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Combined Effects of Exonic Polymorphisms in CRHR1 and AVPR1B Genes in a Case/Control Study for Panic Disorder

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Accumulating evidence from animal studies suggests that the corticotropin releasing hormone (CRH) and arginine vasopressin (AVP) neuropeptide systems, contribute to anxiety behavior. To investigate whether polymorphisms in the genes regulating these two systems may alter susceptibility to anxiety disorders in humans, we genotyped 71 single nucleotide polymorphisms (SNPs) in CRH, CRHR1, CRHR2, AVP, AVPR1A, AVPR1B in a German sample from Munich with patients suffering from panic disorder and matched healthy controls (n = 186/n = 299). Significant associations were then replicated in a second German sample with 173 patients with panic disorder and 495 controls. In both samples separately and the combined sample, SNPs within CHRH1 and AVPR1B were nominally associated with panic disorder. We then tested two locus multiplicative and interaction effects of polymorphisms of these two genes on panic disorder. Fifteen SNP pairs showed significant multiplicative effects in both samples. The SNP pair with the most significant association in the combined sample ($P = 0.00057$), which withstood correction for multiple testing, was rs878886 in CRHR1 and rs28632197 in AVPR1B. Both SNPs are of potential functional relevance as rs878886 is located in the 3′ untranslated region of the CRHR1 and rs28632197 leads to an arginine to histidine amino acid exchange at position 364 of AVPR1B which is located in the intracellular C-terminal domain of the receptor. These data suggest that polymorphisms in the AVPR1B and the CRHR1 genes alter the susceptibility to panic disorder.

KEY WORDS: anxiety disorders; association; SNP; CRHR1; AVPR1B

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INTRODUCTION

Several neurotransmitter systems, including monoaminergic pathways and neuropeptidergic systems have been implicated in the pathophysiology of anxiety disorders [Gorman, 2003]. A plethora of preclinical data points to the corticotropin releasing hormone (CRH) and vasopressin (AVP) systems as two of the major peptidergic candidate systems involved in these disorders [Antoni, 1993; Griebel et al., 2002; Keck et al., 2002, 2003; Müller et al., 2002].

To date, two distinct G protein-coupled receptors have been characterized that mediate the biological actions of CRH: CRHR1 and CRHR2. These two receptors display a markedly different tissue distribution and pharmacological specificity [Lopez et al., 1998; Uhr et al., 2000].

Numerous investigations in animals have described anxiogenic-like effects after central CRH elevation [Dunn and Berridge, 1990; Stenzel-Poore et al., 1994]. These effects are likely to be mediated through the CRH1 receptor, as CRHR1 antagonistic approaches have anxiolytic-like properties in most, but not all anxiety paradigms [Liebsch et al., 1995; Griebel et al., 1998; Muller et al., 2001; Keck et al., 2005]. Moreover, mice lacking the limbic CRHR1 show reduced anxiety-related behavior [Müller et al., 2003]. Beyond CRHR1, recent pharmacological data point towards a complex involvement of the CRHR2 in anxiety. Central administration of urocortin (UCN), an endogenous ligand for

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CRHR2, has been shown to increase anxiety [Slawecki et al., 1999; Spina et al., 2002]. Interestingly, activation of the CRHR2 can result in either anxiolysis or anxiogenesis depending on when the animal is tested and, possibly, in which brain region the receptor is localized [Takahashi, 2001; Reul and Holsboer, 2002].

In the brain, the effects of AVP are also mediated through G-protein-coupled receptors, which have been classified as AVPR1A and AVPR1B subtypes [Barberis and Tribollet, 1996]. AVP itself and both receptors have been shown to be involved in the regulation of anxiety-related behavior [Montkowski et al., 1995; Griebel et al., 2002]. In an animal model of innate hyper-anxiety, the high anxiety-related behavior (HAB) rats, more AVP mRNA is expressed and higher amounts of AVP are released in the hypothalamic paraventricular nucleus (PVN) of hyper-anxious HAB rats than in animals displaying low anxiety (LAB) under both basal and stressful conditions Keck et al., 2002]. The pathophysiological relevance of an overproduction of AVP in this model could be demonstrated by the fact that the pathological outcome of the dexamethasone (DEX) suppression/CRH challenge test in HAB rats (i.e., both elevated basal plasma levels of corticotropin (ACTH) and increased release of ACTH in response to CRH despite prior dexamethasone administration) could be abolished by co-administration of a AVPR1A/AVPR1B receptor antagonist [Keck et al., 2002]. Beyond its role in the regulation of the peripherally measurable hypothalamic-pituitary-adrenocortical (HPA) system, intra-PVN overexpression of AVP is suspected to be critically involved in the regulation of anxiety-related behavior as an increase in anxiety following intracerebroventricular administration of AVP has been reported [Bhattacharya et al., 1998]. Conversely, bilateral intra-PVN administration of a combined AVPR1A/AVPR1B antagonist by inverse microdialysis resulted in an attenuation of hyper-anxiety in HAB rats [Murgatroyd et al., 2004]. Most intriguingly, a polymorphism located in the promotor region of the rat AVP gene seems to account for the increased AVP expression in HAB rats. All HAB rats but none of the LAB rats are homozygous for the allele that disrupts the binding site for the transcriptional repressor CArG binding factor A, which leads to increased AVP mRNA expression [Murgatroyd et al., 2004].

