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5-HT_{1B} receptor modulation of the serotonin transporter *in vivo*: Studies using KO mice

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Abstract

The serotonin transporter (SERT) controls the strength and duration of serotonergic neurotransmission by the high-affinity uptake of serotonin (5-HT) from extracellular fluid. SERT is a key target for many psychotherapeutic and abused drugs, therefore understanding how SERT activity and expression are regulated is of fundamental importance. A growing literature suggests that SERT activity is under regulatory control of the 5-HT_{1B} autoreceptor. The present studies made use of mice with a constitutive reduction (5-HT_{1B}^{+/-}) or knockout of 5-HT_{1B} receptors (5-HT_{1B}^{-/-}), as well as mice with a constitutive knockout of SERT (SERT^{-/-}) to further explore the relationship between SERT activity and 5-HT_{1B} receptor expression. High-speed chronoamperometry was used to measure clearance of 5-HT from CA3 region of hippocampus *in vivo*. Serotonin clearance rate, over a range of 5-HT concentrations, did not differ among 5-HT_{1B} receptor genotypes, nor did [³H]cyanoimipramine binding to SERT in this brain region, suggesting that SERT activity is not affected by constitutive reduction or loss of 5-HT_{1B} receptors; alternatively, it might be that other transport mechanisms for 5-HT compensate for loss of 5-HT_{1B} receptors. Consistent with previous reports, we found that the 5-HT_{1B} receptor antagonist, cyanopindolol, inhibited 5-HT clearance in wild-type mice. However, this effect of cyanopindolol was lost in 5-HT_{1B}^{-/-} mice and diminished in 5-HT_{1B}^{+/-} mice, indicating that the 5-HT_{1B} receptor is necessary for cyanopindolol to inhibit 5-HT clearance. Likewise, cyanopindolol was without effect on 5-HT clearance in SERT^{-/-} mice, demonstrating a requirement for the presence of both SERT and 5-HT_{1B} receptors in order for cyanopindolol to inhibit 5-HT clearance in CA3 region of hippocampus. Our findings are consistent with SERT being under the regulatory control of 5-HT_{1B} autoreceptors. Future studies to identify signaling pathways involved may help elucidate novel therapeutic targets for the treatment of psychiatric disorders, particularly those linked to gene variants of the 5-HT_{1B} receptor.

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Keywords

serotonin transporter; 5-HT_{1B} receptor; hippocampus; uptake; chronoamperometry

1.0 Introduction

The serotonin transporter (SERT) is the primary means for terminating serotonergic neurotransmission and is a key target for many psychotherapeutic and abused drugs. Determining how SERT activity and expression are regulated is therefore of fundamental importance. SERT and 5-HT_{1B} autoreceptors are located on pre-synaptic serotonin nerve terminals, and are therefore anatomically positioned to interact. In rats we showed that 5-HT_{1B} receptor antagonists can inhibit clearance of serotonin (5-HT) *in vivo* and that this is not due to a direct action at SERT (Daws et al., 2000). These data are consistent with the idea that the 5-HT_{1B} autoreceptor can modulate SERT activity. More recently, using rotating disk voltammetry to measure 5-HT clearance in whole brain synaptosomal preparations, Hagan and coworkers (2012) showed that 5-HT_{1B} agonists or virally-mediated over-expression of 5-HT_{1B} receptors increased 5-HT uptake, whereas 5-HT_{1B} antagonists or genetic knockout of 5-HT_{1B} receptors decreased 5-HT uptake (Hagan et al., 2012). Clinically, gene variants of the 5-HT_{1B} receptor have been linked to several psychiatric disorders (Murphy et al., 2003; Banerjee et al., 2012) raising the possibility that functionality of the 5-HT_{1B} autoreceptor may be an important factor in determining therapeutic responsiveness to drugs that target SERT, notably selective serotonin reuptake inhibitors (SSRIs). Here we use constitutive 5-HT_{1B} receptor knockout mice (KO; $-/-$), and 5-HT_{1B} heterozygote ($+/-$) mice, which express half as many 5-HT_{1B} receptors as wild-type ($+/+$) mice, to further explore the relationship between SERT activity and 5-HT_{1B} receptor expression *in vivo*.

