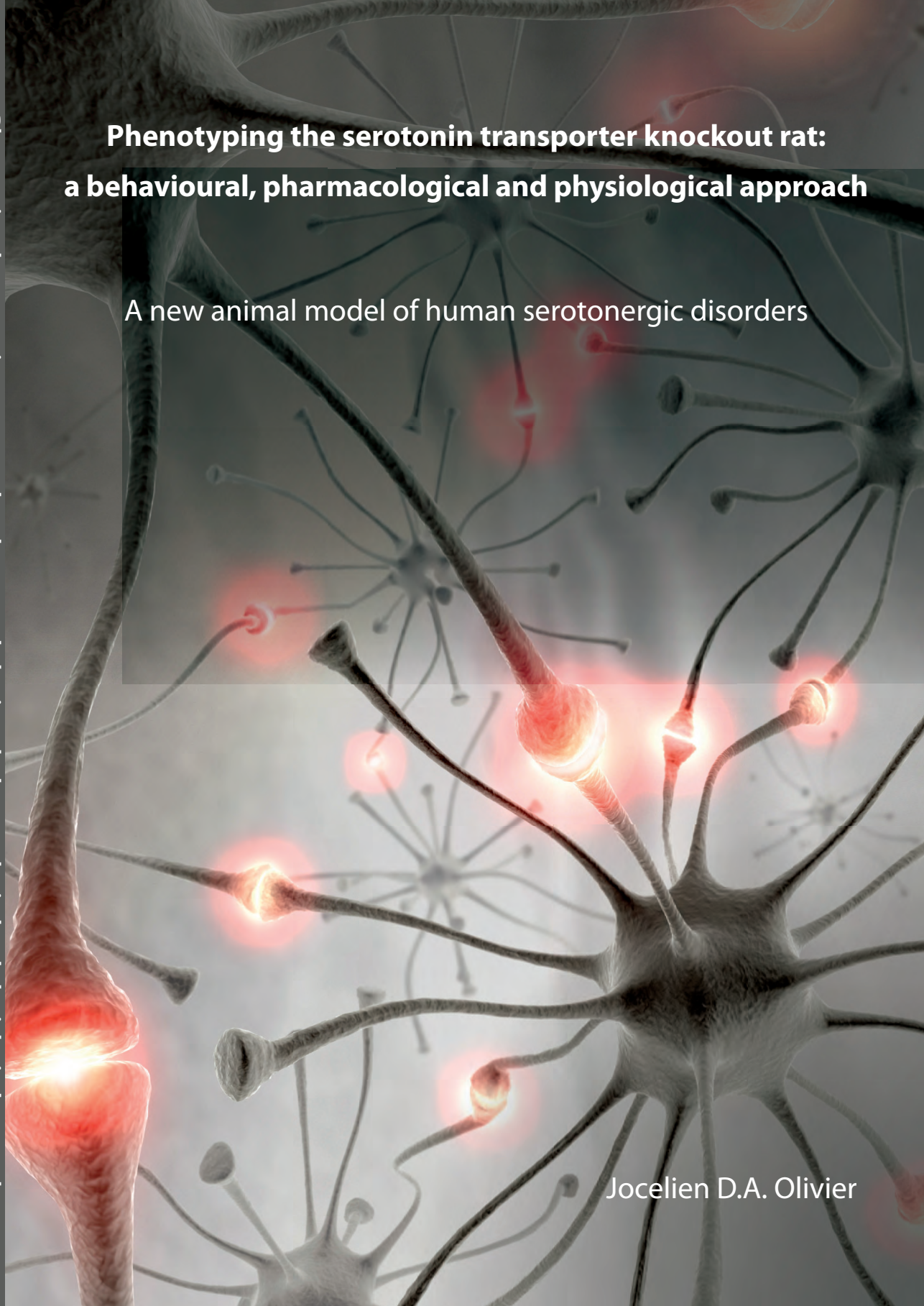

Phenotyping the serotonin transporter knockout rat: a behavioural, pharmacological and physiological approach

A new animal model of human serotonergic disorders

Jocelien D.A. Olivier

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A new animal model of human serotonergic disorders

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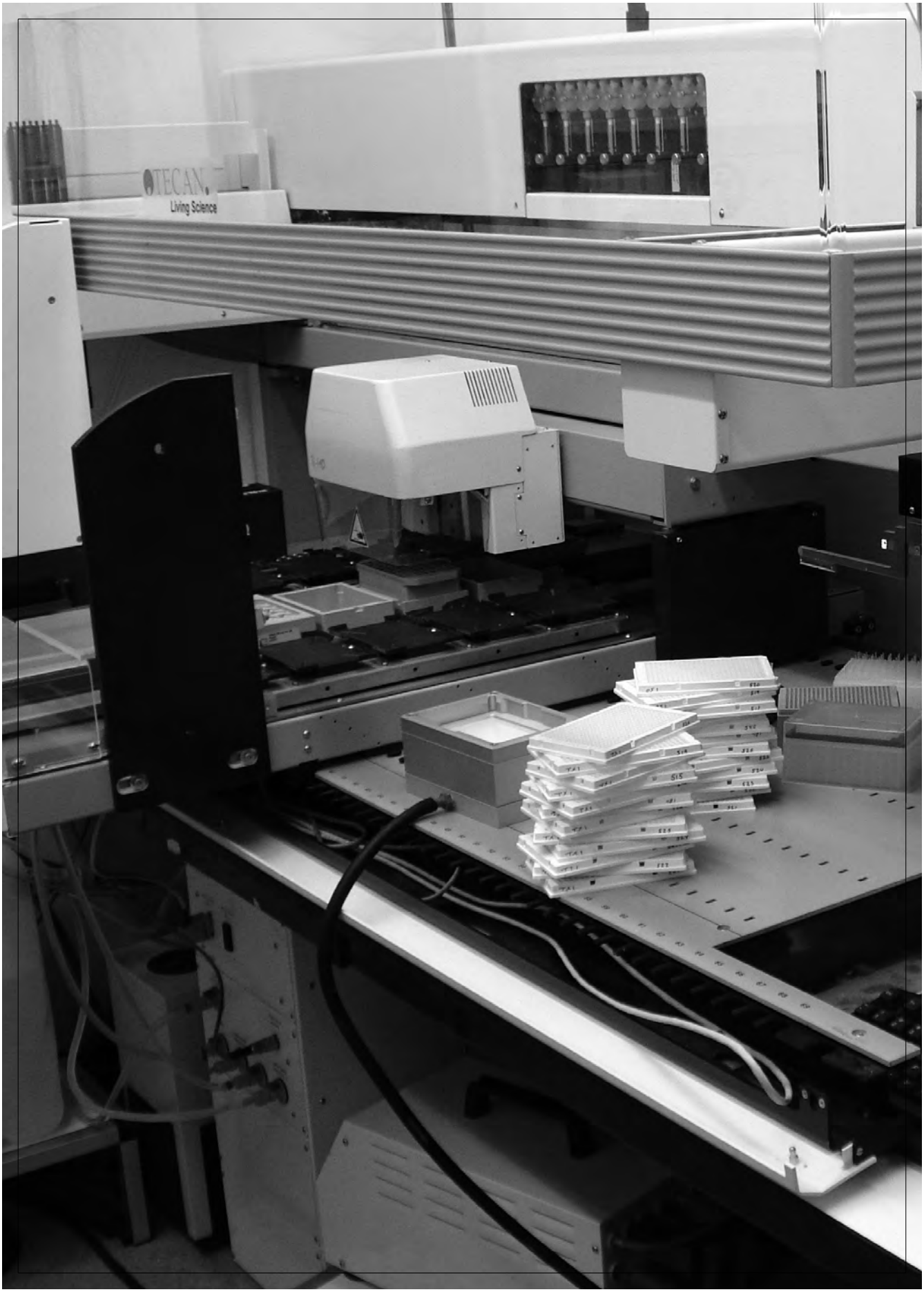
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General introduction

General Introduction

Serotonin (5-hydroxytryptamine or 5-HT) is implicated in many physiological and behavioral processes, including thermoregulation, sleep, feeding and sexual behavior. Moreover, disturbances in the 5-HT system are known to be involved in neuropsychiatric disorders including anxiety, aggression, impulsivity and mood disturbances (Delgado et al., 1990; Griebel, 1995; Barnes & Sharp, 1999; Stain-Malmgren et al., 2001; Neumeister et al., 2002; Moreno et al., 2002; Holmes et al., 2003c); however, the exact role of 5-HT in the etiology and cause of these neuropsychiatric disorders is not known. The pharmacotherapy of patients with these diseases that encompasses agents that modulate serotonergic neurotransmission, is beset by problems of poor efficacy and is marked by many unwanted sideeffects. Therefore, it would be useful to use a model in which these disorders can be studied. The laboratory rat is one of the most extensively studied model organisms for various aspects of human medical research, including neural regeneration, psychiatric disorders, behavioral intervention, and addiction, as well as drug development (Jacob & Kwitek, 2002; Gibbs et al., 2004). The wealth of literature on the rat helps to interpret novel findings. Many behavioral tests have been developed and validated for rats, and rat behavioral repertoires and related neural correlates have been welldescribed. Furthermore, most rat models have phenotypic characteristics that are relevant to a particular human condition. Initially these phenotypes were induced surgically or pharmacologically, but eventually these models were developed by phenotypic selection for certain traits. Rat models have made inestimable contributions to biomedical research in general, and although they do not always recapitulate the clinical outcome of human diseases, animal models are crucial, especially when little is known about the basis of a complex human disease. Thus, animal models can provide important insights into clinically relevant pathways, besides being a means to investigate candidate disease genes.

As the mouse can easily be manipulated genetically, many mouse models are in use nowadays for biomedical research. Among these mouse models are models for human serotonergic disorders, like the 5-HT_{1A} knockout mouse (Ramboz et al., 1998), the 5-HT_{1B} knockout mouse (Saudou et al., 1994), and the serotonin transporter knockout mouse (Bengel et al., 1998). However, mice and rats do differ, for example in their physiological responses to 5-HT_{1A} receptor agonists and antagonists (Goodwin et al., 1985b; Bill et al., 1991; Moser, 1991; Martin et al., 1992; Meller et al., 1992; Millan et al., 1993; De Vry, 1995). Therefore, it is important to generate knockout rats to study human disorders. In this thesis we propose the serotonin transporter knockout ($SERT^{-/-}$) rat as a model for human serotonergic disorders, especially neuropsychiatric disorders such as anxiety and depression. While research on $SERT^{-/-}$ mice has already revealed several novel 5-HT-mediated phenotypes and processes, the $SERT^{-/-}$ rat allows complementary in-depth research through its greater potential of behavioral and neurochemical techniques.

As elaborated in paragraph 2 of this chapter, the serotonin transporter (SERT) has been implicated in several 5-HT disorders and is an important target site for a number of drugs that are given to subjects suffering from neuropsychiatric diseases. In the first five paragraphs of this chapter attention is therefore focussed on (1) the synthesis and metabolism of serotonin as well as its receptors, (2) the SERT, (3) tryptophan depletion as a tool to analyse the vulnerability of individuals (humans and animals) for central serotonergic disorders, (4) the involvement of serotonin in neuropsychiatric disorders such as anxiety and depression, and (5) the involvement

of serotonin in the regulation of hypothermia and stress-induced hyperthermia, i.e. characteristic dysfunction of the autonomic nervous system. In paragraph six the $SERT^{-/-}$ rat is introduced. In the final paragraph of this chapter the aim and outline of this thesis are described.

1. Synthesis and metabolism of serotonin and the involvement of its receptors

5-HT has long been the subject of study in many areas of biomedical sciences. In the body, 95% of total 5-HT is found in the periphery. Therefore, it is no coincidence that initial research was focussed on this area. After it was found in the mammalian brain, 5-HT was recognized as a neurotransmitter, bringing it into the field of neuroscience. These findings were followed by the discovery that 5-HT plays an important role in many physiological and behavioral processes.

5-HT is synthesized in a two-step enzymatic pathway. The hydroxylation of the aromatic amino acid tryptophan forming 5-hydroxytryptophan (5-HTP) by the rate-limiting enzyme tryptophan hydroxylase is the first step in the synthesis of 5-HT. 5-HTP is then converted into 5-HT by the enzyme L-amino acid decarboxylase. As shown in Figure 1, metabolism of 5-HT primarily occurs by deamination via mitochondrial monoamine oxidase (MAO) to form 5-hydroxy-indole acetaldehyde, which in turn is oxidized by aldehyde dehydrogenase to produce 5-hydroxyindole acetic acid (5-HIAA). MAOs are degradative enzymes of monoamines, such as norepinephrine, 5-HT, and dopamine. There are two subtypes of MAO, MAO-A and MAO-B, based on their selectivity to substrate and inhibitors. Whereas MAO-A preferentially metabolizes norepinephrine and 5-HT, MAO-B acts on dopamine. Any cell that can take up 5-HT and possesses MAO has the potential to metabolize 5-HT.

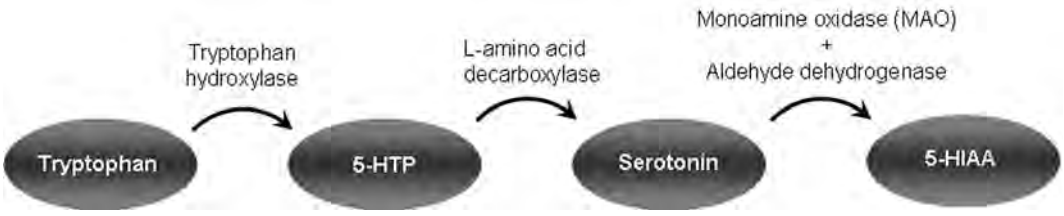


Figure 1
Synthesis and metabolism of 5-HT.

Serotonergic cell bodies are mainly located in the mid- and hindbrain (Tork, 1990). Serotonergic neurons originate from the raphe nuclei and project to many brain areas, including the prefrontal cortex, the amygdala, the hippocampus, the medial preoptic area, the hypothalamus and the ventral striatum. They also project to caudal areas like the cerebellum, medulla and spinal cord (Jacobs & Azmitia, 1992). The effects of 5-HT are mediated by at least 14 different 5-HT receptors: 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D}, 5-HT_{1E}, 5-HT_{1F}, 5-HT_{2A}, 5-HT_{2B}, 5-HT_{2C}, 5-HT_{3}, 5-HT_{4}, 5-HT_{5A}, 5-HT_{5B}, 5-HT_{6}, and 5-HT_{7}. These receptors are anatomically localized at different sites in the CNS (Palacios et al., 1990). Besides all these receptors the SERT plays an important role in the modulation of 5-HT. The SERT is localized both at the terminal portion of the axon and at the cell body of the 5-HT neuron (D'Amato et al., 1987; Hrdina et al., 1990; Chen et al., 1992).

The activity of a serotonergic neuron is presumably regulated via two different presynaptic receptors (5-HT_{1A} and 5-HT_{1B}) and the SERT (Pineyro & Blier, 1999). When the serotonergic neuron fires, 5-HT is released from the terminal and activates all available 5-HT receptors at that time. Several mechanisms are at work in order to regulate the firing and the release of 5-HT. First the SERT in the synaptic terminal and at the cell bodies and the dendrites of the 5-HT neurons return 5-HT back into the neuron via an uptake mechanism, where it is degraded or retained for future release (Murphy et al., 2004). This 5-HT uptake is very important in order to end the 5-HT signalling, thereby avoiding overstimulation of receptors, restoring the resting condition of a cell, and be able to fire again. Besides activation of the SERT, activation of the somatodendritic 5-HT_{1A} (at the level of the cell body and dendrites) and 5-HT_{1B} (at the level of the synaptic terminals) presynaptic receptors also contribute to the inhibition of firing and release of 5-HT. The interaction between these three processes leads to a very fine-tuned system of serotonergic activity.

2. Serotonin transporter

The SERT is important for two reasons. First, genetically determined changes of the SERT gene are associated with certain neuropsychiatric diseases. Second, the SERT is the target site for a number of pharmacotherapeutic agents that are prescribed for patients with various neuropsychiatric disorders. These aspects are elaborated below.

The SERT gene (*SLC6A4*) is located on chromosome 17 and consists of 14 exons. The protein contains 630 amino acids with 12 transmembrane domains. The SERT is associated with several human neuropsychiatric disorders (Murphy et al., 1999; Gingrich & Hen, 2001; Holmes et al., 2003c). Like non-human primates, humans exhibit a polymorphism in the SERT gene, termed the *5-HTTLPR*. The *5-HTTLPR* is a 44-base pair deletion/insertion of a repetitive sequence in the promoter region upstream of the transcription initiation site of the SERT gene. Studies in reporter gene constructs and in human lymphoblastic cell lines have found that the short variant (S) of the polymorphism reduces the transcriptional efficiency of the SERT gene as compared to the long (L) variant (Lesch et al., 1996; Heils et al., 1996; Heils et al., 1997). Moreover, the L variant shows a more rapid initial platelet 5-HT uptake (Greenberg et al., 1999). Originally, this did not hold true for SERT mRNA levels (Parsey et al., 2006) and SERT binding (Naylor et al., 1998; Mann et al., 2000; Willeit et al., 2001; Shioe et al., 2003; Lim et al., 2006) in the brain. Van Dyck et al. (2004) have even found that the SERT availability in young S-S homozygotes is greater than in young L-S heterozygotes. This apparent discrepancy between the periphery and the brain can be understood by considering the findings of Nakamura et al. (2000). They describe several novel variants of S and L, including a functional single nucleotide variant within the L allele, designated L(A) and L(G). Interestingly, only L(A) is associated with high levels of *in vitro* SERT mRNA transcription, whereas L(G) is low expressing and more similar to S (Hu et al., 2006). This also holds for the brain, where the L(A)/L(A) is associated with higher SERT binding potential in the putamen (Praschak-Rieder et al., 2007). Hence, the original failure to observe *5-HTTLPR*-related changes in central SERT binding in previous studies may be explained by the pooling of L(A) and L(G) allelic variants. Anyhow, there is hard evidence that a reduced SERT expression resulting in a reduced SERT function, as seen in the subjects with the S variant, is associated with anxiety- and depression-related personality traits; it exhibits more depressive symptoms, diagnosable depression, and suicidal tendencies in case of stressful events (Mann et al., 2000;

Caspi et al., 2003; Schmitz et al., 2007; Dick et al., 2007; Canli and Lesch, 2007). Not every study has reached the same conclusion in this respect though (e.g. Lang et al., 2004). Overall, *HTTLPR* association studies lead to the hypothesis that the *HTTLPR,* together with other regulatory and structural variations, seems to play a role in neuropsychiatric conditions such as bipolar disorder, depression, anxiety disorders (in particular obsessive-compulsive disorder), suicidal tendencies, eating disorders, substance-abuse disorders, autism, attention-deficit/hyperactivity disorder and neurodegenerative disorders (Greenberg et al., 2000; Murphy et al., 2004; Hu et al., 2006). Hu et al. (2006) found that the L(A)/L(A) genotype was about twice as common in patients with obsessive-compulsive disorder compared to healthy people; Lesch et al. (1996) found a negative association between the S variant and agreeableness. However, most studies reveal an association between the S variant and an increased risk of neuropsychiatric conditions.

Apart from the involvement of SERT in neuropsychiatric diseases, the SERT is the actual target site for a whole subclass of antidepressants, namely the Selective Serotonin-Reuptake Inhibitors (SSRI's). SSRIs like paroxetine, citalopram, fluoxetine, fluvoxamine, and sertraline are widely used in the treatment of depression, anxiety, and impulse control disorders, as well as substance abuse including alcoholism. SSRIs elevate the extracellular 5-HT levels in the CNS through blockade of the SERT (Nutt et al., 1999; Vaswani et al., 2003). The SERT promoter polymorphism also seems to be associated with therapeutic responses and side-effects following treatment with citalopram (Hu et al., 2007). Together, these data imply an important role for the SERT in the CNS.

3. Tryptophan depletion to analyze the vulnerability of individuals for central serotonergic disorders

As mentioned before, 5-HT is synthesized from its amino acid precursor tryptophan (TRP), which is taken up from the blood. While most of the TRP is protein-bound, approximately 5% of the TRP circulates free in the bloodplasma and is available for transport to the brain. Although 5-HT itself is not able to pass the blood-brain barrier, (free) TRP can be actively transported to the central nervous system. However, this transport system also transports five other large neutral amino acids (LNAAs) namely tyrosine, phenylalanine, leucine, isoleucine and valine. These LNAAs also enter the circulation when food is consumed, implying that TRP has to compete with them. Therefore, the access of TRP to the brain depends not only on serum TRP levels itself, but also on the serum concentrations of the other large neutral amino acids that compete with TRP for uptake (Guroff & Udenfriend, 1962; Fernstrom & Wurtman, 1972). Once in the brain, TRP is converted into 5-HT (see figure 2). The availability of free plasma TRP is thus a limiting factor in the synthesis of 5-HT. Therefore, acute tryptophan depletion (ATD) is used as a tool to challenge the 5-HT system by reducing systemic TRP levels and, consequently, central 5-HT concentrations (Biggio et al., 1974; Gessa et al., 1974; Fernstrom, 1981; Moja et al., 1989). To investigate the role of 5-HT in behavioral functions (e.g. anxiety, sleep, aggression, memory, impulsivity) ATD is a validated model that can be used in humans as well as in animals (Young, 1996; Moore et al., 2000; Bell et al., 2001). Administration of an amino acid mixture that lacks TRP has been shown to reduce plasma TRP levels and 5-HT tissue levels in the brain of the rat (Biggio et al., 1974; Gessa et al., 1974; Moja et al., 1989; Brown et al., 1998). Moreover, TRP depletion has been shown to lower extracellular 5-HT levels in the hippocampus (Stancampiano et al., 1997). Previous studies using a gelatin-based-carbohydrate mixture showed a robust reduction in plasma TRP (about 70%) and central

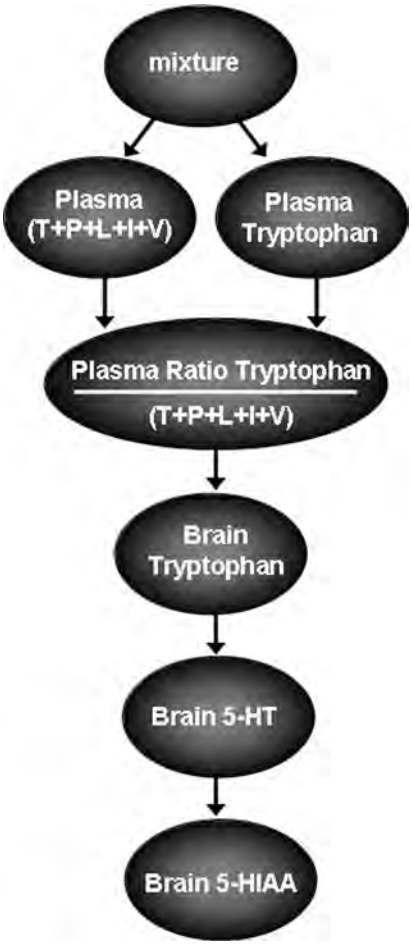


Figure 2
The ratio of total tryptophan to the diet-induced changes in brain serotonin concentration in the rat. Combined levels of tyrosine, phenylalanine, leucine, isoleucine, and valine in serum determines the tryptophan level in brain.

tissue 5-HT (about 40–45%) concentrations in Wistar rats (Lieben et al., 2004a; Jans et al., 2007a). Moreover, this ATD method has been reported to impair object memory, but not affective behavior in rats (Lieben et al., 2004b). This memory impairment has also been shown in healthy human volunteers, whereas other effects of ATD, such as effects on mood, are only found in vulnerable subjects (Riedel et al., 1999; Schmitt et al., 2000; Riedel et al., 2002).

Some controversy has been found in TRP depletion studies. The inconsistency may be related to the degree of TRP depletion used in these studies. It seems like a certain threshold level needs to be exceeded before certain effects will appear that are due to the depletion. An overview of several TRP depletion studies given by Van Der Does (2001) suggests that the threshold for possible mood effects to occur lies somewhere around a 60% reduction of free plasma TRP. High and low TRP depletion turned out to give different results in depressed patients. An impaired processing of positive information was found in all patients after a high dose of TRP depletion, whereas no impairment was found at all after a low dose of TRP depletion (Booij et al., 2005). These results together confirm the idea of a threshold that needs to be exceeded before symptoms occur after TRP depletion.

4. Involvement of serotonin in neuropsychiatric disorders such as anxiety and depression

Mood and anxiety disorders are generally classified as separate types of syndromes; however, depression and anxiety share many overlapping symptoms, including impaired concentration, fatigue, irritability, sleep disturbance, nervousness, worry, and restlessness (Ninan, 1999). It is widely accepted that diminished serotonergic functioning is involved in the onset of depression. Indirect measures of precursor availability and metabolite concentrations provided initial evidence for abnormalities in the system. In depressed patients, alterations such as decreased plasma tryptophan levels (Coppen et al., 1973; Cowen et al., 1989) have been found. In addition, decreased 5-HIAA levels in cerebrospinal fluid (CSF) (Asberg et al., 1976a), suggesting decreased 5-HT metabolism in the CNS, have been found in depression. Moreover, imaging studies have shown that the binding of both pre- and postsynaptical 5-HT_{1A} receptors in the brain is reduced and fails to normalize after remission for depression (Drevets et al., 1999; Sargent et al., 2000). In patients with major depression a decreased availability of SERT has been found in the midbrain and brainstem regions (Malison et al., 1998). Another indication

that 5-HT is involved in the onset of depression is that consequent lowering of brain 5-HT, by means of tryptophan depletion, can cause transient depressive symptoms in individuals that are vulnerable for depression, based on their personal or family history of depression (Ellenbogen et al., 1999; Klaassen et al., 1999; Moreno et al., 2002; Neumeister et al., 2004). Some 5-HT abnormalities have also been found in subjects with a family history of depression or in remitted depressed patients, indicating that either some dysfunction in 5-HT systems or an increased sensitivity of the 5-HT system is a trait abnormality in depression. Further evidence for abnormalities in 5-HT transmission are the findings of reduced SERT binding in the post-mortem cortex of depressed patients (Leake et al., 1991; Mann et al., 2000); however, this was not consistently found (Lawrence et al., 1993). In brain imaging studies fewer SERT have been reported in depressed patients as well (Malison et al., 1998). In addition, reduced 5-HT uptake and reduced SERT binding have been found in platelets (Owens & Nemeroff, 1994). Reduced SERT binding has also been found in anxiety disorders (Iny et al., 1994). Overall, the data is quite consistent indicating that the 5-HT system exhibits decreased activity. Alterations in the 5-HT system likely mediate many of the signs and symptoms of depression and anxiety. Apparently either underactivity or overactivity of the 5-HT system can cause anxiety and mood disorders. Therefore, it is essential that the 5-HT system functions properly during development and adulthood to prevent the development of anxiety and mood disorders.

5. Involvement of serotonin in the regulation of hypothermia and stress-induced hyperthermia

Hypothermia

Autonomic responses to selective 5-HT_{1A} receptor agonists are frequently used to measure the 5-HT_{1A} receptor function (Hjorth, 1985; Millan et al., 1993; De Vry, 1995; Cryan et al., 1999; Ootsuka and Blessing, 2003; Nalivaiko et al., 2005; Ootsuka and Blessing, 2006). Selective 5-HT_{1A} receptor agonists are known to cause hypothermia in both animals (Hjorth, 1985; Millan et al., 1993; De Vry, 1995; Cryan et al., 1999; Bouwknecht et al., 2000) and humans (Pitchot et al., 2002; Pitchot et al., 2004). The hypothermic response to 5-HT_{1A} receptor agonists in rats and humans appear to be mediated by postsynaptic 5-HT_{1A} receptors (Bill et al., 1991; Millan et al., 1993; Blier et al., 2002), although some researchers have suggested that these effects are mediated by presynaptic receptors in rats (Goodwin et al., 1987; Higgins et al., 1988; Hillegaart, 1991) and mice (Goodwin et al., 1985b; Bill et al., 1991; Martin et al., 1992). In addition to 5-HT_{1A} receptor agonists, 5-HT_7 receptor agonists are also able to induce hypothermia (Vinkers et al., in press). Besides the induction of hypothermia, 5-HT receptors are also involved in hyperthermia. 5-HT_2 receptor agonists increase the basal temperature, whereas 5-HT_2 receptor antagonists prevent this hyperthermia and even reduce the temperature (Zethof et al., 1995; Nisijima et al., 2001; Yamada et al., 2001). Overall these data show that multiple 5-HT receptors play a role in the regulation of body temperature.

Stress-induced hyperthermia

Body temperature is not only affected by pharmacological agents, but also increases when an organism is confronted with a stressor. This relatively short-lasting increase in body temperature is generally referred to as stress-induced hyperthermia (SIH) (Friedman & Thayer, 1998; Nijsen et al., 1998a; Olivier et al., 1998; Bouwknecht et al., 2000). SIH is an integral part of an individual's response to situations perceived as threatening or distressing (Reeves et al., 1985). Several

psychological and physical stressors may induce SIH, for example taking an exam (Marazziti et al., 1992) or attending a sports contest (Renbourn, 1960). In animals, a mild disturbance (Bouwknecht et al., 2001), injection (Olivier et al., 2003), clean cage (Spooren et al., 2002; Groenink et al., 2003), predator smell (Rorick-Kehn et al., 2005), or restraint stress (Terlouw et al., 1996) induces hyperthermia. The SIH has a high face validity, since a stress-induced temperature rise is a universal phenomenon that occurs in many species (for a review, see Bouwknecht et al., 2007), including rats (Briese and De Quijada, 1970; Briese and Cabanac, 1991), rabbits (Yokoi, 1966) and humans (Marazziti et al., 1992).

Administration of drugs with anxiolytic properties, like $GABA_A$ and 5-HT receptor agonists, results in a reduction or even ablation of the SIH response; however, as described before, anxiolytic drugs may also induce hypothermia. In this way, especially after high doses of the drug, the SIH is reduced or ablated because of the hypothermia and not because of the anxiolytic mode of action of the drugs (Olivier et al., 2003). Extensive pharmacological testing has shown that the SIH model is suitable for predicting anxiolytic drug properties. Benzodiazepines and $5\text{-}HT_{1A}$ receptor agonists, drugs that have been proven to be effective in the clinic, attenuate the SIH response (Olivier et al., 2003). SSRIs do not seem to have an effect on the SIH acutely (Olivier et al., 2003) or chronically (Roche et al., 2007); however, Conley and Hutson (2007) showed a reduction of the SIH after treatment with fluoxetine. Together these data suggest that the SIH model is sensitive to anxiolytic drugs and the 5-HT system seems to be involved.

6. Serotonin transporter knockout rat

The laboratory rat is one of the most extensively studied model organisms in various areas of human health and disease. The wealth of literature on the rat helps interpreting novel findings. Further, many behavioral tests have been developed and validated for rats, and rat behavioral repertoires and related neural correlates have been well-described. Rats are also preferred because of their relatively large size, which allows for the effective use of a wide variety of tools to study the brain, such as imaging, micromanipulation and *in vivo* sampling. In 2004, the rat joined the mouse and human as the third vertebrate species for which the complete genome sequence (more than 90% coverage) has been determined (Gibbs et al., 2004). For these reasons, knockout rats would be valuable models in, among others, neuroscience research, and could complement knockout mice studies to dissect gene-behavior associations.

As a genetic model, the rat is clearly lagging behind the mouse, primarily because gene knockout technology using pluripotent embryonic stem (ES) cells is still lacking due to the lack of suitable ES cell lines. An alternative approach to the embryonic stem cell technique for generating genetic knockouts is target-selected mutagenesis or TILLING (Targeting Induced Local Lesions in Genomes). Recently, *N*-ethyl-*N*-nitrosourea (ENU)-driven target selected mutagenesis was successfully established for the rat (Zan et al., 2003; Smits et al., 2004; Smits et al., 2006). The approach starts with inducing mutagenesis of the male germline by intraperitoneal injection of ENU. ENU induces random point mutations, primarily in spermatogonial stem cells. These mutations become fixed in the sperm cells during spermatogenesis, which lasts approximately 12 weeks in rats. Mutagenized males are mated with untreated females to generate a large population of F1 animals that harbour many random heterozygous point mutations in their

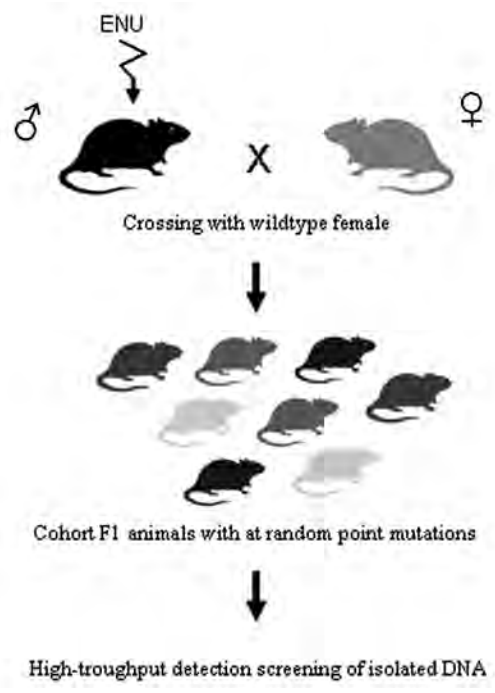


Figure 3
Schematic representation of ENU-driven target selected mutagenesis. Male rats are injected with ENU and crossed with untreated females. A cohort of animals (F1) with at random point mutations is generated, and their DNA is isolated for high-throughput screening re-sequencing of genes of interest.

genome. Next, DNA samples are extracted from each F1 individual. These samples are subsequently screened for induced mutations in exons of interest (see Figure 3).

The most deleterious type of mutation is the nonsense mutation or premature stopcodon, which interrupts the translation process. Mutations can also involve missense mutations, amino acid changes that could lead to structural and/or functional changes of the protein, e.g. when the amino acid change is located in a conserved region of the protein. Splice-site mutations affect alternative splicing processes and are of interest as well. Silent mutations are not expected to have any phenotypic effects (Smits & Cuppen, 2006). The dideoxy resequencing allows the detection of all types of mutations. The dideoxy resequencing method, applied to approximately 400 amplicons (~200 genes) in 1500 rats (mainly Wistars), resulted in the identification of more than 120 ENU-induced mutations. These included fifty-six missense and six nonsense mutations, among which a premature stopcodon in the SERT gene. To be precise, the ENU-induced mutagenesis caused a C to A transversion at position 3924 in the third exon encoding the second extracellular loop of the SERT (ENSRNOG0000003476) in a female F1 rat with a Wistar/Crl background, resulting in a premature stopcodon (TGC > TGA). As described above, the SERT is very important in the CNS, making this rat an interesting model to study dysfunctions in the serotonergic system.

7. Aim and outline of the thesis

The SERT is a major regulator of synaptic 5-HT availability, and is very important in processes in which 5-HT is involved. The aim of the present thesis was to phenotypically characterize a rat that lacks the SERT. Several behavioral paradigms were assessed in the SERT$^{-/-}$ rat, and pharmacological and physiological parameters were measured. Following the introductory chapter, the experimental outcome of our studies is presented in the next five chapters. The final chapter is devoted to a discussion of the results described in **chapters 2-6**.

Disturbances in the 5-HT system are known to be involved in many neuropsychiatric disorders, although the exact role of 5-HT in these disorders is not known. **Chapter 1** gives an overview of the synthesis and metabolism of 5-HT and 5-HT receptors. Moreover, chapter 1 describes

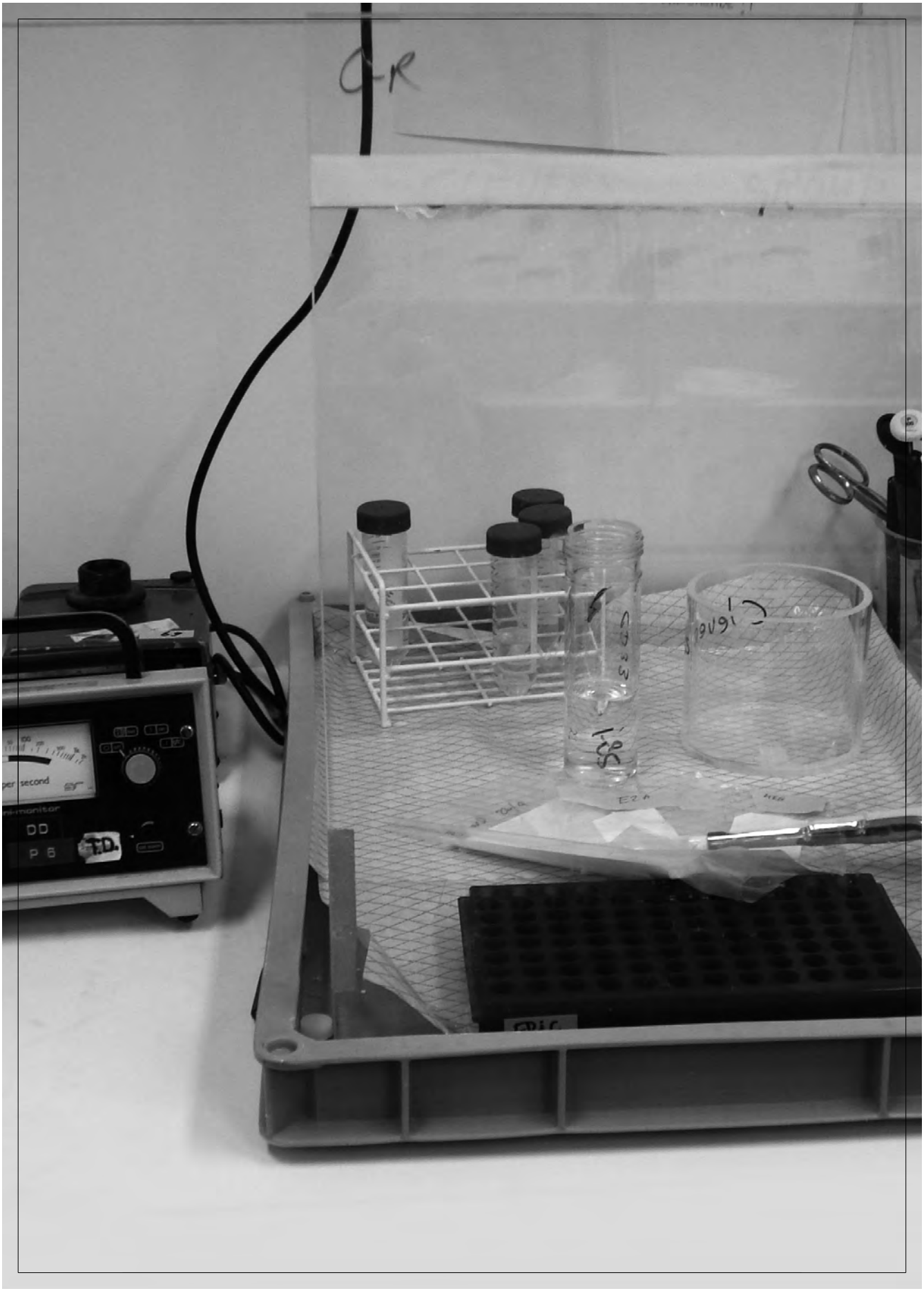
the involvement of the SERT in neuropsychiatric disorders, as well as the 5-HT vulnerability to develop these disorders. To confirm that a premature stopcodon in the SERT gene results into a $SERT^{-/-}$ rat, and to establish whether rats and mice similarly adapt to the absence of the SERT, some experiments that were already performed in the $SERT^{-/-}$ mouse have been replicated in the $SERT^{-/-}$ rat. However, some interesting new insights were obtained by the experiments performed and described in this thesis.

Animal models are very useful in studying both the behavioral and the biochemical consequences of disturbed 5-HT homeostasis. They may reveal the contribution of compensatory adaptations, that in turn might reveal novel targets for medication. To allow for cross-species comparison with (knockout) mouse data and extrapolation to the human situation, a SERT knockout model would complement existing tools and help in understanding the complexity of the serotonergic modulation of major human disease states. We generated such a model by ENU-driven target-selected mutagenesis and the primary biochemical characterization of the $SERT^{-/-}$ rat. The functional consequences for the serotonergic as well as the non-serotonergic systems are described in **chapter 2**.

Genetic alterations in the SERT are associated with multiple neuropsychiatric disorders like anxiety and depression. Moreover, women experience depression about twice as often as men, and are more vulnerable to develop anxiety disorders. We hypothesized that rats lacking the SERT show increased vulnerability to anxiety- and depression-related behaviors, and that females are more vulnerable to develop anxiety- and depression-related behaviors after the SERT deletion than males. Several anxiety- and depression-related tasks were assessed in male and female $SERT^{-/-}$ rats and are described in **chapter 3**.

As individual differences exist in central 5-HT neurotransmission, the impact of a challenge on this system, like ATD, may vary accordingly. ATD results in lower peripheral and central levels of tryptophan and 5-HT and several studies have shown an impaired object recognition after ATD. In **chapter 4** we tested the hypothesis that male $SERT^{-/-}$ rats are more vulnerable to the effects of a normal or low-dose of ATD in an object recognition task than male $SERT^{+/+}$ and $SERT^{+/-}$ rats. Moreover, plasma amino acid concentrations and concentrations of 5-HT and 5-HIAA in the frontal cortex and hippocampus were measured to investigate the effect of ATD in these rats.
In **chapter 5** we focus on the extent to which life-long disturbed 5-HT neurotransmission in $SERT^{-/-}$ rats may result in adaptive changes in the serotonergic system. Chronic administration of SSRIs desensitizes $5\text{-}HT_{1A}$ receptors in rats and humans. As such adaptive changes are known to occur after chronic manipulations of the serotonergic system, we investigated the $5\text{-}HT_{1A}$ receptor pathways in the $SERT^{-/-}$ rat. Autonomic responses to selective $5\text{-}HT_{1A}$ receptor agonists are frequently used to measure $5\text{-}HT_{1A}$ receptor function. Therefore we investigated to what extent the thermoregulatory effects of $5\text{-}HT_{1A}$ receptor agonists and antagonists were altered in the $SERT^{-/-}$ rat, and whether these alterations differed for stress-induced hypothermia (SIH) and core body temperature (T_C). The effects of both the $5\text{-}HT_{1A}$ receptor agonist flesinoxan and the $5\text{-}HT_{1A}$ receptor antagonist WAY100635 were analyzed, as well as the receptor specificity of these effects on Tc, heart rate and SIH, to investigate the role of $5\text{-}HT_{1A}$ receptors in the modulation of these autonomic responses.

Besides studying the adaptive changes in the serotonergic system, we also investigated possible adaptations in other neurotransmitter systems in the SERT$^{-/-}$ rat. The 5-HT system interacts with the GABAergic, the dopaminergic and the noradrenergic system. Alterations in the serotonergic system may affect the sensitivity of these systems. To investigate to what extent life-long disturbed 5-HT neurotransmission may result in adaptive changes of these systems, we challenged the SERT$^{-/-}$ rat with a $GABA_A$ receptor agonist, a dopamine transporter blocker, and a noradrenaline transporter blocker. We studied the resulting changes in body temperature and SIH in **chapter 6**. In **chapter 7**, the outcomes of all preceding chapters are shortly summarized and discussed.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Characterization of the serotonin transporter knockout rat: A selective change in the functioning of the serotonergic system

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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.

Introduction

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 (SSRIs), 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 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 and 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., 2002a; Holmes et al., 2003b; Lira et al., 2003; Adamec et al., 2006), reduced aggression (Holmes et al., 2002b), 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 30 s just above liquid nitrogen and were then plunged into the liquid nitrogen. The brains were stored at −80°C. Coronal sections (16 µm) were cut on cryostat microtome at −20°C, thaw-mounted onto gelatin-coated slides and stored at −20°C until use. The tissue sections (four slides per animal, two animals per genotype) were pre-incubated for 20 min at room temperature in 50 mM Tris–HCl buffer at pH 7.4, containing 120 mM NaCl and 5 mM KCl ($[^3H]$citalopram), in 50 mM Tris–HCl buffer containing 300 mM NaCl, 0.2% bovine serum albumin, 1 µM cis-flupenthixol (Sigma) ($[^3H]$GBR12935), or in 50 mM Tris–HCl buffer containing 300 mM NaCl and 5 mM KCl ($[^3H]$nisoxetine). Subsequently, the sections were incubated in fresh buffer containing 1 nM of $[^3H]$-citalopram (85.0 Ci/mmol, GE Healthcare) for 2 h at room temperature, buffer containing 2 nM $[^3H]$GBR12935 (45.0 Ci/mmol, Perkin Elmer, Boston, MA, USA) for 20 h at 4°C, or in buffer containing 3 nM $[^3H]$nisoxetine (85.0 Ci/mmol, Perkin Elmer) for 4 h at 4°C. The slides were washed in ice cold buffer (4×5 min), rapidly dipped in cold distilled water and dried under a cold stream of air. $[^3H]$citalopram sections together with $[^3H]$Microscales™ standards (GE Healthcare) were exposed to a tritium sensitive screen (GE Healthcare) and scanned after 1 week using a 9200 typhoon scanner (GE Healthcare). $[^3H]$GBR12935 and $[^3H]$nisoxetine sections were exposed to $[^3H]$hyperfilm (GE Healthcare) and developed after 1 week ($[^3H]$GBR12935) or 8 weeks ($[^3H]$nisoxetine). Optical densities were converted into fmol/mg of tissue equivalent using the standard curve. Non-specific binding for $[^3H]$-citalopram binding was determined by binding in $SERT^{-/-}$ rats, and non-specific binding for $[^3H]$GBR12935, and $[^3H]$nisoxetine was determined using 50 µM mazindol and 10 µM desmethylimipramine (desipramine, DMI), respectively.

Body temperature telemetry

Under isoflurane anesthesia rats were surgically equipped with a temperature-sensitive radio-transmitter (TA10TA-F40, Data Sciences Inc., St. Paul, MN, USA) in the peritoneal cavity. Following

surgery the rats were individually housed in test cages that were placed on receivers (Data Sciences Inc.) and allowed to recover for at least 1 week before drug challenge tests. Output signals from each of the implanted transmitters were monitored by the receivers and channeled into a consolidation matrix (BCM 100) connected to a PC-based data acquisition and analysis system (Dataquest Labpro, Data Sciences). This system demodulated the signals and converted the raw telemetry data into degrees Celsius using the factory calibration values, and was configured to sample temperature every 10 min for 10 s on a 24 h basis. Drug challenge tests were performed at 4 h after lights on. Animals received saline (1 ml/kg, s.c.) or *d*-fenfluramine (10 mg/kg, s.c.) in a randomized order.

