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

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Chapter 5

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

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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

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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.

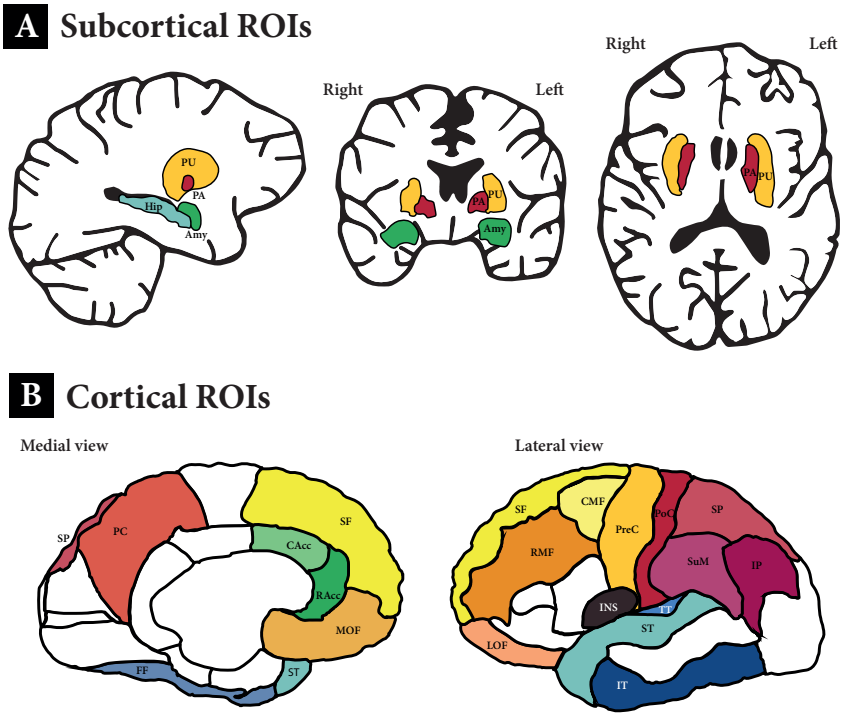


Figure 5.1 Subcortical (A) and cortical (B) regions of interest (ROIs).
Figure 5.1A Amy: amygdala; Hip: hippocampus; PA: pallidum; PU: putamen.
Figure 5.1B **Frontal regions (yellow)**: CMF: caudal middle frontal; LOF: lateral orbitofrontal; MOF: medial orbitofrontal; PreC: precentral; RMF: rostral middle 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

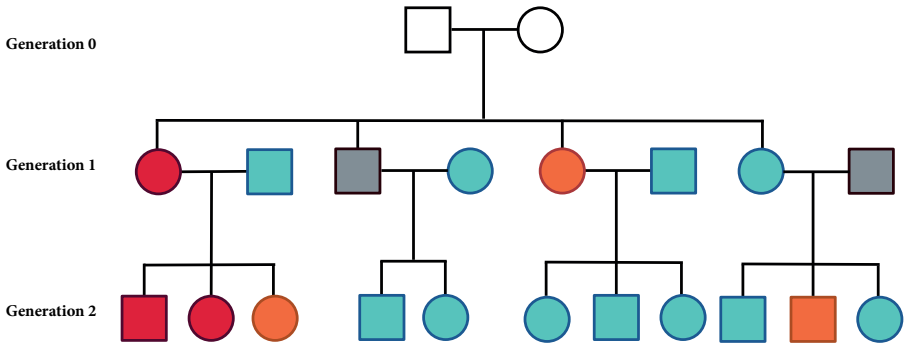


Figure 5.2 Example of a family within the Leiden Family Lab study on Social Anxiety Disorder.
Families were included based on the combination of a parent with SAD ('proband'; depicted in red) and a proband's child with SAD (red) or subclinical SA (orange). In addition, family members of two generations 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.