2.0 Materials and methods

2.1 Animals

Male $+/+$, $+/-$ and $-/-$ 5-HT_{1B} receptor or SERT $-/-$ mice, 5-7 months old, were used for all studies. Mice were bred on either a 129SvEvTac (5-HT_{1B}) or a C57Bl/6J (SERT) background from in-house colonies. Breeders were originally obtained from Drs. Rene Hen and Irwin Lucki (5-HT_{1B}) (Zhuang et al., 1999; Knobelmann et al., 2001) and Dr. Dennis Murphy (SERT) (Bengel et al., 1998; Murphy et al., 2003). Mice were littermates derived from $+/-$ x $+/-$ matings and genotype confirmed by PCR. Mice were housed in groups of 5 in a temperature controlled (24 °C) vivarium with free access to food and water. All procedures were carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals and with the Institutional Animal Care and Use Committee at the University of Texas Health Science Center at San Antonio.

2.2 In vivo chronoamperometry

In vivo chronoamperometry was carried out as described in Montañez et al., (2003). Mice were anesthetized with an intraperitoneal injection (2 ml/kg body weight) of a mixture of chloralose (35 mg/ml) and urethane (350 mg/ml). A microelectrode attached to a multibarrel

micropipette was positioned in the CA3 region of dorsal hippocampus (AP -1.94 mm, ML +2.0 mm, DV -1.8 to -2.0 mm, [Franklin and Paxinos, 1997]). Single carbon fiber electrodes were coated with Nafion (5% solution) to improve selectivity for 5-HT over anionic compounds. High-speed chronoamperometric recordings were made using the FAST-12 system (Quanteon, KY). An oxidation potential of +0.55V versus a Ag/AgCl reference electrode, placed in the cortex rostral to the recording site, was applied for 100 msec. Oxidation potentials were delivered once per second. Oxidation and reduction currents were digitally integrated during the last 80 msec of each 100 msec voltage pulse. Serotonin (200 μ M, 10-200 nl) and drugs (400 μ M, 50 nl or 150 nl) or an equivalent volume of phosphate buffered saline (PBS) vehicle were applied locally by pressure-ejection. Once signals for 5-HT were stable and reproducible, the 5-HT_{1B} antagonist, cyanopindolol (20 pmol), the SSRI, fluvoxamine (60 pmol) or vehicle were pressure-ejected. Two min later 5-HT was applied again, and at 5 min intervals thereafter, until the 5-HT signal returned to its pre-treatment baseline. The pmol amounts of cyanopindolol and fluvoxamine pressure-ejected were selected because they produce maximal inhibition of 5-HT clearance in wild-type mice (data not shown).

2.3 [³H]Cyanoimipramine quantitative autoradiography

[³H]Cyanoimipramine binding to serotonin transporters in dorsal hippocampus was performed by quantitative autoradiography as described by Hensler et al. (1994). Coronal, slide-mounted brain sections (20 μ m) were incubated with [³H]cyanoimipramine (1nM). Nonspecific binding was defined in the presence of 1 μ M paroxetine. At the end of the autoradiographic assay, sections were dried on a slide warmer and apposed to Kodak Biomax MR film (Amersham) for 4 weeks to generate autoradiograms. Analysis of the digitized autoradiograms was performed using the image analysis program NIH Image, version 1.47 (NIH, Bethesda, MD). Autoradiograms of [³H]CN-IMI binding were quantified by the use of simultaneously exposed [³H] standards (ART-123, American Radiochemicals, St. Louis, MO) which had been calibrated using brain-mash sections. The amount of ligand bound was determined by converting optical density measurements to femtomoles per milligram of protein. Specific binding was calculated by subtracting nonspecific binding from total binding on adjacent sections. The concentration of [³H]cyanoimipramine used is approximately eight times its K_d value, so the values obtained approximate maximal binding (B_{max}) values.

