: het verwerken van de informatie die overgebracht wordt door gezichten met een neutrale gezichtsuitdrukking (*Hoofdstuk 9* en *Hoofdstuk 10; Figuur 12.2C),* en het lezen van korte verhaaltjes die sociale situaties met verschillende uitkomsten beschrijven (*Hoofdstuk 8; Figuur 12.2D)*. Opnieuw onderzocht ik of er bewijs was voor twee endofenotype criteria: ik bekeek of veranderingen in hersenfunctie *samen met SAS binnen de families voorkwamen* en of deze veranderingen *erfelijk* waren.

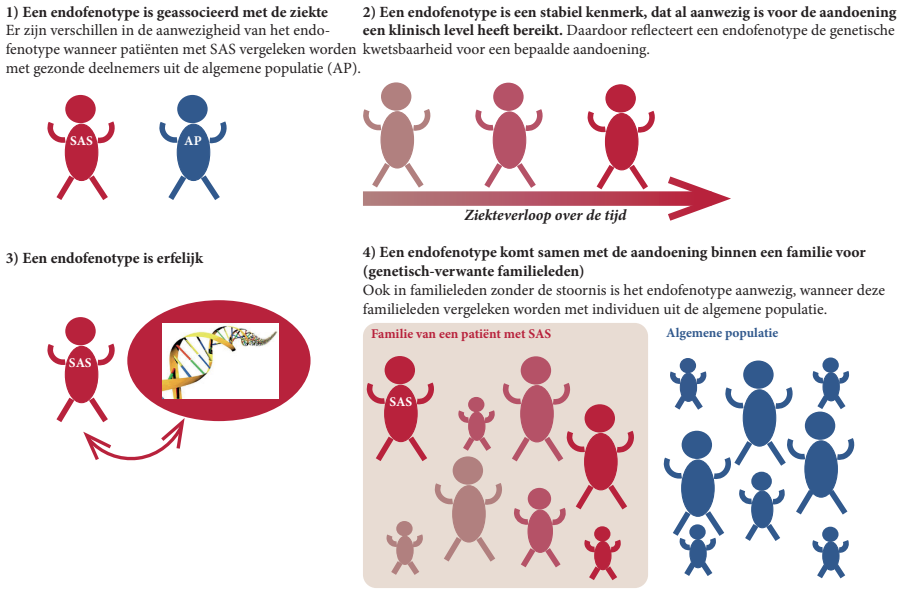
Beeldvormend onderzoek met MRI biedt ook de mogelijkheid om in kaart te brengen hoe gebieden in het brein met elkaar verbonden zijn in zogenaamde netwerken. Deze netwerken kunnen vastgesteld worden door te meten in welke gebieden het patroon van hersenactiviteit een bepaalde mate van synchroniciteit vertoont (Damoiseaux et al., 2006), of door de integriteit van wittestofbanen tussen gebieden te bepalen door middel van diffusie tensor imaging (DTI) (Chanraud et al., 2010). Eerdere onderzoeken in patiënten met SAS hebben veranderingen in beide netwerkmaten laten zien, zoals samengevat door Cremers & Roelofs (2016). In de LFLSAD hebben we ook scans gemaakt om deze netwerken te reconstrueren en binnen sociaal-angstige families te onderzoeken (*Figuur 12.2B, Figuur 12.2E).* Deze onderzoekgegevens worden op het moment geanalyseerd, en de resultaten zijn daarom nog niet in dit proefschrift vermeld.

De belangrijkste bevindingen van dit proefschrift

Het onderzoek dat samengevat is in dit proefschrift heb ik, samen met mijn collega's, uitgevoerd om de kennis over de genetische kwetsbaarheid voor het ontwikkelen van SAS te vergroten. Om dit te bereiken hebben we de Leidse Familiestudie naar Sociale Angststoornis opgezet, en de onderzoeksgegeven van deze studie getoetst aan een aantal belangrijke criteria voor endofenotypes. In de volgende paragrafen vat ik de inhoud van de afzonderlijke hoofdstukken van het proefschrift samen.

In *Hoofstuk 2* beschrijf ik de endofenotype benadering in meer detail, gebaseerd op een literatuur onderzoek (gepubliceerd als Bas-Hoogendam et al., 2016). Omdat het endofenotype concept in de daaropvolgende hoofdstukken steeds terugkomt, zet ik allereerst op een rij aan welke vier criteria een endofenotype moet voldoen. Deze criteria, die al eerder in dit hoofdstuk zijn besproken, zijn geïllustreerd in *Figuur 12.3*, en maken duidelijk dat endofenotypes méér zijn dan *biomarkers.* Biomarkers zijn meetbare karakteristieken die geassocieerd zijn met een bepaalde aandoening, maar hebben niet noodzakelijkwijs een genetische basis. Endofenotypes, daarentegen, zijn per definitie erfelijk én komen voor binnen families met sociale angst; daardoor geven ze inzicht in de genetische kwetsbaarheid voor het ontwikkelen van SAS. Dit onderscheid is kort en krachtig verwoord door Lenzenweger, in een artikel dat in 2013 verschenen is, en ik citeer: *'Alle endofenotypes zijn biomarkers, maar niet alle biomarkers zijn endofenotypes'* (Lenzenweger, 2013a, pagina 187).

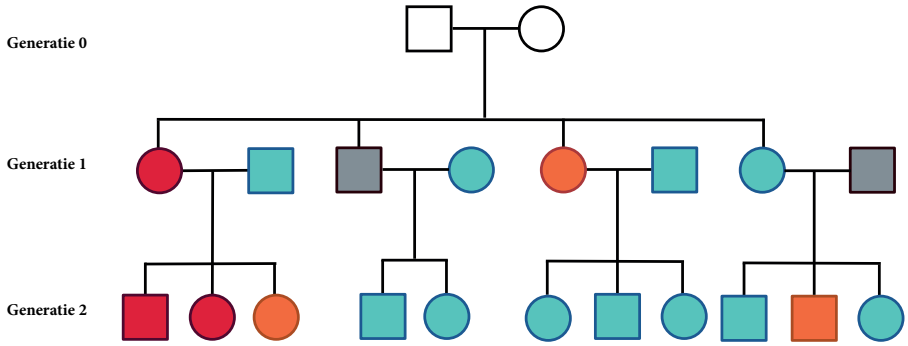
Vervolgens vat ik samen wat de waarde van endofenotype onderzoek is, waarbij het inzicht verschaffen in de genetische kwetsbaarheid voor het ontwikkelen van SAS voor de rest van dit proefschrift de belangrijkste is. Daarna beschrijf ik in hoeverre de resultaten van eerder onderzoek aanwijzingen geven voor een aantal kandidaat endofenotypes, die met MRI te meten zijn. Ik selecteerde vier meetbare eigenschappen van het brein, namelijk de *structuur* van de hersenen, de *functie* van de *amygdala* en de *prefrontale cortex*, en de *verbindingen* tussen de verschillende hersengebieden. Vervolgens ging ik voor elk van deze hersenkarakteristieken na of de bestaande wetenschappelijke literatuur aanwijzingen bood voor de vier criteria voor een endofenotype zoals samengevat in *Figuur 12.3*. Zoals eerder in dit hoofdstuk al werd opgemerkt zijn er nog geen directe endofenotype studies naar SAS uitgevoerd. Daarom maakten we bij het creëren van dit overzicht gebruik van de bevindingen van studies waarin patiënten met SAS vergeleken werden met gezonde deelnemers; ook bekeken we in hoeverre de resultaten van dieronderzoek en van studies in individuen zonder sociale angst indirect bewijs opleverden voor de endofenotype criteria. We vonden vooral bewijs voor het eerste endofenotype criterium: meerdere studies rapporteerden een significante *associatie tussen SAS en de hersenkenmerken* die we geselecteerd hadden. Bewijs voor de andere criteria was echter slechts sporadisch aanwezig, met name door het ontbreken van familiestudies waarin zowel patiënten met SAS als hun familieleden onderzocht werden.



Figuur 12.3 Illustratie van de vier criteria voor een endofenotype. Deze figuur is gebaseerd op Figure 2, eerder gepubliceerd in Bas-Hoogendam et al. (2016, Neuroscience & Biobehavioral Reviews).

In de Leidse Familiestudie naar Sociale Angststoornis (LFLSAD) werden familieleden wél onderzocht, en in *Hoofdstuk 3* van dit proefschrift beschrijf ik de achtergrond, de opzet en de methode van dit onderzoek in meer detail (Bas-Hoogendam et al., 2018a). Kenmerkend voor de LFLSAD is dat er in de families die in het onderzoek meededen *tenminste twee* familieleden last hadden van SAS, als een indicatie dat het een familiale variant van SAS betrof. De belangrijkste vereiste voor deelname was namelijk dat er binnen een gezin tenminste één ouder (leeftijd 25 - 55 jaar) voldeed aan de criteria voor een diagnose van SAS; verder moest tenminste één kind van deze ouder (leeftijd 8 - 21 jaar) last hebben van symptomen van sociale angst. Naast deze ouder-kind combinatie werden alle andere gezinsleden van dit zogenaamde 'kerngezin' uitgenodigd: de partner van de patiënt, en hun eventuele andere kinderen (minimumleeftijd: 8 jaar). Verder vroegen we aan de patiënt met SAS om zijn of haar broers en zussen, met hun partners en kinderen, te informeren over het onderzoek. Wanneer deze familieleden ervoor open stonden nodigden we ook hen uit om deel te nemen.

Door deze uitnodigingsstrategie bestond de onderzoeksgroep van de LFLSAD uit familieleden van twee generaties (*Figuur 12.4).*We kozen voor deze zogenaamde *multiplex* (dat wil zeggen: meerdere individuen met SAS binnen een familie), *multigenerationele* (namelijk: twee generaties per familie) onderzoeksopzet om een stap verder te kunnen gaan dan studies waarin enkel patiënten en gezonde deelnemers onderzocht worden: door het familie design van de LFLSAD konden we onderzoeken of de endofenotypes *samen met sociale*



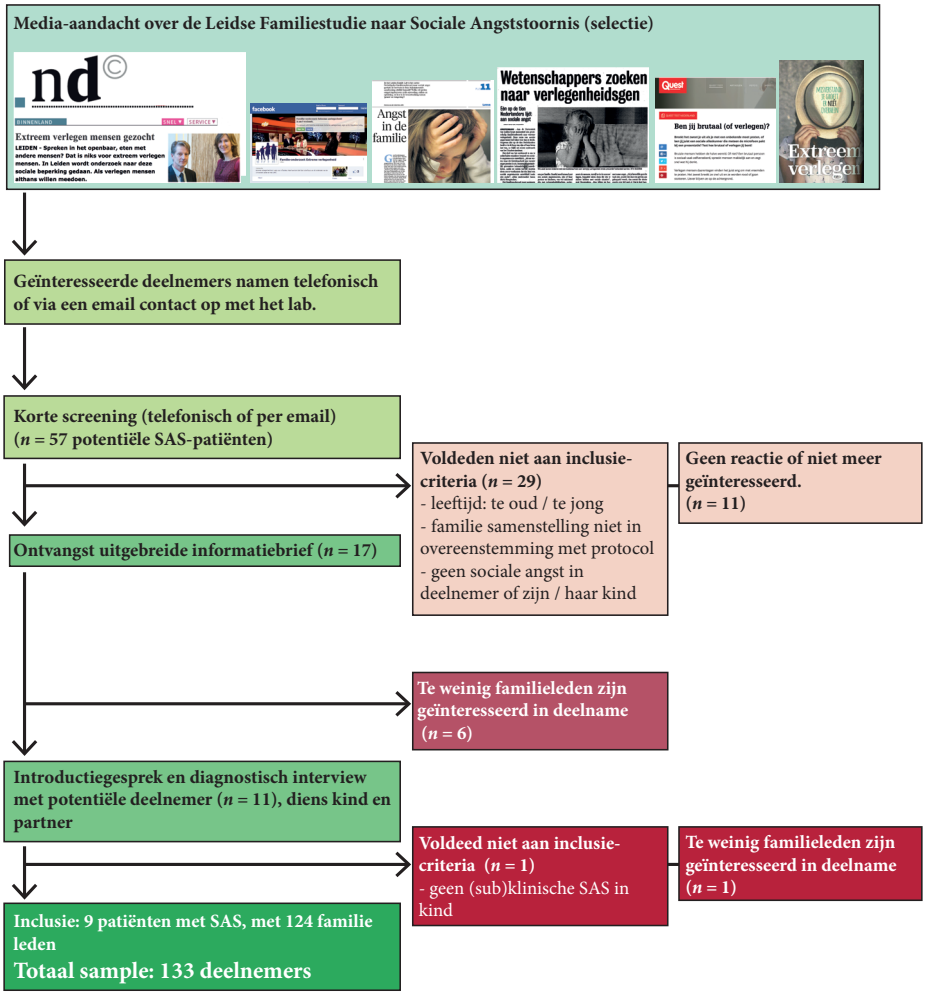
Figuur 12.4 Voorbeeld van een familie binnen de Leidse Familiestudie naar Sociale Angststoornis (LFLSAD).
Families werden geïncludeerd op basis van de combinatie van een ouder met SAS (generatie 1; weergegeven in rood) en een kind van deze ouder (generatie 2) met SAS (rood) of verhoogde symptomen van sociale angst (oranje). Verder werden zoveel mogelijk familieleden uit generatie 1 en generatie 2 uitgenodigd; de hoeveelheid symptomen van SAS in deze familieleden speelde bij de inclusie geen rol (geen SAS: licht blauw; wilde niet deelnemen: grijs). Grootouders (generatie 0; weergegeven in wit) werden niet uitgenodigd om deel te nemen. Deze stamboom is bewerkt om anonimiteit te waarborgen; het aantal familieleden en de aanwezigheid van sociale angst zijn echter waarheidsgetrouw. Vierkanten en cirkels geven mannen dan wel vrouwen weer. Deze figuur is gebaseerd op Figure 1, eerder gepubliceerd in Bas-Hoogendam et al. (2018a, *International Journal of Methods in Psychiatric Research*).

angst binnen de families vóórkwamen; ook konden we de *erfelijkheid* van de endofenotypes onderzoeken.