Considering the above presented data, we hypothesized that genes regulating the function of the CRH or the AVP system may be involved in the pathogenesis of anxiety disorders and that polymorphisms in these genes could contribute to the susceptibility to this disease.

One common anxiety disorder is panic disorder, a disabling psychiatric condition, estimated to affect between 2% and 4% of the population at some time in their lives [Kessler et al., 1994, 1998]. The hallmark symptoms of panic disorder are panic attacks, which are circumscribed episodes of severe state anxiety lasting minutes to hours, with escalating symptoms. The features are associated with an array of physical symptoms of autonomic, primarily sympathetic, arousal as well as disturbances in HPA axis function. An uncoupling of HPA-axis function and noradrenergic tonus has been found [Coplan et al., 1996] as well as elevated overnight cortisol levels [Abelson and Curtis, 1996b] and cortisol response in the Dex-CRH test [Erhardt et al., 2006]. This HPA axis disturbance has been proposed to be involved in higher susceptibility to lactate-induced panic attacks [Coplan et al., 1996] as well as short- and long-term outcome of panic disorder [Coryell et al., 1989; Abelson and Curtis, 1996a] and may be mediated by a dysregulation of the central CRH and AVP systems. Genetic variation in genes regulating these two systems may thus contribute to the development of panic disorder.

In humans, many studies have shown that panic disorder has a significant familial aggregation which is largely explained by genetic effects [Hettema et al., 2001]. Combined evidence from family study and twin data suggests that specific gene/environment interactions account for the liability to develop this disorder. Its heritability is estimated to be between 0.41 and 0.54 [Hettema et al., 2001]. A number of linkage and candidate gene association studies have been published for panic disorder [Hamilton et al., 2001; Sen et al., 2004; Van West and Claes, 2004; Cheng et al., 2006; Zeggini et al., 2007]. While numerous positive candidate gene associations and several linkage loci have been reported, only few of them have been replicated so far [Deckert et al., 1998, 1999; Hamilton et al., 2002; Hosing et al., 2004; Peters et al., 2004; Domschke et al., 2007]. Only three genetic association studies to date have investigated the CRH system in anxiety disorder. Two studies have shown an association of polymorphisms in the CRH gene with behavioral inhibition in children with a family history of panic and phobic disorders, a phenotype that has been shown to predispose to anxiety disorders [Perlis et al., 2003; Smoller et al., 2005]. The third is a negative association study of three polymorphisms in the CRHR2 gene in a Canadian sample with panic disorder [Tharmalingam et al., 2006].

The aim of the present study was to examine the role of single nucleotide polymorphisms (SNPs) in the CRH, CRHR1, CRHR2, AVP, AVPR1A and AVPR1B genes in susceptibility to panic disorder using two independent German case/control samples.

MATERIALS AND METHODS

Sample From the Max Planck Institute of Psychiatry (MPI Sample)

One hundred eighty-six patients consecutively admitted to our Anxiety Disorders Outpatient Clinic for diagnosis and treatment of an anxiety disorder presenting with a panic disorder with agoraphobia (84.8%) or panic disorder without agoraphobia (15.2%) as their primary psychiatric diagnoses were recruited for the study (Table I). The diagnosis was ascertained by trained psychiatrists according to the Diagnostic and Statistical Manual of Mental Disorders (DSM)-IV criteria. All patients underwent the Structured Clinical Interviews for DSM-IV (SCID I and II) [Jacobi et al., 2004]. Anxiety disorders due to a medical or neurological condition or a comorbid Axis II disorder were exclusion criteria. All patients underwent a thorough clinical examination including EEG, ECG, brain and detailed hormone laboratory assessment. The mean age of onset of the disorder was 28.7 (SD: 11.5). The Panic and Agoraphobia scale [Bandelow, 1999] at baseline indicated a moderate severity of panic and agoraphobia (mean score (SD): 30.9 (9.3)). The mean Hamilton Depression Scale score was 13.9 (7.1) and mean Hamilton Anxiety Scale score was 24.3 (10.4), indicating low depression and moderate anxiety at the time of recruitment in the patients [Hamilton, 1959, 1960].

