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

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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

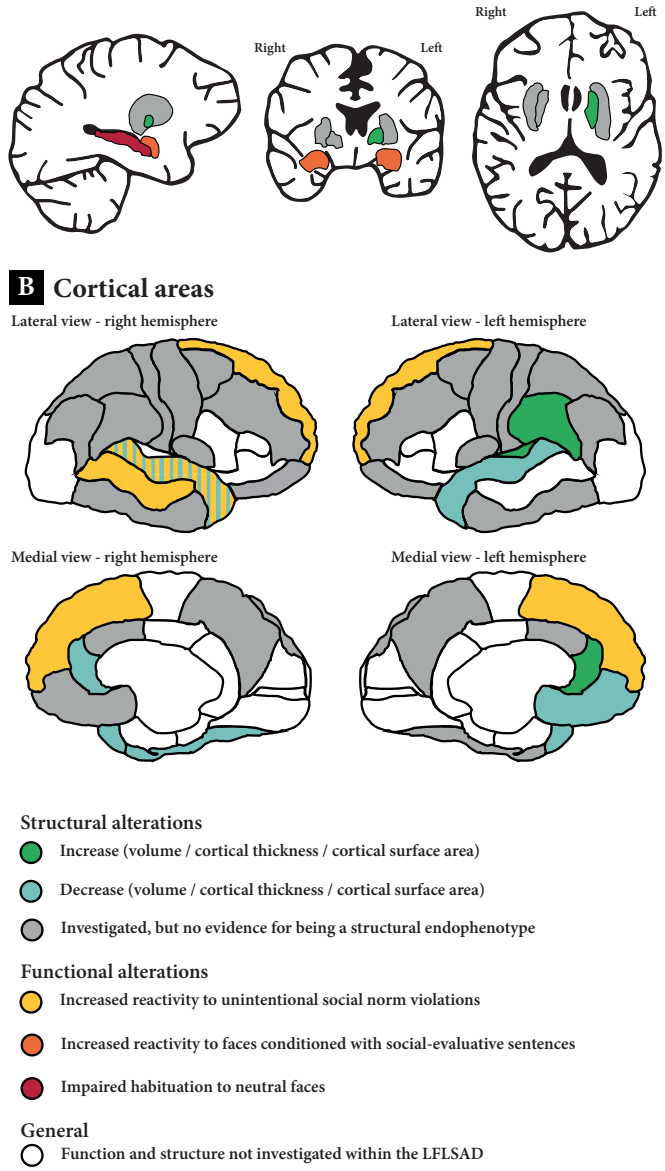

Figure 11.1 Graphical summary of neurobiological endophenotypes of SAD, as revealed by data from the LFLSAD.

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.