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

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Chapter 10

Amygdala hyperreactivity to faces conditioned with a social-evaluative meaning - a multiplex, multigenerational fMRI study on social anxiety endophenotypes

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Abstract

Social anxiety disorder (SAD) runs in families, but the neurobiological pathways underlying the genetic susceptibility towards SAD are largely unknown. Here, we employed an endophenotype approach, and tested the hypothesis that amygdala hyperreactivity to faces conditioned with a social-evaluative meaning is a candidate SAD endophenotype. We used data from the multiplex, multigenerational Leiden Family Lab study on Social Anxiety Disorder (eight families, n = 105) and investigated amygdala activation during a social-evaluative conditioning paradigm with high ecological validity in the context of SAD. Three neutral faces were repeatedly presented in combination with socially negative, positive or neutral sentences. We focused on two endophenotype criteria: *co-segregation of the candidate endophenotype with the disorder within families*, and *heritability*.

Analyses of the fMRI data were restricted to the amygdala as a region of interest, and revealed that bilateral amygdala hyperreactivity in response to the conditioned faces co-segregated with social anxiety within the families. Furthermore, multiple voxels within these amygdala clusters were at least moderately heritable. Taken together, these findings show that amygdala engagement in response to conditioned faces with a social-evaluative meaning qualifies as a neurobiological candidate endophenotype of social anxiety. Thereby, these data shed light on the genetic vulnerability to develop SAD.

Introduction

Social anxiety disorder (SAD), one of the most prevalent anxiety disorders, has a typical onset during adolescence and runs in families (Haller et al., 2015; Isomura et al., 2015). Patients with the disorder have an extreme fear of evaluation by others and avoid social situations as much as possible (Stein & Stein, 2008). Furthermore, SAD is associated with a chronic course, high rates of comorbid psychopathology, reduced quality of life and far-reaching impairments in school, work and relations (Dams et al., 2017; Fehm et al., 2005). Given the severe consequences of the disorder, for patients and their families as well as for society, insight in the neurobiological functional brain alterations underlying the genetic vulnerability to develop SAD is essential.

One of the key structures in the socially-anxious brain is the amygdala (cf. reviews by (Brühl, Delsignore, et al., 2014; Etkin & Wager, 2007; Garner et al., 2009)). The amygdala is essential for processing environmental stimuli and learning their predictive value, as demonstrated in both humans and animals (Hariri & Whalen, 2011; Janak & Tye, 2015; Olsson & Phelps, 2007; Paton, Belova, Morrison, & Salzman, 2006). More specifically, an elegantly designed neuroimaging study by Davis and colleagues (2010) has provided strong evidence for the role of the amygdala in learning the social value of biologically-relevant cues. The authors employed a conditioning paradigm, in which three neutral faces (conditioned stimuli, CS) were consistently paired with either a positive endorsement, a negative comment, or a socially-neutral statement (unconditioned stimuli, US; Davis et al., 2010); importantly, as these sentences were directly addressing the participant, the presentation of these face-sentence combinations created a social-evaluative learning context. Behavioral ratings of likeability indicated that healthy participants learned the social value of the faces, and functional magnetic resonance imaging (fMRI) data revealed the involvement of various amygdala subregions during social-evaluative learning (Davis et al., 2010).

At present, and to the best of our knowledge, this social-evaluative conditioning paradigm has not been used in SAD. Nevertheless, given the heightened fear of negative as well as positive evaluation that characterizes socially-anxious individuals (Reichenberger et al., 2019; Teale Sapach, Carleton, Mulvogue, Weeks, & Heimberg, 2014), investigating the neurobiological underpinnings of social-evaluative learning is of uttermost relevance in SAD (cf. (Pittig, Treanor, LeBeau, & Craske, 2018)). An electromyography (EMG) study in patients with SAD, using a differential fear conditioning paradigm in which neutral faces (CS) were paired with positive, neutral or negative facial expressions and verbal feedback (US) addressing the participant, reported an elevated fear-potentiated startle reflex in response to faces conditioned with critical facial expressions and insults in SAD patients, while no group differences were present with respect to subjective ratings of the conditioned stimuli, nor during extinction learning (Lissek et al., 2008).

Subsequent studies used slightly adapted versions of this differential fear conditioning paradigm. The first, an EMG study on individuals with clinical SAD and participants with high levels of social anxiety (SA) could, however, not replicate fear conditioning in the physiological data, and did not find SA-related differences with respect to self-report measures of anxiety, unpleasantness and arousal due to conditioning (Tinoco-González et al., 2015). The second study (Ahrens, Mühlberger, Pauli, & Wieser, 2015), using electroencephalography (EEG), paired neutral faces (CS) with three types of verbal feedback (positive, neutral or negative; US), and demonstrated impaired electrocortical differentiation in students with high levels of SA: while low socially-anxious individuals showed differential visuocortical processing in relation to the three conditions, with highest cortical activity to faces paired with insults and lowest activity to faces paired with compliments, this distinction was absent in high socially-anxious participants. Again, no group differences were found with respect to ratings of valence (Ahrens et al., 2015).

Due to the methodology used, these studies were, however, not able to investigate amygdala reactivity during social conditioning. To the best of our knowledge, only one fMRI study has explored the relation between SA and amygdala activation during social conditioning using disorder relevant stimuli. In that study, Pejic et al. (2013) paired neutral faces (CS) with film-clips of critical comments (US), and showed positive correlations between SA and amygdala activation during social conditioning; at the behavioral level, participants with higher SA-levels reported stronger increases in unpleasantness and fear following social conditioning (Pejic et al., 2013).

Together, these findings suggest that SA is associated with altered physiological and neural responses during social conditioning, although it should be noted that only one study so far explored amygdala activation. Furthermore, results on the relation between SA and behavioral indices of social conditioning are mixed. In addition, as Pejic and colleagues (2013) used a sample of healthy students with varying levels of SA and only employed negative unconditioned stimuli, the relation between SA and amygdala function related to social conditioning has until now not been investigated in patients with SAD, and the effect of positive and neutral comments as unconditioned stimuli is at present still unknown. Moreover, it has not been examined whether amygdala activation during social-evaluative learning is a candidate endophenotype of SAD. Such research is however, important, as endophenotypes, which are located on the causal pathway from genotype to phenotype, could shed light on the mechanisms by which genetic risk unfolds (Dick, 2018), and as such, could aid in unravelling the genetic susceptibility to SAD and offer new insights in targets for prevention and intervention (Bas-Hoogendam et al., 2016).

By definition, endophenotypes are quantitative characteristics which are *associated with the disorder* (criterion 1), *state-independent and already present in a preclinical state* (criterion 2), *heritable* (criterion 3), and display *co-segregation with the disorder within families of probands, with non-affected family members showing altered levels of the endophenotype in*

comparison to the general population (criterion 4) (Glahn et al., 2007; Lenzenweger, 2013a). The endophenotype approach has yielded promising results in other psychiatric disorders, for example in depression (Goldstein & Klein, 2014), schizophrenia and psychosis (Blakey et al., 2018; Glahn, Williams, et al., 2014; Sutcliffe et al., 2016) and obsessive–compulsive disorder (Taylor, 2012) but research on neurobiological endophenotypes of SAD is still scarce.

Here, we present data from the Leiden Family Lab study on Social Anxiety Disorder (LFLSAD), comprising a unique sample of families genetically enriched for SAD (Bas-Hoogendam, Harrewijn, et al., 2018). This multiplex (i.e. multiple cases of SAD), multigenerational family design is eminently suitable to test two important endophenotype criteria within the same sample, being the heritability and co-segregation of a certain candidate endophenotype within families. Using the social conditioning paradigm developed by Davis and colleagues (2010) for the first time in the context of SAD, we investigated whether amygdala reactivity during social-evaluative learning could serve as a candidate neurobiological endophenotype of SAD. First, we examined evidence for the endophenotype criterion of *co-segregation of the candidate endophenotype with SA within the families* (first element of criterion 4); in case of affirmative results, we established *heritability* (criterion 3). Based on previous research summarized above, we predicted a positive relationship between SA-level and amygdala activation in response to the conditioned stimuli. Furthermore, on a more exploratory basis as research on this subject is still scarce, we examined the relation between SA-level and amygdala activation over time, as well as in response to the three particular conditions. Behavioral ratings were used to validate the paradigm; in addition, their relation with SA-level was explored.

Experimental procedures

Participants

The sample consisted of participants from the LFLSAD, in which families genetically enriched for SAD are included. Families were invited for participation based on the combination of a primary diagnosis of SAD in a parent (aged 25 - 55 years old; 'proband') and a child who met criteria for clinical or subclinical SAD ('proband's SA-child'). The proband's SA-child (age 8 - 21 years) should live at home with the proband; comorbidity other than internalizing disorders or substance abuse was an exclusion criterion for the proband and proband's SA-child. Besides these two SAD-cases, first- and second-degree family members of two generations were invited to participate, being the proband's partner and other children of the nuclear family (age ≥ 8 years), as well as the proband's sibling(s), with their partners and children (age ≥ 8 years). These family members were included independent from the presence of psychopathology. Insufficient comprehension of the Dutch language

was an exclusion criterion for all participants, and general MRI contraindications led to exclusion of the MRI experiment.