Na het uiteenzetten van de onderzoeksopzet beschrijf ik in *Hoofdstuk 3* hoe de steekproef tot stand gekomen is. Dit proces is weergegeven in *Figuur 12.5*. We brachten het onderzoek onder de aandacht van 'extreem verlegen families' door middel van interviews op de radio, televisie, en in kranten; ook zochten we contact met huisartsenpraktijken, klinisch psychologen en patiëntenverenigingen zoals de 'Angst, Dwang en Fobie stichting' en de 'Vereniging van Verlegen Mensen'. Gegeven de aard van sociaal-angstige mensen om terughoudend te zijn in sociale contacten kostte dit proces veel tijd (zomer 2013 – zomer 2015), maar we merkten dat families het prettig vonden om met meerdere familieleden aan het onderzoek deel te nemen. Uiteindelijk namen negen families, met in totaal 133 familieleden, deel aan de Leidse Familiestudie. Binnen de onderzoeksgroep voldeden 19 deelnemers aan de criteria voor een klinische diagnose van SAS, maar geen van deze deelnemers was voor deze klachten behandeld voor aanvang van het onderzoek.

Alle familieleden van de deelnemende families werden uitgenodigd om naar Leiden te komen. In het lab namen zij vervolgens deel aan verschillende onderdelen van het onderzoek: een diagnostisch interview met een psychiater of psycholoog, voor het vaststellen van psychiatrische diagnoses; het invullen van vragenlijsten om de ernst van sociale angstsymptomen en andere gerelateerde karakteristieken in kaart te brengen; twee korte testjes voor

het schatten van IQ. Daarnaast vond het EEG- en MRI onderzoek plaats, en werd speeksel verzameld voor latere DNA analyses. Een klein deel van de deelnemers gaf er de voorkeur aan om thuis een aantal vragenlijsten in te vullen; van deze deelnemers (n = 8) zijn dan ook niet alle gegevens beschikbaar.



Figuur 12.5 Stroomschema van de inclusie van de Leidse Familiestudie naar Sociale Angststoornis.
Deze figuur is gebaseerd op Figure 2, eerder gepubliceerd in Bas-Hoogendam et al. (2018a, *International Journal of Methods in Psychiatric Research*).

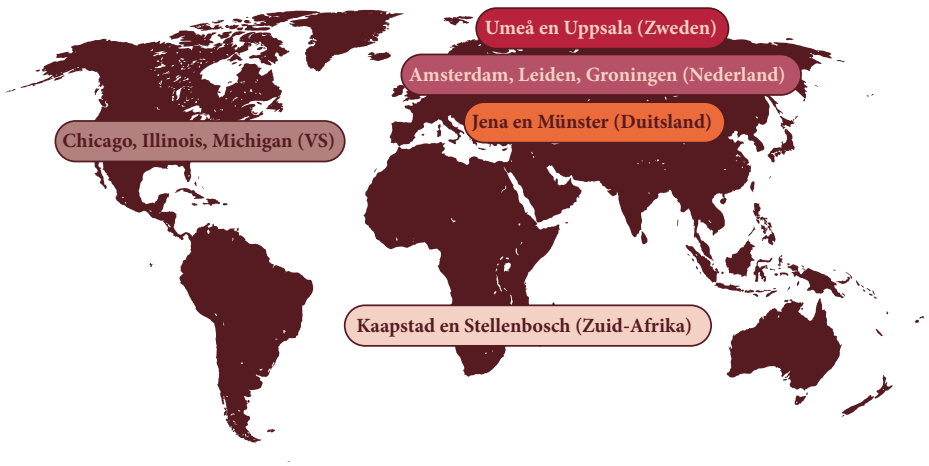
In Hoofdstuk 3 vat ik vervolgens een aantal belangrijke kenmerken van de uiteindelijke LFLSAD onderzoekspopulatie samen. Ten eerste is het belangrijk om te vermelden dat de onderzoeksgroep inderdaad bestond uit families waarin extreme verlegenheid veel

voorkwam: uit de diagnostische interviews bleek dat 38.3 % van de deelnemers in meer of mindere mate last had van SAS (ter vergelijking: in de algemene bevolking ligt dit percentage rond 13 % (Kessler et al., 2012)). Uit de antwoorden op de vragenlijsten bleek verder dat deze familieleden met SAS in het dagelijks leven niet alleen last hadden van sociale angst, maar ook meer gehinderd werden door depressie, angst in het algemeen, en door angst om negatief beoordeeld te worden door anderen. Deze bevindingen bevestigen eerder onderzoek naar SAS (Bas-Hoogendam et al., 2017a; Campbell et al., 2009; Clauss & Blackford, 2012; Goldin, Manber, Hakimi, Canli, & Gross, 2009) en ondersteunen de validiteit van de LFLSAD onderzoekspopulatie.

Karakteristieken van hersenstructuur: endofenotypes van SAS?

In het tweede deel van het proefschrift focus ik op veranderingen in de anatomie van het brein die een relatie laten zien met SAS. In twee verschillende onderzoekspopulaties onderzochten we karakteristieken van grijze stof in de hersenen.

In *Hoofdstuk 4* vat ik de bevindingen samen van een internationale mega-analyse in de grootste dataset van structurele MRI hersenscans die er op dat moment beschikbaar was (Bas-Hoogendam et al., 2017a). Deze hersenscans waren afkomstig van onderzoekscentra over de hele wereld (*Figuur 12.6*); de totale dataset bestond uit 174 MRI scans van patiënten met SAS, en 213 MRI scans van gezonde deelnemers. We onderzochten of het zogenaamde 'grijze stof volume' *geassocieerd was met SAS* (eerste endofenotype criterium), en maakten gebruik van 'voxel-based morphometry (VBM)', een gestandaardiseerde methode die gedetailleerd (op een schaal van millimeters) een inschatting maakt van de verhouding grijze stof in het brein (Ashburner & Friston, 2000, 2001). De resultaten van deze analyse lieten zien dat patiënten met SAS meer grijze stof hadden in twee diepgelegen gebieden in het brein: de



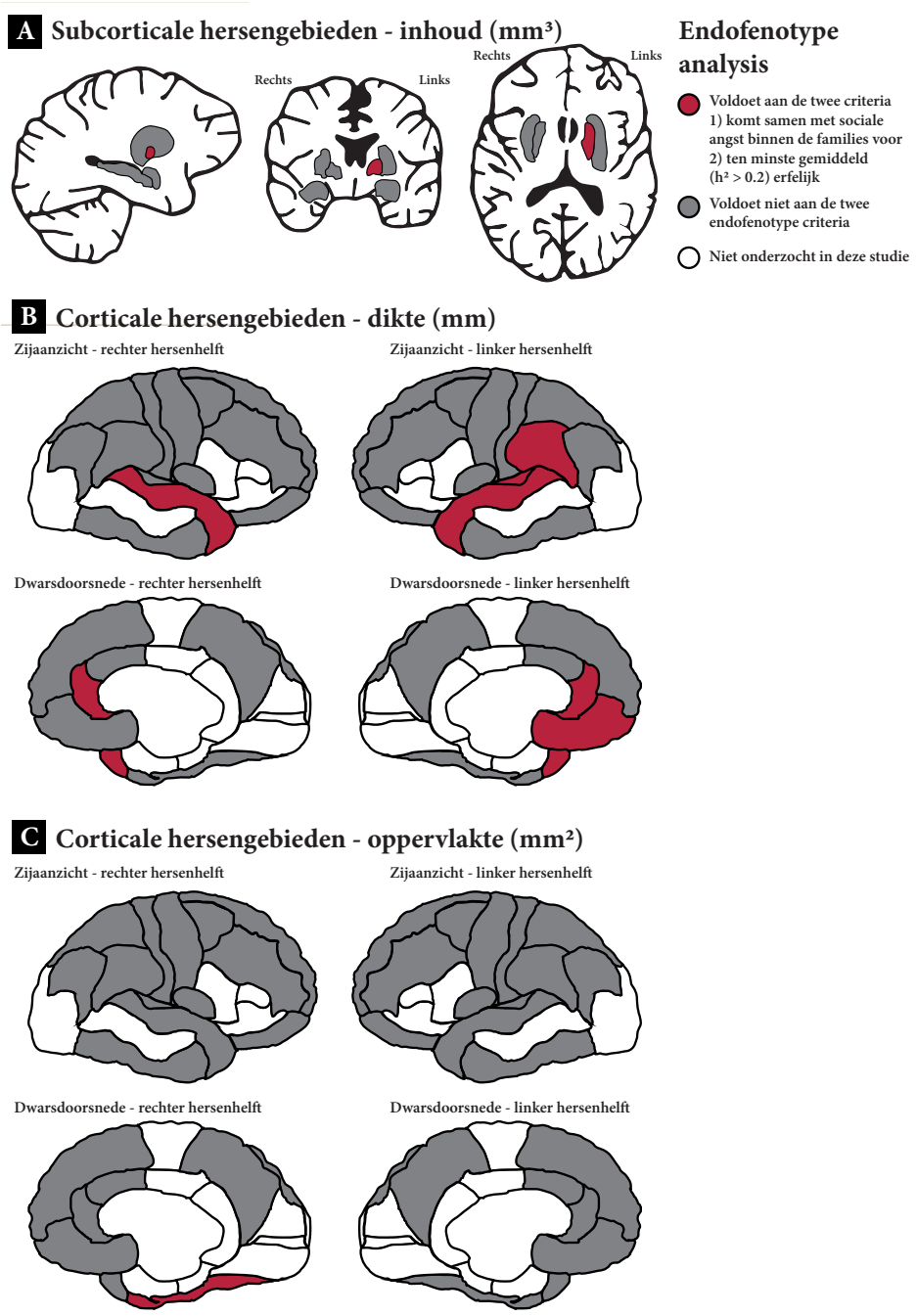
Figuur 12.6 Onderzoekscentra gerepresenteerd in de mega-analyse naar SAS.

putamen en het pallidum, beide onderdeel van het dorsale striatum. Deze toename in grijze stof was bovendien gerelateerd aan de hoeveelheid sociale angstsymptomen die deelnemers rapporteerden. Deze bevindingen geven aan dat grijze stof volume in het dorsale striatum *geassocieerd is met SAS.*

In het onderzoek dat beschreven staat in *Hoofdstuk 5* bouwden we op deze resultaten verder (Bas-Hoogendam et al., 2018b). We onderzochten of veranderingen in grijze stof in het brein beschouwd kunnen worden als endofenotypes van SAS. Om deze vraag te beantwoorden maakten we gebruik van de gedetailleerde anatomische MRI scans van het LFLSAD onderzoek (*Figuur 12.2A)*, en toetsten twee endofenotype criteria. Ten eerste onderzochten we of karakteristieken van grijze stof *samen met SAS binnen de familie vóórkwamen.* Ook maakten we een schatting van de *erfelijkheid* van deze grijze stof karakteristieken. Omdat het statistisch op het moment van het onderzoek niet mogelijk was om de hierboven beschreven VBM-methode toe te passen binnen het familieonderzoek, gebruikten we een andere methode. We kozen voor het software pakket FreeSurfer, dat volgens een aantal geautomatiseerde stappen een schatting maakt van [1)] de *inhoud (*gemeten in kubieke millimeters) van diepgelegen, zogenaamde 'subcorticale' hersengebieden; [2)] de *dikte* van het laagje grijze stof aan de buitenkant van het brein (de zogenaamde 'corticale gebieden'; gemeten in millimeters); en [3)] de *oppervlakte* van deze corticale hersengebieden (uitgedrukt in vierkante millimeters). We beperkten onze analyses tot hersengebieden die in eerder onderzoek geassocieerd waren met SAS, en vonden opnieuw, net als in *Hoofdstuk 4*, een positief verband tussen de inhoud van het pallidum en de mate van sociale angst binnen de families. Bovendien bleek het volume van het pallidum deels erfelijk te zijn. Verder vonden we verspreid over het brein veranderingen in de dikte en het oppervlakte van corticale hersengebieden die *samen met SAS binnen de familie aanwezig waren;* bovendien bleken deze hersenveranderingen veelal *erfelijk* te zijn. Alhoewel deze resultaten statistisch niet sterk genoeg bleken om een correctie voor het aantal testen te doorstaan, bieden ze het eerste, voorlopige bewijs dat karakteristieken van grijze stof in het brein (geïllustreerd in *Figuur 12.7*) endofenotypes van sociale angst zijn.

Karakteristieken van hersenfunctie: endofenotypes van SAS?