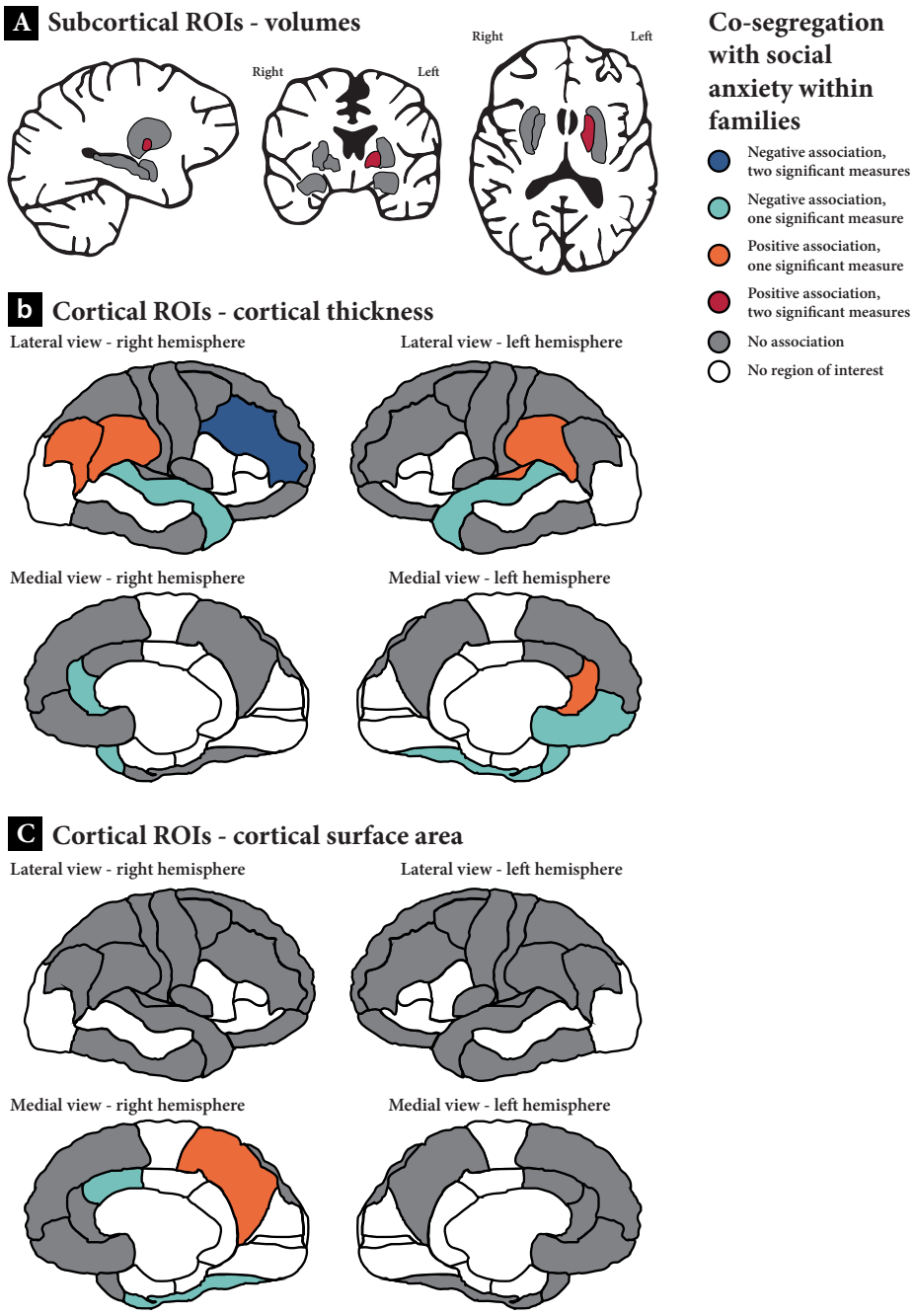


Figure 5.3 Relationship between indices of social anxiety and gray matter characteristics.