Synaptosomal [^{3}H]5-HT uptake

Rats were decapitated and brains were rapidly removed. To obtain brain synaptosomes, hippocampi were rapidly dissected from the brains, transferred into 20 volumes of ice-chilled 0.32 M sucrose, buffered by 5 mM Hepes (pH 7.4), and homogenized a motor-driven Teflon pestle. The synaptosomes were obtained by centrifugation at 1000×*g* (10 min, 4°C) and centrifugation of the supernatant at 12,000×*g* (30 min, 4°C). The resulting pellets were resuspended (50 volumes of original weight) in oxygenated (5%:95% CO_2:O_2) incubation buffer containing 120 mM NaCl, 5 mM KCl, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, 1 mM KH_2PO_4, 10 mM glucose, 100 µM pargyline, 0.05 mM EDTA, 1.3 mM $CaCl_2$, 0.1 mM ascorbic acid, pH 7.4 (adjusted with 1 M Tris). Tubes containing 10–300 nM [^{3}H]5-HT (25 Ci/mmol, GE Healthcare) were pre-incubated in 450 µl incubation buffer for 5 min at 37°C. Subsequently, 50 µl of the synaptosomal suspensions were added and uptake was allowed to occur for 10 min at 37°C under weak agitation. The process was terminated by adding 2 ml of ice-chilled incubation buffer followed by rapid filtration under vacuum through Whatman GF/B filters. The filters were washed with Krebs buffer and the radioactivity trapped on the filters was counted by liquid scintillation. Specific [^{3}H]5-HT uptake was calculated as the difference between total uptake and uptake in the presence of 10^{-4} M fluoxetine and expressed as pmol/mg protein/min [^{3}H]5-HT uptake. Protein concentrations were determined using Bradford protein assay (Biorad, Hercules, CA, USA).

Tryptophan hydroxylase (TPH) activity

Male rats were decapitated, the caudate putamen (CP) and RN (dorsal and medial) were dissected on an ice plate and stored at −80°C until use. TPH activity was determined according to Vrana et al. (1993). In short, brain tissue was sonicated in 20 mM NaH_2PO_4+0.2% Triton X-100 (pH 7.0). Tissue homogenate (25 µl) was added to 25 µl of reaction mixture containing 1 µCi lyophilized [^{3}H]tryptophan (31.0 Ci/mmol, GE Healthcare), 50 mM Hepes (pH 7.0), 5 mM DTT, 0.01 mM $Fe(NH_4)_2(SO_4)_2$, 0.5 mM 6-MPH_4 and 0.1 mg/ml catalase (Sigma), and the reaction mix was incubated for 15 min at 37 °C. Unreacted [^{3}H]tryptophan and 5-hydroxytryptophan (5-HTP) were absorbed with 500 µl of 7.5% charcoal (in 1 M HCL; Crescent Chemical Company Inc., Islandia, NY, USA). The samples were thoroughly vortexed and centrifuged at 14,000×*g* for 2 min at RT. Supernatant (350 µl) was removed and centrifuged at 14,000×*g* for 2 min at RT, and 200 µl supernatant was counted by liquid scintillation. The counts per minute (cpm) were converted to fmol 5-HTP per hour per mg protein by dividing the cpm in the total reaction (corrected for a blank measure in the absence of brain homogenate) through the amount of added cpm that reacted during 15 min per mg protein. The sensitivity of the assay is 1 fmol 5-HTP/mg protein/h. Protein concentrations were determined using Bradford reagent.

Monoamine oxidase (MAO) activity

MAO-A activity was measured using the Amplex™ Red MAO Assay Kit (Invitrogen, UK). This assay is based on the production of H_2O_2 and its detection by an HRP-coupled reaction using 10-acetyl-3,7-dihydroxyphenoxazine (Amplex Red reagent) to yield the fluorescent product resofurin. Male rats were decapitated, and the hippocampus, CP and RN (dorsal and medial) were dissected on an ice plate. As described previously (Mathews et al., 2004), tissue homogenates were obtained by homogenization in 0.1 vol. 0.32 M ice-cold sucrose using a glass pestle, and subsequent centrifugation at 12,000×*g* for 10 min at 4°C. The resulting pellets were resuspended in the same volume of cold 0.32 M sucrose and centrifuged at 21,000×*g* for 20 min at 4°C. Pellets were suspended in 0.2 volumes (of original tissue weight) 0.1 M phosphate-buffered saline (PBS) (pH 7.4), centrifuged at 12,000×*g* for 10 min at 4°C and pellets were stored at −80°C. MAO-A activity was measured using 10^{-4}M p-tyramine as substrate and 1 μM pargyline to inhibit MAO-B. After 30 min of incubation resofurin fluorescence was determined at 530/590 nm. Product amounts were derived from resofurin standard curves (0–10 μM). Protein was determined by the method of Bradford.

Immunohistochemistry

Brain tissues were cut into 15-μm-thick cryosections, collected in antifreeze (5.8 μM NaH_2PO_4, 19.3 μM Na_2HPO_4, 30% ethylene glycol, 20% glycerol) and stored at −20°C. Endogenous peroxidase activity was blocked by incubating the sections in 0.3% H_2O_2 in PBS for 30 min at room temperature, after which normal donkey serum (3% in PBS) was applied for 30 min at room temperature. Subsequently, sections were incubated overnight with primary antibody 1:500 for mouse anti-TPH (Chemicon, Hampshire, UK; AB1541) at 4°C. Next, sections were rinsed three times in wash buffer (0.1% Tween-20 in PBS), followed by incubation with the secondary biotinylated donkey anti-guinea-pig antibody (1:500; Jackson, Cambridgshire, UK) for 120 min. The sections were then stained using the ABC method (Vecta stain, Vector Laboratories, Peterborough, UK) with diaminobenzidine tetrahydrochloride (DAB) as a substrate. As a negative control, the sections were processed without incubation with primary antibody. Stained neurons were counted using AnalySIS, Soft Imaging System (Olympus, Hamburg, Germany).

Neurotransmitter release

Rats were decapitated, the CP, cortex and hippocampus were rapidly dissected from the brain, slices (0.3×0.3×2 mm) were prepared using a McIlwain tissue chopper and incubated and superfused essentially as described before (Schoffelmeer et al., 1988). Slices were washed twice with Krebs–Ringer bicarbonate medium containing 121 mM NaCl, 1.87 mM KCL, 1.17 mM KH_2PO_4, 1.17 mM $MgSO_4$, 25 mM $NaHCO_3$, 1.22 mM $CaCl_2$ and 10 mM d-(+)-glucose, followed by preincubation for 15 min in this medium in a constant atmosphere of 95% O_2–5% CO_2 at 37°C. After preincubation, the slices were rapidly washed with the Krebs–Ringer and incubated for 15 min in 2.5 ml of this medium containing 5μCi [^{3}H]5-HT (15.9 Ci/mmol, GE Healthcare), 5μCi [^{3}H]dopamine (DA) (41 Ci/mmol, GE Healthcare), 5μCi [^{3}H]noradrenaline (NE, GE Healthcare) (35 Ci/mmol, GE Healthcare), 2μCi [^{14}C]choline (56 Ci/mmol, GE Healthcare), [^{3}H]GABA (87 μCi, Ci/mmol, GE Healthcare) or 47 μCi [^{3}H]glutamate (40 Ci/mmol, GE Healthcare) under an atmosphere of 95% O_2–5% CO_2 at 37°C. To prevent accumulation of neurotransmitters into dopaminergic or noradrenergic nerve terminals, 1 μM GBR12935 (Sigma) ([^{3}H]5-HT, [^{3}H]NA, [^{14}C]acetylcholine (Ach)), or 3 μM DMI ([^{3}H]DA), respectively, was added to the medium. For GABA release, the GABA transaminase inhibitor amino-oxyacetic acid (10 μM) (Sigma) was present throughout the

experiment to inhibit [^{3}H]GABA degradation. After labeling, the slices were rapidly washed and transferred to each of 24 chambers of a superfusion apparatus (approximately 4 mg tissue in 0.2 ml volume) and superfused (0.2 ml/min) with medium gassed with 95% O_2–5% CO_2 at 37°C. In each observation, calcium-dependent neurotransmitter release was studied simultaneously in 24 parallel superfusion chambers. After 40 min of superfusion (*t*=40 min), the superfusate was collected as 10-min samples. Neurotransmitter release was induced by exposing the slices to electrical biphasic block pulses (1 Hz, 4 ms at 30 mA) ([^{3}H]5-HT, [^{3}H]DA, [^{3}H]NA, [^{14}C]Ach) for 10 min at *t*=50. Depolarization-induced calcium-dependent release of [^{3}H]GABA and [^{3}H]glutamate was induced by exposing the slices for 10 min to 10^{-4} M 4-aminopyridine (4-AP) (Sigma) since electrical field stimulation of brain slices is not effective in this respect (A.N.M.S., unpublished observations). The radioactivity remaining at the end of the experiment was extracted from the tissue with 0.1 N HCl. The radioactivity in superfusion fractions and tissue extracts was determined by liquid scintillation counting. The efflux of radioactivity during each collection was expressed as percentage of the amount of radioactivity in the slices at the beginning of the respective collection period. The depolarization-induced release of neurotransmitter was calculated by subtracting the spontaneous efflux of radioactivity from the total overflow of radioactivity during stimulation and the next 10 min. A linear decline from the 10-min interval before to that 20–30 min after the start of stimulation was assumed for calculation of the spontaneous efflux of radioactivity. The release was expressed as percentage of the content of radioactivity of the slices at the start of the stimulation period.

Microdialysis

Homemade I-shaped probes (dialyzable surface 4 mm) were inserted into the ventral hippocampus according to the following coordinates: bregma −5.3 mm, lateral to midline +4.8 mm and ventral to dura −8.0 mm (for detailed description see Cremers et al., 2004). Experiments were performed 24–48 h after surgery. On the day of the experiment, animals were connected with flexible PEEK tubing to a microperfusion pump (Harvard Apparatus, South Natick, MA, USA) and perfused with an artificial cerebrospinal fluid, comprising 147 mM NaCl, 3.0 mM KCl, 1.2 mM $CaCl_2$, and 1.2 mM $MgCl_2$ at a flow rate of 1.5 µl/min. Fifteen minute microdialysis samples were collected in HPLC vials containing 7.5 µl of 0.02 M acetic acid and kept at −80°C until analyzed. Citalopram (Sigma) was administered at a dose of 3 mg/kg (s.c.).

Brain tissue monoamine levels

Rats were decapitated and the brains were rapidly removed. The cortex, CP, hippocampus, amygdala and RN (dorsal and medial) were dissected on an ice-plate and immediately frozen on dry-ice and subsequently stored at −80°C until use. Brain tissue was sonicated in 0.5 M perchloric acid and 0.5 mM EDTA. The homogenate was centrifuged at 14,000×*g* for 15 min at 4°C and 20 µl supernatant was used for HPLC analysis.

HPLC

Samples (20 µl) were injected via an autoinjector (Agilent 1100) onto a 150×4.6 mm LC18DB Supelcosil 3 µm diameter column (kept at 20°C) and eluted with a 50 mM isocratic ammonium phosphate buffer (pH 2.5) containing 0.1 mM EDTA, 1.5 sodium octylsulfate, 100 mM sodium perchlorate and 1.5% n-propanol. The HPLC outlet was connected to an electrochemical detection chamber (model 400 of Decade, Antec Leiden) using a glassy carbon fiber set to 410 mV as compared with the reference Ag/AgCl Vt-3 electrode. Oxidation potentials were recorded

and analyzed with a computer. Peaks were quantified using N-methyl-serotonin that was added to each sample as external reference.

Statistics

Data were checked for normality and homogeneity and subjected to a log transformation when necessary to achieve these criteria. Analysis was performed using Students' *t*-test, one-way ANOVA, or two-way ANOVA for repeated measures. ANOVA's were followed by Newman-Keuls post hoc tests when significant ($P<0.05$) genotype/treatment or significant interactions were obtained. Non-significant=n.s.

Results

SERT knockout rat characteristics

High-throughput resequencing of target genes in a library of ENU-mutagenized rats (Smits et al., 2006) revealed a C to A transversion at position 3924 (relative to the start codon in ENSRNOG0000003476) in the SERT gene, resulting in a premature stop codon (TGC>TGA) in the third exon encoding the second extracellular loop (Figure. 1A), and thereby most likely resulting in a non-functional protein product. Northern blot analysis (Figure. 1B and C) revealed that the

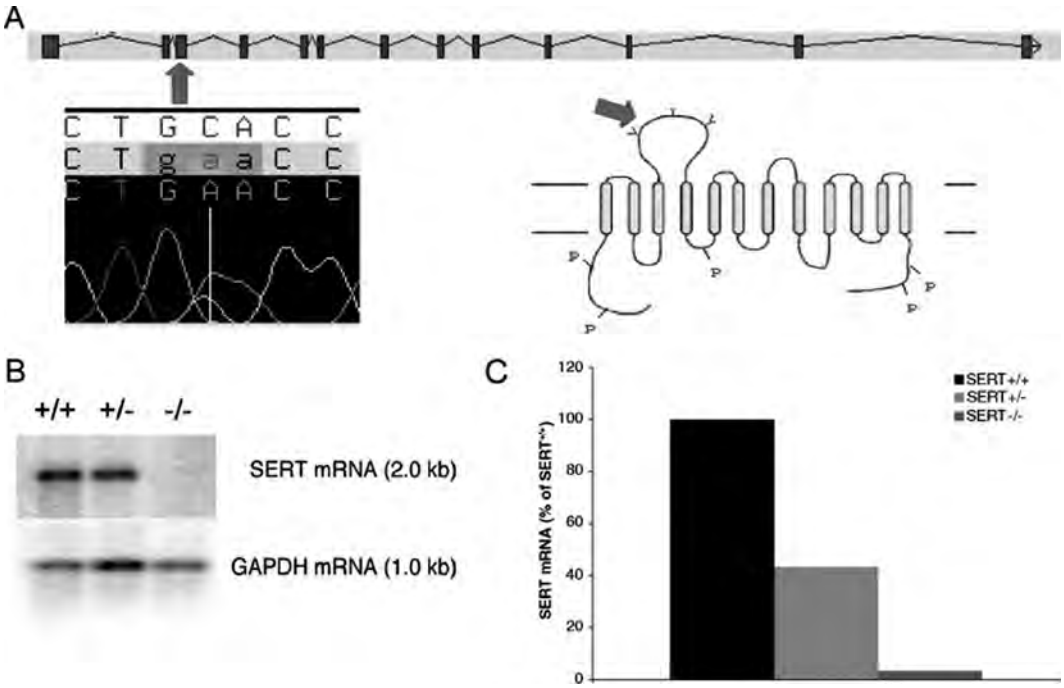

Figure 1

(**A**) Schematic representation of the SERT gene and the induced knockout mutation (Slc6a4^{1Hubr}) obtained by target-selected mutagenesis. The arrow indicates the location of the ENU-induced C to A transversion that results in the change of amino acid 209 from a cysteine to a stopcodon. (**B**) Northern blot analysis and **(C)** densitometric quantification of the SERT transcript in the raphe nucleus of a SERT$^{+/+}$, SERT$^{+/-}$ and SERT$^{-/-}$ rat (n=1). GAPDH mRNA levels were used as internal standards and the optical density of SERT bands was corrected for the amount of RNA using the density of GAPDH bands. The SERT transcript in SERT$^{-/-}$ rats was almost completely absent.

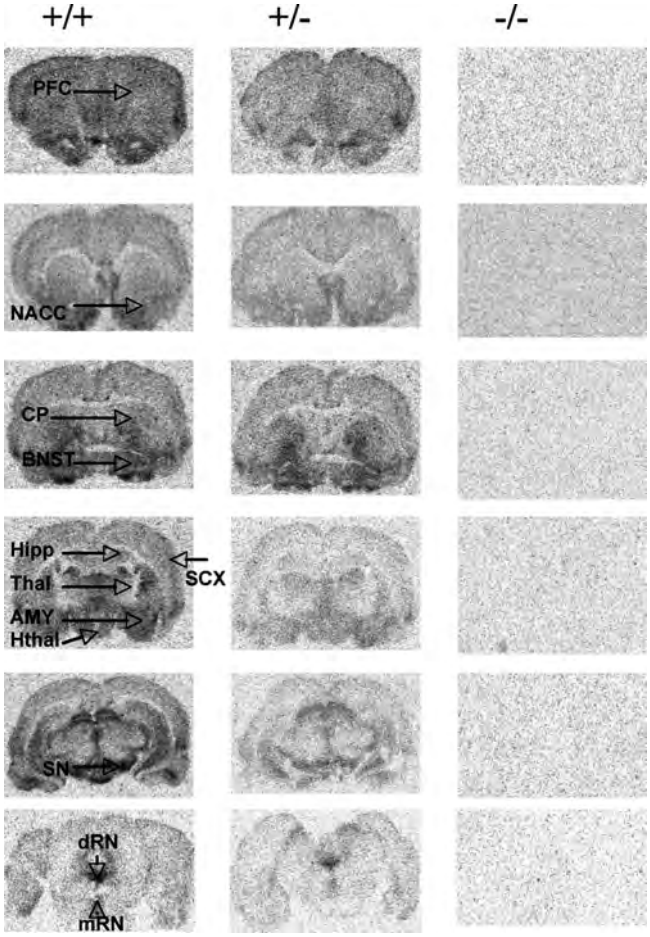

Figure 2
Representative [^{3}H]citalopram autoradiograms of coronal sections from male SERT$^{+/+}$, SERT$^{+/-}$ and SERT$^{-/-}$ rats (n=3) show that SERT protein is completely absent in homozygous knockout animals and is reduced in heterozygous animals. Images for all three genotypes were taken from the same experiment. See table 1 for SERT quantification in the different brain regions. AMY = amygdala, BNST = bed nucleus stria terminalis, CP = caudate putamen, dRN = dorsal raphe nucleus, Hipp = hippocampus, Hthal = hypothalamus, mRN = medial raphe nucleus, NACC = nucleus accumbens, PFC = prefrontal cortex, SN = substantia nigra, SCX = somatosensory cortex, Thal = thalamus.

premature stop codon results in nonsense-mediated decay of the mutant SERT transcript. In addition, absence of [^{3}H]citalopram (SSRI) binding to brain slices of homozygous SERT$^{-/-}$ rats indicates that SERT protein is completely absent (Figure 2 and Table 1), whereas in heterozygous SERT knockout (SERT$^{+/-}$) rats, citalopram binding was reduced by approximately 40% across the measured brain regions (Table 1). At the functional level, *d*-fenfluramine-induced hypothermia was completely absent in SERT$^{-/-}$ rats, and reduced in SERT$^{+/-}$ rats [Figure 3; $F_{(2,497)}$ (genotype, 4 h post *d*-fenfluramine)=14.58, p<0.0005, $F_{(48,497)}$ (time×genotype)=7.57, p=0, post hoc: p<0.05 for all time points between SERT$^{-/-}$ and SERT$^{+/+}$/SERT$^{+/-}$]. From these data it can be concluded that the premature stop codon in the SERT gene results in a full knockout of the SERT, and that SERT expression and function is reduced in SERT$^{+/-}$ rats.

Despite the important role of 5-HT in behavior and the development of the nervous system (Lauder, 1990), homozygous (SERT$^{-/-}$) and heterozygous (SERT$^{+/-}$) knockout rats appear normal, score similar to wild type (SERT$^{+/+}$) littermates on measures of health, and breed normally (litter size 10). Litters consist of wild type, heterozygous and homozygous animals with a Mendelian distribution, indicating that the mutant allele is not associated with embryonic lethality. The weight of female SERT$^{-/-}$ rats is reduced by 10% at the age of 3 weeks and remains reduced, while the weight of male SERT$^{-/-}$ is not different from SERT$^{+/+}$ rats (data not shown).

Table 1. Quantification of SERT, DAT and NET levels in brain regions of $SERT^{+/+}$, $SERT^{+/-}$ and $SERT^{-/-}$ rats using [^{3}H]citalopram, [^{3}H]GBR12935 and [^{3}H]nisoxetine autoradiography

Brain region	Bregma	[^{3}H]citalopram			[^{3}H]GBR12935				[^{3}H]nisoxetine			
		$SERT^{+/+}$	$SERT^{+/-a}$	ANOVA[b]	$SERT^{+/+}$	$SERT^{+/-}$	$SERT^{-/-}$	ANOVA[b]	$SERT^{+/+}$	$SERT^{+/-}$	$SERT^{-/-}$	ANOVA[b]
Nucleus accumbens	2.16	175 ± 9	83 ± 5.3 (48%)*	F(1.18) = 87.45, P<0.001	443 ± 32	445 ± 28	430 ± 24	F(2.11) = 0.11	NS ±	NS	NS	-
Bed nucleus stria terminalis	0.12	106 ± 6	81 ± 7 (80%)*	F(1.11) = 7.75, P<0.05	88 ± 4	88 ± 6	88 ± 6	F(2.9) = 0	79 ± 6	81 ± 7	79 ± 3	F(2.12) = 0.33
Caudate putamen	0.12	82 ± 1.8	38 ± 1.5(46%)*	F(1.18) = 339, P<0.001	713 ± 3	689 ± 7	662 ± 3	F(2.14) = 0.2	7 ± 2	7 ± 3	8 ± 2	F(2.13) = 0.23
Hippocampus	-2.52	85 ± 5.2	51 ± 4.4 (60%)*	F(1.21) = 25.69, P<0.001	128 ± 14	108 ± 21	95 ± 11	F(2.18) = 1.56	56 ± 2	51 ± 2	54 ± 2	F(2.44) = 1.18
Amygdala	-2.52	140 ± 9	127 ± 9 (57%)*	F(1.22) = 23.42,P<0.001	195 ± 17	165 ± 19	143 ± 5	F(2.22) = 2.95	28 ± 7	29 ± 4	30 ± 4	F(2.23) = 0.23
Thalamus	-2.52	147 ± 12	112 ± 12 (76%)*	F(1.18) = 87.45, P<0.001	116 ± 4	124 ± 4	115 ± 12	F(2.7) = 0.89	25 ± 8	26 ± 7	26 ± 8	F(2.23) = 0.69
Somatosensory cortex	-2.52	58 ± 1.6	28 ± 5.5 (54%)*	F(1.14) = 35.05, P<0.001	128 ± 9	108 ± 14	100 ± 6	F(2.29) = 2.31	38 ± 3	30 ± 1	38.8 ± 2	F(2.24) = 0.14
Hypothalamus	-2.52	151 ± 14	105 ± 14 (70%)*	F(2.10) = 5.71, P<0.05	NS	NS	NS	-	22 ± 6	24 ± 2	23 ± 0.1	F(2.9) = 0.48
Substantia nigra	-4.68	222 ± 14	127 ± 15 (57%)*	F(2.13) = 20.35, P<0.001	94 ± 8	95 ± 6	80 ± 16	F(2.10) = 0.42	6 ± 3	7 ± 4	5 ± 2	F(2.22) = 1.61
Raphe nucleus	-6.50	311 ± 18	204 ± 8 (63%)*	F(2.29) = 17.20, P<0.001	ND	ND	ND	-	ND	ND	ND	-

Optical densities were converted into fmol/mg of tissue equivalent using [^{3}H]Microscales^{TM} standards. The data represent mean±S.E.M. fmol/mg tissue. ND, not determined; NS, no signal detected

[a] Percentage of binding as compared to $SERT^{+/+}$ is shown in parentheses.

[b] One-way ANOVA comparing $SERT^{+/+}$ and $SERT^{+/-}$ rats.

* P<0.05.

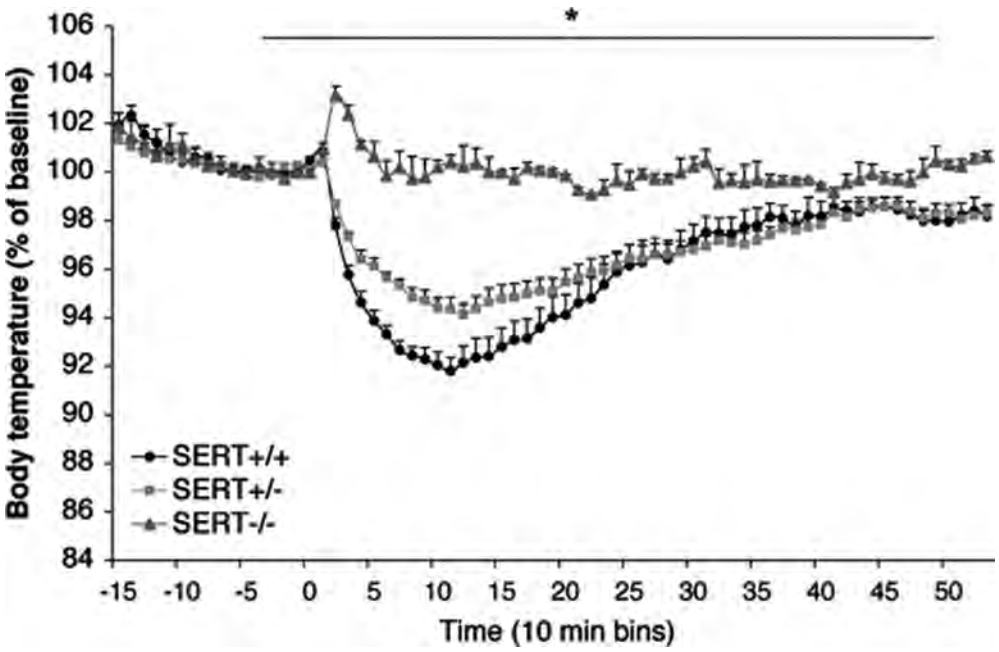

Figure 3
D-fenfluramine-induced hypothermia in male SERT$^{+/+}$, SERT$^{+/-}$ and SERT$^{-/-}$ rats (n = 3-9) as measured by telemetry. The hypothermic response is completely absent in SERT$^{-/-}$ rats and significantly reduced in SERT$^{+/-}$. A very short-lasting increase of body temperature is observed in knockout animals at the time of injection, most likely resulting from a serotonin-independent stress-response. Data are expressed as mean (± S.E.M.) % from baseline temperature (100 %). * $P < 0.005$.

Serotonin homeostasis

The SERT reuptakes 5-HT into the presynaptic nerve terminal, and this process is likely to be reduced in SERT mutant rats, although there may be alternative routes by which 5-HT can be taken up. To investigate this, we measured 5-HT uptake in hippocampal synaptosomes (Figure. 4). The hippocampus was chosen to 'model' the presynaptic adaptations in response to the absence of the SERT because this brain structure is of interest in many types of behavior and is strongly innervated by serotonergic neurons. The maximum rate (V_{max}) of 5-HT uptake in the synaptosomes was reduced by 13.4% in SERT$^{+/-}$ rats, and by 72.2% in SERT$^{-/-}$ rats [Figure. 4A; $F_{(2,111)}$ (genotype)=54.08, P=0]. Application of the noradrenaline transporter (NET) blocker DMI [Figure. 4C; $t_{(1,8)}$ (SERT$^{+/+}$)=0.380, n.s.; $t_{(1,8)}$ (SERT$^{+/-}$)=0.896, n.s.; $t_{(1,8)}$ (SERT$^{-/-}$) 5.97, P=0], but not the dopamine transporter (DAT) blocker GBR12935 [Figure. 4B; $t_{(1,8)}$ (SERT$^{+/+}$)=0.4645; $t_{(1,8)}$ (SERT$^{+/-}$)=0.267, n.s.; $t_{(1,8)}$ (SERT$^{-/-}$)=0.1, n.s.], abolished residual [^{3}H]5-HT uptake, suggesting that 5-HT uptake is partially maintained by noradrenergic neurons in the hippocampus.

Although noradrenergic neurons seem to be capable of taking up 5-HT *in vitro*, the 5-HT K_m for the NET is much lower than for SERT (Daws et al., 1998), raising the question if this activity is sufficient for efficient 5-HT re-uptake *in vivo*. Therefore, we determined the extracellular 5-HT levels in the ventral hippocampus by microdialysis. Basal extracellular 5-HT levels were found to be 9-fold increased in the hippocampus of SERT$^{-/-}$ rats (Figure. 5). Systemic citalopram administration had no effect in SERT$^{-/-}$ rats, while 5-HT levels in SERT$^{+/+}$ rats were increased to basal 5-HT levels found

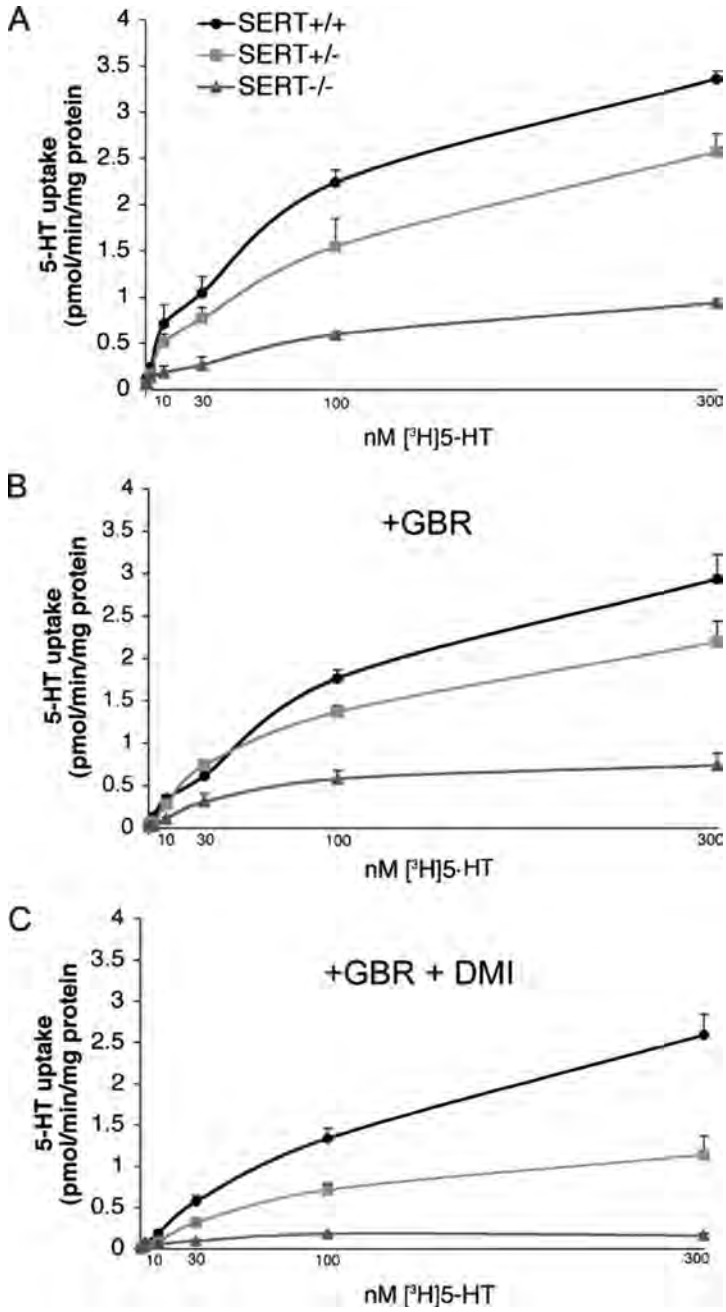

Figure 4

(**A**) [^{3}H]5-HT uptake into hippocampal synaptosomes of male SERT$^{+/+}$, SERT$^{+/-}$ and SERT$^{-/-}$ rats in the absence of additional monoamine transporter inhibitors (n =3) **(B)** in the presence of 1 µM GBR12935 (n = 3-4), and **(C)** in the presence of 1 µM GBR12935 + 1 µM DMI (n = 3-4). 5-HT was gene-dose dependently reduced in SERT$^{-/-}$ and SERT$^{+/-}$ rats, but not completely absent in SERT$^{-/-}$ rats. Inhibition of the NET (DMI), but not DAT (GBR12935) attenuated residual 5-HT uptake. Data are expressed as mean (± S.E.M.) [^{3}H]5-HT (pmol/mg protein/10 min) uptake.

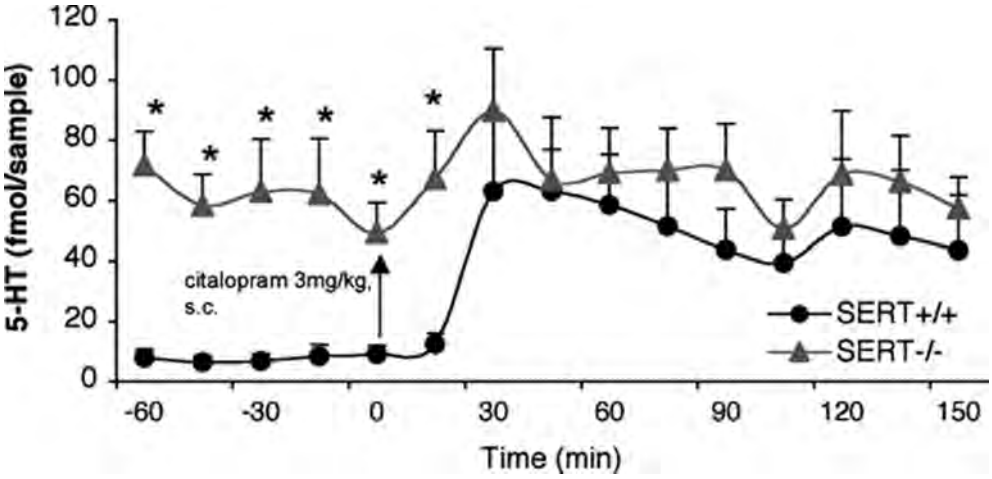

Figure 5
Extracellular 5-HT levels in the ventral hippocampus of male SERT$^{+/+}$ and SERT$^{-/-}$ rats as measured by *in vivo* microdialysis (n = 5). Basal dialysate 5-HT levels are six-fold increased in SERT$^{-/-}$ rats. 3 mg/kg citalopram increased 5-HT levels in SERT$^{+/+}$ rats to that of SERT$^{-/-}$ rats, while citalopram had no effect in SERT$^{-/-}$ rats. Data represent mean (± S.E.M.) extracellular 5-HT (fmol/sample). *P <0.05.

in SERT$^{-/-}$ rats [$F_{(1,146)}$ (genotype)=3.06, p<0.05; $F_{(2,146)}$ (genotype×treatment)=2.62, p<0.005, post hoc: p<0.05 over baseline, t=0 and t=15].

To study whether the reduced 5-HT uptake and increased extracellular 5-HT levels affect 5-HT synthesis and degradation, we measured the activity and immunoreactivity of TPH, the rate-limiting enzyme involved in 5-HT synthesis, and the activity of MAO. TPH activity was not different between genotypes in the RN [$F_{(2,12)}$=1.62, n.s.] and CP [$F_{(2,10)}$=0.18, n.s.] (Figure 6A). Also TPH immunoreactivity in the dorsal RN, which most likely reflects THP2 levels as THP1 is not expressed in the RN (Patel et al., 2004), did not differ between genotypes [Figure 6B; $F_{(2,5)}$=4.64, n.s]. Two MAO isoforms exist, MAO-A and MAO-B. Because 5-HT is mainly oxidized by MAO-A (Shih et al., 1999), we measured MAO-A activity. In neither the cortex [Figure 6C; $F_{(2,11)}$=1.25, n.s.], nor the CP [$F_{(2,12)}$=0.34, n.s.] were differences found in MAO-A activity.

As reduced 5-HT uptake may affect intracellular 5-HT and 5-hydroxyindoleacetic acid (5-HIAA, 5-HT's primary metabolite) levels, we measured 5-HT and 5-HIAA tissue levels, assuming that neurotransmitter tissue levels primarily represent intracellular concentrations. In the hippocampus, CP, cortex and amygdala of SERT$^{-/-}$ rats, but not SERT$^{+/-}$ rats, 5-HT and 5-HIAA levels were reduced by 75–50% and 45–55%, respectively (Table 2). The 5-HIAA/5-HT ratio, was significantly higher in the cortex and CP, but not in the amygdala.

Finally, to study whether the functioning of the presynaptic 5-HT neuron is affected by increased extracellular 5-HT levels we measured [^{3}H]5-HT uptake and depolarization-induced release in superfused brain slices of the hippocampus and cortex. [^{3}H]5-HT uptake was reduced by 90% in SERT$^{-/-}$ rats, and electrically-evoked [^{3}H]5-HT release was reduced by 40% in the CP, by 90% in the hippocampus and by 97% in the hypothalamus and cortex of SERT$^{-/-}$ rats (Table 3). No

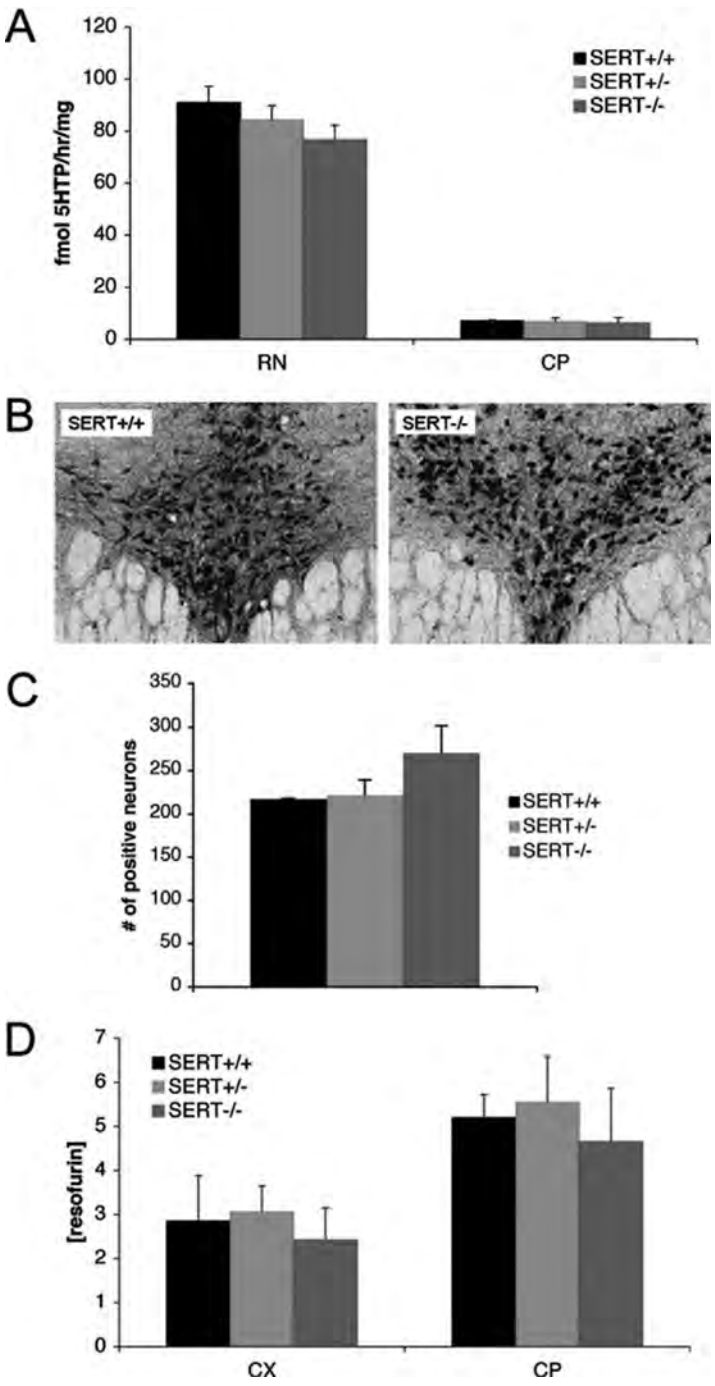

Figure 6

(**A**) TPH activity in the dorsal and medial RN and CP. Data represent mean (± S.E.M.) 5-HTP produced per hour per mg protein (n=4). (**B**) TPH staining in the dorsal RN and (**C**) quantification of neurons positive for TPH binding per representative section (n=2-3). (**D**) MAO-A activity in the CP and cortex. Data represent mean (± S.E.M.) resofurin. For both TPH and MAO-A activity no differences were found between genotypes (n=4).

Table 2. Brain tissue monoamine levels

		SERT$^{+/+}$	SERT$^{+/-}$	SERT$^{-/-}$	ANOVA
5-HT	Hippocampus	664±162	326±36.4	239±39	$F_{(2,15)}$=2.93, n.s.; $T_{(1,9)}$=1.944, P<0.05*
	Cortex	150±7	152±8.1	35±5.2*	$F_{(2,11)}$=87.39, P=0
	CP	487±19.2	543±36.2	225±27*	$F_{(2,11)}$=36.86, P<0.00005
	Amygdala	466±54.6	565±47	193±40*	$F_{(2,15)}$=36.86, P<0.0005
5-HIAA	Cortex	109±6.6	88±2.6	53±9.9*	$F_{(2,11)}$=16.53, P<0.0005
	CP	322±2	343±26.4	179±9*	$F_{(2,11)}$=23.79, P<0.0005
	Amygdala	320±39.7	316±39	142±14.2*	$F_{(2,17)}$=8.62, P<0.005
5-HIAA/5-HT	Cortex	0.74±0.06	0.58±0.02	1.63±0.4*	$F_{(2,10)}$=9.00, P<0.01
	CP	0.66±0.01	0.63±0.01	0.82±0.06*	$F_{(2,10)}$=8.57, P<0.01
	Amygdala	0.64±0.05	0.52±0.06	0.87±0.15	$F_{(2,17)}$=3.18, n.s.
DA	Cortex	84±16	106±22	69±6	$F_{(2,22)}$=1.87
	CP	10815±1478	11373±1189	11957±474	$F_{(2,19)}$=1.13
	Amygdala	1313±406	1884±699	1318±377	$F_{(2,21)}$=0.43
DOPAC	Cortex	13±2	9±2	15±2	$F_{(2,13)}$=2.08
	CP	8180±922	6075±771	6227±1506	$F_{(2,19)}$=2.40
	Amygdala	208±70	208±40	199±47	$F_{(2,18)}$=0.01
HVA	Cortex	9±2.9	12±1.2	8.5±0.6	$F_{(2,11)}$=0.87, n.s.
	CP	730±60.8	757±59.3	575±3.7	$F_{(2,11)}$=2.97, n.s.
	Amygdala	65.7±16.3	64±13	26±4.1	$F_{(2,15)}$=2.41, n.s.
NE	Cortex	205±5.9	207±9.3	198±11.8	$F_{(2,19)}$=0.27, n.s.
	CP	85±6.4	183±55.7	147±40.5	$F_{(2,18)}$=1.57, n.s.
	Amygdala	255±52	384±8.8	407±35*	$F_{(2,15)}$=4.72, P<0.05

Data are expressed as mean pg/mg tissue monoamines and metabolites (±S.E.M.)
* *t*-Test SERT$^{+/+}$ vs SERT$^{-/-}$.
a P<0.05 vs. SERT$^{+/+}$.

differences in [^{3}H]5-HT uptake were observed in SERT$^{+/-}$ animals, despite the observed reduced synaptosomal [^{3}H]5-HT uptake in these animals. This apparent discrepancy may be explained by differences in sensitivity between the two assays (Montañez et al., 2003). Finally, depolarization-induced [^{3}H]5-HT release was not affected in SERT$^{+/-}$ rats.

No changes in presynaptic functioning of non-serotonergic systems

Because 5-HT strongly interacts with other neurotransmitter systems, in particular the dopaminergic and noradrenergic systems, the disturbed 5-HT homeostasis may have consequences for the functioning of these systems. First, we measured the density of the DAT and NET, which may partially take over the function of the SERT. Despite the finding that the NET is responsible for residual 5-HT uptake in the hippocampus of SERT$^{-/-}$ rats *in vitro* (Figure. 4C), NET concentrations, as well as DAT concentrations, were not different between genotypes throughout the brain (Table 1). We further determined the presynaptic functioning of dopaminergic and noradrenergic neurons by [^{3}H]NE and [^{3}H]DA uptake and their depolarization-induced release in superfused brain slices. Again no genotype differences were found (Table 3). In line with these observations, DA, NE, DOPAC and HVA tissue levels were similar in rats of all genotypes (Table 2), except for NE levels in the amygdala, which were increased in SERT$^{-/-}$ rats. Beyond the monoaminergic systems, the uptake and depolarization-induced release of [^{14}C]Ach, [^{3}H]GABA and [^{3}H]glutamate in the hippocampus and cortex was also not different between genotypes (Table 3).

Table 3. Uptake and depolarization-induced calcium-dependent release of radiolabeled 5-HT, DA, NA, ACh, GABA and glutamate in superfused brain slices

		Uptake				Release			
		SERT$^{+/+}$	SERT$^{+/-}$	SERT$^{-/-}$	ANOVA	SERT$^{+/+}$	SERT$^{+/-}$	SERT$^{-/-}$	ANOVA
5-HT	Hippocampus	100±2.6	110±2.6	16.0±1.2*	$F_{(2,125)}$=347.2, P=0	100±2.3	99±4	10±1.7*	$F_{(2,119)}$=89.59, P=0
	Cortex	100±1.3	106±2.5	18±1.5*	$F_{(2,89)}$=191.13, P=0	100±5.5	133±5.7	3±0.5*	$F_{(2,80)}$=937.50, P=0
DA	CP	100±5.2	115±3.7	102±6.2	$F_{(2,23)}$=0.78	100±2.2	112±8.5	112±9.6	$F_{(2,21)}$=0.45
NA	Hippocampus	100±16	122±16	130±5.2	$F_{(2,13)}$=0.48	100±2.9	107±4	111±2.9	$F_{(2,33)}$=3.16
	Cortex	100±3.4	100±4.5	114±8.5	$F_{(2,33)}$=0.27	100±3.4	116±2.9	111±5	$F_{(2,33)}$=1.75
Ach	Hippocampus	100±3	117±8.5	97±9.9	$F_{(2,66)}$=0.63	100±2.3	105±5.4	107±4.8	$F_{(2,36)}$=0.39
	Cortex	100±2.6	117±4.4	102±4.3	$F_{(2,79)}$=2.54	100±1.8	99±2.5	103±3.1	$F_{(2,81)}$=0.56
GABA	Hippocampus	100±16	122±16	130±5.2	$F_{(2,33)}$=0.48	100±1.4	115±5.6	105±6	$F_{(2,36)}$=0.66
	Cortex	100±3.4	118±12.4	95±4.2	$F_{(2,33)}$=0.57	100±1.7	97±5.6	94±5.6	$F_{(2,36)}$=0.11
Glutamate	Hippocampus	100±3.3	106±4.8	108±7.2	$F_{(2,29)}$=0.20	100±3.0	94±5.0	109±6.0	$F_{(2,29)}$=0.67

Neurotransmitter uptake was determined by measuring the total amount of radioactivity in the slices at the onset of depolarization during superfusion, and was expressed as percentage of the accumulation of radiolabeled neurotransmitter in brain slices of SERT+/+ rats. Spontaneous neurotransmitter efflux averaged 2.5–4% of total tissue radioactivity. Depolarization-induced neurotransmitter release in excess of spontaneous efflux was calculated as percentage of total radioactivity in the slices at the onset of depolarization and expressed as percentage of that found in brain slices of SERT+/+ rats (average depolarization-induced [3H]5-HT release: hippocampus 4.9%, cortex 5.3%; [3H]DA release: CP 4.9%; [3H]NA release: hippocampus 8.6%, cortex 11.7%; [14C]ACh release: hippocampus 1.5%, cortex 3.1%; [3H]GABA release: hippocampus 4.9%, cortex 9.8%; [3H]glutamate release: hippocampus 5.3%). Data represent means (±S.E.M.) of three experiments (quadruplicate observations per experiment).

* P<0.05 vs. SERT$^{+/+}$ data.

