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Identification of a Naturally Occurring Polymorphism in the Promoter Region of the Norepinephrine Transporter and Analysis in Major Depression

Peter Zill, Ph.D., Rolf Engel, Ph.D., Thomas C. Baghai, M.D., Georg Juckel, M.D., Thomas Frodl, M.D., Florian Müller-Siecheneder, M.D., Peter Zwanzger, M.D., Cornelius Schüle, M.D., Christo Minov, M.D., Stefanie Behrens, M.T.A., Rainer Rupprecht, M.D., Ulrich Hegerl, M.D., Hans Jürgen Möller, M.D., and Brigitta Bondy, M.D.

Disturbances in the noradrenergic neurotransmission system have been implicated in the etiology of mood disorders. The norepinephrine transporter (NET) is a main target of antidepressant action and was shown to be dysregulated in major depression. Despite the clinical and physiological significance of NET gene regulation, little is known about the transcriptional control mechanisms governing its expression. Since it is well established that affective disorders have a genetic component with many genes of small effect contributing to the genetic susceptibility of depression, the NET gene is an interesting candidate gene for affective disorders. In a search for polymorphisms or mutations in the 5′ flanging region of the NET gene we sequenced approximately 1000 bp upstream of the first codon in the NET gene promoter in 100 patients with major depression and 100 healthy controls. We identified a so far unknown T → C polymorphism 182 bp upstream of the start codon in a transcriptional relevant region. In a case control association study we investigated the newly identified T-182C polymorphism and an already known G1287A polymorphism in exon 9 of the NET gene in a sample of 193 patients with major depression and 136 healthy, non-related controls. No statistical significant differences between patients and controls were found for any of the analyzed polymorphisms, either in the genotype distribution or in the allele frequencies. Our results suggest that the investigated polymorphisms are not major susceptibility factors in the etiology of major depression.
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KEY WORDS: *Norepinephrine; Polymorphisms; Major depression; Association study; Ppromoter*

Monoamine neurotransmitters, particularly norepinephrine (NE) and serotonin play an important, possibly primary, role in pathophysiology and treatment of mood disorders. Many studies have reported altered NE levels in depressed patients (reviewed by Charney 1998). Studies of NE metabolites showed decreased urinary levels of MHPG (3-methoxy-4-hydroxyphenylglycol),

From the Psychiatric Hospital of the Ludwig-Maximilians-University, Munich, Nussbaumstr. 7, D-80336 Munich, Germany.

Address correspondence to: Dr. Peter Zill, Psychiatric Hospital of the Ludwig-Maximilians-University, Munich Nussbaumstr. 7 D-80336 Munich, Germany, Tel.: +49-89-51602741, Fax: +49-89-51604741, E-mail: Peter.Zill@psy.med.uni-muenchen.de

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the major metabolite of NE in unipolar patients, and elevated levels in bipolar patients during manic states (Maas et al. 1972; Muscettola et al. 1984). Investigations of NE metabolites in the cerebrospinal fluid (CSF) of depressed patients compared with healthy individuals led to so far conflicting results. The most consistent finding in the literature is that antidepressant treatment causes decreased NE turnover (DeBellis et al. 1993). This effect occurs consistently with selective NE-specific agents as expected, but also with selective serotonergic-specific agents and with electroconvulsive therapy (Owens 1997). Taken together, these results suggest a decrease in the release and/or production of NE in depressed individuals, which means that a complex dysregulation of the NE system appears to play an important role in the pathophysiology of affective disorders (review by Ressler and Nemeroff 1999). Additionally NE has been implicated in modulating mood, sleep, appetite and neuroendocrine functions, which are often disturbed in affective disorders (Kaye et al. 1990; Stoline 1996).

Norepinephrine functions are mediated by NE binding proteins such as adrenergic receptors and NE transporters. The NE transporter (NET) is a major target for psychoactive drugs such as neuroleptics, psychostimulants and antidepressants. These agents block NE transport via the NET, resulting in enhancement of the synaptic activity of NE. Recently Klimek et al. (1997) reported that the NET levels are reduced in the locus coeruleus in patients with major depression. Given that noradrenergic transmission can be regulated by changes in NET expression, it seems plausible that changes in the level of NET in the brain may contribute to central noradrenergic dysfunction putatively associated with major depression and/or treatment response.

Although environmental factors play an important role in the development of mental illness, there is strong evidence that affective disorders have a genetic component with many genes of small effect contributing to the genetic susceptibility of depression and the gene for the norepinephrine transporter could be a candidate gene for depression. The NET gene is located on chromosome 16q12.2; it spans approximately 45 kb and consists of 14 exons (Porzgen et al. 1995). Recently the sequence and structure of the 5′ flanking promoter region of the NET gene has been reported (Kim et al. 1999). The 5′ flanking promoter region of the NET gene has a length of approximately 4.7 kb and contains several important cis-elements for transcription (Kim et al. 1999). Despite the clinical and physiological significance of NET gene regulation, little is known about the transcriptional control mechanisms governing its expression.

