The effects of selective serotonin reuptake inhibitors on extracellular 5-HT in the hippocampus of 5-HT$_{1B}$ receptor knockout mice

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Abstract

The effects of two selective serotonin reuptake inhibitors (SSRIs) on 5-hydroxytryptamine (5-HT) in the hippocampus were studied in wildtype and in 5-HT$_{1B}$ receptor knockout (KO) mice using in vivo microdialysis. Basal 5-HT levels were not different between the two genotypes. The functional absence of 5-HT$_{1B}$ receptors was examined in the knockout mice by local infusion of the 5-HT$_{1B}$ receptor agonist, CP93129 (1,4-Dihydro-3-(1,2,3,6-tetrahydro-4-pyridinyl)-5H-pyrrolo[3,2-b]pyridin-5-one) into the hippocampus. CP93129 (1 µM) decreased 5-HT levels in wildtype mice, but not in 5-HT$_{1B}$ KO mice. Systemic administration of the SSRI paroxetine (5 mg/kg i.p.) increased extracellular 5-HT levels. The increase of 5-HT in 5-HT$_{1B}$ KO mice was almost twofold higher than in wildtype mice. Systemic administration of SSRIs stimulates both terminal 5-HT$_{1B}$ autoreceptors and somatodendritic 5-HT$_{1A}$ autoreceptors. Therefore, another
SSRI, fluvoxamine, was applied locally into the hippocampus in order to activate only terminal 5-HT$_{1B}$ autoreceptors. Local administration of 0.3 µM fluvoxamine resulted in comparable increases in extracellular 5-HT in both genotypes, whereas 1.0 µM fluvoxamine produced a twofold greater increase in 5-HT levels in 5-HT$_{1B}$ KO as compared to wildtype mice. In conclusion, the differences in hippocampal 5-HT output between wildtype and 5-HT$_{1B}$ KO mice after local or systemic SSRI administration show that 5-HT$_{1B}$ autoreceptors play a significant role in the inhibition of 5-HT release at serotonergic nerve terminals. In addition, the different dose-response to fluvoxamine between the two genotypes also suggest that 5-HT$_{1B}$ KO mice have possible adaptations of 5-HT transporters in order to compensate for the loss of the terminal 5-HT$_{1B}$ autoreceptor.

Introduction

Selective serotonin reuptake inhibitors (SSRIs) are widely used in the treatment of psychiatric conditions like depression and anxiety disorders. SSRIs exert their effects by blocking serotonin (5-HT) reuptake, thereby increasing extracellular 5-HT levels. Since the onset of clinical efficacy is delayed to 2-4 weeks, it is suggested that adaptive processes might be implicated. The release of 5-HT in the forebrain is controlled by autoreceptors on serotonergic cell bodies and on terminals in projection areas. The terminal 5-HT autoreceptor is of the 5-HT$_{1B}$ subtype and stimulation of this receptor results in decreased 5-HT release (Engel et al., 1986, Maura et al., 1986, Hoyer and Middlemiss, 1989). In vivo microdialysis studies have shown that local infusion of a 5-HT$_{1B}$ receptor agonist into the hippocampus results in a decrease of extracellular 5-HT in rat (Hjorth and Tao, 1991, Bosker et al., 1995) and mouse (Trillat et al., 1997). Chronic treatment with antidepressants alters 5-HT autoreceptors in rats (Haddjeri et al., 1999, Le Poul et al., 2000). Terminal 5-HT$_{1B}$ receptors in different brain areas including the hippocampus, hypothalamus and frontal cortex have been found to desensitize after sustained administration of SSRIs (Blier et al., 1984, Moret and Briley, 1990, O'Connor and Kruk, 1994, el Mansari et al., 1995, Newman et al., 2000). Several studies on the role of autoreceptors in the mechanism of action of SSRIs, have used selective 5-HT$_{1A}$ and non-selective 5-HT$_{1B/1D}$ receptor antagonists in combination with SSRIs. The combination of a SSRI with a 5-HT$_{1A}$ and 5-HT$_{1B}$ receptor antagonists synergistically increases 5-HT release in rat frontal cortex (Gobert et al., 1997, Sharp et al., 1997, Dawson and Nguyen, 2000), guinea pig frontal cortex, hippocampus (Roberts et al., 1996) and hypothalamus (Rollema et al., 1996). Due to a lack of selective 5-HT$_{1B}$ receptor antagonists, there is
SSRI-effects in the hippocampus

