NeuroImage: Clinical 27 (2020) 102268

Contents lists available at ScienceDirect

NeuroImage: Clinical

journal homepage: www.elsevier.com/locate/ynicl

Neural correlates of *NOS1* ex1f-VNTR allelic variation in panic disorder and agoraphobia during fear conditioning and extinction in fMRI

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ARTICLE INFO

Keywords:
Panic disorder
NOS1
Fear conditioning
Extinction
Imaging genetics

ABSTRACT

Neuronal nitric oxide synthase (NOS-I) impacts on fear/anxiety-like behavior in animals. In humans, the short (S) allele of a functional promotor polymorphism of *NOS1* (*NOS1* ex1f-VNTR) has been shown to be associated with higher anxiety and altered fear conditioning in healthy subjects in the amygdala and hippocampus (AMY/HIPP). Here, we explore the role of *NOS1* ex1f-VNTR as a pathophysiological correlate of panic disorder and agoraphobia (PD/AG). In a sub-sample of a multicenter cognitive behavioral therapy (CBT) randomized controlled trial in patients with PD/AG (n = 48: S/S-genotype n = 15, S/L-genotype n = 21, L/L-genotype n = 12) and healthy control subjects, HS (n = 34: S/S-genotype n = 7, S/L-genotype n = 17, L/L-genotype = 10), a differential fear conditioning and extinction fMRI-paradigm was used to investigate how *NOS1* ex1f-VNTR genotypes are associated with differential neural activation in AMY/HIPP. Prior to CBT, L/L-allele carriers showed higher activation than S/S-allele carriers in AMY/HIPP. A genotype × diagnosis interaction revealed that the S-allele in HS was associated with a pronounced deactivation in AMY/HIPP, while patients showed contrary effects. The interaction of genotype × stimulus type (CS+, conditioned stimulus associated with an aversive stimulus vs. CS-, unassociated) showed effects on differential learning in AMY/HIPP. All effects were predominately found during extinction. Genotype associated effects in patients were not altered after CBT. Low statistical power due to small sample size in each subgroup is a major limitation. However, our findings provide first preliminary evidence for dysfunctional neural fear conditioning/extinction associated with *NOS1* ex1f-VNTR genotype in the context of PD/AG, shedding new light on the complex interaction between genetic risk, current psychopathology and treatment-related effects.

1. Introduction

Anxiety disorders are highly prevalent and cause a high and chronic individual and societal burden (Gustavsson et al., 2011; Wittchen et al., 2011). In order to improve and prospectively individualize treatment, the neurobiological mechanisms of fear and anxiety, underlying individual differences in fear processing and defensive responses, are of major interest.

Considering the high genetic heritability of anxiety disorders – 30%–50% of the individual variability can be explained by genetic

Clinical Trials Registration: Registry name: ISRCTN registry
URL: https://doi.org/10.1186/ISRCTN80046034

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https://doi.org/10.1016/j.nicl.2020.102268
Received 9 December 2019; Received in revised form 2 April 2020; Accepted 3 April 2020
Available online 23 April 2020

factors (Gordon and Hen, 2004; Leonardo and Hen, 2006; Shimada-Sugimoto et al., 2015) – the functional relevance of potential genetic risk factors on a neural systems level should further to be investigated following the idea of the Research Domain Criteria (RDoC) (Insel et al., 2010; Kozak and Cuthbert, 2016).

Fear and defensive responses are modulated by learning experiences, which can be experimentally modelled by using fear conditioning paradigms. Through differential conditioning, the organism learns to differentiate between discrete signals of threat (conditioned stimuli (CS) that are followed by an aversive unconditioned stimulus (US); CS+) that elicit phasic fear responses vs. safety signals (CS that are never followed by an US; CS-). This information is then integrated in a way that the CS+ alone will also lead to defensive reactions in the future to avoid upcoming threat (Fullana et al., 2016; Lonsdorf et al., 2017; Sehlmeyer et al., 2009; Wendt et al., 2017). The neurofunctional system of defensive responses consist of the cortical forebrain (e.g. (pre-)motor, prefrontal and anterior cingulate cortex; ACC), the insula, limbic (i.e. amygdala, hippocampus) and midbrain structures (Wendt et al., 2017; Schwarzmeier et al., 2019; Carvalho et al., 2010; Fanselow, 1994; Mobbs et al., 2009).

One potential genetic risk factor for anxiety disorders is the neuronal nitric oxide synthase gene (*NOS1*). *NOS1* encodes the neuronal isoform of nitric oxide synthase (NOS-I) which catalyzes the production of the neurotransmitter nitric oxide (NO) and is widely expressed throughout the brain, especially in the cerebellum, basal ganglia, hippocampus and frontal cortex (Blum-Degen et al., 1999). Animal studies have confirmed a wide influence of nitrinergic (dys-)regulation on behavioral domains (Freudenberg et al., 2015), like impulsivity/aggression (Nelson et al., 2006; Carreño Gutiérrez et al., 2017), exploratory vs. anxious behavior, depression-like symptoms, and cognitive performance (Wultsch et al., 2007). Regarding (fear) learning processes, NOS-I has been implicated in anxiety-like behavior, synaptic plasticity, hippocampal long-term potentiation and memory formation in rodents (Schuman and Madison, 1991). Zebrafish and mice showed decreased aggression but increased anxiety-like behavior under decreased NO signaling (Carreño Gutiérrez et al., 2017). Furthermore, the hippocampus features high expression levels of NOS-I (Schuman and Madison, 1991), while *Nos1* knockout (Kelley et al., 2009) and pharmacological manipulations (Kelley et al., 2010) had a marked effect on freezing in rodents, particularly during context conditioning where the hippocampus is assumed to play a key regulatory function (Chang and Liang, 2017; Lang et al., 2009; Pohlack et al., 2012).

In humans, determination of the genotype of the functional promotor polymorphism *NOS1* ex1f-VNTR can be used as a proxy of NO production at least in certain brain regions (hippocampus, cortex, striatum) (Freudenberg et al., 2015; Bros et al., 2006; Reif et al., 2006). The human NOS1 gene has been mapped to chromosome 12q24.2–0.31 (Boissel et al., 1998; Hall et al., 1994) and harbors 12 alternative first exons (1a-1l) along with unique promoters (Wang et al., 1999). Exon 1f is identified to be highly conserved, suggesting that it represents one of the evolutionary ancient first exons (Freudenberg et al., 2015). *NOS1* ex1f-VNTR regulates gene expression and impacts on the neuronal transcriptome (long > short alleles Reif et al., 2011). Based on the complexity of *NOS1*, the wide distribution of NO in the brain and the varying function of NOS-I in different brain regions, it is likely that the impact of *NOS1* ex1f-VNTR genetic variation in humans is also diverse (Freudenberg et al., 2015; Reif et al., 2006; Reif et al., 2009; Kopf et al., 2011; Rife et al., 2009). Most studies on *NOS1* in humans focus on schizophrenia and impulsivity/ADHD (Freudenberg et al., 2015), but some also suggest an involvement of *NOS1* in fear and anxiety. We (Reif et al., 2011; Reif et al., 2009) have previously established that the short alleles of *NOS1* ex1f-VNTR are linked to adult ADHD, suicide, aggression and impulsive personality dimensions as well as higher neuroticism and anxiety in a gene × environment interaction manner and replicated higher anxiety levels in an independent, population based sample (Kurrikoff et al., 2012). Bruenig et al. (2017) investigated NO pathway genes in post-traumatic stress disorder (PTSD) and found the *NOS1* polymorphism rs10744891 to be associated with PTSD severity (G/G-allele less severity), resilience (T/T+ T/G allele less resilience), while another SNP rs7295972 was associated with stress in the PTSD (A/G+ G/G higher stress). Finally, Kuhn et al. (2016) found preliminary evidence for a modulatory role of *NOS1* ex1f-VNTR on sustained contextual fear conditioning in healthy subjects: carriers of at least one S-allele showed significantly higher activation to US-predicting compared to safe contexts at the amygdala/anterior hippocampus junction in an fMRI study. Thus, there is first evidence for the involvement of *NOS1* ex1f-VNTR, and hence NO signaling, in amygdala/hippocampus-dependent conditioning and anxiety-related processes on a neural level.