2.3 Statistical Analyses

Data were analyzed by one-ANOVA with Tukey's post-hoc comparisons, or paired t-tests (pre-versus post-drug clearance times within a subject) using Prism 6 (GraphPad). Non-linear least squares regression was used to calculate apparent maximal velocity (V_{max}) and *in vivo* affinity (K_T) values for serotonin clearance rate. The term K_T is used to differentiate from K_m values, which are calculated using *in vitro* preparations where diffusion through the extracellular matrix is not a factor. Data are presented as the mean and standard error of the mean.

3.0 Results

3.1 SERT expression and rate of serotonin clearance in hippocampus are not affected by constitutive reduction or loss of 5-HT_{1B} receptors

Hippocampus was selected for these studies given its importance in mood and antidepressant drugs effects, including SSRIs, which target SERT (Campbell and McQueen, 2004). We used high-speed chronoamperometry to measure 5-HT clearance *in vivo* by locally applying exogenous 5-HT into CA3 region of hippocampus so as to attain concentrations at the recording electrode (peak signal amplitude) ranging from 0.2 to 10 μ M. Given that synaptic concentrations of 5-HT are estimated to be 6 mM, this concentration range is physiologically relevant (Bunin and Wightman, 1998). Figure 1.A shows that, as expected, rate of 5-HT clearance increased with increasing 5-HT concentration; however, 5-HT clearance rate did not differ among 5-HT_{1B} receptor genotypes. Non-linear regression analyses revealed no significant difference in the apparent V_{max} or K_T values between wild-type and KO mice (V_{max} 259 ± 38 nM/sec and 303 ± 89 nM/sec, K_T 9.8 ± 2.2 μ M and 14.0 ± 6.0 μ M, for 5-HT_{1B}^{+/+} and 5-HT_{1B}^{-/-} mice, respectively). These data indicate that SERT efficiency in CA3 region of hippocampus is not compromised by the absence of 5-HT_{1B} receptors.

Similar 5-HT clearance efficiencies among 5-HT_{1B}^{+/+}, 5-HT_{1B}^{+/-} and 5-HT_{1B}^{-/-} mice do not exclude the possibility that SERT expression may differ among genotypes. For example, to counter loss of 5-HT_{1B} receptor regulation of SERT, one prediction might be that SERT expression increases. However, as shown in Figure 1.B, [³H]cyanoimpramine binding in CA3 region of hippocampus did not differ among genotypes indicating that SERT density in this brain region is not altered by the absence of 5-HT_{1B} receptors.

3.2 5-HT_{1B} receptors are required for cyanopindolol-induced, but not fluvoxamine-induced inhibition of 5-HT clearance in hippocampus

We have previously shown that the 5-HT_{1B} receptor antagonist, cyanopindolol, inhibits 5-HT clearance in CA3 region of rat hippocampus (Daws et al., 2000). Here we found that this effect of cyanopindolol was lost in 5-HT_{1B} receptor KO mice. Figure 2.A shows that the effect of intrahippocampally applied cyanopindolol (20 pmol) to inhibit clearance of 5-HT was 5-HT_{1B} receptor gene dose-dependent; greatest inhibition occurred in 5-HT_{1B}^{+/+} mice, there was no effect in 5-HT_{1B}^{-/-} mice and an intermediate effect in 5-HT_{1B}^{+/-} mice ($F_{2,15} = 5.55$, $P < 0.01$). Data in Figure 2.A are expressed as the percent change in 5-HT clearance time 2 min following intrahippocampal administration of cyanopindolol, where clearance time is defined as the time for 5-HT to be cleared by 80% of peak signal amplitude (T_{80} , in s). Baseline (i.e. before cyanopindolol administration) T_{80} values did not differ among genotypes (94 ± 14 s, 98 ± 8 s and 86 ± 10 s for 5-HT_{1B}^{+/+}, 5-HT_{1B}^{+/-} and 5-HT_{1B}^{-/-} mice, respectively). Cyanopindolol significantly prolonged clearance time for 5-HT in 5-HT_{1B}^{+/+} mice (152 ± 31 s, $t_5 = 2.62$, $P < 0.05$) and 5-HT_{1B}^{+/-} mice (131 ± 16 s, $t_6 = 3.13$, $P < 0.05$), but was without effect in 5-HT_{1B}^{-/-} mice (81 ± 14 s, $t_4 = 0.49$, ns). The effect of cyanopindolol to inhibit 5-HT clearance was maximal 2 min following intrahippocampal administration and returned to baseline T_{80} values within 20 min following intrahippocampal administration.

