ORIGINAL ARTICLE

Influence of Functional Variant of Neuronal Nitric Oxide Synthase on Impulsive Behaviors in Humans

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Context: Human personality is characterized by substantial heritability but few functional gene variants have been identified. Although rodent data suggest that the neuronal isoform of nitric oxide synthase (NOS-I) modifies diverse behaviors including aggression, this has not been translated to human studies.

Objectives: To investigate the functionality of an *NOS1* promoter repeat length variation (*NOS1* Ex1f variable number tandem repeat [VNTR]) and to test whether it is associated with phenotypes relevant to impulsivity.

Design: Molecular biological studies assessed the cellular consequences of *NOS1* Ex1f VNTR; association studies were conducted to investigate the impact of this genetic variant on impulsivity; imaging genetics was applied to determine whether the polymorphism is functional on a neurobiological level.

Setting: Three psychiatric university clinics in Germany.

Participants: More than 3200 subjects were included in the association study: 1954 controls, 403 patients with personality disorder, 383 patients with adult attention-deficit/hyperactivity disorder (ADHD), 151 with familial ADHD, 189 suicide attempters, and 182 criminal offenders.

Main Outcome Measures: For the association studies, the major outcome criteria were phenotypes relevant to impulsivity, namely, the dimensional phenotype conscientiousness and the categorical phenotypes adult ADHD, aggression, and cluster B personality disorder.

Results: A novel functional promoter polymorphism in *NOS1* was associated with traits related to impulsivity, including hyperactive and aggressive behaviors. Specifically, the short repeat variant was more frequent in adult ADHD, cluster B personality disorder, and autoaggressive and heteroaggressive behavior. This short variant came along with decreased transcriptional activity of the *NOS1* exon 1f promoter and alterations in the neuronal transcriptome including *RGS4* and *GRIN1*. On a systems level, it was associated with hypoactivation of the anterior cingulate cortex, which is involved in the processing of emotion and reward in behavioral control.

Conclusion: These findings implicate deficits in neuronal signaling via nitric oxide in moderation of prefrontal circuits underlying impulsivity-related behavior in humans.

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In recent years, considerable evidence has been accrued for a genetic influence in the control of normal human brain function and behavioral disorders. By genome-wide linkage analysis and whole-genome and case-control association studies, a plethora of novel candidate genes have been proposed for all major psychiatric disorders. However, in almost all cases, haplotype blocks of synonymous or intronic single nucleotide polymorphisms in or near the identified genes were associated, while functional variants continue to be elusive. The search for functional gene variants explaining neurobiological functions thus remains a daunting task. For only a few polymorphisms linked to personality traits and resulting behavioral phenotypes, such as the repeat length variations of the serotonin transporter gene, *5-HTTLPR*,[1,2] and the MAO-A gene, *MAO-A* variable number tandem repeat (*u*VNTR),[3,4] molecular functionality has convincingly been demonstrated. Both *5-HTTLPR* and *MAO-A uVNTR* alter the expression of the respective gene, suggesting that this mechanism is likely more common than coding region single nucleotide polymorphisms in the control of human behavior.

Neuronal nitric oxide synthase (NOS-I) has been linked to rodent behavior by nu-

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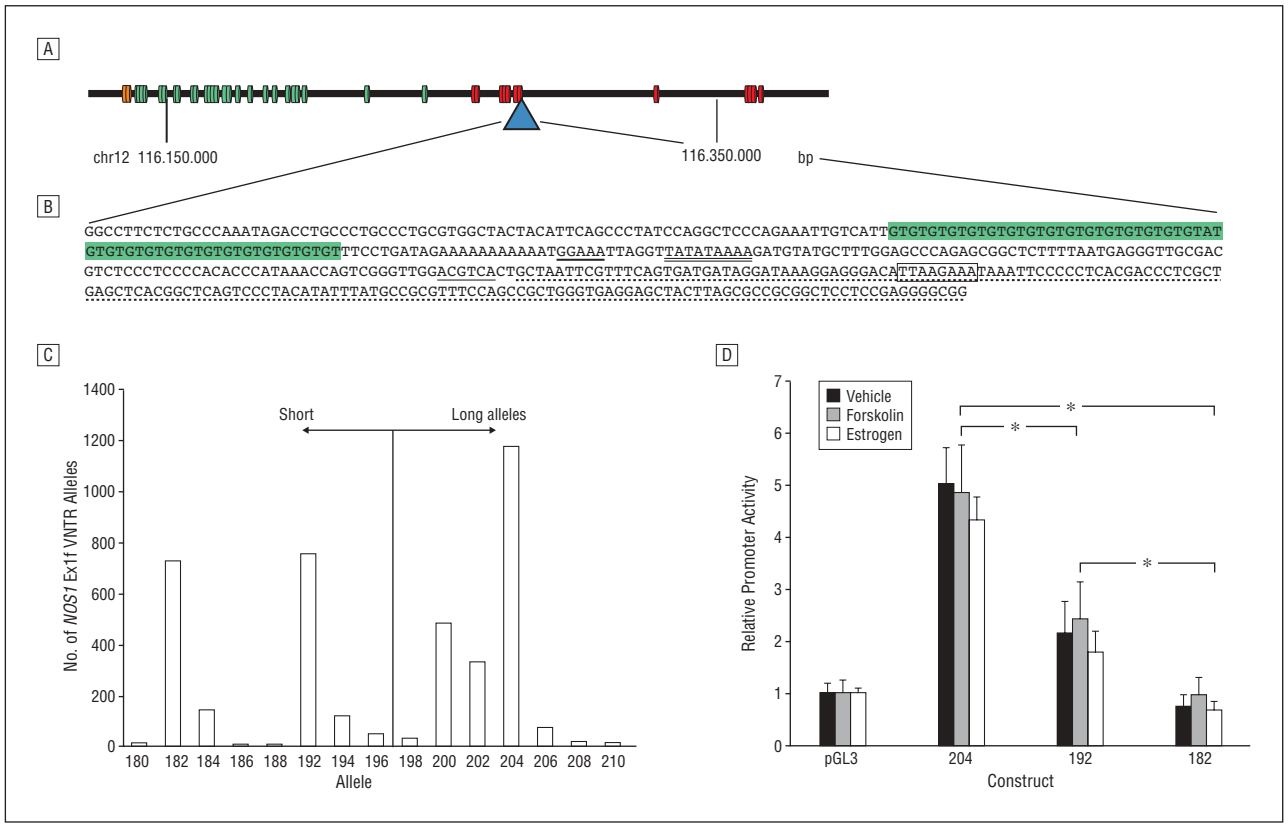