We aimed to take these findings to a next level and investigated the potential role of *NOS1* ex1f-VNTR allelic variation on differential conditioning and extinction in the context of anxiety disorders. Understanding the role of potential genetic risk factors in extinction learning (Myers and Davis, 2007) is important since extinction learning is the key component of successful exposure-based therapy (Milad and Quirk, 2012; Vervliet et al., 2013). In an fMRI fear conditioning and extinction paradigm, we examined the *NOS1* ex1f-VNTR associated neural correlates in patients with current diagnosis of panic disorder (PD) and agoraphobia (AG) and healthy control subjects. We were interested in 1) general *NOS1* ex1f-VNTR associated effects and 2) in *NOS1* ex1f-VNTR associated effects on differential learning of threat (CS+) and safety (CS-) signals reflected in amygdalar and hippocampal BOLD activation due to these regions' implication in the context of gene expression (Bros et al., 2006) and conditioning (Kuhn et al., 2016), as well as emotion (amygdala) and memory (hippocampus). To explore if the current diagnosis of PD/AG interacts with the effects of *NOS1* ex1f-VNTR allelic variation, 3) we examined patients and healthy control subjects together as well as post-hoc separately to determine similarities and differences. Lastly, we 4) explored the stability of *NOS1* ex1f-VNTR associated effects after 12 weeks of highly structured CBT intervention.

2. Experimental procedures

2.1. Participants

Participants represent sub-samples (n = 48 patients S/S-genotype n = 15, mean age 38.3 (*SD* 8.1) years, 11 females; S/L-genotype n = 21, mean age 37.9 (*SD* 10.7) years, 13 females; L/L-genotype n = 12, mean age 36.7 (*SD* 12.6) years, 9 females; n = 34 healthy control subjects (S/S-genotype n = 7, mean age 35.8 (*SD* 11.4) years, 4 females; S/L-genotype n = 17, mean age 34.3 (*SD* 8.9) years, 10 females; L/L-genotype n = 10, mean age 40.4 (*SD* 11.1) years, 7 females; detailed information about the demographic, neuropsychological and clinical characteristics of the whole sample (n = 82) at baseline can be found in the supplementary Table ST1) from the randomized controlled multicenter trial «Mechanism of Action in CBT» (MAC) with a total number of n = 369 enrolled patients (Gloster et al., 2011; Gloster et al., 2009) (the CONSORT diagram for the randomized controlled trial study is given in Gloster et al., 2009). This study was part of the national research network PANIC-NET funded by the German Federal Ministry of Education and Research (BMBF). The study and its sub-projects was approved by the respective local ethical committees; written informed consent of all participants was obtained. Detailed information about inclusion and exclusion criteria, clinical assessment, treatment procedure, and measures of quality control for fMRI data can be found elsewhere (Gloster et al., 2011; Gloster et al., 2009; Straube et al., 2014) and in the Supplementary Methods 1.1 – 1.3. Participants were free from psychotropic medication, unrelated, and patients fulfilled a diagnosis of PD/AG according to DSM-IV-TR criteria (American Psychiatric Association 2000) as diagnosed by the Composite International Diagnostic Interview (CIDI) (Wittchen et al., 1997). Genotype distribution did not deviate from the Hardy-Weinberg

equilibrium ($p = .5$). After the initial data acquisition, patients received 12 sessions of standardized exposure-based CBT (for details see Supplementary Methods 1.2) and afterwards participated again in the fMRI fear conditioning and extinction task. Healthy control subjects also were measured twice with a corresponding time interval. Quality controlled (Kircher et al., 2013) (Supplementary Methods 1.3) pre- and post-data were available of 38 patients (S/S-genotype $n = 12$, S/L-genotype $n = 17$, L/L-genotype $n = 9$) and 26 healthy control subjects (S/S-genotype $n = 6$, S/L-genotype $n = 13$, L/L-genotype $n = 7$).

2.2. Genotyping

Genotyping was performed on blood samples determining *NOS1* ex1f-VNTR by PCR amplification and product size determination as described previously (Reif et al., 2006; Reif et al., 2009). According to previous studies (Freudenberg et al., 2015; Kuhn et al., 2016), the S-allele of *NOS1* ex1f-VNTR is associated with higher anxiety and altered activity in the amygdala and hippocampus. Since the sizes of genetic sub-groups did not allow to reasonably differentiate between S/S-, S/L- and L/L-genotypes, we investigated genetic effects by contrasting the S/S vs. L/L-genotype as our main outcome. The S/L-group was only analysed in an exploratory fashion by extracting their beta-values in the S/S- vs. L/L-contrasts, too.

2.3. fMRI data acquisition and analysis

For details on quality control please refer to the Supplementary Methods 1.3 and for details on the fMRI paradigm, data acquisition and analysis pathways please refer to the Supplementary Methods 1.4. A previously validated (Reinhardt et al., 2010) fear conditioning and extinction task was applied: during the acquisition phase (A), the US (white noise) and one of two previous neutral stimuli (colored sphere) was repeatedly paired (reinforcement rate of 50%; US presentation overlapping with the last 100 msec of CS presentation) to become the fear related conditioned stimulus (CS+) while the other stimulus was never paired and consequently acquired safety signal properties (CS-). In the extinction phase (E), both CS were repeatedly presented again always without the US. Preprocessing and first-level-analyses followed previous publications (Straube et al., 2014; Kircher et al., 2013; Reif et al., 2014; Straube et al., 2014; Lueken et al., 2015). On group level, only those trials in which no US was delivered during acquisition were analyzed to avoid an overlap with neuronal activation directly related to the presentation of the US (Kircher et al., 2013). During the experiment, participants were ask to rate valence and arousal towards CS+ and CS- at the end of the acquisition and extinction phase using a 5-point Likert Scale (1 = 'very unpleasant' to 5 = 'very pleasant' and 1 = 'not arousing' to 5 = 'very arousing') (Reinhardt et al., 2010). Rating data as well as demographic, neuropsychological and clinical characteristics were analyzed by means of IBM SPSS v.21 for Linux.