relatively little data on the role of the terminal 5-HT\textsubscript{1B} autoreceptor in controlling 5-HT release. The selective 5-HT\textsubscript{1B} receptor antagonist, SB-224289, potentiated the effect of fluoxetine in rat frontal cortex (Gobert et al., 2000) and produced an increase in extracellular 5-HT in the hippocampus, but not in the frontal cortex of guinea pigs (Roberts et al., 1998). 5-HT\textsubscript{1B} receptors are located in serotonergic projections to forebrain regions as terminal autoreceptors and as heteroreceptors. Several studies indicate that 5-HT\textsubscript{1B} receptors present in the dorsal raphe nucleus (DRN) are also involved in the release of 5-HT (Starkey and Skingle, 1994, Pineyro et al., 1995a, Moret and Briley, 1997, Davidson and Stamford, 1995). Thus, blockade of 5-HT\textsubscript{1B/1D} receptors in the raphe nuclei after systemic administration of 5-HT\textsubscript{1B/1D} receptor antagonists may also contribute to the effects on 5-HT levels in output areas.

The development of 5-HT\textsubscript{1B} receptor knockout (KO) mice has generated a model to study the importance of the 5-HT\textsubscript{1B} receptor (Saudou et al., 1994) and allows the investigation of SSRI actions in the absence of 5-HT\textsubscript{1B} receptors. Recent studies have found increased 5-HT levels in hippocampus and frontal cortex after systemic SSRI administration in mice lacking 5HT\textsubscript{1B} receptors (Malagie et al., 2001, Knobelman et al., 2001b).

In the present study we report on the effects of SSRIs on 5-HT release in the hippocampus of wild type and 5-HT\textsubscript{1B} KO mice following systemic and local administration. In mice lacking the 5-HT\textsubscript{1B} receptor, augmented 5-HT responses are expected following SSRI administration due to the absence of inhibitory terminal 5-HT\textsubscript{1B} autoreceptors. Since, systemic administration of SSRIs also activates 5-HT autoreceptors in the raphe nuclei, effects of local administration of the SSRI fluvoxamine were studied to assess the role of the terminal 5-HT\textsubscript{1B} autoreceptor.

Material & Methods

Animals

In this study, male wildtype and 5-HT\textsubscript{1B} knockout mice with a 129/SV genetic background were tested. The mice were group-housed, eight per cage and kept on a 12-hour light-dark cycle (6 a.m. on, 6 p.m. off) at constant room temperature (22 ± 2°C) with freely available food and water. For the experiments, bodyweights of the mice were between 25 and 30 grams at an age between 12 and 16 weeks. The two genotypes differ in bodyweight, the 5-HT\textsubscript{1B} KO mice being slightly heavier as compared to the wildtype mice (Bouwknecht et al., 2001b). Wildtype and 5-HT\textsubscript{1B} knockout mice were bred in separate homozygous lines at the animal
facilities, GDL, Utrecht, The Netherlands. The original wildtype and 5-HT\textsubscript{1B} knockout mice were obtained from Dr. René Hen, Columbia University, New York, USA. See for details on the generation of the 5-HT\textsubscript{1B} KO mice the original publication by Saudou et al (1994). The ethical committee for animal research of the University Medical Center Utrecht, The Netherlands, approved the study.