The aim of this study was to screen the promoter region for polymorphisms or mutations, which could affect the transcriptional activity of the NET gene. We sequenced approximately 1000 bp upstream of the start codon, where most of the transcriptional important cis-elements are located. For sequencing, DNA of 100 patients with major depression and of 100 healthy controls was used. Finally, in a case control association study we evaluated whether there is a possible association between the newly identified polymorphisms and an additional silent polymorphism, a G1287A point mutation in exon 9 of the NET gene (Stöber et al. 1996) in a total sample of 193 patients with major depression and 136 healthy controls.

MATERIALS AND METHODS

Subjects

A total of 193 patients with major depression diagnosed according to ICD 10 and DSM IV criteria were included in the study (70 males, 123 females, mean age 50.4 ± 12.9 years, range 21–80 years). Severity of depression was assessed using the 17-item Hamilton Rating Scale for Depression (HAMD-17) and the Clinical Global Impression Scale (CGI). Only subjects with a minimum score of 18 on the HAMD-17 entered the study, and patients with coexisting substance abuse or severe organic disorders were excluded. In 22% of the patients it was the first episode of disease; the other 78% had several episodes (range 2 – 20, mean 3.7 ± 3.6, two and three episodes being the most frequent).

The control group consisted of a sample of 136 healthy probands, recruited from the general population in southern Germany, who were psychiatrically screened using personality questionnaires to rule out psychiatric problems, were included in the study (64 males, 72 females, mean age 48.3 ± 12.6 years, range 22 – 76 years).

All patients and controls gave informed and written consent to participate in the study.

Sequencing

Genomic DNA was isolated from whole blood according to standard procedures. We sequenced a DNA segment of 1140 bp from position −996 in the 5′ region to position 45 in exon 1 (numbering based on GenBank, accession number AF061198; the adenosine of the ATG start codon is indicated as +1). Sequencing was performed by dividing the 1140 bp region in two overlapping fragments, amplified by PCR with the following conditions. Forward primer 1 (pos. –996): 5′-GAT AGT TTA AGT GGC CTG CTG C-3′; reverse primer 1 (pos. –444): 5′-AAA CTC GCT AGC CCT CTG CT-3′; forward primer 2 (pos. –505): 5′-ACA GGG CTA GGT CTG CCT G-3′; reverse primer 2 (pos. 28): 5′-GTT GTT CTC GGG CTG CAC-3′. PCR was carried out in a final volume of 25 μl containing 50 ng genomic DNA, 200 μM of each dNTP, 100 μM 7-deaza-GTP, 5% DMSO, 0.5 μM of the forward and reverse primer and 1.25 units Hot Start Taq DNA polymerase. After an initial denaturation step of 95°C for 10

min, there were 35 cycles of denaturation at 95°C for 30 s, annealing at 68°C for 35 s and extension at 72°C for 45 s. A final step was performed at 72°C for 7 min. PCR products were purified with the Qiaquick PCR Purification Kit according to the manufacturer's instructions. 50 ng of the purified PCR product was used for cycle sequencing with the BigDye Terminator chemistry (ABI Biosystems) in a final volume of 25 μl, 8 μl reaction mix and 3.2 pmol primer. Results were verified by bi-directional sequencing. Cycling conditions were: 25 cycles of denaturation at 96°C for 10 s, annealing at 50°C for 5 s and extension at 60°C for 4 min. The amplified products were purified with Centri-Sep spin columns and sequenced on an ABI 310 capillary sequencer (ABI Biosystems).