In the main fMRI second-level-group-analysis, a flexible factorial design including gender, age, years of education and center as covariates of no interest was used to examine activation differences during presentation of CS+ and CS- separately for A (early, late) and E (early, late) between S/S-, S/L- and L/L-allele carriers in patients and healthy control subjects. Following four contrasts of interest were calculated: (1) main effect of genotype, regardless of diagnosis, to reveal the general influence of allele status on stimulus unspecific brain activation, (2) interaction between genotype and diagnosis to test the general diagnosis specific influence of genotype, (3) interaction between genotype and stimulus type, regardless of diagnosis, to test for the differential learning (CS+/CS-) effects influenced only by genotype, (4) interaction between genotype, CS-type and diagnosis to investigate the effects of genotype on CS-processing in the presence of acute psychopathology. In addition, subordinated post-hoc contrasts were calculated for both groups separately to explore the effect of patients and healthy control subjects more closely: (1) main effect of genotype and (2) the interaction between genotype and stimulus type to test for the differential learning effects.

In a separate second-level-group-analysis (flexible factorial design, including gender, age, years of education and center as covariates of no interest) we explored the pre vs. post CBT differences in patients. We included patients and healthy control subjects in the analysis to gather an idea about the healthy control subjects as well, but built contrasts only on the patients' contrast images. Following contrasts were calculated: (1) interaction between genotype and time and (2) interaction between genotype, time and stimulus type.

Hippocampus and amygdala were chosen a priori as individual regions of interest due to their implication in the context of gene expression (Bros et al., 2006) and conditioning (Kuhn et al., 2016) and used for small volume correction (SVC) based on the predefined masks of the Automated Anatomical Labeling (aal) atlas implemented in SPM5. Multiple comparisons were controlled for by using family-wise error correction ($p < .05$) and a cluster size of $k \geq 4$.

For additional exploratory whole-brain analyses, in accordance with previous analyses (Straube et al., 2014; Kircher et al., 2013; Reif et al., 2014; Straube et al., 2014; Lueken et al., 2015), a Monte Carlo simulation at threshold $p < .005$ (uncorr.) and a minimum cluster size of 142 contiguous voxels was used to correct for multiple comparisons at $p < .05$ for all contrasts of interest. Clusters were localized using the Anatomy Toolbox v1. Beta values from significant clusters were extracted for bar graph visualization. In all analyses, the extreme groups (homozygous S/S vs. L/L) were contrasted and the beta values of all three genetic subgroups were extracted.

3. Results

3.1. Sample characteristics

Genotype associated sample characteristics are given in the Supplementary Table S1. Prior to treatment, genotype groups in patients (S/S-genotype $n = 15$, S/L-genotype $n = 21$, L/L-genotype $n = 12$) did not differ in demographic and clinical characteristics suggesting comparable severity of panic/agoraphobic and depressive symptoms. Patients ($n = 48$) and healthy control subjects ($n = 34$) only differed in education ($p = .03$, unrelated to genotype) but not in their neuropsychological or other demographic characteristics. As expected, patients scored higher than healthy control subjects in the Anxiety Sensitivity Index (ASI; $p < .001$) and the Beck Depression Inventory II (BDI II; $p < .001$), but neither a main effect of genotype nor a genotype × diagnosis effect was found. Distribution of *NOS1* ex1f-VNTR genotypes was not confounded by the distribution of genotypes of other genetic factors in the same sample. So *NOS1* ex1f-VNTR associated effects reported here are not confounded with previously published genetic analyses (Supplementary Results 2.1).

3.2. Rating

Neither main effects of genotype nor interactions of genotype × diagnosis were found in the ratings of valence and arousal towards CS+ or CS- after the end of the acquisition phase as well as after the extinction phase. After acquisition, there was a main effect of diagnosis: healthy control subjects ($n = 34$) reported a higher valence of the CS- rating than patients ($n = 48$) ($p = .012$). Regarding arousal, for both CS + ($p = .029$) and CS- ($p < .001$) patients scored higher than healthy control subjects at baseline, but also after extinction (CS+ ($p = .024$) and CS- ($p = .007$)).

3.3. fMRI

3.3.1. Main effect of genotype

In the combined analysis of patients and healthy control subjects (Table 1A), a main effect of genotype revealed higher activation for the

Table 1
Baseline (t1) effects of *NOS1* ex1f-VNTR genotype on brain activation patterns during fear acquisition and extinction across patients and healthy control subjects (cluster peak voxels are given).

			MNI coordinates				
Contrast/region	hemisphere	n. voxels	x	y	z	t	p
A) Main effect of genotype							
Early extinction: S/S < L/L							
Amygdala	L	5	−28	0	−28	2.74	.032
Late extinction: S/S < L/L							
Amygdala	L	5	−30	4	−18	2.78	.029
Hippocampus	L	36	−30	−16	−22	3.45	.015
Hippocampus	R	45	40	−20	−16	3.47	.014
B) Interaction diagnosis × genotype							
Early extinction: (Pat < HS) > (S/S > L/L)							
Amygdala	R	47	36	2	−26	4.74	< 0.001
Hippocampus	R	53	40	−12	−18	3.74	.006
Late extinction: (Pat > HS) > (S/S > L/L)							
Amygdala	L	23	−30	−4	−22	3.05	.014
Hippocampus	L	53	−30	−10	−24	3.84	.004
C) Interaction diagnosis × genotype × CS							
Early acquisition: (Pat > HS) > (S/S > L/L) > (CS+ < CS-)							
Hippocampus	L	50	−32	−14	−20	3.70	.007
Late extinction: (Pat > HS) > (S/S > L/L) > (CS+ > CS-)							
Amygdala	L	25	−20	−8	−16	3.08	.013
Amygdala	R	31	34	2	−24	3.19	.011

Abbreviations: Pat: PD/AG patients; HS: healthy control subjects; S/S: homozygous short allele; L/L: homozygous long allele; CS+: conditioned stimulus that is followed by the unconditioned stimulus (US) with a reinforcement rate of 50% (only unpaired CS+ were included; CS-: conditioned stimulus that is never followed by an US; L: left; R: right; voxel: number of voxels per cluster; x, y, z: MNI coordinates; results in predefined regions of interest (ROI) using masks of the automatic anatomical labeling (aal) atlas; multiple comparisons were controlled for by using family-wise error (FWE) correction.
Annotation: All contrasts were calculated for all four experimental phases (early/late acquisition/extinction) as well as for all the comparisons S/S > L/L, and S/S < L/L. To reduce complexity, only contrasts with supra-threshold activation is reported.