Following this inclusion strategy, the LFLSAD sample (total sample: n = 132, nine families; MRI sample: n = 110, eight families; more information about recruitment is included in the *Supplemental Methods*) consists of family members of two generations (*Figure 3.1*). Participants completed a number of measurements, such as a diagnostic interview, self-report questionnaires and an MRI scan (Bas-Hoogendam, Harrewijn, et al., 2018). The study was approved by the Medical Ethical Committee of the Leiden University Medical Center and all participants provided informed consent according to the Declaration of Helsinki. Detailed information about the LFLSAD and an a priori power calculation for the study are outlined in a designpaper (Bas-Hoogendam, Harrewijn, et al., 2018); furthermore, the study was pre-registered online (Bas-Hoogendam et al., 2014a, 2014b).

Phenotyping

In order to facilitate extensive phenotyping, the LFLSAD protocol consisted of several measurements (Bas-Hoogendam, Harrewijn, et al., 2018) (cf. *Supplemental Methods*). The following assessments are relevant for the present work.

Experienced clinicians determined the presence of DSM-IV diagnoses using the Mini-International Neuropsychiatric Interview (M.I.N.I.)-Plus (Sheehan et al., 1998) or the M.I.N.I.-Kid interview (Sheehan et al., 2010). Given the nature of the LFLSAD sample, special attention was paid to the presence of (sub)clinical SAD. Clinical SAD was established using the DSM-IV-TR criteria for the generalized subtype of SAD, but the clinician verified whether the DSM-5 criteria for SAD were also met. A diagnosis of subclinical SAD was established when participants met the criteria for SAD as described in the DSM-5, but did not show impairing limitations in important areas of functioning (criterion G) (American Psychiatric Association, 2013). The interviews were recorded.

Furthermore, participants completed age-appropriate questionnaires on the level of SA-symptoms, being the Liebowitz Social Anxiety Scale for adults (Fresco et al., 2001) and the Social Anxiety Scale for adolescents (La Greca & Lopez, 1998), as well as on the level of depressive symptoms (Beck Depression Inventory (Beck et al., 1996) or the Children's Depression Inventory (Kovacs, 1985). To enable interpreting the scores of the age-appropriate questionnaires over the whole sample, z-scores were computed (Bas-Hoogendam, Harrewijn, et al., 2018). Incidental missing values were replaced by the average value of the completed items.

MRI experiment

Scanning was performed using a 3.0T Philips Achieva MRI scanner. The MRI experiment consisted of several structural scans (Bas-Hoogendam, van Steenbergen, Tissier, et al., 2018b) and functional task paradigms (Bas-Hoogendam, van Steenbergen, Blackford, et

al., 2019; Bas-Hoogendam, van Steenbergen, Kreuk, et al., 2017a; Bas-Hoogendam, van Steenbergen, Tissier, et al., 2019); details are provided in the *Supplemental Methods*.

Social-evaluative conditioning paradigm

The social-evaluative conditioning paradigm was part of the neutral faces paradigm (NFP), in which we investigated both the initial habituation response to neutral faces as well as brain activation associated with learning their social-evaluative value (*Figure 10.1*). Neutral faces were selected from the FACES database, a set of well-validated images of naturalistic faces of women and men (Ebner et al., 2010). Participants were presented with faces matching their own sex, so we selected photographs of three young males and three young females (see *Supplemental Methods* for details on these faces). Stimuli were presented using E-Prime software version (2.0.10, Psychology Software Tools).

The NFP consists of two phases, a habituation phase (HP) and the social-evaluative conditioning phase (SCP). Findings on the HP are reported elsewhere (Bas-Hoogendam, van Steenbergen, Blackford, et al., 2019); for reasons of completeness, a description of the HP is included in the *Supplemental Methods*. During the SCP, which was based on the paradigm by (Davis et al., 2010), three neutral faces, which had been shown to the participants already during the HP, were again presented, but now each face was consistently combined with one type of social-evaluative sentence: positive, negative or neutral. That is, after presentation of the face (duration: 1 s; CS) a social-evaluative sentence was presented (duration: 2 s; US) (*Figure 10.1*). One face was always followed by a positive endorsement (for example: 'he/she says you are smart'), the second face was accompanied by a negative comment ('he/she says you are stupid'), while the last face was combined with a socially-neutral statement ('he/she says you are in Leiden'). There were four different sentences within each category (see *Supplemental Table S10.1* for a list of all sentences), and each face-sentence combination was shown three times. This resulted in 12 trials per condition and a total of 36 trials. The order of the face-sentence combinations was pseudorandomized and the combinations of the faces with the type of self-relevant sentences were counterbalanced across the participants.

Participants were instructed to look at the faces and to read the accompanying sentences. As the face presentation always preceded the sentence presentation, participants learned what type of social-evaluative sentence would follow upon presentation of a certain face. The intervals between the presentation of the face and the presentation of the sentence, as well as the intertrial intervals, were jittered in order to optimize the estimation of the blood-oxygen-level-dependent response related to the presentation of the faces and the presentation of the sentences (jitter face-sentence: range 1.5 s – 2.5 s, mean 2.0 s; intertrial interval: range 2.0 s – 3.5 s, mean 2.7 s; cf. (Davis et al., 2010)). Total duration of the SCP was 5 min 41 s.

At three times during the NFP, participants were asked to rate the faces on likeability and arousal in line with the paradigm described by (Davis et al., 2010); the first measurement

was before the HP (T1), the second between the HP and SCP (T2), while the last measurement followed the end of the SCP (T3; *Figure 10.1*). These ratings were used to investigate the initial rating of the faces (T1); furthermore, the ratings were used to assess whether participants learned the social-evaluative value of the faces (i.e. to validate the SCP), and to examine the association between this learning process and social anxiety. The three faces were presented sequentially on the screen, accompanied by the question 'How much do you like this person?' (range from -4, 'not at all' to 4, 'very much'), and, on a second screen, the question 'How much emotion do you experience when seeing this person?' (ranging from 1, 'none' to 9 ,'a lot'). Prior to the start of the MRI scan, participants were familiarized with these ratings by performing a short version of the task (with faces not used in the fMRI task) on a laptop.

Data analysis

Sample characteristics

We compared participants with and without (sub)clinical SAD on demographic variables and on the level of self-reported SA, by performing chi-square tests in SPSS (version 25) and by fitting multi-level regression models in R (R Core Team, 2016). Within these regression models, we modelled genetic correlations between family members by including random effects.

Behavioral data

We examined whether participants learned the social-evaluative value of the faces by performing a repeated measures ANOVA in SPSS, with condition (3 levels: positive, negative, neutral) and time (3 levels: T1, T2 and T3) as within-subjects factors. Significance level was set at $p \leq 0.05$; we applied Greenhouse–Geisser correction when the assumption of sphericity was violated.

Furthermore, we investigated whether the initial behavioral response to the faces, as well as the likeability ratings related to learning their value in the social-evaluative context, were associated with SA. To examine the initial response, we used the average of likeability ratings over the three faces provided at T1 (Likeability_T1); to examine the effect of the social-evaluative context, we calculated the difference in likeability scores between T2 and T3 over the three conditions (ΔLikeability_T3_T2), and for the three conditions separately; furthermore, we calculated difference scores to explore whether SA-level was differentially associated with learning the value of the negative, neutral and positive conditioned faces (ΔLikeability_T3_T2_Neg_vs_Neu; ΔLikeability_T3_T2_Neg_vs_Pos; ΔLikeability_T3_T2_Pos_vs_Neu).

We investigated the association between SA and these likeability ratings using linear mixed models in R (package: *coxme*), with self-reported SA (continuous variable; *z*-score, centered) as predictor of interest. Separate models were used to investigate the initial re-

