A NOS-III haplotype that includes functional polymorphisms is associated with bipolar disorder

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Abstract

The pleiotropic messenger molecule nitric oxide (NO) has been implicated in a variety of higher CNS functions, including learning, memory, and emotionality. In the human brain, NO is predominantly formed by neuronal NO synthase (NOS-I), while the so-called ‘endothelial’ isoform NOS-III also contributes to NO generation. We recently reported that NOS-III knockout mice display decreased adult neurogenesis and reduced responsiveness in a learned helplessness paradigm. To examine whether NOS-III plays a role in affective disorders as well, we tested a NOS-III gene haplotype, consisting of three functional polymorphisms, for an association with bipolar disorder and major depression. A significant global haplotype association with bipolar disorder (n=284 controls; n=91 patients; pglobal=0.021; ptag<0.001), but not unipolar depression (n=45) was detected. Our results thus suggest that the NOS-III genotype may convey a modest genetic risk to develop bipolar disorder. This finding should be further clarified by the use of within-family designs and in samples of other ethnicity.

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Introduction

Affective disorders, consisting mainly of major depression (MD) and bipolar disorder (BD), are frequent and among the 10 most severe diseases with regards to disability adjusted life years. Despite better diagnostic options and increasing awareness of MD and BD, their pathophysiology remains elusive. While there is considerable agreement that MD, and even more so BD, display a substantial genetic background (Berrettini, 2002), the identification of relevant genes continues to be a daunting task. Pertinent candidate genes include mainly those encoding for proteins involved in neurotransmission. Just two genes, G72 (Hattori et al., 2003) and COMT, showing only modest association, were identified or strongly supported respectively, by linkage studies, although ~600 pedigrees with almost 2500 genotyped cases have been analysed using a ‘broad’ disease model of BD (Segurado et al., 2003).

Complementary to linkage analyses, candidate-gene approaches serve as an independent method to uncover the genetic basis of psychiatric diseases. In the pursuit to identify relevant candidates, animal models are a valuable tool in predicting a gene’s impact on human behaviour. Recently, we (Reif et al., 2004) have shown that mice lacking endothelial nitric oxide synthase (NOS-III) feature (a) low rate of neural stem cell proliferation, i.e. decreased adult neurogenesis which has been implicated in the pathophysiology of depressive disorders (Kempermann and Kronenberg, 2003), and (b) reduced responsiveness in a learned helpl...
helplessness paradigm which has been suggested to represent an animal-based model of depression (Vollmayr and Henn, 2001). We thus hypothesized that the NOS-III genotype may contribute to the genetic basis of depressive disorders as well, or – on the contrary – that a certain genotype could protect against affective disorders.

Nitric oxide (NO) is a pleiotropic messenger molecule generated by a family of three NO synthases (NOS), termed NOS-I, NOS-II and the so-called ‘endothelial’ NOS-III. In the human brain, more than 1% of all neurons express NOS-I, and it has been estimated that almost every neuron receives input from a nitrinergic cell (Snyder and Ferris, 2000). While NOS-I is expressed in neurons, neuronal expression of NOS-III, which can be found abundantly in endothelium where it produces NO to regulate vascular tone, remains to be demonstrated unambiguously. Although highly controversial, it has been suggested that NOS-III is present in hippocampal CA1 pyramidal cells (Dinerman et al., 1994; Doyle and Slater, 1997; O’Dell et al., 1994). Likewise, it is unclear whether NOS-III is expressed in astrocytes (Gabbott and Bacon, 1996; Luth et al., 2002; Wiencken and Casagrande, 1999) or not (Burette et al., 2002). These contradictory findings may be explained by regional specificity or differences across species. However, due to the strong expression in CNS endothelium, NOS-III has the physical and biochemical potential to exert influences on neural cells, thus facilitating for a cross-talk between vasculature and excitable tissue.