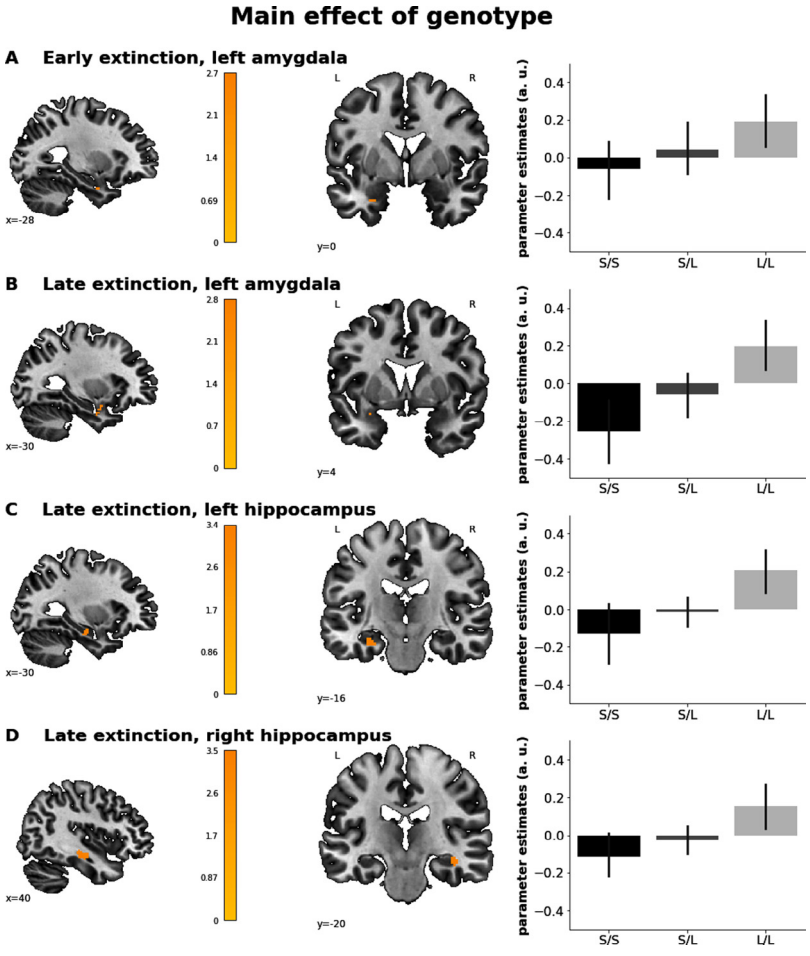

Fig. 1. Main effect of *NOS1* ex1f-VNTR associated BOLD activation across PD/AG patients and healthy control subjects. S/S: carriers of the S/S-allele variant, $n = 22$; S/L: carriers of the S/L-allele variant, $n = 38$; L/L: carriers of the L/L-allele variant, $n = 22$. Bar graphs illustrate the contrast estimates (arbitrary units (a.u.)) of activation. Error bars indicate the standard error of the mean (s.e.m.) in all cases. All Clusters in predefined regions of interest (ROI) using masks of the automatic anatomical labeling (aal) atlas; multiple comparisons were controlled for by using family-wise error (FWE) correction. All beta values were extracted from activation clusters revealed by the S/S- vs. L/L-contrasts. The contrast estimates for the S/L-group was visualized for exploratory purposes by extracting their beta-values from the same clusters as well. **A:** MNI coordinates x, y, z: −28, 0, −28; 5 voxels; $t = 2.74$; $p = .032$. **B:** MNI coordinates x, y, z: −30, 4, −18; 5 voxels; $t = 2.78$; $p = .029$. **C:** MNI coordinates x, y, z: −30, −16, −22; 36 voxels; $t = 3.45$; $p = .015$. **D:** MNI coordinates x, y, z: 40, −20, −16; 45 voxels; $t = 3.45$; $p = .014$.

Table 2
Baseline (t1) effects of *NOS1* ex1f-VNTR genotype on brain activation patterns during fear acquisition and extinction in patients (*A + B*) and healthy control subjects (*C + D*) separately (cluster peak voxels are given).

			MNI coordinates				
Contrast/region	hemisphere	n. voxel	x	y	z	t	p
A) Patients: main effect of genotype							
Early extinction: S/S < L/L							
Amygdala	R	33	36	2	−26	4.47	<0.001
Hippocampus	R	29	38	−8	−20	3.85	.004
Amygdala*	L	19	−26	2	−26	3.16	.010
B) Patients: interaction genotype × CS							
Early extinction: (S/S > L/L) > (CS+ > CS-)							
Amygdala	L	17	−20	−2	−16	3.17	.010
Amygdala	R	51	22	2	−14	3.16	.011
Late extinction: (S/S > L/L) > (CS+ > CS-)							
Amygdala	L	29	−18	−6	−16	3.51	.003
Amygdala	R	21	18	2	−16	3.50	.004
Hippocampus	L	27	−18	−8	−16	3.57	.010
C) Healthy control subjects: main effect of genotype							
Late extinction: S/S < L/L							
Amygdala	L	55	−30	−4	−22	3.45	.004
Hippocampus	L	142	−28	−14	−22	4.44	<0.001
Hippocampus	R	81	38	−20	−16	3.94	.003
D) Healthy control subjects: interaction genotype × CS							
Early acquisition: (S/S > L/L) > (CS+ > CS-)							
Hippocampus	L	17	−32	14	−22	3.37	.019

Abbreviations: S/S: homozygous short allele; L/L: homozygous long allele; CS+: conditioned stimulus that is followed by the unconditioned stimulus (US) with a reinforcement rate of 50% (only unpaired CS+ were included; CS-: conditioned stimulus that is never followed by an US; L: left; R: right; voxel: number of voxels per cluster; x, y, z: MNI coordinates; results in predefined ROIs using masks of the aal-atlas; multiple comparisons were controlled for by using FWE-correction.
* This cluster reveals a *NOS1*-ex1f-VNTR associated effect that can also be found in the main effect over both measurement points (pre + post CBT).
Annotation: All contrasts were calculated for all four experimental phases (early/late acquisition/extinction) as well as for all the comparisons S/S > L/L, and S/S < L/L. To reduce complexity, only contrasts with suprathreshold activation is reported.

L/L-genotype (n = 22) compared to the S/S-genotype (n = 22) in the left amygdala (Fig. 1A/B) and the bilateral hippocampus (Fig. 1C/D) during early and late extinction. Post-hoc analyses of patients (S/S-genotype n = 15, L/L-genotype n = 12) and healthy control subjects (S/S-genotype n = 7, L/L-genotype n = 10) separately revealed this effect to occur in patients (Table 2A) during early extinction but in healthy control subjects (Table 2C) during late extinction.

3.3.2. Interaction diagnosis × genotype

In the contrast genotype × diagnosis (regardless of stimulus type), we also found diagnosis associated effects during extinction (Table 1B). Patients (n = 48) showed higher activation in bilateral amygdala and hippocampus than healthy control subjects (n = 34), driven by a distinct deactivation of the S/S-genotype in healthy control subjects (n = 7). During early extinction, patients and healthy control subjects showed a similar L/L>S/S activation pattern in the right amygdala, however, deactivation in the S/S-genotype was more pronounced in healthy control subjects (patients: S/S-genotype n = 15, L/L-genotype n = 12; healthy control subjects: S/S-genotype n = 7, L/L-genotype n = 10; Fig. 2A). In the right hippocampus, we found in healthy control subjects again reduced activation in the S/S-genotype (L/L>S/S) whereas patients showed higher activation for S/S than the L/L-genotype (Fig. 2B). During late extinction, we found pronounced deactivation in the S/S- vs. L/L-genotype of healthy control subjects and a quite comparable L/L>S/S activation pattern in patients in the left amygdala (Fig. 2C). In the left hippocampus, we found a very similar pattern with a pronounced deactivation in the S/S-genotype in healthy control subjects (Fig. 2D).