Ethnicity was recorded using a self-report sheet for perceived nationality, mother language and ethnicity of the subject itself and all four grandparents. All included patients were Caucasian and 84% of German origin. The most common other ethnicities were Rumanian (German descent) 3.6%, Austrian and Turkish each 1.7% and Bosnia-Herzegovina, Czech and Hungarian each 1.1%. All other ethnicities were only represented by one individual and included Slovenian, Polish, Greek, Italian and USA (German origin). The study has been approved by the Local Ethics Committee. Written informed consent was obtained from all subjects.

Two hundred ninety-nine controls matched for ethnicity (using the same questionnaire as for patients), gender and age were recruited. Controls were selected randomly from a Munich-based community sample and screened for the presence of anxiety and affective disorders using the Composite International Diagnostic-Screener [Wittchen et al., 1998]. Only individuals negative in the screening questions for the

TABLE I. Gender, Age, and Diagnostic Subtype Distribution in the Case/Control Samples

	Cases	Controls	*P*-value
MPI sample			
N	186	299	
Sex	31.2% male 68.8% female	27.4% male 72.6% female	0.41
Age	39.3 (11.9)	39.1 (12.1)	0.39
Diagnosis			
% PD with/without agoraphobia	82.8% with 15.2% without		
Replication sample			
N	173	495	
Sex	39.3% male 60.7% female	39.4% male 60.6% female	0.98
Age in years (SD)	37.7 (10.9)	35.8.0 (10.9)	0.05
Diagnosis			
% PD with/without agoraphobia	65.9% with 34.1% without		

above-named disorders were included in the sample. Recruitment of controls was also approved by the Local Ethics Committee and written informed consent was obtained from all subjects.

German Replication Sample

One hundred seventy-three patients with panic disorder with or without agoraphobia as their primary diagnoses as well as 495 anonymous blood donor controls matched for ethnicity, gender and age were used as a replication sample (see Table I). Patients had been recruited at the Universities of Würzburg, Bonn, Münster, and Göttingen. The diagnosis was ascertained by trained psychiatrists according to the Diagnostic and Statistical Manual of Mental Disorders (DSM)-IIIR (50%) and DSM-IV (50%) criteria on the basis of structured interviews (SADS-LA: [Manuzza et al., 1986], CIDI: [Robins et al., 1988], SCID-I: [Wittchen, 1997]) and clinical records as previously described [Domschke et al., 2007]. Patients with mental retardation, neurological or neurodegenerative disorders where excluded. Patients (100%) were of German descent as ascertained by the clinical records on the ethnic origin of the parents. Controls were matched anonymous blood donors of the University of Münster. German descent was assumed on the basis of the ethnic origin of the last name of the subject. Recruitment of subjects was approved by the Local Ethics Committees and written informed consent was obtained from all subjects.

DNA Preparation

On enrollment in the study, up to 40 ml of EDTA blood were drawn from each subject and DNA was extracted from fresh blood using standard DNA extraction procedures, for example, the Gentra Puregene whole blood DNA-extraction kit (Qiagen Inc., Valencia, CA).

SNP Selection and Genotyping

SNPs were selected within six candidate genes: CRH (NM_000756), CRHR1 (NM_004382), CRHR2 (NM_001883); AVP (NM_000490); AVPR1B (NM_000707); and AVPR1A (NM_000706). A total of 71 SNPs were selected, from public and private databases (dbSNP (http://www.ncbi.nlm.nih.gov:80/) and Celera, Inc. (http://www.celeradiscoverysystem.com/)) and 4 have been identified through resequencing of the DNA of 94 depressed patients [Binder et al., 2004] (see also Table II). The SNP search tool developed at the Institute for Human Genetics, Technical University and GSF-National Research Centre for Environment and Health was used to download SNP sequences from public databases (http://ihg.gsf.de/ihg/snps.html) using the hg16 built of the Genome Browser of the University of Santa Cruz (http://genome.ucsc.edu/). Genotyping was performed on a MALDI-TOF mass-spectrometer (MassArray® System, Sequenom Inc., San Diego, CA) employing the Spectrodesigner software (Sequenom™, San Diego, CA) for primer selection and multiplexing and the homogeneous mass-extension (hMe) process for producing primer extension products. Genotyping was performed at the Genetic Research Center GmbH (Munich, Germany). All primer sequences are available upon request.