Discussion

Using the recently established gene knockout technology (Zan et al., 2003, Smits et al., 2004 and Smits et al., 2006) we obtained a rat knockout for the SERT gene, and here we present the first comprehensive characterization of this novel model. We show that the ENU-induced premature stop codon results into a complete lack of SERT, and interestingly, that SERT$^{+/-}$ rats show a gene-dosage effect with respect to SERT expression and function. The SERT$^{+/-}$ rat may therefore be a novel model system for studying the human 5-HTTLPR polymorphism (Lesch et al., 1996), although a more detailed (behavioral) analysis will be needed to support this. We show that extracellular 5-HT levels are increased in SERT$^{-/-}$ rats and *in vitro* data suggest that this may be counterbalanced by false 5-HT uptake by the NET in the hippocampus and reduced depolarization-induced release of 5-HT. Most importantly, no changes were found in the presynaptic functioning of dopaminergic, noradrenergic, cholinergic, glutamatergic and GABAergic neurons, indicating that compensatory adaptations for the lifelong absence of the SERT are primarily limited to the serotonergic system.

The nature of non-transgenic ENU-induced gene knockouts is fundamentally different from the more common mouse knockouts generated by homologous recombination. In the latter models, genomic fragments encoding important protein domains or the complete protein are replaced by a selection cassette, whereas the model presented here results from a single point mutation that introduces a premature stop codon. Theoretically, it is still possible that an almost intact protein is made, for example resulting from an alternatively spliced transcript. However, we show that the SERT mRNA in SERT$^{-/-}$ rats is completely absent, suggesting that the mutated transcript is subjected to nonsense-mediated decay (Baker and Parker, 2004). At the protein level

the binding of the highly selective SSRI citalopram was completely absent, indicating that there is no properly folded and functionally active SERT protein in the brain of the SERT$^{-/-}$ rats. This is confirmed by the absence of an effect in knockout animals of *d*-fenfluramine, which in wild type animals induces reverse 5-HT transport via the SERT and a reduction in body temperature. Although the exact mechanism of this process is not clear, the absence of a response in SERT$^{-/-}$ rats is most likely due to inability of *d*-fenfluramine to induce 5-HT release via the SERT, as the response in wild type animals can be blocked by citalopram (not shown). SERT function was also directly measured in hippocampal synaptosomes. 5-HT uptake was strongly reduced in SERT$^{-/-}$ rats, but was not completely absent, suggesting that residual 5-HT uptake takes place via other transporters, such as the DAT and NET, or the cation-transporter (Schmitt et al., 2003). We show that the remaining 5-HT uptake activity *in vitro* can efficiently be inhibited by the NET-blocker DMI, but the observed ninefold increased extracellular levels of 5-HT suggest that this activity is not sufficient to maintain low homeostatic extracellular 5-HT levels *in vivo*. In DA rich regions, such as the striatum, the DAT may partially compensate for reduced 5-HT uptake in the rat (Zhou et al., 2002). These and previous observations (Pan et al., 2001; Moron et al., 2002; Vizi et al., 2004) indicate that monoamine transporters are not neurotransmitter selective, insights that are important to understand the neurochemical and behavioral effects of drugs that inhibit (e.g. cocaine) or reverse (e.g. amphetamine) monoamine transporter function.

Interestingly, the SERT$^{+/-}$ rat shows intermediate phenotypes with respect to SERT mRNA levels, SERT protein density and SERT function. Despite these observations, SERT$^{+/-}$ rats did not differ from wild type animals in 5-HT tissue levels and the reactivity of serotonergic neurons. Similarly, in the SERT$^{+/-}$ mouse no changes have been reported for intracellular 5-HT levels (Kim et al., 2005), suggesting that there is at least a redundancy of 40–50% in SERT availability that is not needed for normal presynaptic functioning of serotonergic neurons. Dialysate 5-HT levels, however, are gene-dose dependently increased in the SERT$^{+/-}$ mouse (Mathews et al., 2004), suggesting that extracellular 5-HT levels and SERT function are directly related. This observation is of particular importance in relation to the human 5-HTTLPR, which influences SERT expression and function, and thereby may affect extraneural 5-HT levels. SERT$^{+/-}$ models may therefore be a valuable model to address the biological and behavioral consequences of the 5-HTTLPR, although detection of 5-HTTLPR-linked symptoms of disorders or traits in SERT$^{+/-}$ mice are dependent on the genetic background and not always observed (Holmes et al., 2002a; Holmes et al., 2003b; Adamec et al., 2006). To extend the human relevance of SERT$^{+/-}$ models to the rat we are currently determining extracellular 5-HT levels in SERT$^{+/-}$ rats and testing these animals in advanced human-derived cognitive tasks.

Our results indicate that the adaptations in the presynaptic serotonergic system are a direct or indirect consequence of the reduced 5-HT reuptake and high extracellular 5-HT levels. Thus, reduced 5-HT and 5-HIAA tissue levels are likely to result from reduced 5-HT uptake, as no differences were found in the activity of TPH, the rate-limiting enzyme mediating 5-HT synthesis, and the activity of MAO-A, the enzyme that converts 5-HT into 5-HIAA. However, in the SERT$^{-/-}$ mouse *in vivo* TPH activity is increased, while *in vitro* TPH activity is unchanged (Kim et al., 2005). This raises the possibility that *in vivo* TPH activity is increased in SERT$^{-/-}$ rats as well, despite unchanged TPH density. 5-HT synthesis is negatively controlled by presynaptic 5-HT$_{1B}$ autoreceptors in terminal regions (Moret and Briley, 1990), as well as somatodendritic 5-HT$_{1A}$ autoreceptors that influence the firing rate of serotonergic neurons in the RN (Hamon et al.,

1991). Both types of receptors are desensitized in SERT$^{-/-}$ mice (Fabre et al., 2000; Li et al 2000), and their firing rate of raphe neurons is decreased as well (Gobbi et al., 2001). These adaptations would reduce 5-HT release and relieve tonic autoreceptor-mediated inhibition of synthesis. If TPH activity would be increased, this increase is seemingly not sufficient to compensate the reduced 5-HT uptake. Strikingly, despite unaltered activities of TPH and MAO-A, the 5-HIAA/5-HT ratio was increased in the cortex and CP, but not the amygdala, of SERT$^{-/-}$ rats. An increase in tissue 5-HIAA/5-HT ratios has been observed in the SERT$^{-/-}$ mouse (Kim et al., 2005), but an explanation is lacking. 5-HT is catabolized into 5-HIAA by the intracellular located MAO and 5-HIAA is freely diffusible across the cell membrane. As extraneural 5-HIAA levels are decreased in SERT$^{-/-}$ mice (Mathews et al., 2004), it might be that there is less diffusion of 5-HIAA to the extracellular space. Nevertheless, the increased 5-HIAA/5-HT ratio indicates that 5-HT turnover is increased in SERT$^{-/-}$ rats and that less 5-HT may be available for neurotransmission. Another mechanism to compensate for reduced 5-HT uptake is a reduction in Ca^{2+}-dependent 5-HT release. Indeed, we found that [^{3}H]5-HT release in superfused brain slices was reduced by 40% in the CP, and even more so in the hippocampus, hypothalamus and cortex. In comparison, *in vitro* depolarization-induced [^{3}H]NE release is significantly reduced in cortical slices of the NET knockout mouse (Vizi et al., 2004). The reduced release of 5-HT in SERT$^{-/-}$ rats in these experiments is not due to the observed decreased 5-HT uptake in the same assay, because the release as the fraction of radioactivity present at time of release was increased in the CP. The reduced 5-HT release may be explained by reduced availability of intracellular 5-HT, but may also reflect an adaptation to counteract the high extracellular 5-HT levels. 5-HT$_{1B}$ autoreceptors negatively control the release of 5-HT (Moret and Briley, 1999), and in SERT$^{-/-}$ mice the expression and function of these receptors are reduced (Fabre et al., 2000). Because 5-HT release was measured *in vitro*, without direct tonic regulation by raphe neurons, a reduced reactivity of serotonergic neurons may reflect a local adaptation. Clearly, understanding the mechanism underlying reduced Ca^{2+}-dependent release requires further research. Combining strongly reduced 5-HT uptake and release, and reduced 5-HT tissue levels, 5-HT recycling appears to be attenuated in SERT$^{-/-}$ rats, indicating that the serotonergic system may have lost its dynamics and flexibility. Thus 5-HT homeostasis has changed in such a way that the animal is able to function normally under basal conditions, but when exposed to challenges or stimuli, this system may not be able to adapt appropriately, resulting in aberrant behavioral responses (Bengel et al., 1998; Sora et al., 1998; Holmes et al., 2002a; Holmes et al., 2003b; Lira et al., 2003; Adamec et al., 2006).

Because the serotonergic system interacts strongly with other neurotransmitter systems, in particular the catecholaminergic systems, we extended our analysis on adaptive consequences of the SERT knockout to non-serotonergic systems. As reported previously for the SERT knockout mouse (Shen et al., 2004), no changes were found in DA and NE tissue levels. In addition, we did not find differences between genotypes in tissue levels of DOPAC and HVA (DA metabolites), as well as tissue levels of NE, except for NE levels in the amygdala, which were decreased in SERT$^{-/-}$ rats. Although highly speculative, this observation may relate to the observed hyperreactivity of the amygdala to fearful stimuli in *s/s* carriers of the 5-HTTLPR (Hariri et al., 2002). Also, no differences between SERT$^{+/+}$ and SERT$^{-/-}$ rats were found for DAT and NET densities and for *in vitro* depolarization-induced release of DA, NE, Ach, GABA and glutamate, suggesting that the consequences of the absence of SERT are limited to the serotonergic system and do not result in compensations of non-serotonergic presynaptic neurons. Although *in vivo* microdialysis studies still need to be performed, the lack of adaptations in the presynaptic function of non-

serotonergic systems may imply that extraneural levels of DA, NE, Ach and glutamate are unchanged in SERT$^{-/-}$ rats, as has previously been reported for extracellular DA levels in the striatum, cortex (Mathews et al., 2004) and substantia nigra (Fabre et al., 2000) of SERT$^{-/-}$ mice. In comparison with the DAT and NET knockout mice, it seems unique for the SERT knockout models that compensatory adaptations are limited to the system that is affected, as DAT and NET knockout mice display compensatory adaptations in the serotonergic (Rocha et al., 1998) and dopaminergic (Xu et al., 2000) systems, respectively. Also, DAT knockout mice exhibit severe reductions in tyrosine hydroxylase protein (Jaber et al., 1999) and striatal DA tissue levels, and increased DA synthesis (Gainetdinov et al., 1998), implying that homeostasis is differentially regulated in the dopaminergic and serotonergic systems.

Because one cannot simply assume that SERT knockout rats adapt in a similar fashion to the absence of the SERT as the respective mouse model, we replicated many measurements as done in the SERT$^{-/-}$ mouse, but also performed measurements that have not yet been addressed in the knockout mouse. In general, the compensatory mechanisms associated with the absence of the SERT are comparable to those found in SERT$^{-/-}$ mice (e.g. Bengel et al., 1998; Mathews et al., 2004; Kim et al .,2005). Extracellular 5-HT levels are increased in the hippocampus of SERT$^{-/-}$ rats similar as described for the mouse substantia nigra (Fabre et al., 2000), striatum and frontal cortex (Mathews et al., 2004). Furthermore, SERT knockout rats did not differ in *in vitro* MAO-A and TPH activity as did SERT$^{-/-}$ mice (Mathews et al., 2004; Kim et al., 2005), 5-HT and 5-HIAA tissue levels were decreased in hippocampus, cortex, CP and amygdala of the rat and mouse (Kim et al., 2005), NET density as assessed by autoradiography was unchanged in both SERT$^{-/-}$ rats and mice (Montañez et al., 2003), and DA tissue levels were unchanged in both species (Shen et al., 2004). Differences between SERT$^{-/-}$ mice and rats were also found. For example, in contrast to SERT$^{-/-}$ rats, there was no compensatory 5-HT uptake in hippocampal synaptosomes of SERT$^{-/-}$ mice (Bengel et al., 1998). Also, synaptosomal 5-HT uptake in SERT$^{+/-}$ mice did not differ from SERT$^{+/+}$ mice (Bengel et al., 1998), while SERT$^{+/-}$ rats displayed a 13% reduction in 5-HT uptake. Although the 5-HT concentrations used in the Bengel et al. (1998) study may have been below a threshold concentration of extracellular 5-HT required to maximally recruit SERT to the plasma membrane, intermediate levels of 5-HT uptake in SERT$^{+/-}$ mice have been reported using high-speed chronoamperometry (Montañez et al., 2003). Furthermore, we found that 1 µM of the highly selective NE uptake inhibitor DMI, displaying an EC50 of about 0.3 µM for NET under our experimental conditions (A.N.M.S., unpublished observations), completely abolished residual 5-HT uptake, suggesting that hippocampal synaptosomes partially accumulate 5-HT in NE nerve terminals. In the study of Montañez (2003), however, NET inhibition had no effect on 5-HT clearance in SERT$^{-/-}$ mice. Although the techniques used differ and compensatory effects may depend on the genetic background of the mouse inbred strain used, they may also reflect species-specific differences. These similarities and differences between SERT$^{-/-}$ rats and mice are highly informative for cross-species translation of results obtained in different rodent models and/or the extrapolation to the human situation.

Taken together, we show that genetic inactivation of the SERT gene in the rat results in a selective change of central serotonergic functioning. This makes this animal model very useful to dissect the role of 5-HT in various peripheral and neural processes that are related to 5-HT. The constitutive increased basal dialysate 5-HT levels most likely affect the expression and function of 5-HT receptors, which is currently under investigation. We argue that such adaptations are

not only informative because they play an important role in the regulation of 5-HT homeostasis and may underlie behavioral phenotypes in SERT$^{-/-}$ models, but also because they may serve as targets for medication. Interestingly, chronic SSRI treatment results in a profound reduction in SERT expression and function (Benmansour et al., 1999), and increased extracellular 5-HT levels, which is strikingly similar to the situation in the knockout model described here. This suggests that the SERT$^{-/-}$ rat may be a useful tool to get insight into the long-term effects of chronic SSRI treatment, although it should be mentioned that a genetic knockout model is expected to result in a more complete inactivation than pharmacological intervention, which could be associated with spatial and or temporal heterogeneity in knockout/knockdown levels. In conclusion, the novel rat model described here may help in unraveling the complex role of 5-HT in a wide variety of neurobiological phenotypes.

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3

A study in male and female serotonin transporter knockout rats: An animal model for anxiety and depression disorders

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Abstract

Human studies have shown that a reduction of SERT increases the vulnerability for anxiety and depression. Moreover, women are more vulnerable to develop depression and anxiety disorders than men. For that reason we hypothesized that $SERT^{-/-}$ models, especially female, are valuable and reliable animal models for humans with an increased vulnerability for anxiety- and depression-related disorders. As rats are extensively used in neuroscience research, we used the unique serotonin transporter knockout rat, that was recently generated using ENU-driven mutagenesis, to test this hypothesis. Behavioral testing revealed that male and female $SERT^{-/-}$ rats spent less time in the center of the open field and spent less time on the open arm of the elevated plus maze compared to $SERT^{+/+}$ rats. In the novelty suppressed feeding test, only male $SERT^{-/-}$ rats showed a higher latency before starting to eat in a bright novel arena compared to $SERT^{+/+}$ controls. Both male and female $SERT^{-/-}$ rats showed a higher escape latency from their home cage than $SERT^{+/+}$ littermates. Moreover, $SERT^{-/-}$ rats were less mobile in the forced swim test, and sucrose consumption was reduced in $SERT^{-/-}$ rats relative to $SERT^{+/+}$ rats. Both effects were sex-independent. Neurochemically, basal extracellular 5-HT levels were elevated to a similar extent in male and female $SERT^{-/-}$ rats , which was not influenced by the selective serotonin reuptake inhibitor citalopram. 5-HT immunostaining revealed no difference between $SERT^{+/+}$ and $SERT^{-/-}$ rats in the dorsal raphe nuclei, in both males and females. These findings demonstrate that $SERT^{-/-}$ rats show anxiety and depression-related behavior, independent on sex. Genetic inactivation of the SERT has apparently such a great impact on behavior, that hardly any differences are found between male and female rats. This knockout rat model may provide a valuable model to study anxiety- and depression-related disorders in male and female rats.

Introduction

Serotonin (5-hydroxytryptamine, 5-HT) is a key modulatory neurotransmitter and has been implicated in the pathophysiology and treatment of anxiety and mood disorders (Neumeister et al., 2002). It is widely accepted that disturbances in the 5-HT system are involved in the onset of depression (reviewed in Jans et al., 2007b). Several alterations in the 5-HT system have been reported in depression, including decreased plasma tryptophan levels (Coppen et al., 1973;Cowen et al., 1989) and decreased levels of 5-hydroxyindoleacetic acid (5-HIAA; metabolite of 5-HT) in cerebrospinal fluid (CSF) (Asberg et al., 1976a;Asberg et al., 1976b;Owens and Nemeroff, 1998), suggesting decreased 5-HT metabolism in the central nervous system. Moreover, brain imaging studies have reported a reduction in 5-HT_{1A} receptor binding, which failed to normalize after treatment for depression (Drevets et al., 1999;Sargent et al., 2000). The extracellular level of 5-HT is primarily regulated by the serotonin transporter (Slc6a4; SERT), which reuptakes 5-HT from the extracellular space into the presynaptic neuron where it can be degraded or retained for future release (Blakely et al., 1991;Murphy et al., 1998). Due to this important role, it is not surprising that genetic alterations in the SERT are associated with multiple neuropsychiatric disorders (Murphy et al., 1999;Gingrich and Hen, 2001;Holmes et al., 2003c). For example, the human SERT gene transcription is modulated by a common polymorphism in its upstream regulatory region. Studies in reporter gene constructs and in human lymphoblastic cell lines found that the short variant of the polymorphism (*HTTLPR*s) reduces the transcriptional efficiency of the SERT gene (Lesch et al., 1996;Heils et al., 1996;Heils et al., 1997). Moreover, the long variant was associated with more rapid initial platelet 5-HT uptake (Greenberg et al., 1999). However, the *HTTLPR* genotype was not related to the level of SERT binding by autoradiography in the prefrontal cortex (Mann et al., 2000). It was even showed by van Dyck et al. (2004) that the SERT availability in the short-short homozygotes was greater compared to long-short heterozygotes, in young aged subjects. Several 5-HTTLPR genetic studies have shown a linkage between the short variant and psychiatric conditions. For example carriers of the short version display significantly higher scores in neurotism, an anxiety-related personality trait, and exhibit more depressive symptoms, diagnosable depression, and suicidality in relation to stressful events compared to individuals with the long version (Mann et al., 2000;Caspi et al., 2003;Schmitz et al., 2007;Dick et al., 2007;Canli and Lesch, 2007), although not every study has reached the same conclusions in this respect (e.g. Lang et al. (2004)).

Women experience depression about twice as often as men (Gorman, 2006), and several possible explanations for this phenomenon have been proposed, some of which are directly or indirectly related to 5-HT neurotransmission. In human, women have significantly higher concentrations of both 5HIAA (5-hydroxyindoleacetic acid) and HVA (homovanillic acid) in their CSF (cerebrospinal fluid) than men (Agren et al., 1986), however this provides no direct measure of 5-HT synthesis in the brain. *In vivo* measurements of 5-HT synthesis in the brain by positron emission tomography showed that whole brain rates of 5-HT synthesis is lower in women than in men (Nishizawa et al., 1997). Women also have lower SERT binding in the prefrontal cortex than men (Mann et al., 2000). Moreover, depressed women show a substantial decrease of SERT availability, while hardly any decrease in depressed men was reported (Staley et al., 2006). In view of these data, we hypothesized that rats lacking the SERT show increased vulnerability for anxiety and depression-related behaviors, and that females are more vulnerable to develop anxiety- and depression-related behaviors after the SERT deletion than males.

Because numerous pharmacological and physiological studies on anxiety and depression have been done in rats, we recently generated a SERT knockout rat ($SERT^{-/-}$) by N-ethyl-N-nitrosurea (ENU)-driven target-selected mutagenesis (Smits et al., 2004;Smits et al., 2006). This rat has a pre-mature stop-codon (TGC>TGA) introduced at position 3924 in the third exon encoding the second extracellular loop of the transporter protein. Northern blot analyses have revealed that the transversion from a C to an A results in a nonsense-mediated decay of the mutant SERT transcript. At a functional level, *d*-fenfluramine, a 5-HT releaser, and flesinoxan, a full $5\text{-}HT_{1A}$ agonist, are unable to elicit hypothermia in $SERT^{-/-}$ rats (Homberg et al., 2007a;Olivier et al., 2008a). Moreover, [^{3}H]citalopram (SSRI) binding to brain slices is completely absent in $SERT^{-/-}$ rats (Homberg et al., 2007a).

Using this novel model, we here examined anxiety-related behaviors in several tests, including the open field test (Prut and Belzung, 2003), elevated plus maze test (Hogg, 1996), novelty suppressed feeding test (Bodnoff et al., 1988) and the home cage emergence task (Prickaerts et al., 1996). In addition, the knockout rats were tested in two depression-related tests, namely the sucrose consumption test (anhedonia) (Orsetti et al., 2007), and the forced swim test (Porsolt et al., 1977). Male and female $SERT^{-/-}$ and $SERT^{+/+}$ rats were tested to investigate genotype and sex differences in performance in these tasks. Moreover, we assessed the structure of the dorsal raphe nuclei (DRN) in male and female $SERT^{-/-}$ and $SERT^{+/+}$ rats. Mood and emotion are modulated by the serotonergic midbrain raphe system, which seems to be involved in the pathogenesis of psychiatric disorders like those of the affective spectrum (Gurwitz, 2000). Serotonergic neurons within the DRN give rise to serotonergic projections to forebrain systems involved in anxiety-related behavioral responses, such as the amygdala (Steinbusch H.W.M., 1984;Maier et al., 1993;Graeff et al., 1996). Given these distributions and the knowledge that dysfunction of 5-HT neurons has been implicated in a wide variety of diseases, it is interesting to study whether $SERT^{-/-}$ and $SERT^{+/+}$ rats show differences in the number serotonergic neurons in this brain structure. Finally, we examined extracellular 5-HT levels in the hippocampus to explore how the genetic inactivation of the SERT affects extracellular 5-HT levels in male and female $SERT^{-/-}$ rats, and studied the effect of the SSRI, citalopram on these levels.

Experimental procedures

Subjects

Serotonin transporter knockout rats ($Slc6a4^{1Hubr}$) were generated by ENU-induced mutagenesis (Smits et al., 2004;Smits et al., 2006). All subjects were generated, bred and reared in the Central Animal Laboratory of the Radboud University of Nijmegen. Experimental animals were derived from crossing $SERT^{+/-}$ rats that were outcrossed for 4 or 5 generations. In all experiments, male and female $SERT^{+/+}$ and $SERT^{-/-}$ littermates were compared. After weaning at the age of 21 days, ear cuts were taken for genotyping. All animals were housed two or three per cage in standard Macrolon® type 3 cages (42 x 26 x 20 cm) in temperature-controlled rooms (21 °C ± 1 °C) with standard 12/12-h day/night-cycle (lights on at 7.00 am) and food (Sniff, long cut pellet, Bio Services, Uden, The Netherlands) and water available *ad libitum*. Seven groups of animals were used; the order of experiments was as follows. Group 1: Sucrose consumption + Open field (3-4 months old, open field was performed 2 days after the sucrose consumption experiments ended); Group 2: Elevated plus maze (3-5 months old); group 3: Novelty supressed feeding (4-6 months

old); group 4: Microdialysis (2-2.5 months old); group 5: 5-HT immunohistochemistry (males 8-10 months old, females 3-4 months old); group 6: Home cage emergence task (3-4 months old) and group 7: Forced swim test (3-4 months old). The experiments were performed between 9.00 A.M. and 17.00 P.M.. All experiments were carried out in accordance with institutional, national and international guidelines for animal care and the Dutch law concerning welfare.

Anxiety-related tests

Open field. 24 Male (n=12 $SERT^{+/+}$ and 12 $SERT^{-/-}$) and 24 female rats (n=12 $SERT^{+/+}$ and 12 $SERT^{-/-}$) were isolated 24 hours before testing. The open field test was conducted as reported in (Jans et al., 2007a). In short, the open field is a square arena (100 x 100 x 40 cm), with an open top, dark walls (wood) and a dark floor (polyvinylchloride). The arena was subdivided in 'corner' (four squares each 16 x 16 cm), 'wall' (four rectangles each 16 x 64 cm) and 'center' (one square 64 x 64 cm) zones. Testing was carried out in dimmed white light. A camera was installed above the center of the field. Immediately after a rat was placed in the corner of the open field, the movements and position of the animals were recorded and registered automatically by a computerized system (EthoVision, Noldus Equipment, The Netherlands). Reported are the time (s) spent in the center of the open field and the total distance moved (cm). Testing was carried out on a 5-minute trial. The floor of the open field was cleaned with 70% alcohol solution between trials to prevent transmission of olfactory cues.

Elevated Plus maze. 24 Male (n=12 $SERT^{+/+}$ and 12 $SERT^{-/-}$) and 20 female rats (n=10 $SERT^{+/+}$ and 10 $SERT^{-/-}$) were isolated 24 hours before testing. The test was performed as described by (de Jong et al., 2006). The apparatus was made of polyvinylchloride. It was elevated to a height of 50 cm with two open (50×10) and two enclosed arms (50×10×40) arranged such that the arms of the same type were opposite to each other. The illumination intensity measured in the open arms was 2.5 lux, and in the closed arm 0.2 lux. Rats were placed in the center of the maze, facing one of the open arms, for a free exploration period of 5 min. The movements and position of the animals were recorded and registered automatically by a computerized system (Plus Maze©, Nijmegen, The Netherlands). Results were expressed as the mean of time spent (s) in open arms, the mean time spent in closed arms, and the distance moved (cm) in both open and closed arms.

Novelty suppressed feeding. The novelty suppressed feeding test was performed as described by Lira et al. (2003). 22 Male (n=11 $SERT^{+/+}$ and 11 $SERT^{-/-}$) and 20 female rats (n=10 $SERT^{+/+}$ and 10 $SERT^{-/-}$) were isolated and food deprived. After 24 hours of food deprivation (water available *ad libitum*), rats were placed in a brightly lit (60W incandescent bulb 1.2 m above the arena) open arena (50X50) containing clean wood chip bedding. A round white filter paper, with a radius of 6.25 cm, was placed in the center of the arena, and one home cage food pellet weighing approximately 2 g was placed on the paper. Rats were removed from their home cage, and then placed in one corner of the arena. The latency (s) to begin a feeding episode was recorded (maximum time was 600 s). Bodyweight (g) of the rats was measured before the 24 hours of food deprivation.

Home cage emergence task. 24 Male (n=12 $SERT^{+/+}$ and 12 $SERT^{-/-}$) and 24 female rats (n=12 $SERT^{+/+}$ and 12 $SERT^{-/-}$) were housed socially (two per cage). The test was performed as described by Prickaerts et al. (1996). In short, the home cage was placed in an arena and the lid of the home cage was removed. During testing, the cage mate was placed in another cage for the

duration of the trial. A grid was placed over the edge of the cage to make it easier for the rats to leave the home cage. Testing was carried out during the night period with red light. A stopwatch was used to measure the latency (s) to leave the cage. The latency of the rat to climb out of its cage into the arena was measured. The trial ended when all four paws of the rat were over the edge of the cage. If the rat did not emerge from its home cage within 600 s, the trial was ended, the home cage was closed again and the rat was given a score of 600 s.

Depression-related tests

Sucrose consumption. The procedure was performed as described by van der Kam et al. (van der Kam, 2006). 24 Male (n=12 $SERT^{+/+}$ and 12 $SERT^{-/-}$) and 24 female rats (n=12 $SERT^{+/+}$ and 12 $SERT^{-/-}$) were housed individually and habituated to the two-bottle paradigm by offering them water in two plastic drinking cylinders on top of the cage, one on each side, for a total of 5 days. Immediately after this 5 day period, the two bottles, free-choice, 24 hour sucrose vs. water paradigm started. In short, animals were presented either with water in both bottles or, on alternating days, with water and increasing sucrose percentages (2% to 10%). Bottles were switched on sucrose days to prevent spatial bias. Fluid consumption (g) and bodyweight (g) were measured daily and used to calculate two measurements, namely the preference of sucrose above water (sucrose intake in ml divided by total intake x 100%) and the intake in grams of a 100% sucrose solution per kg bodyweight (intake in ml corrected for the voluminal weight of sucrose and recalculated towards a 100% solution divided by bodyweight in kg).

Forced swim test. 40 Male (n=20 $SERT^{+/+}$ and 20 $SERT^{-/-}$) and 36 female rats (n=17 $SERT^{+/+}$ and 19 $SERT^{-/-}$) were isolated 24 hours before testing. The forced swim test was conducted as reported in Porsolt et al. (1977). (Porsolt et al., 1977). In short, a cylindrical glass tanks (50 cm tall x 18 cm diameter), filled to a depth of 30 cm with 22°C+/-1 water, were used in the forced swimming test. Testing consisted of two phases, the induction phase and the test phase. During the induction phase animals were placed in the water for 15 minutes. After 24 hours the rats are placed in the same tanks for 5 minutes. The movements of the rats were videotaped for off-line measurement of the duration of immobility (s). The behavioral variable 'immobility' was defined as follows: making no movements for at least 2 seconds or making only those movements that were necessary to keep the nose above the water. The rats were allowed to slightly move their forepaws or support themselves by pressing their paws against the wall of the cylinder. Active climbing, diving and swimming along the wall were scored as mobility (s).

Microdialysis

For the microdialyses studies 10 male (n=5 $SERT^{+/+}$ and 5 $SERT^{-/-}$) and 12 female rats (n=6 $SERT^{+/+}$ and 6 $SERT^{-/-}$) were used. Microdialysis was performed as described by Homberg et al. (2007a).

Surgery and microdialysis. Rats were anaesthetised using isoflurane (2.5%, 400 ml/min N_2O, 600 ml/min O_2). Lidocain (10% m/v) was used for local anaesthesia. The animals were fixed in a stereotaxic frame (Kopf Instruments, USA) and I-shaped probes (dialysable surface 4 mm, hospital AN 69, Brainlink, the Netherlands) were inserted into the ventral hippocampus according to the following coordinates: bregma –5.3 mm, lateral to midline + 4.8 mm and ventral to dura –8.0 mm (for detailed description see (Cremers et al., 2004)) and then secured with dental cement and screws. Experiments were performed 24-48 hours after surgery. On the day of the experiment, animals were connected with a flexible PEEK tubing to a microperfusion pump (CMA 102, microdialysis AB, Sweden) and perfused with an artificial CSF, comprising 147 mM NaCl, 3.0 mM

KCl, 1.2 mM $CaCl_2$, and 1.2 mM $MgCl_2$ at a flow rate of 1.5 µl/min. 15 Minutes microdialysis samples were collected in HPLC vials containing 7.5 µl of 0.02 M formic acid and kept at -80°C until analyzed. Baseline samples were taken after a habituation of 150 minutes, for 75 minutes (6 samples).

Drug administration. Citalopram HBr was dissolved in saline. Drugs were separately injected subcutaneously (s.c.) with a volume of 1 ml/kg. Citalopram was injected at a dose of 3 mg/kg directly after baseline measurements.

5-HT analysis. Samples of 20 µl microdialysate were injected via an autoinjector (Gilson 231, France) onto a 100 x 2.0 mm C18 Hypersil 3 µm diameter column (Bester, Amstelveen, The Netherlands) and separated using a mobile phase consisting of 4.2 g/L sodium acetate, 500 mg/L EDTA, 50 mg/L heptane sulfonic acid, 4% methanol v/v, and 30 µl/L of triethylamine, pH was set at 4.75 at a flowrate of 0.4 ml/min (Shimadzu LC-10 AD). 5-HT was detected amperometrically using a glassy carbon electrode at 500 mV vs Ag/AgCl reference electrode (Antec Leyden, Leiden, The Netherlands). The detection limit was 0.5 fmol 5-HT per 20 µl sample (signal to noise ratio of 3).

5-HT immunohistochemistry

Immunohistochemistry was performed as described by de Jong et al. (2005a). 12 Male (n=6 $SERT^{+/+}$ and 6 $SERT^{-/-}$) and 12 female rats (n=6 $SERT^{+/+}$ and 6 $SERT^{-/-}$) were deeply anesthetized with a mixture of N_2O/O_2 (1:2) and isoflurane (2.5%; Rhodia Organique Fine limited, Bristol, United Kingdom). Then, they were perfused transcardially with 0.1 M PBS, pH 7.3, followed by 400 ml 4% paraformaldehyde dissolved in 0.1 M PB, pH 7.2. Subsequently, the brains were removed from the skull and post fixed overnight in 4% paraformaldehyde at 4°C. Before sectioning, the brains were cryoprotected with 30% sucrose in 0.1 M PB. Brain sections were cut on a freezing microtome, thickness 40 µm, and collected in 6 parallel series (6 vials per rat brain with each 1/6 part of the DRN) in 0.1 M PBS containing 0.1% azide. One vial of each rat was used for the staining. The free-floating sections were washed three times in PBS and preincubated with 1% perhydrol (30% H2O2, Merck) for thirty minutes. After washing three times in PBS the sections were presoaked for thirty minutes in an incubation medium consisting out: PBS with 0.1% bovine serum albumin and 0.5% Triton X- 100. Then the sections were incubated overnight at room temperature, on a shaker with a polyclonal anti-5-HT antiserum raised in rabbit (batch 3-9, gift from prof. Dr. H. Steinbusch) diluted 1:10,000 in the incubation medium. The sections were incubated for 90 minutes at room temperature in donkey anti-rabbit (1:1,500 in incubation medium, Jackson ImmunoResearch Laboratories, West Grove, PA) and for 90 minutes at room temperature in ABC-elite (Vector elite 1:800 in PBS). In between incubations, sections were rinsed three times with PBS. The 5-HT-antibody peroxidase complex was visualized by 3,3'-diaminobenzidine tetrahydrochloride (DAB) staining. Sections were incubated for 10 minutes in a chromogen solution consisting of 0.02% DAB and 0.03% Ni-ammonium sulfate in 0.05 M Tris-buffer (pH 7.6), and subsequently for 10 minutes in chromogen solution containing 0.006% hydrogen peroxide. This resulted in a blue-black staining. Following the immunohistochemical staining procedures, the sections were rinsed three times in PBS and mounted on gelatin chrome alum-coated glass slides, dried overnight in a stove at 37°C, dehydrated in an increased serie of ethanol, cleared in xylene, embedded with Entellan (Merck), and coverslipped.

Quantification. Numbers of 5-HT-immunopositive cells were quantified using the software program Neurolucida (MicroBrightfield Inc, Williston, VT, USA). The number of 5-HT-positive cells was counted in the same level of the dorsal raphe nucleus in homologous square fields (using a grid overlay with a size of 100x100 µm) that displayed a representative density of stained cells.

Data analysis

Behavioral data and immunohistochemistry data were analyzed with two-way ANOVA. Genotype and sex were assessed as independent variables. Where appropriate, post hoc comparisons were conducted using one-way ANOVA. For the sucrose consumption test, effects were evaluated using three-way ANOVA with genotype, sex and concentration as repeated measures. If an interaction was found, effect were further determined with a two-way ANOVA, followed by an one-way ANOVA where appropriate. For microdialysis studies, effects were evaluated using three-way ANOVA with genotype, sex and time as repeated measures. Differences between citalopram treatment were analyzed using two way ANOVA for repeated measurements followed by independent sample T-test analysis where appropriate. Level of significance was set at $p<0.05$. All statistical analyses were performed using the Statistical Package for the Social Sciences version 12.0.1 for windows (SPSS Inc., Chicago, IL, USA).

Results

Anxiety-related tests

Open field. To determine whether SERT$^{-/-}$ rats have an increased level of anxiety, they were subjected to the open field test. This test is based on an exploration-conflict and an increase in time spent in the central part of the open field is considered to be an indication of anxiolytic behavior (Walsh and Cummins, 1976;Prut and Belzung, 2003). A genotype effect was found in the open field (Figure. 1A), with SERT$^{-/-}$ rats spending significantly less time in the center compared to SERT$^{+/+}$ rats ($F_{(1,43)}$=13.362; $p<0.001$). There was no genotype effect found in the total distance moved (Figure. 1B; $F_{(1,43)}$=1.187; $p=0.282$). However, there was a significant sex effect (Figure. 1C; $F_{(1,43)}$=10.503; $p<0.002$), with female rats moving a greater distance than male rats. However there was no significant genotype x sex interactions.

Elevated Plus maze. To determine anxiety in a different assay, SERT$^{-/-}$ rats were subjected to the elevated plus maze. Elevated open

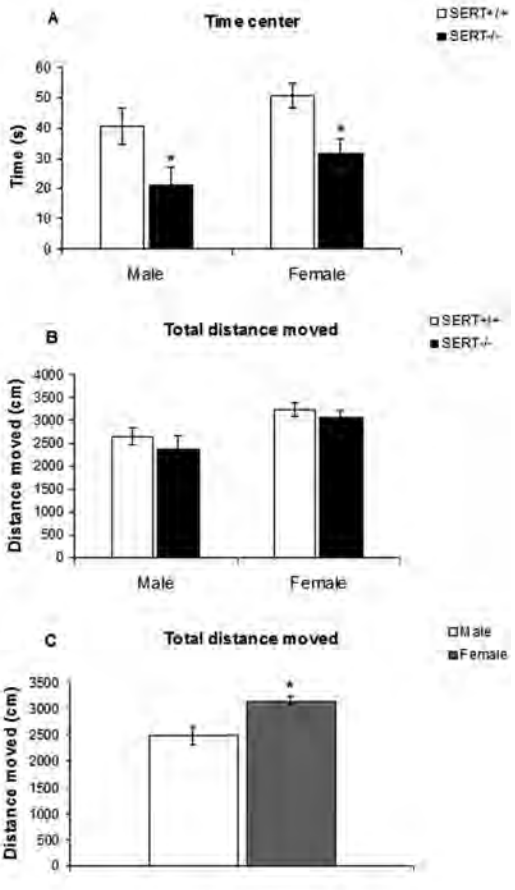

Figure 1.

Open field test. Behavior was recorded for 5 min in SERT$^{+/+}$ and SERT$^{-/-}$ male and female rats. (**A**) Mean ± SEM time (s) spent in the center of the open field. (**B**) Mean ± SEM distance moved (cm). (**C**) Mean ± SEM distance moved (cm) in male and female rats. (males: n=12 SERT$^{+/+}$ and n=12 SERT$^{-/-}$ rats; females: n=12 SERT$^{+/+}$ and n=12 SERT$^{-/-}$ rats. * $p< 0.05$)

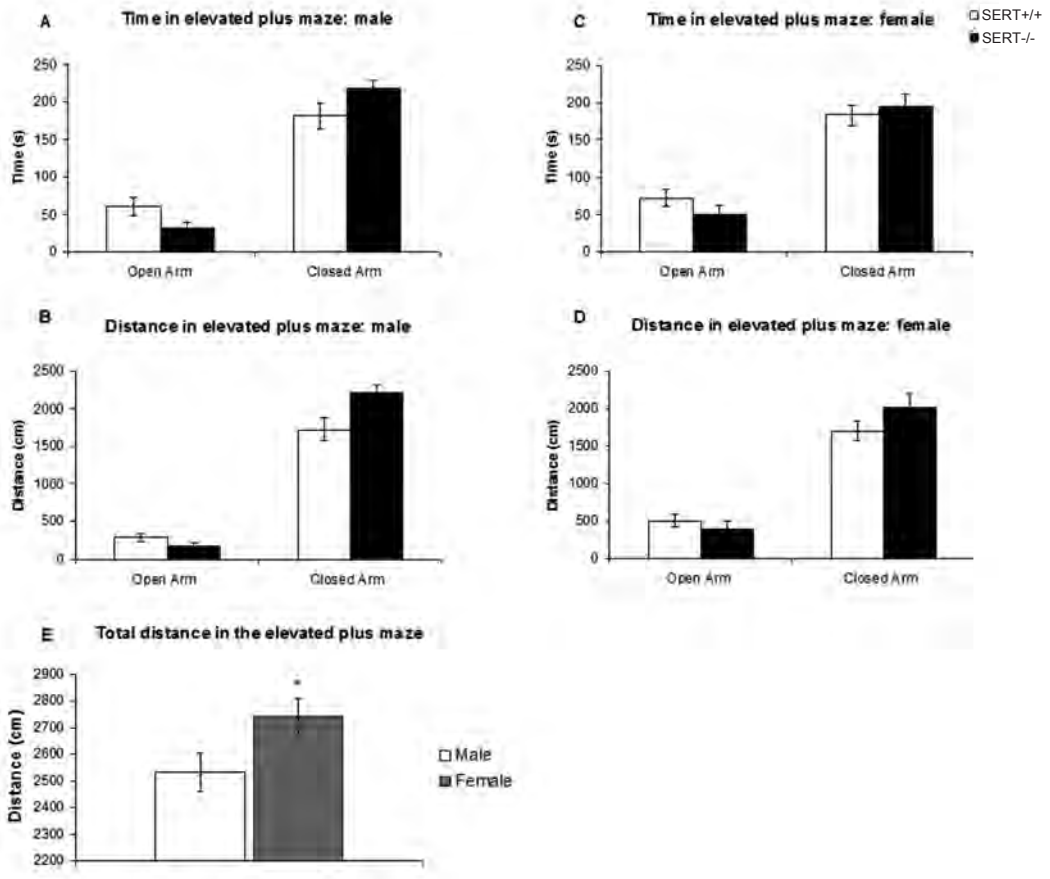

Figure 2.
Elevated plus maze test. Behavior was recorded for 5 min in $SERT^{+/+}$ and $SERT^{-/-}$ male and female rats. (**A**) Mean ± SEM time (s) spent in the open or closed arm in males. (**B**) Mean ± SEM distance moved (cm) in the open or closed arm of the maze in males. (**C**) Mean ± SEM time (s) spent in the open or closed arm in females. (**D**) Mean ± SEM distance moved (cm) in the open or closed arm of the maze in females. (**E**) Mean ± SEM total distance moved (cm) in males and females. (males: n=12 $SERT^{+/+}$ and n=12 $SERT^{-/-}$ rats; females: n=12 $SERT^{+/+}$ and n=12 $SERT^{-/-}$ rats. * p< .05) Note: $SERT^{-/-}$ rats spend significant less time in the open arm [$F_{(1,40)}$=5.194; p<0.028] and moved a greater distance in the closed arms [$F_{(1,40)}$=8.407; p<0.006] compared to $SERT^{+/+}$ rats.

alleys arouse greater avoidance responses than elevated closed alleys. Voluntary passage onto the open arms of an elevated, plus-shaped maze is associated with neurobiological changes indicative of a decreased anxiety (Handley and Mithani, 1984;Hogg, 1996). As shown in Figure. 2, a significant genotype effect was found in the elevated plus maze with $SERT^{-/-}$ rats spending less time in the open arms (Figure 2A+C; F(1,40)=5.194; p<0.028) and moving a greater distance in the closed arms compared to $SERT^{+/+}$ rats (Figure 2B+D; F(1,40)=8.407; p<0.006). In addition, we found a significant sex effect on the total distance moved (Figure 2E; F(1,40)=7.337; p<0.010), with females moving a greater distance compared to males. Moreover, females moved a greater distance in the open arms than males (F(1,40)=9.706; p<0.003). Again, as with the open field test, no significant genotype x sex interactions were found on any of the parameters in the elevated plus maze.

Novelty suppressed feeding. We subjected our SERT$^{-/-}$ rats to a third anxiety test, being the novelty suppressed feeding assay**.** The latency to approach the bright lit center and start eating is considered to be an indication of anxiety (Shephard and Broadhurst, 1982). A significant genotype effect was found for the latency to start eating in a novel environment ($F_{(1,41)}$=17.344; p<0.001). As shown in Figure 3, SERT$^{-/-}$ rats exhibited a longer latency to start eating compared to SERT$^{+/+}$ rats. This was completely due to the male SERT$^{-/-}$ rats, although there was no sex effect, a genotype x sex interaction was found ($F_{(1,41)}$=11.858; p<0.001). Male SERT$^{-/-}$ rats exhibited a longer latency to start eating (Figure. 3) in a novel environment than male SERT$^{+/+}$ rats ($F_{(1,19)}$=27.684; p<0.001), whereas this was not significantly different between female SERT$^{-/-}$ rats and SERT$^{+/+}$ rats (Figure 3; $F_{(1,22)}$=0.274; p=0.606). The bodyweight of all rats was measured before the 24 hours deprivation. There was a significant effect in sex; males weighed more compared to females ($F_{(1,41)}$=166.109; p<0.001). There was no genotype or genotype x sex effect found in weight.

Figure 3
Novelty suppressed feeding test. Behavior was recorded for 10 minutes for male (left) and female (right) SERT$^{+/+}$ and SERT$^{-/-}$ rats. Data are expressed as mean ± SEM latency (s) to start eating. (males: n=11 SERT$^{+/+}$ and n=11 SERT$^{-/-}$ rats; females: n=10 SERT$^{+/+}$ and n=10 SERT$^{-/-}$ rats. * p<0 .05)

Home cage emergence task. The home cage emergence test was used as a last assay to assess the level of anxiety in the SERT$^{-/-}$ rats. An increase in anxiety results in an increased escape latency to leave the home cage (Prickaerts et al., 1996). As shown in Figure 4, a significant genotype effect was found in the home cage emergence task, with SERT$^{-/-}$ rats having a longer latency leaving their home cage than SERT$^{+/+}$ rats ($F_{(1,44)}$=18.025; p<0.001). In addition, a significant sex effect ($F_{(1,44)}$=14.912; p<0.001) was found with females emerging faster from their home cage than males. However, as with the previous tests, we did not find a significant genotype x sex interactions.

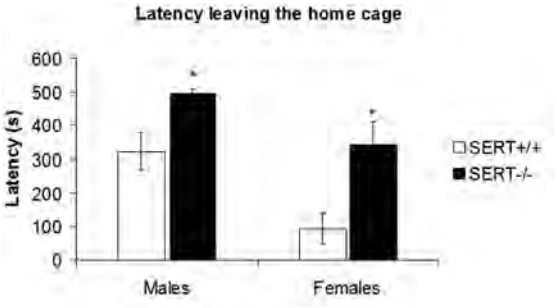

Figure 4
Home cage emergence task. Behavior was recorded for 10 minutes for male (left) and female (right) SERT$^{+/+}$ and SERT$^{-/-}$ rats. Latency (s) leaving the home cage is shown as mean ± SEM. (male: n=12 SERT$^{+/+}$ and n=12 SERT$^{-/-}$ rats; female: n=12 SERT$^{+/+}$ and n=12 SERT$^{-/-}$ rats. * p<0 .05)

Depression-related tests

Sucrose consumption. Loss of interest or pleasure in events that are usually enjoyed (anhedonia) is a core symptom of depression. In animal studies a decreased consumption of palatable solutions is used to measure anhedonia. This decreased consumption can be prevented by antidepressants (Willner et al., 1987;Muscat et al., 1992). Moreover, chronic exposure to mild unpredictable

stress has been found to depress the consumption of palatable sweet solutions (Willner et al., 1987;Orsetti et al., 2007). The preference of sucrose above water and the intake in grams of a 100% sucrose solution per kg bodyweight were measured in the sucrose consumption test as described separately below.