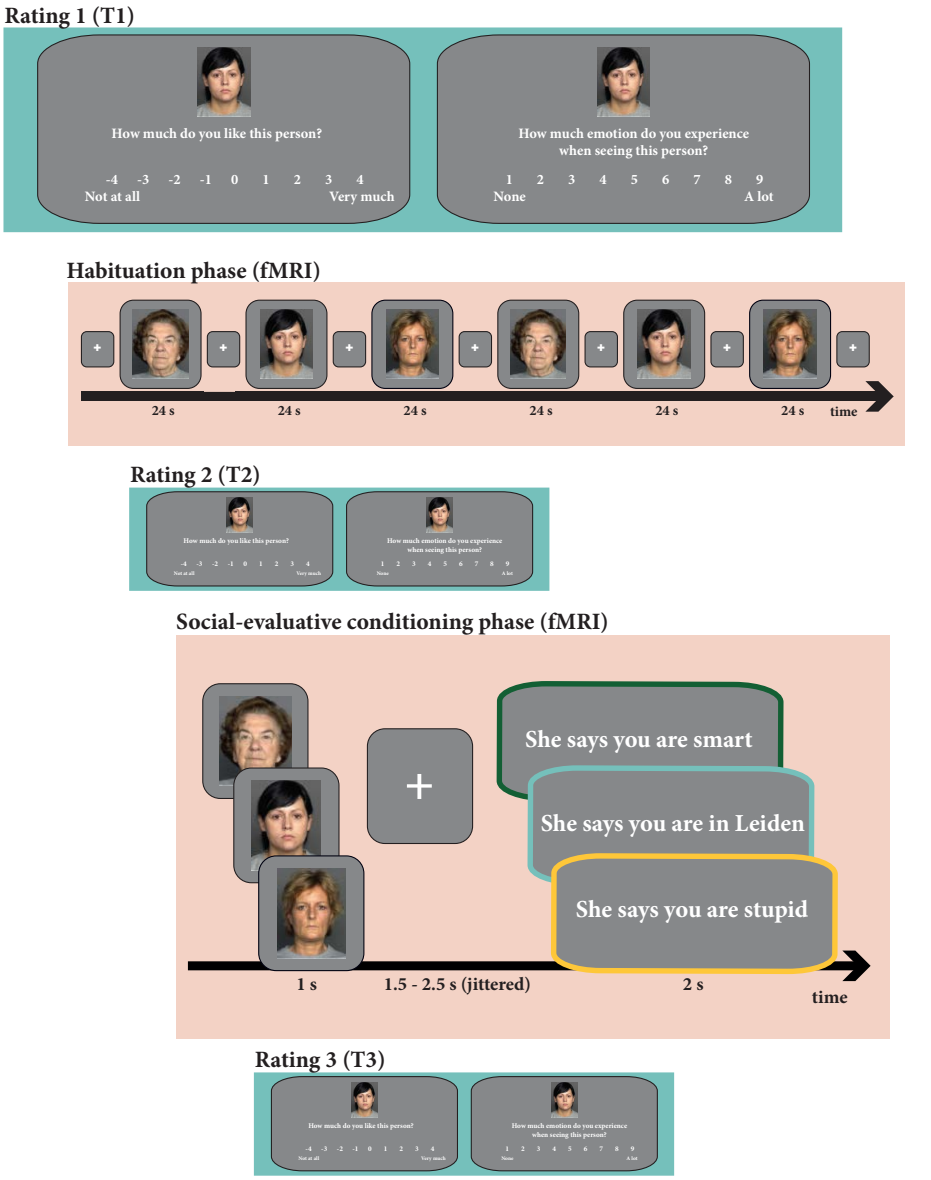


Figure 10.1 Overview of the neutral faces paradigm (NFP).
Stimuli were neutral faces selected from the FACES database (Ebner et al., 2010); the paradigm consists of two fMRI phases, being a habituation phase (described in more detail in *Supplemental Methods* as well as in (Bas-Hoogendam, van Steenbergen, Blackford, et al., 2019) and the social-evaluative conditioning phase (SCP) which is discussed in the present work. During the SCP, three neutral faces were consistently paired with either a positive endorsement, a negative comment or a socially-neutral statement, enabling participants to learn the social value of each face. At three time-points during the NFP, participants rated the faces on likeability and arousal.

sponse to the faces and the difference scores representing learning the value of the faces in the social-evaluative context. Random effects were included to account for the genetic correlations between family members; age (centered) and gender (centered) were added as covariates of no interest. Significance level was set at $p < 0.05$.

fMRI data

General processing steps and statistical analysis

Functional MRI data were pre-preprocessed using standardized procedures in FSL (Jenkinson et al., 2012) – see a detailed description of the processing steps in the *Supplemental Methods* and (Bas-Hoogendam, van Steenbergen, Blackford, et al., 2019; Bas-Hoogendam, van Steenbergen, Tissier, et al., 2019). Event-related statistical analysis was performed in native space, using FILM with local autocorrelation correction (Woolrich et al., 2001). Following previous analyses (Davis et al., 2010), we included twelve explanatory variables (EVs) as well as their temporal derivatives in the general linear model. The EVs represented the presentation of the faces belonging to the three conditions (negative, neutral, positive) and the presentation of the negative, neutral and positive social-evaluative sentences; separate EVs were created for the stimuli presented during the first half and the last half of the SCP, in order to enable investigating social-evaluative learning over time (cf. (Davis et al., 2010)). As the present work focuses on the processing of the conditioned stimuli (the faces), brain responses to the sentences will not be further analyzed. EVs were convolved with a double gamma hemodynamic response function and onset of the EVs for each individual was determined using custom-written scripts in Matlab. The fixation cross between the face and sentence stimuli and the fixation cross between the trials were not modeled and therefore served as the implicit baseline to which EVs could be compared.

We defined several contrasts of interest. First of all, we examined the contrast 'all faces > baseline', in order to investigate brain activation related to viewing the conditioned stimuli (i.e. faces with a social-evaluative meaning). Furthermore, we examined habituation (cf. (Davis et al., 2010)) by contrasting the faces presented during the first half of the SCP with the faces presented during the last half of the SCP; we refer to this contrast as 'all faces early > all faces late'. Next, we investigated valence-effects by contrasting the conditioned stimuli in the three different conditions ('negative > neutral'; 'negative > positive'; 'positive > neutral').

Brain activation at group level

For all contrasts of interest, we determined brain activation over the whole sample in the amygdala, by using masks of the left and right amygdala (mask description included in the *Supplemental Methods* and illustrated in *Supplemental Figure S10.1*; cluster threshold $z > 2.3$, cluster extent threshold $p < 0.05$ within the unilateral regions of interest (ROIs)).

Furthermore, for reasons of completeness, we also report explorative whole-brain analyses (cluster threshold $z > 3.1$, extent threshold $p < 0.05$).

Neurobiological candidate endophenotypes

We tested whether altered amygdala activation in response to conditioned faces could serve as a candidate SAD endophenotype, and investigated the *co-segregation of the candidate endophenotype with the disorder within families* using regression models in R, with self-reported SA-level (*z*-score; centered) as independent variable and individual activation level related to the contrasts of interest as dependent variables. Correlations between family members were modeled by including random effects; age and gender (both centered) were included as covariates of no interest. Furthermore, analyses were corrected for the level of depressive symptoms (*z*-score; centered). Models were ran for each voxel separately and results (*z*-scores) were transformed into a nifti-image with the dimensions of the MNI T1-template brain.

We examined the relation with SA within the clusters representing significant amygdala activation at group level; results were corrected for multiple comparisons using the FSL-tool easythresh (cluster threshold $z > 2.3$, cluster extent threshold $p < 0.05$, minimum of 10 voxels) (Worsley, 2001). For reasons of completeness, we also investigated the association with SA at the level of whole brain activation (*Supplemental Results; Supplemental Figure S10.2*). A subsequent sensitivity analysis was performed to investigate whether the results of the association analyses were driven by (comorbid) psychopathology other than SAD (*Supplemental Methods*).

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

Results

Sample characteristics

Details on quality checking and data availability are provided in the *Supplemental Results*. Characteristics of the samples ($n = 108$ for the behavioral analyses, data on subclinical SAD available for 102 participants; $n = 105$ for the fMRI analyse, data on subclinical SAD available for 98 participants) are presented in *Table 10.1*. Family members with (sub)clinical SAD did not differ from family members without SAD with respect to male / female ratio and age, but they reported higher levels of social anxiety and more depressive symptoms. We refer the reader to *Supplemental Tables S10.2-3* for a detailed characterization of the sample.

Table 10.1 Characteristics of participants with and without (sub)clinical SAD.

	Behavioral samplea			fMRI samplea		
	(Sub)clinical SAD (*n* = 39)	No SAD (*n* = 63)	Statistical analysis	(Sub)clinical SAD (*n* = 38)	No SAD (*n* = 60)	Statistical analysis
Demographics						
Male / Female (n)	20 /19	31 /32	$\chi^2(1) = 0.04$, $p = 0.84$	19 /19	30 /30	$\chi^2(1) = 0.0$, $p = 1.00$
Generation 1 / Generation 2 (n)	19 /20	27 /36	$\chi^2(1) = 0.33$, $p = 0.56$	19 /19	27 /33	$\chi^2(1) = 0.23$, $p = 0.63$
Age in years, (mean ± SD, (range))	30.3 ± 15.5 (9.2 – 59.6)	30.9 ± 15.4 (9.0 – 61.5)	β ± SE = -0.6 ± 3.1, $p = 0.85$	30.9 ± 15.3 (9.2 – 59.6)	31.9 ± 15.0 (9.4 – 61.5)	β ± SE =-1.0 ± 3.1, $p = 0.73$
Diagnostic information						
Clinical SAD (n)	17	0		17	0	
Self-report measures						
Social anxiety (z-score; mean ± SD)	3.0 ± 3.3	0.5 ± 1.6	β ± SE = 2.6 ± 0.5, $p < 0.001$	3.0 ± 3.1	0.6 ± 1.5	β ± SE = 2.6 ± 0.5, $p < 0.001$
Depression (z-score; mean ± SD)	0.0 ± 0.9	-0.5 ± 0.7	β ± SE = 0.5 ± 0.2, $p < 0.001$	0.01 ± 0.8	-0.5 ± 0.7	β ± SE = 0.5 ± 0.2, $p < 0.001$

Abbreviations

SD, standard deviation; SE, standard error.

