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ORIGINAL ARTICLE

Neuropeptide S receptor gene—converging evidence for a role in panic disorder

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Animal studies have suggested neuropeptide S (NPS) and its receptor (NPSR) to be involved in the pathogenesis of anxiety-related behavior. In this study, a multilevel approach was applied to further elucidate the role of NPS in the etiology of human anxiety. The functional NPSR A/T (Asn[107]Ile) variant (rs324981) was investigated for association with (1) panic disorder with and without agoraphobia in two large, independent case–control studies, (2) dimensional anxiety traits, (3) autonomic arousal level during a behavioral avoidance test and (4) brain activation correlates of anxiety-related emotional processing in panic disorder. The more active NPSR rs324981 T allele was found to be associated with panic disorder in the female subgroup of patients in both samples as well as in a meta-analytic approach. The T risk allele was further related to elevated anxiety sensitivity, increased heart rate and higher symptom reports during a behavioral avoidance test as well as decreased activity in the dorsolateral prefrontal, lateral orbitofrontal and anterior cingulate cortex during processing of fearful faces in patients with panic disorder. The present results provide converging evidence for a female-dominant role of NPSR gene variation in panic disorder potentially through heightened autonomic arousal and distorted processing of anxiety-relevant emotional stimuli.
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Introduction

Neuropeptides have been suggested to have a pivotal role in the pathogenesis of stress, arousal and anxiety. In particular, there is converging evidence from animal studies and molecular genetic studies in humans for neuropeptide S (NPS) and its cognate receptor (NPSR) to be involved in the mediation of anxiety-related behavior and anxiety disorders. NPS, a 20 amino-acid peptide, acts as an agonist at the G-protein-coupled NPSR increasing free intracellular calcium and cyclic adenosine monophosphate accumulation.[1–3]

In rodent models, NPS or NPSR agonists have been observed to elicit a robustly increased arousal as indicated by hyperlocomotion, righting reflex and wakefulness.[3–5] Interestingly, these arousal promoting effects seem to be paralleled by an anxiolytic-like profile of NPS because centrally administered NPS has been shown to increase the time mice spent

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exploring less protected or brighter areas of their environment (for example, open field, four-plate test, elevated plus maze, elevated zero maze, light-dark box).[3–5] An overall anxiolytic effect of NPS has furthermore been shown with NPS significantly reducing the time mice spent burying unfamiliar objects (defensive marble-burying test).[3,6] NPS also seems to reduce the physiological response to stress as shown by attenuation of stress-induced hyperthermia in mice.[4,5] Consistently, NPSR knockout mice were found to exhibit increased anxiety-like behavior, and accordingly reduced exploratory activity.[7] On an anatomical level, NPS has been shown to be expressed in the amygdaloid complex[8] and exert a modulatory effect on both afferent and intrinsic transmission in amygdala networks in rodent models.[9–11] On a cellular level, NPS increases glutamatergic transmission to intercalated GABAergic neurons in the amygdala conferring an inhibitory influence on the central amygdaloid nucleus and thereby attenuating anxiety-like responses.[9] Given the crucial role of the amygdala in the elicitation and regulation of anxiety-related responses[12–14] and increased amygdala activity in patients with anxiety disorders,[15–18] this finding underlines the importance of NPS in the processing of acute fear. In addition, acute administration of caffeine, which is a potent anxiogenic and arousal-increasing substance in many participants,[19] has been observed to induce a marked decrease in mRNA levels of NPS itself, but to upregulate NPSR expression levels in the brain stem.[20] Finally, NPS can selectively inhibit the release of serotonin and norepinephrine in the frontal cortex,[21] which might constitute another mechanism through which NPS modulates anxiety- and arousal-related behavior.

The human NPSR gene is located on chromosome 7p14.3 and comprises at least nine exons. The chromosomal region 7p14-15 has been previously found to be linked to panic disorder,[22–24] rendering the NPSR gene a promising functional and positional candidate gene. An A/T single-nucleotide polymorphism (rs324981) at position 107 of the NPSR gene leading to an amino-acid exchange from Asn to Ile ($N^{107}I$) is of functional relevance with the T allele (Ile^{107}) increasing NPSR expression and NPS efficacy at NPSR about tenfold.[25,26] Homozygosity for the less active A allele (Asn^{107}) was found to be underrepresented in the male ($n = 51$) subgroup of 140 Japanese panic disorder patients, suggesting the higher active T (Ile^{107}) allele to constitute a risk factor for panic disorder.[27]

In this study, a multilevel approach was applied to further elucidate the role of NPS in the pathogenesis of human anxiety. In detail, it was investigated whether the functional NPSR A/T ($Asn^{107}Ile$) variant (rs324981) was associated (1) with the categorical phenotype of panic disorder with and without agoraphobia in two independent, large and well-controlled case–control studies; (2) with participants' scores on dimensional anxiety traits such as anxiety sensitivity; (3) with autonomic arousal level as reflected by heart rate and symptom ratings during a behavioral avoidance test (BAT) and (4) with brain activation correlates of anxiety-related emotional processing in patients with panic disorder with and without agoraphobia.

