Characterization of a Functional Promoter Polymorphism of the Human Tryptophan Hydroxylase 2 Gene in Serotonergic Raphe Neurons

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Background: Tryptophan hydroxylase 2 (TPH2) is the rate-limiting enzyme in brain serotonin (5-HT) biosynthesis. Although dysfunction of 5-HT neurotransmission has been implicated in a variety of neuropsychiatric conditions, the human TPH2 promoter has not been characterized in vitro.

Methods: The functional relevance of TPH2 promoter polymorphisms was determined with luciferase assays in primary serotonergic neurons from rat raphe nuclei and in human small cell lung carcinoma cells (SHP-77 cells). We also investigated transcription factor binding to the variant promoter sequence with electrophoretic mobility shift assay (EMSA).

Results: The polymorphism rs11178997 of the human TPH2 promoter significantly reduced TPH2 transcriptional activity by 22% and 7% in primary serotonergic neurons and in SHP-77 cells, respectively. In contrast, no significant differences in promoter activity were observed for the G- and T-alleles of rs4570625. The EMSA revealed reduced binding of the transcription factor POU3F2 (also known as Brn-2, N-Oct-3) to the A-allele of the polymorphism rs11178997. Overexpression of POU3F2 resulted in a robust activation of the TPH2 promoter (2.7-fold).

Conclusions: Our data suggest that the human TPH2 promoter polymorphism rs11178997 impacts on gene expression, which might have implications for the development and function of the serotonergic system in the brain.

Key Words: Brain-2, haplotype, POU transcription factor, serotonin, SHP-77 cells, single nucleotide polymorphism, TPH2

Serotonin (5-hydroxytryptamine or 5-HT) is a monoamine neurotransmitter involved in a variety of behavioral functions, including mood, anxiety, stress, appetite, nociception, alertness, and sleep (Lucki *et al.* 1998). Serotonergic dysfunction has been implicated in a variety of neuropsychiatric conditions, including depression, bipolar disorder, schizophrenia, autism, attention-deficit/hyperactivity disorder (ADHD), aggression, and suicidal behavior (Bondy *et al.* 2006; Lucki *et al.* 1998; Malhotra *et al.* 2004; Young *et al.* 1994). The neurons that produce 5-HT in the central nervous system (CNS) are mostly located in the brainstem raphe nuclei, of which the rostral group (dorsal and median raphe nuclei) provides a dense innervation to the forebrain.

The rate-limiting enzyme in 5-HT biosynthesis is tryptophan hydroxylase (TPH; Walther and Bader 2003). The homotetrameric enzyme catalyzes the hydroxylation of tryptophan to 5-hydroxytryptophan, which is subsequently decarboxylated to 5-HT by aromatic amino acid decarboxylase. Targeted ablation of the *TPH* gene in mice revealed a second TPH isoform (TPH2, neuronal TPH), which was detected exclusively in the brain and in neurons of the myenteric plexus (Côté *et al.* 2003; Walther *et al.* 2003). Expression studies in rat and human brain revealed that *TPH2* messenger RNA (mRNA) is expressed predominantly in the raphe nuclei and is more abundant than *TPH1* mRNA, which is expressed predominantly in the pineal gland (Côté *et al.* 2003; Patel *et al.* 2004; Walther and Bader 2003; Zill *et al.* 2005). No expression of *TPH2* mRNA was found in peripheral tissues, including heart, lung, kidney, duodenum, liver, and adrenal gland (Côté *et al.* 2003; Zill *et al.* 2004b). A functional *TPH2* single nucleotide polymorphism (SNP) was associated with reduced 5-HT levels in the murine CNS, suggesting that TPH2 controls the brain 5-HT synthesis (Zhang *et al.* 2004).