Figure 1. Nitric oxide synthase (*NOS*)1 Ex1f genomic region and functionality. A, Schematic drawing of the *NOS1* region, indicating the 5′UTR (orange), coding region exons (green), and alternative first exons (red). The triangle indicates the promoter region of exon 1f; bp, base pairs. B, Sequence of the variable number tandem repeat (VNTR) polymorphism in the promoter region of exon 1f of *NOS1* (*NOS1* Ex1f VNTR); the displayed region corresponds to the sequence subcloned into the reporter gene constructs. Green background indicates GT repeat region; heavy underlining, nuclear factor of activated T-cell binding site; double underlining, TATA box; single underlining, cyclic adenosine monophosphate response element-binding (CREB) site; boxed and dotted underlining, CCAAT/enhancer binding protein β binding site; and dotted underlining, alternative exon 1f transcript. C, Frequency of *NOS1* Ex1f VNTR alleles in 3916 chromosomes from control subjects. D, Long alleles of *NOS1* Ex1f VNTR (204 repeats) result in significantly increased reporter gene activity compared with intermediate (192 repeats) and, even more so, short (182 repeats) alleles. A pGL3 basic luciferase reporter gene system (Dual-Glo; Promega, Mannheim, Germany) was used, and relative luciferase activity was determined by using a plate photometer. Neither forskolin nor estrogen treatment had an influence on reporter gene expression compared with vehicle treatment. *$P < .001$.

merous pharmacologic studies. Likewise, knockdown of the *Nos1* gene in mice results in behavioral changes involving decreased anxiety, increased impulsivity, and increased aggressiveness.[5] The human gene encoding NOS-I, *NOS1* (OMIM 163731), however, has hitherto not been linked to personality dimensions or behavioral traits. *NOS1* is located at 12q24.3 and displays one of the most complex genes in the human genome.[6] An approximately 125-kilobase (kb) region contains 28 protein-coding exons, whereas another approximately 125-kb region, termed *variable region*, includes at least 11 distinct first exons transcribed into messenger RNA but thereafter removed by splicing (**Figure 1**A).

Transcription of those alternative first exons, designated exons 1a to 1l, is driven by alternative promoters, resulting in a tissue-specific expression pattern.[7] The promoter region of exon 1f, predominantly expressed in the basal ganglia, hippocampus, and cortex, harbors binding motifs for estrogen receptors, cyclic adenosine monophosphate response element-binding (CREB) protein 1, CCAAT/enhancer-binding transport protein (EBP) β-1, and nuclear factor of activated T cells (NFAT) (Figure 1B). Our group previously reported a highly polymorphic dinucleotide repeat with 180 to 210 cytosine-adenosine dinucleotide (CA) units, termed *NOS1* Ex1f VNTR, present 33 base pairs (bp) downstream of the TATA box (Figure 1A and B).[8] The frequency of the repeat length variants is not distributed evenly, because they cluster mainly at the alleles 182/184, 192, and 200/202/204 (Figure 1C). To facilitate genetic studies, the polymorphism was dichotomized in short (180-196 repeats) and long (198-210 repeats) alleles. Short *NOS1* Ex1f VNTR alleles were associated with disease severity and prefrontal functioning in schizophrenia.[8] Furthermore, short-allele carriers showed an increase in the risk of developing Alzheimer disease, with evidence of *NOS1* × *ApoE4* interaction.[9]

Although *NOS1* exon 1f is highly conserved, the promoter repeat appears to be exclusive to humans (eFigure, http://www.archgenpsychiatry.com), although there is considerable linkage disequilibrium around this region (eFigure 1C). We thus hypothesized that *NOS1* Ex1f VNTR might possess functional relevance on a molecular basis and that it is associated with dimensional behavioral traits, also reflected by personality disorders (PDs) and related domains. In analogy to the behavioral phenotype of *Nos1* knockdown mice,[5,10] a role for *NOS1* Ex1f VNTR in impulsivity-related phenotypes (eg, attention-deficit/hyperactivity disorder [ADHD] and cluster B PDs) was assumed.

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METHODS

SUBJECTS

Seven different samples were ascertained for this study, which are described in greater detail in the supplemental "Methods" section[11-13] (also at http://www.archgenpsychiatry.com). The forensic sample was ascertained in the southwest German region of Saarland,[14] while all other cohorts were recruited in Bavaria, a part of southern Germany. All subjects were white. Only participants who gave written informed consent after oral and written explanation about the aims of the investigation were enrolled. The study was approved by the ethics committees of the Universities of Würzburg, Homburg, and Munich.

Control A

This group included a total of 640 subjects; 284 were healthy blood donors originating from the Würzburg area who were not screened for psychiatric disorders; however, all participants were free of medication. An additional 356 individuals were recruited and screened for absence of psychiatric disorders.