Materials and methods

Association studies with panic disorder and dimensional anxiety traits

Samples. The discovery sample (ascertained in Münster, Munich, Würzburg, Bonn and Göttingen and hereafter termed MMWBG sample) comprised 499 unrelated German patients with panic disorder (women 312, men 187; mean age 38.14 ± 11.61 years; panic disorder with agoraphobia: 75.7%). The diagnosis of panic disorder was ascertained by experienced psychiatrists on the basis of medical records and structured clinical interviews (SADS-LA (Schedule for Affective Disorders and Schizophrenia–Lifetime, Anxiety), CIDI (Composite International Diagnostic Interview), SKID-I (Strukturiertes Klinisches Interview für DSM-IV, Achse-I)) according to the criteria of Diagnostic and Statistical Manual of Mental Disorders (DSM)-III-R or DSM-IV, respectively.[28–30] Patients with bipolar disorder, schizophrenia, mental retardation, neurological or neurodegenerative disorders impairing psychiatric evaluation were not included in this analysis. Data regarding comorbid depression were available for 497 of these patients, of whom 15% suffered from a comorbid major depressive episode. The control group consisted of 499 unrelated anonymous blood donors of German descent (women 312, men 187; mean age 40.01 ± 12.55 years), who due to anonymity requirements could not be controlled for the presence of mental diseases. The study was approved of by the respective local ethical committees and informed consent was obtained from all participating subjects.

The replication sample is a subsample of patients from the mechanisms of action in cognitive-behavioral therapy (MAC) study in patients with panic disorder with agoraphobia[31] obtained from the BMBF 'Panic Network' consisting of 277 of the total 369 included patients. In the patient sample ($n = 277$, women 209, men 68; mean age 36.40 ± 10.96 years), the diagnosis of panic disorder with agoraphobia was established by a structured clinical interview and verified by clinical interview (CIDI) according to DSM-IV criteria.[30] Patients with bipolar disorder, schizophrenia, mental retardation, neurological and neurodegenerative disorders were not included in this study, 35% of the patients suffered from a 12-month comorbid diagnosis of depression.[31] All patients were free of psychotropic medication. For categorical association studies, control subjects matched 1:1 by sex ($n = 277$, women 209, men 68; mean age 28.90 ± 7.67 years) were drawn from a larger number of screened healthy controls ($n = 519$, women 361, men 158; mean age 25.61 ± 6.72 years).

The complete control sample ($n = 519$) was used to test for association of NPSR rs324981 with dimensional anxiety traits. Absence of mental axis 1 disorders was established by experienced psychologists on the basis of a structured clinical interview (Mini International Neuropsychiatric Interview) according to the criteria of DSM-IV.[30] For both patient and control groups, panic fear and anxiety sensitivity were evaluated by German versions of the Agoraphobic Cognitions Questionnaire (ACQ)[32,33] and Anxiety Sensitivity Index (ASI).[34,35] Cases and controls were of Caucasian origin. The study was approved of by the ethics committee of Würzburg and written informed consent was obtained from all participating subjects.

Genotyping. To determine the functional NPSR rs324981 A/T ($Asn^{107}Ile$) polymorphism, we amplified DNA isolated from venous blood samples by PCR using the primers F: 5′-GAAGGAAAAAA ATTAAAAATGAACCTCCCCAGGATTTCAT and R: 5′-TTCTACCCAGGAGAAAGCGGGCAGTTTGATGCA, resulting in an amplicon size of 353 bp. Standard PCR was carried out in a 20 µl volume containing 45–60 ng of genomic DNA, 10 pmol of each primer, 200 µM dNTPs, 0.4 U Taq DNA Polymerase (Eppendorf, Hamburg, Germany), 50 mM KCl, 1.5 mM $MgCl_2$ and 10 mM Tris-HCl (pH 8.4). After a 5 min denaturation, 35 cycles were carried out consisting of 30 s at 94 °C, 30 s at 66 °C and 60 s at 72 °C, followed by a final extension time of 10 min at 72 °C. Amplicons were digested with *Tas*I (Fermentas, St Leon-Rot, Germany) (1 U), separated for 2 h on a 15% polyacrylamide gel and visualized by silver-staining. Hardy–Weinberg criteria, as calculated by the online program DeFinetti (http://ihg.gsf.de/cgi-bin/hw/hwa1.pl; Wienker TF and Strom TM), were fulfilled for genotype distributions of NPSR rs324981 in all tested samples (all P-values > 0.05). There were genotyping failures in 10 patients resulting in a total n of 489 patients (women 305, men 184) in the discovery sample available for further analysis.

Statistical analysis. Statistical analyses of allele and genotype distributions were performed by means of χ^2-test as implemented in Haploview version 3.2 and Armitage's trend test as provided by DeFinetti. In addition, grouped genotype (AA vs AT/TT) distribution was statistically analyzed based on our results pointing toward a dominant model for the T allele and in analogy to the initial study by Okamura *et al*.[27] explicitly reporting an underrepresentation of the homozygous AA genotype in patients with panic disorder. Both association studies have also been analyzed jointly using the same procedure; furthermore, meta-analytic treatment of the studies was performed: odds ratios (ORs) were calculated as a measure for effect size; thereafter, the Q-statistic[36,37] was applied to assess heterogeneity. Inconsistency across studies was quantified with I^2 metric ($I^2 = Q\text{-}df/Q$).[38] In absence of heterogeneity, ORs were combined using fixed-effects models;[39] if significant heterogeneity was detected, joint ORs were derived from random-effects models as denoted in Figures 1a–c.[40] Calculations were performed using R version 2.10[41] along with the package metafor version 0.5–7.[42]