3.3.3. Interaction CS × genotype

In the interaction contrast stimulus type (CS+/CS-) × genotype, no cluster above the cluster size threshold of $k \geq 4$ was found.

3.3.4. Differential learning: stimulus type (CS+/CS-) discrimination

In the contrast diagnosis × genotype × stimulus type (Table 1C), during early acquisition, we found for the S/S-genotype in healthy control subjects (n = 7) a specific higher activation for CS- than CS+ in the left hippocampus (Fig. 3A). This CS- > CS+ difference was much less pronounced in the L/L-genotype (n = 10). In patients, there was a higher CS- > CS+ difference in the L/L- (n = 12) compared to the S/S-genotype (n = 15). During late extinction, we found diagnosis specific genotype effects during the processing of the CS+ in the left amygdala, indicating S/S > L/L in patients and L/L > S/S in healthy control subjects. S/S and L/L in patients and healthy control subjects respectively were comparable for the processing of the CS-: patients showed generally higher activation than healthy control subjects (Fig. 3B). In the right amygdala, patients showed specific higher activation towards CS- than towards CS+ in the L/L-genotype. In the S/S-genotype, nearly no CS- > CS+ difference was found. Healthy control subjects again showed higher activation towards CS- than CS+ in the S/S-genotype, but higher activation towards CS+ than CS- in the L/L-genotype (Fig. 3C). Post-hoc tests of genotype x stimulus type in patients and healthy control subjects separately revealed the following: In patients (Table 2B, Supplementary Fig. S1A-D), during early and late extinction, we found the S/S-genotype to show higher activation for CS- vs. CS+ in the bilateral amygdala. The L/L-genotype showed higher CS+ > CS- activation during early but higher CS- > CS+ activation during late extinction. In the left hippocampus, the S/S-genotype showed a CS+ > CS- pattern, whereas the L/L-genotype showed CS- > CS+ activation (Table 2B, Supplementary Fig. S1E). In healthy control subjects (Table 2D, Supplementary Fig. 4F), during early acquisition, we found the S/S- and the L/L-genotype to show higher activation during CS- than CS+ in the left hippocampus.

3.3.5. Pre/post CBT comparison

In the interaction genotype × time as well as genotype × time × stimulus type in patients (n = 38; S/S-genotype n = 12, S/L-genotype

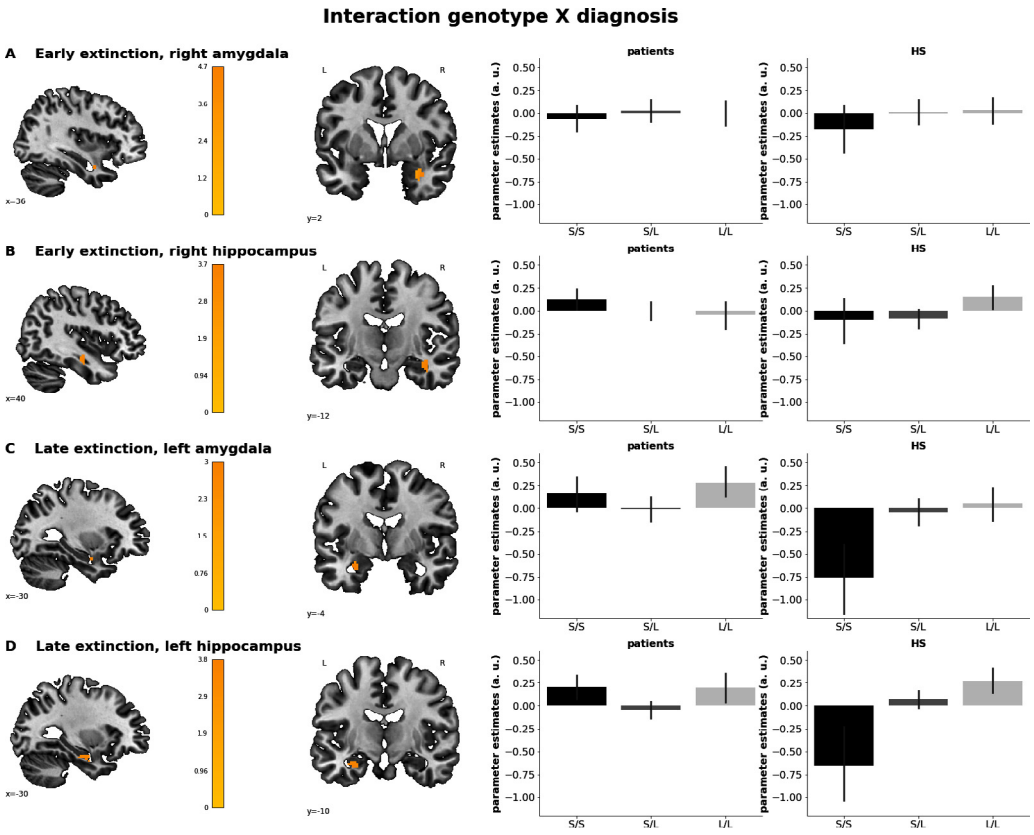

Fig. 2. Differential *NOS1* ex1f-VNTR associated BOLD activation in PD/AG compared to HS. S/S: carriers of the S/S-allele variant, $n = 22$; S/L: carriers of the S/L-allele variant, $n = 38$; L/L: carriers of the L/L-allele variant, $n = 22$; patients (diagnosis of panic disorder and agoraphobia) $n = 48$, HS (healthy control subjects) $n = 34$. Bar graphs illustrate the contrast estimates (a.u.) of activation. Error bars indicate the s.e.m. in all cases. All Clusters in predefined ROIs using masks of the aal-atlas; multiple comparisons were controlled for by using FWE-correction. All beta values were extracted from activation clusters revealed by the S/S- vs. L/L-contrasts. The contrast estimates for the S/L-group was visualized for exploratory purposes by extracting their beta-values from the same clusters as well. **A:** MNI coordinates x, y, z: 36, 2, −26; 47 voxels; $t = 4.74$; $p < .001$. **B:** MNI coordinates x, y, z: 40, −12, −18; 53 voxels; $t = 3.74$; $p = .006$. **C:** MNI coordinates x, y, z: −30, −4, −22; 23 voxels; $t = 3.05$; $p = .014$. **D:** MNI coordinates x, y, z: −30, −10, −24; 53 voxels; $t = 3.84$; $p = .004$.