Sucrose preference. A significant genotype effect was observed for the sucrose preference (Figure 5; $F_{(1,33)}$=4.625; p<0.039), with $SERT^{-/-}$ rats drinking less sucrose solution compared to $SERT^{+/+}$ rats. This effect was independent of the concentration, since no genotype x concentration interaction was found. Although there was no significant sex effect, a significant sex x concentration interaction was found ($F_{(10,330)}$=2.411; p<0.009). Females showed a higher preference to sucrose at lower concentration of sucrose (Figure 5) compared to males. At higher concentration, the preference is similar in males and females. We did not find any significant genotype x sex interactions .
Sucrose intake. As for sucrose preference, there was also a significant genotype effect for sucrose intake ($F_{(1,39)}$=19.779; p<0.001), the sucrose intake being significant lower in $SERT^{-/-}$ rats compared to $SERT^{+/+}$ rats (Figure 5). Moreover a genotype x concentration interaction for sucrose intake was found ($F_{(10,390)}$=6.703; p<0.001). The sucrose intake was lower in $SERT^{-/-}$ rats compared to $SERT^{+/+}$ rats especially at higher concentration of sucrose (Figure 5). In addition a significant sex effect ($F_{(1,39)}$=21.845; p<0.001) as well as a significant sex x concentration interaction was found ($F_{(10,390)}$=6.719; p<0.001) with male rats taking less sucrose compared to female rats, especially at higher concentration of sucrose. However we again did not observe any significant genotype x sex interactions. These data reveal that $SERT^{-/-}$ rats have a decreased consumption of sucrose compared to $SERT^{+/+}$ rats, indicating a more depressive-like phenotype.

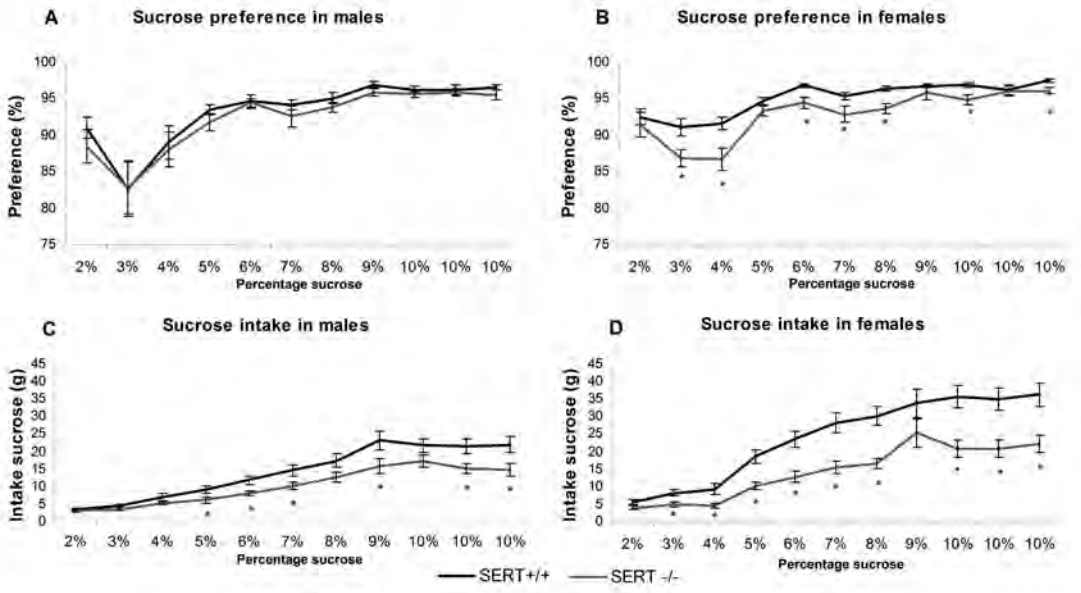

Figure 5
Sucrose consumption test. In a free- choice two bottle paradigm male and female $SERT^{+/+}$ and $SERT^{-/-}$ rats were allowed to consume increasing sucrose solutions (2-10%) on alternating days. Data are expressed as mean ± SEM sucrose preference (sucrose intake/total fluid intake x 100%) in female (**A**) and male (**B**) rats, and as mean ± SEM total sucrose intake (g) in female **(C)** and male (**D**) rats (male: n=12 $SERT^{+/+}$ and n=12 $SERT^{-/-}$ rats; female: n=12 $SERT^{+/+}$ and n=11 $SERT^{-/-}$ rats. * p<0 .05)

Forced swim test. When rats are forced to swim in an inescapable situation, they typically display an immobile posture, which is considered to reflect a state of behavioral despair. Antidepressant treatments are known to reduce immobility time in the forced swim test (Porsolt et al., 1977;Connor et al., 2000), while chronic mild stress and maternal separation increase immobility in the rat (Molina et al., 1994;Huang and Lin, 2006). Therefore increased immobility is considered as 'depression'-like behavior. A significant genotype effect was found in the forced swim test (Figure 6A+B; $F_{(1,72)}$=22.521; p<0.001), with $SERT^{-/-}$ rats spending significantly less time in a mobile state compared to $SERT^{+/+}$ rats. Moreover $SERT^{-/-}$ rats spent a longer time in the immobile phase than $SERT^{+/+}$ rats ($F_{(1,72)}$=22.461; p<0.001). No significant sex or genotype x sex interactions were found. The results of the forced swim test are therefore in line with the data on sucrose consumption and indicate that $SERT^{-/-}$ rats have an enhanced level of depressive-like behavior compared to $SERT^{+/+}$ rats, independent of the sex.

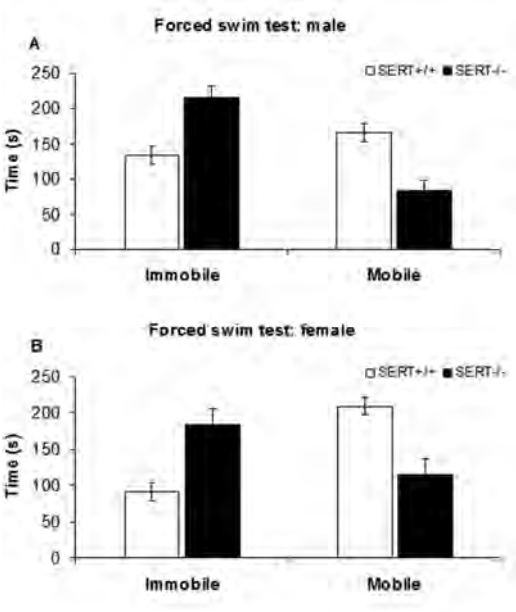

Figure 6
Forced swim test. Behavior was scored for 5 minutes in male (**A**) and female (**B**) $SERT^{+/+}$ and $SERT^{-/-}$ rats on the 2nd day of the test, and expressed as mean ± SEM time spent (s) on mobility and immobility. (male: n=20 $SERT^{+/+}$ and n=20 $SERT^{-/-}$ rats; female: n=17 $SERT^{+/+}$ and n=19 $SERT^{-/-}$ rats. Note: $SERT^{-/-}$ rats were significant less mobile [$F_{(1,72)}$=22.461; p<0.001] and more immobile [$F_{(1,72)}$=22.521; p<0.001] in the forced swim test compared to $SERT^{+/+}$ rats.

Microdialysis

We previously reported that the lack of SERT led to a nine-fold higher level of extracellular 5-HT in male $SERT^{-/-}$ rats (Homberg et al., 2007a). To determine whether this is sex dependent we here investigated the 5-HT levels in male and female $SERT^{-/-}$ rats. As shown in Figure 7, 5-HT levels were seven-fold elevated in the $SERT^{-/-}$ rats compared to $SERT^{+/+}$ rats ($F_{(1,18)}$ =50.227; p< 0.001). These differences were found for male (Figure 7A) and female (Figure 7B) rats. The percentage rise in 5-HT after citalopram treatment was significantly different between $SERT^{+/+}$ and $SERT^{-/-}$ rats ($F_{(1,17)}$ =19.593; p<0.001), because the high basal levels of 5-HT in the $SERT^{-/-}$ rats did not increase, whereas 5-HT levels in the $SERT^{+/+}$ rats were significantly increased after citalopram administration. The difference in rise of 5-HT after citalopram administration was independent of the sex of the rat.

5-HT immunohistochemistry

5-HT neurons within the DRN project to forebrain systems involved in anxiety-related behavioral responses (Steinbusch H.W.M., 1984;Maier et al., 1993;Graeff et al., 1996). We investigated the

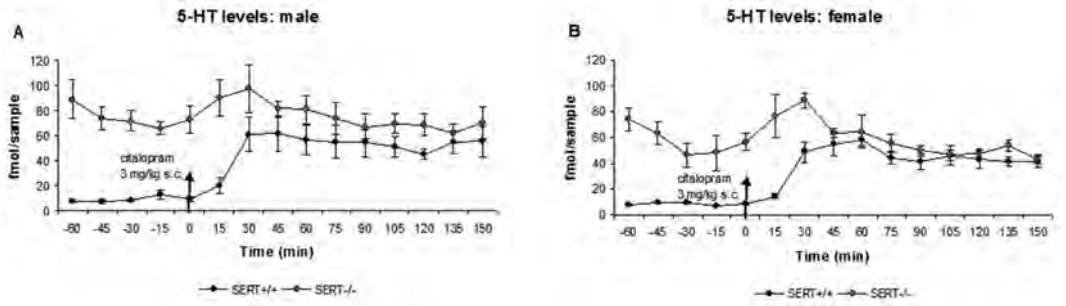

Figure 7
Microdialysis. 5-HT levels were measured in the hippocampus under basal condition and after administration of citalopram in male (**A**) and female (**B**) $SERT^{+/+}$ and $SERT^{-/-}$ rats. Data are expressed as mean ± SEM fmol 5-HT per sample. (male: n=5 $SERT^{+/+}$ and n=5 $SERT^{-/-}$ rats; female: n=6 $SERT^{+/+}$ and n=6 $SERT^{-/-}$ rats. Note: $SERT^{-/-}$ have significant higher basal 5-HT levels $[F_{(1,18)}=50.227; p<0.001]$ compared to $SERT^{+/+}$ rats.

DRN of $SERT^{-/-}$ and $SERT^{+/+}$ rats to reveal possible neural differences. Serotonergic cell numbers in the DRN were quantified as shown in Figure 8. The number of 5-HT immunopositive cells was not significantly different between $SERT^{+/+}$ and $SERT^{-/-}$ rats. Moreover there was not any significant sex or genotype x sex effect.

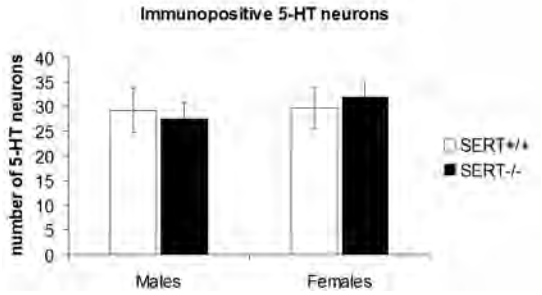

Figure 8
5-HT Immunoreactivity in the dorsal raphe nuclei (DRN) of male and female $SERT^{+/+}$ and $SERT^{-/-}$ rats. Data represent mean ± SEM number of 5-HT immunoreactive neurons (male n=6 $SERT^{+/+}$ and n=6 $SERT^{-/-}$ rats; female n=6 $SERT^{+/+}$ and n=6 $SERT^{-/-}$ rats.

Discussion

In the present study, we analyzed anxiety- and depression-like behaviors in the SERT knockout rat model, with additional focus on possible sex-specific effects. The results showed that $SERT^{-/-}$ rats consistently displayed increased levels of anxiety- and depression-like behaviors, independent of sex and independent of the specific test used.

Compared to $SERT^{+/+}$ rats, $SERT^{-/-}$ rats spent less time in the center part of the open field as well as on the open arm of the plus maze, suggestive of an enhanced level of anxiety in the $SERT^{-/-}$ rats. In this respect, it is important to realize that the total distance moved in the open field did not differ between $SERT^{+/+}$ and $SERT^{-/-}$ rats, indicating that the observed differences are unlikely to be due to differences in exploratory drive per se. The novelty suppressed feeding paradigm, similar to the open field and elevated plus maze, can also be considered as a conflict paradigm, such that hunger becomes the primary drive above exploration (Bodnoff et al., 1988). In this paradigm an increased latency to start eating was found in the $SERT^{-/-}$ rats. This effect was only found in male rats. A latency difference was found between male $SERT^{+/+}$ and female $SERT^{+/+}$ rats (data not

shown). This difference might have arisen by the difference in start body weight of the animals. Moreover the loss of body weight after 24 hours was higher in SERT$^{+/+}$ and in SERT$^{-/-}$ females compared to SERT$^{+/+}$ and in SERT$^{-/-}$ males (data not shown). This may also have influenced the feeding behavior. Nevertheless the increased latency in male SERT$^{-/-}$ rats indicates a higher level of anxiety compared to male SERT$^{+/+}$ rats. Finally, the home cage emergence test was performed to measure anxiety-like behavior in the SERT$^{-/-}$ rats. Again SERT$^{-/-}$ rats showed higher levels of anxiety-like behavior since the latency for escaping from their home cage was higher compared to SERT$^{+/+}$ rats. Taken together these results show that loss of SERT induces anxiety-like behavior in all tests conducted here.

Previous research showed that higher anxiety-like behaviours were also found in several SERT$^{-/-}$ mouse models. Both male and female SERT$^{-/-}$ mice with a 129S6/SvEv background did show an increased anxiety like effect in the novelty suppressed feeding test, but did not display an increased anxiety-like behavior in the open field and elevated plus maze (Lira et al., 2003). On the other hand, male and female SERT$^{-/-}$ mice on a Swiss albino CD-1 strain showed reduced locomotor activity in a novel environment (Alexandre et al., 2006). Moreover, male and female SERT$^{-/-}$ mice with a C57BL/6J background strain exhibited increased anxiety-like behavior in the open field, elevated plus maze, activity patterns and emergence test (Holmes A, 2003a;Holmes et al., 2003b;Zhao et al., 2006;Kalueff et al., 2007a). However, opposite results exist in SERT$^{-/-}$ mice on a C57BL/6J. For example, male SERT$^{-/-}$ mice were only more anxious when they were exposed to predator odor exposure and not on basal levels in the EPM and light/dark box (Adamec et al., 2006). The SERT$^{-/-}$ rats on a Wistar background showed a general increase in anxiety-like behavior, as evident in the open field, the elevated plus maze, the home cage emergence test, and the novelty suppressed feeding. Humans are genetically heterogenous, and in that respect the outbred Wistar background of SERT$^{-/-}$ rats could be an advantage as opposed to inbred mouse strains. However, we did not test the effect of SERT$^{-/-}$ in other rat strains, and as in mice, rat strain background may affect the phenotype.

SERT$^{-/-}$ rats displayed alterations for all depression-like behaviors tested in this study. First SERT$^{-/-}$ rats consumed less sucrose in the two-bottle paradigm compared to SERT$^{+/+}$ rats, being indicative for anhedonia-like . And second, SERT$^{-/-}$ rats showed increased immobility in the forced swim test compared to SERT$^{+/+}$ rats. Taken together, these results indicate a higher 'depression'-like state in SERT$^{-/-}$ rats.

In some SERT$^{-/-}$ mice, depression-like behavior was also present. For example mice with a 129S6 background (male and female) showed an increased immobility in the forced swim test (Holmes A, 2002;Lira et al., 2003), but SERT$^{-/-}$ mice generated on a C57/6J genetic background only a showed an increased immobility when they were repeatedly exposed to the forced swim test (Wellman et al., 2007). In the tail suspension, a test used to measure depressive-like behavior in mice, the immobility of SERT$^{-/-}$ mice on a Swiss albino CD-1 strain (Alexandre et al., 2006) and of SERT$^{-/-}$ mice on a C57BL/6J background (Zhao et al., 2006) was increased. The sucrose consumption test was only performed in male SERT$^{-/-}$ mice with a C57BL/6 background. These mice were not different from the SERT$^{+/+}$ mice in this test (Kalueff et al., 2006), suggesting that genetic ablation of the SERT with this specific genetic background does not induce anhedonia, in line with the unaltered forced swim test performance found in these mice (Holmes A, 2002). However, it does not rule out that SERT$^{-/-}$ mice with other background do not show this behaviour. Thus, SERT ablation leads

to either predominantly anxiety-like behavior in mice (on a C57BL/6 background) or depression-like behavior (on a 129S6 background). In rats on a Wistar background, SERT ablation induces both anxiety and depression-like behaviour, although it remains to be established whether this is a species or a strain dependent phenomenon. The similarities found between SERT$^{-/-}$ rats and mice indicate that gene function is conserved among species and underlines the value that SERT$^{-/-}$ animals represent an interesting model for the anxiety/depression co-morbidity state in humans.

Despite, the higher level of activity of females in the open field, elevated plus maze and home cage emergence task, no differences were found in vulnerability to the deletion of SERT between male and female rats. In fact, if anything, male rats showed a slightly increased vulnerability for the deletion, since the latency to initiate food consumption in the novelty suppressed feeding test was increased in male SERT$^{-/-}$ rats, while in females there is no difference in latency between SERT$^{-/-}$ and SERT$^{+/+}$ rats. Thus, in spite of basal 5-HT differences between males and females found before (Watts and Stanley, 1984;Carlsson and Carlsson, 1988;Haleem et al., 1990;Dominguez et al., 2003) our data show that lifelong absence of SERT in rats, leads to a sex-independent increase of anxiety- or depression-like behavior. This is in line with data from human studies showing an increased risk for both male and female individuals with the short version of the SERT gene promoter polymorphism to develop depression (Mann et al., 2000). No big differences were found between male and female SERT$^{-/-}$ mice in anxiety and depression-like behavior (Holmes A, 2002;Lira et al., 2003;Holmes A, 2003a;Holmes et al., 2003b).

The increase in anxiety- and depression-like behavior is most likely due to alterations in serotonergic neurotransmission in SERT$^{-/-}$ rats. As previously shown for male rats (Homberg et al., 2007a) we found that besides males, also female SERT$^{-/-}$ rats have increased 5-HT levels. Increased extracellular 5-HT is expected to cause excess activity at postsynaptic 5-HT receptors which, in turn, could underlie increased anxiety-like behaviors in SERT$^{-/-}$ rats (Iversen, 1984;Graeff et al., 1996). Also in the SERT$^{-/-}$ mice a fourfold to sixfold increase in basal levels of forebrain extracellular 5-HT has been found (Montanez et al., 2003;Mathews et al., 2004). We can therefore conclude that a lifelong absence of the serotonin transporter in SERT$^{-/-}$ rats causes alterations in the serotonergic neurotransmission, independent of the sex.

As a first attempt to explain the observed effects on anxiety and depression in our SERT$^{-/-}$ rats, we studied the number of serotonergic cells in the dorsal raphe nuclei (DRN) in SERT$^{+/+}$ and SERT$^{-/-}$ rats. The DRN contains the largest number of serotonergic cell bodies and hence is a crucial structure within the 5-HT network related to anxiety and depression. It contains over 50% of all 5-HT neurons projecting to the forebrain (Steinbusch H.W.M., 1984). We used different ages for male and female rats, however a comparison between the groups is possible since aging has no effect on the number of serotonergic cells in the dorsal raphe nucleus (van Luijtelaar et al., 1992). Somewhat surprisingly, we found no significant differences in the number of immunopositive 5-HT neurons in the DRN between SERT$^{-/-}$ and SERT$^{+/+}$ rats, indicating that the absence of SERT has no consequences for the number of serotonergic cells. Obviously, this does not imply that the sensitivity of the 5-HT neurons may not be altered. It would be useful to further assess this for example by electrical recording of neural firing properties. In addition, we have found reduced levels of 5-HT and 5-HIAA tissue levels in SERT$^{-/-}$ rats (Homberg et al., 2007a), presumably reflecting an adaptive reduction of intracellular concentrations. In contrast to SERT$^{-/-}$ rats, SERT$^{-/-}$

mice on a 129S6 background showed an approximately 50% reduction in serotonin neuron number in the DRN (Lira et al., 2003), indicating another clear difference between rats and mice. Since the SERT$^{-/-}$ mice on a 129S6 background, in contrast to the SERT$^{-/-}$ rats, did not display any anxiety like behavior in the elevated plus maze and open field, it is tempting to speculate that the adaptive mechanism at the level of the DRN may somehow protect the SERT$^{-/-}$ mice. However, further research is needed to substantiate this suggestion. Nonetheless, it is important to realize that in post-mortem studies of human individuals suffering from depression no reduction in the number of serotonergic neurons in the DRN was found (Rajkowska, 2000;Hendricksen et al., 2004), suggesting that the SERT$^{-/-}$ rat represents a good animal model for affective disorders in human.

It is paradoxical that SSRIs are effective in the treatment of depression in adults, while neonatal SSRI treatment (Ansorge et al., 2004) or genetic inactivation of the SERT (present study) induce depression-like symptoms. In addition, down-regulation of the SERT with *in vivo* RNAi method (knockdown gene expression) in adult BALB/c mice, results into a reduction in immobility in the forced swim test (Thakker et al., 2005). A possible explanation for this paradox is that either underactivity or overactivity of a neurotransmitter system causes anxiety- and mood disorders. This means that the relationship between 5-HT and 5-HT disorders operates according to an "inverted-U" function, since high 5-HT levels in SERT$^{-/-}$ rats and low 5-HT levels in human patients show similar symptoms (Calabrese and Baldwin, 2001). Chamberlain et al. (2006) used this theory to explain their results in a cognition experiment in which either underactivity or overactivity of the 5-HT system impaired cognition (Chamberlain et al., 2006). Alternatively, a more likely explanation is that the effects of low SERT gene function are present from conception on, also during critical periods of development. Several studies have shown that 5-HT is implicated during the development and organization of the central nervous system (Zhang, 2003). The ontogenic role of monoamines can have a huge impact on the appearance of normal emotional behavior (Meaney et al., 2000;Caspi et al., 2002). In SERT$^{-/-}$ mice, it is already known that postnatal absence of the SERT gene can lead to disorganization of certain cortical regions (Persico et al., 2001;Salichon et al., 2001). Moreover, a greater spine density in the pyramidal neurons in the basolateral amygdala was found, and the length of apical dendritic branches of infralimbic cortex pyramidal neurons of SERT$^{-/-}$ mice was increased compared to SERT$^{+/+}$ mice (Wellman et al., 2007). In SERT$^{-/-}$ rats it is likely that a similar disorganization, or perhaps more widespread neurodevelopment abnormalities, have taken place and it is important to further elucidate this in the near future.

In conclusion, SERT$^{-/-}$ rats showed increased anxiety- and depression-like behavior in a variety of tests. To understand how genetic reduction in SERT contributes to the vulnerability to develop mood or anxiety disorders later in life, more research is needed. Moreover, it is relevant to know if disorganization of brain regions, important for these disorders, takes place during a critical period of development. The SERT$^{-/-}$ rat will be a valuable model for unravelling developmental origins of mood and anxiety disorders. Moreover, since SERT$^{-/-}$ rats do not respond to SSRIs like citalopram, this animal model can be used to develop novel therapies especially useful for the relatively large group of patients that are not responding to current SSRI treatment.

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Acute tryptophan depletion dose-dependently impairs object memory in serotonin transporter knockout rats

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Abstract

Acute tryptophan depletion (ATD) transiently lowers central serotonin levels and can induce depressive mood states and cognitive defects. Previous studies have shown that ATD impairs object recognition in rats. As individual differences exist in central serotonin neurotransmission, the impact of ATD may vary accordingly. In this experiment, we investigated the hypothesis that male serotonin transporter knockout ($SERT^{-/-}$), rats marked by a lower SERT function, are more vulnerable to the effects of ATD in an object recognition task than male wildtype ($SERT^{+/+}$) and heterozygous ($SERT^{+/-}$) rats. Twelve male $SERT^{+/+}$, $SERT^{+/-}$ and $SERT^{-/-}$ rats were treated with standard dose and low-dose ATD using a gelatine-based protein-carbohydrate mixture lacking tryptophan. In the control treatment L-tryptophan was added to the mixture. Four hours after treatment the rats were subjected to the object recognition task. In addition, the effects of ATD on plasma amino acid concentrations were measured and concentrations of 5-HT and 5-HIAA concentrations were measured in the frontal cortex and hippocampus of these rats. Plasma TRP levels and central 5-HT and 5-HIAA levels were decreased in all genotypes after ATD, but effects were stronger in $SERT^{-/-}$ rats. The standard dose of ATD impaired object recognition in all genotypes. $SERT^{-/-}$ and $SERT^{+/-}$ rats were more vulnerable to low-dose of ATD in the object recognition task compared to $SERT^{+/+}$ rats. These results indicate a greater sensitivity to ATD in $SERT^{-/-}$ and $SERT^{+/-}$ rats, which may be related to stronger central depletion effects in these rats.

Introduction

Memory is a multifaceted cognitive function relating to the acquisition and storage of information for shorter or longer periods of time, and the subsequent retrieval of this information. The role of the neurotransmitter serotonin (5-hydroxytryptamine, 5-HT) in learning and memory has been demonstrated in both humans and animals (Buhot 1997;Buhot et al. 2000;Meneses 1999;Riedel et al. 1999;Schmitt et al. 2000). Studies using the method of acute tryptophan depletion (ATD), which results in lower peripheral and central levels of tryptophan and 5-HT (Biggio et al. 1974;Fernstrom and Wurtman 1997;Gessa et al. 1974;Moja et al. 1989;Stancampiano et al. 1997), consistently report impaired memory in healthy volunteers (Park et al. 1994;Riedel et al. 1999;Sambeth et al. 2007;Schmitt et al. 2000) and rats (Jans et al. 2007a;Lieben et al. 2004b;Rutten et al. 2007). More specifically, ATD studies have shown impairments of long-term memory performance (Riedel et al. 2002) and reduced ability to actively recall, as well as recognize, words from a previously presented word list (Riedel et al. 1999). Whereas consolidation of new information into long-term memory appeared to be compromised by a reduction of central 5-HT activity, short-term memory functions were intact. Several studies have subsequently confirmed that 5-HT is specifically involved in long-term memory functioning (for review see Sambeth et al. 2007;Schmitt et al. 2006). Interestingly, ATD did not affect long-term memory retrieval or recognition when the depletion was induced after learning and consolidation of a word list (Schmitt et al. 2000). Thus, these results suggest that consolidation of new information into long-term memory requires normal 5-HT functioning.

The serotonin transporter (SERT) has an important role in the reuptake of 5-HT from the synapse, returning it to the presynaptic neuron where it can be degraded or retained for future release. In fact, the SERT has an essential role in serotonergic neurotransmission as it determines the magnitude and duration of the 5-HT signal in the synaptic cleft. We recently developed a SERT knockout ($SERT^{-/-}$) rat using N-ethyl-N-nitrosurea (ENU) driven mutagenesis (Smits et al. 2004;Smits et al. 2006). This animal has a premature stopcodon (TGC>TGA) introduced at position 3924 in the third exon encoding the second extracellular loop of the SERT protein. Consistent with the absence of SERT in these rats, northern blot analysis revealed that the mutation resulted in nonsense-mediated decay of the mutant SERT transcript, and showed reduced SERT mRNA transcript in the SERT heterozygous knockout ($SERT^{+/-}$) rat (Homberg et al. 2007a). In addition, [^{3}H]citalopram (SSRI) binding to brain slices of $SERT^{-/-}$ rats is completely absent, whereas in $SERT^{+/-}$ rats, citalopram binding was reduced by approximately 40%. Moreover, extracellular 5-HT levels in the hippocampus of $SERT^{-/-}$ are nine-fold elevated (Homberg et al. 2007a; Olivier et al. 2008b), whereas in $SERT^{+/-}$ rats extracellular 5-HT levels are similar to wildtype littermates ($SERT^{+/+}$) (Olivier et al. unpublished data). However, intracellular 5-HT levels were reduced by approximately 75-50% in $SERT^{-/-}$ rats and by 45-55% in $SERT^{+/-}$ rats in several brain areas (Homberg et al. 2007a).

In humans, a polymorphism in the 5-HT transporter gene-linked promoter region (5-HTTLPR) results in individual differences in SERT expression and function (Heils et al. 1996;Lesch et al. 1996). Several studies have shown that 5-HTTLPR genotype can influence behavioral responses to ATD (Marsh et al. 2006;Neumeister et al. 2006;Roiser et al. 2006;Walderhaug et al. 2007). As wildtype ($SERT^{+/+}$), heterozygous ($SERT^{+/-}$) and knockout ($SERT^{-/-}$) rats exhibit large differences in central 5-HT neurotransmission, the impact of ATD may vary accordingly in these rats. In this study, these different types of rat were subjected to two different doses of ATD –a standard dose and a low

dose- and tested for memory in the object recognition task. Animals with lower SERT function were hypothesized to be more vulnerable to the effects of ATD on object recognition memory, as they rely more heavily on 5-HT synthesis than animals in which the re-uptake mechanism is fully functional. The magnitude of the depletion was determined by measuring plasma amino acid concentrations of TRP and five other large neutral amino acids (LNAAs: valine, leucine, isoleucine, phenylalanine, tyrosine) that compete with TRP for transport across the blood brain barrier. The ratio of TRP and these other LNAAs (TRP/ΣLNAA ratio) is thought to be a more sensitive index of brain tryptophan availability than plasma TRP (Fernstrom 1981;Wurtman et al. 1980), because this ratio determines the amount of tryptophan that can enter the brain. Moreover, 5-HT and 5-HIAA concentrations were measured in the frontal cortex and the hippocampus, brain regions involved in cognition and memory (Dalley et al. 2004;Heidbreder and Groenewegen 2003;Squire and Zola-Morgan 1991;Wurtman et al. 1980).

Experimental procedures

Animals

The serotonin transporter knockout rat (Slc6a4^{1Hubr}) has been generated, bred and reared in the Central Animal Laboratory of the Radboud University of Nijmegen. Experimental animals were derived from crossing SERT$^{+/-}$ rats that were outcrossed for 4 or 5 generations. Twelve male SERT$^{+/+}$, SERT$^{+/-}$ and SERT$^{-/-}$ littermates (age: 3 to 5 moths old) were used in this experiment. After weaning at the age of 21 days, ear cuts were taken for genotyping. Genotyping was performed at the Hubrecht Institute (Utrecht, the Netherlands) and the procedure has been described elsewhere (Homberg,et al. 2007a). During the experiment, all animals were individually housed in standard Macrolon® type 3 cages (42 x 26 x 20 cm) in temperature-controlled rooms (21 °C ± 1 °C) with standard 12/12-h day/night-cycle (lights on at 7.00 am) and food (Sniff, long cut pellet, Bio Services, Uden, The Netherlands) and water available *ad libitum*.

Drugs and chemicals

The Gelatin hydrolysate (Solugel P®) was obtained from PB Gelatins (Tessenderlo, Belgium). Glucodry 210 was obtained from Tate & Lyle (Koog aan de Zaan, The Netherlands). Kaliumchloride (KCl) and calciumchloride-dihydrate ($CaCl_2.2H_2O$) were purchased from Merck (Darmstadt, Germany). L-Tryptophan was obtained from Sigma (Zwijndrecht, the Netherlands).

Treatment

During a period of two weeks preceding the experiment, the rats were handled and habituated to oral injections with normal tap water (up to 10 ml/kg). The experiment consisted of blood sample collection (right after the handling period), object recognition task, and brain sample collection (one week after object recognition task). On blood and brain sampling days, rats were treated with a protein-carbohydrate mixture containing L-TRP (TRP+ group, 0.30% TRP of the total protein) or lacking L-TRP (TRP- 100 g group). In all treatment conditions, rats received two oral injections of 10 ml/kg with a 90-minute interval. Blood samples were taken at baseline (10 minutes before the first injection) and 4 hours after the first injection. Brain samples were taken without treatment and 4 hours after the first treatment. The composition of the nutritional mixture is shown in Table 1. In the object recognition task rats were tested without treatment, with TRP+, with TRP- 100 g (standard dose ATD) and with TRP- 40 g (low-dose ATD) respectively with a two-

day interval. The TRP- 40 g condition is a mixture containing 40 g instead of the standard 100 g of Solugel P protein per 100 ml. Because TRP was absent and amino acids were given in a lower concentration this resulted into milder tryptophan depletion. On each testing day, behavioral testing was conducted 4 hours after the first oral administration. Treatment always took place between 8.30 and 12.00 hours and the object recognition task was performed between 12.30 and 17.00 hours. The rats were fasted from 14 hours prior to treatment until the testing period was completed. This was done to minimize the availability of TRP from food. At the end of each testing day, the animals had *ad libitum* access to food.

Table 1. Composition of the treatment mixtures

Substance	Standard dose (g / 100ml Bidest)	Low dose (g / 100ml Bidest)
Solugel P®	100	40
100 grams contains:		
4.8 g Aspartic acid		
10.3 g Glutamic acid		
11.4 g Hydroxyproline		
3.4 g Serine		
23.2 g Glycine		
0.8 g Histidine		
10 g Arginine		
2 g Threonine		
10.4 g Alanine		
12 g Proline		
0.4 g Tyrosine		
2.2 g Valine		
0.7 g Methionine		
1 g Isoleucine		
2.5 g Leucine		
0.9 g Hydroxylysine		
1.1 g Phenylalanine		
2.9 g Lysine		
Carbohydrate (Glucodry 210)	50	50
KCl	0.094	0.094
$CaCl_2.2H_2O$	2.32	2.32
L-tryptophan (TRP- groups)	0	0
L-tryptophan (TRP+ group)	0.28	0.28

Biochemistry

Plasma amino acid levels. For the determination of plasma amino acid levels, blood samples were taken at baseline (T0; i.e., 10 minuters before the first oral administration) and repeated 4 hours after the first administration (T4). Blood sampling was done via a tail-incision method (Fluttert et al. 2000). Promptly after collection of blood in a sodium heparin tube (Microvette® CB 300, Sarstedt, Germany), the samples were kept on ice. After centrifugation of the blood samples (at 4°C for 15 minutes at 3000 g), plasma samples were stored at –70°C. Plasma amino acid concentrations were determined with a fully automated high-performance liquid chromatography (HPLC). The concentrations of the total plasma amino acids are expressed as µmol/L.

Brain 5-HT and 5-HIAA levels. Animals were decapitated and tissue samples (frontal cortex and hippocampus) were dissected from the brain, weighed and stored at -80°C until further use.

The tissue samples were homogenized in 250 µl of an ice-cold solution containing 5 µM clorgyline, 5µg/ml glutathione and 0.6 µM Nω-methylserotonin (NMET, internal standard), using a potter tube. To 100 µl homogenate, 25 µl 2 M $HClO_4$ was added and mixed. Then 20 µl 2.5 M potassium acetate was added and again mixed. After 15 minutes in ice water, the homogenates were centrifuged during 15 minutes at 15000g (4°C). The supernatants were diluted 10 times with water before HPLC analysis. The concentration of 5-HT and 5-HIAA in the tissue extracts were measured by HPLC with ECD. The HPLC system consisted of a pump model P100, an autosampler model AS300 (both from Thermo Separation Products, Waltham, MA, USA), A ERC-3113 degasser (Erma CR. Inc. Tokyo, Japan), an ESA Coulochem II detector with 5011 analytical cell set at potential +450mV (ESA Inc. Bedford MA, USA), a BD 41 chart recorder (Kipp & zn, The Netherlands) and a column (150mm x 4.6mm i.d.) packed with Hypersil BDS C18, 5 µm particle size (Alltech Associates, USA). The mobile phase solution consisted of 50 mM citric acid, 50 mM phosphoric acid, 0.1 mM EDTA, 45 µl/L dibutylamine, 77 mg/L 1-octanesulfonic acid sodium salt, 10% methanol; the pH of the buffer was adjusted to 3.4 with NaOH. Separation was performed at room temperature using a flow rate of 0.7 ml/min. The concentration of each compound was calculated by comparison with both the internal and the external standards. The limit of detection (signal/noise ratio 3:1) was 0.3 nM. Concentrations are expressed as nmol/g. The 5-HIAA/5-HT turnover was calculated, which can be used as an index of 5-HT system activity.

Behavior

Object recognition task. The object recognition task was performed as described elsewhere (Ennaceur and Delacour 1988;Prickaerts et al. 2002). The apparatus consisted of a square arena (100 x 100 x 40 cm), with an open top, dark walls and a dark floor. Testing was carried out in dimmed white light. We used four different sets of objects that could not be displaced by the rat. Each object was available in triplicate. The different objects were: 1) a bowl with handle made of green china (maximal diameter 15 cm and a height of 9 cm), 2) a cubic box (12×12×7 cm) made of polyvinyl, with a pink topping, 3) a china trapezium cylinder (maximal diameter 12 cm and minimum diameter 10.5 cm) with a dish on top (diameter 12 cm), and 4) a brown tinned cylinder (diameter 9.5 cm and height 15 cm).

One day preceding testing, the animals were adapted to the procedure, i.e. they were allowed to explore the apparatus (without any objects) for 3 minutes. In the following days, the rats were tested twice. A testing session comprised two 3-minute trials with a 1 hour interval between trials. Two objects were placed in a symmetrical position about 10 cm away from the black wall. A rat was always placed in the apparatus facing one corner, which was the same for all rats. During the first trial the apparatus contained two identical objects. After the first exploration period the rat was put back in its home cage. One hour later the rat was put back in the apparatus for the second trial, but now with dissimilar objects, a familiar one and a new one. The duration of exploring each object in both trials was recorded manually on a personal computer. Exploration was defined as directing the nose to the object at a distance of no more than 2 cm and/or touching the object with the nose. Sitting on the object was not considered as exploratory behavior. In order to avoid the presence of olfactory trails, the objects were thoroughly cleaned between trials with a 70% ethanol solution. Moreover, each object was available in triplicate so that none of the two objects from the first trial had to be used as the familiar object in the second trial. In addition, all combinations and locations of objects were used in a balanced manner to reduce potential biases due to preferences for particular locations or objects.

The basic measures in the object recognition task were the times spent by rats exploring an

object during trial 1 and trial 2. The discrimination index *d2* ((exploration new object during trial 2 - exploration familiar object during trial 2)/total exploration time during trial 2) was calculated for each treatment condition (see Rutten et al. 2007). The *d2* is a relative index of discrimination between new and familiar object, because it corrects for total exploration time in trial 2 (see Şık et al. 2003). Rats that explored less than 5 s in any of the trials or explored only one of the objects were removed from analysis to avoid possible erroneous conclusions (see Şık et al. 2003).

Statistical analysis

For all variables, treatment effects were analyzed using parametric statistics (ANOVA). Plasma amino acid concentrations were analyzed with repeated measures ANOVA, with factors genotype, treatment, and time. Brain 5-HT, 5-HIAA and 5-HIAA/5-HT turnover were analyzed with 2-way ANOVA, with factors treatment and genotype. Where appropriate, posthoc testing with Bonferroni correction was used. In the object recognition task, effects of treatment and genotype on exploration times in each trial was analyzed using 2-way ANOVA. Where appropriate, posthoc testing with Bonferroni correction was used. We compared the *d2* values of untreated testing and treatment conditions with *d2* values of a virtual control group (see Şık et al, 2003). The virtual control group had a *d2* of zero, meaning there was no object recognition. The number of animals and SEM were similar to those of our treatment groups. Comparison with this virtual control group is used to evaluate more reliably whether discrimination performance differs from zero in a certain treatment condition. The *d2* values were compared with ANOVA and a one-sided Dunnett post-hoc test was used to test whether *d2* in a treatment condition was higher than in the virtual control group, which would indicate that the rats are able to discriminate the objects.

Results

Plasma amino acid concentrations

To determine the effects of the treatment conditions, plasma amino acid concentrations were measured and the TRP/ΣLNAA ratio was calculated for each measurement, treatment and genotype (Figure. 1). Plasma TRP levels and the plasma TRP/ΣLNAA ratio decreased over the four hours [Time: TRP: $F(1,29) = 45.20$, $p<0.001$; Ratio: $F(1,29) = 31.43$, $p<0.001$]. Plasma TRP levels and the TRP/ΣLNAA ratio were lower in the TRP- 100g group compared to the TRP+ group [Treatment: TRP: $F(1,29) = 77.46$, $p<0.001$; Ratio: $F(1,29) = 84.91$, $p<0.001$]. There was no Time x Genotype x Treatment interaction effect and no Genotype x Treatment interaction effect on TRP or on the ratio ($F's < 2.15$, ns). A Time x Treatment interaction effect was found on TRP [$F(1,29) = 206.17$, $p<0.001$] and on the TRP/ΣLNAA ratio [$F(1,29) = 181.17$, $p<0.001$]. Further analysis showed that in the TRP+ condition there was a significant increase of TRP and the TRP/ΣLNAA ratio over the four hours [TRP: $F(1,13) = 26.26$, $p<0.001$; Ratio: $F(1,13) = 23.01$, $p<0.001$], whereas in the TRP- 100g condition these were significantly decreased [TRP: $(1,14) = 223.26$, $p<0.001$; Ratio: $F(1,14) = 222.13$, $p<0.001$]. There was no Time x Genotype effect and no effect of genotype on TRP levels and on the TRP/ΣLNAA ratio. Thus, TRP- 100g ATD resulted in strong depletion of plasma TRP and the TRP/ΣLNAA ratio, whereas TRP+ treatment caused an increase in TRP and the TRP/ΣLNAA ratio. These effects were similar in all genotypes.

Brain 5-HT and 5-HIAA concentrations

To determine the central effects of the treatment, concentrations of 5-HT and 5-HIAA were

determined in the frontal cortex and in the hippocampus and for both structures the turnover (5-HIAA/5-HT) was calculated (Table 2).

5-HT In the frontal cortex, a treatment effect on 5-HT was found [$F(2,66) = 41.46$, $p<0.001$]. Posthoc testing revealed that 5-HT levels were lower in the TRP- 100 g condition than in the untreated condition and the TRP+ condition (Figure 2A). Furthermore, 5-HT concentrations in the TRP+ condition were higher than in the untreated rats. Moreover, a genotype effect on 5-HT in the frontal cortex [$F(2,66) = 73.76$, $p<0.001$] was found. Posthoc analysis showed that 5-HT levels in $SERT^{-/-}$ rats were lower than in $SERT^{+/-}$ and $SERT^{+/+}$ rats. No Treatment x Genotype interaction effect on 5-HT was found in the frontal cortex. In the hippocampus similar effects were found to the prefrontal cortex. In this structure also a treatment effect on 5-HT [$F(2,66) = 17.85$, $p<0.001$] was found, with lower 5-HT levels in the TRP- 100g condition than in the untreated condition and in the TRP+ condition (Figure 2A). Similar to the frontal cortex, 5-HT concentrations in the hippocampus were higher in the TRP+ condition than in the untreated rats. Also, a genotype effect on 5-HT was found in the hippocampus [$F(2,66) = 67.25$, $p<0.001$] with lower 5-HT levels in $SERT^{-/-}$ rats compared with $SERT^{+/-}$ and $SERT^{+/+}$ rats. No Treatment x Genotype interaction effects were found on 5-HT in the hippocampus.

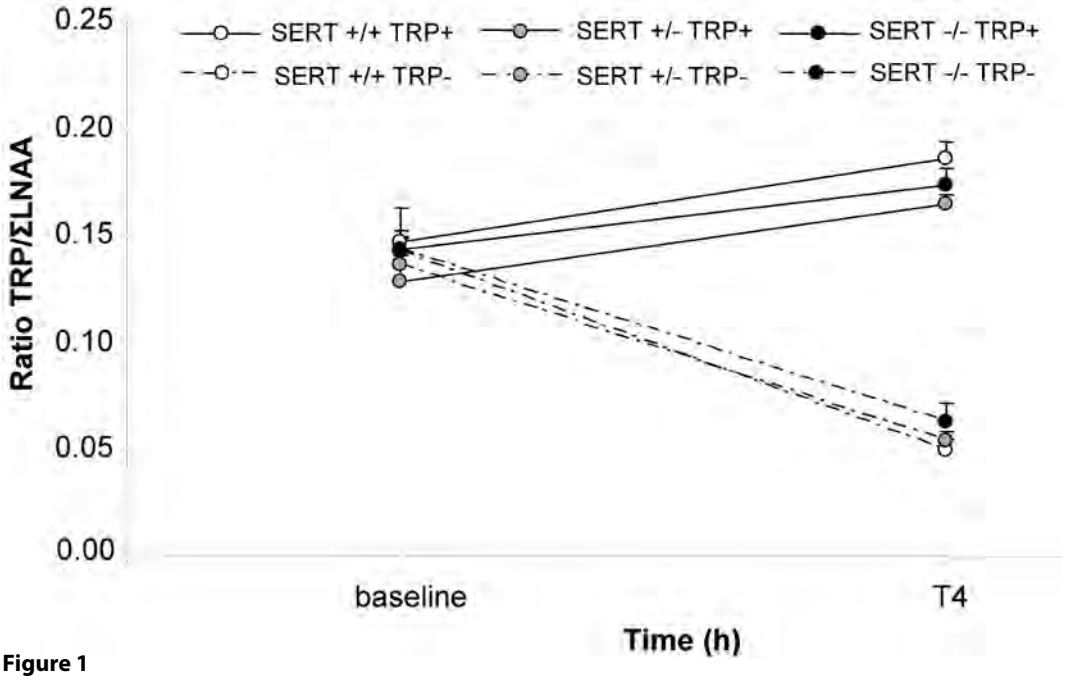

Figure 1
Effects of treatment with TRP+ or TRP- 100g on plasma TRP/ΣLNAA ratio of all groups at baseline (T0) and 4 h after the first administration of the mixture (T4). Percentage difference in TRP/ΣLNAA ratio between the baseline and the TRP+ and TRP- 100g condition after 4 h is indicated in the graph.

In order to test our specific hypotheses, we also analyzed the effects of treatment on behavior in each experimental group separately. In the frontal cortex, 5-HT levels in the TRP- 100g group were significantly lower than in the TRP+ group in all genotypes [Treatment: $SERT^{+/+}$: $F(2,20) = 9.80$, $p<0.001$; $SERT^{+/-}$: $F(2,21) = 12.75$, $p<0.001$; $SERT^{-/-}$: $F(2,21) = 27.88$, $p<0.001$]. In $SERT^{+/+}$ and $SERT^{+/-}$ rats, 5-HT levels were higher in the TRP+ group than in the untreated rats, which was not

the case in SERT$^{-/-}$ rats. In SERT$^{-/-}$ rats 5-HT was lower in the TRP- 100g condition than in untreated rats, which was not the case in SERT$^{+/+}$ and SERT$^{+/-}$ rats. In the hippocampus, there was a treatment effect on 5-HT in SERT$^{+/-}$ [$F(2,21) = 9.29$, $p<0.001$] and in SERT$^{-/-}$ rats [$F(2,21) = 20.88$, $p<0.001$], but not in SERT$^{+/+}$ rats. In SERT$^{+/-}$ rats, 5-HT levels in the TRP+ condition were higher than in untreated and TRP- 100g treated rats. In SERT$^{-/-}$ rats, 5-HT levels in the TRP- 100g condition were lower than in the TRP+ condition and in untreated rats.