Psychophysiological assessment in a behavioral avoidance test

A total of 264 German patients suffering from panic disorder and agoraphobia, who were part of the replication sample (MAC sample), were investigated in a BAT. Thirty patients were excluded from the analysis because they did not enter the small, light-proof chamber, and 29 patients had to be excluded due to measurement failures. From the remaining sample of 205 patients, 55 were homozygous NPSR rs324981 A allele carriers and were compared to the remaining 150 T allele carriers. The groups did not differ in gender, $\chi^2 = 1.58$, $P = 0.209$ (AA: 37 female and 18 male patients; AT/TT: 114 female and 36 male patients), and age, $F(1, 203) < 1$, $P = 0.80$ (AA: 35.18 years ± 1.57 s.e; AT/TT: 35.62 years ± 0.87 s.e.). After arriving at the laboratory, patients were informed about the procedure before the sensors for recording of electrocardiogram were attached. During anticipation, patients were sitting for 10 min in front of a small (75 cm wide, 120 cm long, 190 cm high), dark chamber with the door open. During exposure, patients were locked in the chamber and told to stay as long as possible for a maximum of 10 min. During recovery, patients again sat in front of the opened chamber for 8 min. Patients could terminate the BAT at any time during the procedure. Patients were instructed to rate intensity of 13 panic symptoms and subjective indices of anxiety on a scale from 1 to 10 after each period.

The electrocardiogram was measured using an Einthoven lead II setup with two standard, electrolyte-filled Ag/AgCl electrodes (Marquette Hellige, Freiburg, Germany). The raw signal was filtered (8–13 Hz band pass) and amplified using a Coulbourn V75-04 bioamplifier (Coulborn, Düsseldorf, Germany) and continuously digitized with a sampling rate of 100 Hz. Heart rate was derived from electrocardiogram signal using software provided by ANSLAB.[43] Interbeat intervals were checked and corrected whenever misplaced R-wave triggers had occurred (due to increased T-waves or movement artifacts) and converted to heart rate (beats per minute, b.p.m.) in half-second bins. Mean heart rate was calculated during anticipation, exposure and recovery. This part of the study was approved by the ethics committee of the Technical University of Dresden and informed consent was obtained from all participating subjects.

To test effects of genotype on symptom reports and autonomic arousal, we applied a mixed-model analysis of variance including genotype (AA vs AT/TT) as a between-subjects factor and block (anticipation vs exposure vs recovery) as a within-subjects factor. All statistical tests used a significance level of $P < 0.05$.

a

Complete sample	Cases		Controls		Odds Ratio
	Events	Total	Events	Total	M-H, Fixed, 95% CI
MAC sample	203	277	193	277	1.19 [0.83, 1.73]
MMWBG sample	355	489	319	499	1.49 [1.14, 1.96]
Total (95% CI)		**766**		**776**	1.38 [1.11, 1.72]
Total events	558		512		

Heterogeneity: $Chi^2 = 0.9271$; df = 1 (p = 0.336)
Test for overall effect: Z = 2.92 (p= 0.004)
Cochran-Mantel-Haenzel-Test: CMH =8.20; df = 1 (p = 0.004)

b

Female only subgroup	Cases		Controls		Odds Ratio
	Events	Total	Events	Total	M-H, Fixed, 95% CI
MAC sample	159	209	140	209	1.57 [1.02, 2.41]
MMWBG sample	213	305	192	312	1.45 [1.04, 2.02]
Total (95% CI)		**514**		**521**	1.49 [1.15, 1.94]
Total events	372		332		

Heterogeneity: Chi2 = 0.08; df = 1 (p = 0.774)
Test for overall effect: Z = 2.97 (p = 0.003)
Cochran-Mantel-Haenzel-Test: CMH =8.46; df = 1 (p = 0.004)

c

Male only subgroup	Cases		Controls		Odds Ratio
	Events	Total	Events	Total	M-H, Random, 95% CI
MAC sample	44	68	53	68	0.52 [0.24, 1.11]
MMWBG sample	142	184	127	187	1.60 [1.01, 2.53]
Total (95% CI)		**252**		**255**	0.95 [0.32, 2.85]
Total events	186		180		

Heterogeneity: $Tau^2 = 0.53$; $Chi^2 = 6.16$; df = 1(P = 0.0131); $I^2 = 83.76\%$
Test for overall effect: Z = -0.093 (P=0.926)

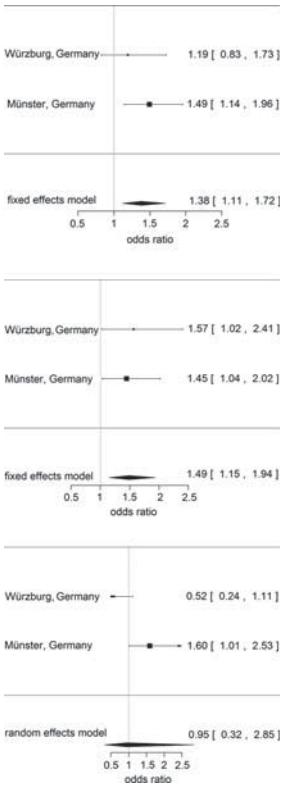

Figure 1 NPSR rs324981 and panic disorder: Forest plot showing analyses of TT/TA vs AA in the combined sample (**a**), females (**b**) and males (**c**).

Functional imaging during presentation of emotional stimuli

Twenty patients with panic disorder were investigated using an imaging genetic approach (women 12, men 8; mean age 36.75 ± 9.39 years). Panic disorder was diagnosed by experienced psychiatrists on the basis of medical records and a structured clinical interview (SKID-I) according to the criteria of DSM-IV.[30] Only patients with primary panic disorder were included, secondary lifetime diagnoses were social phobia in 10 and major depression in 5 patients. Ten patients were treated with a selective serotonin reuptake inhibitor, the other 10 patients were free of medication. This part of the study was approved by the local ethical committee in Muenster and informed consent was obtained from all participating subjects.

