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

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Chapter 9

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

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Abstract

Background

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

Methods

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

Results

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

Conclusions

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

Introduction

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

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

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

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

Materials and methods

Participants

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

Table 9.1 Characteristics of participants within the LFLSAD.

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

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

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

Data acquisition and analyses

Phenotyping

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

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

Demographics and clinical characteristics

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

Habituation paradigm during functional (f)MRI

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

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

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

fMRI data: habituation response

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

fMRI data: endophenotype analysis

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

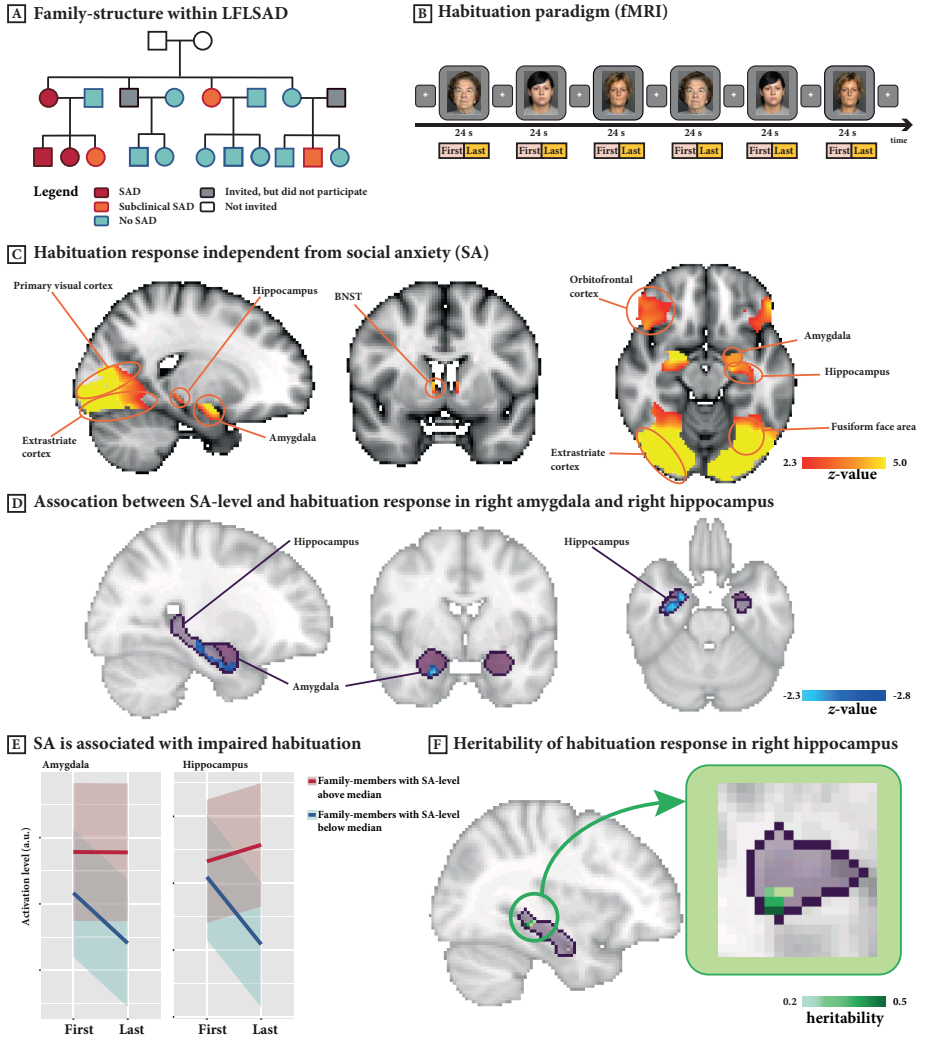

Figure 9.1 Failure to habituate within families genetically enriched for social anxiety disorder.