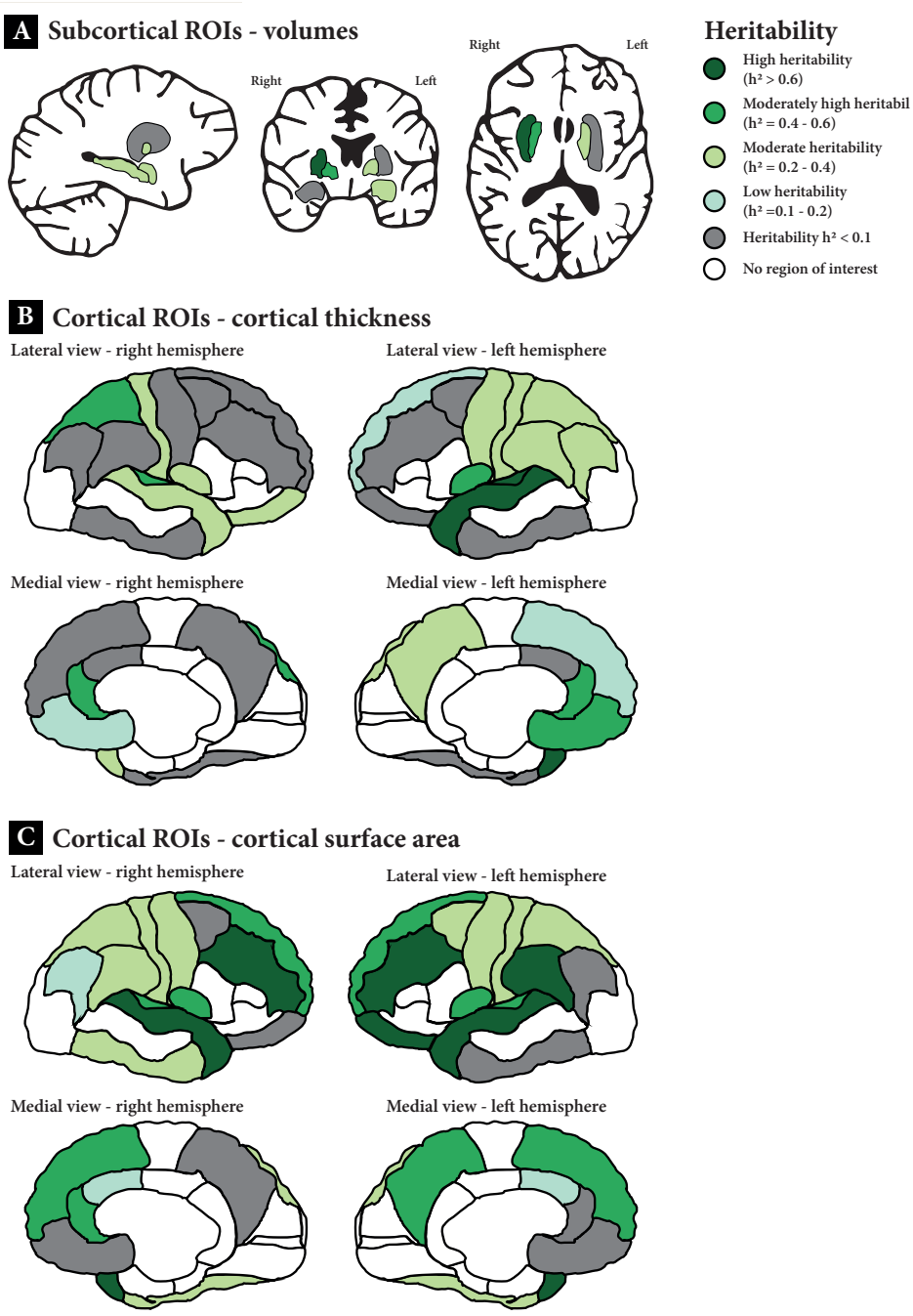


Figure 5.4 Heritability estimates of gray matter characteristics.