Technical details of fMRI data acquisition and processing have been reported in detail previously.[44,45] Briefly, subjects viewed 30 s blocks of alternating emotional (fearful, angry, happy, neutral) faces[46] or a no-face control stimulus (gray rectangle). Emotional stimuli were presented twice per second in a random sequence for 500 ms. T2* functional data were acquired at a 3 Tesla scanner (Gyroscan Intera 3.0 T; Philips Medical Systems, Best, NL, USA; matrix 128 × 128, resolution 1.75 mm × 1.75 m × 3.5 mm; repetition time = 3 s, echo time = 30 ms, flip angle = 90°). Functional imaging data were preprocessed (motion corrected, normalized to standard MNI space and smoothed) with a published protocol using SPM5 (Wellcome Department of Cognitive Neurology, London, UK). Statistical analysis was performed by modeling the different conditions (fearful, angry, happy, neutral, no face) as variables within the context of the general linear model (modeled with a standard hemodynamic response function). Subject-specific contrasts, reflecting emotion-related activation (F, A, H and N vs no face), were entered into a random-effects analysis. Voxel values of 5 × 2 predefined regions of interest (ROIs)[47] were extracted on the basis of *a priori* hypotheses regarding potential involvement of these regions in emotional stimuli processing (amygdala, orbitofrontal cortex, ventromedial prefrontal cortex, dorsolateral prefrontal cortex, anterior cingulate cortex), summarized by mean and tested among the different conditions using the MarsBaR toolbox.[48] Contrast values were used for further analyses. To depict activations on anatomical MRI slices, we applied the Wake Forest University PickAtlas for the same regions. The statistical threshold was set at $P < 0.001$ (uncorrected) with clusters defined by at least 10 contiguous voxels of significant response. To test effects of genotype on activation in brain ROIs, we applied a multivariate analysis of variance including genotype (AA vs AT/TT) as a between-subjects factor and four contrast values (fearful, angry, happy, neutral vs no faces) as a within-subjects factor. There were no statistically detectable differences in medication with selective

serotonin reuptake inhibitors ($P=0.639$), sex ($P=0.085$) and comorbidity with major depression ($P=0.787$) or social phobia ($P=0.639$) across NPSR genotype groups as calculated by χ^2-test. However, because genotype groups differed for age ($T=2.456$, $P=0.024$), age was included as a covariate in all further tests. All statistical tests used a significance level of $P<0.05$.

Results

Association studies with panic disorder

Allele and genotype frequencies of NPSR rs324981 in patient and control samples are given in Table 1. In the discovery MMWBG sample, the NPSR rs324981 AA genotype as compared to carriers of at least one T allele was underrepresented in patients with panic disorder ($P=0.003$). Stratification for gender revealed that this effect held true for both genders; however, it was more pronounced in the female subgroup of patients (women: $P=0.03$; men: $P=0.05$) (see Table 1).

In an attempt to replicate the association of NPSR rs324981 with panic disorder, we interrogated a second, independent case–control sample of 277 patients suffering from panic disorder with agoraphobia (MAC sample), along with matched controls (Table 1). Genotyping procedures and statistical analyses were identical to the discovery sample. Again, the NPSR rs324981 AA genotype was significantly underrepresented in female patients with

Table 1 NPSR rs324981 A/T polymorphism and panic disorder: association studies

	AA	*AT*	*TT*	*Armitage's trend test*	*AA*	*AT/TT*	*χ^2-Test*	*A*	*T*	*χ^2-Test*
MMWBG sample										
Overall sample										
Patients ($N=489$)	134	229	126	OR = 1.32 $\chi^2 = 10.38$ **$P = 0.001$**	134	355	OR = 1.50 95%CI = 1.14–1.96 $\chi^2 = 8.56$ **$P = 0.003$**	497	481	OR = 1.35 95%CI = 1.13–1.62 $\chi^2 = 11.20$ **$P = 0.001$**
Controls ($N=499$)	180	222	97		180	319		582	416	
Females										
Patients ($N=305$)	92	134	79	OR = 1.34 $\chi^2 = 7.13$ **$P = 0.008$**	92	213	OR = 1.45 95%CI = 1.04–2.02 $\chi^2 = 4.71$ **$P = 0.030$**	318	292	OR = 1.38 95%CI = 1.10–1.73 $\chi^2 = 7.95$ **$P = 0.005$**
Controls ($N=312$)	120	135	57		120	192		375	249	
Males										
Patients ($N=184$)	42	95	47	OR = 1.30 $\chi^2 = 3.29$ **$P = 0.069$**	42	142	OR = 1.60 95%CI = 1.01–2.53 $\chi^2 = 3.99$ **$P = 0.046$**	179	189	OR = 1.31 95%CI = 0.98–1.75 $\chi^2 = 3.34$ **$P = 0.068$**
Controls ($N=187$)	60	87	40		60	127		207	167	
MAC sample										
Overall sample										
Patients ($N=277$)	74	140	63	OR = 1.20 $\chi^2 = 2.16$ **$P = 0.141$**	74	203	OR = 1.19 95%CI = 0.83–1.73 $\chi^2 = 0.89$ **$P = 0.347$**	288	266	OR = 1.19 95%CI = 0.94–1.51 $\chi^2 = 2.09$ **$P = 0.148$**
Controls ($N=277$)	84	144	49		84	193		312	242	
Females										
Patients ($N=209$)	50	111	48	OR = 1.36 $\chi^2 = 4.80$ **$P = 0.028$**	50	159	OR = 1.57 95%CI = 1.02–2.40 $\chi^2 = 4.24$ **$P = 0.039$**	211	207	OR = 1.35 95%CI = 1.03–1.77 $\chi^2 = 4.63$ **$P = 0.031$**
Controls ($N=209$)	69	104	36		69	140		242	176	
Males										
Patients ($N=68$)	24	29	15	OR = 0.83 $\chi^2 = 0.74$ **$P = 0.389$**	24	44	OR = 0.51 95%CI = 0.24–1.11 $\chi^2 = 2.91$ **$P = 0.088$**	77	59	OR = 0.81 95%CI = 0.50–1.31 $\chi^2 = 0.73$ **$P = 0.394$**
Controls ($N=68$)	15	40	13		15	53		70	66	