In het derde deel van dit proefschrift beschrijf ik onderzoek naar veranderingen in de *functie* van het brein die gerelateerd zijn aan SAS. Eerder onderzoek, zoals samengevat door Brühl en collega's (2014) en Cremers & Roelofs (2016), liet zien dat SAS vaak samengaat met sterkere hersenactiviteit in subcorticale en corticale (namelijk in frontale, pariëtale en occipitale) hersengebieden. Meestal maakten deze eerdere studies gebruik van onderzoeksparadigma's waarin de reactie van deelnemers op angst-opwekkende stimuli gemeten kon worden. Binnen de Leidse Familiestudie naar Sociale Angststoornis gebruikten we twee paradigma's, elk gericht op een ander aspect van neurocognitief functioneren in SAS. We



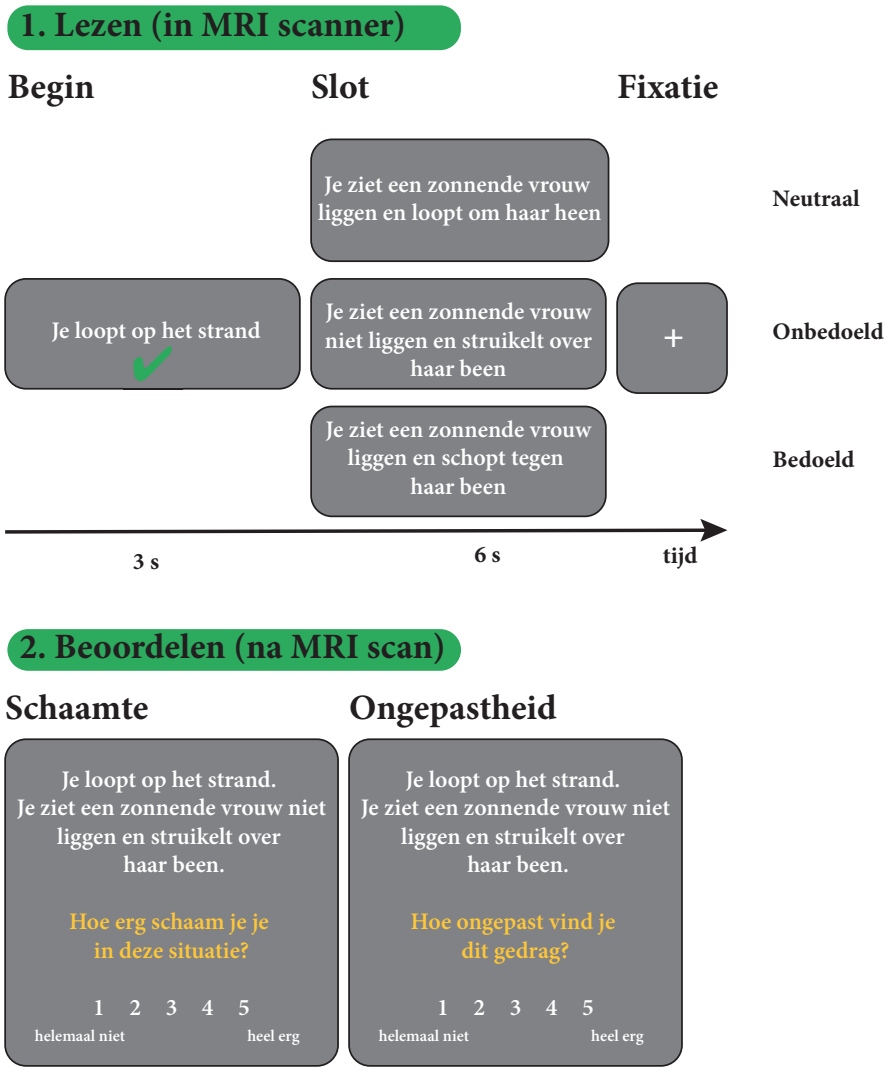
Figuur 12.7 Overzicht van grijze stof karakteristieken die veelbelovende endofenotypes van SAS zijn. Deze figuur is gebaseerd op Figure 5, eerder gepubliceerd in Bas-Hoogendam et al. (2018b, *EbioMedicine*).

onderzochten of hersenactiviteit tijdens deze functionele taken, gemeten met fMRI, een mogelijk endofenotype van SAS is.

De reactie op sociale norm overschrijdingen

Het eerste functionele paradigma dat we gebruikten, de zogenaamde 'Sociale Norm Taak' (in het Engels: Social Norm Processing Task, afgekort SNPT) richt zich op de angst van sociaal-angstige personen om *onbedoeld* een sociale norm te overschrijden in de aanwezigheid van anderen (Moscovitch, 2009); bij deze taak is vooral de mediale prefrontale cortex, het hersengebied net achter het voorhoofd dat van belang is bij het reflecteren op jezelf en op het gedrag van anderen, van belang. De SNPT is geïllustreerd in *Figuur 12.8*. Tijdens de SNPT lezen deelnemers, terwijl ze in de MRI scanner liggen, korte verhaaltjes die geprojecteerd worden op een scherm. We vroegen de deelnemers om zich in de verhaaltjes in te leven en zich voor te stellen wat hun eerste reactie zou zijn in de beschreven situaties. Elk verhaaltje bestond uit twee zinnen; de eerste zin beschreef een bepaalde situatie (bijvoorbeeld: 'Je loopt op het strand'), de tweede zin gaf aan wat er vervolgens gebeurde. Elke beginzin werd (in afwisselende volgorde) gecombineerd met een slotzin die ofwel een *neutrale* situatie weergaf (bijvoorbeeld: 'Je ziet een zonnende vrouw liggen en loopt om haar heen.'), of een situatie waarin een sociale norm *per ongeluk* ('Je ziet een zonnende vrouw niet liggen en struikelt over haar been.'), dan wel *expres* werd overschreden ('Je ziet een zonnende vrouw liggen en schopt tegen haar been.'). Na afloop van de MRI scan lazen de deelnemers alle verhaaltjes nog eens op een laptop, en dit keer vroegen we hen om aan te geven hoe ongepast ze de beschreven situaties vonden, en hoe erg ze zich zouden schamen wanneer ze zich zelf in een dergelijke situatie zouden bevinden.

Door bij elk verhaaltje de drie mogelijke vervolgzinnen te presenteren kan de SNPT gebruikt worden om te onderzoeken welke verschillen in hersenactiviteit en gedrag (beoordelingen van ongepastheid en schaamte) er bestaan bij het inleven in *bedoelde* dan wel *onbedoelde* overschrijdingen van sociale normen, en hoe deze gerelateerd zijn aan sociale angst. In de Engelstalige wetenschappelijke literatuur is de SNPT eerder beschreven (Berthoz et al., 2002, 2006; Blair et al., 2010), en om deze taak binnen de LFLSAD te kunnen gebruiken brachten we een aantal veranderingen in de taak aan. Dit proces is beschreven in *Hoofdstuk 6* van het proefschrift (Bas-Hoogendam, et al., 2017b). Allereerst zorgden we ervoor dat de verhaaltjes ook geschikt zouden zijn voor kinderen en adolescenten; ook creëerden we aparte versies voor mannen en vrouwen, zodat de verhaaltjes waarin deelnemers zich moesten inleven zo goed mogelijk aansloten bij ieders persoonlijke situatie. Op deze manier bestond onze herziening van de SNPT uit vier versies: voor jongens jonger dan 18 jaar, voor meisjes jonger dan 18 jaar, voor mannen vanaf 18 jaar, en voor vrouwen vanaf 18 jaar. De verschillen tussen de versies waren klein, maar betroffen bijvoorbeeld het afzakken van een zwembroek danwel bikinibroekje bij een duik in het zwembad (man / vrouw verschil), en het vervangen van een situatie in een klaslokaal met een leraar (versies voor jongens



Figuur 12.8 Overzicht van de herziene Sociale Norm taak (Social Norm Processing Task; SNPT-R). Tijdens de lees-fase, die plaatsvindt in de MRI scanner (1), lezen deelnemers korte verhaaltjes die uit twee zinnen bestaan. Deze verhalen beschrijven een neutrale sociale situatie, een situatie waarin per ongeluk een sociale norm wordt overgeschreden, of een situatie waarin met opzet een sociale norm geschonden wordt. We vroegen deelnemers om zich in de situaties in te leven. Na afloop van de scan (2) beoordeelden deelnemers de verhalen met het oog op schaamte en ongepastheid. Deze figuur is gebaseerd op Figure 1, eerder gepubliceerd in Bas-Hoogendam et al. (2017b, *PLOS ONE*).

en meisjes) door een werkomgeving met een collega (versie voor volwassen mannen en vrouwen). Verder brachten we een aantal methodologische verbeteringen aan, en zorgden we ervoor dat de verhaaltjes Nederlandstalig waren (originele versie: Engelstalig).

De betrouwbaarheid van deze herziene versie van de SNPT onderzochten we in adolescenten en volwassenen uit de algemene bevolking. Volgens onze verwachtingen, en in lijn met eerder onderzoek, beoordeelden deelnemers de *bedoelde* overschrijdingen van sociale normen als ongepaster, en als meer beschamend, dan de *onbedoelde* sociale norm overschrijdingen. Analyses van de fMRI scans, waarmee we hersenactiviteit tijdens het inleven in bedoelde en onbedoelde sociale norm overschrijdingen konden vergelijken, lieten zien dat bepaalde hersengebieden bij beide processen betrokken waren, terwijl andere gebieden enkel actief waren bij het inleven in onbedoelde overschrijdingen. Deze resultaten zijn beschreven in *Hoofdstuk 6*.

In een vervolgonderzoek zochten we uit wat de relatie was tussen de mate waarin deelnemers sociale angstklachten rapporteerden en de beoordelingen van ongepastheid en schaamte voor de drie verschillende soorten situaties (*Hoofdstuk 7*) (Bas-Hoogendam et al., 2018c). In overeenstemming met onze verwachtingen, die gebaseerd waren op eerder onderzoek in patiënten met SAS (Blair et al., 2010), vonden we een positieve relatie tussen de mate van sociale angst en de beoordelingen. De sterkste resultaten vonden we voor de schaamte-beoordelingen van de situaties die *onbedoelde* sociale norm overschrijdingen weergaven: deelnemers met weinig tot gemiddelde niveaus van sociale angst beoordeelden deze onbedoelde sociale norm overschrijdingen als minder beschamend dan de bedoelde sociale norm overschrijdingen. Dat wil zeggen dat zij onderscheid maakten tussen het bewust en opzettelijk ingaan tegen de sociale standaarden aan de ene kant, en het begaan van een onbedoelde blunder aan de andere kant. Deelnemers met méér dan gemiddelde niveaus van sociale angst vonden de *onbedoelde* overschrijdingen echter net zo beschamend als de *bedoelde* overschrijdingen van sociale normen. Deze bevindingen suggereren dat deze sterkere ervaring van schaamte, die vaak gepaard gaan met een negatief zelfbeeld, een rol speelt in de ontwikkeling van SAS, en ook bijdraagt aan het in stand houden van de aandoening.

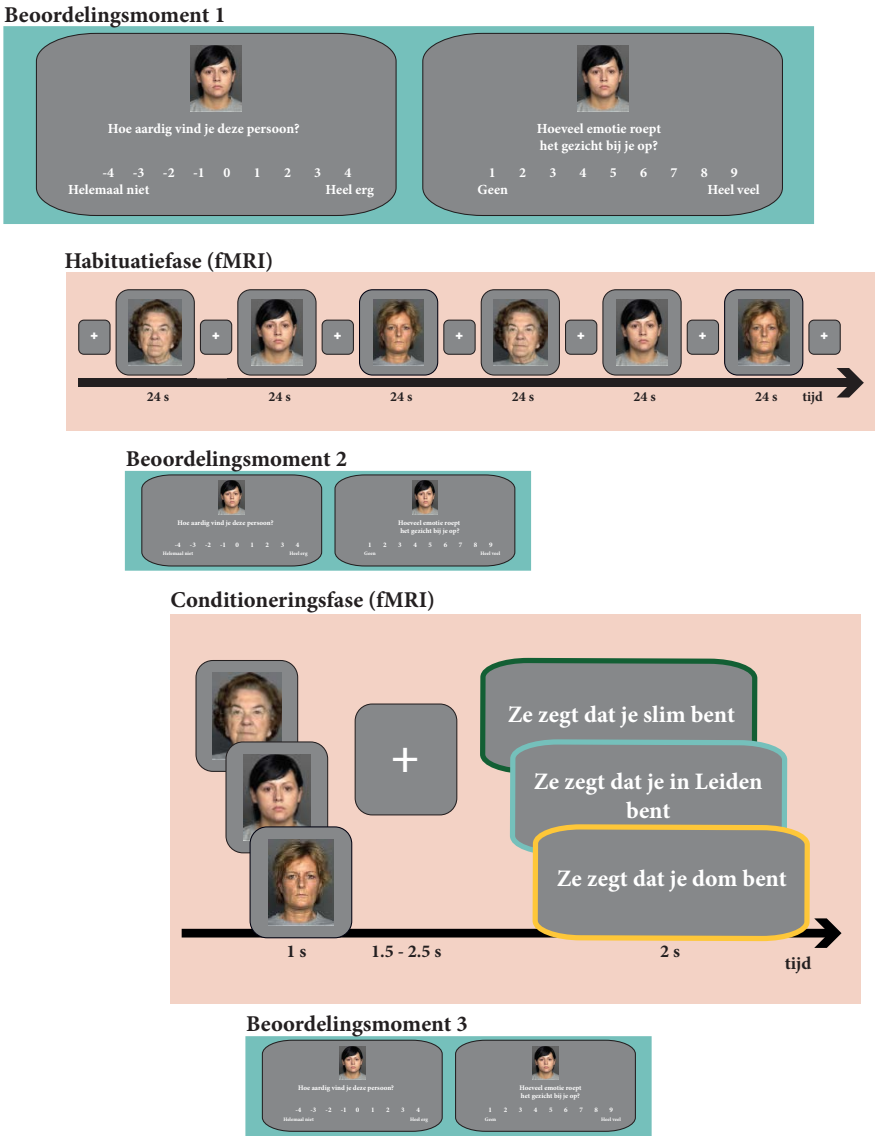
In het volgende hoofdstuk (*Hoofdstuk 8*) beschrijf ik vervolgens het onderzoek waarin we de herziene SNPT gebruikten om in de families van de LFLSAD de reactie op sociale norm overschrijdingen in kaart te brengen (Bas-Hoogendam et al., 2019a). Op basis van onze bevindingen zoals samengevat in *Hoofdstuk 7,* en eerder onderzoek in patiënten met SAS uitgevoerd door Blair en collega's (Blair et al., 2010), onderzochten we of neurobiologische (dat wil zeggen: hersenactiviteit) en gedragsmatige (beoordelingen van schaamte) reacties op met name *onbedoelde* sociale norm overschrijdingen voldeden aan de criteria voor endofenotypes. Net als in *Hoofdstuk 5* beoordeelden we twee endofentoype criteria: het *samen vóórkomen van het endofenotype met sociale angst binnen de families* en de *erfelijkheid* ervan.