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

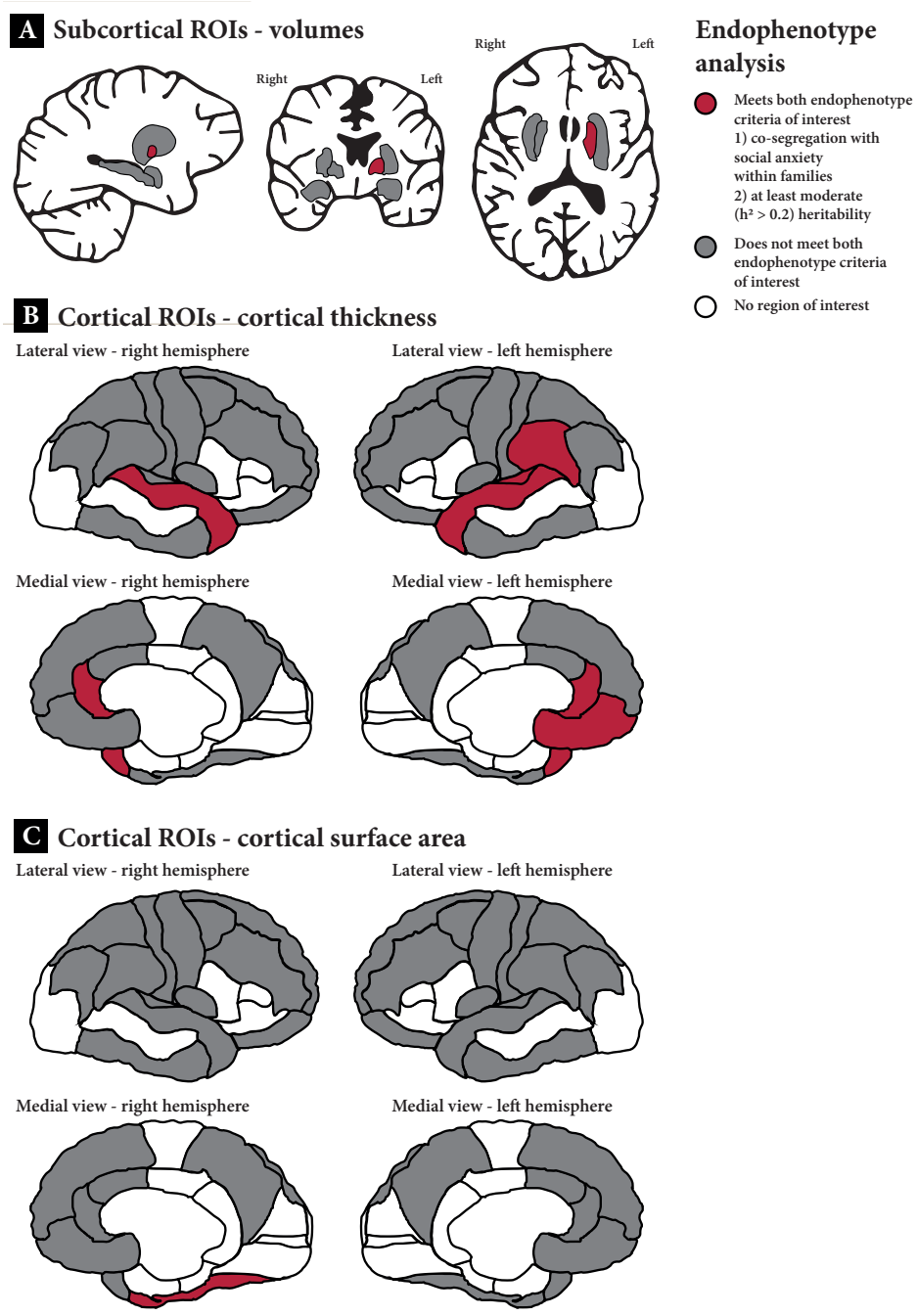


Figure 5.5 Overview of gray matter candidate endophenotypes of social anxiety.