Control B

The control B group consisted of 1314 unrelated healthy volunteers randomly selected from the general population of Munich.[13] Subjects with neuropsychiatric disorders or with first-degree relatives with such disorders were excluded by the Structured Clinical Interview for *DSM-IV* (SCID-I and SCID-II) and the Family History Assessment Module.[11]

Personality Disorder

The PD group included 403 inpatients and outpatients of the Department of Psychiatry, University of Würzburg. Inclusion criteria were PD according to *DSM-IV* (antisocial, histrionic, borderline, narcissistic, avoidant, dependent, and obsessive-compulsive PDs) and age between 18 and 60 years. Exclusion criteria were medical conditions and lifetime diagnosis of schizophrenia or other psychotic disorders. Further details on the sample have previously been reported.[15,16]

Adult ADHD

The adult ADHD (aADHD) group consisted of 383 inpatients and outpatients of the Department of Psychiatry, University of Würzburg, referred for diagnostic assessment and treatment of aADHD and examined with the SCID-I. Inclusion criteria were aADHD according to *DSM-IV*, onset before the age of 7 years, lifelong persistence, current diagnosis, and age at recruitment between 18 and 65 years. Exclusion criteria were the appearance of symptoms restricted to the duration of any other Axis I disorder; current diagnosis of active alcohol or other drug abuse or dependence; lifetime diagnosis of bipolar I disorder, schizophrenia, or any other psychotic disorder; and mental retardation (IQ, <80). Details on the sample may be obtained elsewhere.[12]

Suicide Attempters

We recruited 189 suicide attempters among patients consecutively referred to the Department of Psychiatry, University of Munich. The sample description has previously been published.[13] Current and lifetime diagnoses of mental disorders were assessed close to discharge by applying the SCID-I. Patients with psychiatric disorders due to a general medical condition or with dementia were excluded.

Forensic Group

This sample has formerly been described in detail.[12] Briefly, 182 adult male volunteers, referred for forensic examination to the Institute of Forensic Psychiatry, Saarland University, Homburg, entered the study. Subjects with a severe Axis I diagnosis and mentally retarded subjects (IQ, <70) were excluded. Each subject was assigned to the violent or nonviolent group according to personal police records and recently committed crimes on the basis of careful evaluation of all available documents. Violence, as an outcome variable of predisposition toward offensive aggression, was defined as an abiding "overt and intentional physically aggressive behavior against another person" as suggested by Volavka[17(p308)] and adopted by an expert consensus.[18] By this means, only subjects with habitual aggressive and violent behavior were included in the violent group, whereas offenders with a history of only nonviolent crimes were assigned to the nonviolent group. History of PD and substance abuse was assessed by standardized instruments.[14] Childhood environment was assessed by the adverse childhood environment index, resulting in a mean score of 0 (optimal) to 2 (most adverse childhood environment).

Familial ADHD

The familial ADHD (fADHD) group consisted of 151 inpatients and outpatients aged 8 to 18 years referred to the Department of Child and Adolescent Psychiatry, University of Würzburg. The sample included 118 boys and 33 girls from 102 families. Both parents were ascertained in all families. Included children were characterized by standardized full interview (Schedule for Affective Disorders and Schizophrenia for School-Age Children–Present and Lifetime Version) with a parent and met *DSM-IV* criteria for ADHD. Exclusion criteria were birth weight less than 2000 g, IQ lower than 85, neurologic or severe somatic disorder, drug abuse, and autistic disorder.

GENOTYPING

The *NOS1* Ex1f VNTR was determined as described previously.[8] One of the primers was labeled with a fluorescent dye (cy-5; TIB MolBiol, Berlin, Germany), enabling detection of the polymerase chain reaction (PCR) product. Electrophoretic separation of the PCR products was performed with a DNA sequencer (CEQ8000; Beckman-Coulter, Krefeld, Germany). Primers and PCR conditions are available on request.

MICROARRAY EXPRESSION STUDIES

Approximately 1-cm^3 pieces of cerebellar tissue were obtained from the Stanley Medical Research Institute, corresponding to brains for which BA46 microarray data have been obtained.[19] The DNA was extracted and *NOS1* Ex1f VNTR was determined as described in the previous section. Thereafter, genotype was correlated with microarray data by classifying genotype as 0/1/2 values (long-long/short-long/short-short), in which a fold change reported for a gene would be an average fold change for an increase of 1 in the genotype value. Correction for multiple testing was done thereafter, adjusting α for 20 000 tests. Of the remaining genes, those upregulated or downregulated more than 10% were considered meaningful.

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REPORTER GENE ASSAYS

A fragment of approximately 1.2 kb encompassing *NOS1* Ex1f and its flanking regions was amplified from genomic DNA of humans homozygous for either 182, 192, or 204 CA repeats. The PCR products were digested with *Hae*III and resolved electrophoretically on agarose gel. *Hae*III-*Hae*III amplicon subbands of 558, 568, and 580 bp respective to the different repeat lengths were purified and blunt-end cloned into a pGL3 basic vector (Promega, Mannheim, Germany). XL-10 competent cells (Stratagene, Amsterdam, the Netherlands) were transformed with 1 µL of ligation product and plated. Positive clones were selected and grown before plasmid purification with a kit (Maxiprep; Qiagen, Hilden, Germany). HeLa cells were grown, seeded, and transfected with 400 ng of pGL3-promotor plasmid and 70 ng of phRG-TK-DNA (*Renilla* luciferase as transfection control) per well. Transiently transfected cells were allowed to grow for 24 hours before direct collection or induction with 10µM forskolin or 10nM 17β-estradiol (Sigma-Aldrich Company, Taufkirchen, Germany) for 12 hours. Both firefly and *Renilla* luciferase activities were measured with a kit (Dual-Luciferase Assay Kit; Promega) on a 96-well device (Microlumate; Berthold, Bad Wildbad, Germany). Promoter activities were expressed as relative light units, normalized for protein content and *Renilla* activity.