5-HIAA A treatment effect on 5-HIAA was found in the frontal cortex [$F(2,66) = 23.98$, $p<0.001$], see Figure. 2B. Posthoc testing showed that 5-HIAA concentrations in the TRP- 100g condition were lower than in the untreated rats and in the TRP+ condition. In the frontal cortex a genotype effect was found on 5-HIAA in the frontal cortex [$F(2,66) = 34.03$, $p<0.001$]. Posthoc analysis showed that 5-HIAA levels in SERT$^{-/-}$ were lower than in SERT$^{+/-}$ and SERT$^{+/+}$ rats. No Treatment x Genotype interaction effects on 5-HIAA were found in the frontal cortex. Similar to the frontal cortex, a treatment effect on 5-HIAA was also found in the hippocampus [$F(2,66) = 34.35$, $p<0.001$]. As shown in Figure 2B, 5-HIAA concentrations in the TRP- 100g condition were lower than in the untreated rats and the TRP+ condition. In the hippocampus 5-HIAA levels were higher in untreated rats than in TRP+ treated rats. Moreover, a genotype effect on 5-HIAA was found in the hippocampus [$F(2,66) = 35.30$, $p<0.001$] showing lower 5-HIAA levels in SERT$^{-/-}$ rats compared with SERT$^{+/-}$ and SERT$^{+/+}$ rats. No Treatment x Genotype interaction effects on 5-HIAA levels were found in the hippocampus.

Table 2. An overview of 5-HT, 5-HIAA, and 5-HIAA/5-HT turnover levels in frontal cortex and hippocampus.

Brain area	Treatment	Genotype	5-HIAA (nmol/g)	Sign.	5-HT (nmol/g)	Sign.	5-HIAA/5-HT	Sign.
Fr cortex	untreated	SERT$^{+/+}$	1,19	a	2,74	a	0,46	
Fr cortex	untreated	SERT$^{+/-}$	1,01	a	2,67	a	0,40	
Fr cortex	untreated	SERT$^{-/-}$	0,63		1,39		0,46	
Fr cortex	TRP+	SERT$^{+/+}$	0,99	a	3,38	a	0,31	
Fr cortex	TRP+	SERT$^{+/-}$	0,98	a	3,68	a #	0,27	
Fr cortex	TRP+	SERT$^{-/-}$	0,51		1,90		0,29	
F cortex	TRP- 100g	SERT$^{+/+}$	0,65	a # $	2,21	a # $	0,30	a #
Fr cortex	TRP- 100g	SERT$^{+/-}$	0,63	a #	2,16	a #	0,30	a #
Fr cortex	TRP- 100g	SERT$^{-/-}$	0,35	#	0,51	# $	0,74	# $
Hippocampus	untreated	SERT$^{+/+}$	1,77	a	1,83	a	1,06	
Hippocampus	untreated	SERT$^{+/-}$	1,52	a	1,82	a	0,86	
Hippocampus	untreated	SERT$^{-/-}$	0,92		0,79		1,26	
Hippocampus	TRP+	SERT$^{+/+}$	1,49	a	2,11	a	0,73	
Hippocampus	TRP+	SERT$^{+/-}$	1,33	a	2,48	a #	0,58	
Hippocampus	TRP+	SERT$^{-/-}$	0,51		1,90		0,29	
Hippocampus	TRP- 100g	SERT$^{+/+}$	0,84	a # $	1,56	a	0,56	a #
Hippocampus	TRP- 100g	SERT$^{+/-}$	0,80	a # $	1,37	a $	0,59	a
Hippocampus	TRP- 100g	SERT$^{-/-}$	0,35	#	0,51	# $	0,74	# $

a: significant different from SERT$^{-/-}$ rats. #: significant different from untreated group with the same genotype.
$: significant different from TRP+ treated group with the same genotype.

In all genotypes, there was a treatment effect on 5-HIAA in the frontal cortex [SERT$^{+/+}$: $F(2,20) = 18.69$, $p<0.001$; SERT$^{+/-}$: $F(2,21) = 4.29$, $p<0.05$; SERT$^{-/-}$: $F(2,21) = 10.66$, $p<0.001$]. Posthoc testing revealed that in SERT$^{+/+}$ rats 5-HIAA was lower in the TRP- 100g group compared to the TRP+ group

($p<0.01$) and the untreated group ($p<0.001$). In $SERT^{+/-}$ and $SERT^{-/-}$ rats, 5-HIAA levels were lower in TRP- 100 g compared to untreated rats ($SERT^{+/-}$: $p<0.05$; $SERT^{-/-}$: $p<0.001$). For the hippocampus also, there was a treatment effect in all genotypes in the hippocampus as well [$SERT^{+/+}$: $F(2,20) = 28.19$, $p<0.001$; $SERT^{+/-}$: $F(2,21) = 7.16$, $p<0.001$; $SERT^{-/-}$: $F(2,21) = 11.91$, $p<0.001$]. Posthoc testing showed lower 5-HIAA levels in $SERT^{+/+}$ and $SERT^{+/-}$ rats in the TRP- 100g group compared with the TRP+ ($SERT^{+/+}$: $p<0.001$; $SERT^{+/-}$: $p<0.05$) and untreated group ($SERT^{+/+}$: $p<0.001$; $SERT^{+/-}$: $p<0.01$). In $SERT^{-/-}$ rats, 5-HIAA was higher in the untreated rats compared with TRP+ ($p<0.05$) and TRP- 100g ($p<0.001$) treated rats.

5-HIAA/5-HT As seen in Figure 2C, there was a Treatment x Genotype interaction effect on the 5-HIAA/5-HT turnover in the frontal cortex [$F(4,62) = 9.628$, $p<0.001$] caused by the high 5-HIAA/5-HT turnover rate of $SERT^{-/-}$ rats. A treatment effect was found on the turnover in the frontal cortex [$F(2,62) = 11.60$, $p<0.001$]. Posthoc testing revealed that the turnover was lower in the TRP+ condition than in the untreated rats and in the TRP- 100g condition. Moreover, a genotype effect on 5-HIAA in the frontal cortex [$F(2,62) = 13.64$, $p<0.001$] was found. The turnover was higher in $SERT^{-/-}$ rats than in $SERT^{+/-}$ and $SERT^{+/+}$ rats. Similarly to the frontal cortex, a Treatment x Genotype interaction effect on the 5-HIAA/5-HT turnover was also found in the hippocampus [$F(4,62) = 12.60$, $p<0.001$]. This effect seems to be the result of the high 5-HIAA/5-HT turnover rate in $SERT^{-/-}$ rats. Moreover, a treatment effect on the turnover was found in the hippocampus [$F(2,62) = 9.02$, $p<0.001$]. Again with a lower turnover in the TRP+ condition compared with the untreated rats and the TRP- 100 g condition. Similar to the frontal cortex, a genotype effect on 5-HIAA/5-HT was found in the hippocampus [$F(2,62) = 28.62$, $p<0.001$], with a higher turnover in $SERT^{-/-}$ rats compared with $SERT^{+/-}$ and $SERT^{+/+}$ rats.

In all genotypes, there was a treatment effect on 5-HIAA/5-HT turnover in the frontal cortex [$SERT^{+/+}$: $F(2,20) = 4.64$, $p<0.05$; $SERT^{+/-}$: $F(2,21) = 3.78$, $p<0.05$; $SERT^{-/-}$: $F(2,21) = 16.37$, $p<0.001$]. Posthoc testing showed that in $SERT^{+/+}$ rats the 5-HIAA/5-HT was lower in the TRP- 100 g group compared to the untreated group ($p<0.05$). In $SERT^{+/-}$ rats 5-HIAA/5-HT was lower in TRP+ rats than in untreated rats ($p<0.05$). In $SERT^{-/-}$ rats 5-HIAA/5-HT was higher in TRP- 100 g compared to untreated rats ($p<0.001$) and TRP+ rats ($p<0.001$). In all genotypes, there was a treatment effect in the hippocampus [$SERT^{+/+}$: $F(2,20) = 7.31$, $p<0.01$; $SERT^{+/-}$: $F(2,21) = 4.10$, $p<0.05$; $SERT^{-/-}$: $F(2,21) = 15.12$, $p<0.001$]. Posthoc testing showed that in $SERT^{+/+}$ rats 5-HIAA/5-HT was lower in the TRP- 100g group compared to the untreated group ($p<0.01$). In $SERT^{-/-}$ rats, 5-HIAA/5-HT was higher in the TRP- 100g treated rats compared to untreated rats ($p<0.01$) and TRP+ treated rats ($p<0.001$). In $SERT^{+/-}$ rats no differences in 5-HIAA/5-HT were found.

Object recognition task

In order to test whether animals with lower SERT function are more vulnerable to the effects of ATD we tested $SERT^{+/+}$ $SERT^{+/-}$ and $SERT^{-/-}$ rats on object recognition memory. Exploration times of untreated rats and rats treated with TRP+, TRP- 100 g and TRP- 40 g treated were compared (data not shown). There were no Genotype x Treatment interaction effects on exploration time in trial 1 or in trial 2. There was no effect of Genotype or Treatment on exploration time in trial 1. In trial 2, there was an effect of Genotype [$F(2,136) = 3.27$, $p<0.05$] and Treatment [$F(3,136) = 3.98$, $p<0.01$] on exploration time. Posthoc testing showed that in trial 2, exploration time was lower in the TRP- 100 g and TRP+ condition compared to the TRP- 40 g condition ($p<0.05$). As mentioned before, *d2* values of the untreated rats and the rats treated with TRP+, TRP- 100 g and TRP- 40

g of the present study were compared with *d2* values of a virtual control group with no object recognition (see Şık et al, 2003). The effects of Genotype and Treatment on discrimination index *d2* in the object recognition task are shown in Figure 3.

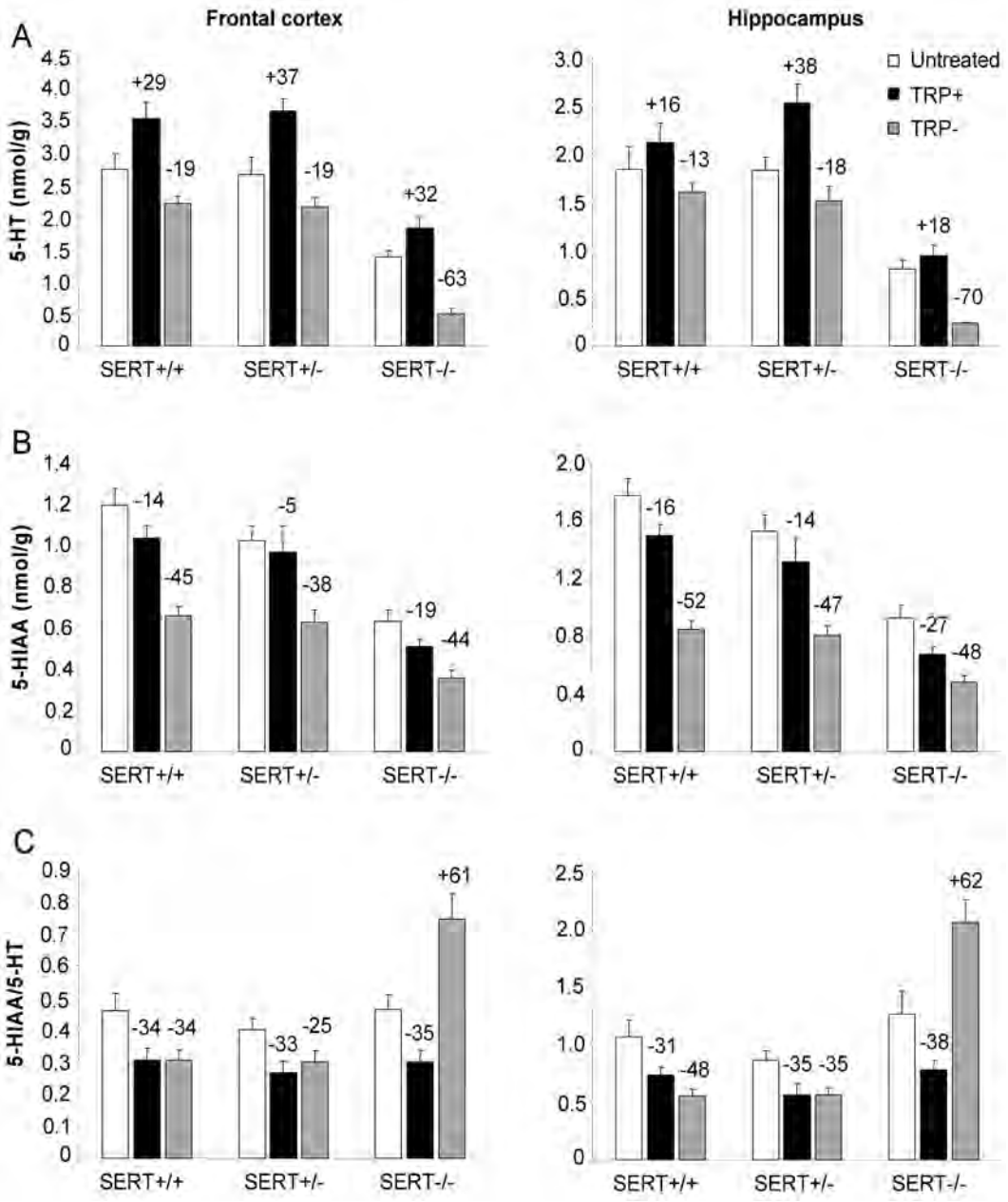

Figure 2
Effect of treatment with TRP+ or TRP- 100 g on basal brain 5-HT (**A**), 5-HIAA (**B**) and 5-HIAA/5-HT turnover (**C**) levels in the frontal cortex and hippocampus. Bars represent mean and standard error. Numbers above the bars represent percentage difference from the untreated condition.

There was no Genotype x Treatment interaction effect [F(8,138) = 1.28, ns] and no effect of Genotype on discrimination index *d2* [F(2,138) = 2.31, ns]. A treatment effect was found on the discrimination index *d2* [F(4,146) = 21.65, p<0.001], post-hoc analysis showed that the untreated *d2* and the *d2* in the TRP+ and TRP- 40 g condition differed from the virtual control group with no object recognition. Only in the TRP- conditions rats were unable to discriminate between the new and familiar object after a one-hour interval. To evaluate whether $SERT^{-/-}$ rats were more vulnerable to ATD treatment than $SERT^{+/-}$ or $SERT^{+/+}$ rats, treatment effects were analyzed within each genotype group. There was a treatment effect on *d2* in all genotypes [$SERT^{+/+}$ F(4,50) = 17.61, p<0.001; $SERT^{+/-}$ F(4,43) = 6.00, p<0.01; $SERT^{-/-}$ F(4,45) = 4.92, p<0.01]. In the $SERT^{+/+}$ untreated rats and rats treated with TRP+ and TRP- 40 g were different from the virtual controls, but TRP- 100 g was not. In the $SERT^{+/-}$ only untreated rats and TRP+ treated rats were different from the virtual controls, but rats treated with TRP- 100 g and TRP- 40 g were not. In the $SERT^{-/-}$ rats only the untreated rats were different from the virtual controls, whereas rats treated with TRP+, TRP- 100 g and TRP- 40 g were not.

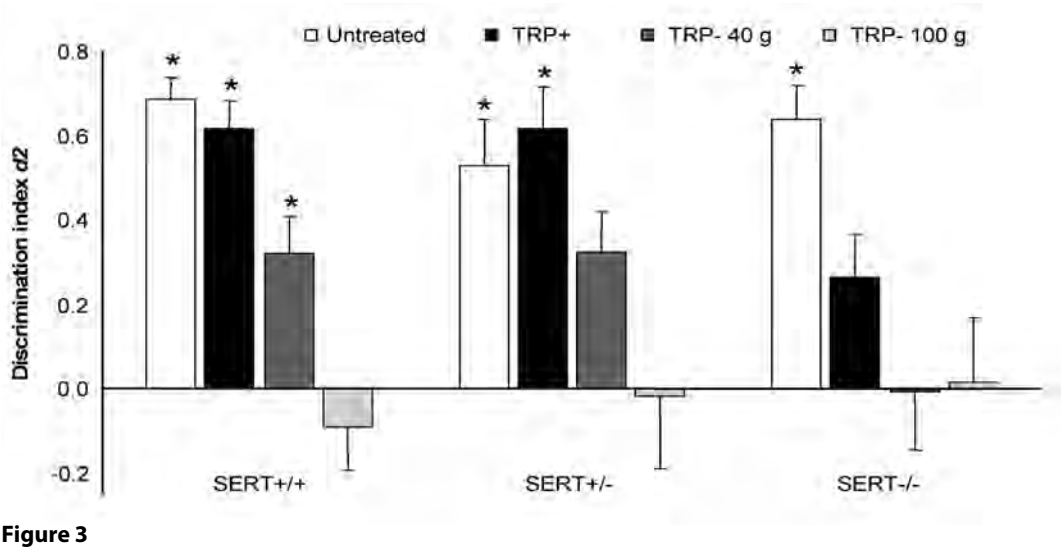

Figure 3
Treatment effects in $SERT^{+/+}$, $SERT^{+/-}$, and $SERT^{-/-}$ on discrimination index *d2*. Treatment effect * p<0.05 difference from virtual control group with no object recognition.

Discussion

In the present study, the effects of standard dose (TRP- 100 g) and low dose (TRP- 40 g) ATD were examined in $SERT^{+/+}$, $SERT^{+/-}$ and $SERT^{-/-}$ rats in the object recognition task. The biochemical data showed plasma TRP depletion of 65% in $SERT^{+/+}$, 61% in $SERT^{+/-}$ and 55% in $SERT^{-/-}$ rats four hours after standard dose TRP-. This decrease in plasma TRP levels is in agreement with other ATD studies (Jans et al. 2007a;Lieben et al. 2004b). $SERT^{-/-}$ rats showed stronger depletion of 5-HT in the frontal cortex (63%) than $SERT^{+/+}$ and $SERT^{+/-}$ (both 19%) rats. Similar results were found in the hippocampus, where $SERT^{-/-}$ rats also showed a stronger depletion (70%) compared to $SERT^{+/-}$ rats (18%) and $SERT^{+/+}$ rats (13%). Rats treated with standard dose TRP- showed lower 5-HT levels in both brain structures compared to TRP+ treated rats and untreated rats. These

effects were most pronounced in SERT$^{-/-}$ rats, as only these rats showed significantly lower 5-HT in the standard dose TRP- condition than in the untreated condition. In both the frontal cortex and hippocampus, standard dose ATD decreased 5-HIAA to a similar extent in all genotypes. In SERT$^{+/+}$ and SERT$^{+/-}$ rats, 5-HIAA/5-HT turnover was a bit lower in rats treated with TRP+ and standard dose TRP- compared with the untreated condition, but in SERT$^{-/-}$ rats, this turnover ratio was much higher in the standard dose TRP- group than in the TRP+ group and the untreated group. In previous studies, SERT$^{-/-}$ animals exhibited an increased 5-HT turnover at basal levels in the cortex and caudate putamen, but not in the amygdala medial prefrontal cortex, and orbitofrontal cortex (Homberg et al. 2007a;Homberg et al. 2007b). In line with this, untreated SERT$^{-/-}$ animals in this study did not have increased 5-HIAA/5-HT turnover in the frontal cortex. Although the central effects of ATD were stronger in SERT$^{-/-}$ rats, plasma TRP and TRP/ΣLNAA levels decreased to a similar extent in all genotypes. Thus, different effects seen in SERT$^{+/+}$, SERT$^{+/-}$ and SERT$^{-/-}$ rats after ATD were found only in the brain and not in the periphery. Previous ATD studies reported a similar dissociation between peripheral and central effects (Jans et al 2007a; Jans et al. unpublished data). Although the reason for this difference is not known, it is interesting to note that 5-HT synthesis in brain and pheripheral tissues function differently. For example, tryptophan hydroxylase (TPH), the rate-limiting enzyme to form 5-hydroxytryptophan (5-HTP) from tryptophan, is controlled by a different isoform in the brain than in the periphery (for review, see Walther and Bader, 2003). This difference in 5-HT synthesis might play a role in the different effects seen after ATD in brain and periphery.

The standard ATD dose (TRP- 100 g) is known to impair object recognition in Wistar rats (Jans et al. 2007a;Lieben et al. 2004b) and was thus expected to impair object recognition in all genotypes. The re-uptake mechanism in SERT$^{+/+}$ rats is fully functional, whereas this mechanism is partly functional in SERT$^{+/-}$ rats, and not functional in SERT$^{-/-}$ rats. Therefore, SERT$^{+/-}$ and SERT$^{-/-}$ rats rely more heavily on 5-HT synthesis than SERT$^{+/+}$ rats. Consequently, it was hypothesized that differences in object recognition between the genotypes would occur only after the low dose ATD treatment. It was found that all genotypes showed impaired object recognition after standard dose ATD. The relatively mild depletion of the TRP- 40 g treatment impaired object recognition in SERT$^{+/-}$ and especially in SERT$^{-/-}$ rats but not in SERT$^{+/+}$ rats, suggesting that SERT$^{-/-}$ and SERT$^{+/-}$ rats are more sensitive to the memory-impairing effects of low-dose ATD. In SERT$^{+/+}$ rats, the *d2* in the TRP- 40 g condition was lower than in the untreated condition, but still different from the virtual control group with impaired object recognition. The stronger effects of the TRP- 40 g treatment in SERT$^{-/-}$ rats may be related to the stronger effects of TRP- treatment on central 5-HT levels. The same dose of TRP- 100 g had a stronger effect on central 5-HT levels in SERT$^{-/-}$ rats compared to SERT$^{+/-}$ and SERT$^{+/+}$ rats. Notably, SERT$^{-/-}$ rats showed impaired object recognition after TRP+ treatment as well, although their untreated *d2* indicated normal object recognition when untreated. This may be related to stress associated with the ATD procedure, such as the repeated oral injections. Chronic stress is known to impair object memory (Beck and Luine 1999), and there is some evidence to suggest that diminished SERT function is associated with increased stress responsivity in humans (for review see Canli and Lesch 2007) and mice (Wellman et al. 2007). In this way, the injection stress may have resulted in mildly impaired object recognition memory in TRP+ treated SERT$^{-/-}$ rats.

ATD has been reported to impair cognitive performance in humans (Park et al. 1994;Riedel et al. 1999;Riedel et al. 2002;Sambeth et al. 2007;Schmitt et al. 2000), and impaired object recognition

has been found in Wistar rats (Lieben et al., 2004b;Jans et al., 2007a). The effects of low-dose ATD on memory in groups that differ in serotonergic functioning have not been studied extensively. Booij et al. (2005) found that ATD had a dose-dependent effect on selective attention (Stroop color-word interference) in remitted depressed patients, but no other cognitive effects of low-dose ATD were observed. The magnitude of the reduction of plasma tryptophan concentrations following ATD depends on the amount and composition of the amino acid mixture (Young et al. 1989). In human studies, it has been suggested that a threshold exists that needs to be exceeded before any behavioral effects occurs, since studies in which the plasma tryptophan reduction was lower than 70% generally do not find any symptomatic effects (Van Der Does 2001). A similar threshold may also exist in the rat, although the level of the threshold is likely to be lower, as rat studies in general show lower plasma TRP depletion than human studies. In the present study $SERT^{-/-}$, and to a smaller extent $SERT^{+/-}$ rats, showed impairment in recognition memory after low-dose ATD. Thus, it may be possible that in $SERT^{-/-}$, and to a smaller extent in $SERT^{+/-}$ rats, the threshold is lower than in $SERT^{+/+}$ rats, and less depletion of TRP is required to cause impaired memory.

The results of this study indicate higher serotonergic vulnerability in $SERT^{-/-}$ and to a lesser extent $SERT^{+/-}$ rats than in $SERT^{+/+}$ rats, as low-dose ATD only affected memory in subjects that already have a disturbed 5-HT system. Serotonergic vulnerability means that minor changes in serotonergic functioning do not cause symptoms but make the system more vulnerable, so that additional challenges of the serotonergic system may result in the occurrence of psychiatric symptoms (Jans et al. 2007b). Thus, serotonergic vulnerability can be demonstrated by challenging the 5-HT system, as is done with ATD; only vulnerable subjects will react to such manipulations with changes in behavior. Challenging the 5-HT system in vulnerable subjects can have stronger effects on the memory function of these subjects. ATD in $SERT^{-/-}$ rats may therefore be a good tool to investigate serotonergic vulnerability.

Some possible limitations of this study have to be mentioned. First, plasma amino acid concentrations and brain 5-HT and 5-HIAA levels after the low dose TRP- 40g treatment could not be determined in this study due to the limited number of rats that were available for this study. Previous standard-dose ATD studies have shown a robust reduction in plasma TRP (about 70%) and central tissue 5-HT (about 40-45%) concentrations in male Wistar rats (Lieben et al. 2004a). Lowering the concentration of Solugel protein in the TRP- mixture had dose-dependent effects on the plasma TRP/ΣLNAA ratio in previous research, and there was a positive correlation between the plasma TRP/ΣLNAA ratio and performance in the object recognition task (Lieben et al. unpublished data). We can therefore assume that the low-dose TRP- 40g treatment resulted in milder TRP and 5-HT depletion. Further research may elucidate the exact level of peripheral and central depletion that is required to impair object recognition memory in $SERT^{+/+}$, $SERT^{+/-}$ and $SERT^{-/-}$ rats. Secondly, the TRP+ treatment resulted in increased levels of plasma TRP and brain 5-HT, suggesting an active control. Essentially, this means that we compared TRP depletion and mild TRP suppletion in all rats. This may have affected behavior in the TRP+ condition. Although these treatment effects in the TRP+ condition occurred to a similar degree in all genotypes, it cannot be excluded that the mild TRP suppletion had different effects in $SERT^{+/+}$, $SERT^{+/-}$, and $SERT^{-/-}$ rats. A third concern could be that the same group of rats were repeatedly subjected to ATD treatment (for the measurement of plasma levels, for the ORT, and eventually for the measurement of brain levels), although the treatment condition varied between tests. Previous

ATD studies have shown that repeated treatment did not alter the effect of the treatment on the plasma TRP/ΣLNAA ratio (Jans et al. 2007a) and that plasma levels returned to baseline levels on the treatment day (Jans et al., unpublished data). We therefore assume that the treatment has had comparable biochemical effects throughout the study.

The aim of the present study was to investigate whether male $SERT^{+/+}$, $SERT^{+/-}$, and $SERT^{-/-}$ serotonin transporter rats differed in their response to ATD with respect to object memory. Without treatment, all rats showed normal object recognition memory. After a standard 100g dose of ATD, object recognition memory was impaired in all genotypes. However, in the low-dose ATD condition, $SERT^{+/-}$ and $SERT^{-/-}$ rats showed increased responsiveness to the treatment than $SERT^{+/+}$ rats by showing impaired object recognition after this relatively mild ATD treatment. Therefore, ATD in $SERT^{-/-}$ rats might be a valuable animal model to investigate serotonergic vulnerability. It can be concluded that there is a SERT gene-dosage effect with respect to the behavioral response to TRP depletion in the object recognition task. Because ATD decreased 5-HT levels in brain structures that play a crucial role in memory, such as the hippocampus and the frontal cortex, the outcome of the present study underlines the relevance of the serotonergic system in memory.

Acknowledgements

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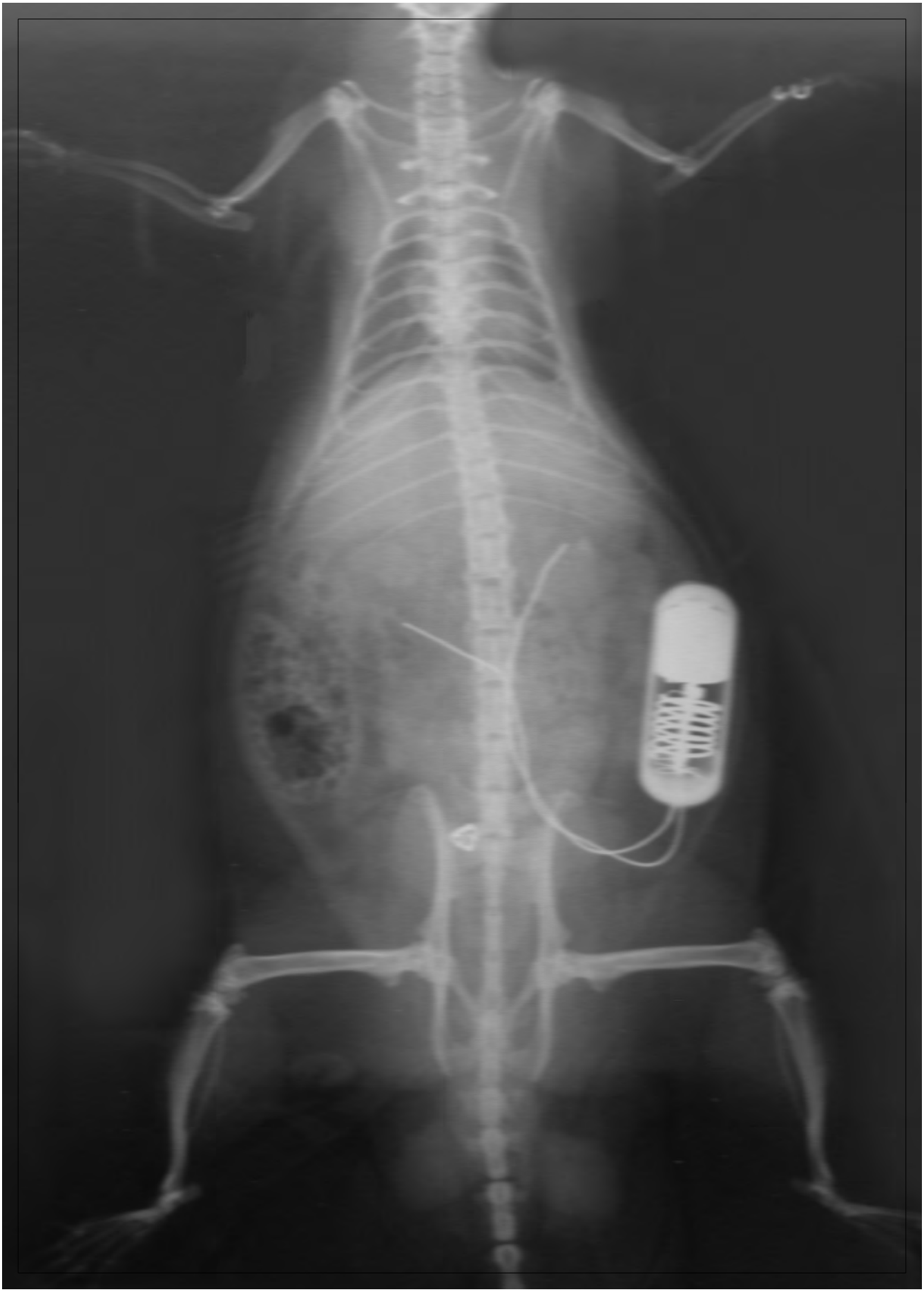

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Stress-induced hyperthermia and basal body temperature are mediated by different 5-HT_{1A} receptor populations: a study in SERT Knockout rats.

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Abstract

Disturbances in the serotonergic system are implicated in many CNS disorders. The serotonin transporter (SERT) regulates the serotonin homeostasis in the synapse. We recently developed a rat which lacks the serotonin transporter ($SERT^{-/-}$). It is likely that adaptive changes take place at the level of pre- and postsynaptic 5-HT receptors. Because autonomic responses are often used to measure $5\text{-}HT_{1A}$ receptor function, we analysed these responses by examining the effects of a $5\text{-}HT_{1A}$ receptor agonist and antagonist under *in vivo* conditions in the $SERT^{-/-}$ rat. Moreover, we studied the effect of a mild stressor on the temperature (stress-induced hyperthermia: SIH) because of the known involvement of $5\text{-}HT_{1A}$ receptors in this phenomenon. Results show that core body temperature did not differ between genotypes under basal, non-stressed conditions. Compared to $SERT^{+/+}$ rats, SIH was reduced in $SERT^{-/-}$ rats. The $5\text{-}HT_{1A}$ receptor agonist [R(+)-*N*-(2[4-(2,3-dihydro-2-2-hydroxy-methyl-1,4-benzodioxin-5-yl)-1-piperazininyl]ethyl)-4-fluorobenzoamide HCL (flesinoxan) reduced SIH in both genotypes. The flesinoxan-induced hypothermia in $SERT^{+/+}$ rats was blocked by the $5\text{-}HT_{1A}$ receptor antagonist [*N*-(2-[4-(2-methoxyphenyl)-1-piperazinyl] ethyl)-*N*-(2-pyridinyl) cyclohexanecarboxamide 3HCL (WAY100635). Moreover, WAY100635 induced hyperthermia in $SERT^{-/-}$, but not in $SERT^{+/+}$ rats. In $SERT^{-/-}$ rats, WAY100635 completely blocked the flesinoxan-induced reduction of SIH and induced hyperthermia. Interestingly, flesinoxan induced hypothermia was absent in $SERT^{-/-}$ rats. It is concluded that the SERT knockout rat allows us to reveal that $5\text{-}HT_{1A}$ receptors modulating SIH belong to a population of receptors that differs from that involved in hypothermia.

Introduction

Several studies support the involvement of the central serotonergic system in feeding and sexual behaviour as well as in systems affected by psychiatric disorders including anxiety, aggression, impulsivity and mood disturbances (Delgado et al., 1990;Stain-Malmgren et al., 2001;Neumeister et al., 2002;Moreno et al., 2002;Holmes et al., 2003c). Serotonergic (5-hydroxytryptamine, 5-HT) neurotransmission is primarily regulated by the serotonin transporter (SERT), which after its release into the synaptic cleft, transports 5-HT back into the presynaptic neuron where it is degraded or retained for future release (Murphy et al., 1998). A polymorphism in the human SERT has been described which leads to changes in SERT functioning early in life, and remains functional in adolescence and adulthood (Caspi et al., 2003;Beitchman et al., 2006;Covault J. et al., 2007;Schmitz et al., 2007). This SERT polymorphism has been linked to vulnerability for various personality characteristics and psychiatric disorders (Hahn and Blakely, 2002). Given the prominent role of the SERT in the regulation of 5-HT neurotransmission, it is likely that this polymorphism will lead to adaptations in the serotonergic system, although such changes have not yet been studied.

Selective serotonin re-uptake inhibitors (SSRIs) are widely used in the treatment of mood disorders, obsessive-compulsive disorder, eating disorders, and substance abuse (Murphy, 1990)(Graham et al., 1989). Chronic administration of SSRIs produces adaptive changes in the serotonergic system (Briley and Moret, 1993;Blier and De Montigny, 1998)(Blier and de, 1998;Briley and Moret, 1993). (Blier et al., 1997)However, such chronic treatments are still relatively short-lasting and provide only limited insight into the consequences of life-long adaptations in serotonergic functioning such as occurring in individuals with the above-mentioned SERT polymorphisms. Therefore, the goal of the present study was to investigate to what extent life-long disturbed 5-HT neurotransmission in SERT gene knockout (SERT$^{-/-}$) rats may result in adaptive changes in the serotonergic system. We especially focused on the 5-HT$_{1A}$ receptor pathways, because adaptive changes in these pathways, notably at the level of their receptors, are known to occur after chronic manipulations with the serotonergic system: e.g. chronic administration of SSRIs desensitizes 5-HT$_{1A}$ receptors in rats (Le Poul et al., 1995;Li et al., 1996;Li et al., 1997;Davidson and Stamford, 1998) and humans (Sargent et al., 1997;Berlin et al., 1998)(Sargent et al., 1997;Berlin et al., 1998). Although SERT$^{-/-}$ mice are available (Bengel et al., 1998), physiological responses to 5-HT$_{1A}$ receptor ligands strongly differ between mice and rats (see discussion) indicating that these responses are mediated by different 5-HT$_{1A}$ receptor populations in these species. SERT$^{-/-}$ rats were generated by ENU (N-ethyl-N-nitrosourea)-driven target-selected mutagenesis (Smits et al., 2004;Smits et al., 2006). Northern blot analyses have revealed that the premature stop codon results in a nonsense-mediated decay of the mutant SERT transcript. Compared to SERT$^{+/+}$ littermates, SERT$^{-/-}$ rats have nine-fold higher extracellular 5-HT levels in brain structures such as the hippocampus (Homberg et al., 2007a). Both *d*-fenfluramine-induced hypothermia and [^{3}H] citalopram binding to brain slices are completely absent in SERT$^{-/-}$ rats (Homberg et al., 2007a). In this recently developed rat model, we examined the functional status of the 5-HT$_{1A}$ receptor pathways under *in vivo* conditions.

Autonomic responses to selective 5-HT$_{1A}$ receptor agonists are frequently used to measure 5-HT$_{1A}$ receptor function (Hjorth, 1985;Millan et al., 1993;De Vry, 1995;Cryan et al., 1999;Ootsuka and Blessing, 2003;Nalivaiko et al., 2005;Ootsuka and Blessing, 2006). 5-HT$_{1A}$ receptor agonists

cause hypothermia in both animals (Hjorth, 1985;Millan et al., 1993;De Vry, 1995;Cryan et al., 1999;Bouwknecht et al., 2000) and humans (Pitchot et al., 2002;Pitchot et al., 2004). Several studies have suggested that the hypothermic responses to 5-HT_{1A} receptor agonists in rats and humans are mediated by postsynaptic 5-HT_{1A} receptors (Bill et al., 1991;Millan et al., 1993;Blier et al., 2002), although others have suggested that these effects are mediated by presynaptic receptors in rats (Goodwin et al., 1987;Higgins et al., 1988;Hillegaart, 1991) and mice (Goodwin et al., 1985b;Bill et al., 1991;Martin et al., 1992).

We analyzed the effects of both the 5-HT_{1A} receptor agonist flesinoxan, a potent and selective 5-HT_{1A} full agonist (Olivier et al., 1991), and the 5-HT_{1A} receptor antagonist WAY100635 as well as the receptor specificity of these effects on core body temperature (T_c), heart rate and stress-induced hypothermia (SIH) to investigate the role of 5-HT_{1A} receptors in the modulation of these autonomic responses. When an organism is confronted with a stressor it responds with a short-lasting increase of body temperature, the so-called SIH (Friedman and Thayer, 1998;Olivier et al., 1998;Nijsen et al., 1998a;Nijsen et al., 1998b;Bouwknecht et al., 2000). 5-HT_{1A} receptors are, amongst others, involved in both the modulation of SIH (for review see Adriaan Bouwknecht et al., 2007) and the modulation of T_C, although it is unclear whether both phenomena are regulated by the same populations of 5-HT_{1A} receptors. Given the nine-fold higher extracellular 5-HT level in the hippocampus of the $SERT^{-/-}$ rats (Homberg et al., 2007a), we hypothesized that the responsiveness of 5-HT_{1A} receptors is changed in these animals, due to fully occupation by 5-HT, or by downregulation of these receptors.

The aim of the present study was therefore to investigate to what extent the thermoregulatory effects of 5-HT_{1A} receptor agonists and antagonists are altered in the $SERT^{-/-}$ rat, and whether these alterations are (dis)similar for SIH and T_C. To study this, we first measured the circadian rhythms in T_C and heart rate of $SERT^{+/+}$ and $SERT^{-/-}$ animals under unchallenged conditions to detect any significant genotype differences in this respect. Additionally, we determined the reaction of these animals to a mild stressor in order to detect putative differences in basal responses of both genotypes in SIH and stress-induced tachycardia. Once these control experiments were performed, we initiated the study on the drug-induced challenges.

Experimental procedures

Subject

Serotonin transporter knockout rats ($Slc6a4^{1Hubr}$) were generated by ENU-induced mutagenesis (Smits et al., 2004;Smits et al., 2006). All subjects have been bred and reared in the Central Animal Laboratory of the University of Nijmegen. Experimental animals were derived from crossing heterozygous SERT knockout rats that were out crossed for 5 generations. In all experiments, male $SERT^{+/+}$ and $SERT^{-/-}$ littermates were compared. After weaning at the age of 21 days, ear cuts were taken for genotyping. All animals were housed two or three per cage in standard Macrolon® type 3 cages (42 x 26 x 20 cm) in temperature-controlled rooms (21°C) with standard 12/12-h day/night-cycle (lights on at 7.00 am) and food and water available *ad libitum*. Two groups of animals were used. The first group (9 months old) was used for (a) measuring the circadian rhythms, two weeks after surgery (see below), (b) measuring the responses to different doses of flesinoxan, given in an at random order from 4 weeks after surgery till 9 weeks after surgery (the saline

injection was used for measuring the hyperthermia induced by a mild stressor) and (c) measuring the responses to different doses of WAY100635, 11 weeks after surgery; the various doses were given in an at random order. The second group (4 months old) was used for (a) measuring the hyperthermia induced by a mild to intermediate stressor, one week after surgery (see below), (b) measuring the receptor-specificity of the drug-induced effects by giving WAY100635 immediately followed by flesinoxan, two weeks after surgery, and (c) measuring the response to the highest doses of flesinoxan (3 weeks after surgery) and WAY100635 (4 weeks after surgery) tested in the first group in order to study the possible occurrence of drug-induced changes in the sensitivity of the receptors. All experiments were carried out in accordance with institutional, national and international guidelines for animal care and the Dutch law concerning animal welfare.

Surgery

The telemetry ECG transmitter was chronically implanted following a surgical procedure that was described before (Sgoifo et al., 1996). Briefly rats were deeply anesthetized with a mixture of N_2O/O_2 (1:2) and isoflurane (2.5%; Rhodia Organique Fine limited, Bristol, United Kingdom). ECG transmitters (Data Sciences International™, type TA11CTA-F40, St. Paul, MN, USA) were implanted in the abdominal cavity with one electrode subcutaneously placed on the muscle of the lower right limb of the animal, and the other electrode on the muscle bundle of the left shoulder. After surgery, animals were isolated and allowed to recover for 14 days; rats were checked daily during that period.

Radio-telemetry system

The radiotelemetry system consisted of an implantable transmitter (model TA11CTA-F40) with two flexible leads, a telemetric receiver (model RPC-1 and RLA1020), a Data Exchange Matrix collecting input from the receivers and an in-line analog ECG adaptor, all purchased from Data Sciences International™ (St. Paul, MN, USA). The matrix was connected to an IBM Compaq 486/66 computer. Signals from the transmitters were passed on *via* a radio signal to the receiver, localized under the animal cage, transforming it into a digital signal. Digital information from the telemetry receivers was collected by the data matrix and provided to the computer. Raw data were collected and analyzed by the software package Dataquest A.R.T. version 3.11 (Data Sciences International™, St. Paul, MN, USA).

Experimental protocol radiotelemetric studies

Data of all animals were sampled every 5 minutes, collecting body temperature (°C) and heart rate (HR, beats per minute).

Circadian Rhythm, baseline – Two weeks after recovery from surgery, T_C and heart rate (HR) were recorded continuously during seven days. On days 1, 3 and 5 the experimenter performed a 5-min inspection for animal health, water and food supplies.

Stressful stimuli– To investigate basal differences in stress response between $SERT^{-/-}$ and $SERT^{+/+}$ rats, the autonomic response (HR and T_C) to two different stressors was measured. A saline injection was used as a mild stressor (Baumgartner et al., 1998) in the first group of animals: this stressor is known to cause an increase in temperature and HR (Baumgartner et al., 1998). In the second group of animals a mild to intermediate stressor, namely exposure to a novel cage (standard Macrolon® type 3 cages (42 x 26 x 20 cm) with clean sawdust in it) was used. These two types of stressor were assessed, because it is known that the response to a stressor varies across stressors with different intensity (van Bogaert et al., 2006a;Adriaan Bouwknecht et al., 2007).

The difference between basal temperature or HR (average of one hour before the injection) and the maximal temperature or HR increase after a saline injection is referred to as SIH and stress-induced tachycardia, respectively.
Injection with drugs –Sampling data of the $SERT^{-/-}$ and $SERT^{+/+}$ rats started at 9:00 AM the day prior to the administration of an intraperitoneal injection to obtain undisturbed baseline values. All doses of flesinoxan (0, 0.3, 1, 3 and 10 mg/kg) and WAY100635 (0, 0.1 and 1 mg/kg) were randomly administered at one-week intervals to each rat (within-animal design). The doses of WAY100635 (1 mg/kg) and flesinoxan (10 mg/kg) to assess the receptor-specificity of the drug-induced effects were chosen on the basis of the outcome of the dose response curve made of the individual drugs. All injections were given at 11.00 AM. In case of the antagonism experiment, WAY100365 was IP injected to the left side, and immediately thereafter flesinoxan IP to the right side of the belly. The effects were measured till five hours after the injection.

Drugs
Flesinoxan HCL [R(+)-*N*-(2[4-(2,3-dihydro-2-2-hydroxy-methyl-1,4-benzodioxin-5-yl)-1-piperazininyl] ethyl)-4-fluorobenzoamide was kindly provided by the department of Psychopharmacology, Utrecht University, Utrecht, the Netherlands. WAY100635 3HCL [*N*-(2-[4-(2-methoxyphenyl)-1-piperazinyl] ethyl)-*N*-(2-pyridinyl) cyclohexanecarboxamide was derived from SIGMA, Lyon, France. All drugs were dissolved in saline (0.9% NaCl), and intraperitoneally injected in a volume of 1 ml/kg.

Data analysis
Circadian Rhythm, baseline – Data (T_C and HR) were sampled every five minutes on undisturbed days (2, 4, 6 and 7). The data were averaged per hour and, subsequently, averaged across the four days, proving thereby a mean ± SEM for each hour of the day. Differences in baseline conditions between genotypes were analyzed using two-way ANOVA, with genotype and time as repeated measures. Where appropriate, an independent sample T-test was used for post-hoc analyses.
Stressful stimuli – Data collected during the period starting one hour before the exposure to the stressor and ending five hours after exposure to the stressor were analyzed. Genotype differences in T_C and HR were analyzed by means of a two-way ANOVA for repeated measures with the factors genotype and time (repeated). Where appropriate, data were further analyzed by means of a post-hoc independent sample T-test on genotype.
Injection with drugs – The effects of the drugs on SIH and T_C were analyzed using a three-way ANOVA for repeated measures with the factors genotype, dose and time (repeated). In case of statistical significance a two-way ANOVA for repeated measures with the factors dose and time (repeated) was performed. Where appropriate, data were further analyzed by means of a LSD post-hoc test for doses, and independent sample T-test for time.
The level of significance was set at $p < 0.05$. All statistical analyses were performed using the Statistical Package for Social Sciences for Windows version 12.0.1 (SPSS, Chicago, IL, USA).

Results

Body temperature and heart rate are presented to illustrate putative differences between $SERT^{+/+}$ and $SERT^{-/-}$ rats in circadian rhythms and stress-induced hyperthermia/tachycardia. Pharmacological data are presented only for body temperature, because HR and T_c showed largely similar results.

Circadian Rhythm.

Figures. 1A and 1B show a clear day-night pattern in SERT$^{+/+}$ and SERT$^{-/-}$ rats, with a higher T_C and HR during the night, the active period of rats. No significant differences between T_C (Figure. 1A) and HR (Figure. 1B) of SERT$^{+/+}$ and SERT$^{-/-}$ rats were found.