The human gene for NOS-III, NOS3, is located on chromosome 7q35.1 and comprises 26 exons, spanning 21 kb (Marsden et al., 1993). Of several known NOS3 polymorphisms, three have been extensively studied in cardiovascular disease. Although results were inconsistent, probably due to ethnic stratification and single-SNP association designs, the association of NOS-III genotype and ischaemic heart disease was confirmed in a recent meta-analysis incorporating >20,000 subjects (Casas et al., 2004). Since these three polymorphisms are functional with evidence for epistasis, they have been selected in the present study: (1), a promoter single nucleotide polymorphism (SNP) (T-786C), (2), an intronic 27-bp variable number of tandem repeats polymorphism (VNTR) in intron 4, and (3) a coding SNP (G894T) which causes an amino-acid change from Glu to Asp in exon 7. G894T has been suggested to alter the enzyme’s susceptibility to proteolytic cleavage (Tesauro et al., 2000), T-786C has been found to influence the NOS3 promoter activity as assessed by the luciferase reporter assay (Nakayama et al., 1999), and intron 4 VNTR is thought to function as a cis-acting enhancer element in an epistatic manner depending on T-786C genotype (Wang et al., 2002). The haplotype which can be constructed from those three polymorphisms displays marked inter-ethnic variation (Tanus-Santos et al., 2001), which has to be taken into account when comparing different studies.

Since we found that NOS-III knockout mice display diminished adult neurogenesis but reduced responsiveness in a learned helplessness paradigm (Reif et al., 2004), and no study has as yet addressed the role of the NOS-III in affective disorders, we have tested this NOS3 haplotype for association with MD or BD respectively.

Materials and methods

Subjects

A total of 136 patients from the Lower Franconia area in Germany were enrolled in the study, the patients were ascertained from the Department of Psychiatry and Psychotherapy. Ninety-one patients (27 males) suffered from bipolar affective disorder (BD) according to DSM-IV criteria, and only patients with at least one manic and one depressive episode were classified as BD (i.e. strict bipolar I criteria). A further 45 patients with unipolar depression (MD, 16 males) were included in the study, however, only when no signs of bipolarity were present throughout the whole course of the disease; those patients fulfilled the DSM-IV criteria of recurring depressive disorder, and had at least two depressive episodes. All patients were in-patients at the Department of Psychiatry at least once. Diagnoses were made by an extensive, semi-structured interview analogous to the AMDP interview (Arbeitsgemeinschaft für Methodik und Dokumentation in der Psychiatrie, 2000) performed by an experienced psychiatrist (A.R., C.P.J. or K.P.L.), along with chart reviews. If possible, further information was retrieved from family informants and case records from other hospitals to ensure consistent diagnoses. Chart reviews of every patient were done by A.R. None of the subjects showed significant neurological comorbidity, epilepsy, mental retardation, or a history of substance addiction, or other organic disorders suggesting organic affective disorder. The average age was $50 \pm 3$ yr for the BD group and $59 \pm 14$ yr for the MD group respectively. While 67 BD subjects had a positive family history for BD or depression in first- or second-degree relatives, only 12 MD patients had a positive family history ($\chi^2=27.27$, d.f. = 1), in almost all cases for depression.
DNA samples were collected from 284 control subjects (158 males) consisting of healthy blood donors from the same area as the patient group; the sample was not screened for a history of psychiatric disorders. All control subjects however were free of any regular medication, and the study was explained to them, so that the chance that severe psychiatric disorders were present in the control sample was low. Mean age of controls was $35 \pm 13$ yr. Both patients and controls were of Caucasian origin. Only patients and volunteers who gave written informed consent after oral as well as written explanation about aim and scope of the investigation were enrolled in the study. The study was approved by the Ethics Committee of the University of Würzburg.