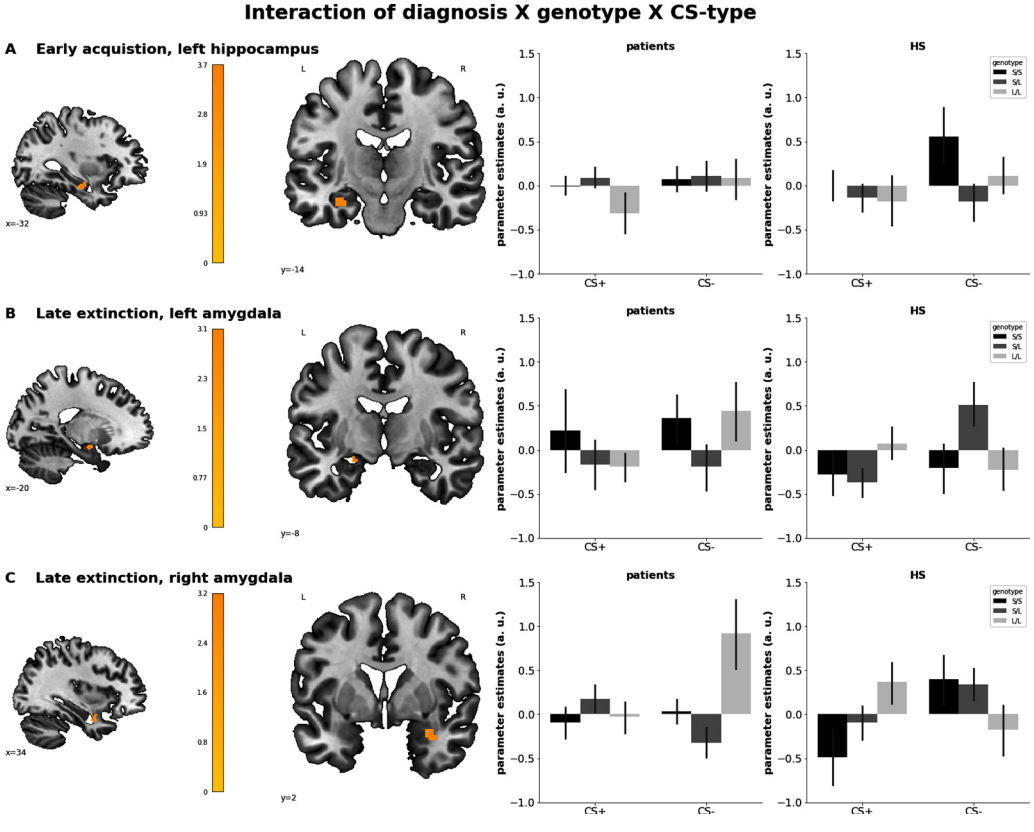

Fig. 3. Interaction of *NOS1* ex1f-VNTR associated BOLD activation with diagnosis of PD/AG and stimulus type. S/S: carriers of the S/S-allele variant, $n = 22$; S/L: carriers of the S/L-allele variant, $n = 38$; L/L: carriers of the L/L-allele variant, $n = 22$; patients (diagnosis of panic disorder and agoraphobia) $n = 48$, HS (healthy control subjects) $n = 34$; CS+: conditioned stimulus that is followed by the unconditioned stimulus (US) with a reinforcement rate of 50% (only unpaired CS+ were included; CS-: conditioned stimulus that is never followed by an US. Bar graphs illustrate the contrast estimates (a.u.) of activation. Error bars indicate the s.e.m. in all cases. All Clusters in predefined ROIs using masks of the aal-atlas; multiple comparisons were controlled for by FWE-correction. All beta values were extracted from activation clusters revealed by the S/S- vs. L/L-contrasts. The contrast estimates for the S/L-group was visualized for exploratory purposes by extracting their beta-values from the same clusters as well. **A:** MNI coordinates x, y, z: −32, −14, −20; 50 voxels; $t = 3.70$; $p = .007$. **B:** MNI coordinates x, y, z: −20, −8, −16; 25 voxels; $t = 3.08$; $p = .013$. **C:** MNI coordinates x, y, z: 34, 2, −24; 31 voxels; $t = 3.19$; $p = .011$.

n = 17; L/L-genotype n = 9; n = 26 healthy control subjects were included in the second-level-analysis as well, see Methods 2.3, S/S-genotype n = 6, S/L-genotype n = 13, L/L-genotype n = 7) no differential activation was found. A main effect of genotype in patients across both measurement points (pre + post CBT) revealed a cluster in the left amygdala during early extinction (ROI analysis with aal mask; 28 voxels; $x = -22$; y = 2; $z = -24$; t = 3.12; $p_{FWE} = .012$) (Table 2; Supplementary Fig. S2). In the differential CS-processing across both measurement points only a very small cluster in the left amygdala (ROI analysis with aal mask; 4 voxels; $x = -30$; y = 4; $z = -18$; t = 2.78; $p_{FWE} = .031$) showed a persistent CS- > CS+ tendency during early extinction in the S/S-genotype in patients and an CS+ > CS- pattern in the L/L-genotype (Supplementary Fig. S3).

3.3.6. Exploratory whole-brain analyses

Exploratory whole-brain analyses ($p < .005$ uncorr., 142 contiguous voxels) additionally revealed – amongst others – clusters in the somatosensory and motor cortices, the striatum (where *NOS1* ex1f is widely expressed Reif et al., 2006) and the cingulate cortex during acquisition in patients (n = 48) as well as in healthy control subjects (n = 34). During extinction, pronounced clusters were found in the medial, inferior and middle temporal gyri. Details can be found in the Supplementary Tables ST2-ST4.

4. Discussion

Present preliminary findings on neural activation patterns associated with *NOS1* ex1f-VNTR allelic variation extend evidence on the relevance of this polymorphism in the context of emotional-associative learning, fear, and anxiety to the clinical level and corroborate previous results in healthy subjects. Our results indicate for the first time, that *NOS1* ex1f-VNTR allelic variation differentially impacts on the amygdala and hippocampus during fear conditioning and extinction in patients suffering from PD/AG vs. healthy control subjects: particularly during late extinction learning, the S-allele in healthy control subjects was associated with a pronounced deactivation of the amygdala and hippocampus, while contrary effects were observed in patients. These findings implicate that «risk alleles» have to be revisited in the context of a chosen target population. Genotype associated effects in patients were not altered after 12 weeks of CBT, which points to a trait marker in patients.

We found similarities between patients and healthy control subjects in the main effect of *NOS1* ex1f-VNTR genotype especially in the amygdala as well as diagnosis associated differences in the processing of threat (CS+) and safety (CS-) signals during fear conditioning and extinction, regarding the neural activation patterns in two key regions (amygdala, hippocampus) for fear processing. The amygdala is part of the core and the hippocampus part of the extended emotional regions (Pessoa, 2008). While the hippocampus is highly involved in memory formation (Rothschild et al., 2017), the amygdala, on the one hand, is often categorized as an affective region strongly linked to fear processing. On the other hand however, it is also involved in functions that are closely linked to cognition, including attention and associative learning (Pessoa, 2008). Our exploratory whole brain analyses revealing *NOS1* ex1f-VNTR allelic variation associated activation differences in temporal regions also support the hypothesis that these are neural key regions during fear extinction processes both in patients with PD/AG and healthy control subjects.