De fMRI resultaten lieten inderdaad een positief verband zien tussen sociale angst en hersenactiviteit in de mediale prefrontale cortex en temporale hersengebieden (mediale en bovengelegen temporale gyrus, met daar tussen de temporale sulcus), gerelateerd aan het zich inleven in onbedoelde overschrijdingen van sociale normen. Verder bleken deze patronen van hersenactiviteit deels erfelijk te zijn. Onze verwachtingen met betrekking tot de beoordelingen van schaamte werden slechts deels door de onderzoeksgegevens ondersteund: we vonden een positief verband tussen de mate van sociale angst en de hoeveelheid schaamte die deelnemers rapporteerden, maar dit verband was niet specifiek voor de *onbedoelde* overschrijdingen, en was bovendien niet erfelijk. Samengevat bieden de resultaten van deze studie sterke aanwijzingen dat hersenactiviteit gerelateerd aan het verwerken van sociale norm overschrijdingen een endofenotype van SAS kan vormen.

De reactie op neutrale gezichten

Het tweede paradigma dat we toepasten om binnen de families van de LFLSAD naar de relatie tussen sociale angst en de mate van hersenactiviteit te kijken maakt gebruik van foto's waarop gezichten met een neutrale uitdrukking te zien zijn. Neutrale gezichten zijn, in sociaal opzicht, relevante stimuli, die echter een onduidelijke betekenis hebben: neigen ze naar positief, of hebben ze een negatieve lading? De amygdala (amandelkern) in het brein is van groot belang voor het interpreteren van de emotionele inhoud van gezichten, en door middel van het 'neutrale gezichten paradigma' (NGP) brachten we de functie van de amygdala in sociaal-angstige families in kaart. Het NGP dat we speciaal voor de LFLSAD ontwierpen bestaat uit twee fases, waardoor we de functie van de amygdala in twee verschillende processen konden bestuderen (*Figuur 12.9*).

In de eerste fase, de zogenaamde 'habituatie fase', onderzochten we het verloop van hersenactivatie tijdens het herhaaldelijk presenteren van drie neutrale gezichten. Habituatie houdt een afname in hersenactiviteit in, die meetbaar is als een bepaalde stimulus herhaaldelijk getoond wordt zonder dat er een betekenisvolle consequentie aan verbonden is. Deze habituatie-reactie heeft een belangrijke functie; door na verloop van tijd minder hersenactiviteit te spenderen aan een stimulus die toch niet werkelijk van belang blijkt te zijn, worden individuen in staat gesteld om zich te richten op andere, mogelijk belangrijkere zaken. Eerder onderzoek, in jongeren met een gedragsvermijdend temperament en in jongvolwassenen met sociale angst (Avery & Blackford, 2016; Blackford et al., 2013), heeft laten zien dat habituatie van hersenactiviteit in deze groepen niet, of slechts in mindere mate, optreedt. Gebaseerd op deze resultaten verwachtten wij dat afwijkingen in de habituatiereactie een endofenotype van sociale angst zou kunnen zijn. Inderdaad lieten de resultaten van de familiestudie zien dat er in familieleden met meer sociale angst, minder habituatie meetbaar was in de amygdala en hippocampus: deze hersenstructuren bleven actief reageren op de neutrale gezichten, tijdens de gehele tijdspanne van de taak, en de gebruikelijke afname in activiteit bleef uit. Verminderde habituatie kwam dus *samen met sociale angst*



Figuur 12.9 Overzicht van het neutrale gezichten paradigma (NGP).
Het paradigma bestaat uit twee fMRI fases, een habituatiefase en een conditioneringsfase. Tijdens de habituatiefase worden drie neutrale gezichten herhaaldelijk gepresenteerd; tijdens de conditioneringsfase worden de gezichten gecombineerd met positieve, negatieve, of neutrale beoordelingen, waardoor deelnemers de sociale waarde van de gezichten kunnen leren. Op drie momenten tijdens het NGP beoordelen de deelnemers de gezichten, en geven daarbij onder andere aan hoe aardig ze de persoon op de foto vinden. De foto's van de neutrale gezichten zijn afkomstig uit de FACES database (Ebner et al., 2010).

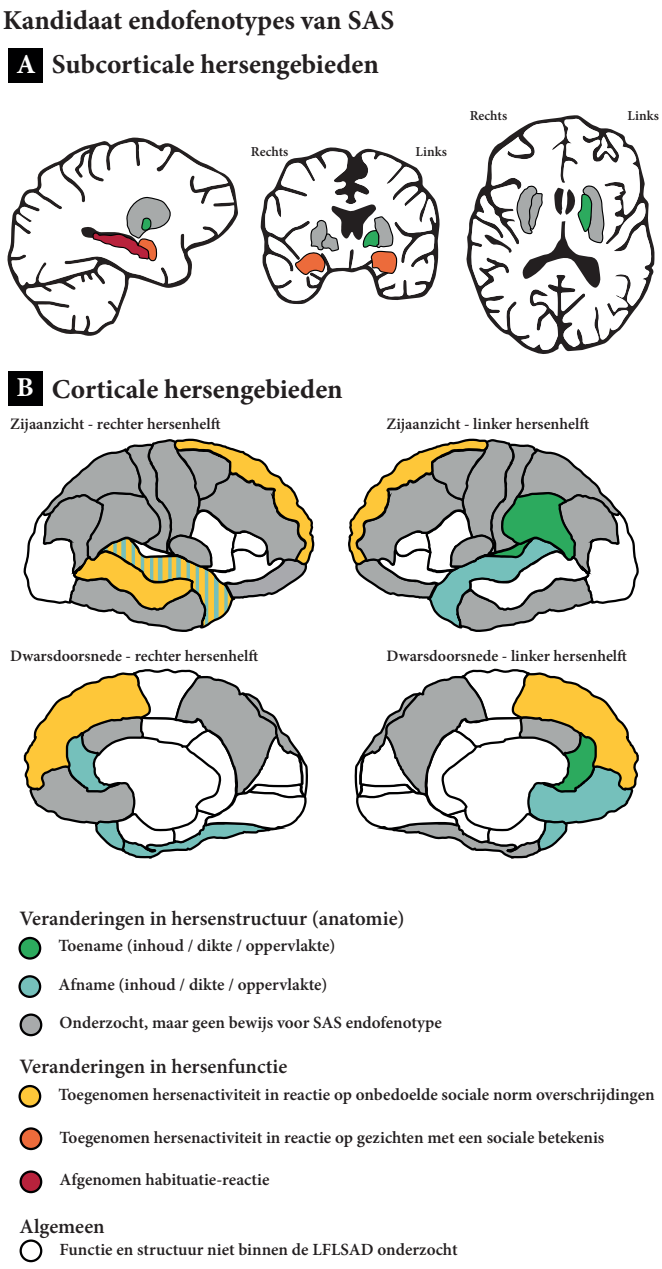
binnen de families voor; bovendien bleek deze verminderde habituatie-reactie, specifiek in de rechter hippocampus, deels erfelijk te zijn. Deze resultaten, beschreven in *Hoofdstuk 9* van het proefschrift, laten zien dat verminderde habituatie een endofenotype van SAS is (Bas-Hoogendam et al., 2019b).

In de tweede fase van het NGP werden de drie neutrale gezichten opnieuw gepresenteerd, maar dit keer werd elk gezicht direct gevolgd door een zin die betrekking had op de deelnemer zelf: het waren beoordelende zinnen met een positieve ('zij zegt dat je slim bent'), negatieve ('zij zegt dat je dom bent') of neutrale ('zij zegt dat je in Leiden bent') inhoud. Doordat elk neutraal gezicht consequent gevolgd werd door één van deze drie soorten feedback konden deelnemers de betekenis van de drie gezichten leren kennen. Dat dit leerproces (ook wel *conditionering* genoemd) inderdaad plaatsvond bleek uit de onderzoeksgegevens: op verschillende momenten tijdens het NGP vroegen we deelnemers om aan te geven hoe aardig ze de personen op de foto's vonden (*Figuur 12.9*). Na de conditioneringsfase vonden de deelnemers het gezicht gecombineerd met de negatieve feedback significant minder aardig, terwijl ze het gezicht dat voorafging aan de positieve feedback juist aardiger waren gaan vinden.

Eerder onderzoek waarin deze conditioneringstaak werd gebruikt liet zien dat de amygdala een belangrijke functie heeft tijdens dit leerproces (Davis et al., 2010), maar de relatie tussen amygdala-functioneren tijdens dit conditioneringsproces en sociale angst is niet eerder onderzocht, laat staan in families met sociale angst. In *Hoofdstuk 10* beschrijf ik het onderzoek waarin we de functie van de amygdala tijdens conditionering onderzochten, in de families van de LFLSAD (Bas-Hoogendam et al., 2019c). We vonden verhoogde amygdala activiteit in reactie op de geconditioneerde gezichten, die *samen met sociale angst binnen de families voorkwam,* en die voor een deel *erfelijk bepaald* bleek te zijn. Een interessante bevinding betreft het resultaat dat deze overactiviteit van de amygdala niet, zoals we van te voren gedacht hadden, specifiek was voor het bekijken van het gezicht met een negatieve lading; ook de andere gezichten leidden tot overactiviteit in de amygdala, waarschijnlijk doordat de deelnemers in alle situaties direct aangesproken werden ('Zij zegt dat je…'). Kortom, deze bevindingen geven aan dat de verhoogde activiteit van de amygdala in reactie op gezichten met een aangeleerde sociale betekenis een neurobiologisch endofenotype van SAS zou kunnen zijn.

Samenvatting van de neurobiologische genetische kwetsbaarheid voor het ontwikkelen van SAS

De hierboven beschreven bevindingen van de LFLSAD heb ik samengevat in *Figuur 12.10*. Deze grafische weergave representeert de neurobiologische veranderingen in het brein die *samen met sociale angst binnen de families vóórkomen* en ten minste deels *erfelijk* ($h^2 \geq 0.20$)



Figuur 12.10 Grafische samenvatting van neurobiologische endofenotypes van SAS, op basis van onderzoeksgegevens van de Leidse Familiestudie naar Sociale Angststoornis (LFLSAD).
Hersengebieden in *felle kleuren* geven regio's aan waarin de functie en / of de structuur samen met sociale angst binnen de families voorkomt, en die ten minste deels erfelijk zijn. Regio's in *grijs* geven gebieden aan waarin geen veranderingen gevonden werden, terwijl regio's weergegeven in *wit* niet specifiek onderzocht werden in de studie.

zijn, en zodoende als endofenotypes van SAS beschouwd kunnen worden. Deze veranderingen in structuur dan wel functie zijn aangegeven met heldere kleuren. Regio's waarin we geen veranderingen vonden met betrekking tot de anatomie van het brein (zoals beschreven in *Hoofdstuk 5*) zijn grijs van kleur; hersengebieden die niet expliciet onderzocht zijn in het proefschrift zijn weergeven in wit. Ik wil benadrukken dat deze samenvatting een momentopname is van 'werk in uitvoering', een punt dat ik later verder zal uitwerken. Desondanks biedt deze grafische samenvatting aanleiding voor een aantal opvallende observaties, die ik kort zal samenvatten.

Meerdere hersengebieden zijn betrokken bij de genetische kwetsbaarheid voor SAS

Allereerst maakt *Figuur 12.10* duidelijk dat de hersenkarakteristieken die gerelateerd zijn aan de genetische kwetsbaarheid voor het ontwikkelen van SAS, verspreid zijn over het hele brein. Deze regio's, waarvan de functie en / of de structuur gezien kan worden als een potentieel endofenotype van SAS, komen grotendeels overeen met de regio's die beschreven werden in een eerder neurobiologisch model van SAS (Brühl, Delsignore, et al., 2014). Dit model, dat gebaseerd was op een kwalitatief onderzoek en een meta-analyse van 76 eerder verschenen neuroimaging onderzoeken in volwassen patiënten met SAS, beschrijft veranderingen in hersenfunctie in subcorticale, frontale, pariëtale en occipitale hersengebieden, en ook afwijkingen in de netwerkstructuur van het brein. Met hun model breidden Brühl en collega's destijds een ouder model van Etkin & Wager uit, dat functionele hersenveranderingen in het zogenaamde 'angst netwerk' van het brein beschreef in patiënten met angststoornissen (Etkin & Wager, 2007); de resultaten van dit proefschrift stellen ons in staat om op onze beurt het model van Brühl uit te breiden, door aan te geven dat sommige van deze hersenveranderingen niet alleen maar *biomarkers* zijn (dat wil zeggen: gerelateerd aan de aandoening maar niet noodzakelijkwijs betrokken bij de genetische kwetsbaarheid voor SAS), maar ook gezien kunnen worden als *endofenotypes* van de aandoening, en als zodanig onderdeel uitmaken van de manier waarop genetische veranderingen leiden tot SAS (Meyer-Lindenberg & Weinberger, 2006). Dit onderscheid tussen biomarkers en endofenotypes is belangrijk, omdat het aangeeft dat de neurobiologische veranderingen die samengevat zijn in *Figuur 12.10* niet het resultaat zijn van het (vaak chronische) verloop van SAS, en ook niet toe te schrijven zijn aan de effecten van psychologische behandeling, medicatie, of door het samen vóórkomen van SAS met aan SAS gerelateerde psychiatrische stoornissen (Beauchaine & Constantino, 2017; Lenzenweger, 2013a). Onze resultaten geven daarmee aan dat SAS al in een vroeg stadium (namelijk, al op het niveau van aangeboren kwetsbaarheid) meerdere hersengebieden omvat.