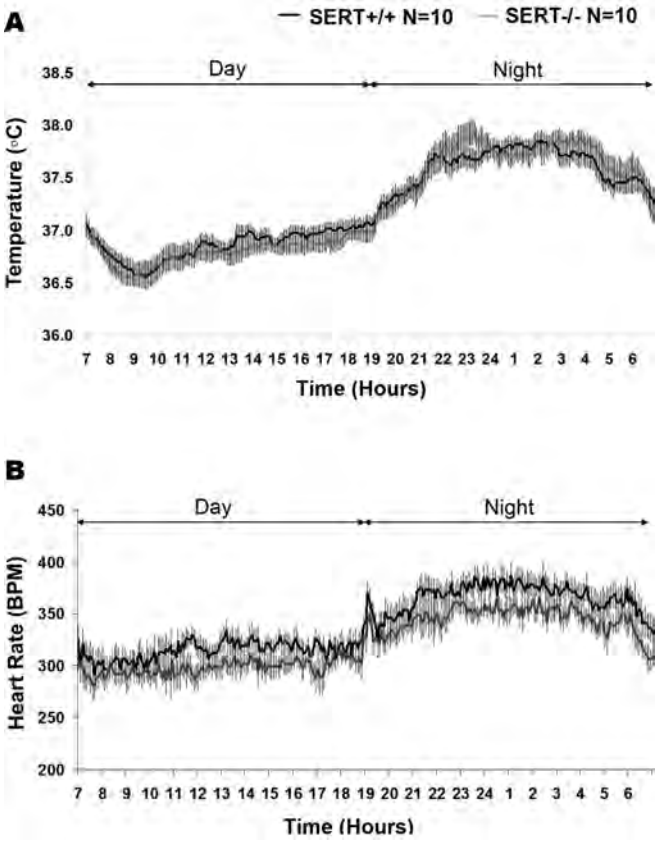

Figure 1
(**A**) Baseline temperature Circadian rhythm of T_C over 12 h light/ 12 h dark cycle (lights on from 7:00 am to 7:00 pm). Data are based on mean values of 4 days in SERT$^{+/+}$ and SERT$^{-/-}$ rats. Data present mean group values over 24 hours. Circadian rhythm is found in both genotypes. No differences are found between genotypes. (**B**) Baseline heart rate Circadian rhythm of HR over 12 h light/ 12 h dark cycle (lights on from 7:00 am to 7:00 pm). Data are based on mean values of 4 days in SERT$^{+/+}$ and SERT$^{-/-}$ rats. Data present mean group values of 24 hours. Circadian rhythm is found in both genotypes. No differences are found between genotypes.

Stress-induced Hyperthermia (SIH) and Tachycardia (SIT).

The stress response to a single saline injection was significantly lower in SERT$^{-/-}$ rats compared to SERT$^{+/+}$ rats (Figure. 2A). An overall genotype x time effect was found for temperature (Figure. 2A; 2-way ANOVA: $F_{(72,1152)}=1.668$; $p<0.001$). Post hoc analyses revealed a significant difference (SERT$^{+/+}$ > SERT$^{-/-}$ rats) from 30 till 55 min, at 95 min, and from 105 till 125 min after injection. Stress-induced tachycardia was also lower in SERT$^{-/-}$ compared to SERT$^{+/+}$ rats (Figure. 2B). An overall genotype x time effect was found for the heart rate (2-way ANOVA: $F_{(72,1224)}=1.448$; $p<0.01$); post hoc analyses revealed significant differences (SERT$^{+/+}$ > SERT$^{-/-}$ rats) after 20 till 30 min, at 50 min and at 70 min after saline injection (Figure 2B). Interestingly, exposing the rats to a novel cage did not result in any difference between both genotypes (data not shown).

5-HT$_{1A}$ receptor agonist flesinoxan.

In SERT$^{+/+}$ rats flesinoxan dose-dependently decreased SIH and elicited a dose-dependent hypothermic effect at the same time (Figure 3A). However, in SERT$^{-/-}$ rats flesinoxan produced a dose-dependent decrease in SIH that was slightly smaller than that seen in SERT$^{+/+}$ rats, but had no hypothermic effect (Figure 3B). A highly significant genotype x dose x time interaction (3-way

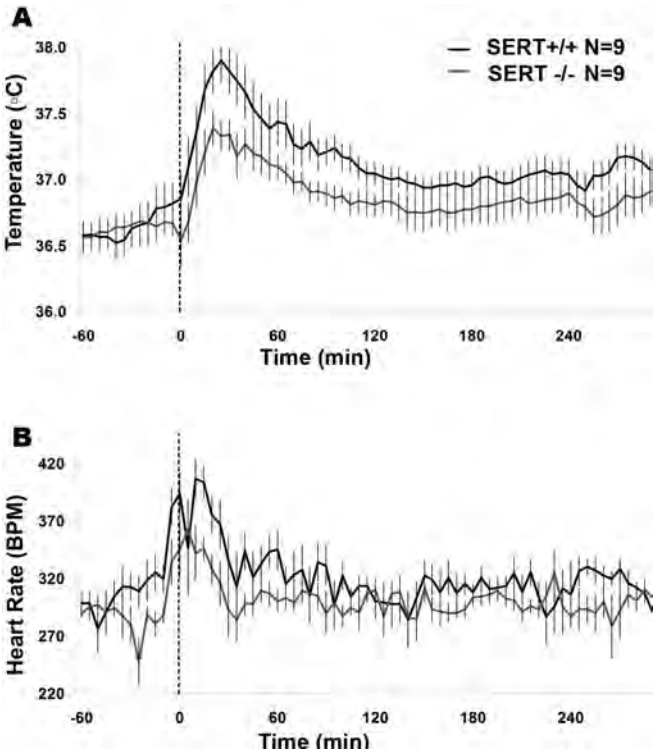

Figure 2
(**A**) Stress induced hyperthermia, mean ± SEM temperature after saline injection. $SERT^{+/+}$ rats have significantly higher SIH ($F_{(172,1152)}$=1.668 p<0.001) compared to $SERT^{-/-}$ rats from 30 till 55, at 95 and from 105 till 125 minutes after injection. (**B**) Stress induced tachycardia; mean ± SEM heart rate after saline injection. $SERT^{+/+}$ rats have significantly higher SIT ($F_{(172,1224)}$=1.448 p<0.01) compared to $SERT^{-/-}$ rats from 20 till 30 minutes, at 50 minutes and at 70 minutes after saline injection.

ANOVA: $F_{(288,6048)}$ =9.918; p<0.001) was found. A subsequent 2-way ANOVA revealed a significant dose x time interaction in $SERT^{+/+}$ rats ($F_{(288,2952)}$ =19.422; p<0.001), as well as in $SERT^{-/-}$ rats ($F_{(288,3096)}$ =2.074; p<0.001). 3 mg/kg flesinoxan significantly decreased the temperature in $SERT^{+/+}$ rats compared to the saline injection from 15 min till 180 min after injection (p<0.05). 10 mg/kg flesinoxan also decreased the temperature in $SERT^{+/+}$ rats compared to saline (p<0.05) from 20 min post-injection until the end of the measurement (5 hours after injection). Furthermore, 3 mg/kg flesinoxan decreased the temperature in $SERT^{+/+}$ rats compared to 0.3 mg/kg flesinoxan (p<0.05), 10 mg/kg flesinoxan decreased the temperature compared to 0.3 mg/kg flesinoxan (p<0.05), compared to 1 mg/kg flesinoxan (p<0.05) and compared to 3 mg/kg flesinoxan (p<0.05). In $SERT^{-/-}$ rats only 10 mg/kg flesinoxan significantly decreased the SIH compared to saline (p<0.05) from 25 min till 145 min after injection.

5-HT$_{1A}$ receptor antagonist WAY100635.
WAY100635 had no effect on the SIH response in $SERT^{+/+}$ rats (Figure. 4A), but it dose-dependently increased the body temperature in the $SERT^{-/-}$ rats (Figure. 4B). A highly significant genotype x dose x time interaction (3-way ANOVA: $F_{(144,3384)}$ =3.958; p<0.001) was found. A subsequent 2-way ANOVA revealed a significant dose x time effect for $SERT^{-/-}$ rats ($F_{(144,1728)}$ =10.764; p<0.001), while no dose-dependent effects in $SERT^{+/+}$ rats were found. The temperature in $SERT^{-/-}$ rats after 1 mg/kg WAY100635 was significantly higher (p<0.05) compared to saline injected $SERT^{-/-}$ rats (25 min after injection till the end of the experiment). Temperature was also significantly higher in 1 mg/kg WAY100635 injected $SERT^{-/-}$ rats, compared to 0.1 mg/kg WAY100635 injected $SERT^{-/-}$ rats (p<0.05). This difference was found at 35, 40, 65 and 85 min after injection till the end of the experiment.

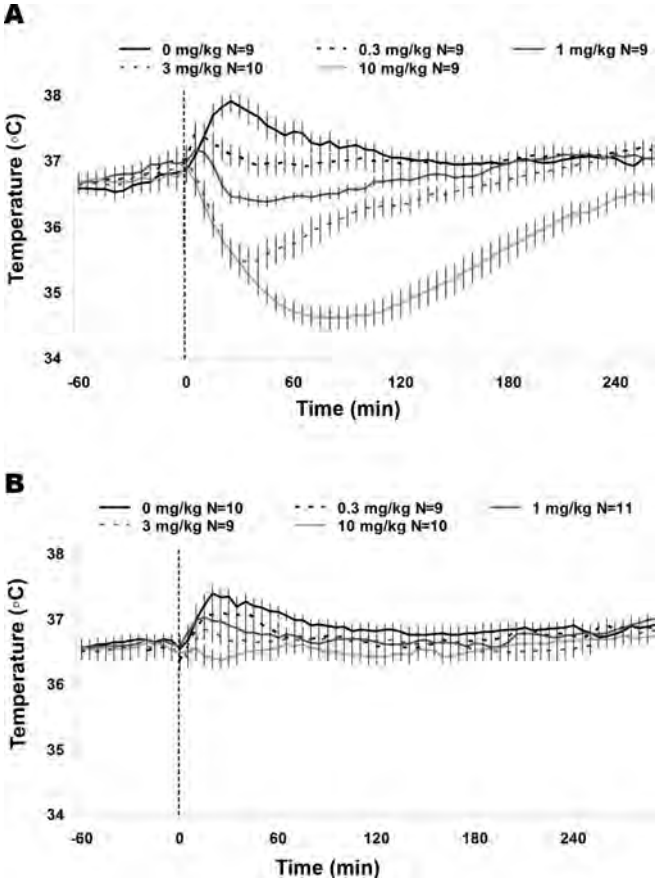

Figure 3.
(**A**) Dose response curve of body temperature after flesinoxan in SERT$^{+/+}$ rats. Effects of flesinoxan on mean ± SEM SIH and T_c in SERT$^{+/+}$ rats. Temperature was significantly different between doses ($F_{(288,2952)}$ =19.422 p<0.001). Difference were found between saline and 3 mg/kg flesinoxan (p<0.001 from 15 minutes till 180 minutes after injection), between saline and 10 mg/kg flesinoxan (p<0.001 from 20 minutes post-injection until the end of the measurement), between 0.3 mg/kg and 3 mg/kg flesinoxan (p<0.004), between 0.3 mg/kg and 10 mg/kg flesinoxan (p<0.001), between 1 mg/kg and 10 mg/kg flesinoxan (p<0.001) and between 3 mg/kg and 10 mg/kg flesinoxan (p<0.001). (**B**) Dose response curve of body temperature after flesinoxan in SERT$^{-/-}$ rats. Effects of flesinoxan on mean ± SEM SIH in SERT$^{-/-}$ rats. Temperature was significantly different between doses ($F_{(288,3096)}$ =2.074 p<0.001). A difference was found between saline and 10 mg/kg flesinoxan (p<0.046) from 25 minutes till 145 minutes after injection.

5-HT$_{1A}$ Receptor-specificity of the response to flesinoxan.

As observed earlier (Figure. 3A) the stress response to a saline injection differed between SERT$^{+/+}$ and SERT$^{-/-}$ rats (Figure. 5A); an overall genotype x time effect was found ($F_{(60,1020)}$=1.995; p<0.001). The SIH seen after the saline injection in SERT$^{-/-}$ was significantly lower than that seen in SERT$^{+/+}$ rats from 10 till 25 min after injection, at 45 till 140 min, at 150 min after injections and from 220 till 260 min (independent T-test) after injections compared to SERT$^{+/+}$ rats. As shown in Figure. 5A, the effect of WAY100635 in flesinoxan-treated SERT$^{-/-}$ rats fully differed from that seen in identically treated SERT$^{+/+}$ rats. Indeed, a highly significant genotype x treatment x time interaction (3-way ANOVA: $F_{(60,1980)}$=9.320; p<0.001) was found. No significant treatment x time interaction in SERT$^{+/+}$ rats ($F_{(60,900)}$ =1.306; p=0.064) was found: the hypothermic effect of flesinoxan in the SERT$^{+/+}$ rats was completely blocked by WAY100635 (Figure 5A); In contrast, the treated SERT$^{-/-}$ rats showed an increase in temperature (2-way ANOVA: $F_{(60,1080)}$ =13.101; p<0.001). This hyperthermic effect was found from 45 min till 240 min after injection, compared to the saline injections (Figure 5A). Also a significant genotype x time interaction (2-way ANOVA: $F_{(60,900)}$= 9.063; p<0.001) was found. SERT$^{-/-}$ rats showed a higher hyperthermia compared to SERT$^{+/+}$ rats from 35 min after injection till the end of the experiment.

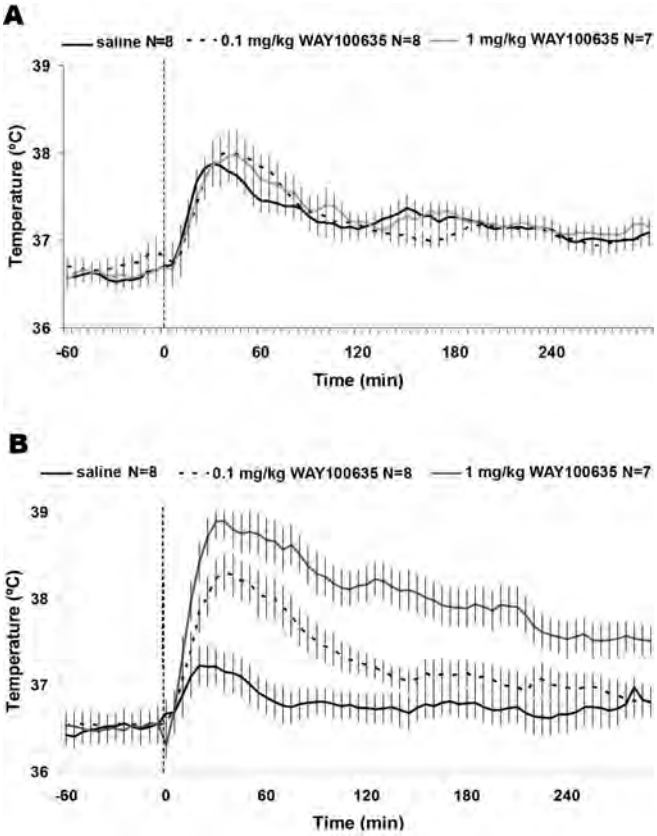

Figure 4.
(**A**) Dose response curve of temperature after WAY100635 in $SERT^{+/+}$ rats. Effect of WAY100635 on mean ± SEM SIH in $SERT^{+/+}$ rats. No differences were found between doses.
(**B**) Dose response curve of temperature after WAY100635 in $SERT^{-/-}$ rats. Effect of WAY100635 on mean ± SEM SIH in $SERT^{-/-}$ rats. Significant differences were found between doses ($F_{(144,1728)}$ =10.764 p<0.001); temperature after 1 mg/kg WAY100635 was significant higher (p<0.001) compared to saline injected $SERT^{-/-}$ rats (25 minutes after injection till the end of the experiment). Differences were also found between 1 mg/kg and 0.1 mg/kg WAY100635 (p<0.019; found at 35, 40, 65 and 85 minutes after injection till the end of the experiment).

Drug-induced priming effects.
To study whether drug-induced changes in the sensitivity might have occurred during the study, the experiments on the highest doses of flesinoxan and WAY100635 were replicated at the end of the study in group 2. After 10 mg/kg flesinoxan again a genotype x treatment x time interaction was found (3-way ANOVA: $F_{(60,1800)}$=16.850; p<0.001) showing that this dose of flesinoxan had a much stronger effect in $SERT^{+/+}$ than in $SERT^{-/-}$ rats. In $SERT^{+/+}$ rats a treatment x time effect was found (2-way ANOVA: $F_{(60,900)}$=44.416; p<0.001) from 15 minutes after injection till the end of measurement (Figure 5B). In $SERT^{-/-}$ rats a treatment x time effect was found (2-way ANOVA: $F_{(60,900)}$=14.813; p<0.001) from 20 minutes till 140 minutes after injection. Also a genotype x time was found (2-way ANOVA: $F_{(60,960)}$=1.626; p<0.001) after flesinoxan. $SERT^{+/+}$ rats were significant different from $SERT^{-/-}$ rats from 15 minutes till 225 minutes after injection of flesinoxan. These results are equal to the results found in the first group of animals which were treated ad randomlywith different doses of flesinoxan, indicating that no priming effects were found after administration of flesinoxan. After treatment of 1 mg/kg WAY100635 we also found a genotype x treatment x time interaction (3-way ANOVA: $F_{(60,1800)}$=13.353; p<0.001). In $SERT^{+/+}$ rats no treatment x time effect was found (Figure 5C; 2-way ANOVA: $F_{(60,900)}$=0.596; p<0,994), but in $SERT^{-/-}$ rats we again found a treatment x time effect (2-way ANOVA: $F_{(60,900)}$=29.269; p<0,001) from 55 minutes after injection till the end of the measurement. Also here a genotype x time effect was found (2-way ANOVA: $F_{(60,840)}$=15.534; p<0,001). $SERT^{+/+}$ rats significantly differed from $SERT^{-/-}$ rats from the injection till 10 minutes after injection and from 25 minutes after injection till the end of measurement (Figure 5C). The response to

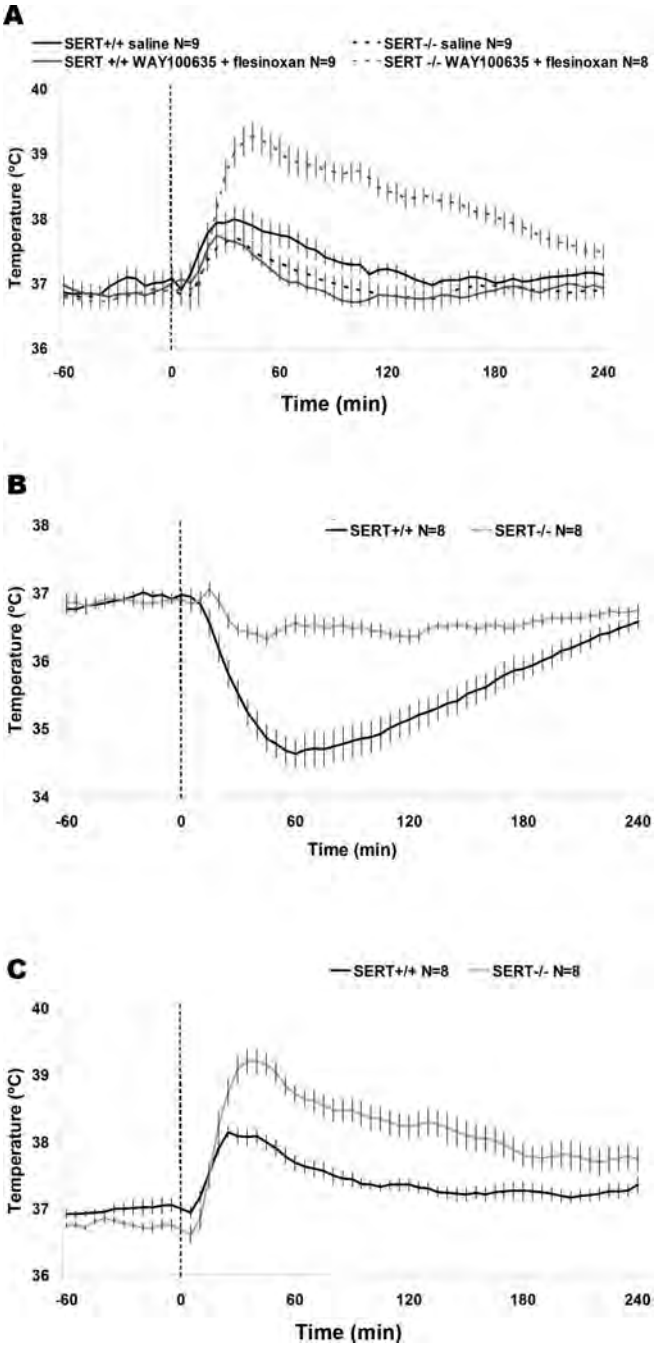

Figure 5
(**A**) Temperature after saline and after administration WAY100635 followed by flesinoxan. Effect of the combination WAY100635 and flesinoxan on mean ± SEM T_c in SERT$^{+/+}$ and SERT$^{-/-}$ rats. A difference was found between SERT$^{+/+}$ and SERT$^{-/-}$ after the combination of WAY100635 and flesinoxan ($F_{(60,1980)}$=9.320 p<0.001). In SERT$^{+/+}$ rats no differences were found between the combination of WAY100635 and flesinoxan compared to the control. A significant difference was found in SERT$^{-/-}$ rats ($F_{(60,1080)}$ =13.101 p<0.001). The combination of WAY100635 and flesinoxan gave a hypothermic effect, this effect was found from 45 minutes till 240 minutes after injection, compared to the double saline injections. (**B**) Temperature after 10 mg/kg flesinoxan. Effect of 10 mg/kg flesinoxan on mean ± SEM SIH and T_c in SERT$^{+/+}$ and SERT$^{-/-}$ rats. A difference was found between SERT$^{+/+}$ and SERT$^{-/-}$ after flesinoxan ($F_{(60,840)}$=17.213; p<0.001) from 15 minutes till 225 minutes after the injection of flesinoxan. (**C**) Temperature after 1 mg/kg WAY100635. Effect of 1 mg/kg WAY100635 on mean ± SEM SIH in SERT$^{+/+}$ and SERT$^{-/-}$ rats. A difference was found between SERT$^{+/+}$ and SERT$^{-/-}$ after WAY100635 ($F_{(60,840)}$=15.534; p<0.001) from the injection till 10 minutes after injection and from 25 minutes after the injection till the end of the measurement.

WAY100635 are found to be the same as found in the first group of animals, again indicating that priming, or other alterations, did not occur in these animals after treatment of different doses of WAY100635.

Discussion

The 5-HT-transporter is the primary modulator of the tone of the serotonergic system in the CNS. Here we show that rats constitutionally lacking the SERT have altered 5-HT_{1A} receptor reactivity. More importantly, however, the data show that this alteration in 5-HT_{1A} receptor functioning is not a global phenomenon, but limited to some specific subpopulations of 5-HT_{1A} receptors. This became clear by comparing autonomic responses like body temperature (T_C) and heart rate (HR) under basal conditions, the effects seen in animals challenged by stressors, and the effects seen in animals treated with 5-HT_{1A} receptor ligands. These phenomena are discussed below. In the experiments drugs were administered randomly over the whole experimental period. The effects of the drugs and controls found in the beginning of the study were similar to those found in the end, and we therefore conclude that the effect of the drugs remained stable during this study, indicating that priming or other alterations did not occur in our animals over the course of the experiments.

Circadian Rhythms.

Both $SERT^{+/+}$ and $SERT^{-/-}$ rats displayed a clear circadian rhythm. During the dark period both T_C and HR were higher in $SERT^{+/+}$ and $SERT^{-/-}$ rats compared to the day; in line with the fact that nocturnal animals have their active period during the night (Benstaali et al., 2001). Apparently, a deletion of the SERT did not lead to disturbances in day-night rhythms in the autonomic parameters under an undisturbed 12-hour light/dark cycle. The suprachiasmatic nucleus (SCN) of the hypothalamus orchestrates circadian rhythms (Reppert and Weaver, 2002). Input to the SCN is, amongst others, provided by serotonergic projections originating in midbrain raphe nuclei, and disruption of 5-HT afferents to the SCN alters circadian behaviour in rodents (Banky et al., 1988). Either constitutional absence of serotonergic transporters does not lead to disturbances in these serotonergic projections to the SCN or, alternatively, compensatory mechanisms may have taken place in $SERT^{-/-}$ rats. Because $SERT^{-/-}$, 5-HT_{1A}, 5-HT_{1B} and 5-HT_7 receptor knockout mice neither show differences in circadian rhythms (Li et al., 1999;Bouwknecht et al., 2001;Hedlund et al., 2003;van Bogaert et al., 2006b), we conclude that there are no basal differences in the circadian rhythm between rats and mice with regard to the serotonergic involvement. Apparently, the serotonergic system does not play a primary regulatory role in the circadian rhythm of rodents, although it may exert modulatory influences under certain conditions such as changed light/dark phases as shown for example in mice lacking 5-HT_{1B} receptors (Sollars et al., 2002;Sollars et al., 2006).

Like in $SERT^{-/-}$ mice (Li et al., 1999), no differences in basal T_c were found between $SERT^{+/+}$ and $SERT^{-/-}$ rats, suggesting that serotonin in mice and rats plays no major role in the modulation of T_c. Still, both 5-HT_{1B} knockout mice have a relatively enhanced T_c compared to wild types (Bouwknecht et al., 2001) and 5-HT_{1A} knockout mice in a 129/Sv strain have a relatively low Tc compared to wild types (van Bogaert et al., 2006b), revealing that the serotonergic system at least in mice is nevertheless involved in the regulation of T_c.

Stress Induced Hyperthermia.

Under normal conditions, exposure to a mild stressor elicits SIH in rodents which is modulated, amongst others, by 5-HT_{1A} receptors (Lesch, 1991;Groenink et al., 1996;van der Heyden et al., 1997;Friedman and Thayer, 1998;Olivier et al., 1998;Nijsen et al., 1998a;Nijsen et al., 1998b;Bouwknecht et al., 2000). Compared to $SERT^{+/+}$ wild type rats, $SERT^{-/-}$ rats displayed an

attenuated SIH response to a saline injection being a mild stressor according to Baumgartner et al., 1998. Because extracellular 5-HT levels have been shown to be nine times higher in the hippocampus of SERT$^{-/-}$ rats compared to SERT$^{+/+}$ rats (Homberg et al., 2007a), it is likely that a relative hyperstimulation of 5-HT$_{1A}$ receptors underlies the relatively small SIH in SERT$^{-/-}$ rats. However, our present data do not allow a conclusion about the exact location of these receptors: it remains open for investigation whether these 5-HT$_{1A}$ receptors are pre- or postsynaptically located, or in which brain areas they are concentrated.

Exposure to a novel cage, which is considered to be a mild to intermediate stressor (van Bogaert et al., 2006a; Vinkers et al., 2008) did not result in any SIH difference between SERT$^{+/+}$ and SERT$^{-/-}$ rats. Therefore, the difference in SIH between the two genotypes studied appears to be present only after subtle stressors. As a final remark, exposure to a mild stressor such as a saline injection does elicit identical effects on body temperature in SERT$^{+/+}$ and SERT$^{-/-}$ mice (Li et al., 1999), underlining the earlier mentioned notion that mice do differ from rats.

Flesinoxan-induced reduction of SIH.
Both SERT$^{+/+}$ and SERT$^{-/-}$ rats showed a clear reduction in SIH after administration of the full 5-HT$_{1A}$ receptor agonist flesinoxan, although the reduction in SERT$^{+/+}$ rats was stronger than that in SERT$^{-/-}$ rats. This stronger reduction may be caused (partially) by the additional hypothermic effects of flesinoxan in SERT$^{+/+}$ rats that was not present in SERT$^{-/-}$ rats (see below), but does not rule out that 5-HT$_{1A}$ receptors involved in SIH are slightly desensitized in SERT$^{-/-}$ rats. To investigate whether the flesinoxan effect was 5-HT$_{1A}$ receptor specific, the highly selective 5-HT$_{1A}$ receptor antagonist, WAY100635, was tested. WAY100635 itself did not alter the core body temperature in SERT$^{+/+}$ rats, but it dose-dependently increased the temperature in SERT$^{-/-}$ rats. It is unclear whether this hyperthermia (enhanced both in amplitude and duration) has to be ascribed to the enhancement of SIH per se, or involves an associated hyperthermia adding to the normal SIH. Nevertheless, WAY100635 completely antagonized the flesinoxan-induced reduction of SIH in SERT$^{+/+}$ rats, providing compelling evidence that the effects of flesinoxan on SIH were mediated by 5-HT$_{1A}$ receptors. The increase of temperature seen in the antagonism experiment with SERT$^{-/-}$ rats can be ascribed to WAY100635 (blockade of 5-HT$_{1A}$ receptors), because WAY100635 alone caused a similar effect. More importantly, these results also show that the 5-HT$_{1A}$ receptors involved in the regulation of SIH were apparently not fully occupied, or desensitized, in SERT$^{-/-}$ rats, because flesinoxan was still effective. This is highly remarkable in view of the earlier reported finding that the 5-HT levels in at least the hippocampus were greatly increased in SERT$^{-/-}$ rats compared to SERT$^{+/+}$ rats. Apparently, such a 5-HT increase is limited to certain brain areas or other adaptational changes took place (see also below); future studies are required to investigate these possibilities.

Flesinoxan-induced Hypothermia.
The phenomenon that flesinoxan caused a dose-dependent hypothermia in the SERT$^{+/+}$ rats, but not in SERT$^{-/-}$ rats, shows that changes occurred within the 5-HT$_{1A}$ receptor pathways involved in thermoregulation in SERT$^{-/-}$ rats. There are at least three processes that may give rise to these changes: first, the serotonergic tone is altered; second, the (sensitivity of the) receptor is altered; and, third, the postsynaptic machinery is changed. In view of the above mentioned notion that the 5-HT level is increased in at least some parts of the brain, the most likely explanation is that the involved receptors were fully occupied, preventing thereby flesinoxan to produce any

effect. Although the occurrence of desensitization and changes in the postsynaptic machinery are theoretically alternative explanations, this is unlikely in view of the fact that the antagonist WAY100635 was still fully effective (see above).

Summarizing the above mentioned conclusions about the occupancy of the 5-HT_{1A} receptors involved in either the SIH or the thermoregulation results in the evident notion that there are at least two distinct subpopulations of 5-HT_{1A} receptors: one subpopulation of receptors involved in SIH which are at best partially occupied and one population of receptors involved in thermoregulation which are fully occupied. Future research is required to investigate whether these two subpopulations are differentially located at pre- and postsynaptic sites and/or located in different brain areas, each of them possibly marked by its specific 5-HT level in $SERT^{-/-}$ rats. An attractive explanation for differences in the occupancy of the 5-HT_{1A} receptors might be that there is a site-specific difference in density of dopaminergic and noradrenergic terminals in the vicinity of the serotonergic terminals: these catecholaminergic terminals contain dopamine transporters (DAT) and noradrenergic transporters (NET) which can also transport serotonin into their terminals, removing thereby the surplus of serotonin in the serotonergic synapses of $SERT^{-/-}$ rats. Indeed, Homberg et al. (2007a) have shown that blockade of NET and DAT in $SERT^{-/-}$ rats blocks the [^{3}H]5-HT uptake into hippocampal synaptosomes. In other words, it is suggested that the serotonergic tone may vary across brain regions that vary in number of catecholaminergic terminals in the vicinity of serotonergic terminals in $SERT^{-/-}$ rats.

Conclusion.
5-HT_{1A} receptors of $SERT^{-/-}$ rats that are involved in flesinoxan-induced hypothermia, do not respond to 5-HT_{1A} receptor agonists in contrast to the 5-HT_{1A} receptors involved in SIH, which are completely active. Whether elimination of the SERT affects the sensitivity of other 5-HT receptors and systems in the brain remains to be investigated. The finding that exposure to a mild, but not mild to intermediate, stressor results in a SIH in $SERT^{-/-}$ rats that is smaller than that seen $SERT^{+/+}$ rats is suggested to be due to the relatively high serotonergic tone in the $SERT^{-/-}$ rats. The present study clearly indicates that rats with a lifelong 5-HT disturbance ($SERT^{-/-}$ rats) have regional specific changes in 5-HT_{1A} receptor occupancy. It is highly interesting to see whether comparable changes occur in humans that suffer from lifelong 5-HT disturbances.

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Blockade of dopamine, but not noradrenaline, transporters produces hyperthermia in rats that lack serotonin transporters.

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Abstract

Given the modulatory role of 5-HT in emotional and cognitive processes, putatively adaptational changes in GABA-ergic, dopaminergic and noradrenergic systems in $SERT^{-/-}$ rats were investigated by assessing the stress-induced hyperthermia paradigm and measuring core body temperature. In response to the $GABA_A$ receptor agonist chlordiazepoxide, $SERT^{-/-}$ rats showed a small increased sensitivity with respect to the core body temperature, indicating a small change in sensitivity in the GABA-ergic system. $SERT^{-/-}$ rats were slightly more sensitive to the NET blocker atomoxetine; the 1 mg/kg dose reduced core body temperature in $SERT^{-/-}$ rats, but not in $SERT^{+/+}$ rats. The highest dose reduced core body temperature in both genotypes. Interestingly, there was a robust differential response to the DAT blocker GBR12909; while hyperthermic response was induced in $SERT^{-/-}$ rats, no response was found in $SERT^{+/+}$ rats. Moreover, $SERT^{-/-}$ rats were more sensitive to blockade of both NET and DAT transporters with bupropion than $SERT^{+/+}$ rats. The drug-induced changes in stress-induced hyperthermia did not differ between $SERT^{+/+}$ and $SERT^{-/-}$ rats. We conclude that permanent changes in the serotonergic system lead to compensatory changes in the GABA-ergic, noradrenergic and dopaminergic system with regard to core body temperature regulation, but not stress-induced hyperthermia.

Introduction

Serotonin (5-hydroxytryptamine, 5-HT) plays an important modulatory role in a variety of processes such as emotion, motivation, cognition, feeding and sexual behaviour and a disturbance in the serotoninergic system can contribute to psychiatric disorders including affective disorders, violent aggression, impulsivity-related disorders, drug addiction, and eating disoders (Delgado et al., 1990;Stain-Malmgren et al., 2001;Neumeister et al., 2002;Moreno et al., 2002;Holmes et al., 2003c;Murphy et al., 2004). Serotonin neurotransmission is primarily regulated by the serotonin transporter (SERT), which returns 5-HT from the synaptic cleft into the presynaptic neuron where it is degraded or retained for future release (Murphy et al., 1998). Selective serotonin re-uptake inhibitors (SSRIs) block the SERT and are widely used in the treatment of affective disorders, obsessive-compulsive and eating disorders as well alcoholism (Murphy, 1990; Graham et al., 1989). Chronic administration of SSRIs produces adaptive changes in the serotonergic system(Blier and de Montigny, 1998;Briley and Moret, 1993; Blier et al., 1997). To investigate to what extent life-long disturbed 5-HT neurotransmission may result in adaptive changes of GABA-ergic, dopaminergic and noradrenergic systems, we challenged the serotonin transporter knockout rat ($SERT^{-/-}$) with drugs affecting these neurotransmitter systems and studied the resulting changes in body temperature. The choice for these systems is elaborated below.

$SERT^{-/-}$ rats were generated by ENU (N-ethyl-N-nitrosourea)-driven target-selected mutagenesis (Smits et al., 2004;Smits et al., 2006). Northern blot analyses revealed that the premature stop codon results in a nonsense-mediated decay of the mutant SERT transcript (Homberg et al., 2007a). Compared to $SERT^{+/+}$ littermates, $SERT^{-/-}$ rats have nine-fold higher extracellular 5-HT levels in brain structures such as the hippocampus (Homberg et al., 2007a;Olivier et al., 2008b). Both *d*-fenfluramine-induced hypothermia and [^{3}H]citalopram binding to brain slices are completely absent in $SERT^{-/-}$ rats (Homberg et al., 2007a).
Body temperature is not only affected by pharmacological agents, but also increases when an organism is confronted with a stressor. This relatively short-lasting increase in body temperature is generally referred to as stress-induced hyperthermia (Friedman and Thayer, 1998;Nijsen et al., 1998a;Olivier et al., 1998;Bouwknecht et al., 2000) and is thought to be important for survival as preparation for fight-or-flight. Different psychological and physical stressors induce stress-induced hyperthermia, for example taking an exam (Marazziti et al., 1992) or attending a sport contest (Renbourn, 1960). In animals, a mild disturbance (Bouwknecht et al., 2001), an injection (Olivier et al., 2003), a clean cage (Spooren et al., 2002;Groenink et al., 2003), predator smell (Rorick-Kehn et al., 2005), or restraint stress (Terlouw et al., 1996) induces hyperthermia. The reproducible and robust stress-induced hyperthermia effects, combined with the relative ease of testing, makes the stress-induced hyperthermia paradigm very suitable for drug screening. Rats that lack the SERT display enhanced anxiety-like behaviour in an open field, an elevated plus maze, a novelty suppressed feeding test and an escape latency test (Olivier et al., 2008b). As a result of the changes in the serotonergic system of $SERT^{-/-}$ rats it is likely that adaptations have taken place outside the serotonergic system. GABA is known to have a negative influence on the firing of serotoninergic neurons (Gallager and Aghajanian, 1976;Ferraro et al., 1996), and $GABA_A$ receptors have been shown to mediate this inhibitory effect (Colmers and Williams, 1988;Pan et al., 1989). Moreover, the 5-HT system strongly interacts with the dopaminergic (DA) and the noradrenergic (NE) system (Korsgaard et al., 1985;Benloucif et al., 1993;Iyer and Bradberry, 1996;Salomon et al., 2006;Guiard et al., 2008;Olivier et al., 2008b), raising the possibility that consititutive increased

extraneuronal 5-HT levels are compensated by adaptations in these catecholaminergic systems. The finding that application of the NE transporter (NET) blocker desimipramine, but not the DA transporter (DAT) blocker GBR12909 abolished residual [^{3}H]5-HT uptake in the hippocampus of SERT$^{-/-}$ rats, suggests that 5-HT uptake is partially maintained by noradrenergic neurons in this region (Homberg et al., 2007a). In dopamine rich regions the DAT could be responsible for residual 5-HT uptake, as has been reported in the substantia nigra and VTA of SERT$^{-/-}$ mice (Pan et al., 2001;Zhou et al., 2002). Taken all these findings together, a life-long disturbed 5-HT neurotransmission may affect the sensitivity of the GABA-ergic, noradrenergic and/or the dopaminergic system. Brain catecholamines are involved in thermoregulation; however, no differences were found in the core body temperature of SERT$^{-/-}$ rats compared to SERT$^{+/+}$ rats (Olivier et al., 2008a), indicating that adaptations have taken place with respect to the body temperature. We therefore investigated the pharmacological effects of the GABA$_A$ receptor agonist chlordiazepoxide, the NET blocker atomoxetine, the DAT blocker GBR12909, and the NET + DAT blocker bupropion in SERT$^{+/+}$ and SERT$^{-/-}$ rats on body temperature to study putative sensitivity differences in these genotypes.

Experimental procedures

Subjects

Serotonin transporter knockout rats (Slc6a4^{1Hubr}) were generated by ENU-induced mutagenesis (Smits et al., 2004;Smits et al., 2006). All subjects have been bred and reared in the Central Animal Laboratory of the Radboud University of Nijmegen, the Netherlands. Experimental animals were derived from crossing heterozygous SERT knockout rats that were out crossed for 5 generations. In all experiments, male SERT$^{+/+}$ and SERT$^{-/-}$ littermates were compared. After weaning at the age of 21 days, ear cuts were taken for genotyping at the Hubrecht Institute (Utrecht, the Netherlands). All animals were housed individually in standard Macrolon® type 3 cages (42 x 26 x 20 cm) in temperature-controlled rooms (21°C ± 1) with 12/12-h day/night-cycle (lights on at 7.00 am) and food and water available *ad libitum*. Three groups of animals were used. The first group (4-5 months old; SERT$^{+/+}$ n=8, SERT$^{-/-}$ n=8) was used for measuring the response to different doses of the GABA$_A$ receptor agonist chlordiazepoxide, the second group (4-6 months old; SERT$^{+/+}$ n=8, SERT$^{-/-}$ n=9) was used for measuring different doses of the NET blocker atomoxetine, and the third group (6-8 months old: SERT$^{+/+}$ n=7, SERT$^{-/-}$ n=9) for the effects of the DAT blocker GBR12909 and the DAT + NET blocker bupropion. Only in this latter group the two drugs were given in a random order in the same group of animals. All experiments were carried out in accordance with institutional, national and international guidelines for animal care and the Dutch law concerning animal welfare.

Surgery

The telemetry ECG transmitter was chronically implanted following a surgical procedure that was described before (Sgoifo et al., 1996;Olivier et al., 2008a). Briefly, rats were deeply anesthetized with a mixture of N_2O/O_2 (1:2) and isoflurane (2.5%; Rhodia Organique Fine limited, Bristol, United Kingdom). ECG transmitters (Data Sciences International™, type TA11CTA-F40, St. Paul, MN, USA) were implanted in the abdominal cavity with one electrode subcutaneously placed on the muscle of the lower right limb of the animal, and the other electrode on the muscle bundle of the left shoulder. After surgery, animals were isolated and allowed to recover for 14 days; rats were checked daily during that period.

Radio-telemetry system

The radiotelemetry system consisted of an implantable transmitter (model TA11CTA-F40) with two flexible leads, a telemetric receiver (model RPC-1 and RLA1020), a Data Exchange Matrix collecting input from the receivers and an in-line analog ECG adaptor, all purchased from Data Sciences International™ (St. Paul, MN, USA). The matrix was connected to an IBM Compaq 486/66 computer. Signals from the transmitters were passed on *via* a radio signal to the receiver, localized under the animal cage, transforming it into a digital signal. Digital information from the telemetry receivers was collected by the datamatrix and provided to the computer. Raw data were collected and analyzed by the software package Dataquest A.R.T. version 3.11 (Data Sciences International™, St. Paul, MN, USA).

Experimental protocol radiotelemetric studies

Data of all animals were sampled every 5 minutes, collecting body temperature (°C). Pharmacological effects of chlordiazepoxide, atomoxetine, GBR12909 and bupropion were measured on core body temperature and stress-induced hyperthermia in $SERT^{-/-}$ and $SERT^{+/+}$ rats. Sampling data of the rats started at 18:00 hours the day prior to the administration of an intraperitoneal injection (vehicle, or the drug) to obtain undisturbed baseline values. The injection was used as a stressor (Olivier et al., 2003). All doses of chlordiazepoxide (0.0, 2.5, 5.0 and 10.0 mg/kg), atomoxetine (0.0, 1.0 and 3.0 mg/kg), GBR12909 (0.0, 5.0 and 10.0 mg/kg), and bupropion (0.0, 7.5, 15.0 and 30.0 mg/kg) were randomly administered at one-week intervals to each rat (within-animal design). All injections were given at 11.00 AM.

Drugs

Atomoxetine-HCL (Strattera 40 mg, Koster Pharmacy Lelystad, The Netherlands) and chlordiazepoxide-HCL (Pharbita, Zaandam, the Netherlands) were kindly provided by the department of Psychopharmacology (Utrecht University, Utrecht, the Netherlands). GBR12909 dihydrochloride (vanoxerine= 1-(2-[bis(4-fluorophenyl)-[methoxy]ethyl)-4-(3-phenylpropyl) piperazine dihydrochloride) was derived from SIGMA-ALDRICH, (Steinheim, Germany). Bupropion-HCL (Zyban) was derived from GlaxoSmithKline (8037). Atomoxetine and bupropion were suspended in 0.5%/5% gelatin-mannitol (Pharmacy Utrecht University), GBR12909 was dissolved in 1% methylcellulose, further dilutions were dissolved in saline (0.9% NaCl), and chlordiazepoxide was dissolved in saline (0.9% NaCl). All drugs were intraperitoneally injected in a volume of 1 ml/kg.

Data analysis

The pharmacological effect of the drugs on the core body temperature was measured in 5-minute intervals from 1 hour before the injection till 4 hours after the injection. For the stress-induced hyperthermia, data from one hour before the injection were averaged to one measurement (baseline). This baseline was subtracted from the temperature measured 30 minutes (peak response) after the injection (T_1). Thus, stress-induced hyperthermia = T_1- baseline. The pharmacological effects on stress-induced hyperthermia were analyzed using a two-way ANOVA with genotype and dose as independent variables. Where appropriate, data were further analyzed by means of a LSD post-hoc test for doses. The area under the curve was calculated for three time periods: 1) from 65 minutes after injection till 120 minutes after the injection, 2) from 125 minutes after the injection till 180 minutes after the injection and 3) from 185 minutes after the injection till 240 minutes after the injection. The first hour includes the stress-induced

hyperthermia; therefore, the area under the curve was not calculated for this hour. The pharmacological effects on the area under the curve were analyzed using a two-way ANOVA with genotype and dose as independent variables. Where appropriate, data were further analyzed by means of a LSD post-hoc test for doses. The level of significance was set at $p<0.05$. All statistical analyses were performed using the Statistical Package for Social Sciences for Windows version 16.0 (SPSS, Chicago, IL, USA).

Results

$GABA_A$ receptor agonist chlordiazepoxide (CDP).

Overall, CDP decreased the body temperature of both $SERT^{+/+}$ (figure 1A) and $SERT^{-/-}$ rats (figure 1B).

Stress-induced hyperthermia. Figure 1C shows no differences between genotypes ($F_{(1,55)}$ =0.215; p=0.644) in the stress-induced hyperthermia. However, a dose-effect of CDP was found ($F_{(3,55)}$ =6.396; p=0.001). Rats treated with 10.0 mg/kg CDP showed a significantly smaller stress-induced hyperthermia than rats treated with the vehicle ($p<0.001$). Moreover, 10.0 mg/kg CDP reduced the stress-induced hyperthermia more than that elicited by 5.0 mg/kg ($p<0.05$) or 2.5 mg/kg ($p < 0.005$) CDP.