The discovery of the neuronal-specific *TPH2* provided a novel candidate gene for 5-HT–related neuropsychiatric disorders. An SNP in intron 5 of *TPH2* was associated with major depression and suicide, and the findings were supported by haplotype analysis (Harvey *et al.* 2004; Zill *et al.* 2004a, 2004c). Two SNPs in introns 1 and 4 of *TPH2* were associated with autism, and several haplotypes including an SNP in intron 5 were more prevalent in ADHD (Coon *et al.* 2005; Sheehan *et al.* 2005).

Previous studies of variants in other genes involved in serotonergic neurotransmission have underscored the importance of examining regulatory regions. Thus, the short variant promoter polymorphism of the human 5-HT transporter (*5-HTT*) was associated with a 30%–40% reduction in *5-HTT* mRNA expression and a 50% reduction in 5-HT uptake (Lesch *et al.* 1996). This frequent promoter polymorphism has been associated with anxiety-related personality traits, depression, and enhanced responses of the human amygdala to environmental threats (Caspi *et al.* 2003; Hariri *et al.* 2002; Lesch *et al.* 1996). Interestingly, a common SNP of the *TPH2* promoter, rs4570625, was also associated with increased amygdala reactivity to emotional stimuli (Brown *et al.* 2005; Canli *et al.* 2005). Moreover, two polymorphisms of the *TPH2* promoter, rs4570625 and rs11178997, are included in a haplotype block, which was linked to anxiety disorder, major depression, and suicide attempts in US and Finnish whites (Zhou *et al.* 2005).

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The aim of the present study was to establish a cell culture system for the characterization of the human *TPH2* promoter and to shed light on the functional relevance of human *TPH2* promoter variants. With luciferase assays in primary rat serotonergic raphe neurons and in human small cell lung carcinoma cells, we identified a functional polymorphism of the human *TPH2* promoter.

Methods and Materials

Subjects

A total of 58 patients with major depression and a history of one or more suicide attempts were selected from a larger pharmacogenetic study population (Kirchheiner *et al.* 2006). Diagnoses were determined by the Mini International Neuropsychiatric Interview. Patients gave written informed consent. The institutional review board of the Charité approved the study.

Genotyping

Genomic DNA was extracted from human blood according to standard procedures. A 1-kilobase (kb) fragment of the 5′ regulatory region of *TPH2* was amplified by polymerase chain reaction (PCR) with primer pair 1 and 2 (Supplement 1). Amplicons were sequenced (DLMBC Berlin, Germany). We used D′ to describe the magnitude of linkage disequilibrium (LD) (Lewontin *et al.* 1988). For calculation of pairwise LD between the SNPs, we used the program HAPLOVIEW (Barrett *et al.* 2005).

Vector Construction

Luciferase reporter plasmids were generated, which included the different haplotypes of the *TPH2* promoter: G/T/A (wild type), T/A/G, T/T/A corresponding to SNPs: rs4570625/rs11178997/rs11178998. The 1-kb amplicons of the *TPH2* promoter were cloned into the pGL3-Basic plasmid (Promega, Madison, Wisconsin). Additionally, we created the promoter construct G/A/A, which only contains the SNP rs11178997 in the core sequence of the POU3F2 binding site, by digesting the pGL3-Basic plasmid containing the T/A/G promoter haplotype with *Eco*R V and *Pvu* II and cloning the promoter fragment into the wild type promoter construct G/T/A digested with *Eco*R V and *Pvu* II.

Two expression vectors were generated, in which the red fluorescent protein, DsRed-Express, is driven by a 4-kb fragment and a 1-kb fragment of the human *TPH2* promoter. For the generation of the 4-kb *TPH2* promoter fragment we used primer pair 3 and 4 (Supplement 1). Blunt-ended DNA fragment were cloned into the pDsRed-Express-N1 vector (Clontech, Mountain View, California), which had been digested with *Pci* I and *Bgl* II to remove the cytomegalovirus (CMV) promoter. The human *POU3F2* sequence was excised from pEV-NOct-3 vector (kindly provided by J. Burbach, University of Utrecht) and cloned into pcDNA3.1 vector (Invitrogen, Carlsbad, California). The CMV promoter-driven *PQBP1* expression vector was kindly provided by V. Kalscheuer (Max-Planck-Institute for Molecular Genetics, Berlin, Germany).