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

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

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

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

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

Results

Demographics and clinical characteristics

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

fMRI analyses

Habituation response

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

Endophenotype analyses

Voxelwise regression analyses revealed that SA-level was associated with reduced neural habituation in the right amygdala (cluster characteristics: 27 voxels, p = 0.013; max z-value: 2.82) and right hippocampus (cluster characteristics: 136 voxels, p = 0.04; max z-value: 3.13) (*Figure 9.1D*). Follow-up analyses on the individual activation levels within the significant

clusters indicated that family members with high SA-levels showed a failed habituation response in the late part of the task and not a heightened novelty response in the early part of the task (*Figure 9.1E*). To specify, SA-level was positively related with brain activation levels during the presentation of the faces in the *last half* of the blocks (amygdala: β ± SE = 1.23 ± 0.67, $p = 0.07$; hippocampus: β ± SE = 1.53 ± 0.57, $p = 0.007$), while there was no relation between SA and activation during the *early* presentation of the faces in the *first half* of the blocks (amygdala: β ± SE = -0.39 ± 0.72, $p = 0.59$; hippocampus: β ± SE = -0.25 ± 0.72, $p = 0.73$; regression analyses corrected for age, gender and family structure).

Voxelwise heritability analyses within the clusters showing an association with SA-level revealed that the neural habituation response within the right hippocampus was heritable, with 13 voxels showing at least moderate heritability ($h^2 > 0.20$)(*Figure 9.1F*). In the other ROIs, no association with SA was present at the pregnificance level.

Table 9.2 Neural habituation in regions of interest (ROIs) at group level.

			Peak coordinates (MNI space)			
ROI	**Left / right**	***Z*-score**	**x**	**y**	**z**	**Cluster size**
Amygdala	Left	5.26	-20	-12	-12	191
	Right	5.88	20	-4	-12	225
BNST	Left	3.53	-8	4	6	5
	Right	4.50	8	4	6	9
Extrastriate cortex	Left/right	9.49	-30	-86	-14	8736
FFA	Left	8.99	-26	-84	-18	964
	Right	8.67	30	-78	-2	1200
Hippocampus	Left	5.26	-20	-12	-12	244
	Right	5.88	20	-4	-12	233
Orbitofrontal cortex	Left	4.76	-52	36	-12	952
	Right	5.44	34	26	-26	1205
V1	Left/right	9.42	6	-90	0	2880
vmPFC	*No significant clusters*					

Abbreviations
BNST: bed nucleus of stria terminalis; FFA: fusiform face area; V1: primary visual cortex; vmPFC: ventromedial prefrontal cortex.

Discussion

The present findings provide evidence that altered habituation is an endophenotype of social anxiety disorder (SAD). First, we showed that impaired habituation to neutral faces in neural structures supporting threat (amygdala) and memory-related processes (hippocampus) is associated with SA within families genetically enriched for SAD, supporting

the endophenotype criterion of *co-segregration within families* (criterion 4, first element). Next, our data indicated that the habituation response to neutral faces in the hippocampus is partly *heritable* (endophenotype criterion 3). Thereby, these results from the multiplex, multigenerational Leiden Family Lab study on Social Anxiety Disorder add substantially to prior work indicating an *association* between impaired habituation and SA (endophenotype criterion 1) and studies on the *trait-stability* of the habituation response (endophenotype criterion 2) (Avery & Blackford, 2016; Blackford et al., 2013, 2011) (cf. (Bas-Hoogendam et al., 2016)), and shed light on the genetic pathways leading to SAD.

