Neuroscience 146 (2007) 1662–1676

CHARACTERIZATION OF THE SEROTONIN TRANSPORTER KNOCKOUT RAT: A SELECTIVE CHANGE IN THE FUNCTIONING OF THE SEROTONERGIC SYSTEM

J. R. HOMBERG,[a] J. D. A. OLIVIER,[b] B. M. G. SMITS,[a1] J. D. MUL,[a] J. MUDDE,[a] M. VERHEUL,[a] O. F. M. NIEUWENHUIZEN,[c] A. R. COOLS,[b] E. RONKEN,[c] T. CREMERS,[d] A. N. M. SCHOFFELMEER,[e] B. A. ELLENBROEK,[b1] AND E. CUPPEN[a]*

[a]*Hubrecht Laboratory, Uppsalalaan 8, 3584 CT Utrecht, The Netherlands*

[b]*Department of Psychoneuropharmacology, Radboud University Nijmegen, The Netherlands*

[c]*Solvay Pharmaceuticals Research Laboratories, Weesp, The Netherlands*

[d]*Department of Pharmacy, Groningen University, Groningen, The Netherlands*

[e]*Department of Anatomy and Neurosciences, VU Medical Center, Amsterdam, The Netherlands*

Abstract—Serotonergic signaling is involved in many neurobiological processes and disturbed 5-HT homeostasis is implicated in a variety of psychiatric and addictive disorders. Here, we describe the functional characterization of the serotonin transporter (SERT) knockout rat model, that is generated by N-ethyl-N-nitrosurea (ENU)-driven target-selected mutagenesis. Biochemical characterization revealed that SERT mRNA and functional protein are completely absent in homozygous knockout ($SERT^{-/-}$) rats, and that there is a gene dose-dependent reduction in the expression and function of the SERT in heterozygous knockout rats. As a result, 5-HT homeostasis was found to be severely affected in $SERT^{-/-}$ rats: 5-HT tissue levels and depolarization-induced 5-HT release were significantly reduced, and basal extracellular 5-HT levels in the hippocampus were ninefold increased. Interestingly, we found no compensatory changes in *in vitro* activity of tryptophan hydroxylase and monoamine oxidase, the primary enzymes involved in 5-HT synthesis and degradation, respectively. Similarly, no major adaptations in non-serotonergic systems were found, as determined by dopamine and noradrenaline transporter binding, monoamine tissue levels, and depolarization-induced release of dopamine, noradrenaline, glutamate and GABA. In conclusion, neurochemical changes in the SERT knockout rat are primarily limited to the serotonergic system, making this novel rat model potentially very useful for studying the behavioral and neurobiological consequences of disturbed 5-HT homeostasis.

Key words: target-selected mutagenesis, serotonin transporter, 5-HT homeostasis, 5-HTTLPR.

5-HT, the most ancient neurotransmitter, plays an important modulatory role in emotion, motivation and cognition, and has a function in gut and neuroendocrine systems. Hence, disturbed 5-HT homeostasis contributes to many disorders, including affective disorders, drug addiction, schizophrenia, eating disorders, impulse control disorders and irritable bowel syndrome (Murphy et al., 2004). Because of the complexity of the serotonergic system, which consists of at least 14 different receptor subtypes, it is still not yet completely understood how 5-HT contributes to these disorders, which hampers the development of effective medication.

Extracellular 5-HT levels are increased upon activation of serotonergic neurons that originate from the raphe nuclei (RN) and project to almost all parts of the brain. 5-HT homeostasis is primarily regulated by the Na^+/Cl^--dependent serotonin transporter (SERT), which reuptakes 5-HT from the extracellular space into the presynaptic serotonergic nerve terminal to recycle 5-HT for future release (Murphy et al., 1998). The SERT is of interest in many research areas as it is the target for selective serotonin reuptake inhibitors (SSRI's), which have therapeutic efficacy in several neuropsychiatric disorders, including depression (Murphy et al., 1998). Furthermore, psychostimulants such as cocaine, amphetamine and related drugs act on it (Ritz and Kuhar, 1989; Ritz et al., 1990). Interestingly, humans carry a polymorphism in the promoter region of the SERT gene (5-HTTLPR), which involves a common 44-base pair insertion/deletion of a repetitive sequence (Lesch et al., 1996). The dominant short (s) allelic variant reduces transcriptional efficiency of the SERT as compared with the long (l) allelic variant (Lesch et al., 1996), and is associated with reduced brain SERT mRNA levels (Little et al., 1998), reduced SERT binding sites (Heils et al., 1996), and a 40% decrease in 5-HT re-uptake in blood platelets (Greenberg et al., 1999). In line with the wide range of processes mediated by 5-HT and the effects

[1] Present address: Evotec Pharmaceutical Division, Neuropharmacology, Hamburg, Germany (B. A. Ellenbroek); McArdle Laboratory for Cancer Research, University of Wisconsin, Madison, WI, USA (B. M. G. Smits).