In these experiments 5-HT was locally applied to CA3 region of hippocampus to attain signals with peak amplitudes of less than 1 μ M. We have previously shown that clearance of 5-HT in this concentration range is largely driven by SERT (Daws and Toney, 2007). Baseline signal amplitudes among genotypes did not differ and averaged $0.62 \pm 0.04 \mu\text{M}$ ($n = 18$, all genotypes combined) and were not changed following intrahippocampal administration of cyanopindolol. Vehicle had no effect on 5-HT signal parameters in any genotype.

To investigate the requirement of SERT for cyanopindolol-induced inhibition of 5-HT clearance, we also studied mice constitutively lacking SERT. Cyanopindolol was without effect on 5-HT clearance in SERT $^{-/-}$ mice (Figure 2.A). As expected, and consistent with our published findings (Montañez et al., 2003) the SSRI fluvoxamine, was also without effect in SERT $^{-/-}$ mice (Figure 2.B).

To assess SERT activity in 5-HT $_{1B}$ mutant mice we examined the effect of fluvoxamine to inhibit 5-HT clearance. Consistent with our published findings in wild-type C57Bl/6 mice (Montañez et al., 2003; Baganz et al., 2010), fluvoxamine inhibited 5-HT clearance time in 5-HT $_{1B}^{+/+}$ mice (T_{80} pre-fluvoxamine 84 ± 8 s, vs 2 min post-fluvoxamine 110 ± 8 s, $t_5 = 4.61$, $P < 0.01$). Similarly, fluvoxamine inhibited 5-HT clearance time in 5-HT $_{1B}^{+/-}$ mice (pre- 75 ± 15 s vs post- 106 ± 15 s, $t_5 = 3.28$, $P < 0.05$) and 5-HT $_{1B}^{-/-}$ mice (pre- 104 ± 36 s vs post- 160 ± 41 s, $t_4 = 6.27$, $P < 0.01$). The percent change from baseline T_{80} values is shown in Figure 2.B and did not differ significantly among 5-HT $_{1B}$ genotypes.

4.0 Discussion

The key finding from the present data is that both the 5-HT $_{1B}$ receptor and SERT are necessary for the action of the 5-HT $_{1B}$ antagonist, cyanopindolol, to inhibit 5-HT clearance in CA3 region of hippocampus. Constitutive KO of either of these important regulatory proteins for serotonergic neurotransmission abolishes the ability of cyanopindolol to inhibit 5-HT clearance. These results add to a growing literature supporting the view that SERT is under the regulatory control of 5-HT $_{1B}$ receptors (Daws et al., 2000, Ase et al., 2001; Malagié et al., 2001; Hagan et al., 2012).