Genotyping

Following DNA extraction, the three NOS-III polymorphisms were determined using standard protocols (Tanus-Santos et al., 2001): the promoter polymorphism T-786C SNP, an intronic 27-bp VNTR in intron 4, and the coding SNP G894T. Briefly, PCR reactions were performed in a reaction volume of 25 $\mu$l, including ~50 ng of template genomic DNA, 7 pmol of each primer (Table 1), 2.5 mM of each dNTP, 1 mM MgCl$_2$ (0.75 mM MgCl$_2$ for G894T), 50 mM KCl, 10 mM Tris–HCl (pH 8.3 at 25 °C), 0.025 mg/ml BSA, 0.025% Tween-20, and 1 U of Taq DNA polymerase. Annealing temperature was 58 °C for intron 4 VNTR (35 cycles) and G894T (40 cycles), and 64 °C for T-786C (35 cycles). For the determination of T-786C, PCR products were digested with MspI (overnight at 37 °C; fragment sizes: wild type, 140 and 40 bp; SNP, 90, 50 and 40 bp). G894T PCR amplicons were digested with MboI (overnight at 37 °C; fragments: wild type, 129 bp; SNP, 71 and 58 bp). Fragments, or PCR products in the case of the intron 4 VNTR (4a, 393 bp; 4b, 420 bp; 4c, 447 bp), were subsequently visualized on a 4% agarose gel.

Statistical analysis

Deviation of genotype frequencies from Hardy–Weinberg equilibrium was determined separately for the two patient populations and the controls by calculating $\chi^2$ statistics with d.f. = 1 for T-786C and G894T and with d.f. = 3 for the intron 4 polymorphism using an online calculator software (http://kursus.kvl.dk/shares/vetgen/_Popgen/genetik/applets/kitest.htm). Single association tests were performed by means of $\chi^2$ tests using SPSS for Windows version 9.0 (SPSS Inc., Chicago, IL, USA). Pairwise linkage disequilibrium between the three polymorphisms was assessed using 2 LD (Zhao, 2004). Haplotype analyses and tests for significance of differences in estimated haplotype frequencies between controls and patients were performed using the GENECOUNTING/PERMUTE utility of the GENECOUNTING software (Zhao, 2004). Both programs are available from the Institute of Psychiatry, King’s College, London (IOP, 2005).

GENECOUNTING implements an EM algorithm which can handle missing genotypes and ambiguously phased data to estimate haplotype frequencies in unrelated subjects (Dempster et al., 1977). GENE_COUNTING/PERMUTE performs permutation tests for global association by randomly reassigning case and control labels in the actual data. The resulting $p$ values reflect the proportion of replicates that produce values of statistics at least as large as those observed. In the current study, 10000 permutations were performed. The significance of specific haplotypes was assessed by a proportion test also.

Results

Ninety-one patients with BD, 45 patients with MD, and 284 controls were genotyped for three polymorphisms of NOS3: T-786C, intron 4ab, and G894T. Genotype frequencies at all loci were in Hardy–Weinberg equilibrium for cases and controls (all

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>T-786C forward</td>
<td>5'-TGG AGA GTG CTG CTG TAC CCC A-3'</td>
</tr>
<tr>
<td>T-786C reverse</td>
<td>5'-GCC TCC ACC CCC ACC CTG TC-3'</td>
</tr>
<tr>
<td>Intron 4 VNTR forward</td>
<td>5'-AGG CCC TAT GGT AGT GCC TTT-3'</td>
</tr>
<tr>
<td>Intron 4 VNTR reverse</td>
<td>5'-TCT CTT AGT GCT GTG TGC AC-3'</td>
</tr>
<tr>
<td>G894T forward</td>
<td>5'-GCA TTC AGC ACG GCT GGA-3'</td>
</tr>
<tr>
<td>G894T reverse</td>
<td>5'-GCT CCA GGG GCA CCT CAA-3'</td>
</tr>
</tbody>
</table>

Table 1. Sequences of nucleotide primer pairs used for NOS3 genotyping
All three polymorphisms were in significant linkage disequilibrium with each other (T-786C/intron 4 VNTR: $D' = 0.83$, S.D. = 0.05; T-786C/G894T: $D' = 0.56$, S.D. = 0.04; intron 4 VNTR/G894T: $D' = 0.96$, S.D. = 0.03; all $p < 0.0001$). Interestingly, two control subjects had a c allele of the intron 4 VNTR, which is very rare in the Caucasian population and seems to convey a risk for ischaemic heart disease (Sigusch et al., 2000).