Statistical Analysis

Only SNPs with a minor allele frequency (MAF) equal or greater than 10% were included in the analyses and all of them were in Hardy–Weinberg Equilibrium (HWE) in the MPI control group once correcting for multiple testing. Only SNPs with a call rate of 90% or higher were included. Average call rates in the MPI samples were 98.5% and 98.2% in the replication sample. Call rates for all CRHR1 and AVPR1B SNPs for both samples are listed in Supplemental Table I. Genotypes of rs110402 and rs3785877 were also available in 174 MPI cases from genotyping on the Illumina 317k SNP-array. Comparing the genotypes generated by the Sequenom vs. the Illumina platform, zero discrepancies could be detected. In the MPI sample, 34 controls did not have valid AVPR1B genotypes due to technical reasons and were excluded from call rate calculation for these genotypes.

Group differences for the case and control samples were tested using contingency tables or ANOVA using SPSS software version 13. Analyses for case/control associations and the two locus models were performed using logistic regression using R (version 2.5.1) testing effects on the genotypic level. Each two locus models included terms for the main effect for a CRHR1 and an AVPR1B SNP and their interaction term. *P* values for the multiplicative effects were then computed by combining the two main SNP effects. Case/control associations were tested for SNPs in all six genes in the MPI samples. For the two genes (CRHR1 and AVPR1B) with nominally significant associations, all SNPs within these loci were genotyped in the replication sample and tested for association with panic disorder in the replication sample as well as the combined sample. In a second step, multiplicative and interaction effects were tested for all possible combination of AVPR1B (6 SNPs) and CRHR1 (13 SNPs) SNPs in the two samples separately as well as the combined sample.

TABLE II. SNP Specifications: Location According to the July 2003 Human Reference Sequence (UCSC Version hg16) (http://genome.ucsc.edu/), Location Within Gene and MAF for all SNPs Genotyped in This Study

Gene	SNP ID	Origin	Location on hg16	Location within gene	AA exchange	MAF in controls MPI
AVP						
NM_000490 chromosome 20	rs2740194	dbSNP	3054364	3′ of gene		0.295
	rs2740192	dbSNP	3054396	3′ of gene		0.174
	rs2740204	dbSNP	3057467	3′ of gene		0.408
	rs1051744	dbSNP	3058415	Exon 3	Val/Gly	0.000
	AVP5UTRe	Binder et al. [2004]	3060759	Promoter/5′UTR		0.246
	AVP5UTRa	Binder et al. [2004]	3061259	Promoter/5′UTR		0.012
	rs3761249	dbSNP	3061362	Promoter		0.103
	AVPprom	Binder et al. [2004]	3061609	Promoter		0.099
	rs7351339	dbSNP	3066430	Promoter		0.000
	rs6037484	dbSNP	3069224	Promoter		0.000
	rs857240	dbSNP	3070629	Promoter		0.083
AVPR1B						
NM_000707 chromosome 1	hcv1845028	Celera	202774331	3′UTR		0.128
	rs28529127	dbSNP	202774219	3′UTR		0.105
	rs28632197	dbSNP	202774025	Exon 2	His/Arg	0.115
	rs28607590	dbSNP	202773615	Intron 1		0.128
	rs28575468	dbSNP	202772666	Intron 1		0.128
	rs3883899	dbSNP	202770175	Intron 1		0.126
	rs3891059	dbSNP	202768630	Intron 1		0.000
AVPR1A						
NM_000706 chromosome 12	rs1057616	dbSNP	61822856	3′UTR		0.000
	rs1042615	dbSNP	61830476	Exon 1	Phe/Phe	0.250
	rs3741865	dbSNP	61831125	Exon 1		0.004
	rs3021529	dbSNP	61831947	Exon 1		0.130
	rs7488628	dbSNP	61838862	5′ of gene		0.000
CRH						
NM_000756 chromosome 8	rs1054108	dbSNP	67136927	RFN29 3′UTR		0.000
	rs6159	dbSNP	67139386	Exon 2	Gly/Gly	0.151
	rs6158	dbSNP	67140514	Exon 1 5′UTR		0.000
	rs6157	dbSNP	67140551	Exon 1 5′UTR		0.000
	rs6156	dbSNP	67140557	Exon 1 5′UTR		0.000
	rs3176921	dbSNP	67141340	Promoter		0.083
	CRHprom	Binder et al. [2004]	67141360	Promoter		0.070
	rs7843797	dbSNP	67144911	5′ of gene		0.000
	rs6472258	dbSNP	67146440	5′ of gene		0.069
	rs1870392	dbSNP	67148472	5′ of gene		0.037
	rs1870393	dbSNP	67148698	5′ of gene		0.143
CRHR1						
NM_004382 chromosome 17	rs4077813	dbSNP	44330593	Promoter		0.037
	rs4076452	dbSNP	44331299	Promoter		0.180
	rs7207992	dbSNP	44337460	Intron 1		0.000
	rs7209436	dbSNP	44345552	Intron 1		0.449
	rs4792885	dbSNP	44351521	Intron 1		0.104
	rs4792886	dbSNP	44352252	Intron 1		0.108
	rs110402	dbSNP	44355457	Intron 1		0.474
	rs2664008	dbSNP	44358522	Intron 1		0.089
	rs242925	dbSNP	44364285	Intron 2		0.493
	rs3785877	dbSNP	44367607	Intron 2		0.043
	rs171440	dbSNP	44368906	Intron 2		0.494
	rs242937	dbSNP	44373788	Intron 3		0.287
	rs717312	dbSNP	44377848	Intron 4		0.000
	rs1396862	dbSNP	44378417	Intron 4		0.178
	rs1876831	dbSNP	44383165	Intron 6		0.182
	rs242950	dbSNP	44386073	Intron 9		0.121
	rs878886	dbSNP	44387910	Exon 13 3′UTR		0.177
	rs242948	dbSNP	44388964	3′ of gene		0.318
CRHR2						
NM_001883 chromosome 7	rs3735430	dbSNP	30434258	3′UTR		0.015
	rs8192492	dbSNP	30435434	Exon 12	Arg/End	0.000
	rs38027	dbSNP	30435877	Intron 11		0.011
	rs2240403	dbSNP	30437474	Exon 10	Ser/Ser	0.089
	rs2270007	dbSNP	30442244	Intron 8		0.193
	rs8192498	dbSNP	30444084	Exon 7	Val/Ile	0.014
	rs2270008	dbSNP	30444516	Intron 6		0.123