Core body temperature. A genotype difference was found in the periods from 65 minutes till 120 minutes after the injection ($F_{(1,54)}$=13.13; $p<0.001$), from 125 minutes till 180 minutes after injection ($F_{(1,54)}$=24.18; $p<0.001$) and from 185 minutes till 240 minutes after injection ($F_{(1,54)}$=28.33; $p<0.001$). Actually, the effects of CDP lasted longer in $SERT^{-/-}$ rats than in $SERT^{+/+}$ rats (figure 1D + E). Indeed, when the data were split into genotypes, there was among others a significant treatment effect in the period of 185 till 240 minutes in $SERT^{-/-}$ rats (F(3,27)

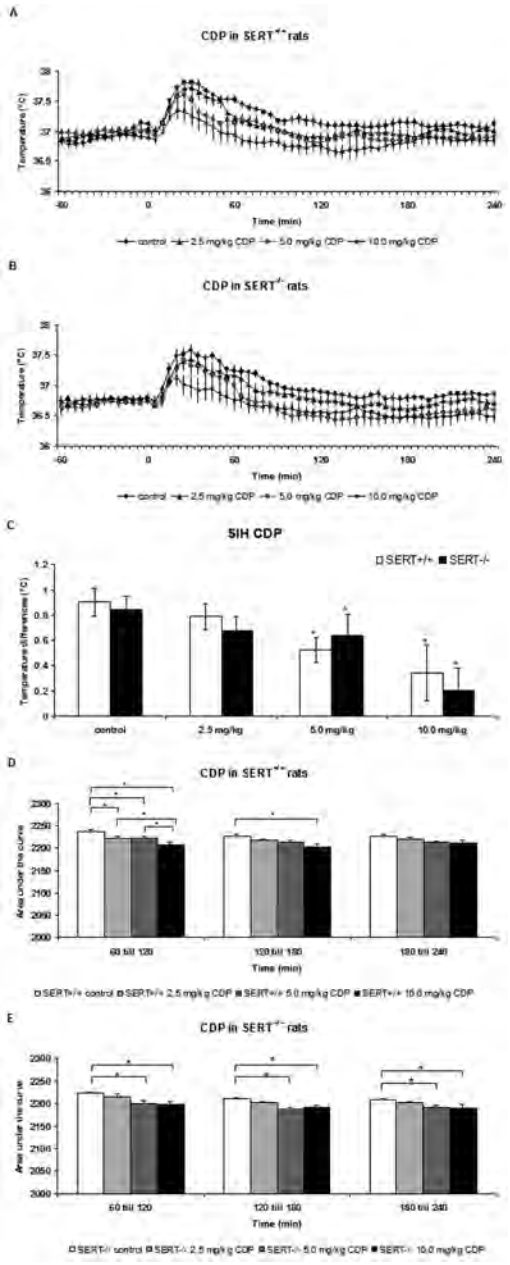

Figure 1

Effect of $GABA_A$ ligand chlordiazepoxide on body temperature in $SERT^{+/+}$ **(A)** and $SERT^{-/-}$ **(B)** rats. Temperature is plotted in 5-min periods starting 60 min before injection of chlordiazepoxide. Effect of $GABA_A$ ligand chlordiazepoxide on the stress-induced hyperthermia (**C**: * significant different from saline group) and on the area under the curve in $SERT^{+/+}$ (**D**) and $SERT^{-/-}$ rats (E). * significant difference ($p<0.05$).

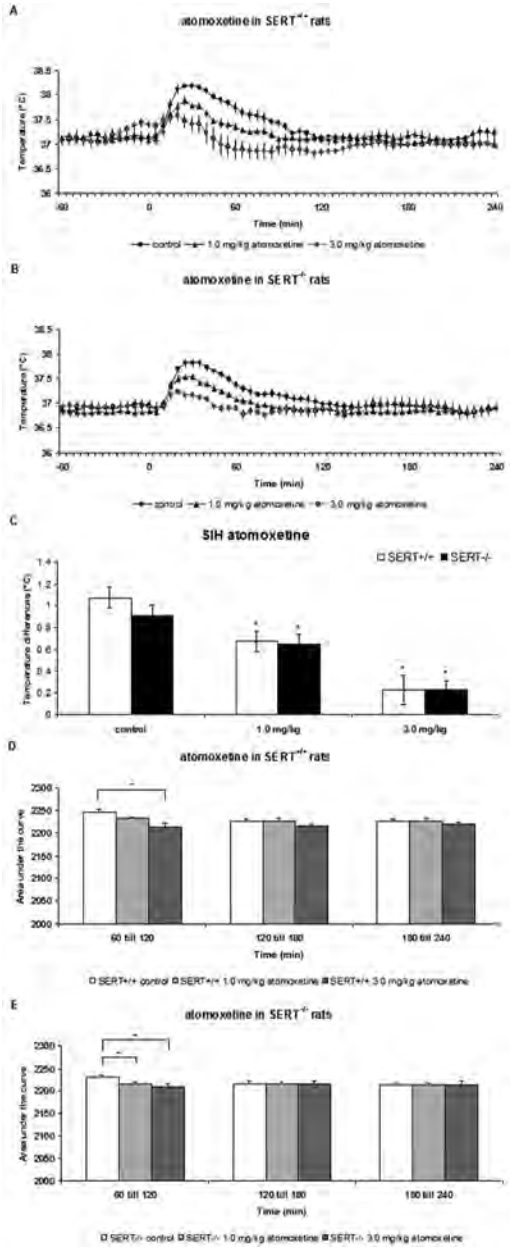

Figure 2
Effect of NET blocker atomoxetine on body temperature in SERT$^{+/+}$ **(A)** and SERT$^{-/-}$ **(B)** rats. Temperature is plotted in 5-min periods starting 60 min before injection of atomoxetine. Effect of NET blocker atomoxetine on the stress-induced hyperthermia (**C**: * significant different from saline group) and on the area under the curve in SERT$^{+/+}$ (**D**) and SERT$^{-/-}$ rats (**E**). * significant difference ($p<0.05$).

= 3.44; $p < 0.05$), but not the SERT$^{+/+}$ rats. The LSD posthoc test showed that the temperature of SERT$^{-/-}$ rats treated with 5.0 and 10.0 mg/kg CDP still significantly differed from that measured in control SERT$^{-/-}$ rats treated with 0.0 mg/kg CDP in the period of 180 till 240 min after the injection (figure 1D; $p> 0.05$), whereas the temperature of SERT$^{+/+}$ rats treated with CDP (2.5 till 10.0 mg/kg) did not anymore differ from that measured in control SERT$^{+/+}$ rats treated with 0.0 mg/kg CDP during that time-interval (figure 1E; $p<0.05$). In the time period from 65 till 120 minutes the temperature of SERT$^{+/+}$ rats treated with CDP was lower from that measured in control SERT$^{+/+}$ rats treated with 0.0 mg/kg CDP during that time-interval ($F_{(3,27)}=7.58$; $p<0.001$). Doses from 2.5 mg/kg till 10.0 mg/kg CDP significantly lowered the temperature ($p>0.05$) of rats treated with CDP. Moreover, the temperature of SERT$^{+/+}$ rats treated with 10.0 mg/kg CDP was significant lower compared to SERT$^{+/+}$ rats treated with 2.5 mg/kg CDP and 5 mg/kg CDP ($p<0.05$). In the time period 125 minutes till 180 minutes after the injection another effect of CDP was found ($F_{(3,27)}=3.29$; $p<0.05$), temperature of SERT$^{+/+}$ rats which were treated with 10.0 mg/kg CDP was significant lower compared to SERT$^{+/+}$ rats which were treated with 0.0 mg/kg CDP ($p<0.05$).
In SERT$^{-/-}$ rats (figure 1E) similar effects were found compared to SERT$^{+/+}$ rats in the time period from 65 till 120 minutes ($F_{(3,27)}=4.40$; $p<0.01$) and from 125 till 180 minutes ($F_{(3,27)}=5.22$; $p<0.01$). In both time periods the temperature of SERT$^{-/-}$ rats which were treated with 5.0 mg/kg CDP and 10.0 mg/kg CDP were lower compared to SERT$^{-/-}$ rats treated with 0.0 mg/kg CDP ($p<0.05$).

NET blocker atomoxetine.
As shown in Figure 2, atomoxetine reduced the body temperature in both SERT$^{+/+}$ (figure 2A) and SERT$^{-/-}$ (figure 2B) rats.

Stress-induced hyperthermia. No genotype differences were found in the stress-induced

hyperthermia after treatment with atomoxetine ($F_{(1,46)}$=0.603; n.s.). As seen in Fig 2C, atomoxetine dose-dependently reduced the stress-induced hyperthermia ($F_{(2,46)}$=30.063; p=0.001) in both genotypes. Atomoxetine (1.0 mg/kg and 3.0 mg/kg (p<0.05)) significantly lowered the stress-induced hyperthermia when compared with rats treated with 0.0 mg/kg atomoxetine. Moreover, 3.0 mg/kg atomoxetine decreased the stress-induced hyperthermia significantly more than 1.0 mg/kg atomoxetine did.

Core body temperature. A genotype difference was found in the time period from 65 minutes till 120 minutes after the injection ($F_{(1,45)}$=6.22; p<0.05) and from 185 minutes till 240 minutes after injection ($F_{(1,45)}$=4.47; p<0.05). Figure 2 clearly shows that the effect of a lower dose atomoxetine on core body temperature was more effective in SERT$^{-/-}$ rats than in SERT$^{+/+}$ rats. When the data were split into genotypes, there was a significant treatment effect in the time period from 65 minutes till 120 minutes after the injection in both the SERT$^{-/-}$ rats ($F_{(2,24)}$=5.38; p<0.01) and SERT$^{+/+}$ rats ($F_{(2,21)}$=6.56; p<0.01). More importantly, the LSD posthoc test showed that a dose of 1.0 mg/kg atomoxetine already produced a significant decrease of the temperature in SERT$^{-/-}$ rats (figure 2E; p< 0.05), whereas a dose of 3.0 mg/kg atomoxetine was required to lower the temperature in SERT$^{+/+}$ rats (figure 2D; p< 0.05).

DAT blocker GBR12909

In figure 3 the effects of GBR12909 are shown. GBR12909 increased the body temperature, but only in SERT$^{-/-}$ rats. When the stress-induced hyperthermia effect waned around 60 minutes after the injection of GBR12909 in SERT$^{+/+}$ rats, the core body temperature remained high or, even increased in SERT$^{-/-}$ rats treated with this compound.

Stress-induced hyperthermia. No genotype ($F_{(1,35)}$=0.04; n.s.) or dose effect ($F_{(2,35)}$=2.42; n.s.) on the stress-induced hyperthermia was found after treatment with GBR12909 (figure 3C).

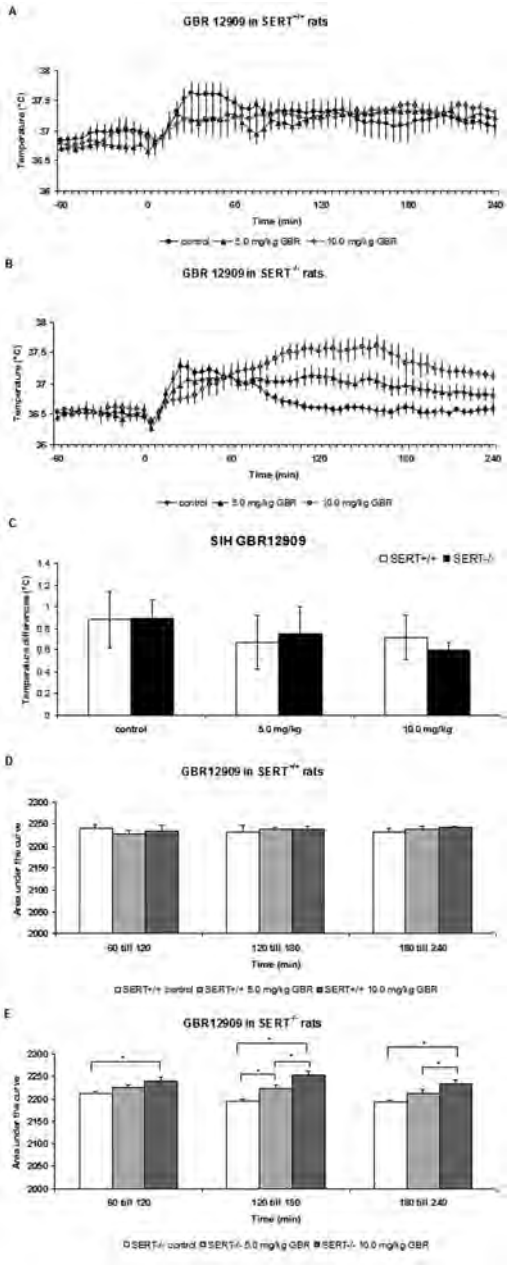

Figure 3
Effect of DAT blocker GBR12909 on body temperature in SERT$^{+/+}$ **(A)** and SERT$^{-/-}$ **(B)** rats. Temperature is plotted in 5-min periods starting 60 min before injection of GBR12909. Effect of DAT blocker GBR12909 on the stress-induced hyperthermia (**C**: * significant different from saline group) and on the area under the curve in SERT$^{+/+}$ (**D**) and SERT$^{-/-}$ rats (**E**). * significant difference (p<0.05).

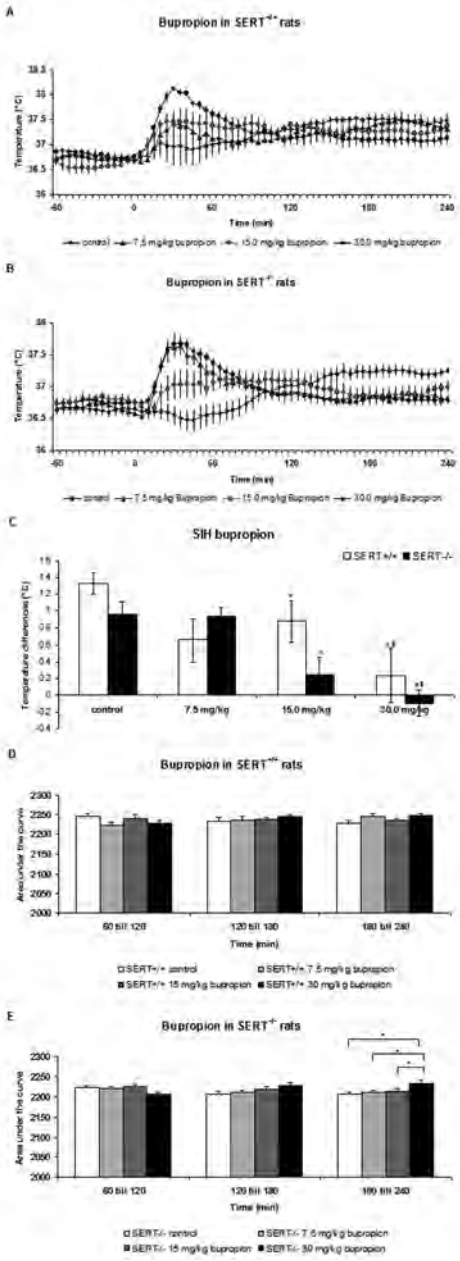

Figure 4
Effect of DAT + NET blocker bupropion on body temperature in SERT$^{+/+}$ **(A)** and SERT$^{-/-}$ **(B)** rats. Temperature is plotted in 5-min periods starting 60 min before injection of bupropion. Effect of DAT + NET blocker bupropion on the stress-induced hyperthermia (**C**: * significant different from saline group; $ significant different from the 15.0 mg/kg bupropion) and on the area under the curve in SERT$^{+/+}$ (**D**) and SERT$^{-/-}$ rats (**E**). * significant difference ($p<0.05$).

GBR12909 treated rats did not significantly differ at any time point from the vehicle treated rats; this held for both genotypes.

Core body temperature. A genotype difference was found in the time period from 185 minutes till 240 minutes after the injection ($F_{(1,35)}=14.03$; $p<0.001$). Figure 3 indeed shows that both doses of GBR12909 were still effective in SERT$^{-/-}$ rats, but not anymore in SERT$^{+/+}$ rats during that period.
Indeed, when the data were split into genotypes, only the SERT$^{-/-}$ rats, but not the SERT$^{+/+}$ rats, displayed a significant treatment effect during the period of 65 till 120 min after the injection ($F_{(2,17)}=5.12$; $p<0.05$), the period 125 till 180 min after the injection ($F_{(2,17)}=15.38$; $p<0.001$) and the period of 185 till 240 min after the injection ($F_{(2,17)}= 8.54$; $p<0.01$; figures 3D and E). The LSD posthoc tests showed that (a) during the period of 65 till 120 min after the injection the temperature of SERT$^{-/-}$ rats treated with 10.0 mg/kg GBR12909 was significantly different from SERT$^{-/-}$ rats treated with 0.0 mg/kg GBR12909, (b) during the period of 125 till 180 min after the injection the temperature of SERT$^{-/-}$ rats treated with 10.0 mg/kg GBR12909 was significantly higher than that measured in SERT$^{-/-}$ rats treated with 5.0 mg/kg and 0.0 mg/kg GBR12909 and that 5.0 mg/kg elicited a significantly higher temperature than that measured in SERT$^{-/-}$ rats treated with 0.0 mg/kg GBR12909, and that (c) during the period of 185 till 240 min after the injection the temperature of SERT$^{-/-}$ rats treated with 10.0 mg/kg GBR12909 was significantly higher than that measured in SERT$^{-/-}$ rats treated with 5.0 mg/kg or 1.0 mg/kg GBR12909.

DAT + NET blocker bupropion

Figure 4 shows the effects of bupropion in SERT$^{+/+}$ (Figure 4A) and SERT$^{-/-}$ (Figure 4B) rats. In SERT$^{-/-}$, but not SERT$^{+/+}$, rats there was a small hyperthermia found after the stress-induced hyperthermia effects waned at the highest dose of bupropion.

Stress-induced hyperthermia. No genotype effect was found after treatment with bupropion in the stress-induced hyperthermia paradigm (Figure 4C). Bupropion dose-dependently reduced the stress-induced hyperthermia in both genotypes ($F_{(3,56)}$ = 9.598; p<0.001). Both 15.0 mg/kg (p<0.006) and 30.0 mg/kg (p<0.001) bupropion significantly reduced stress-induced hyperthermia compared with vehicle treated rats. In addition, 30.0 mg/kg bupropion reduced stress-induced hyperthermia stronger than 15.0 mg/kg (p<0.022) and 7.5 mg/kg (p<0.001) bupropion. No genotype x dose interaction was found.

Core body temperature. A genotype difference was found in the time period from 65 minutes till 120 minutes after the injection ($F_{(1,56)}$=8.34; p<0.01), from 125 minutes till 180 minutes after injection ($F_{(1,56)}$=19.05; p<0.001) and from 185 minutes till 240 minutes after injection ($F_{(1,56)}$=28.44; p<0.001). Figure 4 shows that a hyperthermia was only found in $SERT^{-/-}$ rats after treatment with bupropion. When the data were split into genotypes, different effects were found. In $SERT^{+/+}$ rats no differences were found in the core body temperature. Interestingly, in $SERT^{-/-}$ rats a difference was found in the time period from 185 minutes till 240 minutes after the injection ($F_{(3,33)}$= 4.81; p<0.01). A hyperthermia was found after treatment with 30.0 mg/kg bupropion (p<0.01) compared to $SERT^{-/-}$ rats treated with 0.0 mg/kg bupropion, but also compared to $SERT^{-/-}$ rats treated with 7.5 mg/kg bupropion (p<0.01) and compared to $SERT^{-/-}$ rats treated with 15.0 mg/kg bupropion (p<0.01).

Discussion

The SERT regulates the serotonergic neurotransmission and is therefore the primary modulator for 5-HT in the CNS. In the present study we pharmacologically manipulated GABA-ergic, dopaminergic and noradrenergic systems and measured the drug-induced changes in the core body temperature and stress-induced hyperthermia in rats lacking constitutionally the SERT. The serotonergic system shows drastic changes upon genetic manipulation of the SERT, both in rats and mice (see for an extensive discussion on $SERT^{-/-}$ mice and rats: Murphy and Lesch, 2008 & Olivier et al., *in press*). Previous results in rats have shown strongly increased extracellular 5-HT (Homberg et al., 2007a) and changed 5-HT_{1A} receptor functioning (Homberg et al., 2008;Olivier et al., 2008a). Besides adaptive changes in the 5-HT system it is likely that adaptations in other neurotransmitters systems are also present (see Introduction). Our experiments using radiotelemetric measurements of body temperature showed that CDP decreased the body temperature in both $SERT^{+/+}$ and $SERT^{-/-}$ rats; however, the effects seemed to last longer in $SERT^{-/-}$ rats than in $SERT^{+/+}$ rats. Our results also showed that $SERT^{-/-}$ rats were slightly more sensitive to blockade of the NET than $SERT^{+/+}$ rats, since both doses reduced the body temperature, compared to only the highest dose in $SERT^{+/+}$ rats. Moreover, blocking the DAT showed a stronger hyperthermic response in $SERT^{-/-}$ rats compared to $SERT^{+/+}$ rats. Combined blockade of both NET and DAT resulted in a stronger effect in the $SERT^{-/-}$ rats compared to the $SERT^{+/+}$ rats.

$GABA_A$ receptors play an important role in autonomic stress and anxiety responses, and $GABA_A$ receptor agonists dose-dependently reduce stress-induced hyperthermia across species (Olivier et al., 2002;Adriaan Bouwknecht et al., 2007). This effect was also found in $SERT^{+/+}$ and $SERT^{-/-}$ rats; CDP dose-dependently and similarly reduced the stress-induced hyperthermia in both genotypes. Further, CDP dose-dependently reduced the body temperature in both genotypes; however, the

effect lasted longer in SERT$^{-/-}$ rats, suggesting an adaptation in this system. Chlordiazepoxide, a non-subunit selective $GABA_A$ receptor agonist reduces stress-induced hyperthermia in mice and rats probably via activation of α_2 and/or α_3 subunits (van Bogaert et al., 2006b), whereas (at higher doses) core body temperature is decreased via activation of α_1 subunits (van Bogaert et al., 2006b). It can be concluded that the absence of SERT does not lead to substantial changes in these $GABA_A$ subunits. Still, other GABAergic receptors than the $GABA_A$ receptor remains to be investigated. In SERT$^{-/-}$ mice for example, $GABA_B$ receptors, but not $GABA_A$ receptors, are functionally desensitized in the DRN (Mannoury la et al., 2004). This might also be the case in other parts of the brain, including parts involved with core bodytemperature.

5-HT strongly interacts with other neurotransmitter systems, in particular the DA and the NE system (Korsgaard et al., 1985;Benloucif et al., 1993;Iyer and Bradberry, 1996;Salomon et al., 2006). SSRIs are known to inhibit the spontaneous firing rate of noradrenergic neurons in the locus coeruleus (Szabo et al., 2000;Seager et al., 2004;Dremencov et al., 2007), and decrease the firing rate of dopaminergic neurons in the ventral tegmental area (Prisco and Esposito, 1995;Di et al., 1998). Although the SERT$^{-/-}$ rat is no longer able to take up 5-HT into the presynaptic neuron through the SERT, alternative routes by which 5-HT is taken up in the SERT$^{-/-}$ rat seem to exist. Hippocampal synaptosomes of SERT$^{-/-}$ rats take up 5-HT, which can be blocked by NET + DAT blockade, but not by DAT blockade alone suggesting that NETs take over the role of SERTs (Homberg et al., 2007a).

Brain catecholamines are involved in thermoregulation, but NET inhibitors have not been tested before in the stress-induced hyperthermia paradigm. When the NET was blocked with atomoxetine, SERT$^{-/-}$ rats appeared slightly more sensitive compared to SERT$^{+/+}$ rats. The highest dose (3.0 mg/kg) significantly lowered the body temperature in both SERT$^{+/+}$ and SERT$^{-/-}$ rats, but in SERT$^{-/-}$ rats the effect was also apparent with 1.0 mg/kg atomoxetine. This indicates some mild adaptation of the NE system where SERT$^{-/-}$ rats seem to have a little more activated NET system compared to SERT$^{+/+}$ rats. Upregulation of the NET seems a logical explanation for more 5-HT uptake, however the NET concentration is similar to SERT$^{+/+}$ rats (Homberg et al., 2007a). It has been reported that in rats intrahypothalamic injection of NE decreases body temperature in rats (Cox and Lee, 1980). In addition, i.p. injection of 0.5 mg/kg NE lowered body temperature in rats for three hours, whereas in mice an i.p. injection of 1.0 mg/kg lowered body temperature for 30 minutes (Clark and Lipton, 1986). Thus NE lowers the stress-induced hyperthermia and the core body temperature , with a milder effect in SERT$^{+/+}$ rats compared to SERT$^{-/-}$ rats.

In the present study, DA influenced the body temperature in the SERT$^{-/-}$ rats, but not in SERT$^{+/+}$ rats where GBR12909 had no consistent effects, neither on stress-induced hyperthermia nor on body temperature. The GBR12909-induced blockade of the DAT increased the body temperature in SERT$^{-/-}$ rats. It has been reported that intrahypothalamic injection of DA decreases body temperature in rats (Cox and Lee, 1980). Moreover, ethanol, that interacts amongst others with the DA system, produces no differences in hypothermia between SERT$^{+/+}$ and SERT$^{-/-}$ mice (Boyce-Rustay et al., 2006;Fox et al., 2007). The hyperthermic effects found in SERT$^{-/-}$ rats suggest that the SERT$^{-/-}$ rats are more sensitive to blockade of the DAT compared to SERT$^{+/+}$ rats, and that the effects are opposite to the hypothermic effects seen in mice after ethanol, and to the hypothermic effects of intrahypothalamic injected DA in rats, indicating a shifted sensitivity to DA in SERT$^{-/-}$ rats Because the DAT concentrations are similar in SERT$^{+/+}$ and SERT$^{-/-}$ rats (Homberg et al., 2007a),

these results suggest a higher activation of the DAT in SERT$^{-/-}$ rats. A possibility is that in the SERT$^{-/-}$ rats the DATs take up DA and 5-HT, and with blockade of the DAT levels of DA and 5-HT increase, causing a hyperthermia in these rats.

Bupropion is a weak monoamine reuptake inhibitor that shows selectivity for NET and DAT but has no effect on SERT (Hyttel, 1982;Richelson and Pfenning, 1984;Ascher et al., 1995). Administration of bupropion caused differential effects on body temperature of SERT$^{+/+}$ and SERT$^{-/-}$ rats. As mentioned above SERT$^{-/-}$ rats appeared more vulnerable for blockade of DAT compared to SERT$^{+/+}$ rats. After treatment with bupropion, some hyperthermia was also seen in SERT$^{-/-}$ rats at the highest dose used. These findings support the involvement of the DAT in the 5-HT uptake in SERT$^{-/-}$ rats. Only when the DAT is blocked (in the SERT$^{-/-}$ rats) this effect is found. The non-selective inhibitor bupropion has been tested before on body temperature in rats. Hasegawa et al. (2005) have shown that body temperature is increased after administration of bupropion and that, using *in vivo* microdialysis, the increase of NE was higher compared to DA in the hypothalamus (preoptic area and anterior hypothalamus) after administration of bupropion (Hasegawa et al., 2005). It is likely that 5-HT uptake in the SERT$^{-/-}$ rat is taken over by different transporters in different brain areas, depending presumably on the relative density of the re-uptake mechanisms. The 5-HT uptake of SERT$^{-/-}$ rats in the brain area involved with temperature regulation (probably hypothalamus) might be regulated by the DAT, while in the hippocampus, a brain area not important for core body temperature regulation, the 5-HT uptake may be mainly regulated by the NET (Homberg et al., 2007a). Reasoning along this line, the increase in body temperature after GBR12909 and bupropion could then be explained by blockade of the DAT in the brain area involved in the regulation of body temperature, thereby blocking the 5-HT uptake via the DAT and, subsequently, increasing the extracellular 5-HT in this brain area. However, it is well known that an increase of extracellular 5-HT normally leads to hypothermia. Drugs like the 5-HT releaser d-fenfluramine (Cryan et al., 2000) and the SSRI citalopram (Oerther and Ahlenius, 2001) are known to reduce core body temperature in rats, although d-fenfluramine induced hypothermia is reduced in SERT$^{-/-}$ (Homberg et al., 2007a). One possible explanation for this apparent discrepancy may lie in the fact that the extracellular 5-HT in SERT$^{-/-}$ rats is in the vicinity of the DAT and not the SERT and hence may induce an anomalous effect. Alternatively, the possibility exists that compensatory changes may also have occurred in the uptake of DA as well. Previous *in vitro* studies in the SERT$^{-/-}$ rats, however, have suggested that the consequences of the absence of SERT are limited to the serotonergic system and do not result in compensations of non-serotonergic neurons (Homberg et al., 2007a)

For the GABA system, uptake and depolarization-induced release in superfused brain slices in SERT$^{-/-}$ rats is not different from SERT$^{+/+}$ rats. Here we showed that the $GABA_A$ system, at least the response to $GABA_A$ receptor agonist in the stress-induced hyperthermia paradigm, was not affected in SERT$^{-/-}$ rats. However, the core body temperature was slightly changed. Blockade of the DAT, NET, or both, increases levels of DA, NE, or both respectively. Increasing the neurotransmitter levels will have an impact mainly on the postsynaptic neurons. In SERT$^{-/-}$ rats the NET and DAT concentrations are not significantly changed (Homberg et al., 2007a). Furthermore, [^{3}H]NE and [^{3}H]DA uptake and their depolarization-induced release in superfused brain slices are not different between SERT$^{+/+}$ and SERT$^{-/-}$ rats (Homberg et al., 2007a). Moreover, DA and NE tissue levels are similar in both genotypes (Homberg et al., 2007a). Therefore, we can conclude that basal NE and DA levels are similar in these rats, at least *in vitro*. However, the sensitivity of the DA and NE

system *in vivo* is changed, as shown by the change in sensitivity of the transporter blockers in the regulation of body temperature. It is possible that regional difference exist in the brain for the sensitivity of the DA and NE system. In conclusion, the SERT$^{-/-}$ rat shows adaptive changes in the catecholaminergic system. These changes might be shared with humans carrying the short allele of the serotonin transporter promoter length polymorphism, making the SERT$^{-/-}$ rat a good *in vivo* model to investigate targets for treatment in human psychiatric disorders.

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7

General discussion

Serotonin transporter knockout rats

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Introduction

Currently, ENU mutagenesis is the only approach to generate genetic knockouts in the rat (Zan et al., 2003;Smits et al., 2004;Smits et al., 2006). Using this method, we developed a rat with a premature stopcodon in the SERT gene (TGC > TGA transversion at position 3924 in the third exon). Since this technique will most likely lead to additional, unwanted mutations, rats carrying the SERT gene premature stopcodon were first outcrossed on commercially available wildtype rats (Harlan, The Netherlands) background for at least five generations before the first experiments began. This procedure led to a homozygous SERT knockout rat line and at the same time the risk for potential additional mutations was reduced. Unwanted mutations may seem to be a disadvantage of this rat knockout approach, but thus far target-selected mutagenesis is the only method available to generate knockout rats. Because the phenotypes of the SERT knockout rat described in this thesis are stable across generations and laboratories, and several phenotypes are concurrent with literature findings and SERT knockout mouse phenotypes, it is assumed that any phenotype in the knockout rat is most likely attributable to the absence of the SERT and not other induced mutations.

The aim of the present thesis was to phenotypically characterize the SERT knockout rat by studying a variety of behavioral paradigms and measuring pharmacological and physiological parameters. In this chapter, the results obtained in the various studies underlying this thesis are briefly summarized and discussed.

Confirmation of the SERT knockout rat at the molecular level

The nature of non-transgenic ENU-induced gene knockouts is fundamentally different from the homologous recombination method commonly used to generate mouse knockouts. In the latter models, genomic fragments encoding important protein domains or the complete protein are replaced by a selection cassette, whereas the SERT knockout rat presented here results from a single point mutation that introduces a premature stopcodon. It is theoretically possible that, due to an alternatively spliced transcript, the normal SERT protein is nonetheless produced; however, Northern blot analysis (**chapter 2**) and sequencing of RT-PCR products (unpublished observations) revealed that the SERT transcript is almost completely absent in $SERT^{-/-}$ rats. This is most likely due to a process called 'nonsense mediated decay' (Baker and Parker, 2004), a mechanism by which nonsense transcripts are degraded. The expression level of the SERT transcript in $SERT^{+/-}$ rats is intermediate to that of $SERT^{-/-}$ and $SERT^{+/+}$ rats. Autoradiography, using the tritium labeled form of the highly selective serotonin reuptake inhibitor (SSRI) citalopram, revealed that SERT binding was completely absent in $SERT^{-/-}$ rats (**chapter 2**). In $SERT^{+/-}$ rats, SERT binding was reduced by approximately 40% as compared to $SERT^{+/+}$ littermates (**chapter 2**). At the functional level, *d*-fenfluramine-induced hypothermia, which can be blocked by citalopram (unpublished observations), was completely absent in $SERT^{-/-}$ rats, and reduced in $SERT^{+/-}$ rats relative to $SERT^{+/+}$ littermates (**chapter 2**). Finally, the *in vitro* maximum rate (V_{max}) of [^{3}H]5-HT uptake in synaptosomes prepared from the hippocampus was reduced by 13.4 % in $SERT^{+/-}$ rats, and by 72.2 % in $SERT^{-/-}$ rats (**chapter 2**). These data strongly argue in favor of the hypothesis that the premature stopcodon in the SERT gene resulted in a full knockout of the SERT. Furthermore, the $SERT^{+/-}$ rat studies showed that there was a gene-dose effect for most molecular and neurochemical findings.

General appearance of the SERT knockout rat

Despite the important role of 5-HT in the development of the nervous system (Lauder, 1990), $SERT^{-/-}$ and $SERT^{+/-}$ knockout rats appear normal, and score similarly to $SERT^{+/+}$ littermates on measures of health and neurological functions. This has been shown by using an adapted SHIRPA protocol. The SHIRPA protocol has been developed to characterize the phenotype of transgenic and knockout mice (Roger et al., 1997). Several components of this protocol have been adapted to evaluate our rat subjects, including behaviors in the viewing Jar (body position, spontaneous activity, respiration rate, and tremor), behavior in an open arena (transfer arousal, locomotor activity, palpebral closure, piloerection, startle response, gait, pelvic elevation, tail elevation, touch escape, and positional passivity), behaviors recorded on or above the arena (trunk curl, limb grasping, visual placing, grip strength, body tone, pinna reflex, corneal reflex, toe pinch, and wire movement) and, finally, physiological conditions and behaviors were recorded during supine restraint (skin color, heart rate, limb tone, abdominal tone, lacrimation, salivation, prevoked biting, righting reflex, contact righting reflex, negative geotaxis, fear, irritability, aggression, vocalization and body temperature). Experimenters performing the SHIRPA protocol were blind to the genotype of the animals. No differences were found in the general appearance of male $SERT^{+/+}$ and $SERT^{-/-}$ rats (unpublished observations); however, the body weight of female $SERT^{-/-}$ rats was reduced by 10% from the age of 3 weeks compared to female $SERT^{+/+}$ rats, while the weight of male $SERT^{-/-}$ rats was not different from $SERT^{+/+}$ rats (**chapter 2**).

To generate experimental animals, $SERT^{+/-}$ x $SERT^{+/-}$ crossings are maintained, with litter sizes of 10 on average and a Mendelian distribution of $^{+/+}$, $^{-/-}$ and $^{+/-}$ alleles. After 8-10 generations of outcrossings, it is ideal to choose for $SERT^{-/-}$ x $SERT^{-/-}$ and $SERT^{+/+}$ x $SERT^{+/+}$ crossings to maintain the line and producing proper control animals, although the reproductivity may be slightly reduced in $SERT^{-/-}$ x $SERT^{-/-}$ crossings (unpublished observations); however, one should realize that the genetic background of $SERT^{-/-}$ rats and control $SERT^{+/+}$ rats may differ in a more systematic way than when using littermates as controls, which may confound measurements.

Serotonin homeostasis

Throughout development, neuroplastic events are likely to have taken place which compensate for the lifelong reduced 5-HT uptake in $SERT^{+/-}$ and $SERT^{-/-}$ rats. An overview of the possible adaptations, and consequences of the absence of SERT, that have been studied in the $SERT^{-/-}$ rats are listed in table 1. The most obvious consequence of the absence of the SERT is that extra-neuronal 5-HT levels were strongly increased in both male and female $SERT^{-/-}$ rats, as measured by microdialysis with the dialysis probe located in the hippocampus (**chapter 2 and 3**), a strongly serotonergic innervated region of interest for many types of behavior.
Compensatory adaptations are likely to have taken place to enable the organism to 'cope' with this extreme condition. Presynaptically, the serotonergic system would be expected to be 'silenced', as the serotonergic tone is already high. However, tryptophan hydroxylase (5-HT synthesis) and monoamine oxidase (MAO-A; 5-HT degradation) enzymatic activity were not different between genotypes across several brain regions, including the raphe nuclei. These findings were supported by the absence of genotype differences in tryptophan hydroxylase immunoreactivity in the dorsal raphe nuclei (**chapter 2**). While the 5-HT synthesis and degradation machinery seem to function

normally, 5-HT tissue levels were reduced by approximately 55-75% and 5-hydroxyindoleacetic acid (5-HIAA) levels by approximately 45-50% in various brain regions (**chapter 2**; Homberg et al., 2007b). We assume that 5-HT tissue levels predominantly reflect intracellular 5-HT content, suggesting that intracellular 5-HT levels were decreased in neurons of SERT$^{-/-}$ rats, while extracellular 5-HT levels were increased. If less 5-HT is available for release and 5-HT release will have little effect on neurotransmission processes because of the high endogenous 5-HT tone, it is expected that 5-HT release would be attenuated. That is exactly what has been found for electrically-evoked Ca^{2+}-dependent 5-HT release in superfused brain slices (**chapter 2**). Together, the findings of substantially reduced 5-HT uptake and release as well as reduced 5-HT tissue levels indicate that 5-HT recycling might be attenuated in SERT$^{-/-}$ rats, which further implies that the serotonergic system has lost its dynamics and flexibility. Thus, it is tempting to suggest that 5-HT homeostasis may be changed in such a way that the animal is able to function normally under basal conditions, but when exposed to challenges or stimuli, this system may not be able to adapt appropriately, resulting in aberrant behavioral responses.

Interestingly, 5-HT levels in CSF were significantly reduced and 5-HT turnover (as reflected by the 5-HIAA/5-HT ratio) in CSF was increased in SERT$^{-/-}$ knockout rats (Homberg et al., 2007b). Because selected subsets of serotonergic dorsal raphe neurons project to the ependymal wall of the ventricular system in the rat (Simpson et al., 1998), CSF 5-HIAA and 5-HT levels may particularly reflect 5-HT homeostasis processes in the raphe nuclei. Otherwise, the CSF may collect the freely diffusible 5-HIAA from diverse brain regions. Overall, the CSF measurements suggest that 5-HT turnover is increased in SERT$^{-/-}$ rats, which is in line with the increased serotonergic tone in this mutant rat model (**chapter 2 and 3**).

Despite a 40% reduction in SERT binding (**chapter 2**), there were no differences in 5-HT tissue levels and 5-HT release in SERT$^{+/-}$ rats, probably because 5-HT uptake was only slightly reduced (**chapter 2**) and extraneuronal 5-HT levels are only slightly increased (unpublished observations) in these animals compared to SERT$^{+/+}$ rats. Hence, there may be at least a redundancy of 40-50% in SERT availability.

5-HT uptake in hippocampal synaptosomes was strongly decreased in SERT$^{-/-}$ rats, but not completely absent, which suggests that there are alternative routes by which 5-HT can be taken up. The monoamine transporters are neuron-specific, but not neurotransmitter-specific, and have low affinity and reuptake capacity for monoamines that do not match the monoamine transporter. Under extreme conditions, e.g. high levels of monoamines, the high affinity 'monoamine-own' uptake process will be overruled by the abundancy of other monoamines, resulting in the uptake of 'false' neurotransmitters (Vizi et al., 2004). The hippocampus is noradrenaline enriched. In this region, combined dopamine (DAT) and noradrenaline (NET) transporter blockade, but not DAT blockade alone, prevented the reuptake of residual 5-HT in SERT$^{-/-}$ rats (**chapter 2**), suggesting that the NET is responsible for residual 5-HT uptake in the hippocampus of SERT$^{-/-}$ rats. In dopamine-rich regions, e.g. the striatum, it is expected that residual 5-HT uptake will take place via the DAT, as has been reported in SERT$^{-/-}$ mice (Zhou et al., 2002). Due to false 5-HT uptake, DAT or NET blockade in SERT$^{-/-}$ models will not only result in increases in extracellular DA or NE levels, but also 5-HT levels (Shen et al., 2004).

The 5-HT system interacts with several other (neurotransmitter) systems and, secondary to the changes in 5-HT homeostasis in the knockout rat, these systems may adapt as well. The

Table 1. An overview of serotonin homeostasis and adaptive presynaptic processes tested in $SERT^{-/-}$ rats. All results are indicated as $SERT^{-/-}$ rat versus $SERT^{+/+}$ rat.

Measurement	Tissue	Method	Results $SERT^{-/-}$ rats versus $SERT^{+/+}$ rats
Extra-neuronal 5-HT levels	hippocampus	Microdialyses	Nine-fold higher
Tryptophan hydroxylase	Several brain regions	Enzymatic reactivity	No difference
Tryptophan Hydroxyase	DRN	Immunoreactivity	No difference
MAO-A activity	Various brain regions	Enzymatic reactivity	No difference
5-HT tissue levels	Various brain areas	HPLC	55-75% reduced
5-HIAA tissue levels	Various brain areas	HPLC	45-50% reduced
Ca^{2+} dependent 5-HT release	Superfused brain slices	Superfused electrically evoked [^{3}H]5-HT release	Reduced
5-HT levels	CSF	HPLC	Reduced
5-HIAA/5-HT ratio	CSF	HPLC	Increased
5-HT uptake with DAT blocker	Hippocampal synaptosomes	[^{3}H]5-HT uptake	Residual 5-HT uptake in $SERT^{-/-}$ rats
5-HT uptake with DAT and NET blocker	Hippocampal synaptosomes	[^{3}H]5-HT uptake	Residual uptake abolished in $SERT^{-/-}$ rats
Ca^{2+} dependent DA release	Various brain areas	Superfused electrically evoked [^{3}H]DA-HT release	No difference
Ca^{2+} dependent NE release	Various brain areas	Superfused electrically evoked [^{3}H]NE-HT release	No difference
SERT binding	Various brain areas	Autoradiography	Completely absent
DAT binding	Various brain areas	Autoradiography	No difference
NET binding	Various brain areas	Autoradiography	No difference
DA tissue levels	Various brain areas	HPLC	No difference
NE tissue levels	Various brain areas	HPLC	Deceased in amygdala only
HVA tissue levels	Various brain areas	HPLC	Deceased in amygdala only
DOPAC tissue levels	Various brain areas	HPLC	No difference
Ca^{2+} dependent Ach, GABA, glutamate release	Various brain areas	Superfused electrically/4A-P evoked [^{3}H] neuro-transmitter release	No difference

presynaptic function of dopaminergic and noradrenergic neurons has been studied by measuring the electrically-evoked Ca^{2+}-dependent dopamine (DA) and noradrenaline (NE) release. Despite the high endogenous 5-HT tonus, no change was observed in DA and NE release (**chapter 2**). The absence of presynaptic adaptations in dopaminergic and noradrenergic neuron function was further supported by the lack of genotype differences in DAT and NET binding and tissue levels of DA, NE, and their metabolites HVA and DOPAC (**chapter 2**). The exception was NE levels in the amygdala, which were decreased in $SERT^{-/-}$ rats, along with HVA levels. Also, the reactivity of cholinergic, glutamatergic and GABAergic neurons was not changed in $SERT^{-/-}$ rats (**chapter 2**). Together, these *in vitro* data suggest that compensatory adaptations in response to the constitutive absence of the SERT are predominantly found in the serotonergic system; however, these data do not exclude changes in the responsiveness of the dopaminergic or noradrenergic system which can only be found *in vivo*. This observation makes the $SERT^{-/-}$ rat very valuable to study the role of the serotonergic system in any system of interest, although it should be kept in mind that, due to the lifelong increased 5-HT tonus, the $SERT^{-/-}$ condition is not directly comparable to the consequences of acute rises in 5-HT.

Pharmacological responses

5-HT_{1A} receptor agonists and antagonists

$SERT^{-/-}$ rats exhibit nine-fold increases in extra-neuronal 5-HT levels (**chapter 2 and 3**) that could potentially affect the functioning or responsivity of 5-HT receptors, as has been reported after chronic SSRI treatment (Blier et al., 1990;Briley and Moret, 1993). To address this issue, the effect of flesinoxan (5-HT_{1A} receptor agonist) on body temperature and stress-induced hyperthermia (SIH) have been measured, processes that are well known to be modulated by 5-HT_{1A} receptor ligands (Hjorth, 1985;Lesch, 1991;Millan et al., 1993;De, 1995;Friedman and Thayer, 1998;Olivier et al., 1998;Nijsen et al., 1998a;Nijsen et al., 1998b;Cryan et al., 1999;Bouwknecht et al., 2000). Flesinoxan-induced (10 mg/kg) hypothermia was completely absent in $SERT^{-/-}$ rats (**chapter 5**). While flesinoxan dose-dependently (0.3-10 mg/kg) inhibited SIH and additionally induced hypothermia in $SERT^{+/+}$ rats, a three-fold higher dose was needed to inhibit SIH in $SERT^{-/-}$ rats, and no additional hypothermia was observed (**chapter 5**). This higher dose needed to reduce SIH may be due to the absence of an additional effect of hypothermia as found in $SERT^{+/+}$ rats. Moreover, WAY100635 alone strongly enhanced SIH in $SERT^{-/-}$ rats, but not in $SERT^{+/+}$ rats (**chapter 5**). These findings clearly indicate that the responsivity to 5-HT_{1A} receptor ligands is changed in $SERT^{-/-}$ rats. The lower responsivity to 5-HT_{1A} receptor agonists may be explained either by constitutive occupation of 5-HT_{1A} receptors due to the high endogenous 5-HT tonus, or by desensitization of the receptors as a compensatory adaptive response to the high endogenous 5-HT tonus, or both. The remarkable response to WAY100635 in $SERT^{-/-}$ rats is most likely explained by the high endogenous 5-HT tonus, such that WAY100635 is competing with endogenous 5-HT occupation of 5-HT_{1A} receptors. In rats (e.g. Goodwin et al., 1987;Bill et al., 1991;Millan et al., 1993) and humans (Blier et al., 2002) 5-HT_{1A} receptor-mediated hypothermia is thought to be mediated by postsynaptic 5-HT_{1A} receptors, while in mice the somatodendritic 5-HT_{1A} receptors seem to be involved (Goodwin et al., 1985a;Martin et al., 1992). However, some studies also suggest that 5-HT_{1A} receptor mediated hypothermia in rat is mediated by somatodendritic 5-HT_{1A} receptors (Higgins et al., 1988;Hillegaart, 1991). Flesinoxan-mediated hypothermic responses may be exerted via postsynaptic 5-HT_{1A} receptors. Hence, the response profile of $SERT^{-/-}$ rats may suggest

that postsynaptic 5-HT_{1A} receptors are fully occupied in $SERT^{-/-}$ rats and/or (slightly) desensitized. Regarding the SIH, it is not clear whether this short-lasting thermogenic effect is mediated by somatodendritic or postsynaptic 5-HT_{1A} receptors, or both. The finding that flesinoxan reduced the SIH in $SERT^{-/-}$ rats suggests that 5-HT_{1A} receptors involved in SIH are still sensitive to 5-HT_{1A} agonists and hence must represent a different population of 5-HT_{1A} receptors than those involved in hypothermia. While the 5-HT_{1A} receptor-mediated autonomic responses have not provided sufficient data from which a firm conclusion can be drawn regarding enhanced 5-HT_{1A} receptor occupation or receptor desensitization, autoradiography studies reveal slight, but significant decreases in 5-HT_{1A} receptor density in the raphe nuclei and several terminal regions (unpublished observations). Hence, reduced expression of the 5-HT_{1A} receptor may partially explain the reduced responsivity to 5-HT_{1A} receptor agonists in $SERT^{-/-}$ rats.