**SNP association analyses**

Individual SNP association analyses by means of $\chi^2$ tests did not yield significant results (see Table 2). Similarly, no significant association between any of the polymorphisms and BD, and MD respectively, was obtained when comparing allele distributions (data not shown).

**Haplotype analyses**

Eight of 12 possible haplotypes were present in our dataset. Two haplotype analyses were performed to test for global haplotype association with BD and MD respectively. A significant global haplotype association with BD was observed ($p = 0.02$), whereas no haplotype association was observed for MD ($p = 0.54$). After correction for multiple testing (Bonferroni-adjusted level of significance: $\alpha' = 0.05/8 = 0.006$), the global association with BD remained significant. Table 3 shows the haplotype frequency estimates for BD patients and controls for the eight non-zero haplotypes observed in our sample as well as the permutation-based significance of frequency differences. For the control subjects, the obtained haplotype frequencies were similar to previously published results (Table 3).

As shown in Table 3, the estimated c-b-g and t-a-g haplotypes were under-represented in BD patients ($p = 0.022$, and $p < 0.001$ respectively). Furthermore, the estimated t-b-g haplotype had a tendency to be over-represented in BD patients ($p = 0.052$). After correction for multiple testing (Bonferroni-adjusted level of significance: $\alpha' = 0.05/8 = 0.006$), the frequencies differed significantly only for the estimated t-a-g haplotype.

**Discussion**

In the present study, we detected an association of NOS3 haplotypes with BD: two haplotypes, c-b-g and t-a-g, were found to be under-represented in BD patients (c-b-g just failed to reach significance following Bonferroni-adjustment) and thus may be protective against the disorder. MD was not found to be associated with NOS3 haplotypes. This might be a false-negative finding due to the low MD sample size.
size. Furthermore, the average age of the control group was 35 yr, which renders it likely that at least some subjects may be at risk to develop MD in later life. Also, the control sample was not explicitly screened for psychiatric disorders (however free of medication); therefore, it cannot be excluded that some subjects have had suffered from an episode of affective disorder earlier in life. Both aspects further decrease power to detect association with the disorder. Assessment of the association with MD and replication of the BD finding in a larger sample, compared to an age- and sex-matched control group screened for psychiatric disorders is, therefore, required before generalization on a possible protective nature of the \textit{NOS3} haplotypes can be made.

Unlike many other haplotype analyses in psychiatric genetics, emphasis was put on the investigation of a set of functional polymorphisms in the present study. However, it cannot be deduced from previous studies which functional consequences the protective \textit{NOS3} haplotypes convey. Only a few haplotype analyses using this three-marker haplotype have been published to date (Amoli et al., 2003, 2004; Hassan et al., 2004). While examination of the protective mechanism of the t-a-g/c-b-g haplotypes is beyond the scope of the present study, the relevance of the \textit{NOS3} genotype in cardio- and cerebrovascular disease (Casas et al., 2004; Hassan et al., 2004) may render it worthwhile to further investigate its functional implications.