Impaired habituation in families genetically enriched for SAD

As habituation is an adaptive process, reflecting a basic, non-associative learning mechanism that acts like 'an intelligent firewall' that filters out irrelevant sensory information (Poon & Young, 2006), the failure to habituate likely contributes to the feelings of uncertainty that characterize individuals with high SA levels: at the neurobiological level, these individuals keep considering neutral social stimuli as being alarming, which makes them feel uncomfortable in social situations and contributes to aberrant social behavior. Although previous neuroimaging studies on habituation in patients with SAD yielded divergent results (Campbell et al., 2007; Sladky et al., 2012), potentially due to differences in task characteristics (cf. the extended discussion in (Bas-Hoogendam et al., 2016)), our findings are in line with work on participants with high levels of behavioral inhibition (Blackford et al., 2013; Schwartz et al., 2012; Schwartz, Wright, Shin, Kagan, & Rauch, 2003), as well as with the results of a study in individuals with high levels of social fearfulness (Avery & Blackford, 2016). Interestingly, impairments in neural habituation have also been reported in other neuropsychiatric disorders in which social behavior is altered, like autism and schizophrenia (Blackford, Williams, & Heckers, 2015; Kleinhans et al., 2009; Williams, Blackford, Luksik, Gauthier, & Heckers, 2013, and review by McDiarmid, Bernardos, & Rankin, 2017), indicating that impaired habituation is not specifically related to SA. However, as argued more extensively in Bas-Hoogendam et al. (2016), specificity is not a prerequisite for an endophenotype, as endophenotypes that are related to more than one disorder could advance transdiagnostic research on the shared genetic background of these disorders (Bearden & Freimer, 2006).

Habituation response in hippocampus, but not the amygdala, is heritable

The dissociation in heritability of the habituation response between the amygdala and hippocampus was unexpected, as previous studies indicated genetic influences on both hippocampus activation (Kauppi, Nilsson, Persson, & Nyberg, 2014) and amygdala reactivity (Lonsdorf et al., 2011; Munafò et al., 2008; Murphy et al., 2013). However, the results presented here are in line with findings from a multigenerational family study in rhesus monkeys, revealing significant heritability of metabolic activity predictive of anxious temperament in hippocampal regions, but not in the amygdala (Oler et al., 2010). Together, these findings

suggest that the impaired habituation response in the amygdala, although associated with SA, does not meet the endophenotype criterion of *heritability*, illustrating the distinction between disease-related neurobiological traits (biomarkers) and endophenotypes, with the latter having a genetic link with the disorder (cf. (Lenzenweger, 2013a)), and underscoring the value of studies using a family-design (Glahn et al., 2018). Furthermore, these findings support the notion that both genes and environment play a role in the development of SAD (Bas-Hoogendam, Roelofs, Westenberg, & van der Wee, 2019), and indicate that research on the interaction between these factors is important.

Habituation in other ROIs

Although the brain response to neutral faces habituated in several ROIs besides the amygdala and hippocampus, namely in the BNST, extrastriate cortex, FFA, orbitofrontal cortex and V1, we did not find an association with SA within these regions. Thereby, we could not replicate previous work demonstrating a relationship between social fearfulness and a slower habituation response to neutral faces in the orbitofrontal cortex, FFA, extrastriate cortex and V1 (Avery & Blackford, 2016). It should, however, be noted that this study employed a task paradigm in which the neutral faces were presented 1, 3, 5 or 7 times; as a result, their design allowed for investigation of habituation within specific repetition windows, for example from first to third presentation, third to fifth presentation and fifth to seventh presentation (Avery & Blackford, 2016). Interestingly, the effect of social fearfulness on habituation in the hippocampus was present over the whole paradigm, in line with our findings. The effects in the other ROIs were, however, only present in specific time windows (first to third and third to fifth presentation) which, arguably, could explain why we did not find associations with SA within these regions in the present study. Future studies, using the same task parameters and analysis methods as described by Avery & Blackford (2016), are needed to explore whether the associations between social fearfulness and neural habituation at specific timing intervals are also present in families genetically enriched for SAD.