**Surgery**
Mice were anaesthetized with chloralhydrate (400 mg/kg, i.p.) and lidocaine (2%) was applied on the skull. For surgery, the mice were placed in a stereotaxic frame using a mouse adaptor (Stoelting, Germany) with modified earbars. The mice were on a heating pad during surgery. A small hole was drilled in the skull for the implantation of the probe and two small holes for anchor screws. A self-constructed microdialysis probe with an AN filtral 69 membrane, outer diameter 310 µM (Hospal, Uden, The Netherlands), was placed in the right hippocampus. The coordinates were: AP -2.8 mm from bregma, ML -3.5 mm, DV -4.0 mm from dura, according to the stereotaxic atlas of the mouse brain (Franklin and Paxinos, 1997). The exposed membrane tip length was 2 mm. The probe was secured in place with dental cement and two anchor screws in the skull. After surgery, mice were injected with saline (0.5 ml i.p.) to prevent dehydratation and the mice were housed separately.

**Microdialysis**
Microdialysis experiments started the day after surgery. Mice were tested on two subsequent days to reduce the number of animals. The treatment groups were randomized over the two days. Mice were in their homecage during the experiments. The animals were connected to a high precision pump (Harvard PHD2000, Harvard Scientific, USA) using mouse swivels (Type 375/25, Instech Laboratories, Inc, USA) and PEEK-tubing (ID0.005", OD0.020") to allow free movements of the mice. Ringer solution (147 mM NaCl, 2.3 mM KCl, 2.3 mM CaCl\textsubscript{2}, 1.0 mM MgCl\textsubscript{2}) was perfused through the microdialysis probe with a flow of 1.13 µl/min. The experiments were performed during the light phase and started after three hours equilibration. Samples were collected every 20 minutes in vials containing 7.5 µl acetic acid, using fraction collectors (type 142, CMA, Sweden). Samples were stored at −80°C until High Performance Liquid Chromatography (HPLC) analysis. At the end of the experiment the mice were decapitated, the brains were removed and fixed in 4% formaldehyde. The brains were cut in 50 µm slices on a vibratome to verify the position of the probe. In case of improper probe placement, data was excluded.
**Drugs**

Paroxetine (donated by GSK, Harlow, UK) was dissolved in sterile 0.9 % saline on the day of the experiment. Fluvoxamine (donated by Solvay Pharmaceuticals, Weesp, The Netherlands) and CP93129 dihydrochloride (1,4-Dihydro-3-(1,2,3,6-tetrahydro-4-pyridinyl)-5H-pyrrolo[3,2-b]pyridin-5-one, obtained from Tocris, UK) were dissolved in Ringer solution.

**HPLC-ECD analysis**

5-HT was analyzed by HPLC with electrochemical detection. Samples (25 µl) were injected onto a LUNA 3 µm RP18 column (100x2 mm, Phenomenex, Bester, The Netherlands) using a Triathlon autosampler and a Gynkotek P580 pump (Separations, The Netherlands). Separation was performed at 40ºC. The electrochemical detector (Intro, ANTEC Leyden, The Netherlands) was set at a potential of 600 mV against an Ag/AgCl reference electrode. The signal was analyzed using Gynkotek software. The mobile phase consisted of 5 g/l (NH₄)₂SO₄, 150 mg/l heptane sulphonic acid sodium salt, 0.5 g/l EDTA, 5% methanol, 30 µl/l triethylamine, 30 µl/l acetic acid, pH 4.6. Flow rate was 0.3 ml/min. The detection limit for 5-HT was 0.5 fmol/25 µl sample (signal to noise ratio 2).