Present findings are in agreement with reports from other investigators using 5-HT $_{1B}$ KO mice. Hagan and co-workers (2012) using rotating disk voltammetry in whole brain synaptosomal preparations *in vitro*, found that the highly selective 5-HT $_{1B}$ receptor antagonist, SB224289, dose-dependently inhibited 5-HT uptake in 5-HT $_{1B}^{+/+}$ mice, but not in 5-HT $_{1B}^{-/-}$ mice. Likewise, these investigators also found that V_{max} and K_m for 5-HT uptake did not differ between 5-HT $_{1B}^{+/+}$ and 5-HT $_{1B}^{-/-}$ mice, nor did [^3H]citalopram binding to SERT in synaptosomes prepared from whole brain. Ase and co-workers (2001), using quantitative autoradiography to measure [^3H]citalopram binding to SERT in regionally distinct brain regions also found no differences between 5-HT $_{1B}^{+/+}$ and 5-HT $_{1B}^{-/-}$ mice in the majority of regions studied, including cortex, thalamus, hypothalamus, amygdala, brainstem and dorsal hippocampus. However, the ventral hippocampus was one region (of few) where [^3H]citalopram binding was found to be significantly increased. The reason for this highly brain-region specific effect of constitutive 5-HT $_{1B}$ KO on SERT

density remains unclear, though is associated with hyperinnervation of 5-HT fibers to these regions (Ase et al., 2001). Of note, Malagié and co-workers (2001) found that microdialysate levels of 5-HT in 5-HT_{1B} receptor KO mice following a single injection of the SSRI, paroxetine, were increased in ventral hippocampus, but not in cortex, relative to wild-type mice. The greater increase in extracellular 5-HT in ventral hippocampus in 5-HT_{1B} receptor KO mice following administration of paroxetine is consistent with the greater density of SERT in 5-HT_{1B} KO mice compared to 5-HT_{1B} wild-type mice. Thus, data for SERT density and the effect of SSRIs to increase extracellular 5-HT, or inhibit 5-HT clearance (present study) are in good agreement, and suggest that SERT density increases to compensate for constitutive loss of 5-HT_{1B} receptors in ventral, but not dorsal hippocampus.

Related to these observations, are reports that 5-HT_{1B} receptor expression and functional coupling is reduced in globus pallidus and substantia nigra of SERT KO mice (Fabre et al., 2000; Shanahan et al., 2009). While there are no published reports for 5-HT_{1B} receptor density in the hippocampus of which we are aware, in preliminary studies we found that [³H]cyanopindolol binding to 5-HT_{1B} receptors in hippocampus did not vary among SERT genotypes, but did follow the expected 5-HT_{1B} receptor gene-dose relationship in 5-HT_{1B}^{+/+}, 5-HT_{1B}^{+/-} and 5-HT_{1B}^{-/-} mice. Interestingly, SERT KO mice have markedly attenuated responses to 5-HT_{1B} agonist-induced behaviors (Shanahan et al., 2009). Thus, although our preliminary data indicate no differences in 5-HT_{1B} receptor expression in hippocampus among SERT genotypes, the functionality of these receptors may be compromised in SERT KO mice. Based on information in hand it appears, at least in dorsal hippocampus, that 5-HT_{1B} receptor expression is not influenced by constitutive KO of SERT, and SERT expression is not influenced by constitutive KO of 5-HT_{1B} receptors.