Clinical implications

In addition to providing insight into the genetic susceptibility to SAD, our results might have potential clinical relevance, for example when considering the effect of exposure therapy. Exposure therapy, targeted at diminishing anxiety levels by repeated confrontations with the feared stimulus (i.e. a social situation), is often applied in SAD as part of cognitive behavioral therapy, with typically only small to moderate effects (Carpenter et al., 2018; Klumpp & Fitzgerald, 2018). Importantly, the effect of exposure therapy is thought to rely (at least partly) on habituation responses. Indeed, a recent meta-analysis showed a positive association between both within-session as well as between-session habituation on the one hand, and treatment outcome on the other (Rupp, Doebler, Ehring, & Vossbeck-Elsebusch, 2017). Furthermore, a research paper on adults with speaking anxiety indicated

that less amygdala activation during extinction learning predicted greater reduction in SA symptoms two weeks after a session of exposure (Ball, Knapp, Paulus, & Stein, 2017), while another study in SAD patients indicated that a decrease in regional cerebral blood flow in the amygdala was associated with anxiety reduction following repeated stress exposure (Åhs, Gingnell, Furmark, & Fredrikson, 2017). These results suggest that impaired habituation might have a negative consequence on the outcome of exposure therapy, but more research is needed to test this hypothesis. In this light, the role of inhibitory learning is also relevant: inhibitory learning, involving the amygdala, hippocampus, as well as the prefrontal cortex, and aimed at inhibiting the original feared association by a newly formed association representing safety, has been proposed as an alternative mechanism underlying exposure therapy (Craske, Liao, Brown, & Vervliet, 2012; Craske, Treanor, Conway, Zbozinek, & Vervliet, 2014). We hypothesize that a focus on inhibitory learning in exposure therapy might yield better outcomes in anxiety patients with impaired habituation responses.

Limitations and future studies

As the LFLSAD had a cross-sectional design and was intended to investigate the endophenotype criteria with respect to *co-segregation* and *heritability* (Bas-Hoogendam, Harrewijn, et al., 2018), we were not able to establish the *trait stability* of the candidate endophenotype (endophenotype criterion 2), nor could we examine the *difference in neural habituation between nonaffected family members within the sample and participants from the general population* (endophenotype criterion 4, second element). To investigate whether neural habituation meets these criteria, longitudinal studies, including families enriched for SAD as well as control families from the general population, are necessary. Furthermore, the present promising results paved the way for future analyses on the genetics underlying neural habituation: we did collect genetic data on the LFLSAD sample (Bas-Hoogendam, Harrewijn, et al., 2018), but we have not yet investigated whether specific genetic variations or epigenetic changes (cf. (Alisch et al., 2014; Dannlowski et al., 2011; Domschke et al., 2012; Ziegler et al., 2015)) are associated with the impaired neural habituation response. Such an investigation would be the following stage in disentangling the genetic vulnerability to SAD.

Finally, given work reporting changes in functional and structural connectivity of the amygdala in SAD (Brühl, Delsignore, et al., 2014), future studies should explore whether these aberrant connectivity patterns meet criteria for being candidate SAD endophenotypes.

Conclusion

The findings reported here support the hypothesis that impaired neural habituation to neutral faces is a promising neural candidate endophenotype of SAD, as our data revealed that

impaired habituation to neutral faces, expressed as a prolonged response to these faces in the right hippocampus and right amygdala, *co-segregated with social anxiety within families of probands*. Next, our data indicated that brain activation related to habituation displayed moderate to moderately-high heritability in the right hippocampus, providing support for the endophenotype criterion of *heritability*. Thereby, the present results offer novel insights in the neurobiological pathways leading to SAD.

Supplemental Methods

Participants

Exclusion criteria

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

Recruitment

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

Ethics

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

A priori power calculation and sample size

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

Phenotyping

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

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

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

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

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

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

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

MRI experiment

Prior to the MRI scan, participants were informed about the safety procedures and they were told that they could refrain from continuing the experiment at any time. Children and adolescents were familiarized with the MRI scanner using a mock scanner (Galván, 2010) and all participants received instructions about the task paradigms presented during the scan session. Scanning was performed using a 3.0 T Philips Achieva MRI scanner (Philips

Medical Systems, Best, The Netherlands), equipped with a 32-channel Sensitivity Encoding head coil. During the habituation paradigm, fMRI scans were acquired using T2*-weighted echo-planar imaging (EPI). These scans had the following characteristics: 38 axial slices, 2.75 mm x 2.75 mm x 2.75 mm + 10 % interslice gap, field of view (FOV) = 220 mm x 115 mm x 220 mm, repetition time (TR) = 2200 ms, echo time (TE) = 30 ms. The first six volumes of each fMRI scan were dummy volumes; these volumes were removed to allow for equilibration of T1 saturation effects.