**Data analysis and statistics**

Values for the first four consecutive samples were averaged to calculate the basal levels of extracellular 5-HT. Student’s t-tests were used to compare basal 5-HT values between the two genotypes. In the figures, all values are expressed as percentages of basal levels ± SEM. The data were statistically analyzed with SPSS (Version 8.0). Effects of 5-HT response to SSRI treatment were analyzed by multivariate analysis of variance (ANOVA) with time as ‘within’ and dose and genotype as ‘between’ factors. When appropriate, data were broken down and effects of drug dosages or genotype were analyzed by pairwise comparison. For the treatment period, the area under the curve (AUC) was calculated. AUC values were analyzed with ANOVA and post hoc analyses when appropriate. The significance level for all analyses was set at 5%. In the figures the point of injection or start of the local infusion (timepoint zero) is corrected for the lagtime of the microdialysis system.
Results

Basal 5-HT levels
Basal levels of extracellular 5-HT in the hippocampus were not different between the two genotypes (ANOVA; F(1,68)=2.0, p=0.17, NS). The mean basal levels were $5.66 \pm 0.47$ fmol/sample for wildtype (n=34) and $6.63 \pm 0.54$ fmol/sample for 5-HT$_{1B}$ KO mice (n=35).

Effects of the 5-HT$_{1B}$ receptor agonist CP93129
The 5-HT$_{1B}$ receptor agonist, CP93129 (1 µM), was infused by reversed microdialysis for a period of 60 minutes (Fig. 1). ANOVA revealed an effect of dose by genotype (F(1,26)=6.8, p<0.05). In wildtype mice, local administration of 1 µM CP93129 significantly decreased extracellular 5-HT to $58 \pm 11\%$ from baseline levels. Local infusion of CP93129 had no effect in 5-HT$_{1B}$ KO mice, demonstrating the functional absence of 5-HT$_{1B}$ receptors in the knockout mice.

Effects of systemic administration of paroxetine
Acute administration of paroxetine (5 mg/kg, i.p.) increased extracellular 5-HT in the hippocampus of wildtype and 5-HT$_{1B}$ KO mice (Fig. 2). ANOVA revealed significant effects of time (F(8,186)=9.7, p<0.001), dose (F(1,21)=20.9, p<0.001) and of genotype (F(1,21)=7.8, p<0.05). wildtype mice (F(1,11)=21.9, p<0.01) and in 5-HT$_{1B}$ KO mice (F(1,10)=8.7, p<0.05). Contrast analyses revealed a genotype effect for paroxetine treatment (F(1,11)=9.0, p<0.05). The paroxetine-induced
Fig 2. Systemic injection of paroxetine. Data are expressed as mean percent change in 5-HT from basal levels ± S.E.M. Saline (O) or paroxetine (5mg/kg, i.p.) (●) was injected at timepoint zero in wildtype mice (A) or 5-HT$_{1B}$ knockout mice (B). (C) Mean percent change in 5-HT from basal levels is expressed as the AUC ± S.E.M. for the treatment period after i.p. injection of saline or paroxetine (5 mg/kg) injection. Symbols: * indicates a significant drug effect P < 0.05) and ‡ indicates a significant genotype effect (P<0.05). For each group n=6-8 mice.
increase in extracellular 5-HT was almost twofold higher in 5-HT<sub>1B</sub> KO mice as compared to the wildtype mice. Taking the AUC as outcome variable similar results were obtained (Fig. 2C).

**Effects of local administration of fluvoxamine**

The local administration of fluvoxamine (0.3 and 1.0 µM) induced an increase in extracellular 5-HT in both genotypes (Fig. 3). ANOVA revealed an effect of time (F(12,408)=10.6, p<0.001), of dose (F(2,34)=57.8, p<0.001) and of dose by genotype (F(2,34)=10.4, p<0.001). In wildtype mice, administration of 0.3 and 1.0 µM fluvoxamine induced a significant increase in 5-HT as compared to vehicle (F(2,17)=39.0, p<0.001), but no difference between the two fluvoxamine dosages. In 5-HT<sub>1B</sub> KO mice, both dosages of fluvoxamine increased 5-HT levels as compared to vehicle (F(2,17)=29.6, p<0.001), the difference being greater with the highest dosage. Statistical analyses revealed a dose-dependent effect (F(1,11)=14.6, p<0.05). When data were broken down on dosage, a significant difference was found for the highest dosage of fluvoxamine between the genotypes (F(1,12)=9.1, p<0.05). The highest dosage of fluvoxamine induced an almost twofold greater increase in extracellular 5-HT in the knockout as compared to the wildtype mice. No differences in 5-HT response were found between the genotypes for the lower dosage of fluvoxamine or vehicle. Taking the AUC as outcome variable, similar results were obtained. The highest dosage of fluvoxamine (1.0 µM) induced in an almost twofold increase in 5-HT in the knockouts as compared to the wildtype mice (Fig. 3C).