Het striatum: een nieuwe speler in beeldvormend onderzoek naar angst

Een ander opvallend resultaat van dit proefschrift betreft de rol van het dorsale striatum, een diepgelegen hersenkern waarvan onder andere het pallidum en het putamen deel uit maken. Dit hersengebied, dat pas in de laatste jaren geleidelijk meer aandacht heeft gekregen in het kader van beeldvormend onderzoek naar angst, was nog geen onderdeel van het neurobiologische model van Brühl en collega's (2014). In twee onafhankelijke studies, namelijk de internationale mega-analyse beschreven in *Hoofdstuk 4* van dit proefschrift, en in de endofenotype studie in families van de LFLSAD (*Hoofdstuk 5),* vonden we positieve associaties tussen sociale angst en de hoeveelheid grijze stof in deze hersengebieden. Deze bevindingen zijn recent door andere studies gerepliceerd. Zo vond een onderzoek in gezonde individuen een omgekeerd verband tussen de mate waarin deze personen om kunnen gaan met onzekere situaties en het volume van het striatum (Kim et al., 2017), terwijl de resultaten van een andere studie lieten zien dat gezonde vrouwen die meer sociale angst rapporteren een groter striatum hebben (Günther et al., 2018). Verder is het interessant om te vermelden dat in een recent onderzoek, in een groep van honderden patiënten met een psychiatrische stoornis (depressie, post-traumatische stressstoornis, obsessieve-compulsieve stoornis of schizofrenie) en een kleinere groep familieleden van deze patiënten, een vergroting van het putamen gerelateerd was aan de erfelijke kwetsbaarheid om een psychiatrische stoornis te ontwikkelen (Gong et al., 2019).

Deze bevindingen sluiten aan bij onze resultaten, en maken tegelijkertijd duidelijk dat een vergroot striatum niet uitsluitend te vinden is in SAS, maar ook in andere psychiatrische aandoeningen meetbaar is. Dit is echter niet in tegenspraak met de bewering dat een vergroot striatum een endofenotype van SAS is. In een veelgeciteerd overzichtsartikel zetten Cannon en Keller namelijk uiteen dat specificiteit geen vereiste is voor een endofenotype; bepaalde endofenotypes kunnen bijdragen aan de kwetsbaarheid voor meerdere angst- en stemmingsstoornissen (Cannon & Keller, 2006). Voortbordurend op deze uitspraak is mijn hypothese, op basis van de hierboven beschreven bevindingen, dat een vergroot dorsaal striatum de algemene genetische kwetsbaarheid voor angst- en stemmingsstoornissen weerspiegelt (Ohi et al., 2019; Shimada-Sugimoto et al., 2015). Deze verwachting is in lijn met onlangs gepubliceerde onderzoeksresultaten, die aantoonden dat de genetische variaties die geassocieerd zijn met verschillende psychiatrische aandoeningen, onderling sterk overeenkomen (Anttila et al., 2018). Verder wordt mijn hypothese ondersteund door een recent artikel dat beschrijft dat het striatum een essentiële rol speelt in drie psychologische processen die vaak aangetast zijn in angst- en stemmingsstoornissen, namelijk aandachtsprocessen, leerprocessen, en motivatie (Lago et al., 2017). Deze bevindingen geven aanleiding om de anatomische variatie en de functionele veranderingen in het striatum in toekomstige onderzoeken verder onder de loep te nemen, om daardoor meer inzicht te verkrijgen in de genetische kwetsbaarheid voor psychiatrische aandoeningen in het algemeen, en voor sociale angst in het bijzonder.

Een overactief, emotioneel brein

De laatste observatie met betrekking tot de samenvatting van de genetische kwetsbaarheid voor SAS (*Figuur 12.10),* die tegelijkertijd ook een overeenkomst met het model van Brühl (2014) weergeeft, betreft de bevinding dat beide modellen enkel *toenames* van hersenactiviteit in SAS impliceren. Op basis van de onderzoeksgegevens van de LFLSAD vonden wij *meer* amygdala activiteit in reactie op neutrale gezichten met een sociaal-relevante betekenis (*Hoofdstuk 10), meer* hersenactiviteit bij het inleven in sociale situaties waarin een sociale norm onbedoeld werd overschreden (*Hoofdstuk 8),* en een *langer aanhoudende* hersenactiviteit bij het herhaaldelijk zien van neutrale gezichten (*Hoofdstuk 9).* Dit komt overeen met de resultaten van de meta-analyse van Brühl en collega's (Brühl et al., 2014), die lieten zien dat meerdere hersengebieden *meer* actief zijn in patiënten met SAS dan in gezonde deelnemers. In hun overzichtsartikel beschrijven Brühl en anderen (2014) dat deze toegenomen activiteit waarschijnlijk toe te schrijven is aan het feit dat emotionele stimuli, bijvoorbeeld gezichten, meer gevoelens van opwinding teweeg brengen in patiënten met SAS. De auteurs zetten vervolgens een aantal mogelijke onderliggende mechanismen op een rij (Brühl, Delsignore, et al., 2014), maar het voert te ver om die in deze samenvatting weer te geven. Wel is het van belang om op te merken dat er wel degelijk fMRI onderzoeken bekend zijn waarin gevonden werd dat bepaalde hersengebieden juist mínder actief zijn in patiënten met SAS (Brühl et al., 2011; Sareen et al., 2007), tijdens het uitvoeren van opdrachten die geen deel uitmaakten van het protocol van de LFLSAD.

Werk in uitvoering en suggesties voor toekomstig onderzoek

Deze opmerking brengt mij haast automatisch bij een belangrijke algemene kanttekening met betrekking tot de samenvatting zoals die weergegeven is in *Figuur 12.10,* namelijk, dat dit overzicht van de neurobiologische genetische kwetsbaarheid voor het ontwikkelen van SAS nog niet compleet is, en door vervolgonderzoek aangevuld moet worden. Ik schets kort vier onderzoekslijnen die in dit verband van belang zijn.

Ten eerste is het van belang om aanvullende neurocognitieve onderzoeksparadigma's te gebruiken om hersenactiviteit in sociaal-angstige families te onderzoeken. Binnen het protocol van LFLSAD was ruimte voor twee zorgvuldig geselecteerde fMRI taken (het Neutrale Gezichten Paradigma en de Sociale Norm Taak, *Figuur 12.2),* maar het was niet mogelijk om extra scans aan het protocol toe te voegen: de MRI sessie zou dan te lang duren en onprettig worden voor de deelnemers. Recent gepubliceerde onderzoeken hebben echter laten zien dat andere, nieuwe onderzoeksparadigma's ook veranderingen in hersenfunctie in SAS patiënten in beeld kunnen brengen; dit betreft bijvoorbeeld een studie die hersenactiviteit in reactie op het horen van menselijke stemmen onderzocht (Kreifelts et al., 2019),

en een onderzoek dat zich richtte op het verwerken van positieve en negatieve feedback door patiënten met SAS (Peterburs et al., 2016). De resultaten van deze en andere studies (bijvoorbeeld Figel et al., 2019; Kim et al., 2018) maken duidelijk dat SAS geassocieerd is met veranderingen in hersenfunctie in gebieden die niet binnen de LFLSAD onderzocht zijn; bovendien is het belangrijk om ervan bewust te zijn dat de context bepaalt of een bepaald hersengebied meer of minder actief is in patiënten met SAS (Kraus et al., 2018). Toekomstig onderzoek is nodig om vast te stellen of deze veranderingen in hersenfunctie die geassocieerd zijn met SAS, ook voldoen aan de endofenotype criteria.

Ten tweede is het interessant om de verbindingen tussen hersengebieden in kaart te brengen, en te onderzoeken in hoeverre veranderingen in deze netwerken endofenotypes van SAS zijn. Binnen de LFLSAD maakten we scans om deze netwerken in kaart te brengen (*Figuur 12.2B* en *Figuur 12.2E),* maar deze scans zijn nog niet geanalyseerd. Onderzoek dat erop gericht is om deze netwerken in SAS en in familieleden van SAS patiënten vast te stellen is van groot belang, omdat veranderingen in deze netwerken bij kunnen dragen aan de ontwikkeling en het verloop van psychiatrische stoornissen (Bassett & Sporns, 2017; Buckholtz & Meyer-Lindenberg, 2012; Morgan et al., 2018). Ook heeft eerder onderzoek vastgesteld dat genetische variaties van invloed zijn op de stabiliteit van deze hersennetwerken (Thompson, Ge, Glahn, Jahanshad, & Nichols, 2013). Endofenotype studies zijn van belang om vast te stellen of veranderingen in de integriteit van hersennetwerken bijdragen aan de genetische kwetsbaarheid voor het ontwikkelen van SAS.

Verder stel ik vervolgonderzoek voor dat erop gericht is om vast te stellen hoe stabiel de endofenotypes van SAS, die we in dit proefschrift beschrijven, zijn (endofenotype criterium 2; *Figuur 12.3*). De onderzoeksgegevens van de LFLSAD zijn niet geschikt om dit te bepalen, aangezien de deelnemers slechts één keer onderzocht zijn. Langlopend onderzoek met meerdere meetmomenten is essentieel om na te gaan of de gevonden endofenotypes inderdaad *stabiele kenmerken zijn,* en om vast te stellen in hoeverre therapie kan bijdragen aan het verminderen van de mate waarin het endofenotype tot uitdrukking komt. Het is in het bijzonder van belang om zulk langlopend onderzoek te doen bij adolescenten, aangezien SAS vaak in deze leeftijdsgroep voor het eerst tot uiting komt (Haller et al., 2018) en er in de adolescentie belangrijke veranderingen plaatsvinden in de functie en structuur van hersengebieden die een rol spelen in sociaal-emotionele processen (Blakemore, 2008; Crone & Dahl, 2012; Mills et al., 2012; Nelson et al., 2015). Daarnaast verbreden adolescenten letterlijk en figuurlijk hun leefwereld, waardoor een gevoeligheid voor sociale angst meer op de voorgrond kan komen te staan (Caouette & Guyer, 2014; Haller et al., 2015). Het is echter nog onbekend op welke manier deze processen inwerken op de genetische kwetsbaarheid voor het ontwikkelen van SAS. Daarom ben ik een sterk voorstander van langlopende onderzoeken in grote onderzoeksgroepen van adolescenten met en zonder een familiaire kwetsbaarheid voor het ontwikkelen van SAS. Dergelijk onderzoek stelt ons in staat om beter te begrijpen hoe de ontwikkeling van het brein tijdens de adolescentie en de genetische

kwetsbaarheid voor SAS interacteren in de ontwikkeling van sociale angst, en om vast te stellen welke individuele karakteristieken hierbij van belang zijn (Crone & Elzinga, 2015).

Ten slotte is het interessant om in vervolgonderzoek te verkennen welke genetische variaties geassocieerd zijn met de endofenotypes die in dit proefschrift beschreven zijn. Op basis van het buisje speeksel dat de deelnemers van de LFLSAD hebben afgestaan kunnen we gericht onderzoek doen naar DNA variaties die bijdragen aan de genetische kwetsbaarheid voor het ontwikkelen van SAS.

Methodologische overwegingen

In de afzonderlijke hoofdstukken van dit proefschrift heb ik, voor elk onderzoek, de sterke kanten en de beperkingen samengevat. Hier wil ik een aantal prominente eigenschappen van de LFLSAD in het algemeen voor het voetlicht brengen.

Allereerst is de LFLSAD, voor zover wij weten, de eerste en enige studie waarin, met behulp van beeldvormend onderzoek, het brein van meerdere generaties van familieleden van SAS patiënten in kaart gebracht wordt. Zoals al eerder vermeld kozen we voor een familiestudie om te kunnen onderzoeken in hoeverre hersenkarakteristieken *samen met sociale angst vóór komen binnen de families* en *erfelijk* zijn. Familiestudies leveren zo unieke informatie op over de genetische kwetsbaarheid voor het ontwikkelen van SAS, hebben veel statistische zeggingskracht en zijn kosteneffectief (Glahn et al., 2018). Er worden echter maar weinig familiestudies uitgevoerd, alhoewel we op de hoogte zijn van enkele familiestudies die in kaart brachten hoe en in welke mate symptomen van sociale angst en gerelateerde psychiatrische aandoeningen binnen meerdere generaties aanwezig waren (Knappe et al., 2009; Lawrence et al., 2019; Merikangas et al., 2003). Deze onderzoeken lieten zien dat SAS inderdaad vaak binnen families voorkomt, maar omdat er geen beeldvormend onderzoek plaatsvond verschaften deze onderzoeken geen inzicht in de neurobiologische veranderingen die hierin een rol spelen. Aan de andere kant zijn er enkele interessante MRI studies die kinderen van ouders met een (sociale) angststoornis hebben onderzocht (Christensen, Van Ameringen, & Hall, 2015; Suffren et al., 2019), maar in deze onderzoeken werden geen MRI scans gemaakt bij de ouders of andere familieleden, waardoor het niet mogelijk is om te bepalen of de hersenveranderingen die bij deze kinderen gevonden werden, endofenotypes zijn.