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

Habituation paradigm

Faces

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

fMRI data

General processing steps

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

temporal filtering (Gaussian-weighted least-squares straight line fitting, with sigma = 30.0 s) and registration. Functional scans of each participant were registered to the individual 3D T1-weighted anatomical scan using FLIRT (Jenkinson et al., 2002; Jenkinson & Smith, 2001) and subsequently registered to the MNI T1-template brain (resolution 2 mm) using FNIRT nonlinear registration (warp resolution 10 mm) (Andersson et al., 2007).

We checked whether the individual scans were registered correctly and confirmed that relative motion parameters did not exceed 2.5 mm.

Definition of the regions of interest (ROI)

The amygdala ROI was defined using the Harvard-Oxford atlas implemented in FSLview, using a threshold of 50 %. Replicating the methods by (Avery & Blackford, 2016), the hippocampus, primary visual cortex (V1) and extrastriate cortex ROI were defined using the AAL (automated anatomical labeling) standard masks (Tzourio-Mazoyer et al., 2002); for V1, we selected the calcarine fissure mask, while the extrastriate cortex ROI consisted of the lingual gyrus, the inferior occipital cortex and the middle occipital cortex. Because we did not perform a standard fusiform face area (FFA) localizer task as described by (Avery & Blackford, 2016), the FFA ROI was based on the AAL atlas as well. The ventromedial prefrontal cortex and orbitofrontal cortex ROI were defined following the population masks described by (Mackey & Petrides, 2010) – see Avery & Blackford (2016).

For the BNST ROI we used a mask that was previously created using an ultra-high field (7T) MRI and a specialized GRASE sequence to trace the BNST (Avery et al., 2014).

Endophenotype analyses with (sub)clinical SAD as predictor

For reasons of completeness, we performed voxelwise analyses using (sub)clinical SAD as a discrete predictor, in addition to the main analyses using self-reported SA-level (continuous variable) as a predictor. In these analyses, individual activation level related to the contrast 'first > last' was used as dependent variable. Correlations between family members were modeled by including random effects; age and gender (both centered) were included as covariates. Models were run for each voxel separately. Results (z-scores) were transformed into a nifti-image with the same dimensions of the MNI T1-template brain. Clusters within the ROIs were corrected for multiple comparisons using the FSL tool easythresh (cluster threshold: $z > 2.3$, cluster extent threshold $p < 0.05$) (Worsley, 2001).

Sensitivity analyses

We performed two sensitivity analyses to examine whether the results of the association analysis (effect of self-reported social anxiety on brain activation related to 'first half > last half') were driven by the severity of depressive symptoms as measured by the BDI-II or the CDI, or by (comorbid) psychopathology other than SAD (cf. (Bas-Hoogendam, van Steenbergen, Tissier, et al., 2018b, 2019)). To this aim, we added the z-score of the level

of depressive symptoms as a covariate in the voxelwise analysis (sensitivity analysis 1) or excluded all family members with past and / or present psychopathology other than SAD and repeated the association analysis (sensitivity analysis 2). Note however, that this latter analysis may yield biased and weaker results, as the majority of the probands, on which the selection of the families was based, had comorbid psychopathology and were thus excluded. We used the same statistical threshold as for the main analyses, within the bilateral ROIs ($z > 2.3$, cluster-threshold $p < 0.05$).