Genotyping

All genotypings were performed by the fluorescence resonance energy transfer method (FRET) using the Light Cycler (Roche Diagnostics). A detailed description of the theoretical background and methodology is given by Toyota et al. (2000). For the two investigated polymorphisms the following conditions were applied. G1287A polymorphism (exon 9): forward primer: 5′-TTC AGG GAG ACC CTA ATT CC-3′; reverse primer: 5′-TTG ACT TTA TTG AAA TGC GGC-3′; donor hybridization probe: 5′-GTC ATC ACG GGC CTG GCA-fluorescein-3′; acceptor hybridization probe: 5′-LCRed 705-TGA CTT CCA GGT CCT GAA GC-3′. PCR was performed with 50 ng DNA in a total volume of 20 μl containing 2 μl reaction mix, 0.5 μM each primer, 0.2 μM each hybridization probe and 2 μM $MgCl_2$ according to the manufacturer's instructions for 40 cycles of denaturation (95°C, 0 s, ramp rate 20°C/s), annealing (53°C, 10 s, ramp rate 20°C/s) and extension (72°C, 10 s, ramp rate 20°C/s). After amplification a melting curve was generated by holding the reaction at 40°C for 20 s and then heating slowly to 95°C with a ramp rate of 0.2°C/s. The fluorescence signal was plotted against temperature to give melting curves for each sample. Peaks were obtained at 66°C for the G-allele and at 63°C for the A-allele. T–182C polymorphism (5′-region): forward primer: 5′-GAA CGA GGA AAA GTG CTG C-3′; reverse primer: 5′-TTG ACT TTA TTG AAA TGC GGC-3′; donor hybridization probe: 5′-GAC GCG CGC TCT TTT CTG GGA-fluorescein-3′; acceptor hybridization probe: 5′-LCRed640-CCC TGC GTC CGC TCA GCG CGC GCT CAT CCC-3′. PCR conditions were the same as for G1287A. Amplification was done for 40 cycles of denaturation (95°C, 0 s, ramp rate 20°C/s), annealing (60°C, 10 s, ramp rate 20°C/s) and extension (72°C, 10 s, ramp rate 20°C/s). After amplification a melting curve was generated by holding the reaction at 55°C for 20 s and then heating slowly to 95°C with a ramp rate of 0.1°C/s. The T-allele led to melting peaks at 68°C, the C-allele at 65°C.

All laboratory procedures were carried out blind to case control status.

Statistical Analysis

General statistical analyses were performed using SPSS for Windows (Version 9.0; SPSS; Chicago, IL). Haplotype estimation and their association with the disorder were calculated with the computer programs pm (Zhao et al. 2000) and eh (Xie and Ott 1993). Power was estimated with the help of Table 8 in Longman (2001).

RESULTS

Identification of a new polymorphism in the 5′ promoter region of the NET gene: A fragment of 1040 bp of the NET gene upstream of the start codon in the 5′ flanking promoter region was PCR amplified in two overlapping pieces from genomic DNA and sequenced in 100 patients with major depression and 100 healthy controls to screen for new polymorphisms or mutations. Direct sequencing of the two amplified genomic DNA fragments revealed a new T → C polymorphism in the promoter region at position –182 (numbering based on GenBank, accession number AF061198; the adenosine of the ATG start codon is indicated as +1). All reactions were performed by bi-directional sequencing. The sequence surrounding the variant is: 5′-GAC GCGCGC(T/C)CTTTTCTGG. The T-182C polymorphism is a relative common base exchange with genotype frequencies of 50% (TT), 39% (TC), 11% (CC) and an allele frequency of 69% (T-allele) and 31% (C-allele) in healthy controls.

Case Control Association Study

Primers surrounding the new T-182C polymorphism were created and further 93 patients and 36 healthy probands were genotyped for the new polymorphism using the fluorescence resonance energy transfer method (FRET). Additionally the already known G1287A polymorphism in exon 9 of the NET gene was also investigated. The results of the genotype distribution and allele frequencies for the two investigated polymorphisms in the NET gene in patients with major depression and healthy controls are summarized in Table 1, the estimated haplotypes in Table 2. No statistical significant differences were found for the allele or the genotype frequencies between patients and controls. T-182C: (genotypes: $p = .951$, $\chi^2 = 0.099$, df = 2; alleles: $p = .865$, OR = 1.03, 95% CI 0.74 – 1.49, Fisher's Exact Test, two sided); G1287A: $p = .378$, $\chi^2 = 1.945$, df = 2; alleles: $p = .305$, OR = 1.20, 95% CI 0.86–1.67, Fisher's Exact Test, two sided. We could not detect a linkage disequilibrium between the two polymorphisms, either in pa-

tients (χ^2 = 1.11, df = 3, p = .77) or in controls (χ^2 = 1.59, df = 3, p = .66). Cases had similar haplotype frequencies as controls (χ^2 = 1.94, df = 4, p = .75, m;odel free analysis). To detect a main effect for each polymorphism with a relative risk of 2 or greater in an additive mode, given the disorder related gene frequency of 0.30, a test size of α = 0.05 and a power of 80 percent, a sample size of 193 subjects would have been large enough. A joint effect of both polymorphisms could have been detected with an even smaller number of subjects.