ELECTROPHYSIOLOGIC EXAMINATIONS

One hundred sixty-seven healthy controls, genotyped for *NOS1* Ex1f VNTR, were investigated by means of a continuous performance test (CPT) as described previously.[8] Subjects were matched regarding mean age ($F_{2,163}=0.18$, $P=.84$) and sex distribution ($\chi^2=1.13$, $P=.57$). For the CPT, 400 letters were presented sequentially in a pseudo-randomized order; participants were instructed to press a button whenever the primer condition "O" was directly followed by an "X" ("go" condition). "no-go" was defined as "O" followed by any letter other than an "X." Parallel to this task, the electroencephalogram was recorded from 21 scalp electrodes. The data were segmented into different conditions of the CPT, and go and no-go epochs were further analyzed. Segments were averaged to 1 go and 1 no-go event-related potential per subject. For topographic analysis, the global field power peaks were determined within a P300 time frame. At individual global field power peaks, amplitude, latency, and anterior-posterior location of the positive centroid were calculated. From these data, the no-go anteriorization, defined as the distance between the individual go and no-go centroid, was determined. For the statistical analysis, analysis of variance was used to determine the effect of genotype on the go and the no-go centroid. Post hoc analyses were conducted by the *t* test for independent samples.

STATISTICAL ANALYSES

Case-Control Comparisons for Categorical Phenotypes (PD, Suicide Attempt, aADHD)

Association of *NOS1* genotype with aADHD were tested by means of χ^2 tests comparing *NOS1* genotype frequencies in the aADHD sample with those in the fully psychiatrically screened control sample B. The association remained significant when tested against control sample A and the combined control sample. To control for comorbidity with cluster B PD, a second analysis was performed comparing only those aADHD participants who did not exhibit a cluster B PD (n=194). Tests for association between *NOS1* Ex1f VNTR and PD, or suicide attempt, were examined by means of χ^2 tests with $df=2$.

Personality Measures in Sample B

Possible effects of *NOS1* Ex1f VNTR genotype on personality traits assessed by the NEO Personality Inventory–Revised (NEO-PI-R)[20] were examined in control sample B (complete data available for 1099 subjects). Association tests were performed by means of analysis of variance. Taking into account possible sex effects and interactions between sex and genotype, *NOS1* genotype and the participants' sex were entered as between-subjects factors. The analysis of variance model comprised sex and *NOS1* genotype as well as their interactions. Effects were considered significant if their type 1 error probability was less than $\alpha=.05$ (uncorrected). Numerator degrees of freedom in both samples were 1 for sex and 2 for *NOS1* and *NOS1* × sex; denominator degrees of freedom were 1087. Power calculations using G*Power 3[21] indicated that the healthy control sample was large enough to detect effects explaining more than 1% variance in personality traits at a significance threshold of $\alpha=.05$ and with a power of $1-\beta=0.80$.

Forensic Sample

Subjects were compared with regard to the presence or absence of pervasive violent behavior as previously described.[14] A logistic regression model with violent behavior as the dependent variable and *NOS1* Ex1f VNTR as the main effect of interest was performed, controlling for 4 possible confounding variables: adverse childhood experience, history of substance abuse, PD, and age.

fADHD Sample

A family-based association test, valid for families with an arbitrary number of affected children, was performed by using a permutation test implemented in FAMHAP software.[22]

RESULTS

EFFECT OF *NOS1* Ex1f VNTR ON REPORTER GENE EXPRESSION

Because the functional relevance of this polymorphism has not been investigated, we determined expression of *NOS1* Ex1f VNTR short (182 repeats), intermediate (192 repeats), and long (204 repeats) alleles fused to a luciferase reporter vector. The sequence included a CREB binding site, the TATA box, and the beginning of exon 1f (Figure 1B). The full-length insert with long repeats of *NOS1* Ex1f VNTR resulted in maximal gene expression, whereas intermediate and, to a greater extent, short repeats decreased expression of the reporter gene (Figure 1D). Although the construct contains a CREB binding site, forskolin treatment failed to increase gene expression; relative promoter activity was also not affected by estrogen.

EFFECT OF *NOS1* Ex1f VNTR ON THE NEURONAL TRANSCRIPTOME

To assess whether *NOS1* Ex1f VNTR also influences the regulation of other genes, we examined the impact of length variation on global gene transcription by expression profiling of human postmortem brain (Brodmann area [BA] 46) collected from 105 individuals.[19] Microarray data in-

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Table 1. Effect of *NOS1* Ex1f VNTR on the Transcriptome of Human Brodmann Area 46[a]