Wanneer we breder kijken en ons niet beperken tot het onderzoek naar SAS zijn er een klein aantal onderzoeken die, tot op zekere hoogte, een vergelijkbare opzet hebben als de LFLSAD. Ten eerste wil ik de 3G studie noemen, waarin de mate waarin opvoedstijlen, emotie-regulatie en stressverwerking in meerdere generaties familieleden doorgegeven worden, onder andere met behulp van beeldvormend onderzoek, in kaart gebracht wordt (van den Berg et al., 2018, 2019). Verder is er een langlopende familiestudie naar depressie,

die in 1982 werd gestart en waarin EEG- en MRI gegevens werden verzameld in drie opeenvolgende generaties van families waarin depressie veel voorkomt, of juist niet aanwezig is (Talati, Weissman, et al., 2013). Ten slotte kennen we familiestudies die zich richten op het vaststellen van de neurobiologische veranderingen die geassocieerd zijn met schizofrenie, bipolaire stoornis, depressie en obsessieve-compulsieve dwangstoornis (een aantal recente voorbeelden zijn beschreven in Blakey et al., 2018; Goghari, MacDonald, & Sponheim, 2014; Miskowiak et al., 2018; Vaghi et al., 2017; Yalin et al., 2019); deze onderzoeken betreffen vaak echter 'slechts' patiënten, enkele gezonde familieleden, en gezonde deelnemers die niet genetisch verwant zijn aan de patiënten, maar omvatten niet, zoals de LFLSAD, hele families. Kortom, de opzet van de LFLSAD is uniek, niet alleen binnen het onderzoek naar SAS, maar ook breder in het onderzoeksveld dat zich richt op de neurobiologische veranderingen die geassocieerd zijn met het ontstaan van psychiatrische stoornissen.

Het is van belang om stil te staan bij de vraag of de patiënten met SAS die deel uit maakten van de LFLSAD in bepaalde opzichten afwijken van SAS patiënten die in andere onderzoeken 'in hun eentje' werden onderzocht. Een eerder onderzoek toonde namelijk aan dat patiënten met schizofrenie die deel uit maakten van een familiestudie (waarbij het dus van belang was dat ook hun familieleden bij het onderzoek betrokken waren) gemiddeld genomen jonger en hoger opgeleid waren, en beter presteerden op neurocognitieve opdrachten, in vergelijking met patiënten die deelnamen aan studies waarin 'enkel' patiënten en gezonde controle deelnemers betrokken waren (Gur et al., 2015). Gebaseerd op deze bevinding stelden Glahn en collega's (2018) dat 'studies die gebruik maken van een patiënt-controle opzet mogelijk deelnemers bevatten die blootgesteld zijn aan factoren die een negatieve invloed hebben op het ziektebeloop, in vergelijking met deelnemers die deel uit maken van studies waarin het van belang is dat ook hun familieleden betrokken zijn'. Deze vertekening zou ook binnen de LFLSAD een rol kunnen spelen, aangezien families geselecteerd waren op basis van de combinatie van een ouder met SAS en een kind met symptomen van SAS; verder streefden we ernaar om minimaal acht deelnemers per familie in de studie te includeren, wat in de praktijk vaak betekende dat de patiënt met SAS tenminste één van zijn of haar broers / zussen moest willen en kunnen benaderen voor deelname aan het onderzoek. Gegeven bevindingen uit eerder onderzoek dat patienten met SAS gemiddeld genomen minder vaak getrouwd zijn (Wells et al., 1994), en de observatie dat patiënten met SAS vaker ontevreden zijn over hun familieleven (Stein & Kean, 2000) en meer moeilijkheden rapporteren in het onderhouden van relaties (Wenzel et al., 2005), kunnen we niet uitsluiten dat de deelnemers met SAS die deel uit maakten van de LFLSAD leden aan een minder ernstige variant van SAS.

Daar staat echter tegenover, en ik maak opnieuw gebruik van het werk van Glahn en anderen (2018), dat familieonderzoeken waarin meerdere familieleden aan een bepaalde aandoening lijden vaak patiënten betreffen die een *ernstiger* ziektebeeld laten zien, in vergelijking met patiënten die geen genetische kwetsbaarheid voor de desbetreffende

ziekte hebben. Dit is ook van toepassing op de LFLSAD, aangezien we families selecteerden waarin SAS veel voorkwam (zie de resultaten samengevat in *Hoofdstuk 3).* Verder is het van belang om op te merken dat de patiënten met SAS die deelnamen aan de LFLSAD allemaal voldeden aan de DSM-5 criteria voor de stoornis, en dus in hun dagelijks leven daadwerkelijk gehinderd werden door hun klachten. Daarom zijn we van mening dat de deelnemers binnen de LFLSAD geen selectie zijn van patiënten die lijden aan een mildere variant van SAS.

Ethische overwegingen met betrekking tot de LFLSAD

In de afzonderlijke hoofdstukken van dit proefschrift beschrijf ik dat het onderzoeksprotocol van de Leidse Familiestudie naar Sociale Angststoornis goedgekeurd is door de Medisch Ethische Commissie van het Leids Universitair Medisch Centrum. Ook ontvingen alle deelnemers uitgebreide informatie over het onderzoek, en gaven zij persoonlijk toestemming voor deelname aan de studie (deze procedure is beschreven in *Hoofdstuk 3).* Desalniettemin brengen familiestudies, waarin familieleden afkomstig uit meerdere generaties deelnemen, specifieke ethische overwegingen met zich mee. Geïnspireerd door de discussie in het werk van Mesman (2015) wil ik een aantal ethische aspecten voor het voetlicht brengen.

Ik begin met een aantal kritische kanttekeningen die bij het familieonderzoek geplaatst zouden kunnen worden. Allereerst zou men kunnen stellen dat het uitnodigen van familieleden om deel te nemen aan een onderzoek naar 'extreme verlegenheid binnen families' leidt tot gevoelens van stress, omdat familieleden zich door deze uitnodiging meer bewust worden van het feit dat zij een verhoogd risico hebben om aan een sociale angststoornis te lijden. Verder is het mogelijk dat er sprake is van bepaalde groepsdruk: we benadrukten immers dat het voor het interpreteren van de onderzoeksgegevens van belang was dat zoveel mogelijk familieleden aan het onderzoek deelnamen. Hierdoor bestaat de mogelijkheid dat familieleden zich niet vrij gevoeld hebben om te beslissen of ze aan de studie wilden deelnemen.

In de praktijk ontvingen we echter vooral positieve feedback van deelnemers, alhoewel we niet systematisch in kaart hebben gebracht hoe familieleden het onderzoek ervaren hebben. Een aantal elementen uit de spontane feedback wil ik hier verder noemen. Ten eerste vertelden meerdere deelnemers ons dat het onderzoek hen ervan bewust maakte dat de 'extreme verlegenheid', waar ze in hun dagelijks leven hinder van ondervonden, geen persoonlijke zwakte betrof; ze leerden dat het een erkende psychiatrische aandoening is, die bovendien geassocieerd is met veranderingen in het brein én onderzocht wordt door een wetenschappelijk team van neurowetenschappers, artsen en psychologen. Deze erkenning was voor veel deelnemers aan de LFLSAD van groot belang, en hielp hen om het gesprek over hun angsten aan te gaan. Meerdere deelnemers vertelden ons dat ze zich door het onderzoek realiseerden dat ze 'niet de enige zijn' die te kampen hebben met gevoelens van

sociale angst. In sommige families wisten familieleden onderling niet van elkaars angsten, en deelname aan het onderzoek hielp hen om hun ervaringen met elkaar te delen.

Verder vertelden meerdere deelnemers ons dat ze gemotiveerd waren om aan het onderzoek deel te nemen omdat ze wilden weten wat ze konden doen om te voorkómen dat hun kinderen ook gehinderd zouden worden door sociale angst. Daarom denken we dat de uitnodiging voor deelname aan de familiestudie hen niet vanuit het niets in een zogenaamde 'hoog-risico positie' bracht, zoals hierboven beschreven: hun motivatie impliceert dat deze deelnemers zich al bewust waren van sociaal-angstige karaktertrekken in hun kinderen, en dat ze zich zorgen maakten dat deze klachten zouden verergeren.

Ook denken we dat de Leidse Familiestudie de drempel om hulp te zoeken heeft verlaagd, zowel voor ouders als hun kinderen. Geen van de deelnemers met SAS was behandeld voor de aandoening voordat de studie van start ging; na afloop informeerden meerdere deelnemers echter naar mogelijkheden voor therapie. Dit is van groot belang, omdat patiënten met SAS niet snel hulp zoeken, waarschijnlijk veroorzaakt door schaamte over hun klachten of een onderschatting van de ernst ervan. Hierdoor duurt het vaak opvallend lang (soms wel 10 tot 15 jaar) voordat patiënten behandeling ontvangen (Alonso et al., 2018; Dingemans et al., 2001; Iza et al., 2013). Verder vestigde een recent artikel de aandacht op barrières die kinderen met angstklachten ervaren in hun zoektocht naar hulp, waarbij het gegeven dat 'ouders niet weten waar en bij wie ze hulp moeten zoeken' de belangrijkste belemmering bleek te zijn (Salloum et al., 2016). Ook bleek dat minder dan 1 % van de kinderen van patiënten met een ernstige angst en / of stemmingsstoornis deelnam aan een preventief interventie programma dat speciaal voor deze hoog-risico groep ontwikkeld was, zoals beschreven in Potijk, Drost, Havinga, Hartman, & Schoevers (2019). Dit is zorgelijk, omdat een adequate en tijdige behandeling van angstklachten in kinderen en jongeren, bij voorkeur voordat er sprake is van een daadwerkelijke angst*stoornis*, de ontwikkeling van een ernstigere aandoening later in het leven kan voorkomen (Hirshfeld-Becker & Biederman, 2002; Marín, 2016; Sylvester et al., 2018; Talati, Weissman, et al., 2013). Een recent onderzoek liet veelbelovende resultaten zien: een kort preventief behandelprogramma leidde tot verminderde angstsymptomen in kinderen van ouders met een angststoornis, wat duidelijk werd toen deze kinderen een jaar na afloop van het programma opnieuw onderzocht werden (Ginsburg et al., 2015). Preventieve behandelingsprogramma's, liefst ingebed in de standaard psychiatrische zorg en met oog voor de hele familie, zijn van groot belang om toekomstig lijden in kinderen met een hoog genetisch risico voor het ontwikkelen van een angststoornis te verminderen (Knappe, Beesdo-Baum, & Wittchen, 2010; Potijk et al., 2019).

Ik wil afsluiten met het uitspreken van de hoop dat de bevindingen van de LFSLAD niet alleen in wetenschappelijk opzicht waardevol zijn, maar dat de familiestudie ook op persoonlijk vlak positieve gevolgen heeft voor de deelnemers. Ik ben ervan overtuigd dat het van groot belang is dat we wetenschappelijk onderzoek naar SAS blijven doen, waarbij

we sociaal-angstige personen 'in the spotlight' zetten; niet letterlijk, maar door onderzoek te doen naar hun brein, zodat we deze patiënten, die vaak zo ongemerkt lijden, in de toekomst beter kunnen helpen.

Conclusie

Samengevat bieden de onderzoeken die in dit proefschrift opgenomen zijn nieuwe inzichten in de neurobiologische kwetsbaarheid voor het ontwikkelen van SAS. Door gebruik te maken van onderzoeksgegevens van de Leidse Familiestudie naar Sociale Angststoornis konden we diverse veranderingen in de structuur en functie van de hersenen vast stellen die *samen met sociale angst binnen de families vóórkwamen* en bovendien *erfelijk* waren. Dat maakt deze karakteristieken kansrijke endofenotypes van SAS. Vervolgonderzoek is nodig om openstaande vragen te beantwoorden, maar de veelbelovende resultaten die in dit proefschrift beschreven zijn bieden hiervoor een waardevol uitgangspunt.

Part 5

Appendices

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Contributing authors

Prof. Gerhard Andersson, PhD.

Department of Clinical Neuroscience, Karolinska Institutet, Stockholm, Sweden; Department of Behavioural Sciences and Learning, Psychology, Linköping University, Linköping, Sweden.

Prof. Jennifer U. Blackford, PhD.

Department of Psychiatry and Behavioral Sciences, Vanderbilt University Medical Center, Nashville, TN, United States of America; Research Service, Research and Development, Department of Veterans Affairs Medical Center, Nashville, TN, United States of America.

Karina S. Blair, PhD.

National Institute of Mental Health, National Institutes of Health, Department of Health and Human Services, Bethesda, MD, United States of America; *Present: Center for Neurobehavioral Research, Boys Town National Research Hospital, Boys Town, NE, United States of America.*

Prof. Carl-Johan Boraxbekk, PhD.

Umeå Centre for Functional Brain Imaging (UFBI), Umeå University, Umeå, Sweden; Danish Research Centre for Magnetic Resonance (DRCMR), Centre for Functional and Diagnostic Imaging and Research, Copenhagen University Hospital Hvidovre, Denmark.

Annette B. Brühl, PhD.

Behavioural and Clinical Neuroscience Institute, Department of Psychiatry, University of Cambridge, Cambridge, United Kingdom; Department of Psychiatry, Psychotherapy, and Psychosomatics, Psychiatric Hospital, University of Zurich, Zurich, Switzerland.

Prof. Per Carlbring, PhD.

Department of Psychology, Stockholm University, Stockholm, Sweden.

Henk R. Cremers, PhD.