Cell Culture and DNA Transfection

Primary serum-free cultures of serotonergic raphe neurons were generated from fetal Wistar rats at embryonic ages E14–E15 as described (Lautenschlager *et al.* 2000). The human small cell lung carcinoma line, SHP-77 (ATCC), was maintained in RPMI 1640 medium supplemented with 10% fetal calf serum and 100 U/mL penicillin/streptomycin at 37°C with 5% carbon dioxide.

Primary neurons and SHP-77 cells were transfected with firefly luciferase and DsRed-Express constructs with lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Primary neurons were transfected between 8 and 14 days in vitro. Cells were harvested and analyzed 48 hours after transfection.

Luciferase Assays

Reporter activities were measured with a Lumat LB 9501 (Berthold Technologies, Bad Wildbad, Germany) and the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions. Cotransfection with PRL-null vector served as an internal standard to adjust for the differences in transfection efficiency. Promoter activity is expressed as relative luciferase units (RLU), which is defined as the value of the firefly luciferase/renilla luciferase ratio of each construct related to the empty pGL3-Basic vector.

Immunohistochemistry/Immunocytochemistry

Rats were perfused transcardially with saline followed by 4% paraformaldehyde in phosphate-buffered saline. The brains were postfixed at 4°C overnight, and 30-μm coronal sections were obtained from the caudal midbrain. A polyclonal antibody against TPH2 was generated by immunization of rabbits with an aminoterminal peptide (PAMMMFSSKYWARRG; BioGenes, Berlin, Germany) and affinity purification of the obtained antisera. Incubation with the antibody against TPH2 was performed at a dilution of 1:100 at 4°C overnight. A donkey anti-rabbit secondary antibody conjugated to Alexa Fluor 594 (1:600, Molecular Probes, Eugene, Oregon) was used. For POU3F2 immunofluorescence staining, we used a goat polyclonal antibody against POU3F2 (Santa Cruz Biotechnology, Santa Cruz, California) at a dilution of 1:200, which was visualized with a donkey anti-goat secondary antibody conjugated to Alexa Fluor 488 (1:600, Molecular Probes). Nuclei were stained with Vectashield Mounting Medium with DAPI (Vector Laboratories, Burlingame, California). In all cases, omission of primary antibodies served as negative control.

Western Blot Analysis

Western blots were performed according to standard protocols with the rabbit polyclonal antibody against TPH2 (1:1000) or a sheep polyclonal antibody against TPH (1:1000, Chemicon, Temecula, California), which does not discriminate between TPH1 and TPH2.

Secondary antibodies and reagents were: a donkey anti-rabbit secondary antibody conjugated to horseradish peroxidase (1:3000, Amersham Biosciences, Pittsburgh, Pennsylvania), a secondary biotinylated rabbit anti-sheep antibody (1:3000, Vector), streptavidin-conjugated horseradish peroxidase (1:3000, Chemicon). Antibody binding was visualized with luminol reagent (Santa Cruz Biotechnology).

Northern Blot and Electrophoretic Mobility Shift Assays

Northern hybridization was performed as described with the same probe as in previous RNAse protection assays (Priller *et al.* 1995; Walther *et al.* 2003).

For electrophoretic mobility shift assay (EMSA), nuclear extracts from SHP-77 cells containing endogenous POU3F2 were prepared according to standard procedures. Recombinant POU3F2 was obtained after transfection of COS-7 cells with a POU3F2 expression vector. Cell extracts were generated by homogenization of the cells and differential centrifugation. All buffers contained protease inhibitors free of ethylene diamine tetraac-