Table 2. Estimated Haplotype Frequencies of the T-182C and G1287A Polymorphisms in the NET Gene among Patients with Major Depression and Controls under the Assumption of Linkage Disequilibrium (Association) or Non Linkage Disequilibrium (No Association) between the Alleles

	Patients		Controls	
Haplotypes	no association	association	no association	association
C, A	0.093	0.108	0.107	0.086
C, G	0.220	0.206	0.213	0.234
T, A	0.203	0.187	0.228	0.248
T, G	0.484	0.499	0.453	0.432

DISCUSSION

The NET gene is an interesting candidate gene, which contributes to the susceptibility for major depression and the response to antidepressant treatment. The findings that the expression of the NET gene is altered in the brain of depressed patients compared with healthy controls (Klimek et al. 1997) and its role as pharmacological target for antidepressants support the theory that the NET gene might be involved in the pathophysiology of depression. It is important to note that to our knowledge in only one study were several non-functional polymorphisms in the coding region of the NET gene reported (Stöber et al. 1996). Little is known about possible transcriptional mechanisms leading to changes in the expression of the NET gene. Because of these links we performed a sequencing scan of the 5′ flanking promoter region of the NET gene in patients with major depression and healthy controls. We identified a so far unknown T → C point mutation 182 bp upstream of the first codon. The 5′ flanking promoter region first described by Kim et al. (1999) comprises approximately 4.7 kb and contains an additional intron of 476 bp. In this intron several potential transcriptional elements are located, which seem to have an important meaning as enhancer of transcription and correct splicing (Kim et al. 1999). The T → C point mutation lies in this intron and is located 20 bp downstream of a CCAAT-box, 84 bp downstream of a SP1 binding site and 98 bp downstream of a binding site for the transcription factor C/EBP. A polymorphism in this region could probably lead to an altered transcriptional activity by changes in the DNA structure. Many other genes are known to have polymorphisms in their promoter region, not directly in cis-elements, but neighboring altering the transcriptional activity (e.g. Tarkowski et al. 2000). Therefore we cannot exclude this possibility also for the T-182C polymorphism, but further studies of the promoter activity in dependence of this gene variant are needed to analyze potential effects on NET expression.

Table 1. Genotype Distribution and Allele Frequencies of the T-182C and G1287A Polymorphisms in the Promoter Region of the NET Gene among Patients with Major Depression and Controls (Percentages in Parentheses)

		Genotypes			Allele Frequencies	
	N	TT	TC	CC	T	C
T-182C						
Major Depression	193	94 (49)	77 (40)	22 (11)	0.69	0.31
Controls	136	66 (50)	53 (39)	17 (11)	0.68	0.32
		GG	GA	AA	G	A
G1287A						
Major Depression	193	101 (53)	70 (36)	22 (11)	0.70	0.30
Controls	136	61 (44)	59 (44)	16 (12)	0.66	0.34

T-182C: Genotypes: p = 0.951, χ^2 = 0.099, df = 2

Alleles: p = 0.865 (OR = 1.03, 95% CI 0.74 – 1.49, Fisher's Exact Test, two sided) G1287A: Genotypes: p = 0.378, χ^2 = 1.945, df = 2

Alleles: p = 0.305 (OR = 1.20, 95% CI 0.86 – 1.67, Fisher's Exact Test, two sided)

In order to determine a possible role of the T-182C polymorphism in the development of major depression we performed a case control association study with 155 depressed patients and 136 healthy controls. Additionally a silent polymorphism in exon 9 of the NET gene (G1287A) was also genotyped. Our study failed to detect an association between the two investigated polymorphisms and major depression or the response to antidepressant treatment evaluated by the 17-item Hamilton Score. These results are in concordance with previously reported findings. Stöber et al. (1996) found no evidence for an association between major depression and five missense mutations, including the G1287A polymorphism in the coding region of the NET gene. Another study investigated the G1287A polymorphism in a sample of 105 patients with major depression and 74 unrelated matched controls with negative results (Owen et al. 1999). Hadley et al. (1995) detected no linkage between NET gene polymorphisms and bipolar disorder. Taken together these results make it very improbable that the NET gene has a major effect (i.e. a relative risk of 2 or

more) on depression. However, we cannot exclude a minor contribution of the NET gene in the pathophysiology of major depression, because the sample size of the present study does not provide statistical power to detect differences of small effect size. Although our samples were from the same geographical area the effect of ethnic stratification cannot be excluded. Moreover, the genetic contribution of depression is attributable to multiple genes with incomplete penetrance, each of small effect, and only large samples of patients and controls may show evidence for association or linkage if the effect size is small.

In summary our findings that genetic variants of the NET gene are not causally related to major depression should be replicated in different population samples and family studies. Especially the new identified T-182C polymorphism in the 5′ flanking promoter region of the NET gene would require further investigations of the pattern of NET expression in dependence of the genotype to confirm the results of this study. Finally, future research might seek to uncover new functional NET gene variants in coding or promoter regions.

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