Department of Clinical Psychology, University of Amsterdam, Amsterdam, The Netherlands.

Jonas Engman, MSc.

Department of Psychology, Uppsala University, Uppsala, Sweden.

Jean-Paul Fouche, PhD.

Department of Psychiatry and Mental Health, University of Cape Town, Observatory, Cape Town, South Africa.

Prof. Mats Fredrikson, PhD.

Department of Psychology, Uppsala University, Uppsala, Sweden; Department of Clinical Neuroscience, Karolinska Institutet, Stockholm, Sweden.

Andreas Frick, PhD.

Department of Psychology, Uppsala University, Uppsala, Sweden; Department of Clinical Neuroscience, Karolinska Institutet, Stockholm, Sweden.

Prof. Tomas Furmark, PhD.

Department of Psychology, Uppsala University, Uppsala, Sweden.

Anita Harrewijn, PhD.

Developmental and Educational Psychology, Institute of Psychology, Leiden University, Leiden, The Netherlands; Leiden Institute for Brain and Cognition, Leiden University, Leiden, The Netherlands. *Present: Department of Human Development and Quantitative Methodology, University of Maryland, College Park, United States of America; Emotion and Development Branch, National Institutes of Mental Health, Bethesda, United States of America.*

Coenraad J. Hattingh, PhD.

Department of Psychiatry and Mental Health, University of Cape Town, Observatory, Cape Town, South Africa.

Prof. Jeanine J. Houwing-Duistermaat, PhD.

Department of Statistics, University of Leeds, Leeds, United Kingdom.

Heide Klumpp, PhD.

Departments of Psychiatry and Psychology, University of Illinois at Chicago, Chicago, IL, United States of America.

Tanja Kreuk, MSc.

Developmental and Educational Psychology, Institute of Psychology, Leiden University, Leiden, The Netherlands.

Prof. Christine Lochner, PhD.

SU/UCT MRC Unit on Anxiety & Stress Disorders, South Africa; Department of Psychiatry, Stellenbosch University, Tygerberg, South Africa.

Kristoffer N.T. Månsson, PhD.

Department of Psychology, Stockholm University, Stockholm, Sweden; Department of Clinical Neuroscience, Karolinska Institutet, Stockholm, Sweden.

Melle J.W. van der Molen, PhD.

Developmental and Educational Psychology, Institute of Psychology, Leiden University, Leiden, The Netherlands; Leiden Institute for Brain and Cognition, Leiden University, Leiden, The Netherlands.

J. Nienke Pannekoek, PhD.

Neuropsychopharmacology Unit, Centre for Psychiatry, Division of Brain Sciences, Imperial College London, United Kingdom; *Present: MRC Unit on Risk & Resilience in Mental Disorders, Stellenbosch University, Stellenbosch, South Africa.*

Jutta Peterburs, PhD.

Institute of Medical Psychology and Systems Neuroscience, University of Münster, Münster, Germany; *Present: Department of Biological Psychology, Institute for Experimental Psychology, Heinrich-Heine- University, Düsseldorf, Germany.*

Prof. K. Luan Phan, PhD.

Departments of Psychiatry, Psychology and Anatomy and Cell Biology, and the Graduate Program in Neuroscience, University of Illinois at Chicago, Chicago, IL, United States of America.

Catrien G. Reichart, PhD.

Curium, Leiden University Medical Center, Leiden, The Netherlands.

Prof. Karin Roelofs, PhD.

Behavioural Science Institute, Radboud University, Nijmegen, The Netherlands; Donders Institute for Brain, Cognition and Behaviour, Radboud University, Nijmegen, The Netherlands.

Prof. P. Eline Slagboom, PhD.

Section of Molecular Epidemiology, Department of Medical Statistics and Bioinformatics, Leiden University Medical Center, Leiden, The Netherlands.

Prof. Thomas Straube, PhD.

Institute of Medical Psychology and Systems Neuroscience, University of Münster, Münster, Germany.

Renaud L.M. Tissier, PhD.

Developmental and Educational Psychology, Institute of Psychology, Leiden University, Leiden, The Netherlands; *Present: Department of Epidemiology and Biostatistics, Vrije Universiteit Medical Center, Amsterdam, The Netherlands.*

Marie-José van Tol, PhD.

Department of Neuroscience, University Medical Center Groningen, University of Groningen, Groningen, The Netherlands.

Prof. Dick J. Veltman, PhD.

Department of Psychiatry, Neuroscience Campus Amsterdam, VU University Medical Center, Amsterdam, The Netherlands.

Irene M. Van Vliet, PhD.

Department of Psychiatry, Leiden University Medical Center, Leiden, The Netherlands.

Henk van Steenbergen, PhD.

Cognitive Psychology, Institute of Psychology, Leiden University, Leiden, The Netherlands; Leiden Institute for Brain and Cognition, Leiden University, Leiden, The Netherlands.

Prof. Dan J. Stein, PhD.

Department of Psychiatry and Mental Health, University of Cape Town, Observatory, Cape Town, South Africa.

Prof. Nic J.A. van der Wee, PhD.

Department of Psychiatry, Leiden University Medical Center, Leiden, The Netherlands; Leiden Institute for Brain and Cognition, Leiden University, Leiden, The Netherlands.

Prof. P. Michiel Westenberg, PhD.

Developmental and Educational Psychology, Institute of Psychology, Leiden University, Leiden, The Netherlands; Leiden Institute for Brain and Cognition, Leiden University, Leiden, The Netherlands.

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Curriculum vitae

Janna Marie Bas-Hoogendam was born on November 21, 1985 in Gouda, The Netherlands. In 2003, she graduated from Gymnasium Camphusianum in Gorinchem (cum laude / with honours) and started studying Medicine at the Erasmus Medical Center, Rotterdam. After finishing the Bachelor's phase (cum laude / with honours, 2004), she continued with the Master's phase of this study. During several research internships at the department of Psychiatry of the Erasmus Medical Center, she got acquainted with brain research and became more and more enthusiastic about the possibilities of neuroimaging, especially in relation to psychopathology. She wrote a review on the effect of antipsychotics on the blood-oxygen-level-dependent (BOLD) signal (published in revised form in *Current Pharmaceutical Design*) and decided to focus on brain research after her graduation. She was admitted to the prestige research master Neuroscience and Cognition at Utrecht University (2007). As part of this master, she completed an internship involving the combination of functional Magnetic Resonance Imaging (fMRI) and repetitive Transcranial Magnetic Stimulation (rTMS) at the Psychiatry department of the University Medical Center (UMC) Utrecht, and an internship concerning a magneto-encephalogram experiment in patients with Parkinson's disease and obsessive-compulsive disorder at the VU Medical Center in Amsterdam. In addition, she wrote a master's thesis on the physiological basis of the effects of rTMS on the brain, with a focus on synaptic plasticity (published as a review in *Brain Stimulation*).

She obtained her master's degree (cum laude / with honours) in 2009 and started working as a research assistant at the Psychiatry department of the UMC Utrecht on the BRain Imaging, Development & Genetics (BRIDGE) project. In this project, she investigated the structural and functional brain development of children (10 - 16 years of age) who were genetically at high risk for the development of schizophrenia and bipolar disorder. In 2012, she moved to the United Kingdom to work as a Marie Curie Early Stage Researcher at the Cognitive Neuroimaging Laboratory of the University of Birmingham within the Adaptive Brain Computations research program, a training network funded by the European Union.

In 2013, Janna Marie Bas-Hoogendam started her PhD project at the unit Developmental and Educational Psychology of the Institute of Psychology (Leiden University) and the department of Psychiatry of the Leiden University Medical Center (LUMC). This PhD project, embedded within the Leiden Family Lab study on Social Anxiety Disorder (LFLSAD) and supervised by prof. dr. P. Michiel Westenberg, prof. dr. Nic. J. A. van der Wee and dr. Henk van Steenbergen, aimed to determine endophenotypes of social anxiety disorder using structural and functional MRI. The results of this study are included in this thesis.

In addition to the data collection and analysis of the LFLSAD project, she was involved in the Netherlands Study of Depression and Anxiety (NESDA) and worked on a study on the neurobiological effects of cortisol on the brain, a collaboration between the departments of Psychiatry and Endocrinology (LUMC). Furthermore, Janna Marie was part-time

employed as a Junior staff-member at the Netherlands Organization for Scientific Research (NWO), the Hague (2015), and she was actively involved in teaching: she supervised Bachelor's and Master's theses, was coordinator and working group leader of Clinical Child and Adolescent Psychology and a mentor of first year's students of the International Bachelor in Psychology. She obtained her University Teaching Qualification in 2017.

Furthermore, Janna Marie Bas-Hoogendam is a coordinator of the ENIGMA-ANXIETY working group (since 2016) and associate faculty member of F1000prime (since 2017). Currently, Janna Marie is appointed as a researcher at the unit Developmental and Educational Psychology of the Institute of Psychology (Leiden University) and the department of Psychiatry (LUMC).

Janna Marie is married to Matthijs Bas and they have two sons: David (2016) and Manuel (2018).

Curriculum vitae (*Nederlands*)

Janna Marie Bas-Hoogendam is geboren op 21 November 1985 in Gouda. In 2003 behaalde ze haar diploma aan het Gymnasium Camphusianum in Gorinchem (*cum laude*) en in datzelfde jaar startte ze met de opleiding Geneeskunde aan het Erasmus Medisch Centrum, Rotterdam. Na het afronden van de propedeutische fase (*cum laude*, 2004), zette ze haar studie voort met de doctoraal fase van de opleiding. Tijdens meerdere onderzoeksstages bij de afdeling Psychiatrie van het Erasmus MC maakte ze kennis met hersenonderzoek, en werd daarbij steeds enthousiaster over de mogelijkheden van beeldvormende technieken om inzicht te krijgen in de neurobiologische basis van psychopathologie. Ze schreef een overzichtsartikel over het effect van antipsychotica op hersenactiviteit (in herziene vorm gepubliceerd in het wetenschappelijke tijdscrift *Current Pharmaceutical Design*) en besloot om zich na het afronden van haar doctoraal te richten op het doen van wetenschappelijk onderzoek naar het brein. Ze werd geselecteerd voor de prestige researchmaster Neuroscience and Cognition aan de Universiteit Utrecht (2007). Tijdens deze master rondde Janna Marie twee stages af. In de eerste stage, die plaatsvond op de afdeling Psychiatrie van het Universitair Medisch Centrum (UMC) Utrecht werkte ze mee aan een project waarin functionele Magnetische Resonantie Imaging (fMRI) gecombineerd werd met repetitieve Transcraniële Magnetische Stimulatie (rTMS); tijdens de tweede stage hield ze zich bezig met een magneto-encefalografie in patiënten met de ziekte van Parkinson en patiënten met een obsessieve-compulsieve dwangstoornis (VU Medisch Centrum, Amsterdam). Daarnaast schreef ze haar master scriptie over de fysiologische basis van de effecten van rTMS op het brein, waarbij ze zich met name richtte op synaptische plasticiteit. Deze scriptie werd in 2010 in het blad *Brain Stimulation* gepubliceerd.

Na het behalen van haar masterdiploma (*cum laude*) in 2009 werkte Janna Marie een aantal jaar als onderzoeksassistent op de afdeling psychiatrie van het UMC Utrecht aan het *BRain Imaging, Development & Genetics* (BRIDGE) onderzoek. In dit project onderzocht ze de structurele en functionele ontwikkeling van de hersenen in kinderen (10 - 16 jaar oud) die een hoog genetisch risico hadden voor het ontwikkelen van schizofrenie of een bipolaire stoornis. In 2012 verhuisde ze naar het Verenigd Koninkijk om daar als een *Marie Curie Early Stage Researcher* te werken bij het *Cognitive Neuroimaging Laboratory* van de Universiteit van Birmingham, in het kader van het *Adaptive Brain Computations* onderzoeksprogramma (financieel mogelijk gemaakt door de Europese Unie).

In 2013 begon Janna Marie Bas-Hoogendam met haar promotieonderzoek bij de afdeling Ontwikkelings- en onderwijspsychologie (Instituut Psychologie, Universiteit Leiden) en de afdeling Psychiatrie (Leiden Universitair Medisch Centrum, LUMC), onder begeleiding van prof. dr. P. Michiel Westenberg, prof. dr. Nic. J. A. van der Wee en dr. Henk van Steenbergen. Het project was onderdeel van de Leidse Familiestudie naar Sociale Angststoornis (LFLSAD) en was erop gericht om endofenotypes van sociale angststoornis vast te stellen

met behulp van structurele en functionele MRI. De resultaten van het onderzoek zijn in dit proefschrift beschreven.

Naast het verzamelen en analyseren van de onderzoeksgegevens van de LFLSAD was Janna Marie betrokken bij de Nederlandse Studie naar Depressie en Angst (NESDA), en werkte ze aan een onderzoek naar de neurobiologische effecten van cortisol op het brein, een samenwerking tussen de afdelingen Psychiatrie en Endocrinologie van het LUMC. Verder werkte ze parttime als Junior Beleidsmedewerker bij de Nederlandse Organisatie voor Wetenschappelijk Onderzoek (NWO; Den Haag; 2015) en vervulde ze diverse onderwijstaken. Ze begeleidde studenten bij het schrijven van hun bachelor- en masterscripties, was coördinator en werkgroepdocent van het vak *Clinical Child and Adolescent Psychology* en mentor van eerstejaarsstudenten van de *International Bachelor in Psychology*. Ze behaalde haar Basiskwalificatie Onderwijs in 2017.