Both haplotypes were present in the control population in exactly the same frequency as previously published for Caucasians (Tanus-Santos et al., 2001). Because of the inter-ethnic differences in \textit{NOS-III} haplotype frequency it is of critical importance to carefully match patient and control samples. Accordingly, the patient sample was entirely of Western European descent, as was the control group. Since both haplotypes are relatively rare in the general population, there might either be evolutionary pressure against them, or alternatively, they might be haplotypes of recent origin. In contrast to the c-b-g haplotype, which is rare in populations of different ethnicity, t-a-g is found in 27\% of African-Americans, but only in 2–3\% of Caucasians and Asians (t-b-g is the haplotype of combined wild types; Tanus-Santos et al., 2001). Thus, the t-a-g haplotype, or the intron 4a variant respectively, was suggested to exert a major effect in the African-American ethnic group; this variant was shown to result in lower NO metabolite levels in a gene/dose-dependent manner (Tsukada et al., 1998). Likewise, the -786C allele (i.e. c-b-g compared to t-b-g) was suggested to lower \textit{NOS-III} expression and thus presumably activity (Nakayama et al., 1999). Thus, both haplotype variants should result in lower \textit{NOS-III} activity compared to the wild-type haplotype t-b-g. In agreement with our finding that those presumably low-activity \textit{NOS3} haplotypes protect against BD, it was previously shown that BD patients display significantly higher plasma nitrite (a metabolite of NO) concentrations (Savas et al., 2002; Yanik et al., 2004), which probably reflects \textit{NOS-III} activity due to the endothelial localization of the enzyme.

### Table 3. Estimated \textit{NOS3} haplotype frequencies for MD patients, BD patients, and controls

<table>
<thead>
<tr>
<th>\textit{NOS3} haplotype</th>
<th>Controls$^a$</th>
<th>BD</th>
<th>$p^b$</th>
<th>MD</th>
<th>$p^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>c-a-g</td>
<td>0.130 (0.14)</td>
<td>0.150</td>
<td>0.515</td>
<td>0.133</td>
<td>0.949</td>
</tr>
<tr>
<td>c-a-t</td>
<td>0.000 (0.00)</td>
<td>0.003</td>
<td>0.212</td>
<td>0.001</td>
<td>0.484</td>
</tr>
<tr>
<td>c-b-g</td>
<td>0.040 (0.04)</td>
<td>0.006</td>
<td>0.022</td>
<td>0.073</td>
<td>0.182</td>
</tr>
<tr>
<td>c-b-t</td>
<td>0.228 (0.24)</td>
<td>0.197</td>
<td>0.408</td>
<td>0.249</td>
<td>0.696</td>
</tr>
<tr>
<td>c-c-t</td>
<td>0.004 (0.00)</td>
<td>0.000</td>
<td>0.523</td>
<td>0.000</td>
<td>0.291</td>
</tr>
<tr>
<td>t-a-g</td>
<td>0.027 (0.02)</td>
<td>0.000</td>
<td>$&lt;0.001$</td>
<td>0.000</td>
<td>0.148</td>
</tr>
<tr>
<td>t-b-g</td>
<td>0.478 (0.46)</td>
<td>0.563</td>
<td>0.052</td>
<td>0.438</td>
<td>0.523</td>
</tr>
<tr>
<td>t-b-t</td>
<td>0.094 (0.10)</td>
<td>0.080</td>
<td>0.601</td>
<td>0.106</td>
<td>0.673</td>
</tr>
</tbody>
</table>

Global $p = 0.021$  
Global $p = 0.542$

$^a$ Values within parentheses, from a previously published study (Tanus-Santos et al., 2001), are given for comparison.  
$^b$ Permutation-based significance of frequency differences between BD patients and controls, and between MD patients and controls respectively (10000 permutations).
In a series of studies in NOS-III knockout mice, we found that animals lacking NOS-III exhibit reduced responsiveness in a learned helplessness paradigm compared to wild-type controls (Reif et al., 2004). Since the learned helplessness paradigm has been suggested to represent an animal model of behavioural despair and depression (Vollmayr and Henn, 2001), NOS-III knockout mice display an antidepressant-like phenotype. The notion that NOS-III impacts on behavioural control is further substantiated by several recent studies investigating the behavioural phenotype of NOS-III knockout mice. Taken together, the main findings of these investigations are that animals lacking NOS-III are (a) less helpless, (b) better learners (Frisch et al., 2000), and (c) not as aggressive (male mice; Demas et al., 1999). Thus, several key syndromal dimensions of affective disorders including emotionality, impulsivity, aggression, and cognition are altered in NOS-III knockout mice.