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).

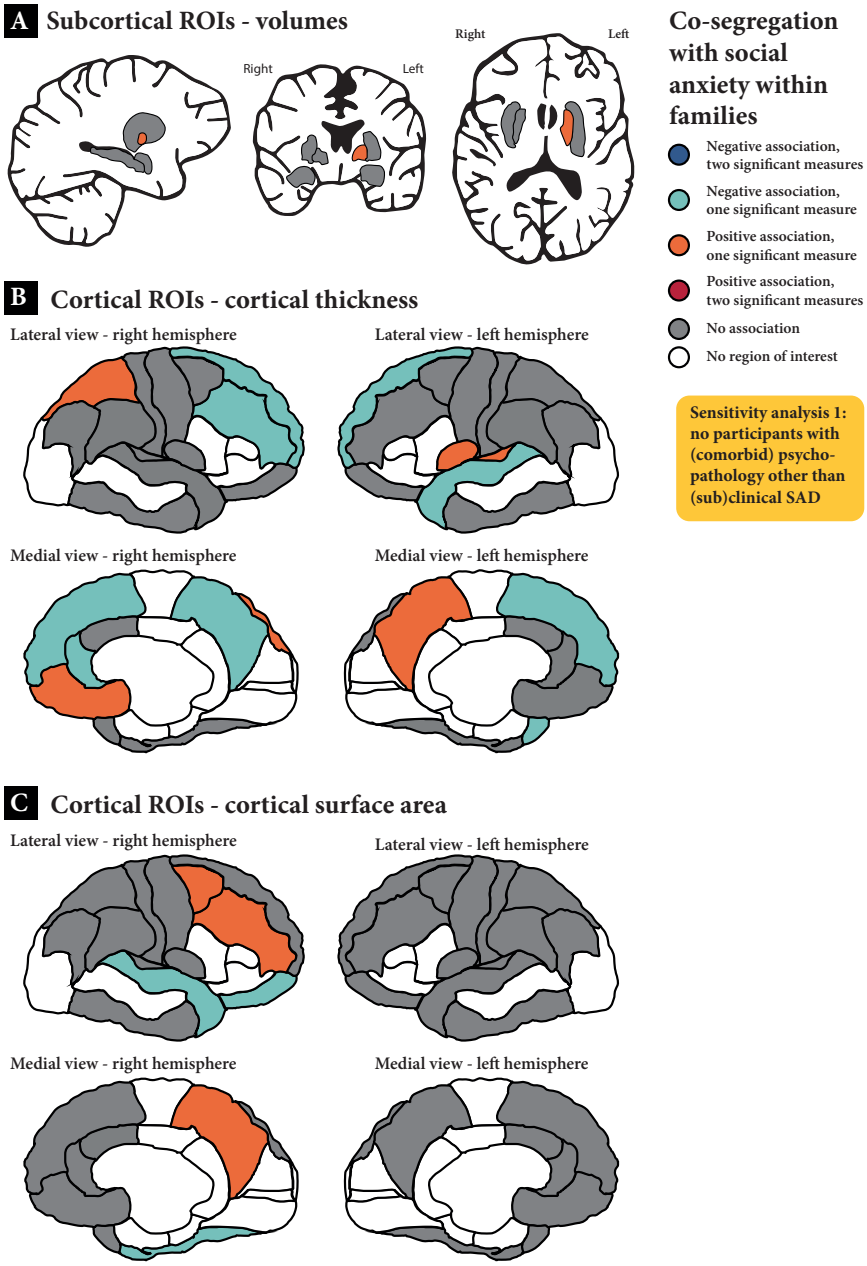
Supplemental Table S5.4

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).