*Corresponding author. Tel: +31-30-2121969; fax: +31-30-2516554. E-mail address: ecuppen@niob.knaw.nl (E. Cuppen).

Abbreviations: Ach, acetylcholine; CP, caudate putamen; cpm, counts per minute; DA, dopamine; DAT, dopamine transporter; DMI, desmethylimipramine; ENU, N-ethyl-N-nitrosurea; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MAO, monoamine oxidase; NE, noradrenaline; NET, noradrenaline transporter; PBS, phosphate-buffered saline; RN, raphe nuclei; SERT, serotonin transporter; $SERT^{-/-}$, homozygous serotonin transporter knockout rat; $SERT^{+/-}$, heterozygous serotonin transporter knockout rat; $SERT^{+/+}$, wildtype littermate of heterozygous serotonin transporter knockout and homozygous serotonin transporter knockout rats; SSRI, selective serotonin reuptake inhibitor; TPH, tryptophan hydroxylase; 5-HIAA, 5-hydroxyindoleacetic acid; 5-HTP, 5-hydroxytryptophan.

0306-4522/07$30.00+0.00 © 2007 IBRO. Published by Elsevier Ltd. All rights reserved.
doi:10.1016/j.neuroscience.2007.03.030

of drugs targeting the SERT, this common polymorphism has been linked to various, mostly psychiatric, disease states, from affective disorders to alcoholism, obsessive compulsive disorder and drug dependence (Bengel et al., 1999; Caspi et al., 2003; Gerra et al., 2004).

Animal models are highly informative to study both the biochemical as well as the behavioral consequences of disturbed 5-HT homeostasis and may reveal the contribution of compensatory adaptations that could serve as novel targets for medication. The SERT knockout mouse has revealed that ablation of the SERT increases extracellular 5-HT levels (Fabre et al., 2000; Mathews et al., 2004) and induces several behavioral phenotypes, which include anxiety, and depression-like symptoms (Holmes et al., 2002b, 2003; Lira et al., 2003; Adamec et al., 2006), reduced aggression (Holmes et al., 2002a), and altered responses to drugs of abuse (Bengel et al., 1998; Sora et al., 1998). In some research areas the rat is traditionally one of the preferred animal models. Especially in the area of complex cognitive tasks, addiction, and pharmacology, a lot of data in the rat model have been acquired over the last decades. To allow for cross-species comparison with mouse (knockout) data and extrapolation to the human situation, a SERT knockout model in the rat would complement existing tools and help in understanding the complexity of the serotonergic modulation of major human disease states.

Recently, we generated such a model by N-ethyl-N-nitrosurea (ENU)-driven target-selected mutagenesis (Smits et al., 2006) and here we describe the primary biochemical characterization of this novel model and the functional consequences for the serotonergic as well as the major non-serotonergic neurotransmitter systems. We show that, in line with the central role of SERT in regulating extracellular 5-HT levels, 5-HT homeostasis is dramatically disturbed in homozygous SERT knockout ($SERT^{-/-}$) rats. Interestingly, no adaptations were observed in the presynaptic functioning of non-serotonergic systems, indicating that the primary consequences of the absence of SERT are restricted to the serotonergic system.

EXPERIMENTAL PROCEDURES

Animals

All experiments were approved by the Animal Care Committee of the Royal Dutch Academy of Science, the Free University of Amsterdam, the Radboud University Nijmegen, and the University of Groningen according to the Dutch legal ethical guidelines. Experiments were designed to minimize the number of required animals and their suffering.