Footnote

[a]: Due to technical reasons, data on the presence of subclinical SAD were lost for several family members. Data from these participants were, however, included in the endophenotype analyses using SA-level (*z*-score) as a predictor (behavioral sample: *n*=108; fMRI sample: *n*=105).

Behavioral data

Validation of the SCP

Likeability ratings for the faces, provided at three timepoints during the NFP, are provided in *Table 10.2* and illustrated in *Figure 10.2A*. As expected, a repeated measures ANOVA with condition and time as within-subject factors indicated a significant interaction between time and condition ($F(3.4,362.8) = 37.2$, $p < 0.001$, $\eta^2 = 0.18$). Subsequent repeated measures ANOVAs separately for each timepoint, with condition as within-subjects factor, indicated that the faces did not differ with respect to likeability at T1 ($F(2,214) = 1.0$, $p = 0.38$, $\eta^2 = 0.009$) and T2 ($F(2,214) = 0.9$, $p = 0.40$, $\eta^2 = 0.009$), which validated the use of these faces for the subsequent SCP. Indeed, after the SCP (T3), a significant effect of condition was present ($F(1.8,194.5) = 34.5$, $p < 0.001$, $\eta^2 = 0.24$), indicating that participants learned the social-evaluative value of the faces; this finding is in line with the original report on this paradigm (Davis et al., 2010).

Table 10.2 Behavioral ratings on the neutral faces paradigm.

Likeability ratings (mean ± SD)	**T1**	**T2**	**T3**
Average	0.7 ± 1.1	0.8 ± 1.1	0.7 ± 1.1
Negative	0.8 ± 1.6	0.8 ± 1.6	-0.3 ± 1.8
Neutral	0.7 ± 1.6	0.9 ± 1.6	1.0 ± 1.5
Positive	0.6 ± 1.6	0.7 ± 1.6	1.3 ± 1.6
Effect of social anxiety (*z*-score)[a]	β ± SE	*p*	
Likeability_T1	0.07 ± 0.04	0.07	
ΔLikeability_T3_T2	-0.08 ± 0.03	0.003	
ΔLikeability _T3_T2_positive	-0.06 ± 0.06	0.27	
ΔLikeability_T3_T2_ negative	-0.11 ± 0.06	0.07	
ΔLikeability-T3_T2_ neutral	-0.07 ± 0.05	0.15	

Abbreviations
SD, standard deviation; SE, standard error.

Footnote
[a] Corrected for age, gender and family structure.

Association analyses showed that the initial response to the neutral faces (Likeability_T1) was not significantly related to SA-level within the families (*Table 10.2*). SA-level was, however, associated with the change in likeability ratings due to social-evaluative conditioning: there was a significant negative relation between SA-level and ΔLikeability_T3_T2, indicating that the addition of the social-evaluative sentences (US) was aversive for family members with higher SA-levels (*Table 10.2; Figure 10.2B upper half*). This effect was present regardless of the valence of the comments: follow-up analyses indicated that the negative association between SA and ΔLikeability_T3_T2 was present in all three conditions (*Table 10.2; Figure 10.2B lower half*), while subsequent regression analyses on the difference scores

between the conditions confirmed that the relationship between SA and ΔLikeability_T3_T2 was not different between the conditions (ΔLikeability_T3_T2_Neg_vs_Neu: β ± SE = -0.04 ± 0.07, p = 0.57; ΔLikeability_T3_T2_Neg_vs_Pos: β ± SE = -0.05 ± 0.09, p = 0.60; ΔLikeability_T3_T2_Pos_vs_Neu: β ± SE = 0.007 ± 0.08, p = 0.93). A sensitivity analysis on the difference in likeability between T1 and T2 confirmed that the effect of SA was specific for the SCP of the NFP (*Supplemental Results*).

In addition to these likeability ratings, we included ratings of arousal in the NFP in line with the task description by Davis et al. (2010). However, it was hard to find a good transcription of the term 'arousal' when translating the question from English to Dutch. Indeed, participants indicated during debriefing that they struggled to interpret the question with

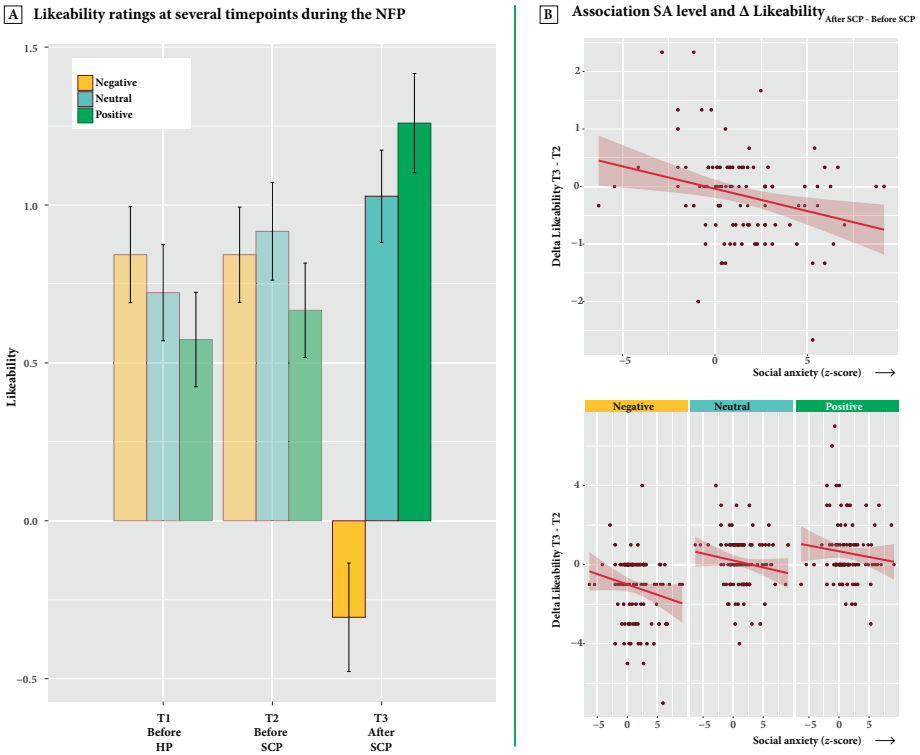


Figure 10.2 Behavioral ratings on the NFP.

Figure 10.2A Ratings of likeability for the three conditions at the three timepoints. Faded colors at T1 and T2 indicate that the faces were not conditioned yet; at T3, participants had learned the social-evaluative value of the faces, as indicated by a significant interaction between time and condition, as well as an effect of condition at T3. Errorbars represent standard errors of the mean.

Figure 10.2B Association between the level of social anxiety (SA) and learning the social-evaluative value of the faces (ΔLikeability_T3_T2), depicted over all conditions (upper half) and separate for the three conditions (lower half).

respect to arousal. Data showed that the changes in the arousal ratings due to conditioning resembled the pattern of the likeability ratings (i.e. increase for the positive condition and decrease for the negative condition), and did not, as expected based on the findings by Davis et al. (2010), reflect increased levels of arousal for the faces conditioned with the positive and negative social-evaluative sentences when compared to the neutrally-conditioned faces. Therefore, the arousal ratings will not be further considered; for reasons of completeness, they are available in *Supplemental Table S10.4*.

fMRI data

Brain activation at group level

Significant activation related to the contrasts of interest is summarized in Table 10.3 and illustrated in *Figure 10.3* (amygdala ROIs). These results confirmed the role of the amygdala during social-evaluative learning, previously described by Davis et al., (2010). In short, the ROI-analyses on the contrast 'all faces > baseline', 'negative > neutral', and 'negative > positive' revealed bilateral amygdala activation, while the contrast 'all faces early > all faces late' showed activation in the right amygdala. No amygdala activation was present for the contrast 'positive > neutral'. The latter contrast was therefore not further investigated in the endophenotype analysis.

Neurobiological candidate endophenotypes

Voxelwise regression analyses on the association between self-reported SA and amygdala activation related to viewing the conditioned stimuli (faces with a social-evaluative meaning) revealed significant positive associations within both the left and right amygdala (*Table 10.4; Figure 10.4*). The amygdala findings were replicated in a sensitivity analysis, in which data from participants with (comorbid) psychopathology other than SAD were excluded (*Supplemental Table S10.5; Supplemental Figure S10.3*). Within the right amygdala cluster, 22 voxels had at least moderate heritability (range: $h^2 = 0.20$ (moderate heritability) – 0.63 (high heritability)); in the left amygdala, only one voxel survived the threshold of $h^2 \geq 0.20$ (*Table 10.4*).

Analyses on the association with SA for the other contrasts of interest('all faces early >all faces late'; 'negative > neutral'; negative > positive') did not yield significant results within the amygdala.

Table 10.3 Brain activation independent from level of social anxiety.