The finding that kinetics (V_{max} , K_m/K_T) for 5-HT uptake/clearance do not differ among 5-HT_{1B} receptor genotypes, at least in brain regions where SERT density is not affected by constitutive KO of 5-HT_{1B} receptors, might seem at odds with the notion the SERT is under regulatory control of the 5-HT_{1B} receptor. However, it is possible that transporters for 5-HT, other than SERT, may increase their expression or function to compensate for this loss of 5-HT_{1B} receptor regulatory control of SERT. We, and others, have shown that 5-HT uptake is governed by multiple transporters and is contingent upon the concentration of 5-HT in the extracellular milieu (Daws, 2009). The findings of Hagan and co-workers (2012) suggest that the activity of non-SERT clearance mechanisms (e.g. organic cation transporters, plasma membrane monoamine transporter) may be enhanced in 5-HT_{1B} KO mice and may account for the equivalent V_{max} values for 5-HT uptake between genotypes. Although not significant, they found K_m values tended to be greater (i.e. lower affinity) in 5-HT_{1B}^{-/-} mice, than in 5-HT_{1B}^{+/+} mice (Hagan et al., 2012), and our data are consistent with this finding (also see Daws and Toney, 2007; Daws, 2009). Lower affinity values for 5-HT uptake/clearance are in line with the idea that low-affinity, but high-capacity transporters for 5-HT may be a compensatory mechanism to maintain 5-HT homeostasis in brain regions of mice with a constitutive KO of the 5-HT_{1B} receptor where SERT activity, if not expression, is compromised. Future studies will be important in this regard; likewise studies to understand the remarkable distinction in brain regions where SERT expression and innervation of 5-HT fibers is increased in 5-HT_{1B} KO mice.

Collectively, studies to date suggest that SERT is subject to regulation by 5-HT_{1B} autoreceptors. Although 5-HT_{1B} receptors are also heteroreceptors (e.g. located on cholinergic neurons) and therefore their involvement cannot be ruled out, studies in synaptosomal preparations suggest a primary role for 5-HT_{1B} autoreceptors (Hagan et al., 2012). SERT has already been shown to be under the regulatory influence of a number of receptors (including alpha-2 adrenoceptors, adenosine-3 and interleukin-1 β receptors) (see Blakely and Edwards 2012 for review); present data, together with existing literature indicate that the 5-HT_{1B} receptor is another element that fine-tunes activity of SERT. Future studies to identify the signaling mechanisms involved in 5-HT_{1B} receptor regulation of SERT will be important avenues to help elucidate novel therapeutic targets as well as help understand the relationship between gene variants of the 5-HT_{1B} receptor and psychiatric disorders.

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Highlights

- Serotonin clearance rate is not affected by constitutive KO of 5-HT_{1B} receptors.
- SERT expression is similar among 5-HT_{1B} receptor +/+, +/- and -/- mice.
- 5-HT_{1B} receptors are required for cyanopindolol-induced inhibition of 5-HT clearance.
- SERTs are required for cyanopindolol-induced inhibition of 5-HT clearance.
- Data provide further evidence for 5-HT_{1B} receptor regulation of SERT.

Figure 1.

Serotonin clearance rate and SERT expression in CA3 region of hippocampus are not different among 5-HT_{1B} receptor genotypes. **A.** Clearance rate of 5-HT applied locally into the CA3 region of hippocampus increased as a function of increasing extracellular concentration of 5-HT, and was not different among 5-HT_{1B} receptor +/+, +/- and -/- mice. Data are mean and SEM, n=4-10 mice/genotype/5-HT concentration. **B.** Specific [³H]cyanoimipramine binding to SERT in CA3 region of hippocampus did not vary among 5-HT_{1B} receptor genotypes. Data are mean and SEM.

Figure 2.

The ability of cyanopindolol, but not fluvoxamine, to inhibit 5-HT clearance is dependent on 5-HT_{1B} receptor genotype and requires the presence of the 5-HT_{1B} receptor and SERT. **A.** Effect of cyanopindolol to inhibit 5-HT clearance time (T_{80}) in 5-HT_{1B} receptor +/+, +/- and -/- mice (1-way ANOVA, $F_{2,15} = 5.55$, $P < 0.01$; Tukey's post-hoc comparisons [pentastar2] $P < 0.05$, 5-HT_{1B}+/+ vs 5-HT_{1B}-/-). The hatched bar shows no effect of cyanopindolol in SERT-/- mice. **B.** Effect of fluvoxamine to inhibit 5-HT clearance time (T_{80}) in 5-HT_{1B} receptor +/+, +/- and -/- mice (1-way ANOVA, ns). The hatched bar shows no effect of fluvoxamine in SERT-/- mice. Data are mean and SEM.