Bold values are significant *P*-value at significance level of <0.05.

panic disorder, whereas there was no significant effect in the male or the total sample (see Table 1).

When both samples were analyzed jointly in a meta-association approach leading to a combined sample size of 766 patients and 776 controls, there was a highly significant association of the NPSR rs324981 risk genotypes containing at least one T allele with disease in the total sample (Armitage's trend test OR 1.28, $\chi^2 = 12.11$, $P = 0.0005$) as well as in the female (Armitage's trend test OR 1.35, $\chi^2 = 11.95$, $P = 0.00055$), but not the male subsample (Armitage's trend test OR 1.15, $\chi^2 = 1.25$, $P = 0.263$). As such a meta-association approach might bear several problematic issues, such as ethnic background admixture (although this is unlikely as both samples were ascertained in Germany), we also treated the data in a meta-analytic manner (see Figure 1). Association of the NPSR rs324981 T risk allele with panic disorder was confirmed in this meta-analysis, yielding an OR of 1.38 ($P = 0.004$), which held true in the female (OR 1.49, $P = 0.004$), but not the male subsample (OR 0.95, $P > 0.1$).

Association study with dimensional anxiety traits

Next, we examined whether the NPSR rs324981 T allele also influences dimensional constructs associated with panic disorder and agoraphobia. To do so, we tested whether the ACQ and ASI were associated with NPSR rs324981 in patients from the MAC study as well as 519 healthy controls. Although there was not a significant association in control subjects (ACQ, $P = 0.41$; ASI, $P = 0.99$), the ASI (but not the ACQ; best $P = 0.31$) score was significantly associated with genotype in the expected direction in patients: whereas NPSR rs324981 AA carriers had an ASI score of 27.5 ± 11.1, T allele carriers displayed a score of 32.1 ± 11.7 ($P = 0.002$) (see Figure 2). Again, the difference was due to the female subsample (27.0 ± 11.7 vs 32.6 ± 11.8, $P = 0.001$) and not present in the male subsample (28.6 ± 11.8 vs 30.2 ± 11.2, $P = 0.29$).

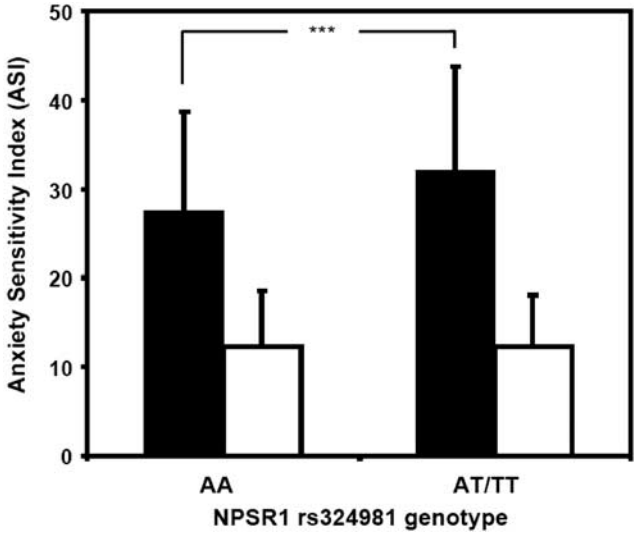

Figure 2 NPSR rs324981 A/T polymorphism and anxiety sensitivity. Closed bars, patients; open bars, controls; *** = significant at $P = 0.002$.

Genetic modulation of heart rate and symptom reports: behavioral avoidance test

Genotype groups (AA = 55, AT = 102, TT = 48) did not differ significantly in duration of exposure, Group $F(1, 203) < 1$, $P = 0.33$ (AA: 539.98 s ± 22.74 s.e; AT/TT: 514.00 s ± 13.77 s.e). As expected, mean heart rate and symptom reports significantly increased from anticipation to exposure and decreased again during recovery (Block $F(2, 406) = 28.69$, $P < 0.001$; Block $F(2, 406) = 90.42$, $P < 0.001$ for heart rate and symptom reports, respectively) showing that the BAT reliably induced intense fear in this group of patients.