**Discussion**

The main finding of the present study is that extracellular levels of 5-HT are regulated differently in mice lacking the 5-HT<sub>1B</sub> receptors as compared to wildtype mice. Basal extracellular 5-HT levels in 5-HT<sub>1B</sub> KO and wildtype mice were similar, but blockade of 5-HT reuptake sites by systemic paroxetine administration resulted in a greater increase in hippocampal 5-HT in the 5-HT<sub>1B</sub> KO than in wildtype mice. Inhibition of the uptake sites in the hippocampus by local administration of fluvoxamine resulted also in augmented 5-HT levels in the 5-HT<sub>1B</sub> KO mice, but the effect appeared to be dose-related in that it was only apparent at the higher SSRI concentration. In addition, local administration of the 5-HT<sub>1B</sub> receptor agonist CP93129 into the hippocampus decreased absence of the 5-HT<sub>1B</sub> autoreceptor in the latter strain.
Fig 3. Local administration of fluvoxamine into the hippocampus. Data are expressed as mean percent change in 5-HT from basal levels ± S.E.M.. Ringer solution (○), 0.3 µM (●) or 1.0 µM fluvoxamine (▲) was infused for 240 minutes starting at timepoint zero in wildtype mice (A) or 5-HT₁B knockout mice (B). (C) Mean percent change in 5-HT from is expressed basal levels as the AUC ± S.E.M. after infusion of Ringer solution (0 µM), 0.3 µM or 1.0 µM fluvoxamine. Symbols: * indicates a significant drug effect (P<0.05) and ‡ indicates a significant genotype effect (P<0.05). For each group n=6-8 mice.
These data confirm and extend previous findings using systemic administration of SSRIs, indicating that 5-HT$_{1B}$ autoreceptor stimulation limit the effects of acute SSRI administration (Malagie et al., 2001, Knobelman et al., 2001b). A confounding factor of studies using systemic administration of SSRIs is that an effect of somatodendritic 5-HT$_{1A}$ autoreceptors on 5-HT output cannot be excluded. There is circumstantial evidence that stimulation of 5-HT$_{1B}$ receptors in the raphe nuclei may affect 5-HT levels in projection areas (Pineyro and Blier, 1996, Davidson and Stamford, 1995). Studies with mixed 5-HT$_{1B/1D}$ receptor antagonists have revealed differences between the dorsal raphe and medial raphe nucleus innervated brain areas (Roberts et al., 1997, Roberts et al., 1998). The hippocampus receives innervation from both the dorsal raphe and medial raphe nucleus, although there is a preferential input from the medial raphe nucleus to the dorsal part of the hippocampus (Steinbusch, 1981, Mokler et al., 1998). Moreover, systemic administration of SSRIs may also affect other 5-HT modulating receptors in the raphe nuclei, such as the 5-HT$_{1A}$ autoreceptor and the 5-HT$_{1D}$ receptor. To exclude these effects, we applied the SSRI fluvoxamine and the 5-HT$_{1B}$ receptor agonist, CP93129, locally into the hippocampus by reversed microdialysis. Although the microdialysis probe was placed mainly in the ventral part of the hippocampus, we refer to the hippocampus rather than to the ventral hippocampus, due to the relative small size of the mouse brain. We used fluvoxamine, rather than paroxetine, because the former SSRI was found to have better properties to cross the dialysis membrane used in this study. After local administration of fluvoxamine 5-HT levels were stable after one hour of infusion, whereas after local infusion of paroxetine (1 µM) 5-HT levels increased slowly and were not stable after three hours of infusion (data not shown). Our results confirm the view that release of 5-HT in the hippocampus is