Cluster	Region	*Z*-score	Peak coordinates (MNI space)			Cluster size
			x	y	z	
All faces > baseline						
Whole brain						
1	Occipital pole, fusiform gyrus	12.1	14	-94	2	81562
	Middle temporal gyrus	8.30	-62	-46	6	
	Middle frontal gyrus	9.90	-40	0	48	
	Orbitofrontal cortex	7.60	-46	28	-8	
	Amygdala, left	7.47	-20	-6	-14	
	Amygdala, right	6.27	22	-4	-18	
2	Caudate, right	4.93	16	8	8	397
Amygdala ROI						
1	Amygdala, left	7.47	-20	-6	-14	738
2	Amygdala, right	6.27	22	-4	-18	884
All faces early > all faces late						
Whole brain						
1	Occipital pole	4.77	16	-92	-4	2335
Amygdala ROI						
1	Amygdala, right	3.02	16	-4	-12	44
Negative > neutral						
Whole brain						
1	Anterior cingulate gyrus	4.35	8	44	8	963
2	Supramarginal gyrus, right	4.33	64	-40	8	513
3	Precentral gyrus, right	4.80	44	8	26	377
4	Cerebellum, left	3.81	-32	-86	-32	367
5	Superior temporal gyrus, right	5.04	44	-26	-2	347
Amygdala ROI						
1	Amygdala, left	3.87	-16	-8	-10	182
2	Amygdala, right	3.36	16	-12	-10	64
Negative > positive						
Whole brain						
1	Inferior frontal gyrus, right	4.36	50	16	20	572
Amygdala ROI						
1	Amygdala, left	3.37	-16	-10	-12	109
2	Amygdala, right	2.75	18	-4	-14	48
Positive > neutral						
Whole brain analysis						
1	Lingual gyrus, right	5.16	20	-66	-12	3876
	Lingual gyrus, left	4.63	-18	-74	-2	
	Lateral occipital cortex	4.59	30	-80	12	
Amygdala ROI		*No significant clusters*				

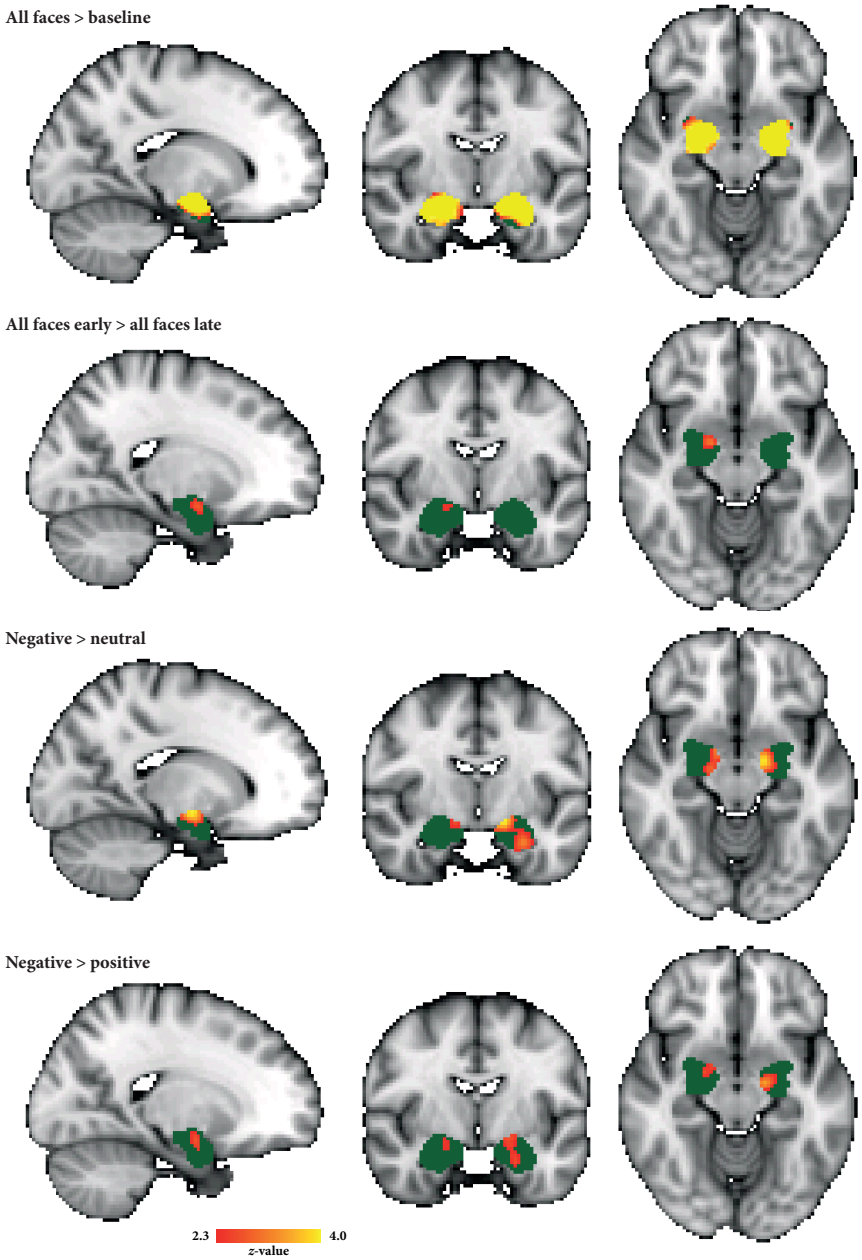


Figure 10.3 Amygdala activation (group level).
Activation related to contrasts of interest within the amygdala regions of interest (depicted in green), over the whole sample (n = 105). The contrast 'positive > neutral' did not yield significant amygdala activation. Coordinates displayed slices (MNI space, x,y,z): -16,-8,-12 (contrasts 'all faces > baseline' and 'negative > neutral') and 20,-6,-12 (contrasts 'all faces early > all faces late' and 'negative > positive'). Images are displayed according to radiological convention: right in the image is left in the brain.

Table 10.4 Effect of self-reported social anxiety on neutral face processing.

Region		*Z*-score	Peak coordinates (MNI space) x	y	z	Cluster size	Number of voxels with $h^2 > 0.20$	Mean h^2, range
All_faces > baseline								
Amygdala	Left	2.65	-28	-6	-14	36	1	0.27, n.a.
	Right	3.01	28	-10	-14	164	22	0.31, 0.20–0.63

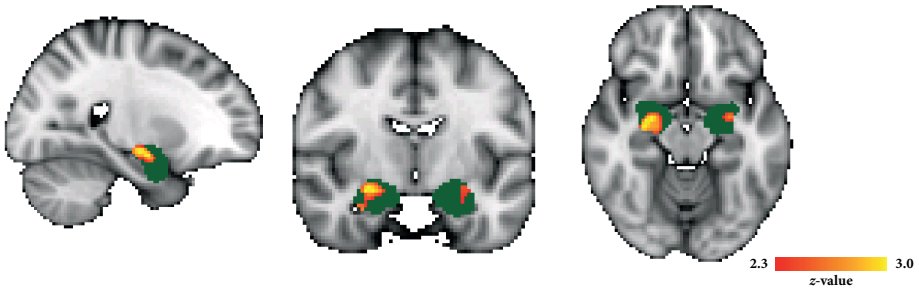


Figure 10.4 Association between social anxiety and brain activation in the amygdala.
Amygdala activation related to viewing faces conditioned with a social-evaluative meaning (versus baseline) co-segregates with social anxiety within families. Significant positive associations between social anxiety and activation were present in both the left (36 voxels) and right (164 voxels) amygdala.
Coordinates displayed slices (MNI space, x,y,z): 24,-8,-14. Images are displayed according to radiological convention: right in the image is left in the brain.

Discussion

Here, we demonstrated substantial evidence for amygdala hyperactivation, in response to faces conditioned with a social-evaluative meaning, as a putative neurobiological social anxiety disorder (SAD) endophenotype. Using a conditioning paradigm with high ecological validity in the context of SAD, in a unique sample of families genetically enriched for SAD (n = 105) (Bas-Hoogendam, Harrewijn, et al., 2018), we showed that amygdala reactivity *co-segregated with social anxiety within families of probands* (endophenotype criterion 4, first element); furthermore, multiple voxels within these amygdala clusters displayed at least moderate ($h^2 \geq 0.20$) *heritability* (endophenotype criterion 3). Thereby, we extend previous work on the role of the amygdala in SAD (see summary by (Bas-Hoogendam et al., 2016)), and offer novel insights into the genetic vulnerability to SAD.

Amygdala hyperreactivity during social-evaluative learning

The positive association between SA-level and amygdala activation to social-evaluative conditioned faces (conditioned stimuli, CS) confirmed our a priori prediction, which

was based on a previous neuroimaging study reporting increased SA-related amygdala activation during conditioning of socially threatening stimuli (Pejic et al., 2013). Here, we extend these findings, by using a paradigm which included three types of social evaluation (negative, neutral and positive; unconditioned stimuli, US), and demonstrated amygdala hyperreactivity within SAD patients as well as their family members.