NPSR rs324981 genotype systematically modulated these response patterns. Homozygous A allele carriers showed significantly lower overall heart rate than T allele carriers, Group $F(1, 203) = 6.39$, $P < 0.05$, which was not modulated by conditions (Figure 3), thus depicting an overall larger arousal response during all three phases of the BAT. Symptom reports were also significantly modulated but showed a different

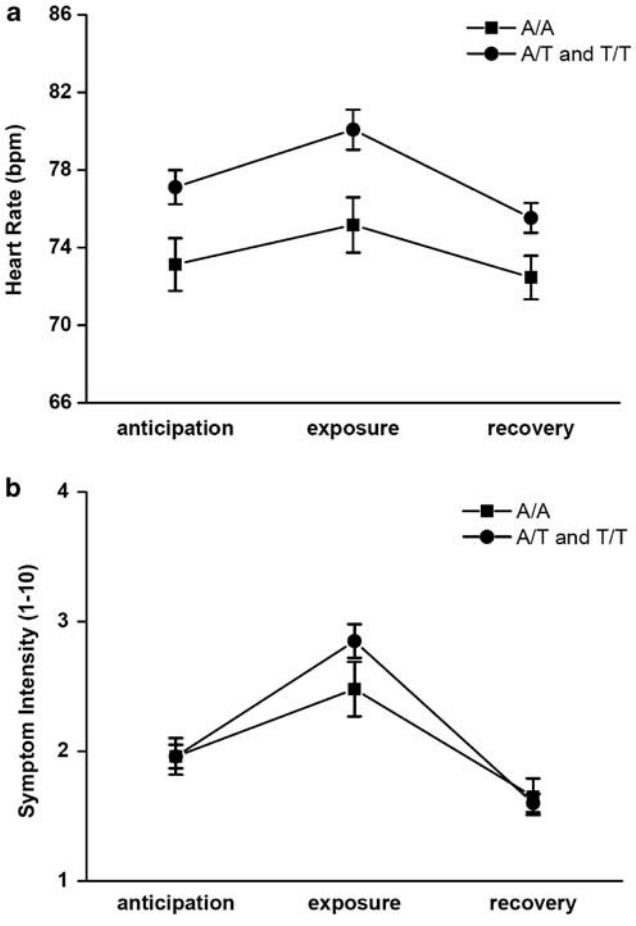

Figure 3 Mean heart rate (upper panel; **a**) and mean symptom intensity (lower panel; **b**) during anticipation, exposure and recovery in homozygous A allele carriers and T allele carriers, respectively.

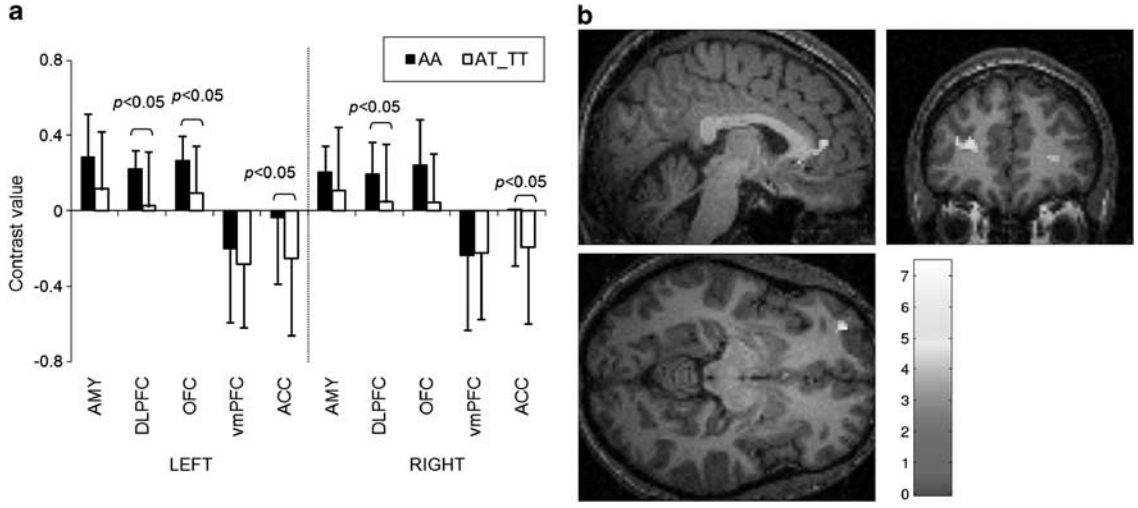

Figure 4 NPSR rs324981 A/T (Asn[107]Ile) effects on brain activation during processing of fearful faces (vs no face). (**a**) Contrast values analyzed by MarsBaR; (**b**) ROI analyses of the same regions using the Wake Forest University Pick Atlas (patients homozygous for the A allele vs patients with AT and TT genotypes at $P<0.001$). AMY, amygdala; DLPFC, dorsolateral prefrontal cortex; OFC, orbitofrontal cortex; vmPFC, ventromedial prefrontal cortex; ACC, anterior cingulate cortex.

pattern. While A and T allele carriers did not differ in their symptom reports during anticipation and recovery, T allele carriers reported significantly more intense symptoms during acute exposure, Block × Group $F(2, 406) = 4.26$, $P<0.05$.

Genetic modulation of fear circuit activation

We observed association of NPSR rs324981 with brain activation responses to fearful faces in contrast to the no-face condition (Figure 4): the protective AA genotype was found to be associated with an increased activation in the dorsolateral prefrontal cortex (left: AA: mean = 0.221, s.d. = 0.097; AT/TT: mean = 0.025, s.d. = 0.285; $F = 3.857$, $P = 0.042$; right: AA: mean = 0.193, s.d. = 0.170; AT/TT: mean = 0.048, s.d. = 0.306; $F = 4.182$, $P = 0.033$) and lateral orbitofrontal cortex (left: AA: mean = 0.267, s.d. = 0.124; AT/TT: mean = 0.092, s.d. = 0.251; $F = 2.621$, $P = 0.102$; right: AA: mean = 0.243, s.d. = 0.243; AT/TT: mean = 0.044, s.d. = 0.259; $F = 3.674$, $P = 0.047$), whereas carriers of at least one T risk allele exhibited a significantly decreased activity in the anterior cingulate cortex (left: AA: mean = −0.037, s.d. = 0.352; AT/TT: mean = −0.255, s.d. = 0.408; $F = 4.880$, $P = 0.021$; right: AA: mean = 0.009, s.d. = 0.301; AT/TT: mean = −0.197, s.d. = 0.402; $F = 5.962$, $P = 0.011$).