A main effect of genotype revealed carriers of the L/L-genotype in patients and healthy control subjects to show heightened activation in bilateral regions of the amygdala and hippocampus during the extinction phase. As indicated by the genotype × diagnosis interaction, this effect was however mainly driven by a pronounced deactivation in S-allele carriers of healthy control subjects. This interaction of genotype and diagnosis emphasizes the importance to investigate this potential genetic risk factors not only in healthy control subjects but also in the disorder of interest, to understand its mechanism in this particular critical context.

In the analysis of differential learning, we detected the extinction phase as important for *NOS1* ex1f-VNTR associated effects over threat and safety signals. This is a new aspect of *NOS1* ex1f-VNTR associated effects since previous work focused on acquisition processes only (Kelley et al., 2009; Kelley et al., 2010; Kuhn et al., 2016). During late extinction, clusters in the bilateral amygdala revealed a CS- > CS+ activation pattern in carriers of the S/S- and L/L-genotype in patients. In healthy control subjects, the S/S-genotype showed a CS- > CS+ pattern whereas the L/L-genotype showed the reversed pattern. Post-hoc tests revealed diagnosis specific differences in threat (CS+) and safety (CS-) signal processing in healthy control subjects during early acquisition whereas in patients only during extinction.

Kuhn et al. (2016) found distinct effects of higher amygdala/hippocampus activation in carriers of at least one S-allele to US-predicting (equivalent to the CS+ in a cue conditioning paradigm) compared to safe contexts (equivalent to CS-) in the acquisition phase of a context conditioning paradigm. In contrast, we consistently found higher activation towards the safety than towards the threat signal, especially in carriers of the S/S-genotype in both patients and healthy control subjects during acquisition. This might be due to the fact that cue conditioning elicits stimulus-dependent phasic fear whereas context conditioning elicits sustained anxiety responses to the global situation (Davis et al., 2010). Fear and anxiety are similar but not the same and it is plausible that they are influenced differently by modulatory factors like *NOS1* ex1f-VNTR allelic variation due to slightly different neural pathways (Davis et al., 2010). In context conditioning, the US-predicting context is only predictable in terms of “that” the US will happen. In fact, this context is an unpredictable stimulus in terms of “when” and “how (often)” the negative event will happen. In Kuhn et al. (2016) the US was administered on average two times within fixed time windows during the context (UCXT). In contrast, cue conditioning is predictable in terms of “that, when and how (often)” the US will appear. Differences in neural activation due to different expectations therefore are plausible. The experiment of Kuhn et al. (2016) did not include extinction. With this study we could provide first preliminary evidence for *NOS1* ex1f-VNTR associated effects during fear extinction, too. This is of particular relevance since extinction learning is the key component of successful exposure-based therapy (Milad and Quirk, 2012; Vervliet et al., 2013). However, in the comparison over time our data suggest that the *NOS1* ex1f-VNTR-associated effects we found during the extinction phase could not be significantly altered by 12 sessions of successful exposure-based CBT (Gloster et al., 2011; Kircher et al., 2013). Therefore, this could indicate *NOS1* ex1f-VNTR allelic variation to be a persistent risk factor.

The pronounced evidence for higher activation towards CS- that we found consistently in carriers of the S/S-genotype – especially in the amygdala in patients during extinction – might be an indication for a dysfunctional attenuated fear inhibition in the face of safety signals. Increased brain activity towards CS- in conditioning paradigms in patients with panic disorder has been reported (Tuescher et al., 2011) and interpreted as an impaired ability of stimulus discrimination (Lissek et al., 2009) which, on a neural level, may be associated with a poor prognosis to sufficiently respond to exposure-based CBT (Lueken et al., 2013). Higher activation towards the CS- is discussed to be an overgeneralization in fear to neutral stimuli (Laufer et al., 2016; Lissek et al., 2010), that might be compatible with the immanent bias of anxiety disorders to respond with excessive fear to stimuli that are actually not harmful. Results from a meta analysis strongly support the idea of impaired ability to inhibit fear in the presence of safety cues and an increased tendency to generalize fear responses in patients (Duits et al., 2015). A meta-analysis on neural signatures of human fear conditioning (Fullana et al., 2016) confirms the evidence for CS- > CS + activation in the hippocampus and lateral inferior and middle temporal cortex. Together, this supports the hypothesis of the *NOS1* ex1f-

VNTR S-alleles being a potential risk factor for PD/AG for now as it is associated with pathologically altered neural activation. At present, however, this is not yet shown in genome wide association studies and has to be investigated in future studies.

Of note – although we found consistently higher rated arousal towards our stimuli in patients vs. healthy control subjects (see also Kircher et al., 2013) – we failed to find genotype associated ratings of higher anxiety (ASI) and arousal and lower valence (both during fMRI paradigm) in patients as well as in healthy control subjects as previously expected according to the behavioral results of Kuhn et al. (2016). However, Kuhn et al. (2016) had much more powerful samples for their questionnaires and behavioral studies.

Several limitations must be considered. Since genetic variance cannot be manipulated in human samples, the relationship between neural activation and *NOS1* ex1f-VNTR allelic variation can only be correlative. Additionally, diagnosis also represents an unrandomized factor. Also, the healthy control group was free of any lifetime mental disorders. As such, it may represent a «super-healthy» control group and it remains to be shown whether the pronounced inhibitory effects in S-allele carriers can be translated to the general population. Furthermore, our results have to be interpreted with caution and classified as exploratory because of the small sample sizes in the genotype sub-groups especially when conducting interaction analyses. We cannot exclude that our results could either represent a false positive effect or that important differences might have been missed due to false negative findings. This low number of participants may have introduced type 2 errors that may explain why we observed no CBT-related changes in activation at the second measurement. However, although this sample is likely underpowered, complex analyses on available clinical samples of interest are needed to provide at least preliminary information about group specific activation to better understand the role of genes in the complex environment of factors possibly influencing psychopathologies. Due to the current lack of studies on the intermediate *NOS1* ex1f-VNTR-phenotype in PD/AG, exploratory, analyses on existing samples are a starting point to pave the way for future research on systematic conducted larger samples. Our data benefit from coming from a large and controlled clinical trial with rigorous treatment adherence. Further longitudinal investigations on larger clinical samples are needed on how *NOS1* ex1f-VNTR allelic variation contributes to the development of PD/AG and how the brain is shaped by both genetic risk and environmental factors (e.g., life events/learning). This is part of our research in an ongoing clinical multicenter trial (Heinig et al., 2017).