Interestingly, although the analyses using other contrasts of interest, defined to determine amygdala activation during the course of the social-evaluative conditioning phase (SCP; contrast 'all faces early > all faces late') and related to the three different US conditions ('negative > neutral'; 'negative > positive'), revealed overall amygdala engagement at the group level, in line with the results of Davis and colleagues (2010), they did not yield significant associations with SA. These results suggest that the SA-related amygdala hyperreactivity seems not to differ between the first and last half of the social-evaluative conditioning phase (SCP), nor was this amygdala hyperreactivity specific for faces conditioned with negative, neutral or positive sentences, although we obviously cannot exclude that the lower statistical power inherent to these difference contrasts (i.e. containing less trials) limited us to detect significant effects of SA. We argue that these results reflect that the amygdala hyperreactivity in family members with high SA-levels is related to the social-evaluative context of the SCP, in which participants were directly addressed ('He says you are …'), rather than to the valence of the sentences (for example, 'He says you are boring' (negative), 'He says you are smart' (positive) or 'He says you are in Leiden'(neutral)). This idea is supported by the behavioral data, as these showed that family members with higher SA-levels rated all faces as less likeable after conditioning, independent from the value of the conditioning sentences (US). Together, these findings underscore the increased saliency of social information, being it negative, positive, or neutrally loaded, in social anxiety, which was present even without a cover story (note that we did not pretend that the faces belonged to 'real people' who did judge the participants in real-life; cf. (Harrewijn et al., 2018)).

The present results concur with contemporary models of social anxiety, acknowledging the multidimensional nature of the disorder (Reichenberger & Blechert, 2018). For example, as illustrated by a recent study, SAD patients displayed elevated scores on fear of negative evaluation as well as on fear of positive evaluation, combined with altered psychophysiological responses to negative as well as to positive social-evaluative videos (Reichenberger et al., 2019). Our findings support the view that social anxiety involves fear and avoidance of all potential social-evaluative interpersonal interactions (Miskovic & Schmidt, 2012), and emphasize that, although the fear of negative evaluation is especially prominent in SAD, the central fear in socially anxious individuals concerns the view that their self-characteristics are deficient or contrary to perceived societal expectations (Moscovitch, 2009). It is of importance to acknowledge this comprehensive fear in cognitive-behavioral therapy for SAD.

Furthermore, our results broaden the knowledge with respect to amygdala overreactivity in SAD. A recent meta-analysis indicated that SA is associated with increased amygdala

responsiveness related to face perception processing (Gentili et al., 2016), and it is commonly hypothesized that amygdala hyperreactivity is reflective of the heightened threat processing that characterizes SAD (Brühl, Delsignore, et al., 2014). Indeed, hyperactivation of the amygdala in response to socially-relevant stimuli has been repeatedly reported in SAD patients, as well as in children and adolescents with anxiety disorders (Blair, Geraci, Korelitz, et al., 2011; Ferri, Bress, Eaton, & Proudfit, 2014; Figel et al., 2019; Kraus et al., 2018;. Williams et al., 2015). However, to the best of our knowledge, the present results are the first demonstrating amygdala hyperreactivity in response to conditioned faces with a social-evaluative meaning, and the first to detect amygdala overreactivity within a sample of patients with SAD as well as their family members of two generations.

Co-segregation within families

The unique multiplex and multigenerational family-design of the LFLSAD enabled us to investigate two endophenotype criteria within the same sample, namely the *co-segregation within families* and the *heritability* of the candidate endophenotype. In addition to the association between amygdala hyperreactivity and the level of SA within the families, our data revealed that amygdala hyperactivation displayed moderate to even high heritability. Thereby, our results extend previous work reporting genetic influences on amygdala activation (cf. (Bas-Hoogendam et al., 2016)) and indicate that amygdala hyperreactivity is not just a biomarker of SAD (a characteristic associated with the disorder, which is not necessarily positioned on the pathway from genotype to phenotype; cf. (Beauchaine & Constantino, 2017; Lenzenweger, 2013a)), but reflective of the genetic vulnerability to SAD, thus providing a starting point for the development of preventive and therapeutic interventions (Beauchaine et al., 2008).

Amygdala function, structure and connectivity

In the present study, we used a mask of the extended amygdala, based on previous work using this paradigm (Davis et al., 2010), and in line with theories on the role of the extended amygdala in conditioning and treat processing (Fox, Oler, Tromp, et al., 2015; Shin & Liberzon, 2010). The amygdala consists of several subnuclei, being the laterobasal, centromedial, and superficial nucleus, with distinct connectivity patterns with other brain regions (Kerestes, Chase, Phillips, Ladouceur, & Eickhoff, 2017; Roy et al., 2009); furthermore, these connectivity patterns display different relationships with anxiety-related temperamental traits (Blackford et al., 2014; Roy et al., 2014).

According to a probabilistic atlas (Amunts et al., 2005), the hyperreactivity of the amygdala in the present study maps to the bilateral laterobasal nuclei. These nuclei receive information from sensory cortical regions, frontal brain areas and subcortical regions, and play a role in associative processing of environmental cues and the integration of this information with self-relevant cognition (Bzdok, Laird, Zilles, Fox, & Eickhoff, 2013). Future studies

could explore if there are SA-related changes in connectivity of these nuclei (cf. (Pannekoek et al., 2013; Prater et al., 2013)), and whether such alterations are heritable.

Furthermore, it is interesting to note that, in contrast to the consistent findings with respect to amygdala hyperactivation in SAD, findings on SAD-related alterations in amygdala structure are inconclusive (Brühl, Delsignore, et al., 2014). However, both a recent mega-analysis (Bas-Hoogendam, van Steenbergen, Pannekoek, et al., 2017) as well as a recent meta-analysis ((Wang et al., 2018) cf. the commentary by (Bas-Hoogendam, 2019)) did not report structural alterations in the amygdala in SAD patients, while we, in a previous study on the LFLSAD sample, did not detect SA-related differences in amygdala volume in socially-anxious families (Bas-Hoogendam, van Steenbergen, Tissier, et al., 2018b). Together, these findings suggest that alterations in amygdala function, rather than in its structure, are associated with SAD.

Limitations and future research

The LFLSAD was especially designed to investigate the endophenotype criteria of *co-segregation* and *heritability*. Longitudinal studies involving control families from the general population are essential to assess other endophenotype criteria, like the *trait-stability of the candidate endophenotype* (criterion 2) and the *difference between non-affected family members and participants from the general population* (criterion 4, second element). Furthermore, as the present work focused on the amygdala as an a priori defined, hypothesis-based region of interest, and we only performed an exploratory whole-brain analysis on the association with SA with a stringent statistical threshold, we might have missed functional SA-related alterations in other brain areas. For example, a recent study on reversal learning indicated that trait SA influenced learning rate-related activation of the dorsal anterior cingulate cortex (Piray, Ly, Roelofs, Cools, & Toni, 2018), while Blair et al. (2016) reported, besides amygdala hyperactivation, increased responsiveness of frontal and parietal cortices during social reference learning in SAD patients. Future studies could explore whether these regions display SA-related functional alterations during social conditioning as well.

In conclusion, the results of the present study provide evidence for bilateral amygdala hyperactivation in response to conditioned faces with a social-evaluative meaning as a candidate neurobiological SAD endophenotype. As such, these findings shed novel light on the genetic susceptibility to SAD.

Supplemental Methods

Participants

Recruitment and ethics

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

Both parents signed the informed consent form for their children, and children between 12 and 18 years of age signed the form themselves as well. Participants received a financial compensation. Confidentiality of the data was maintained by the use of a unique research ID number for each family member.

Phenotyping

The presence of DSM-IV diagnoses was determined using the Mini-International Neuropsychiatric Interview (M.I.N.I.)-Plus (version 5.0.0) (Sheehan et al., 1998; van Vliet & de Beurs, 2007) or the M.I.N.I.-Kid interview (version 6.0) (Bauhuis et al., 2013; Sheehan et al., 2010); these interviews were administered by experienced clinicians and recorded.

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

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

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

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

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

Two subscales of the Wechsler Adult Intelligence Scale-IV (WAIS-IV) (Wechsler et al., 2008) or Wechsler Intelligence Scale for Children-III (WISC) (Wechsler, 1991), the

similarities (verbal comprehension) and block design (perceptual reasoning) subtests, were administered to obtain an estimate of cognitive functioning.

MRI experiment: detailed description

Prior to the MRI scan, participants were informed about the safety procedures and they were told that they could refrain from continuing the experiment at any time. Children and adolescents were familiarized with the MRI scanner using a mock scanner (Galván, 2010) and all participants received instructions about the task paradigms presented during the scan session. Scanning was performed using a 3.0 T Philips Achieva MRI scanner (Philips Medical Systems, Best, The Netherlands), equipped with a 32-channel Sensitivity Encoding head coil. The total duration of the MRI scan protocol was 54 min 47 s.

During the neutral faces paradigm, fMRI scans were acquired using T2*-weighted echo-planar imaging (EPI). Characteristics of these scans with the following characteristics: 38 axial slices, 2.75 mm x 2.75 mm x 2.75 mm + 10 % interslice gap, field of view (FOV) = 220 mm x 115 mm x 220 mm, repetition time (TR) = 2200 ms, echo time (TE) = 30 ms. The first six volumes of each fMRI scan were dummy volumes; these volumes were removed to allow for equilibration of T1 saturation effects.