(*Continued*)

TABLE II. (*Continued*)

Gene	SNP ID	Origin	Location on hg16	Location within gene	AA exchange	MAF in controls MPI
	rs929377	dbSNP	30446431	Intron 5		0.329
	rs8192495	dbSNP	30447502	Exon 4	Arg/His	0.000
	rs2008003	dbSNP	30450811	Intron 2		0.226
	rs2284216	dbSNP	30454233	Intron 2		0.093
	rs2267715	dbSNP	30458359	Intron 2		0.409
	rs2267716	dbSNP	30458915	Intron 2		0.222
	rs6965973	dbSNP	30460732	Intron 2		0.163
	rs8175360	dbSNP	30464042	Intron 1		0.038
	rs6967702	dbSNP	30464768	Promoter		0.000
	rs2097911	dbSNP	30466835	Promoter		0.000
	rs255098	dbSNP	30469599	Promoter		0.402
	rs255102	dbSNP	30473436	Promoter		0.346

Correction for multiple testing. The first association analysis with SNPs from all six genes in the MPI sample was considered exploratory, so that the level of significance was set to 0.05. For the analysis of single SNP associations in CRHR1 and AVPR1B in the combined sample, we used a Bonferroni-type correction, correcting for the 19 SNP with an MAF $\geq$ 10%, so that the alpha level was set to 0.0026. For the test of the multiplicative and interaction models, we corrected for all 78 possible two-way combinations of the 6 AVPR1B and 13 CRHR1 SNPs, so that the alpha level was set to 0.00064. Our strategy was to not correct in the discovery sample in order to not overlook potentially relevant associations due to overly strict correction for multiple testing since several CRH and AVP-related genes were tested. Once the candidate genes from this first stage were selected, we then applied conservative correction for multiple testing in the combined sample.

Power calculations for single SNP associations. Power was calculated using the Quanto software, version 1.1.1 (http://hydra.usc.edu/gxe). In the discovery sample, we had at least 83% power to detect an additive genetic effect with a genetic relative risk greater or equal to 1.65 (alpha set to 0.05, MAF = 0.15 or greater, population prevalence to 3%). In the combined sample, we had at least 83% power to detect an additive genetic effect with a genetic relative risk greater or equal to 1.60 (alpha level set to 0.0026, MAF = 0.15 or greater).