The SERT knockout rat ($Slc6a4^{1Hubr}$) was generated by target-selected ENU-induced mutagenesis (for detailed description, see Smits et al., 2006). Briefly, high-throughput resequencing of genomic target sequences in progeny from mutagenized rats revealed an ENU-induced premature stop codon in exon 3 of the *SERT* gene in a female rat (Wistar/Crl background). The heterozygous mutant animal was outcrossed for at least six generations to a Wistar background to eliminate confounding effects from other mutations that may have been induced by the ENU mutagenesis. Under the used mutagenesis conditions the mean mutation frequency was roughly 1 in 1.2 million base pairs (about 1 cM). Although the chance for the occurrence of a strongly linked mutation with a phenotypic effect is very small, this possibility should be taken into account in the design and interpretation of experiments. To control for this possibility as much as possible, we always generated experimental animals by incrosses between outcrossed heterozygous SERT knockout ($SERT^{+/-}$) rats. Furthermore, we compared as much as possible wild type ($SERT^{+/+}$) and mutant littermates. Finally, we have repeated several measurements in multiple backcross generations and could perfectly replicate previous findings. At the age of 3 weeks ear cuts were taken and used for genotyping. Genotypes were reconfirmed after experimental procedures were completed. Animals were housed under standard conditions in groups of two to four per cage per gender under controlled experimental conditions (12-h light/dark cycle, 21±1 °C, 60% relative humidity, food and water *ad libitum*). Only male rats at the age of 11–15 weeks were used for experiments.

Genotyping

DNA isolation and genotyping were performed as described previously (Smits et al., 2006). In brief, a 670 bp fragment of the *SERT* gene, containing the ENU-induced mutation was amplified using gene-specific primers (forward, TCACAAAGCACTGAGACCAG; reverse, AACCTGCCAAGAGAGAGTTG) and a touchdown PCR cycling program (92 °C for 60 s; 12 cycles of 92 °C for 20 s, 65 °C for 20 s with a decrement of 0.6 °C per cycle, 72 °C for 30 s, followed by 20 cycles of 92 °C for 20 s, 58 °C for 20 s and 72 °C for 30 s; 72 °C for 180 s; GeneAmp9700, Applied Biosystems, Foster City, CA, USA). The PCR reactions were diluted with 25 μl water, and 1 μl was used as template for the dideoxy sequencing reactions, which were preformed according to the manufacturer instructions (Applied Biosystems). Sequencing products were purified using Sephadex G50 (superfine, coarse, Sigma, Zwijndrecht, The Netherlands) mini-columns and analyzed on a 96-capillary 3730XL DNA analyzer (Applied Biosystems). Sequences were analyzed for polymorphisms using polyphred (Nickerson et al., 1997) and manual inspection of the mutated position.

Northern blot analysis

The dorsal and medial RN, which contain large amounts of SERT mRNA, were dissected quickly from male $SERT^{+/+}$, $SERT^{+/-}$ and $SERT^{-/-}$ rats with sterilized instruments on an ice plate. Poly A^+ RNA was isolated using a MicroPoly(A) Purist Isolation Kit (Ambion Inc., Austin, TX, USA) according to the manufacturer's instructions. Poly A^+ RNA (0.75 μg) was fractionated by electrophoresis on an agarose gel (1%), containing 0.6 M formaldehyde, and transferred by capillary elution during 16–24 h to nylon hybridization membranes (Hybond-N, GE Healthcare, Chalfont St. Giles, UK). The RNA was UV-crosslinked to the membrane by a UV-stratalinker 1800 (Stratagene, Amsterdam, The Netherlands) (2×1200 μJ×100). Following pre-hybridization of the membrane for 1 h at 65 °C in pre-hybridization buffer (0.36 M $Na_2HPO_4.2H_2O$, 0.14 M $NaH_2PO_4.2H_2O$, 0.5 M EDTA, 20% SDS), a radiolabeled PCR-generated SERT probe (forward primer, GCCTAGCCAAATATCCAATG; reverse primer, GCCAGTTGGGTTTCAAGTAG), generated using Megaprime DNA labeling system (GE Healthcare) in the presence of [α-32P]dCTP (3000 Ci/mmol), was heat denaturated together with 50 μg/ml salmon sperm DNA at 98 °C for 10 min. The samples were added to the hybridization buffer for overnight hybridization (65 °C). Membranes were exposed to a phosphor screen and results were visualized using a 9200 Typhoon scanner (GE Healthcare). Densitometric analysis of autoradiograms was performed using ImageQuant 5.2 software. After stripping the same membrane was re-used for a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe hybridization that served as internal standard.

Quantitative autoradiography

Rats were killed by decapitation and brains were rapidly removed. The brains were frozen in aluminum cups, which were held for