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

Neutral faces paradigm

Habituation phase

The first phase of the NFP paradigm, the habituation phase (HP), was inspired by the paradigm described by Wedig and colleagues (Wedig et al., 2005) and by several other paradigms on habituation (Blackford et al., 2013; Schwartz, Wright, Shin, Kagan, Whalen, et al., 2003; Schwartz, Wright, Shin, Kagan, & Rauch, 2003). This habituation phase started with the presentation of a fixation cross (24 s), followed by the presentation of three neutral faces. The faces were presented in blocks of 24 s, and within each block a neutral face was repeatedly presented (48 times) for 200 ms with a 300 ms interstimulus interval. There were six face blocks (two blocks for each face), in order to resemble the design described previously (Wedig et al., 2005), and face blocks were separated by the presentation of a fixation cross (duration 12 s). An additional 12 s fixation cross was presented at the end of the habituation phase. Faces were presented in pseudo-random order and participants were instructed to keep looking at the faces and the fixation crosses. Total duration of the habitu-

ation phase was 2 min 42 s. Results of the HP are reported elsewhere (Bas-Hoogendam, van Steenbergen, Blackford, et al., 2019).

Faces

We selected the following faces from the FACES database (Ebner et al., 2010): M049, M072 and M089 (faces of men; mean age: 24 y) and F069, F152 and F171 (faces of women; mean age: 25.7 y).

fMRI data

General processing steps

FMRI data were denoised using FIX (FMRIB's ICA-based X-noiseifier), a publicly available plugin for FSL (FMRIB Software Library, version 5.0.9) (Jenkinson et al., 2012), which provides an automatic solution for denoising fMRI data via accurate classification of ICA components (Griffanti et al., 2014; Salimi-Khorshidi et al., 2014). Next, data underwent several preprocessing steps using FEAT (FMRI Expert Analysis Tool; version 6.00) (Jenkinson et al., 2012; Smith et al., 2004), including motion correction using MCFLIRT (Jenkinson et al., 2002), spatial smoothing using a Gaussian kernel of full-width half-maximum (FWHM) 6.0 mm and grand-mean intensity normalization of the entire 4D dataset by a single scaling factor in order to enable higher-level analyses and registration. Scans were first registered to high-resolution EPI images, which were registered to T1 images, which in turn were registered to the Montreal Neurological Institute (MNI) T1-template brain (resolution 2 mm) using FNIRT nonlinear registration (warp resolution 10 mm) (Andersson et al., 2007; Jenkinson et al., 2002; Jenkinson & Smith, 2001). Next, ICA-AROMA (ICA-based Automatic Removal of Motion Artifacts) was used to remove motion-related artefacts (Pruim, Mennes, van Rooij, et al., 2015; Pruim, Mennes, Buitelaar, et al., 2015). Data were then submitted to FEAT to perform non-brain removal using BET (Smith, 2002), high-pass temporal filtering (Gaussian-weighted least-squares straight line fitting, with sigma = 30.0 s) and registration. Functional scans of each participant were registered to the individual 3D T1-weighted anatomical scan using FLIRT (Jenkinson et al., 2002; Jenkinson & Smith, 2001) and subsequently registered to the MNI T1-template brain (resolution 2 mm) using FNIRT nonlinear registration (warp resolution 10 mm) (Andersson et al., 2007). We checked whether the individual scans were registered correctly and confirmed that relative motion parameters did not exceed 2.5 mm.

Region of interest – amygdala mask

Masks for the amygdala were based on the Harvard-Oxford Subcortical Structural Atlas implemented in FSLview. We used a liberal threshold of 5 %, based on the findings by Davis et al. on the social conditioning paradigm (Davis et al., 2010): we transformed the coordinates of their results (reported in Talairach-space) to MNI space and chose the threshold of

our mask in such a way that the coordinates of their findings (in medial ventral amygdala, dorsal amygdala/substantia innominate and lateral ventral amygdala) were included in our ROIs. Furthermore, because of the laterality of the results reported by Davis and colleagues (2010), we used unilateral masks and investigated effects within the left and right amygdala separately. Masks are depicted in *Supplemental Figure S10.1.*

Sensitivity analysis

We performed a sensitivity analysis to examine whether the results of the association analyses (effect of self-reported social anxiety (*z*-score) on brain activation related to the contrast 'all faces > baseline') were driven by (comorbid) psychopathology other than SAD (cf. (Bas-Hoogendam, van Steenbergen, Tissier, et al., 2018b)). To this aim, we excluded all family members with past and / or present psychopathology other than SAD and repeated the association analysis. Note however, that this analysis may yield biased results, as the majority of the probands, on which the selection of the families was based, had comorbid psychopathology and were thus excluded. We used the same statistical threshold as for the main analyses (within the amygdala ROIs: $z > 2.3$, cluster-threshold $p < 0.05$).

Supplemental Results

Data availability

We collected MRI data from nine families ($n = 113$) (Bas-Hoogendam, Harrewijn, et al., 2018), but we had to exclude data from one family ($n = 3$ family members) as this family's proband was not able to participate in the MRI experiment due to an MRI contraindication. Due to technical problems, behavioral data were lost for two participants (behavioral sample: $n = 108$), while 110 imaging data sets were available for fMRI pre-processing and quality control. Two datasets could not be used due to an imaging artefact, while the relative motion parameters of three other participants exceeded 2.5 mm. As a result, 105 fMRI datasets were available for further analysis of brain activation related to the social conditioning phase. Furthermore, data on the presence of (sub)clinical SAD were lost for several family members.

Sample characteristics

We refer to *Supplemental Table S10.2* and *Supplemental Table S10.3* for detailed information about the sample. In line with the design of the study, participants originated from two generations, which differed significantly in age (behavioral sample: $\beta \pm SE = -30.3 \pm 0.7$, $p < 0.001$; fMRI sample: $\beta \pm SE = -30.1 \pm 0.7$, $p < 0.001$), but not in male / female ratio (behavioral sample: $\chi^2(1) = 0.57$, $p = 0.56$; fMRI sample: $\chi^2(1) = 0.76$, $p = 0.44$). In line with previous reports on this sample (Bas-Hoogendam, van Steenbergen, Tissier, et al.,

2019), family members with and without (sub)clinical SAD did not differ with respect to male / female ratio, age and estimated IQ. Groups did differ, however, in comorbidity rates: family members with (sub)clinical SAD were more often diagnosed with depression (past), dysthymia (present) and panic disorder. These differences were, however, only significant at an uncorrected significance level. Furthermore, family members with (sub)clinical SAD reported higher levels of fear of negative evaluation, more depressive symptoms, higher levels of trait anxiety and behavioral inhibition (BIS), as well as lower levels of behavioral activation (BAS).

Behavioral data

Ratings of arousal

Arousal ratings are summarized in *Supplemental Table S10.4*. As several participants indicated during debriefing that they struggled to interpret the arousal question correctly, results of these ratings will therefore not be further considered.

Behavioral candidate endophenotypes: sensitivity analysis

A sensitivity analyses, investigating the effect of SA-level on the difference in likeability between T1 and T2 (ΔLikeability_T2_T1) showed no significant association between these scores and social anxiety ($\beta \pm SE = -0.02 \pm 0.03$, $p = 0.51$), confirming that the effect of SA was specific for the SCP of the NFP.

fMRI data

Whole brain analyses on association with SA

For reasons of completeness, we investigated the association between SA-level and brain activation at the whole-brain level (cluster threshold $z > 3.1$, extent threshold $p < 0.05$), in addition to the ROI analyses within the amygdala. There were no significant clusters for the contrasts 'all faces > baseline', 'all faces early > all faces late', and 'negative > neutral'. For the contrast 'negative > positive', we found a positive relation between SA-level and brain activation in the right frontal pole (cluster size: 392 voxels, $p = 0.008$, max z-value 5.05, MNI coordinates (x,y,z) peak voxel: 24, 52, -14) (*Supplemental Figure S10.2*).

Sensitivity analyses

In the sensitivity analysis, we excluded all participants with past and/or present comorbid psychopathology other than SAD; this resulted in a sample of 58 participants, of which 14 in the (sub)clinical SAD group. Next, we repeated the association analysis with self-reported social anxiety as predictor (corrected for age, gender and level of depressive symptoms). These analyses confirmed the amygdala findings for the contrast 'all faces > baseline' (*Supplemental Table S10.5; Supplemental Figure S10.3).*

Supplemental Tables

Supplemental Table S10.1 Sentences included in the SCP.

Negative comments	Positive endorsements	Socially-neutral statements
He / she thinks you are lazy	He / she thinks you are active	He / she thinks you are in the MRI scanner
He / she thinks you are boring	He / she thinks you are nice	He / she thinks you speak Dutch
He / she says you are stupid	He / she says you are smart	He / she says you are in Leiden
He / she says you are greedy	He / she says you are generous	He / she says you are righthanded / lefthanded*

Footnote

* This sentence was adapted based on the scores on the Edinburgh Handedness Inventory (Oldfield, 1971).

Supplemental Table S10.2 Detailed characteristics of participants with and without (sub)clinical SAD: demographics and clinical information.