RESULTS

A total of 71 SNPs were genotyped in 6 candidate genes: CRH (NM_000756), CRHR1 (NM_004382), CRHR2 (NM_001883); AVP (NM_000490); AVPR1A (NM_000706); and AVPR1B (NM_000707). Of these SNPs, 53 turned out to be polymorphic and 36 had MAF equal or greater than 10%. Only the latter SNPs were included in the analysis (see Table II). For AVPR1A, spanning 6.7 kb on chromosome 12, only 2 SNPs were included in the analysis. To test whether these adequately cover the genetic variation of this gene in Caucasians, we investigated the linkage disequlibrium (LD) structure of this locus plus 3 kb upstream and downstream in the HapMap phase II data of Caucasian Utah Mormons (CEPH) (www.hapmap.org) using the Haploview software [Barrett et al., 2005]. In this region, 10 SNPs are reported with a MAF of $\geq$10% in CEPHs, including our 2 SNPs rs1042615 and rs3021529. All ten SNPs are all located in one large haplotype type block. The two SNPs genotyped in our sample, tag all but two SNP located in the promoter region with a mean r^2 of 0.96. The two promoter SNP rs10877969 and rs7298346 are both tagged by our SNP rs3021529 with an r^2 of 0.65. Due to the high degree of LD in this locus in Caucasians, the two SNPs genotyped in our sample thus capture most of the genetic variation reported for this gene. The second gene for which we only included two SNPs in the main analysis was the 2 kb spanning CRH gene which had two SNPs with a MAF $\geq$ 10%. For this gene, in a region of 10 kb including the CRH gene, no SNP in the HapMap project phase II had a MAF greater than 10% in the CEPHs. For the AVPR1B no HapMap genotype data are available to date. The LD structure in the MPI controls of the selected AVPR1B and CRHR1 SNPs is depicted in Supplemental Figure 1.

Association of all SNPs in MPI Sample

All polymorphic SNPs were first tested for association with panic disorder in the MPI case/control sample. Nominally significant associations were only observed with SNPs within the AVPR1B and the CRHR1 genes (see Table III). The smallest *P*-value was observed with rs242937 ($P = 0.0046$) located in CRHR1. The strongest association within AVRP1B was seen with rs28575468 ($P = 0.0052$).

Association of CRHR1 and AVPR1B SNPs in the German Replication Sample and Combined Sample

All SNPs in the CRHR1 and AVPR1B gene were then tested for association with panic disorder in the replication sample. Again, several SNPs in these two genes showed a nominally significant association with panic disorders, although there was not a 100% overlap in SNPs with significant association in the two samples (see Table III). In the replication sample, the most significant SNP in AVPR1B was rs28529127 ($P = 0.001$) and in CRHR1 rs878886 ($P = 0.011$).

In the combined sample, the strongest associations were seen with rs28529127 in AVPR1B ($P = 0.0055$) and rs878886 in CRHR1 ($P = 0.0012$), whereby the CRHR1 SNP withstood correction for multiple testing (corrected $P = 0.024$; see Table III). For this SNP, the rarer G allele had an Odds ratio (OR) of 1.36 (95% CI: 1.094–1.694) and was present in 18.6% of controls and 23.8% of cases. The full genotype distributions for all SNPs listed in this table can be viewed in Supplemental Table I.

Two Locus Models for CRHR1 and AVPR1B SNP Genotypes

We then investigated the combined effects of CRHR1 and AVPR1B SNPs looking at all possible two-way combinations between CRHR1 and AVPR1B SNP genotypes in both samples separately as well as in the combined sample, evaluating multiplicative and interaction effects. Table II Supplemental data show a representation of the investigated 2-way combinations and the respective *P* values. Fifteen SNP genotype pairs showed multiplicative effects that were significantly associated with panic disorder independently in both samples.

TABLE III. *P* Values for Association With Case/Control With SNPs Within AVPR1B and CRHR1 in the MPI and Replication Sample and the Combined Sample

SNP ID	Gene	*P*-value MPI sample	*P*-value replication sample	*P*-value combined sample
hcv1845028	AVPR1B	0.057	**0.011**	**0.031**
rs28529127	AVPR1B	0.361	**0.001**	**0.0052**
rs28632197	AVPR1B	**0.046**	0.197	**0.015**
rs28607590	AVPR1B	0.079	0.053	**0.026**
rs28575468	AVPR1B	**0.0053**	0.085	**0.016**
rs3883899	AVPR1B	0.198	0.066	0.124
rs4076452	CRHR1	0.687	0.285	0.224
rs7209436	CRHR1	0.727	0.643	0.528
rs4792885	CRHR1	0.242	0.165	**0.017**
rs4792886	CRHR1	0.248	0.465	0.062
rs110402	CRHR1	0.568	0.890	0.565
rs242925	CRHR1	0.413	0.493	0.277
rs171440	CRHR1	0.490	0.454	0.266
rs242937	CRHR1	**0.0047**	0.892	**0.036**
rs1396862	CRHR1	0.060	0.138	**0.013**
rs1876831	CRHR1	**0.045**	0.101	**0.012**
rs242950	CRHR1	**0.036**	0.983	0.141
rs878886	CRHR1	0.052	**0.011**	**0.00129***
rs242948	CRHR1	0.140	0.750	0.197

Association with a nominal *P*-value smaller than 0.05 are bolded, * denominates association withstanding correction for multiple testing for single SNP associations.