No other ROIs including the amygdala were found to be associated with NPSR rs324981 at the corrected threshold in response to fearful faces. There was no significant impact of NPSR rs324981 on responsiveness in any of the ROIs to happy, angry or neutral faces.

Discussion

In this study, association of the more active NPSR rs324981 T allele with the categorical diagnosis of panic disorder with and without agoraphobia was observed in two independent, large case–control studies as well as in a meta-analytic approach. This finding strengthens recent evidence arising from animal models for a pivotal role of NPS in the expression of anxiety[3–7,9–11,49] and for a risk locus on chromosome 7p14 containing the NPSR gene in panic disorder.[22–24,27]

Interestingly, in both samples comprising a total of 766 patients association of NPSR gene variation with panic disorder strongly originated from or was even restricted to the female subgroup of patients ($n = 514$). This finding is in line with previous molecular genetic reports of female-specific association findings in panic disorder possibly through hormonal interactions or female-specific transcriptional patterns.[50–52] From an evolutionary perspective, one might speculate that the evolutionarily 'newer' T allele variant might convey a beneficial effect by optimizing the fight-or-flight reaction through increased arousal levels, which might be positive in more male-dominant predatory environments such as hunting, but not in comparatively safe agricultural or household environments. The present female-dominant finding is contrary to the observation by Okamura *et al*.[27] who reported an association of NPSR rs324981 with panic disorder in the male subgroup of patients only. However, as their male subsample of patients was very small ($n = 51$) and NPSR rs324981 genotype distribution in male control subjects did not conform to Hardy–Weinberg equilibrium, this observation is finally inconclusive. Nevertheless, population-specific differences across Caucasian and Asian samples, as already observed for other polymorphisms, may explain this discrepancy.[53]

Association of the gain-of-function NPSR rs324981 T allele with panic disorder in humans seems inconsistent with findings in rodent models, where

NPS and agonists at NPSR have been shown to rather exert a dose-dependent anxiolytic effect in the open field, the light-dark box, the elevated plus maze and marble-burying paradigms[3] (although it should be noted that pharmacological interventions during adulthood do not readily mimic genetically driven alterations during ontogeny). A possible explanation of this paradox might be that panic disorder is to a great extent conferred through an increased level of arousal,[54–56] which in animal models has been found to be driven by increased NPS activity with a potentially differential dose-dependent effect on arousal and anxiety, respectively.[1,3–5] Increased arousal mediated by the more active T allele would be in line with Smith *et al*.[57] reporting NPS to cause a significant stimulation of the hypothalamic-pituitary-adrenal axis with an increase in plasma adrenocorticotropic hormone and corticosterone along with increased arousal-like behavior in rats. This hypothesis of increased arousal possibly mediating the anxiety-increasing effect of NPSR gain-of-function gene variation is substantiated by several translational findings at different levels in this study.

The NPSR rs324981 T risk allele was observed to be associated with increased anxiety sensitivity as measured by the ASI. Anxiety sensitivity is a dimensional construct measuring the extent to which individuals believe that symptoms of arousal and anxiety can have harmful somatic, social or psychological consequences.[35] Several studies provide evidence for anxiety sensitivity predicting fear responses to bodily sensations increasing physiological arousal[58]; for example, following a stair-stepping task[59] or hyperventilation challenge,[60] during detection of changes in pulse transit time[61] or in response to caffeine administration[62] and ASI seems to be a predictor for the development of panic disorder after periods of stress.[63] So, the observed association of the NPSR rs324981 T risk allele with increased anxiety sensitivity might in part reflect an increased level of arousal. In addition, individual anxiety sensitivity profiles seem to discriminatively characterize specific panic disorder subtypes because in patients with panic disorder high scores on the ASI have been reported to be associated with the respiratory subtype of panic disorder[64] and to predict symptomatological reaction to CO_2[65] as well as increased subjective fear during hyperventilation challenge.[66] Given that patients with the respiratory subtype of panic disorder are characterized by a particularly high familial loading of panic disorder and thereby a potentially higher genetic vulnerability,[67] NPSR gene variation associated with high anxiety sensitivity in patients with panic disorder might in part reflect vulnerability toward this particular subtype of patients with panic disorder, which, however, remains to be elucidated in future studies. Finally, women with panic disorder score higher than men on the AS physical concerns subscale,[68] and anxiety sensitivity has been shown to be heritable particularly in women.[69] So, the present finding of NPSR gene variation to be associated with the categorical diagnosis of panic disorder particularly in female patients might in part be explained by its association with the dimensional phenotype of anxiety sensitivity restricted to female patients.