Gene Symbol	Gene Name	Fold Change	*P* Value
AP1S1	Adaptor-related protein complex 1, σ 1 subunit	1.1083	1.42×10^{-5}
BHLHB2	Basic helix-loop-helix domain containing, class B, 2	1.1348	6.27×10^{-6}
CACNB2	Calcium channel, voltage-dependent, β 2 subunit	1.1107	5.59×10^{-6}
CHI3L1[b,c]	Chitinase 3–like 1 (cartilage glycoprotein-39)	1.1016	3.00×10^{-7}
CLPTM1	Cleft lip and palate–associated transmembrane protein 1	1.1082	2.27×10^{-5}
DTX3	Deltex 3 homologue (*Drosophila*)	1.1068	1.67×10^{-7}
GHITM	Growth hormone–inducible transmembrane protein	1.1308	1.62×10^{-5}
GPX3[b]	Glutathione peroxidase 3 (plasma)	1.1214	2.01×10^{-5}
GRIN1[b,c]	Glutamate receptor, ionotropic, *N*-methyl D-aspartate 1	1.1248	3.07×10^{-10}
HDGFRP3	Hepatoma-derived growth factor, related protein 3	1.1308	5.79×10^{-8}
KIAA0182	KIAA0182 protein (genetic suppressor element 1)	1.1226	5.32×10^{-6}
MAP1LC3B	Microtubule-associated protein 1 light-chain 3 β	1.1008	2.30×10^{-5}
MAPK8[b,c]	Mitogen-activated protein kinase 8 (alias JNK)	1.1010	2.56×10^{-6}
MEF2C	MADS box transcription enhancer factor 2, polypeptide C (myocyte enhancer factor 2C)	1.1066	1.90×10^{-6}
NEFL[b,c]	Neurofilament, light polypeptide 68 kDa	1.1272	1.91×10^{-5}
RGS4[c]	Regulator of G-protein signaling 4	1.1148	1.06×10^{-5}
SNCA[b,c]	Synuclein, α (non-A4 component of amyloid precursor)	1.1271	4.83×10^{-6}
SNX3	Sorting nexin 3	1.1129	5.29×10^{-7}
SOD2[b,c]	Superoxide dismutase 2, mitochondrial	1.1148	3.05×10^{-6}
SRD5A1	Steroid-5-α-reductase, α polypeptide 1 (3-oxo-5 α-steroid δ 4-dehydrogenase α 1)	1.1126	5.17×10^{-6}
TPD52	Tumor protein D52	1.1046	4.60×10^{-6}
TUBB[c]	β 5-tubulin	1.1072	3.57×10^{-8}
TUBB4	β 4-tubulin	1.1197	1.36×10^{-8}
WARS	Tryptophanyl-tRNA synthetase	1.1216	1.73×10^{-7}
XIST	X (inactive)-specific transcript	–1.21854	6.17×10^{-6}

Abbreviation: *NOS1* Ex1f VNTR, nitric oxide synthase (*NOS*) 1 variable number tandem repeat polymorphism in the promoter region of exon 1f of *NOS1*.
[a] Significantly (uncorrected $P<.0000025$) and meaningfully (upregulation or downregulation by >10%) dysregulated genes were calculated as a function of *NOS1* Ex1f VNTR. The genotypes were analyzed as 0/1/2 values (long-long/short-long/short-short); a fold change reported for a gene would be an average fold change for an increase of 1 in the genotype value.
[b] Genes known to be directly involved in nitric oxide pathways.
[c] Genes that have already been implicated in neuropsychiatric disease.

cluding a total of 19 500 genes were stratified by the presence of 0, 1, or 2 short promoter alleles. We applied strict correction for multiple testing, ie, used an α adjustment of 2.5×10^{-6}, and considered genes that were upregulated or downregulated more than 10% as functionally meaningful. Twenty-five genes were identified as significantly altered (**Table 1**). The complete list of genes with significant changes in expression is given in eTable 1.

ASSOCIATION OF *NOS1* Ex1f VNTR WITH IMPULSIVITY-RELATED PHENOTYPES

On the basis of our findings indicating that *NOS1* Ex1f VNTR alleles affect the brain transcriptome and decrease promoter activity, and previous data in *Nos1* knockdown mice arguing for increased impulsivity and resulting aggression,[5] we predicted an influence of *NOS1* Ex1f VNTR on human impulsivity (ie, instant acting without consideration of consequences, including aggressive, emotional, and hyperactive behaviors) and related disorders. The primary aim was to link *NOS1* both to dimensional variations of human behavior, most likely underlying psychopathological disease states, and to disease categories. Because genotyping of large and well-characterized cohorts is critical in this respect, we determined frequencies of *NOS1* Ex1f VNTR variants in several distinct samples (**Table 2**), including healthy controls with data on personality questionnaires, a PD sample, a cohort of patients with aADHD, suicide attempters, and criminal offenders. The total number of genotyped individuals was 3111 (3466 including the trio analysis).

Both examined control populations (which were ascertained independently) displayed an almost identical distribution of *NOS1* Ex1f VNTR (χ^2, $P=.33$), whereas a consistent significant excess of short alleles was detected in adequately powered patient populations (Table 2; for the comparison of NOS genotype frequencies in PD, suicide attempters, and aADHD, the respective sample sizes permitted detection of relatively small effects with a power of $1-\beta>0.95$). On a categorical level, short *NOS1* Ex1f VNTR alleles were not associated with anxious-avoidant cluster C PD ($P=.33$), but were associated with the presence of emotional-dramatic cluster B PD ($P=.01$), especially histrionic ($P=.009$) PD, and with the presence of aADHD ($P=.001$). Because in the latter sample a high comorbidity with cluster B PD has been reported,[12] we investigated in a separate, family-based sample of children with ADHD (n=355)[23] whether the short *NOS1* Ex1f VNTR allele contributes to genetic risk toward ADHD per se. However, short *NOS1* Ex1f VNTR alleles were not preferentially transmitted to affected offspring ($P=.39$). We then controlled for absence or presence of cluster B PD in aADHD; after exclusion of all aADHD participants with cluster B PD, the association of *NOS1* with aADHD remained significant ($P=.04$), arguing for the notion that the polymor-

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Table 2. Association of *NOS1* Ex1f VNTR SS Genotype With Disorders Featuring Externalizing, Disinhibited Behavior

Sample[a]	Size (N=3466)	Phenotype of Primary Interest	Psychiatric Assessment/Screening	SCID-II	% LL	% SL	% SS	*P* Value[b]
Control A	640	Population-based control	356 Yes, 284 no	No	28	51	21	
Control B	1314	Healthy controls	Yes	Yes	27	53	20	
PD[c]	403	Personality disorder	Yes	Yes	28	46	26	.01
aADHD	383	Adult ADHD	Yes	Yes	28	44	28	.002
Suicide	189	Suicide attempt	Yes	No	20	55	25	.02
Forensic	182	Aggression and violence	Yes	No	26	51	23	.04
fADHD	355	ADHD, family-based sample	Yes	No	27	49	24	.16

Abbreviations: aADHD, adult attention-deficit/hyperactivity disorder (ADHD); fADHD, familial ADHD; % LL, % SL, and % SS, percentage of the dichotomized variable number tandem repeat (VNTR) polymorphism in the promoter region of exon 1f of nitric oxide synthase (*NOS*) 1 (*NOS1* Ex1f VNTR) repeat allele (long-long, short-long, and short-short) in this sample; PD, personality disorder; SCID, Structured Clinical Interview for *DMS-IV*.