We could provide first preliminary evidence for *NOS1* ex1f-VNTR associated effects during fear extinction in healthy control subjects and patients with PD/AG which is of particular relevance since extinction learning is the key component of successful exposure based therapy. Importantly, we were able to provide an initial insight in the association between *NOS1* ex1f-VNTR allelic variation with neural correlates in a fear conditioning and extinction paradigm in patients with PD/AG. We could expand insights from context to cue condition as well as to extinction processes, while in general confirming evidence from healthy control subjects for a modulatory role of *NOS1* ex1f-VNTR allelic variation in functional neuroimaging (Kuhn et al., 2016). Our findings support the hypothesis that *NOS1* ex1f-VNTR allelic variation might play a role in anxiety disorders and could be involved in dysfunctional neural processes in hippocampus and amygdala. However, since the major limitation of this study is its statistical power, our results must necessarily be understand as preliminary. Future research on considerably larger samples is needed to further clarify the role of *NOS1* ex1f-VNTR allelic variation in the gene x environment context.

Funding/Support

This work is part of the German multicenter trial “Mechanisms of Action in CBT (MAC)”. The MAC study is funded by the German Federal Ministry of Education and Research (BMBF; project no. 01GV0615; neuroimaging study: project no. 01GV0611) as part of the BMBF Psychotherapy Research Funding Initiative. Additional funding was obtained from the DFG (TRR CRC 58, subproject Z02, to AR and JD).

Centers

Principal investigators (PI) with respective areas of responsibility in the MAC study are V. Arolt (Münster: Overall MAC Program Coordination), H.U. Wittchen (Dresden: Principal Investigator (PI) for the Randomized Clinical Trial and Manual Development), A. Hamm (Greifswald: PI for Psychophysiology), A.L. Gerlach (Münster: PI for Psychophysiology and Panic subtypes), A. Ströhle (Berlin: PI for Experimental Pharmacology), T. Kircher (Marburg: PI for functional neuroimaging), and J. Deckert (Würzburg: PI for Genetics). Additional site directors in the RCT component of the program are G.W. Alpers (Würzburg), T. Fydrich and L. Fehm (Berlin-Adlershof), and T. Lang (Bremen).

Data access and responsibility

All principle investigators take responsibility for the integrity of the respective study data and their components. All authors and co-authors had full access to all study data. Data analysis and manuscript preparation were completed by the authors and co-authors of this article, who take responsibility for its accuracy and content.

Acknowledgements and staff members by site

Greifswald (coordinating site for Psychophysiology): Christiane Melzig, Jan Richter, Susan Richter, Matthias von Rad; Berlin-Charité (coordinating Center for Experimental Pharmacology): Harald Bruhn, Anja Siegmund, Meline Stoy, André Wittmann; Berlin-Adlershof: Irene Schulz; Münster (Overall MAC Program Coordination, Genetics and Functional Neuroimaging): Andreas Behnken, Katharina Domschke, Adrianna Ewert, Carsten Konrad, Bettina Pfleiderer, Christina Uhlmann, Peter Zwanzger; Münster (coordinating site for psychophysiology and subtyping): Judith Eidecker, Swantje Koller, Fred Rist, Anna Vossbeck-Elsebusch; Marburg/Aachen (coordinating center for functional neuroimaging): Barbara Drüke, Sonja Eskens, Thomas Forkmann, Siegfried Gauggel, Susan Gruber, Andreas Jansen, Thilo Kellermann, Isabelle Reinhardt, Nina Vercamer-Fabri; Dresden (coordinating site for data collection, analysis, and the RCT): Franziska Einsle, Christine Froehlich, Andrew T. Gloster, Christina Hauke, Simone Heinze, Michael Hoefler, Ulrike Lueken, Peter Neudeck, Stephanie Preiß, Dorte Westphal; Würzburg Psychiatry Department (coordinating center for genetics): Andreas Reif, Caro Gagel; Würzburg Psychology Department: Julia Duerner, Hedwig Eisenbarth, Antje B. M. Gerdes, Harald Krebs, Paul Pauli, Silvia Schad, Nina Steinhäuser; Bremen: Veronika Bamann, Sylvia Helbig-Lang, Anne Kordt, Pia Ley, Franz Petermann, Eva-Maria Schroeder. Additional support was provided by the coordinating center for clinical studies in Dresden (KKS Dresden): Xina Graehlert and Marko Käppler.

Dr. Sabine Herterich (Würzburg) performed the sequencing of *NOS1* ex1f-VNTR genotypes.

The RCT project was approved by the Ethics Committee of the Medical Faculty of the Technische Universität Dresden (EK 164082006). The neuroimaging components were approved by the Ethics Committee of the Medical Faculty of the Rheinisch-Westfaehlische Hochschule University Aachen (EK 073/07) and at all local sites. The study was registered with the ISRCTN: ISRCTN80046034.

Contributors

V. Arolt, H.U. Wittchen, A. Ströhle, B. Pfleiderer and T. Kircher designed the study and wrote the protocol. H.U. Wittchen designed and

supervised the clinical trial. A. Reif designed and supervised the genetic trial and undertook the genotyping together with S. Herterich and H. Weber. T. Kircher and B. Straube supervised the MRI subproject. Y. Yang undertook the preprocessing and first-level analysis of the fMRI data. I.C. Ridderbusch generated the hypotheses of the paper, undertook the second-level analysis of the fMRI data and wrote the original manuscript. B. Straube supervised the analyses and writing of the paper. U. Lueken helped reviewing and editing the paper. All authors contributed to and have approved the final manuscript.

Declaration of Competing Interest

The following authors report no conflicts of interest concerning the content of this paper: I. C, Ridderbusch, Y. Yang, H. Weber, S. Herterich, B. Pfleiderer, U. Lueken, B. Straube. T. Kircher received fees for educational programs from Janssen-Cilag, Eli Lilly, Servier, Lundbeck, Bristol Myers Squibb, Pfizer, and Astra-Zeneca; travel support/sponsorship for congresses from Servier; speaker's honoraria from Janssen-Cilag; and research grants from Pfizer and Lundbeck. H.-U. Wittchen has been member of advisory boards of several pharmaceutical companies. He received travel reimbursements and research grant support from Essex Pharma, Sanofi, Pfizer, Organon, Servier, Novartis, Lundbeck, Glaxo Smith Kline. A. Reif is member of advisory boards and/or gave presentations for the following companies: Medice, Shire, Janssen, Servier and neuraxpharm. V. Arolt is member of advisory boards and/or gave presentations for the following companies: Astra-Zeneca, Janssen-Organon, Lilly, Lundbeck, Servier, Pfizer, and Wyeth. He also received research grants from Astra-Zeneca, Lundbeck, and Servier. He chaired the committee for the “Wyeth Research Award Depression and Anxiety”. A. Stroehle received research funding from the German Federal Ministry of Education and Research, the European Commission (FP6) and Lundbeck, and speaker honoraria from AstraZeneca, Boehringer Ingelheim, Eli Lilly & Co, Lundbeck, Pfizer, Wyeth and UCB. Educational grants were given by the Stifterverband für die Deutsche Wissenschaft, the Berlin Brandenburgische Akademie der Wissenschaften, the Boehringer Ingelheim Fonds and the Eli Lilly International Foundation.

Ethical standards

The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national and institutional committees on human experimentation and with the Helsinki Declaration of 1975, as revised in 2008.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.nicl.2020.102268.

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