Manipulation of the 5-HT system with acute tryptophan depletion

In humans, a polymorphism in the SERT gene-linked promoter region (5-HTTLPR) exists which causes individual differences in SERT expression and function (Lesch et al., 1996;Heils et al., 1996). Behavioral responses to acute tryptophan depletion (ATD) are influenced by this 5-HTTLPR genotype (Neumeister et al., 2006;Marsh et al., 2006;Roiser et al., 2006;Walderhaug et al., 2007). In line with this, the impact of ATD may vary in $SERT^{+/+}$, $SERT^{+/-}$ and $SERT^{-/-}$ rats as they exhibit large differences in central 5-HT neurotransmission. Biochemically, the standard dose TRP- depletion caused a decrease of 65% in plasma TRP in $SERT^{+/+}$, 61% in $SERT^{+/-}$ and 55% in $SERT^{-/-}$ rats (**chapter 4**). Interestingly, $SERT^{-/-}$ rats showed stronger depletion of 5-HT in the frontal cortex (63%) than $SERT^{+/+}$ and $SERT^{+/-}$ (both 19%) rats (**chapter 4**). Similar results were found in the hippocampus, where $SERT^{-/-}$ rats showed a depletion of 70%, whereas $SERT^{+/-}$ rats showed a 18% depletion and $SERT^{+/+}$ rats a 13% depletion (**chapter 4**). The standard dose ATD decreased the 5-HIAA in frontal cortex and hippocampus to a similar extent in all genotypes (**chapter 4**). The 5-HIAA/5-HT ratio (reflecting turnover) in $SERT^{+/+}$ and $SERT^{+/-}$ rats was somewhat lower in rats treated with TRP+ and standard dose TRP- compared with the untreated condition; however, in $SERT^{-/-}$ rats, the turnover in the standard dose TRP- group was much higher than in the TRP+ and the untreated group (**chapter 4**). The different effects seen in $SERT^{+/+}$, $SERT^{+/-}$ and $SERT^{-/-}$ rats after ATD were found only in the brain and not in the periphery. Although the reason for this difference is not known, it is interesting to note that 5-HT synthesis in brain and peripheral tissues functions differently. For example, tryptophan hydroxylase (TPH), which forms 5-hydroxytryptophan (5-HTP) from tryptophan, is controlled by different isoform in the brain and periphery (for review, see Walther and Bader, 2003). This difference in 5-HT synthesis might play a role in the different effects seen after ATD in brain and periphery. Another possibility could be that there is more compensatory 5-HT in the intestine compared to the brain. It is known that compensatory systems exist in the intestine of $SERT^{-/-}$ rat (Linder et al., 2008) and $SERT^{-/-}$ mouse (Chen et al., 2001).

In the object recognition test, two different doses of depletion were tested, the standard dose (TRP- 100g) and a mild depletion (TRP-40g). The standard ATD dose impairs object recognition in Wistar rats (Lieben et al., 2004b;Jans et al., 2007a) and was thus expected to impair object recognition in $SERT^{+/+}$, $SERT^{+/-}$ and $SERT^{-/-}$ rats. As expected, all genotypes showed impaired object recognition after treatment with the standard TRP depletion (**chapter 4**). Interestingly, mild TRP depletion impaired object recognition in $SERT^{+/-}$ and especially in $SERT^{-/-}$ rats but not in $SERT^{+/+}$ rats, indicating that $SERT^{-/-}$ and $SERT^{+/-}$ rats are more sensitive to mild ATD (**chapter 4**). These data suggests that $SERT^{-/-}$ and to a lesser extent $SERT^{+/-}$ rats show higher serotonergic

vulnerability than SERT$^{+/+}$ rats, as low-dose ATD only affected memory in subjects that already have a disturbed 5-HT system. ATD in SERT$^{-/-}$ rats may therefore be a good tool to investigate serotonergic vulnerability in humans.

Pharmacological responses to GABAergic, noradrenergicand dopaminergic systems

Adaptive changes in the serotonergic system occur after chronic administration of SSRIs (Blier and de Montigny, 1998;Briley and Moret, 1993). Besides the serotonergic system (Blier et al., 1997), adaptations also occur at the level of other neurotransmitters systems (Smith et al., 2000;Kugaya et al., 2003). In SERT$^{-/-}$ rats, small adaptations were found in body temperature after treatment with chlordiazepoxide, a $GABA_A$ receptor agonist (**chapter 6**). The effects of chlordiazepoxide lasted longer in SERT$^{-/-}$ rats than in SERT$^{+/+}$ rats. After treatment with atomoxetine, a NET blocker, a slightly increased response was found in the SERT$^{-/-}$ rat (**chapter 6**). This result was very modest, the low dose of atomoxetine reduced core body temperature in SERT$^{-/-}$ rats, but not in SERT$^{+/+}$ rats. The highest dose reduced core body temperature in both genotypes. These results indicate that NE receptors in SERT$^{-/-}$ rats are slightly more sensitive than in SERT$^{+/+}$ rats. Treatment with the DAT blocker GBR12909 resulted in a hyperthermia in SERT$^{-/-}$ rats, which was not found in SERT$^{+/+}$ rats (**chapter 6**). This might suggest a higher sensitivity of SERT$^{-/-}$ rats to blockade of the DAT in regions involved in body temperature. Interestingly, this hyperthermia was also found after treatment of the dual DAT and NET blocker bupropion (**chapter 6**). The drug-induced changes in stress-induced hyperthermia did not differ between SERT$^{+/+}$ and SERT$^{-/-}$ rats. Together, these results show that permanent changes in the 5-HT system leads to an adaptation in responsivity of the GABAergic, the noradrenergic and the dopaminergic system, with regard to body temperature, but not stress-induced hyperthermia.

Behavioral studies

Anxiety-like and depression-like behaviors of SERT$^{-/-}$ rats

In humans the 5-HTTLPR short variant, which results in reduced transcriptional efficiency of the SERT gene, is strongly associated with anxiety-related, harm-avoidance, negative personality traits, and exhibit more depressive symptoms, diagnosable depression, and suicidality in relation to stressful events compared with individuals with the long version (Lesch et al., 1996;Mann et al., 2000;Caspi et al., 2003;Anguelova et al., 2003;Sen et al., 2004;Kendler et al., 2005;Schmitz et al., 2007;Dick et al., 2007;Canli and Lesch, 2007). SERT$^{-/-}$ rats were therefore predicted to exhibit increased anxiety-like and depression-like behaviors. For anxiety-like behavior, both male and female SERT$^{-/-}$ rats spent significantly less time in the central part of the open field and less time on the open arms of the elevated plus maze as compared to their SERT$^{+/+}$ littermates (**chapter 3**). SERT$^{-/-}$ rats have also been subjected to the novelty suppressed feeding assay. In this test, only male SERT$^{-/-}$ rats showed a longer latency to start eating compared to SERT$^{+/+}$ controls (**chapter 3**). The home cage emergence test has been used as a last assay to assess anxiety-related behaviors in the SERT$^{-/-}$ rats. In this test, both male and female SERT$^{-/-}$ rats showed a higher escape latency than SERT$^{+/+}$ littermates (**chapter 3**). Taken together, these results indicate that anxiety-like behaviors are intensified in SERT$^{-/-}$ rats. For depression-like behavior, SERT$^{-/-}$ rats displayed an increased immobility time in the forced swim test compared to SERT$^{+/+}$ rats. Moreover, SERT$^{-/-}$ rats consumed less sucrose in the two-bottle paradigm compared with SERT$^{+/+}$ rats. These results suggest a higher depression-like state in SERT$^{-/-}$ rats.

Male versus female

In humans, women experience depression about twice as often as men (Gorman 2006). Possible explanations for this phenomenon are directly or indirectly linked to 5-HT neurotransmission. For example, *in vivo* measurements of 5-HT synthesis in the brain by positron emission tomography showed that whole brain rates of 5-HT synthesis is lower in women than in men (Nishizawa et al 1997). Moreover, women have lower SERT binding in the prefrontal cortex than men (Mann et al., 2000). Finally, depressed women show a decrease of SERT availability, while almost no decrease has been reported in men. In the $SERT^{-/-}$ rat, only anxiety- and depression-related behaviors were analyzed in male and female rats to find out if females were more sensitive to the loss of the SERT compared to males (**chapter 3**). Despite a higher general activity of females, no sex differences in anxiety- and depression-related behaviors have been found. Statistical analysis revealed no genotype x gender interactions (**chapter 3**). If anything, male rats show a slightly increased expression of anxiety-related behaviors, since the latency to initiate food consumption in the novelty suppressed feeding test was increased in male, but not female, $SERT^{-/-}$ rats compared to $SERT^{+/+}$ rats. Thus, regardless of the basal 5-HT differences between males and females (Watts and Stanley, 1984;Carlsson and Carlsson, 1988;Haleem et al., 1990;Dominguez et al., 2003) these data show that lifelong absence of SERT in rats leads to a sex independent increase of anxiety- or depression-like behaviour. Apparently, the behavioural consequences of genetic inactivation of the SERT are so robust that any difference between male and female knockout rats might be completely masked. These results are in line with a human study, which reported that both male and female individuals with the short version of the SERT gene are more prone to develop depression (than those with the longer version) (Mann et al., 2000).

Synthesis

Adaptive processes

The $SERT^{-/-}$ rat is one of the various genetic and pharmacological models available to study the role of 5-HT, and in particular of the SERT, in any process in which 5-HT is potentially involved. A major concern of knockout models is that the absence of a gene affects early development and that compensatory processes could have taken place. As such, phenotypes of a knockout model may not be exclusively attributable to the gene that has been knocked-out, but also to secondary adaptations in interacting systems. Studying the function of the SERT itself may be best accomplished by using selective serotonin reuptake inhibitors, e.g. citalopram. $SERT^{-/-}$ models including their adaptive mechanisms are, however, of major relevance when studying stable (genetic) individual differences in central serotonergic activity as an endophenotype underlying personality, such as harm avoidance (Gerra et al., 2000) and social impulsivity (Fairbanks et al., 2001), and those associated with the serotonin transporter promoter polymorphism (Dragan and Oniszczenko, 2006). Insight into these processes is of importance because they may increase our understanding of interactions between systems, and adaptive capabilities of the central nervous system. Further, due to such adaptive processes the impact of pharmacological compounds may change. Hence, individual differences in genetic, and consequently neurochemical make-up may determine the efficacy of pharmacotherapies. When considering the $SERT^{-/-}$ rat as potential model for inherited increased 5-HT levels, the pharmacological profile of these animals may have predictive value for pharmacological effects in humans exhibiting inherited increased central serotonergic activity.

Rat versus Mouse

Until now, rat genetic tools are clearly lagging behind those that are available for mice. Homologous recombination in embryonic stem (ES) cells, which is used to generate knockout mice (for review see Capecchi, 1989), fails in rats. As an alternative, target-selected ENU driven mutagenesis has been used to generate knockout rats (Zan et al., 2003;Smits et al., 2004;Smits et al., 2006). ENU induces random point mutations, and the currently available mutation-detection techniques are insufficient to screen the entire genome for mutations, raising the possibility that additional mutations in the SERT$^{-/-}$ rats could have gone undetected. Using the homologous recombination technique in ES cells, the first SERT$^{-/-}$ mouse was generated 10 years ago (Bengel et al., 1998), which has been characterized extensively afterwards. To confirm that a premature stopcodon in the SERT gene results into a SERT$^{-/-}$ rat, and to establish whether rats and mice similarly adapt to the absence of the SERT, many experiments done in the SERT$^{-/-}$ mouse have been replicated in the SERT$^{-/-}$ rat. Interestingly, several phenotypes in SERT$^{-/-}$ mice and rats are comparable. For instance, both SERT$^{-/-}$ mice (Fabre et al., 2000;Holmes A, 2002a;Holmes et al., 2002b;Lira et al., 2003;Montanez et al., 2003;Holmes et al., 2003b;Holmes et al., 2003c;Mathews et al., 2004;Shen et al., 2004;Kim et al., 2005;Zhao et al., 2006;Carroll et al., 2007;Fox et al., 2007;Kalueff et al., 2007a;Kalueff et al., 2007b) and rats (Homberg et al., 2007b; **chapter 2 and 3**) display strongly enhanced extra-neuronal 5-HT levels, no change in *in vitro* MAO-A and TPH activity, reduced 5-HT and 5-HIAA tissue levels, unchanged NET density, no changes in DA, NE, DOPAC and HVA tissue levels in the PFC, nucleus accumbens and caudate putamen, reduced responsivity to 5-HT$_{1A}$ ligands and reduced 5-HT$_{1A}$ receptor binding, thigmotaxis, increased anxiety and depression-related symptoms. These similarities in neurochemistry and behaviours indicate that phenotypes of SERT$^{-/-}$ rats are valid in the sense that the phenotypes in SERT$^{-/-}$ rats generally meet our expectations and are consistent with the literature. Moreover, they indicate that the influence of unknown additional mutations, if present, is minimal. Furthermore, these similarities between SERT$^{-/-}$ rat and mice models strengthen the assumption that gene function is conserved among species and thereby highlight the value of genetic animal models to understand gene function in humans.

Interestingly, differences between SERT$^{-/-}$ mice and rats were also found. For example, in contrast to SERT$^{-/-}$ rats (**chapter 2**), there is no compensatory 5-HT uptake in hippocampal synaptosomes of SERT$^{-/-}$ mice (Bengel et al., 1998), oral free-choice sucrose intake is decreased in SERT$^{-/-}$ rats (**chapter 3**), but not in SERT$^{-/-}$ mice (Kalueff et al., 2006), and no changes in 5-HT$_{1B}$ receptor binding are found in SERT$^{-/-}$ rats (unpublished observations), while regional changes in 5-HT$_{1B}$ receptor binding have been found in SERT$^{-/-}$ mice (Fabre et al., 2000). Finally, there are no differences in the number of immunopositive 5-HT neurons in the dorsal raphe nuclei between SERT$^{-/-}$ and SERT$^{+/+}$ rats (**chapter 3**), while a 50% reduction of serotonergic cell number has been found in the SERT$^{-/-}$ mouse (Lira et al., 2003). It should be noted that techniques or experimental conditions used in mice and rat studies differ, which hamper direct rat-mouse comparisons. In addition, the SERT$^{-/-}$ mouse has been generated in two different inbred lines, which differ among themselves (Holmes et al., 2003b;Adamec et al., 2006;Altamura et al., 2007), thereby further complicating comparisons with the SERT$^{-/-}$ rat that has been generated on a outbred Wistar background. Whether and how SERT$^{-/-}$ rat phenotypes are affected by genetic background is currently investigated. Together, studying similarities and differences between SERT$^{-/-}$ rats and mice are highly informative for cross-species translation of results obtained in different rodent models and/or the extrapolation to the human situation.

SSRIs

Interestingly, chronic SSRI treatment results in a profound reduction in SERT expression and function (Benmansour et al., 1999), and increased extracellular 5-HT levels. Furthermore, SSRI-induced reduction in 5-HT_{1A} receptor function has been reported (Dawson et al., 2002). Adaptations that have been observed in $SERT^{-/-}$ rats are strikingly similar. This may suggest that the $SERT^{-/-}$ rat can be considered as a model for studying the consequences of chronic SSRI treatment. However, $SERT^{-/-}$ rats paradoxically show anxiety- and depression-like phenotypes. Early postnatal SSRI treatment induces rather than ameliorates anxiety- and depression-like symptoms during adulthood, which are comparable to symptoms found in $SERT^{-/-}$ mice (Ansorge et al., 2004) and rats (**chapter 3**). It has been hypothesized that hyperserotonemia at early stages of development can cause loss of 5-HT terminals in the brain that persists throughout subsequent development (Whitaker-Azmitia, 2005). Hence, early SSRI treatment or the constitutive loss of the SERT is expected to have profound effects on brain development (Persico et al., 2001;Salichon et al., 2001), which obviously differs from the impact of chronic SSRI on the mature brain (Taravosh-Lahn et al., 2006). The finding that prenatal exposure to chronic fluoxetine increases anxiety-like behaviour in adult mice (Ansorge et al., 2004), supports this developmental hyperserotonemia hypothesis. Otherwise, chronic SSRI treatment decreases aggressive (Fuller, 1996) and sexual behavior (de Jong et al., 2005b) in rats, phenotypes that are also observed in $SERT^{-/-}$ rats (Homberg et al., 2007b; unpublished observations). Apparently, not all $SERT^{-/-}$-related behavioral phenotypes are opposite of those seen after chronic SSRI treatment during adulthood. Although highly speculative, it is possible that some effects of chronic SSRI treatment directly relate to SERT inhibition and increased extra-neuronal 5-HT levels, while others depend on associated compensatory adaptations or are the result of developmental changes induces by the constitutive absence of the SERT.

Conclusion

Although the ENU-driven target selected mutagenesis approach to generate knockout rats could theoretically be associated with unknown mutations additional to the premature stopcodon in the SERT gene, the $SERT^{-/-}$ rat has proven to exhibit both expected and novel phenotypes that may contribute to the further understanding of SERT function and its role in diseases. Furthermore, the $SERT^{-/-}$ rat models several important endophenotypes, and can be used as such. While research on $SERT^{-/-}$ mice has already revealed several novel 5-HT-mediated phenotypes and processes, the $SERT^{-/-}$ rat allows complementary in-depth research through its greater accessibility of behavioral and neurochemical techniques. The $SERT^{-/-}$ rat has only recently been generated (Smits et al., 2006), and based on the output the knockout rat has already provided, we expect that this mutant will become a very valuable research tool in neuroscience research and beyond.

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Abbreviations

Ach:	acetylcholine
AMY:	amygdale
ATD:	acute tryptophan depletion
BNST:	bed nucleus stria terminalis
CDP:	chlordiazepoxide
CNS:	central nerve system
CP:	caudate putamen
Cpm:	counts per minute
CSF:	cerebrospinal fluid
DA:	dopamine
DAT:	dopamine transporter
DOPAC:	dihydroxyphenylacetic acid
DMI:	desmethylimipramine
DRN:	dorsal raphe nucleus
ECG:	electrocardiogram
ENU:	*N*-ethyl-*N*-nitrosurea
GAPDH:	glyceraldehyde-3-phosphate dehydrogenase
Hipp	hippocampus
HPLC:	high-performance liquid chromatography
Htal:	hypothalamus
HVA:	homovanillic acid
LNAA:	large neutral amino acids
MAO:	monoamine oxidase
mRN:	medial raphe nucleus
NACC:	nucleus accumbens
NE:	noradrenaline
NET:	noradrenaline transporter
PBS:	phosphate-buffered saline
PFC:	prefrontal cortex
RN:	raphe nuclei
SERT:	serotonin transporter
$SERT^{-/-}$:	homozygous serotonin transporter knockout rat
$SERT^{+/-}$:	heterozygous serotonin transporter knockout rat
$SERT^{+/+}$:	wildtype serotonin transporter knockout rats
Sc:	subcutaneously
SCX:	somatosensory cortex
SIH:	stress-induced hyperthermia
SN:	substantia nigra
SSRI:	selective serotonin reuptake inhibitor
T_c:	core body temperature
Thal:	thalamus
TLLING:	targeting induced local lesions in genomes
TPH:	tryptophan hydroxylase
TRP:	tryptophan
VTA:	ventral tegmental area
5-HIAA:	5-hydroxyindoleacetic acid
5-HTP:	5-hydroxytryptophan
5-HTTLPR:	polymorphism in the promotor region of the SERT gene

Nederlandse samenvatting

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Serotonine is een neurotransmitter die een belangrijke rol speelt in processen die in de hersenen plaats vinden. De serotonerge huishouding wordt primair gereguleerd door de serotonine transporter (SERT). Recent moleculair genetisch onderzoek heeft aangetoond dat afwijkingen in de SERT het risico op het ontstaan van neuropsychiatrische aandoeningen zoals angststoornissen, pathologische agressie en depressie sterk verhoogd. Vooralsnog is niet duidelijk wat de consequenties van deze genetische verandering in de SERT voor de serotonine huishouding heeft. Meestal worden deze aandoeningen behandeld met medicijnen die aangrijpen op de SERT. Alhoewel deze therapieën de serotonerge neurotransmissie moduleren, is de efficiëntie vaak niet optimaal en gaat een dergelijke behandeling vaak gepaard met ongewenste bijwerkingen. Een goed model voor deze neuropsychiatrische aandoeningen en meer specifiek de serotonerge aandoeningen zou perspectieven kunnen bieden voor de ontwikkeling van medicijnen tegen deze stoornissen. Het doel van dit proefschrift is daarom het karakteriseren van een diermodel dat onderzoek naar humane serotonerge aandoeningen mogelijk maakt

Serotonine transporter

De SERT is een transporteiwit dat de extracellulaire serotonine terug naar het presynaptische neuron transporteert. Hier wordt serotonine verder afgebroken of opgeslagen voor hergebruik. Veel medicijnen die worden voorgeschreven tegen neuropsychiatrische aandoeningen blokkeren de SERT. Hierdoor is heropname van serotonine niet meer mogelijk en wordt de extracellulaire concentratie van serotonine in de synaps verhoogt. Selectieve serotonineheropnameremmers (SSRI) zijn stoffen die de SERT blokkeren. Zij worden veel voorgeschreven in de behandeling van onder meer depressie. Er zijn verschillende polymorfismen bekend in het humane SERT-gen. De *5-HTTLPR* is een insertie of deletie van 44 baseparen in een repetitieve sequentie van de promotor van het SERT-gen. Bij een deletie van de baseparen spreekt men van de korte variant (S), bij een insertie van de lange variant (L). Bij de S-variant komt de SERT minder vaak tot expressie, waardoor de SERT minder goed functioneert. Mensen met de S-variant lijken gevoeliger voor het ontwikkelen van angststoornissen en depressieve symptomen dan mensen met de L-variant, vooral na blootstelling van stress. Hieruit blijkt dat de serotonine transporter een belangrijke rol speelt in het centrale zenuwstelsel.

Serotonine transporter knock-outrat

De laboratoriumrat (*Rattus norvegicus*) is een van de meest gebruikte proefdieren voor het bestuderen van humane aandoeningen. Veel gedragsmatige testen, die model staan voor humane ziekten, zijn ontwikkeld en gevalideerd voor ratten. Ook genetische variaties in het genoom, zoals single nucleotide polymorphism, worden bestudeerd. Nu het complete genoom van de rat bekend is, is het interessant om genetische variaties, zoals een gen dat uitgeschakeld is (knock-out), in deze diersoort te bestuderen. Deze variaties kunnen een belangrijke rol spelen in het onderzoek naar associaties tussen genen en gedrag.

In **hoofdstuk 1** wordt beschreven hoe de serotonine transporter knock-outrat ($SERT^{-/-}$) is gemaakt. Kort samengevat vindt dit plaats met behulp van de mutagene stof *N*-ethyl-*N*-nitrosourea (ENU). ENU is in staat zijn ethylgroep over te dragen aan de nucleobasen in nucleïnezuren; hierdoor ontstaan op willekeurige basis puntmutaties in het DNA van spermatogenale stamcellen. Bij een puntmutatie wordt één nucleotide uitgewisseld voor een andere. Als deze mutatie optreedt in een coderend gedeelte van een gen, kunnen er duidelijk zichtbare gevolgen zijn. In de $SERT^{-/-}$

-rat is door zo'n puntmutatie een premature stopcodon ontstaan, waardoor translatie van het DNA niet meer mogelijk is. Het gen is uitgeschakeld (knock-out) en zal niet worden afgelezen, waardoor er geen functioneel eiwit gevormd wordt. Zoals eerder genoemd speelt de SERT een belangrijke rol in het centrale zenuwstelsel. De $SERT^{-/-}$-rat is daarom een interessant model om dysfuncties in het serotonerge systeem mee te onderzoeken.

Als het SERT-gen niet meer functioneert, heeft dit gevolgen voor de rat. In **hoofdstuk 2** wordt een primaire karakterisatie gegeven van de $SERT^{-/-}$-rat. Door de puntmutatie in de SERT blijkt dat het SERT-mRNA en het functionele eiwit niet langer aanwezig zijn. Daarnaast kan het tritium gelabeld citalopram (een SSRI) niet meer binden aan de SERT. Door de afwezigheid van het SERT- eiwit is het logisch dat de serotoninehomeostase wordt verstoord. De verstoring blijkt uit het verlaagde serotoninegehalte in verschillende hersengebieden, maar ook uit de verlaagde elektrisch opgewekte [^{3}H]serotonineafgifte in meerdere hersengebieden.
D-fenfluramine is een serotonine-releaser die werkzaam is middels de SERT. Na toediening van d-fenfluramine vindt een uitstoot plaats van serotonine, die een verlaging van de lichaamstemperatuur (hypothermie) veroorzaakt. Bij de $SERT^{-/-}$-rat is deze temperatuurverlaging niet meer aantoonbaar. Nu de SERT niet langer functioneel blijkt, is heropname van serotonine in het presynaptische neuron niet mogelijk. Daardoor is het extracellulaire serotoninegehalte tot ongeveer negen keer verhoogd. *In vitro* is de opname van serotonine in hippocampale synaptosomen met ongeveer 72% verlaagd; er treden echter geen veranderingen op in de activiteit van de tryptofaanhydroxylase, een enzym dat betrokken is bij de synthese van serotonine, en in de activiteit van de monoamine-oxidase, een enzym dat betrokken is bij de afbraak van serotonine. Buiten het serotonerge systeem zijn ook geen verschillen gevonden aangaande de noradrenalinetransporter (NET) en de dopaminetransporter (DAT) concentraties, de monoamineniveaus en de door depolarisatie geïnduceerde afgifte van dopamine, noradrenaline, glutamaat en GABA. Omdat de opname van serotonine verlaagd, maar niet compleet afwezig is, is het aannemelijk dat de dopamine- of noradrenalinetransporter een gedeelte van de serotonine opname voor zijn rekening neemt. In de hippocampus blijkt dat remming van de DAT niet leidt tot een verandering in serotonineopname. Wanneer de DAT en NET beide geremd worden, blijkt dat de serotonineopname afneemt. Dit duidt erop dat de NET verantwoordelijk is voor serotonineopname in de hippocampus. De hippocampus is rijk aan noradrenaline, waardoor het aannemelijk is dat de serotonine door de NET wordt opgenomen. Dit sluit echter niet uit dat in een gebied als het striatum, waar dopamine de overhand heeft, de serotonineopname door de DAT wordt gereguleerd, zoals eerder in $SERT^{-/-}$-muizen is aangetoond.

Angst en depressie
Angststoornissen en depressie hebben veel overeenkomsten, zoals een verminderde concentratie, vermoeidheid, verhoogde irritatie, slaapstoornissen, nervositeit en rusteloosheid. Het is algemeen bekend dat het verminderd functioneren van serotonine een rol speelt bij de ontwikkeling van depressies. Bij patiënten met een depressie is er bijvoorbeeld een verlaging van 5-HIAA (metaboliet van serotonine) gevonden in de cerebrale vloeistof, hetgeen duidt op een verlaagd serotoninemetabolisme. Daarnaast is een vermindering gevonden van bepaalde serotoninereceptoren, met name in het aantal 5-HT_{1A}-receptoren. Deze receptoren herstellen zich niet meer na remissie van depressie. In beeldverwerkingsstudies van depressieve patiënten is aangetoond dat bij hen minder serotonineopname mogelijk is. Daarnaast is de beschikbaarheid van SERT aantoonbaar verminderd bij zowel depressieve patiënten als patiënten met een

angststoornis. Hieruit valt te concluderen dat veranderingen in het serotonerge systeem bijdragen aan symptomen van depressies en angststoornissen. Als het serotonerge systeem goed functioneert, is de kans op het ontwikkelen van deze verschijnselen kleiner.

In **hoofdstuk 3** komt aan de orde of SERT$^{-/-}$-ratten gevoeliger zijn in angst- en depressiegerelateerde testen. Omdat vrouwen vaker depressies en angststoornissen ontwikkelen dan mannen, is in dit hoofdstuk ook gekeken naar geslachtsverschillen. Vergeleken met SERT$^{+/+}$-ratten bleken zowel mannelijke als vrouwelijke SERT$^{-/-}$-ratten minder tijd door te brengen in het centrum van het open veld en op de open armen van een verhoogde plus-maze. Daarnaast duurde het langer voordat SERT$^{-/-}$-ratten begonnen met eten in een nieuwe omgeving en uit hun thuiskooi ontsnapten. Samengevat lijkt het erop dat SERT$^{-/-}$-ratten meer angstgerelateerde gedragingen vertonen dan SERT$^{+/+}$-ratten. De zwemtest waarin geen ontsnapping mogelijk is, wordt gebruikt om antidepressiva te testen. Zodra een rat doorheeft dat het onmogelijk is om uit de zwemtest te komen, zal het dier blijven drijven en zo min mogelijk zwemmen (immobiel worden). Na toediening van antidepressiva blijven ratten langer proberen om te ontsnappen (ze blijven mobiel). SERT$^{-/-}$-ratten bleken eerder immobiel te worden dan SERT$^{+/+}$-ratten, wanneer ze aan deze test werden blootgesteld. Depressieve mensen beleven minder plezier aan dingen waarvan ze eerst wel genoten; een symptoom dat ook wel anhedonia wordt genoemd. In ratten is dit te meten door suikerwater naast gewoon water aan te bieden en te meten hoeveel suikerwater er wordt ingenomen. SERT$^{-/-}$-ratten bleken minder suikerwater te drinken dan SERT$^{+/+}$-ratten. Samen met de verhoogde immobiliteit in de zwemtest duidt dit erop dat SERT$^{-/-}$-ratten ook gevoeliger zijn in depressiegerelateerde testen.

Verschillen tussen mannelijke en vrouwelijke ratten zijn in dit model niet naar voren gekomen. Alleen in de voedseltest hadden SERT$^{-/-}$-mannelijke ratten een hogere latentietijd, terwijl er bij de vrouwelijke ratten geen verschil was tussen SERT$^{+/+}$- en SERT$^{-/-}$-ratten. In de andere testen bleken SERT$^{-/-}$-mannelijke en vrouwelijke ratten hetzelfde te reageren. Tevens is gekeken naar de extracellulaire niveaus van serotonine. Deze bleek omstreeks negen maal verhoogd in SERT$^{-/-}$-ratten en niet onderling te verschillen tussen mannen en vrouwen. Daarnaast is een immunokleuring uitgevoerd om te onderzoeken of het aantal serotonerge neuronen verschilt tussen SERT$^{+/+}$- en SERT$^{-/-}$-ratten. Dit bleek niet het geval te zijn. Bovendien waren er geen verschillen tussen mannelijke en vrouwelijke ratten. Deze bevindingen tonen aan dat SERT$^{-/-}$-ratten angst- en depressiegerelateerd gedrag vertonen, onafhankelijk van hun geslacht. Blijkbaar heeft de genetische inactivatie van de SERT een dermate grote impact op de neurotransmissie van serotonine dat mogelijke verschillen tussen mannen en vrouwen worden gemaskeerd.

Tryptofaandepletie

Serotonine wordt gemaakt uit tryptofaan, dat we binnenkrijgen via ons voedsel. Als tryptofaan zich in de bloedbaan bevindt, moet het de competitie aangaan met vijf andere grote aminozuren om actief door de bloed-hersenbarrière getransporteerd te worden. Hoe minder concurrerende eiwitten, hoe hoger het tryptofaangehalte in het brein. Eenmaal in het brein wordt tryptofaan omgezet in serotonine. De beschikbaarheid van vrij tryptofaan is dus de beperkende factor in de synthese van serotonine. Acute tryptofaandepletie is een manier om het serotonerge systeem uit te dagen. Door een verlaging van tryptofaan in het bloed zal het serotoninegehalte in de hersenen dalen. Toediening van een aminozuurmix zonder tryptofaan leidt tot een verlaging van tryptofaanniveaus in het plasma en tot een verlaging van serotonineniveaus in de hersenen.

Daarnaast is gebleken dat acute tryptofaandepletie leidt tot een verminderd geheugen; dit is gevonden bij zowel mensen als ratten.

In hoofdstuk 4 komt de bepaling van concentraties plasmatryptofaan, centrale serotonine en 5-HIAA van SERT$^{-/-}$-, SERT$^{+/-}$- en SERT$^{+/+}$-ratten na acute tryptofaandepletie aan bod. Bij alle drie de genotypen was het plasmatryptofaan, het serotoninegehalte in de hippocampus en frontale cortex en de 5-HIAA niveaus verlaagd, met de sterkste daling bij de SERT$^{-/-}$-ratten. Met een standaarddosering van tryptofaandepletie was de objectherkenning van de SERT$^{+/+}$-, de SERT$^{+/-}$- en de SERT$^{-/-}$-ratten verslechterd. Bij een lagere dosering bleken de SERT$^{+/-}$- en de SERT$^{-/-}$-ratten gevoelig voor de depletie (geen objectherkenning), maar de SERT$^{+/+}$-ratten niet (volledige objectherkenning). Deze resultaten laten zien dat SERT$^{-/-}$- en SERT$^{+/-}$-ratten gevoeliger zijn voor acute tryptofaandepletie dan SERT$^{+/+}$-ratten.

Farmacologische respons

Een verandering in het serotonerge systeem zou de gevoeligheid van serotonine receptoren kunnen beïnvloeden. Daarnaast zou een verandering in het serotonerge systeem ook een invloed kunnen hebben op de gevoeligheid van het GABAerge, het dopaminerge en het noradrenerge systeem, daar het interacteert met deze systemen.

Het is bekend dat na chronische toediening van SSRI's de gevoeligheid van serotonerge receptoren verandert. De verhoogde extracellulaire serotonine van de SERT$^{-/-}$-rat zou vergelijkbare gevolgen kunnen hebben op het functioneren en de gevoeligheid van serotoninereceptoren. Daarom is in **hoofdstuk 5** gekeken naar het effect van flesinoxan, een 5-HT$_{1A}$ receptor agonist, op de lichaamstemperatuur en stressgeïnduceerde hyperthermie (SIH). Beide processen kunnen worden gemoduleerd door 5-HT$_{1A}$ receptor(ant)agonisten. De hypothermie, die door flesinoxan geïnduceerd wordt, bleek afwezig in de SERT$^{-/-}$-rat. Interessant genoeg werd de SIH in SERT$^{-/-}$-ratten wel verminderd door flesinoxan, ofschoon de dosering om dit te bewerkstelligen hoger lag bij SERT$^{-/-}$-ratten dan bij SERT$^{+/+}$-ratten. Deze verhoging in dosering zou verklaard kunnen worden door het ontbreken van hypothermie bij SERT$^{-/-}$-ratten, terwijl bij SERT$^{+/+}$-ratten de hypothermie bij een lagere dosering op gang was gebracht. Toediening van WAY100635, een 5-HT$_{1A}$ receptor antagonist, resulteerde in een verhoogde SIH-respons bij SERT$^{-/-}$-, hetgeen bij SERT$^{+/+}$-ratten niet zichtbaar was. Deze resultaten tonen aan dat de gevoeligheid van de 5-HT$_{1A}$-receptor veranderd is bij SERT$^{-/-}$-ratten. De verlaagde gevoeligheid van de 5-HT$_{1A}$-receptor kan verklaard worden door de hoge endogene tonus, door desensitisatie van de 5-HT$_{1A}$-receptor, of door beide. De opmerkelijke hyperthermie in SERT$^{-/-}$-ratten die ontstaat na toediening van WAY100635 is hoogstwaarschijnlijk te verklaren door de hoge endogene serotonineconcentratie, die de receptoren continu bezet. Doordat WAY100625 de serotonine van de receptor verdringt, is de compensatie duidelijk zichtbaar. Dat flesinoxan de hypothermie niet in gang kan zetten, maar wel een verlaging van de SIH kan geven, duidt erop dat deze processen, in ieder geval bij SERT$^{-/-}$-ratten, gereguleerd worden door verschillende populaties van 5-HT$_{1A}$-receptoren. Immers, de ene populatie (de receptoren betrokken bij SIH) is gevoelig voor een 5-HT$_{1A}$ receptoragonist, terwijl dit bij de andere populatie (de receptoren betrokken bij hypothermie) in het geheel niet het geval is.

Serotonine interacteert met andere neurotransmittersystemen. Een verandering in het serotonerge systeem zou hun functioneren dus kunnen beïnvloeden. In **hoofdstuk 6** is bij de

SERT$^{-/-}$-rat gekeken naar de effecten van toediening van een DAT-blokker, een NET-blokker en een $GABA_A$-receptor-agonist op SIH en lichaamstemperatuur.

$GABA_A$-receptoren spelen een belangrijke rol in autonome stress en angst. Bij veel diersoorten kan de SIH gereduceerd worden door $GABA_A$-receptor-agonisten. Chlordiazepoxide (CDP) is een $GABA_A$-receptor-agonist en verlaagt de SIH in zowel de SERT$^{+/+}$- als de SERT$^{-/-}$-ratten. De hypothermie, die ontstaat na CDP, is ook terug te vinden in beide genotypen, alleen duurt het effect bij SERT$^{-/-}$-ratten langer. Hieruit blijkt dat er een adaptatie heeft plaats gevonden in het GABAergic systeem.
Naast het GABA systeem beïnvloedt serotonine ook het dopamine- en noradrenalinesysteem. Onderzoek heeft aangetoond dat door gebruik van SSRI's de spontane vuursnelheid van noradrenerge neuronen in de locus coeruleus en dopaminerge neuronen in het ventrale tegmentaalgebied vermindert. Zoals in hoofdstuk 2 is beschreven, kan serotonine langs alternatieve routes worden opgenomen door de SERT$^{-/-}$-mutant. Het is de vraag wat het effect zal zijn van een blokkade van de NET, de DAT, of beide tegelijk. Na blokkade van de NET met atomoxetine is te zien dat SERT$^{-/-}$-ratten gevoeliger zijn voor deze blokkade. Bij een lagere dosering is al duidelijke hypothermie te zien bij de SERT$^{-/-}$-rat; bij de SERT$^{+/+}$-rat zien we dit pas bij een hoge dosering. Hieruit blijkt dat een milde adaptatie heeft plaatsgevonden en dat de SERT$^{-/-}$-rat een iets actiever NET-systeem heeft dan de SERT$^{+/+}$-rat. Na toediening van de DAT-blokker GBR12909 was er bij de SERT$^{+/+}$-rat geen effect op de lichaamstemperatuur, terwijl er een duidelijke hypothermie te zien was bij de SERT$^{-/-}$-rat. Hetzelfde resultaat werd bereikt na blokkade van zowel de NET en de DAT door toediening van bupropion. Opnieuw was een hypothermie zichtbaar bij de SERT$^{-/-}$-rat en niet bij de SERT$^{+/+}$-rat, vermoedelijk als gevolg van de blokkade van de DAT. Ook hieruit blijkt dat een adaptatie in het dopaminerge systeem heeft plaatsgevonden. De onderzoeksresultaten maken duidelijk dat een verandering in het serotonerge systeem ook veranderingen teweeg brengt in het dopaminerge, het noradrenerge en het GABAerge systeem, zoals is gebleken bij de SERT$^{-/-}$-rat.

Een goed diermodel voor humane serotonerge aandoeningen

In **hoofdstuk 7** worden de bevindingen bediscussieerd en wordt antwoord gegeven op de vraag of de SERT$^{-/-}$-rat een goed diermodel is voor humane serotonerge aandoeningen. De SERT$^{-/-}$-rat is een van de beschikbare genetische en farmacologische modellen waarmee de rol van serotonine, en in het bijzonder van de SERT, in bepaalde centrale processen bestudeerd kan worden. Het SERT-gen functioneert bij de SERT$^{-/-}$-rat vanaf de conceptie niet. Daarnaast hebben er verschillende compensaties in het dier plaatsgevonden. Het fenotype van een knock-outrat hoeft daarom niet per se een oorzaak te zijn van het missen van het gen, maar kan ook een gevolg zijn van secondaire adaptaties in het systeem. De functie van de SERT zou misschien het beste bestudeerd kunnen worden met een stof die de SERT zeer selectief blokkeert, zoals een SSRI. Dan nog blijft de SERT$^{-/-}$-rat met zijn adaptaties zeer belangrijk om stabiele (genetische) individuele verschillen in centrale serotonerge activiteit te onderzoeken. Het is belangrijk om deze processen te begrijpen, zodat duidelijker wordt hoe de interacties tussen bepaalde systemen plaatsvinden en hoe adaptaties in het centrale zenuwstelsel ontstaan. Daarnaast is het aannemelijk dat door bepaalde adaptaties in het lichaam de gevoeligheid voor bepaalde medicijnen verandert.

Chronische behandeling met een SSRI resulteert in een verminderde expressie en functie van de SERT en verhoogde extracellulaire serotonineniveaus. Daarnaast is aangetoond dat de functie

van de 5-HT_{1A}-receptor vermindert na gebruik van SSRIs. Deze adaptaties zijn vergelijkbaar met de bevindingen bij de $SERT^{-/-}$-rat. Er zijn echter belangrijke verschillen. De $SERT^{-/-}$-rat vertoont angst- en depressieachtig gedrag, terwijl SSRI's vaak worden gebruikt om dit gedrag tegen te gaan. Het is bekend dat kinderen van vrouwen die tijdens hun zwangerschap SSRI's hebben gebruikt op volwassen leeftijd gevoeliger zijn voor het ontwikkelen van angst- en depressiestoornissen. Een verklaring hiervoor zou kunnen zijn dat de verminderde SERT-functie vanaf de zwangerschap aanwezig is en daarom ook de verstoorde serotonineniveaus. Het is dus belangrijk dat de serotoninehuishouding normaal functioneert tijdens de foetale ontwikkeling, de kindertijd, maar ook in het volwassen leven. Er zijn gedragingen bekend na het gebruik van SSRI die overeenkomen met fenotypen in de $SERT^{-/-}$-rat. Chronisch gebruik van SSRI's vermindert bijvoorbeeld agressiviteit en seksueel gedrag, wat ook gevonden werd bij $SERT^{-/-}$-ratten. Blijkbaar zijn niet alle $SERT^{-/-}$-gerelateerde gedragsfenotypes tegengesteld aan het gedrag na chronisch gebruik van SSRI's. Het is mogelijk dat sommige effecten van chronische SSRI-behandeling direct relateren aan de inhibitie van de SERT, terwijl andere gedragingen wellicht relateren aan compensaties in het lichaam, of het resultaat zijn van veranderingen tijdens de foetale ontwikkeling door systematisch gemis van de SERT.

De ENU-geïnduceerde mutagenese levert in theorie, naast de premature stopcodon in het SERT-gen, ook onbekende mutaties op. Maar omdat de $SERT^{-/-}$-rat zowel verwachte als nieuwe fenotypes laat zien, wordt aangenomen dat deze mutaties zijn uitgekruist. De fenotypen van de $SERT^{-/-}$-rat kunnen gebruikt worden om de functie en met name de rol van de SERT in het ontwikkelen van bepaalde stoornissen te onderzoeken. Daarnaast is de $SERT^{-/-}$-rat een goed model voor verschillende fenotypen. De $SERT^{-/-}$-muis heeft vele nieuwe serotonine-gerelateerde fenotypen en processen aan het licht gebracht. De $SERT^{-/-}$-rat kan hieraan bijdragen, omdat het repertoire van gevalideerde, gedragsmatige testen en gevalideerde, neurochemische technieken voor ratten vele malen groter is dan voor muizen. Gezien de uitkomsten van onderzoek met de $SERT^{-/-}$-rat lijkt deze mutant een waardevol model in de neurowetenschappen. Toekomstig onderzoek met de $SERT^{-/-}$-rat zou een fundamentele bijdrage aan het onderzoek naar serotonerge stoornissen kunnen leveren.

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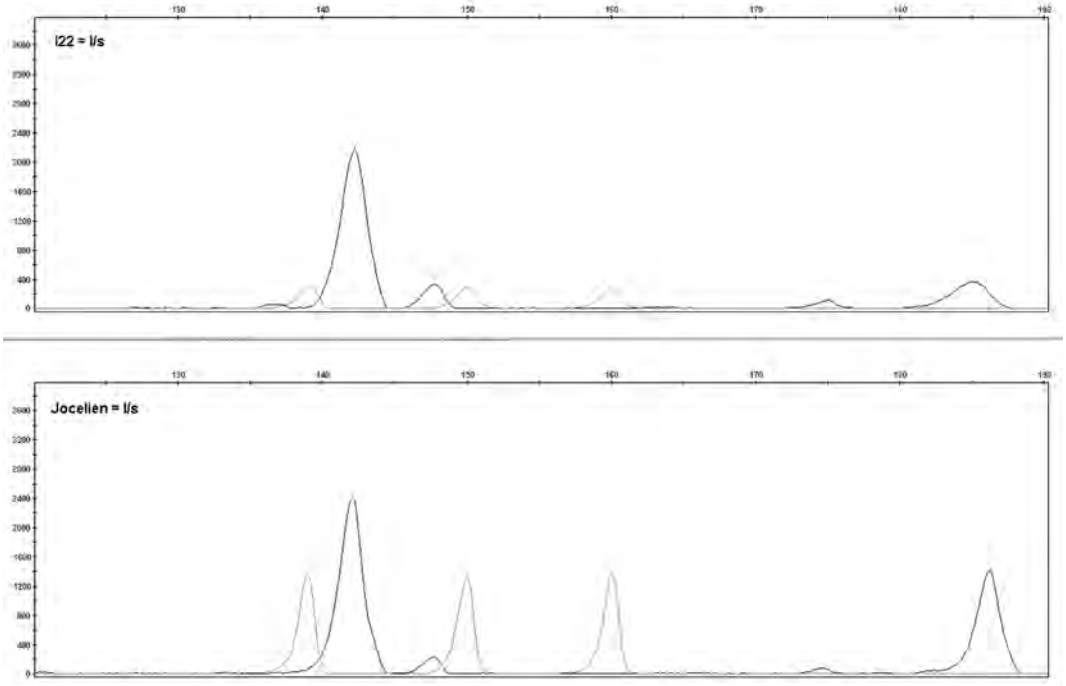
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Curriculum vitae

Curriculum vitae

Jocelien Danielle Attalie Olivier was born on 4 April 1978 in Kampen, the Netherlands. In 1995 she passed her final exams at the Christelijke College Nassau Veluwe in Harderwijk and studied from 1995 till 2000 higher laboratorial education at the Hogeschool van Utrecht. In order to receive her Bachelor degree in zoology, she investigated the effects of CREB activity in the nucleus accumbens on rat behavior and investigated the influence of the retinoid receptor RXRγ on adaptive changes in the nucleus accumbens caused by opiate addiction. This internship was performed under supervision of dr. Michel Barrot and Professor dr. Eric Nestler at Yale University, New Haven, U.S.A.. In 2000 Jocelien started her study in Medical Biology at the Vrije Universiteit in Amsterdam. During her Master degree she investigated the effects of apomorphine on sexual behavior of male rats and studied the brain areas activated in sexual behaviour. This internship was performed under supervision of dr. Jan Veening at the Radboud University Medical Centre Nijmegen. In 2002 she worked one year as a Research Associate at Utrecht University. Since 2003 Jocelien worked as a PhD-student on the research project "Reverse genetics by resequencing of mutagenized animals; Making a knock-out library of the rat" at the department of psychoneuropharmacology of the Radboud University Medical Centre in Nijmegen, under supervision of dr. Bart Ellenbroek and prof. dr. Alexander Cools. Most of the results obtained in this project were described and discussed in the present thesis.

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