[a] Several samples were investigated in this study, all of which were ascertained in southern Germany; in the forensic sample (total n=233), only male subjects were further analyzed, and the percentages for violent subjects are given. *NOS1* Ex1f VNTR was found to be in Hardy-Weinberg equilibrium in all samples ($P>.1$).

[b] Significant difference from the respective control sample (PD, aADHD, suicide: control B; forensic: internal control sample and multivariate logistic regression analysis; fADHD, internal control sample and transmission disequilibrium test analysis). In all cases, the SS genotype was associated with the trait under investigation.

[c] Cluster B PD only; cluster C PD was not significantly associated with *NOS1* Ex1f VNTR.

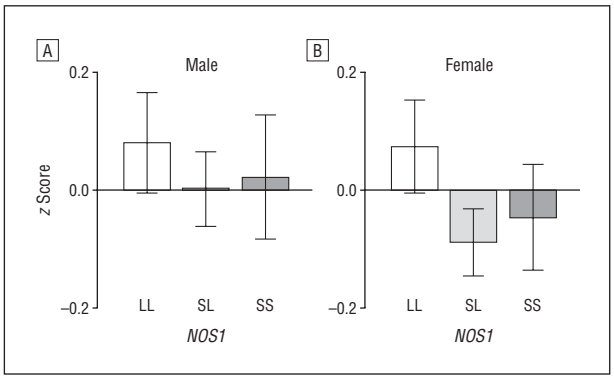

Figure 2. Influence of the variable number tandem repeat polymorphism in the promoter region of exon 1f of nitric oxide synthase (*NOS*) 1 (*NOS1* Ex1f VNTR) on the *conscientiousness* personality domain. Whereas in healthy male controls (A), *NOS1* Ex1f VNTR short allele carriers (SS) descriptively showed lower conscientiousness levels on the NEO Personality Inventory–Revised (NEO-PI-R), this effect was statistically significant in healthy female controls (B; $P=.001$), indicating that women with either 1 or 2 short *NOS1* Ex1f VNTR alleles tend to behave with less persistent goal-directed behavior, ie, they feature increased impulsivity. Bars represent estimated marginal means; limit lines, SEM. LL indicates long-long; SL, short-long.

phism conveys risk for persistence of ADHD into adulthood independently of comorbid PD. In the forensic sample, childhood adversity was controlled for because it was shown to be a risk factor for later-life violence.[14] Short *NOS1* Ex1f VNTR alleles were independently associated with later-life violent crime ($P=.04$; eTable 2). Finally, suicide attempters also carried short alleles more frequently ($P=.02$).

Taken together, these findings provide evidence of an association between short *NOS1* Ex1f VNTR alleles and disorders characterized by impulsive, hyperactive, and aggressive behaviors. To uncover possible underlying dimensional variation in personality, the revised NEO-PI-R[20] was applied in controls. In healthy controls (n=1099), an *NOS1* Ex1f VNTR × sex interaction on the personality domain conscientiousness was demonstrated (eTable 3). There was a nominal trend to lower scores in male short-allele carriers, whereas this effect was statistically significant for women ($P=.001$; **Figure 2**).

EFFECT OF *NOS1* Ex1f VNTR ON PREFRONTAL BRAIN FUNCTIONING

Diminished activity of the prefrontal cortex is suggested to play a role in increased impulsivity. This prompted us to investigate a subset of control subjects (n=167) by using a CPT with parallel recording of event-related potential and subsequent topographical analysis. The no-go centroid, a measure of medial prefrontal activity during response inhibition, was localized significantly more posteriorly in carriers of the short *NOS1* Ex1f VNTR allele, in a gene–dose-dependent fashion ($F_{2,167}=3.29$, $P<.05$; **Figure 3**), as was nominally also the case for schizophrenic patients.[8] This indicates that activation of the anterior cingulate cortex (ACC), which is the major neural source for the specific topography of the no-go centroid,[24] is diminished in short-allele carriers, suggesting impaired medial prefrontal functioning in these subjects.

COMMENT

Rodent studies using pharmacologic methods and genetically modified animals[5,10] suggest that NOS-I has a role in regulating impulsive-aggressive behaviors. We thus investigated whether genetic variation in *NOS1* influences analogous behaviors in humans as well. This was indeed the case because a functional promoter polymorphism (*NOS1* Ex1f VNTR) was associated with personality dimensions related to impulsivity as well as psychiatric disorders that share impulsive behavior as a common phenotype (namely, aADHD, cluster B PD, and aggression). Together with *MAO-Au*VNTR[3] and *5HTT*LPR,[1] *NOS1* Ex1f VNTR thus belongs to a class of promoter repeat polymorphisms that alter gene expression *and* have been shown to be associated with variation in human behavior.