Supplemental Results

Data availability

We acquired MRI data from nine families ($n = 113$) (Bas-Hoogendam, Harrewijn, et al., 2018), but data from one family ($n = 3$ family members) had to be excluded as the proband from this family was not able to participate in the MRI experiment due to an MRI contra-indication. As a result, 110 datasets were available for further fMRI pre-processing and quality control. Two datasets could not be used due to an imaging artefact, while the relative motion parameters of three other participants exceeded 2.5 mm. So, 105 fMRI datasets were available for further analysis of brain activation related to habituation. Furthermore, data on the presence of (sub)clinical SAD were lost for several family members due to technical reasons.

Sample characteristics

In line with the design of the study, participants originated from two generations, which differed significantly in age ($\beta \pm SE = -30.1 \pm 0.7$, $p < 0.001$), but not in male / female ratio ($\chi^2(1) = 0.75$, $p = 0.38$). In line with previous reports on this sample (Bas-Hoogendam, van Steenbergen, Tissier, et al., 2018b, 2019), family members with and without (sub)clinical SAD did not differ with respect to male / female ratio, age and estimated IQ. Groups did differ, however, in comorbidity rates: family members with (sub)clinical SAD were more often diagnosed with depression (past) and dysthymia (present). These differences were, however, only significant at an uncorrected significance level. Furthermore, family members with (sub)clinical SAD reported higher levels of fear of negative evaluation, more depressive symptoms, higher levels of trait anxiety and behavioral inhibition (BIS), as well as lower levels of behavioral activation (BAS) (*Supplemental Table S9.1*).

fMRI data

Endophenotype analyses with (sub)clinical SAD as predictor

The regression analysis using discrete (sub)clinical SAD as a predictor did not yield clusters within the ROIs surviving the predefined threshold. So, although we did find an association

between the habituation response and self-reported SA (continuous predictor), there was no relation with (sub)clinical SAD (discrete predictor). We speculate that this lack of a correlation is power-related, as the fMRI sample only contained 37 (sub)clinical SAD cases. This indicates the need for replication of the present findings in a larger sample.

Sensitivity analyses

Results of the first sensitivity analysis, with the level of depressive symptoms as an additional covariate, confirmed the relationship between SA and reduced neural habituation in the right amygdala (cluster characteristics: 29 voxels, $p = 0.014$; max z-value: 2.83), while the relationship between SA and habituation in the right hippocampus was not significant.

In the second sensitivity analysis, we excluded all participants with past and/or present comorbid psychopathology other than SAD; this resulted in a sample of 58 participants, of which 12 in the (sub)clinical SAD group. Next, we repeated the association analysis with self-reported social anxiety as predictor; this analysis confirmed the relation between SA level and impaired habituation in the right hippocampus (cluster characteristics: 89 voxels, $p = 0.047$; max z-value: 3.86); no significant clusters were present in the right amygdala.

Supplemental Table

Supplemental Table S9.1 Detailed characteristics of participants with and without (sub)clinical SAD: scores on self-report questionnaires.

	(Sub)clinical SAD (*n* = 37)[a]	No SAD (*n* = 61)	Statistical analysis
Self-report measures			
Social anxiety symptoms (z-score; mean ± SD)	2.9 ± 3.3	0.6 ± 1.5	β ± SE = 2.5 ± 0.5, $p < 0.001$
Fear of negative evaluation (mean ± SD)	23.6 ± 12.4	12.7 ± 8.0	β ± SE = 10.9 ± 2.0, $p < 0.001$
Depressive symptoms (z-score; mean ± SD)	0.1 ± 0.9	-0.5 ± 0.7	β ± SE = 0.5 ± 0.2, $p < 0.001$
STAI – trait (mean ± SD)	38.9 ± 9.6	33.0 ± 8.6	β ± SE = 5.6 ± 1.9, $p = 0.003$
BIS (z-score; mean ± SD)	0.3 ± 1.3	-0.4 ± 0.9	β ± SE = 0.7 ± 0.2, $p < 0.001$
BAS (z-score; mean ± SD)	-1.0 ± 0.8	-0.6 ± 1.0	β ± SE = -0.5 ± 0.2, $p = 0.007$

Footnote

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