Verder is Janna Marie sinds 2016 coördinator van de ENIGMA-ANXIETY werkgroep en *associate faculty member* van de website F1000prime (sinds 2017). Momenteel is Janna Marie werkzaam als onderzoeker bij de afdeling Ontwikkelings- en onderwijspsychologie (Instituut Psychologie, Universiteit Leiden) en de afdeling Psychiatrie (LUMC).

Janna Marie is getrouwd met Matthijs Bas en samen hebben ze twee zoons: David (2016) en Manuel (2018).

List of publications

Papers in peer-reviewed journals

Janna Marie Bas-Hoogendam, Henk van Steenbergen, Jennifer U. Blackford, Renaud L.M. Tissier, Nic. J.A. van der Wee, P. Michiel Westenberg (2019b). Impaired neural habituation to neutral faces in families genetically enriched for Social Anxiety Disorder. *Depression & Anxiety* (available online, in press).

Janna Marie Bas-Hoogendam, Henk van Steenbergen, Renaud L.M. Tissier, Nic. J.A. van der Wee, P. Michiel Westenberg (2019a). Altered neurobiological processing of unintentional social norm violations: a multiplex, multigenerational fMRI study on social anxiety endophenotypes. *Biological Psychiatry: Cognitive Neuroscience and Neuroimaging* (available online, in press).

Janna Marie Bas-Hoogendam (2019). Commentary: Gray Matter Structural Alterations in Social Anxiety Disorder: a Voxel-Based Meta-Analysis. *Front. Psychiatry* 10:1.

Janna Marie Bas-Hoogendam, Henk van Steenbergen, Renaud L.M. Tissier, Jeanine J. Houwing-Duistermaat, P. Michiel Westenberg, Nic J.A. van der Wee (2018b). Subcortical brain volumes, cortical thickness and cortical surface area in families genetically enriched for social anxiety disorder – a multiplex multigenerational neuroimaging study. *EBioMedicine, 36,* 410-428

Janna Marie Bas-Hoogendam, Anita Harrewijn, Renaud L.M. Tissier, Melle J.W. van der Molen, Henk van Steenbergen, Irene M. Van Vliet, Catrien G. Reichart, Jeanine J. Houwing-Duistermaat, P. Eline Slagboom, Nic J.A. van der Wee, P. Michiel Westenberg (2018a). The Leiden Family Lab study on Social Anxiety Disorder: a multiplex, multigenerational family study on neurocognitive endophenotypes. *International Journal of Methods in Psychiatric Research*: e1616.

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Janna Marie Bas-Hoogendam*, Henk van Steenbergen* , J. Nienke Pannekoek, Jean-Paul Fouche, Christine Lochner, Coenraad J. Hattingh, Karin Roelofs, Tomas Furmark, Kristoffer N.T. Månsson, Andreas Frick, Jonas Engman, Carl-Johan Boraxbekk, Per Carlbring, Gerhard Andersson, Mats Fredrikson , Thomas Straube, Jutta Peterburs, Heide Klumpp, K. Luan Phan, Henk R. Cremers, Dick J. Veltman, Marie-José van Tol, Dan J. Stein, Nic

J.A. van der Wee (2017a). Voxel-Based Morphometry Multi-Center Mega-Analysis of Brain Structure in Social Anxiety Disorder. *NeuroImage: Clinical, 16*, 678-688. (*: shared first authorship)

Janna Marie Bas-Hoogendam, Henk van Steenbergen, Tanja Kreuk, Nic J.A. van der Wee, P. Michiel Westenberg (2017b). How embarrassing! The behavioral and neural correlates of processing social norm violations. *PLOS ONE, 12 (4)*, e0176326.

Janna Marie Bas-Hoogendam, Jennifer U. Blackford, Annette B. Brühl, Karina S. Blair, Nic J.A. van der Wee, P. Michiel Westenberg (2016). Neurobiological Candidate Endophenotypes of Social Anxiety Disorder. *Neuroscience and Biobehavioral Reviews* , *71*, 362-378.

Janna Marie Bas-Hoogendam*, Cornelie D. Andela*, Steven J.A. van der Werff, J. Nienke Pannekoek, Henk van Steenbergen, Onno C. Meijer, Mark A. van Buchem, Serge A.R.B. Rombouts, Roos C. van der Mast, Nienke R. Biermasz, Nic J.A. van der Wee, Alberto M. Pereira (2015). Altered neural processing of emotional faces in remitted Cushing's disease. *Psychoneuroendocrinology* 59, 134–146. (*: shared first authorship)

Matthijs Vink, Bram B. Zandbelt, Thomas Gladwin, Manon Hillegers, **Janna Marie Hoogendam**, Wery P.M. van den Wildenberg, Stefan Du Plessis, René S Kahn (2014). Frontostriatal activity and connectivity increase during proactive inhibition across adolescence and early adulthood. *Human Brain Mapping 35 (9)*, 4415-4427.

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Bram B. Zandbelt, Mirjam Bloemendaal, **Janna Marie Hoogendam**, René S. Kahn, Matthijs Vink (2013). Transcranial magnetic stimulation and functional MRI reveal cortical and subcortical interactions during stop-signal response inhibition. *Journal of Cognitive Neuroscience, 25 (2)*, 157-174.

N.M.J. van Veelen, M. Vink, N.F. Ramsey, M. van Buuren, **J.M. Hoogendam**, R.S. Kahn (2011). Prefrontal lobe dysfunction predicts treatment response in medication-naive first-episode schizophrenia. *Schizophrenia Research 129 (2-3)*, 156-162.

N.M.J. van Veelen, M. Vink, N.F. Ramsey, I.E.C. Sommer, M. van Buuren, **J.M. Hoogendam**, R.S. Kahn (2011). Reduced language lateralization in first-episode medication-naive schizophrenia. *Schizophrenia Research 127 (1-3),* 195-201.

C.H. Röder, **J.M. Hoogendam**, F.M. van der Veen (2010). FMRI, antipsychotics and schizophrenia. Influence of different antipsychotics on BOLD-signal. *Current Pharmaceutical Design 16 (18)*, 2012-2025.

J.M. Hoogendam, G.M.J.Ramakers, V.Di Lazzaro (2010). Physiology of repetitive transcranial magnetic stimulation of the human brain. *Brain Stimulation 3 (2)*, 95-118.

Papers under review

Paul Thompson, Neda Jahanshad, Christopher R. K. Ching, Lauren E. Salminen, Sophia I. Thomopoulos, Joanna Bright, Bernhard T. Baune, …., **Janna Marie Bas-Hoogendam**, …, Nic J. A. van der Wee, Ysbrand D. van der Werf, Theo G. M. van Erp, PhD, Neeltje E. M. van Haren, Daan van Rooij, Laura S. van Velzen, Ilya M. Veer, Dick J. Veltman, Julio E. Villalon-Reina, Henrik Walter, Christopher D. Whelan, Elisabeth A. Wilde, Mojtaba Zarei, and Vladimir Zelman, for the ENIGMA Consortium (2019). *ENIGMA and Global Neuroscience: A Decade of Large-Scale Studies of the Brain in Health and Disease across 43 Countries.* ***PsyArXiv.*** *July 4*.

Janna Marie Bas-Hoogendam, Henk van Steenbergen, Nic. J.A. van der Wee, P. Michiel Westenberg (under review). Amygdala hyperreactivity to faces conditioned with a social-evaluative meaning – a multiplex, multigenerational fMRI study on social anxiety endophentoypes.

Laura S. van Velzen, Marie-José van Tol, Martijn P. van den Heuvel, **Janna Marie Bas-Hoogendam**, Nic J. A. van der Wee, Dick J. Veltman, Brenda W. J. H. Penninx, Lianne Schmaal (in revision). Multimodal graph theoretical brain networks and the 9-year cumulative disease load of depression and anxiety.

Book chapters

Janna Marie Bas-Hoogendam, Eline F. Roelofs, P. Michiel Westenberg, Nic J. A. van der Wee. Pathogenesis of SAD; chapter in 'The American Psychiatric Association Textbook of Anxiety, Trauma and OCD related Disorders', edited by Naomi Simon, Eric Hollander, Barbara O. Rothbaum, and Dan J. Stein (2019, in press).

Dick J. Veltman, **Janna Marie Bas-Hoogendam**, Nic J. A. van der Wee en Odile A. van den Heuvel. Het angstige brein: beeldvormend onderzoek; hoofdstuk in 'Handboek angst- en

dwangstoornissen', onder redactie van Ton van Balkom, Désirée Oosterbaan, Saka Visser en Irene van Vliet. Uitgeverij de Tijdstroom (2018).

Conference abstracts *(selection)*

Janna Marie Bas-Hoogendam, Henk van Steenbergen, Nic J.A. van der Wee, P. Michiel Westenberg (2019). Neuroimaging results from the Leiden Family Lab study on Social Anxiety Disorder: a multiplex, multigenerational endophenotype study. *Journal of Neural Transmission* (in press).

Janna Marie Bas-Hoogendam, Henk van Steenbergen, Nic J.A. van der Wee, P. Michiel Westenberg (2019). Social conditioning of neutral faces in families genetically enriched for social anxiety disorder. *European Neuropsychopharmacology* (in press). ***WINNER TRAVEL AWARD 32nd ECNP Congress (2019), and selected for 'Poster Jam Session: Anxiety and stress related disorders' dedicated to four high-scoring posters.***

Laura van Velzen, Marie-José van Tol, Martijn van den Heuvel, **Janna Marie Bas-Hoogendam**, Nic van der Wee, Dick Veltman, Brenda Penninx, Lianne Schmaal (2019). Multimodal Graph Theoretical Brain Networks and the 9-Year Cumulative Disease Load of Depression and Anxiety. *Biological Psychiatry 85 (10),* S264.

Nynke A. Groenewold, **Janna Marie Bas-Hoogendam**, Alyssa R. Amod, Laura van Velzen, Moji Aghajani, Courtney A. Filippi, Andrea L. Gold, Christopher R.K. Ching, Karin Roelofs, Tomas Furmark, Kristoffer N.T. Månsson, Thomas Straube, Jutta Peterburs, Heide Klumpp, K. Luan Phan, Christine Lochner, Alexander Doruyter, Jesus Pujol, Narcis Cardoner, Laura Blanco-Hinojo, Katja Beesdo-Baum, Kevin Hilbert, Benjamin Kreifelts, Michael Erb, Qiyong Gong, Su Lui, Jair C. Soares, Mon-Ju Wu, P. Michiel Westenberg, Dominik Grotegerd, Elisabeth J. Leehr, Udo Dannlowski, Peter Zwanzger, Dick J. Veltman, Daniel S. Pine, Neda Jahanshad, Paul M. Thompson, Dan J. Stein, and Nic J.A. van der Wee, on behalf of the ENIGMA-Anxiety Working Group (2018). Subcortical volumes in Social Anxiety Disorder: Preliminary Results from ENIGMA-Anxiety. *Biological Psychiatry 83 (9),* S247-S248.

Janna Marie Bas-Hoogendam, Henk van Steenbergen, Nic J.A. van der Wee, P. Michiel Westenberg (2017); Subcortical brain volumes as endophenotypes of social anxiety disorder – preliminary findings from the Leiden Family Study on Social Anxiety Disorder. *Journal of Neural Transmission 124 (10),* 1300. ***WINNER TRAVEL AWARD WASAD Congress (2017).***

Janna Marie Bas-Hoogendam, Henk van Steenbergen, Nic J.A. van der Wee, P. Michiel Westenberg (2017); Subcortical brain volumes as endophenotypes of social anxiety disorder

– preliminary findings from the Leiden Family Study on Social Anxiety Disorder. *European Neuropsychopharmacology 27 (4),* s1021.

Janna Marie Bas-Hoogendam, Henk van Steenbergen, J. Nienke Pannekoek, Jean-Paul Fouche, Christine Lochner, Coenraad J. Hattingh, Henk R. Cremers, Tomas Furmark, Kristoffer N.T. Månsson, Andreas Frick, Jonas Engman, Carl-Johan Boraxbekk, Per Carlbring, Gerhard Andersson, Mats Fredrikson, Thomas Straube, Jutta Peterburs, Heide Klumpp, K. Luan Phan, Karin Roelofs, Dan J. Stein, Nic J.A. van der Wee (2017). Sample size matters: A voxel-based morphometry multi-center mega-analysis of gray matter volume in Social Anxiety Disorder. *Biological Psychiatry 81 (10),* S7-S8.

Janna Marie Bas-Hoogendam, Henk van Steenbergen, Nic J.A. van der Wee, P. Michiel Westenberg (2017). Social norm processing as an endophenotype of social anxiety disorder - a family study in two generations. *European Neuropsychopharmacology 27*, S49-S50. ***WINNER TRAVEL AWARD ECNP Workshop on Neuropsychopharmacology for Junior Scientists in Europe (2017).***

Janna Marie Bas-Hoogendam, Henk van Steenbergen, P. Michiel Westenberg, Nic J.A. van der Wee (2015). Social conditioning of neutral faces: A pilot-study on brain functioning in social anxiety patients and their unaffected first-degree relatives. *European Neuropsychopharmacology 25*, S573-S574. ***WINNER TRAVEL AWARD 28th ECNP Congress (2015).***

Patients with social anxiety disorder (SAD) are 'extremely shy': they are afraid of a negative evaluation and avoid social situations as much as possible, with negative influences on their lives. SAD runs in families: being 'genetically close' to a patient with SAD substantially increases the risk to develop the disorder.

The studies summarized in this thesis aim to broaden our knowledge of this genetic vulnerability to SAD. Using MRI scans from the unique Leiden Family Lab study on Social Anxiety Disorder, we demonstrate that alterations in the structure and function of the brain are genetically linked to the disorder. These results offer novel insights in the neurobiological pathways leading to SAD.