To demonstrate functionality of the polymorphism, we established that short variants of the repeat result in decreased reporter gene expression. It thus can be assumed that short promoter repeats also decrease the expression of the alternative *NOS1* exon 1f. The consequences, however, remain unclear. In enteric neurons, it was shown that

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reduced *NOS1* exon 1c expression was compensated for by upregulation of *NOS1* exon 1f,[25] suggesting a reciprocal regulation of both alternative first exons. It can therefore be speculated that, on reduced *NOS1* exon 1f transcription, a counter-regulatory increase of *NOS1* exon 1c occurs. Further consequences of diminished *NOS1* exon 1f expression might include altered messenger RNA stability, differential messenger RNA maturation, or changes in the intracellular trafficking of messenger RNA. Although the detailed molecular action of *NOS1* Ex1f VNTR is not known yet, it has a role in intracellular mechanisms as reflected by changes in the transcriptome of human brain tissue, indicated by microarray data from BA46 (Table 1). In a recent meta-analysis incorporating 18 studies,[26] this brain region belongs to the neurocircuitry of impulsivity, as assessed by functional magnetic resonance imaging using no-go tasks, a method comparable to our event-related potential study (Figure 3), suggesting that these expressional changes might indeed have a role in the regulation of impulsive behavior.

Interestingly, several of the genes identified to be dysregulated are directly related to nitric oxide (NO) function. Some of these genes code for enzymes involved in reactive oxygen detoxification mechanisms and form a common pathway: superoxide dismutase 2, glutathione peroxidase 3, and mitogen-activated protein kinase (*MAPK8*). The last of these is activated by stressful stimuli,[27] negatively regulated by NO,[28] and in turn suppresses superoxide dismutase 1 expression.[29] Thus, *NOS1* Ex1f VNTR seems to be associated with an imbalance of genes related to NO function. A second set of genes corresponds to cytoskeletal proteins (β4- and β5-tubulin, MAP1LC3B, and NEFL).

Most interestingly, however, a number of genes were previously implicated in psychiatric disorders: *CHI3L1*, involved in Akt signaling, has been proposed as a schizophrenia susceptibility gene,[30] probably by counteracting pro-inflammatory cytokines. α-Synuclein is a key player in the pathogenesis of neurodegenerative disorders.[31] Probably by nitrosylation,[32] NO increases the number of α-synuclein aggregates, thereby probably promoting disease progression. With respect to impulsive behaviors, it appears noteworthy that α-synuclein has also been suggested as a candidate gene for alcoholism.[33] Several upregulated genes were already shown to have a role both in glutamatergic signaling and schizophrenia: *RGS4* can be considered one of the most promising schizophrenia candidate genes, as underscored by a recent meta-analysis,[34] and affects *N*-methyl D-aspartate 1 (NMDA) signaling.[35] Also converging on the glutamatergic synapse, NEFL increases the cell surface expression of the NMDA receptor and consecutive NMDA-mediated toxicity.[36] Finally, and most notably, *GRIN1*, the gene encoding the upstream NOS-I activator NMDA receptor 1, is dysregulated as a function of *NOS1* Ex1f VNTR. Our previous finding that this polymorphism affects disease severity in schizophrenia[8] thus might at least in part be explained by its effects on glutamatergic genes. Taken together, these data indicate a deficit in specific molecular pathways in short-allele carriers, all of which represent important molecular cascades in psychiatric disease: oxidative stress, neuronal cytoskeleton, and glutamatergic signaling.

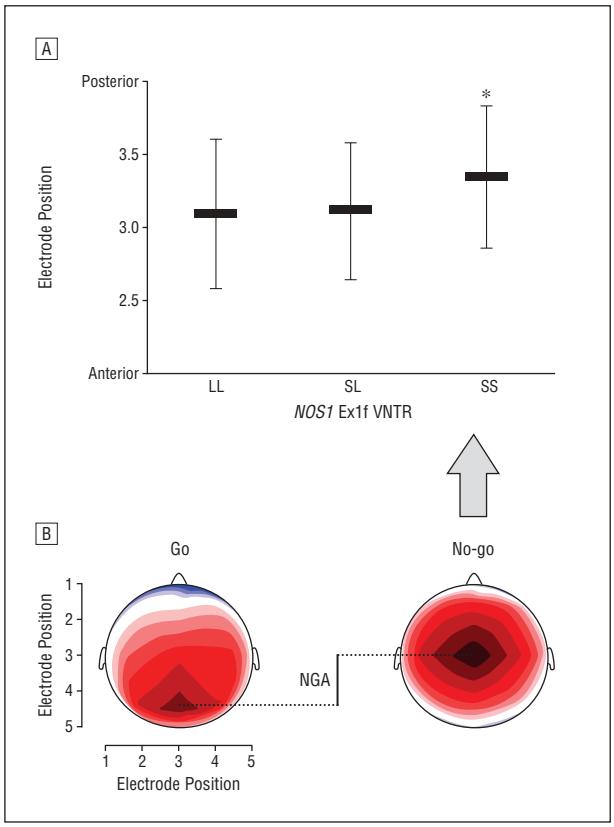

Figure 3. Influence of the variable number tandem repeat (VNTR) polymorphism in the promoter region of exon 1f of nitric oxide synthase (*NOS*) 1 (*NOS1* Ex1f VNTR) on the function of the anterior cingulate cortex (ACC). A, A total of 167 healthy controls were genotyped for *NOS1* Ex1f VNTR and underwent a continuous performance test with parallel recording of event-related potentials; those were thereafter analyzed topographically by the centroid method. The "no-go" centroid was localized significantly (*) more posteriorly in homozygous *NOS1* Ex1f VNTR short allele carriers (SS) than in both homozygous long allele carriers (LL) (t_{74}=2.17, *P*=.03) and subjects with a heterozygous genotype (SL) (t_{125}=2.42, *P*=.02). Because the ACC is the major source for the no-go centroid topography, this points toward impaired function of the medial prefrontal cortex in these subjects, which probably underlies an improper cognitive control of initiated responses resulting in impulsive behaviors. Horizontal bars indicate mean values; limit lines, SD. B, Topographic maps illustrating the position of the "go" centroid compared with the no-go centroid; the more anterior localization of the no-go centroid during the continuous performance test is commonly referred to as the *no-go anteriorization* (NGA).