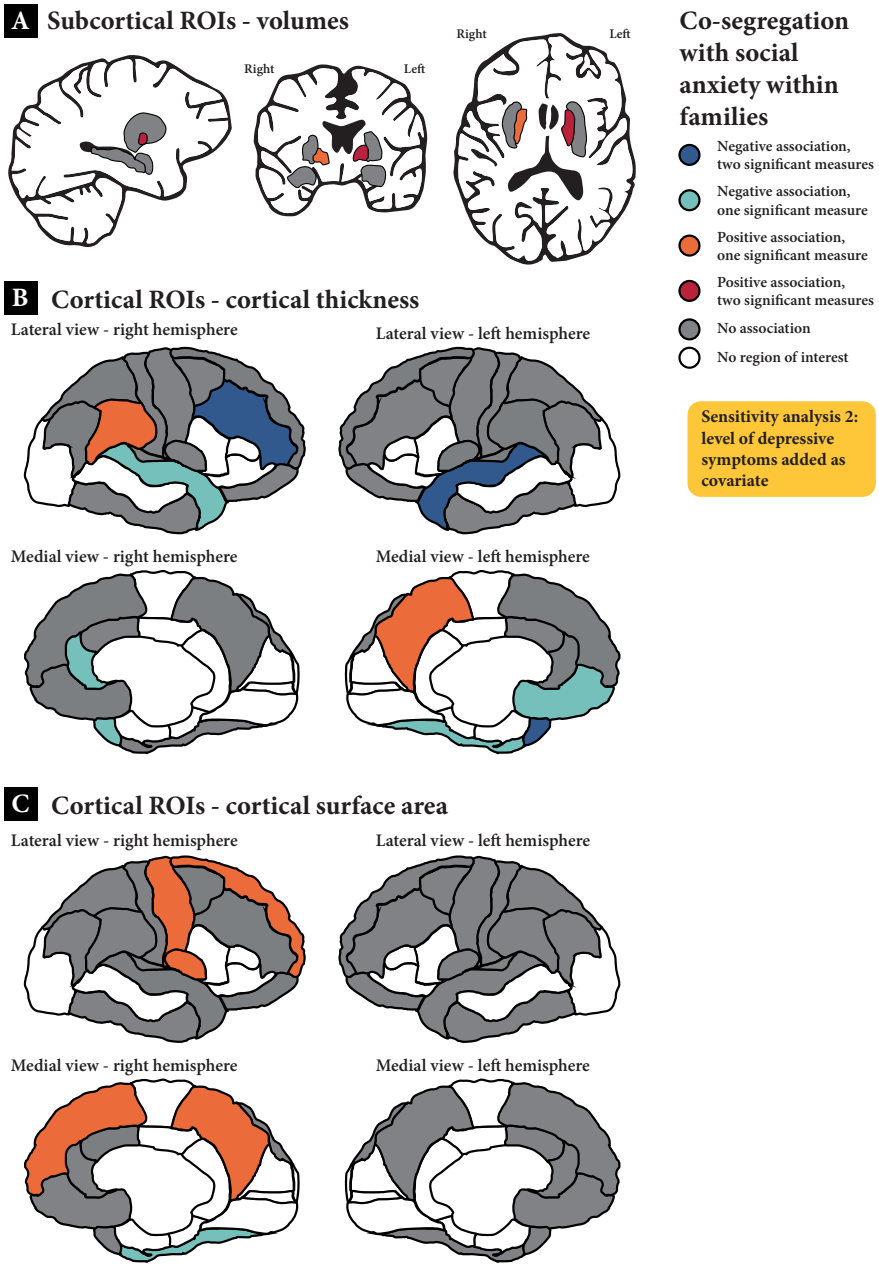
Supplemental Figure S5.4 QR code for easy access to Supplemental Tables belonging to Chapter 5.

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).