Pharmacological studies provide further evidence for an involvement of NOS in affective illness. Although not isoform-specific and thus, not discriminatory between NOS-I and NOS-III, inhibitors of NOS were shown to have antidepressive properties in the elevated plus maze (Volke et al., 1995) and the forced swim test (Harkin et al., 1999; Volke et al., 2003), which seems to be mediated by the serotonergic system. Serotonin (5-HT) depletion resulted in a complete loss of the antidepressant actions of NOS inhibitors (Harkin et al., 2003), whereas their effect was further augmented by 5-HT reuptake inhibitors (Harkin et al., 2004). This corresponds well with neurochemical studies in which NOS-III knockout mice were found to display increased 5-HT turnover in the cortex and the striatum (Frisch et al., 2000).

As there are no animal models of BD, the investigation of behavioural traits related to affective disorders – e.g. helplessness and cognitive deficits related to depression; aggression and hyperactivity occurring in mania – without regarding them as specific for a given diagnostic entity (BD, MD, etc.) is the only way to obtain candidate genes from animal studies. Nevertheless, this approach obviously has drawbacks. Apart from the limitations of the present study mentioned above, which have to be borne in mind, there is, however, converging evidence from NOS-III knockouts and human genetic findings pointing in the same direction, that is, a distinct role for NOS-III in affective disorders.

Interestingly, the 5-HT transporter (5-HTT) can be modulated by NO in that binding of NO increases 5-HT uptake (Kilic et al., 2003). A recently reported coding region variant of 5-HTT, segregating with a complex phenotype related to obsessive-compulsive and affective disorders (Ozaki et al., 2003), results in continuously elevated 5-HT uptake and concomitant loss of 5-HTT regulation by NO (Kilic et al., 2003). These findings strongly point further towards an interaction of both the nitrinergic and serotonergic systems in humans (Kiss and Vizi, 2001), and that dysregulation of this interplay may be involved in the pathogenesis of affective disorders.

In the recent years, there has been an increasing interest in a hypothetical disease entity designated ‘vascular depression’ (Alexopoulos et al., 1997) thought to delineate a subgroup of depressive disorders, in which vascular dysfunction is proposed to underlie psychiatric symptoms especially in the elderly (Baldwin and O’Brien, 2002; Camus et al., 2004) suffering from cerebrovascular disease. The finding that NOS3 haplotypes are associated with the risk to develop cerebral small vessel disease (Hassan et al., 2004) suggests that NOS-III may contribute to the risk towards vascular depression. While the connection between cerebrovascular disorder and depression might still be explained by vascular lesions in critical brain regions and not by a common pathophysiology, it remains an unresolved question why depression – with prognostic value – also occurs more frequently in cardiovascular disease.

NOS-III could be a possible link between cardio-vascular risk and affective disorder on a genetic basis, which makes it an apparent candidate gene in coronary disease with depression.

In conclusion, there is a small but significant contribution of NOS3 to the genetic risk towards BD which does not explain the evidence for possible major gene effects found in informative pedigrees. As the protective haplotypes are relatively rare in the general population, other genetic influences are likely to play an important role in the pathogenesis of affective disorders, with the NOS-III variation probably modulating this genetic risk only in only a few subjects. NOS-III is, therefore, likely to represent a disease modifier gene influencing the penetrance of other genes. Given a prevalence of ~1% (Waraich et al., 2004) of BD in the general population, a protective factor of modest impact, however, can also become important on a population-based level. Whether NOS3 influences the genetic risk towards affective disorders should be further tested in family-based studies, which would be a worthwhile future task as NO synthases represent a novel and innovative drug target.
Acknowledgements

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Statement of Interest

None.

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