Our case-control association studies in several independent, large samples argue for a role of *NOS1* Ex1f VNTR in disorders with the shared feature of impulsive behaviors,[37] a core feature and diagnostic criterion of aADHD and cluster B PD[38] alike. Furthermore, increased impulsivity is thought to underlie or facilitate at least some forms of suicidal[39] and aggressive[40] behavior. The aforementioned categorical phenotypes are not independent and should therefore be viewed from a quantitative standpoint, as aADHD has a high comorbidity with cluster B PD[12]; both conditions go along with an increased risk for autoaggressive and heteroaggressive behavior. Thus, *NOS1* Ex1f VNTR might be a genetic factor underlying those conditions, partially explaining their interrelatedness with emphasis on impulsivity as a common factor. Given that those disorders feature complex genetics, this possible explanation for their interrelatedness strongly argues for a dimensional view of these phe-

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notypes. This argument, however, should also be reflected by parallel alteration of personality dimensions, as assessed, eg, by personality questionnaires; indeed, this was the case because *NOS1* Ex1f VNTR influenced conscientiousness.

Low conscientiousness levels indicate high impulsivity, ie, they correlate negatively with impulsivity.[41,42] Despite the fact that impulsivity is a facet of NEO-PI-R neuroticism, the biological trait of impulsivity might rather be mirrored by the conscientiousness domain.[43] Underscoring this notion, both cluster B PD and aADHD feature significantly decreased conscientiousness scores.[12,44] A complex effect of *NOS1* Ex1f VNTR on conscientiousness became evident as we observed a gene × sex interaction. Female carriers of the short *NOS1* Ex1f VNTR allele tend to act less diligent and persistent, with an overall lower motivation in goal-directed behavior. The biological rationale behind the sex specificity might be a *cis*-acting estrogen binding site upstream of the *NOS1* Ex1f VNTR–containing region, outside the reporter gene construct, which itself was not sensitive to estrogen. Because age correlates positively with conscientiousness,[45] and because early adversity also affects this personality dimension[46] as well as later-life violence,[14] these variables should be assessed for their interaction with *NOS1* Ex1f VNTR genotype in future studies.

Taken together, *NOS1* Ex1f VNTR is associated with a personality dimension related to cognitive control and inhibition, relevant to the categorical phenotypes associated with *NOS1* (ie, aADHD, cluster B PD, and aggression), and thereby contributes to the genetic risk of psychiatric disorders that have dysfunctional impulsivity[47] as a common feature. Molecular mechanisms might include dysregulation of glutamatergic neurotransmission but also interactions of NO with the serotonergic system. Serotonin levels, which are regulated by serotonin transporter, have been suggested to be crucial in the regulation of impulsivity[48]; accordingly, selective serotonin reuptake inhibitors are the treatment of choice for disorders of impulse control. Interestingly, NOS-I reciprocally interacts with serotonin transporter, causing a reduction of serotonin uptake.[49] High *NOS1* expression thus might have the same functional consequences as selective serotonin reuptake inhibitor treatment. Alternative explanations for the association findings are, however, also possible. For example, deficits in cognitive functioning underlie most of the conditions examined in this study: *NOS1* Ex1f VNTR may not influence impulsivity per se but may influence cognitive abilities, a hypothesis that is well worth further investigation. However, in the control sample (n=1216), *NOS1* Ex1f VNTR was not associated with overall IQ ($F_{1,1208}=1303$, $P=.27$), even if this does not necessarily rule out the possibility that NOS-I influences more specific cognitive functions.

Although impulsivity is considered neurobiologically heterogeneous,[48] behavioral inhibition is regarded to represent an important endophenotype that can be assessed by go/no-go tasks.[50] Because the prefrontal cortex is implicated in behavioral inhibition and impulsivity, and because our previous data suggested impaired prefrontal functioning due to *NOS1* Ex1f VNTR in schizophrenic patients, the approach of imaging genetics by means of event-related potentials that has been developed and applied in our laboratory[51] was used to probe the hypothesis that genetic variation of *NOS1* Ex1f VNTR affects ACC functioning. Indeed, carriers of risk alleles displayed decreased activation of the ACC, as evidenced by more posterior localization of the no-go centroid. This might well be a neural circuitry–dependent mechanism in how *NOS1* Ex1f VNTR genotype exerts its influence on cognitive control and psychiatric disease states. The ACC is thought to be involved not only in error detection but also in the recognition of effort-reward correlations, thus comprising an outcome monitoring system.[52] Genetically driven hypoexcitability of the ACC might thus lead to blunted response toward long-term behavioral consequences, thereby resulting in more immediate and uncontrolled actions underlying disinhibited, impulsive, and impaired goal-directed behavior as reflected by the personality domain conscientiousness. Further support for involvement of *NOS1* in prefrontal functioning comes from a recent study[53] demonstrating an association of a 3′UTR *NOS1* single nucleotide polymorphism with frontotemporal dementia; nevertheless, the underlying molecular mechanisms remain to be established.

In conclusion, we have demonstrated that a repeat polymorphism in the alternative promoter of exon 1f of neuronal *NOS1* (1) affects promoter activity, (2) influences the transcriptome of the human brain, (3) is associated with psychiatric disorders with the shared feature of impulsivity, (4) is associated with personality traits related to cognitive control, and (5) affects electrocortical measures of prefrontal functions involved in cognitive control. Translational phenotyping of low *NOS1* expression in mice and humans thus converges to the notion that deficits in neuronal signaling via gaseous NO are associated with impulsive behaviors and impaired prefrontal brain functioning.

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