The NPSR rs324981 T risk allele was related to overall increased heart rate during exposure but also during anticipation and even recovery during a BAT. These data are consistent with the notion that patients who carry the T risk allele have an enhanced sympathomimetic activation and consequently arousal level. In challenging situations in which the arousal response further increases over an already elevated baseline level, these patients are more prone to experience panic symptoms. These findings are in line with modern learning theories of panic disorder,[55] postulating that internal cues of elevated arousal increase the chance of experiencing another panic attack once they have been associated with aversive responses.[70] These data suggest that not only conditioned anxious apprehension but also genetic risk factors contribute to the increased arousal level and might therefore uncover an important missing link in the understanding of the mechanisms involved in the development of a panic disorder.

We observed an NPSR rs324981 genotype-dependent brain activation pattern in response to anxiety-relevant emotional stimuli. The NPSR rs324981 T risk allele was found to be associated with significantly decreased activity in the anterior cingulate cortex. This result is in line with previous studies reporting anterior cingulate cortex volume reduction in patients with panic disorder[71,72] and diminished cingulate activity in patients with panic disorder during identification of fearful facial affect compared to healthy controls.[73] Decreased cingulate cortex activity has been hypothesized to be due to a chronic hyperarousal in patients with panic disorder,[74] because decreased cingulate cortex activity appears to be associated with engagement of arousal networks particularly during processing of threat-related stimuli also in other anxiety disorders such as simple phobias and posttraumatic stress disorder.[75–78] Given that NPS has been found to dose-dependently elicit increased arousal response as detailed above, the NPSR rs324981 T allele increasing NPSR expression and NPS efficacy at NPSR might confer a heightened risk of panic disorder through an elevated level of arousal as reflected by decreased cingulate cortex activity. In addition, homozygosity for the A allele was associated with significantly increased activity in the dorsolateral prefrontal and orbitofrontal cortex during the processing of fearful faces compared to activation in T risk allele carriers. Given the well-established role of the prefrontal and orbitofrontal cortex to attenuate an emotional response to fear/threat-related stimuli in anxiety disorders by inhibition of amygdala activation thorough a top-down mechanism,[13–15,78] increased activity in those regions might contribute to the observed protective effect of the NPSR rs324981 AA genotype with respect to panic disorder.

Several limitations of this study have to be considered: Patients and controls in the discovery and replication samples were not completely matched for age in that some of the control subjects still are at risk to develop panic disorder. This, however would bias against an association finding and not favor a false-positive finding. Indeed, this may even explain the somewhat weaker association in the replication sample as here the age difference was larger. Also, the discovery and replication sample differed with respect to the ratio of panic disorder patients with and without agoraphobia with a smaller agoraphobia ratio in the discovery sample possibly. Furthermore, comorbid depression might constitute a confounding factor, particularly, because the discovery and the replication samples differed in the rate of comorbid depression. A further limitation of this study is that no non-Caucasian populations were investigated rendering comparison to the study by Okamura *et al*.[27] difficult. However, although in all samples Caucasian ancestry was ascertained, ethnic stratification even within the German population cannot be excluded,[79] particularly because no genomic control has been performed in the presently investigated samples. The effect of genotype on the ASI is only found in patients, but not in controls. One possible explanation may be the small variation of anxiety sensitivity in our supranormal control sample. Finally, caution and restraint is necessary in the interpretation of the imaging genetic part of the study, given a relatively small number of patients and an explorative statistical approach not corrected for multiple testing. Apart from significantly decreased activity in the anterior cingulate cortex in response to fearful faces, *P*-values would not withstand Bonferroni correction for the four multivariate analysis of variances that were performed for the four different contrasts (fearful, angry, happy, neutral vs no faces) over all ROIs resulting in a corrected *P*-value of 0.0125 (two-sided) or 0.025 (one-sided), respectively, which might reflect a possible type I error. However, as largely debated, Bonferroni correction might be too conservative for strictly hypothesis-driven studies as the present one and actually increase the probability of a type II error. A further limitation is that the small sample size did not allow for meaningful statistical analyses in subsamples stratified for gender, which, given a female-dominant result in the categorical association studies, would have been of interest also in the imaging genetic part of this study. Furthermore, although no biased distribution of gender, medication with selective serotonin reuptake inhibitors and comorbidity with major depression or social phobia across NPSR genotype groups could be discerned, these factors still could constitute possible confounding factors in the evaluation of emotional processing. For instance, either single dose or subchronic (7–21 days) medication with selective serotonin reuptake inhibitors has been shown to attenuate amygdala, hippocampus and medial prefrontal cortex response to aversive facial expression.[80–82] Thus, besides a potential ceiling effect due to generally increased baseline amygdala activity in patients with panic disorder, this may partly explain why no amygdala activation could be observed as with previous studies in this sample.[44] In addition, patients with social phobia tend to evaluate neutral faces as potentially threatening and more aversive than healthy controls or possibly also patients with pure panic disorder,[83] which might have influenced the present results. Future studies will have to elucidate the mechanisms by which genetic variation of NPSR modulates the sympathomimetic system, cingulate and orbito/prefrontal cortex activity and their possible functional interactions. One such mechanism may be mediated by the startle reflex, which therefore is currently under investigation in our group.

In summary, the present body of research provides converging lines of evidence for a role of NPSR gene variation in the pathogenesis of panic disorder. The more active NPSR rs324981 T allele (Ile^{107}) has been found to be associated with the categorical phenotype of panic disorder in a female-dominant manner, possibly mediated through increased arousal levels as reflected by elevated anxiety sensitivity, increased autonomic arousal as well as distorted processing of fear-related emotional stimuli. In synopsis with previous evidence from animal models, these findings might nourish future studies exploring a potentially beneficial use of therapeutic agents targeting the NPS system in anxiety disorders.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

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