No interaction term showed significant effects in both samples (12 pairs showed nominally significant interaction effects in the MPI sample and 1 in the replication sample, 19 in the combined sample, but for none there was an independent nominal association in both samples). Of the 15 SNP pairs with significant multiplicative effects, 10 showed a more significant association in the combined than in the two separate samples, of which 2 SNP pairs withstood correction for multiple testing in the combined sample (rs28632197 of AVPR1B and rs878886 or rs1876831 of CRHR1, $P = 0.00057$ and $P = 0.00059$ in the combined sample, respectively). The *P*-value for the best multilocus effect in the MPI sample was 0.025, in the replication sample 0.022 and in the combined sample 0.00057. This association effect seems to be carried by an overrepresentation of CRHR1 rs878886 G allele (OR = 1.36 (1.09–1.69)) and of rs28632197 TT homozygotes (OR = 5.60 (1.47–21.26)).

In a single SNP model, the power to detect the effect size observed with the CRHR1 SNP in the combined sample is 50% (with alpha 0.0026, MAF 1.8, additive model). For the AVPR1B SNP the power to detect the reported association was over 92% (with an alpha of 0.0026, MAF = 0.11, recessive model). Even when using an overly conservative Bonferroni correction, the observed associations are well within the expected range.

Both the MPI and the replication sample showed very similar distributions of these two genotypes in cases versus controls (see Table IV). Interestingly, in the combined sample four cases showed the combination of the CRHR1 rs878886 G allele and rs28632197 TT homozygotes, thus carrying both risk factors, while this was not observed in the combined control sample of more than twice the size. In fact, in the combined sample, an interaction model showed a trend significance ($P = 0.052$), see Supplemental Table II.

DISCUSSION

The results of this study suggest a multiplicative genetic effect of polymorphisms within the CRHR1 and AVPR1B genes on the susceptibility for panic disorder.

TABLE IV. Distribution of rs878886 and rs28632197 Genotypes in Patients and Controls From the MPI and Replication Sample as well as the Combined Sample

	AVPR1B rs28632197			CRHR1 rs878886		
	CC	TC	TT	CC	CG	GG
MPI cases (N/%)	156	24	3	106	68	9
	85.25	13.11	1.64	57.92	37.16	4.92
MPI controls (N/%)	206	49	1	176	66	14
	80.47	19.14	0.39	68.75	25.78	5.47
Replication cases (N/%)	133	30	5	96	63	9
	79.17	17.86	2.98	57.14	37.50	5.36
Replication controls (N/%)	401	90	2	325	151	17
	81.34	18.26	0.41	65.92	30.63	3.45
Combined cases (N/%)	289	54	8	202	131	18
	82.34	15.38	2.28	57.55	37.32	5.13
Combined controls (N/%)	607	139	3	501	217	31
	81.04	18.56	0.40	66.89	28.97	4.14

For the combined analysis of these two SNPs in both samples, 351 cases were compared to 749 controls. The *P* values for HWE in the combined controls sample were 0.093 for rs28632197 and 0.226 for rs878886.

The significant contribution of multiplicative effects of SNPs in CRHR1 and AVPR1B to panic disorder was observed in two independent samples of patients and matched controls and the multiplicative effects of two CRHR1/AVPR1B SNP pairs withstood correction for multiple testing in the combined sample. The distribution of the genotypes of the most significant SNP pair (AVPR1B SNP rs28632197 + CRHR1 SNP rs878886) in patients versus controls was very similar in both samples. In patients with panic disorder, we observed an overrepresentation of the rare homozygote genotype of the AVPR1B SNP rs28632197 and the rare allele of the CRHR1 SNP rs878886 (see Table IV). Due to the smaller sample size of the two studies, independent replications in larger samples are, however, necessary to confirm these genetic associations.

Interestingly, single SNP associations showed nominally significant results for both genes in both samples but there was no exact overlap in the observed strength of association of each CRHR1 and AVPR1B SNP across samples. These discrepancies may be due to subtle differences between the two samples, such as the slightly different diagnostic criteria (DSM-IV versus DSM-IIIR in 50%), the slightly different gender ratio and proportion of patients suffering from agoraphobia or the differential control groups (healthy controlled vs. anonymous blood donors). However, the association with rs878886 in CRHR1 withstood correction for multiple testing in the combined sample.

Within the CRH system, polymorphisms in the gene encoding the neuropeptide CRH have been previously reported to be associated with behavioral inhibition in children with a family history of anxiety disorders [Smoller et al., 2005]. We did, however, not observe any associations with the two CRH SNPs with a MAF $> 10\%$ with PD. We then also tested the additional four SNPs with a MAF $< 10\%$ in this gene for association with panic disorder in the MPI sample but did not observe any significant associations with these SNPs either.