	Behavioral samplea		
	(Sub)clinical SAD (*n* = 39)	**No SAD (*n* = 63)**	**Statistical analysis**
Demographics			
Male / Female (n)	20 / 19	31 /32	$\chi^2(1) = 0.04$, $p = 0.84$
Generation 1 / Generation 2 (n)	19 / 20	27 / 36	$\chi^2(1) = 0.33$, $p = 0.56$
Age in years (mean ± SD)	30.3 ± 15.5	30.9 ± 15.4	β ± SE = -0.6 ± 3.1, $p = 0.85$
Estimated IQ (mean ± SD)	104.3 ± 12.2	105.7 ± 10.4	β ± SE=-2.2 ± 2.2, $p = 0.32$
Diagnostic information (*n*)			
Clinical SAD	17	0	$\chi^2(1) = 32.9$, $p < 0.001$
Depressive episode present	1	1	$\chi^2(1) = 0.2$, $p = 0.69$
Depressive episode past	12	9	$\chi^2(1) = 4.9$, $p = 0.03$
Dysthymia present	3	0	$\chi^2(1) = 5.4$, $p = 0.02$
Dysthymia past	1	1	$\chi^2(1) = 0.2$, $p = 0.65$
Panic disorder lifetime	5	2	$\chi^2(1) = 4.0$, $p = 0.05$
Agoraphobia present	3	2	$\chi^2(1) = 1.3$, $p = 0.26$
Agoraphobia past	0	2	$\chi^2(1) = 1.2$, $p = 0.28$
Separation anxiety	0	1	$\chi^2(1) = 0.8$, $p = 0.39$
Specific phobia	2	3	$\chi^2(1) = 0.02$, $p = 0.89$
Generalized anxiety disorder	1	0	$\chi^2(1) = 1.8$, $p = 0.18$
Obsessive-compulsive disorder	1	0	$\chi^2(1) = 1.8$, $p = 0.18$
Attention deficit hyperactivity disorder (ADHD)	3	1	$\chi^2(1) = 2.5$, $p = 0.11$
Alcohol dependency present	1	1	$\chi^2(1) = 0.2$, $p = 0.70$
Alcohol dependency lifetime	1	3	$\chi^2(1) = 0.2$, $p = 0.63$
Present psychotropic medication (*n*)	4	3	$\chi^2(1) = 1.1$, $p = 0.30$
Antidepressants, not otherwise specified	3	0	
ADHD medication, not otherwise specified	1	3	

Abbreviations

SD, standard deviation; SE, standard error.

Footnote

[a]: Due to technical reasons, data on the presence of subclinical SAD were lost for six family members. Data from these participants were, however, included in the endophenotype analyses using SA-level (*z*-score) as a predictor (behavioral sample: $n = 108$).

Supplemental Table S10.3 Detailed characteristics of participants with and without (sub)clinical SAD: scores on self-report questionnaires.

	Behavioral sample[a]		
	(Sub)clinical SAD (*n* = 39)	No SAD (*n* = 63)	Statistical analysis
Self-report measures			
Social anxiety symptoms (z-score; mean ± SD)	3.0 ± 3.3	0.5 ± 1.6	β ± SE = 2.6 ± 0.5, $p < 0.001$
Fear of negative evaluation (mean ± SD)	23.3 ± 12.3	12.9 ± 8.0	β ± SE = 10.4 ± 2.0, $p < 0.001$
Depressive symptoms (z-score; mean ± SD)	0.0 ± 0.9	-0.5 ± 0.7	β ± SE = 0.5 ± 0.2, $p < 0.001$
STAI – trait (mean ± SD)	38.8 ± 9.4	33.2 ± 8.5	β ± SE = 5.3 ± 1.8, $p = 0.003$
BIS (z-score; mean ± SD)	0.4 ± 1.3	-0.4 ± 0.9	β ± SE = 0.8 ± 0.2, $p < 0.001$
BAS (z-score; mean ± SD)	-0.9 ± 1.0	-0.6 ± 1.0	β ± SE = -0.5 ± 0.2, $p = 0.02$

Abbreviations
SD: standard deviation; SE: standard error.

Footnote
[a]: Due to technical reasons, data on the presence of (sub)clinical SAD were lost for six family members. Data from these participants were, however, included in the endophenotype analyses using SA-level (*z*-score) as a predictor (behavioral sample: $n = 108$).

Supplemental Table S10.4 Arousal ratings (*n* = 108).

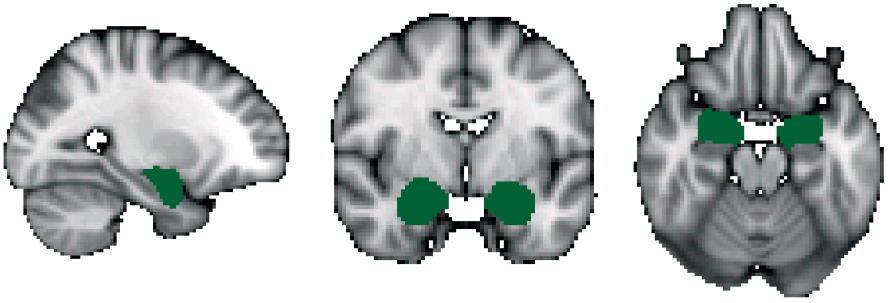
Condition	T1	T2	T3
Negative	3.1 ± 1.6	3.2 ± 1.6	2.9 ± 1.6
Neutral	2.9 ± 1.7	3.0 ± 1.7	3.1 ± 1.8
Positive	2.9 ± 1.5	2.9 ± 1.6	3.4 ± 1.8

Footnote
Values represent mean ± standard deviation.

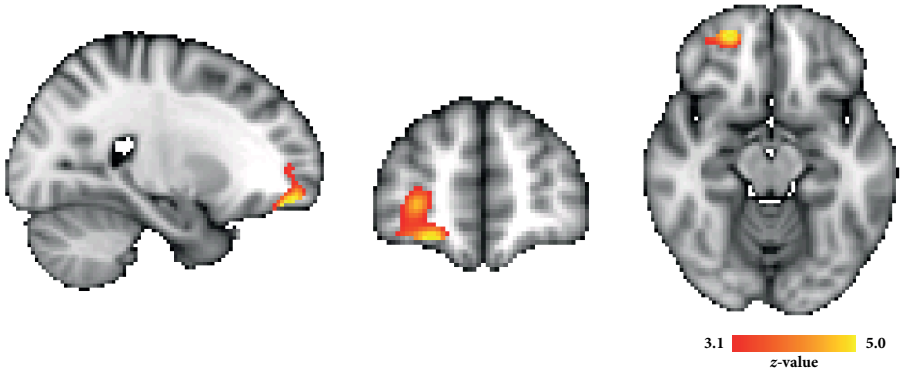
Supplemental Table S10.5 Sensitivity analyses in sample without (comorbid) psychopathology other than SAD.

			Peak coordinates (MNI space)			
Region		***Z*-score**	**x**	**y**	**z**	**Cluster size**
All faces > baseline						
Amygdala	Left	2.98	-24	-12	-18	38
	Right	4.61	26	-8	-14	161

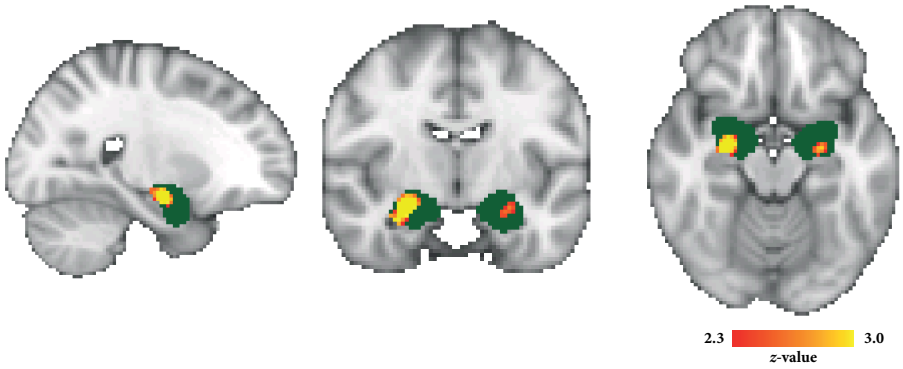
Supplemental Figures



Supplemental Figure S10.1 Mask of the amygdala regions of interest.
Coordinates displayed slices (MNI space, x,y,z): -26, -4, -20. Masks are displayed on the template MNI_T1_152_2mm_brain and images are displayed according to radiological convention: right in the image is left in the brain.



Supplemental Figure S10.2 Results whole-brain analysis on the relation between SA-level and brain activation (contrast 'negative > positive').
Positive association between SA-level and brain activation related to the contrast 'negative > positive' in the left frontal pole. Coordinates displayed slices (MNI space, x,y,z): 24, -52, -14.
Clusters are displayed on the template MNI_T1_152_2mm_brain and images are displayed according to radiological convention: right in the image is left in the brain.



Supplemental Figure S10.3 Results sensitivity analyses in sample without (comorbid) psychopathology other than SAD (*n* = 58).

Positive association between SA-level and brain activation related to the contrast ' all faces > baseline' in both the left and right amygdala. Coordinates displayed slices (MNI space, x,y,z): 24, -8, -16. Clusters are displayed on the temple MNI_T1_152_2mm_brain and images are displayed according to radiological convention: right in the image is left in the brain.