Both the CRH receptor type 1 and the AVP receptor 1B are localized in behaviorally relevant brain regions such as the limbic system and at the level of the anterior pituitary where they mediate the corticotropin (ACTH)-releasing effects of CRH and AVP [Barberis and Tribollet, 1996; Lopez et al., 1998]. The two SNPs showing the strongest interaction effects are potential candidates for being polymorphisms affecting receptor function. rs28632197 is a non-synonymous SNP in exon 2 of AVPR1B that results in an arginine to histidine substitution in the C-terminal intracellular loop of the receptor. This could lead to an altered intracellular coupling of the receptor to second messenger cascades. rs878886 is located in the 3′UTR of the CRHR1 mRNA and may alter translation efficiency. In the absence of additional in vitro and in vivo functional data both SNPs could, however, only be markers in linkage disequlibrium with the actual causal mutation and further experiments will be needed to clarify this issue.

In addition to the data from animals studies described in the introduction, results supporting a role of these peptides in anxiety disorder also come from linkage studies. Attempts to map the genetic factors involved in the regulation of anxiety-related behavior and fear conditioning in mice have primarily utilized the quantitative trait loci (QTL)-mapping technique, in which the degree of association between genetic loci and quantitative measures are estimated. Overlapping loci influencing anxiety-like behaviors have been consistently found on the distal region of mouse chromosome 1 where the avpr1b gene is located and which is roughly syntenic with human 1q22–32, where the human AVPR1B gene is located [Finn et al., 2003]. In fact, three human genome scans found suggestive linkage of panic disorder on chromosome 1q: Gelernter et al. [2001] performed a linkage analysis in a set of families which segregate panic disorder, agoraphobia and several other anxiety disorders whereas Smoller et al. [2001] used regions identified by QTL-mapping of anxiety phenotypes in mice to guide a linkage analysis of a large multiplex pedigree segregating panic disorder/agoraphobia. Crowe et al. [2001] published a full genome scan for panic disorder with 23 families. The region found by Gelernter et al. [2001], which contains the human AVPR1B gene, had a LOD score of 2.04 and coincides with a region that generated a LOD score of 1.1 in the study by Crowe et al. [2001]. A locus for panic disorder on human chromosome 17q, where the CRHR1 gene is located, has not been reported so far.

Anatomical, functional, and behavioral interactions of the AVP and CRH systems have been reported, suggesting the possibility of interactions of risk polymorphisms from both systems: In neuroendocrine parvocellular neurons of the hypothalamic PVN, AVP, and CRH are co-localized and, when secreted into the hypophyseal portal circulation, act synergistically to release ACTH [Antoni, 1993; Jessop, 1999; Tamiya et al., 2005]. Also in behaviorally relevant limbic brain regions, AVP and CRH systems are overlapping and while direct co-localization of CRHR1 and AVPR1B has not been reported yet, their overlapping expression pattern in limbic brain regions such as hippocampus and amygdala is suggestive of it [Swanson et al., 1983; Barberis and Tribollet, 1996; Lopez et al., 1998; Sanchez et al., 1999]. Extrahypothalamic CRH/AVP synergistic effects could be demonstrated in the regulation of hippocampal corticosteroid receptor expression [Hügin-Flores et al., 2003]. Most interestingly, the combined, but not separate administration of a CRHR1 and an AVPR1B antagonist, effectively blocked the rise in serum ACTH induced by three different types of stressors [Ramos et al., 2006]. In addition, both these antagonists were effective in animal models of anxiety [Hodgson et al., 2007].

Interestingly, in the combined sample four individuals with panic disorder carried the CRHR1 SNP risk G allele and the AVPR1B TT risk genotype, while this was never observed in the more numerous control sample, suggesting a possible interaction effects on the genetic level. While this observation and nominally significant interaction effects in the MPI sample may be suggestive of potential genetic interactions between polymorphisms in CRHR1 and AVPR1B, larger studies with more power are needed to address this issue, especially when investigating rarer coding SNPs.

Taken together, our results complement a plethora of data suggesting that perturbations of CRH and AVP neurocircuitries contribute to abnormal neuronal communication in conditions of pathological anxiety. Compounds selectively targeting CRHR1 and AVPR1B have already been described [Griebel, 1999; Zobel et al., 2000; Muller et al., 2001; Griebel et al., 2003] and may not only have great therapeutical value in patients suffering from depression but also in patients with panic disorder.

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to medicaments. International application number: PCT/EP2005/005194.

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