regulated by terminal 5-HT$_{1B}$ autoreceptors. The data are also in line with studies in other species using 5-HT$_{1B}$ receptor antagonists. Thus, GR127935, a mixed 5-HT$_{1B/1D}$ receptor antagonist, potentiated the effects of SSRIs in rat frontal cortex, presumably by blocking terminal 5-HT$_{1B}$ autoreceptors, after systemic (Gobert et al., 1997, Roberts et al., 1997 or local administration (Hertel et al., 1999). SB224289, a more selective 5-HT$_{1B}$ receptor antagonist, also augmented the SSRI-induced increase of extracellular 5-HT levels in rat frontal cortex, and guinea pig frontal cortex and hippocampus (Gobert and Millan, 1999, Roberts et al., 1999). Finally, local administration of a new selective 5-HT$_{1B}$ receptor antagonist, NAS-181, in the presence of a SSRI, resulted in increased extracellular 5-HT levels in rat hippocampus and prefrontal cortex (Hjorth et al., 2000). Taken together, these findings support the notion that terminal 5-HT$_{1B}$ autoreceptors negatively regulate 5-HT release and thereby restrain the effects of acute SSRI administration. Thus,
increased 5-HT levels were expected in mice lacking 5-HT$_{1B}$ receptors. In line with previous studies, we found that basal 5-HT levels in 5-HT$_{1B}$ KO mice were not different from those in the wildtype mice (Malagie et al., 2001, Knobelman et al., 2001a). This would suggest that either 5-HT$_{1B}$ receptors do not display endogenous activity under basal conditions or that compensatory changes have taken place during neurodevelopment. Interestingly, we found that two concentrations of fluvoxamine (0.3 and 1.0 µM) resulted in similar increases in extracellular 5-HT in the wildtype mice, suggesting that a maximum blockade of 5-HT reuptake was already achieved after the lower concentration of fluvoxamine. In 5-HT$_{1B}$ KO mice, on the other hand, the increase in extracellular 5-HT was not different from the wildtypes at the lower concentration of fluvoxamine, but augmented at the higher concentration, indicating a different dose-response relationship. A likely explanation could be an adaptation of the 5-HT transporter in the 5-HT$_{1B}$ KO mice during development, resulting in a different functional capacity. A higher uptake capacity could compensate for the lack of terminal 5-HT autoinhibition and may account for the normal basal 5-HT levels in the 5-HT$_{1B}$ KO mice as well as the higher concentration of the SRRI necessary to completely block the uptake of 5-HT. Developmental alterations have been previously described for the 5-HT transporter KO mice. These 5-HT transporter KO mice have upregulated 5-HT$_{1A}$ and 5-HT$_{1B}$ receptors in some brain areas to compensate for the lack of 5-HT transporters (Fabre et al., 2000). There is evidence for adaptation of the 5-HT$_{1A}$ receptors based on the finding that 5-HT$_{1B}$ KO mice show a reduced 5-HT response in the hippocampus after systemic injection of a 5-HT$_{1A}$ receptor agonist (Knobelman et al., 2001a). The authors propose based on this finding that 5-HT$_{1B}$ KO mice have desensitized 5-HT$_{1A}$ receptors in the MRN to compensate for the loss terminal 5-HT$_{1B}$ autoreceptors. These finding are not supported by other studies, since densities of 5-HT$_{1A}$ receptors (Malleret et al., 1999, Evrard et al., 1999) and raphe neuronal firing after stimulation of 5-HT$_{1A}$ receptors are normal in 5-HT$_{1B}$ KO mice (Evrard et al., 1999). Furthermore, postsynaptic 5-HT$_{1A}$ receptor function as measured by corticosterone release was normal in 5-HT$_{1B}$ KO mice (Bouwknecht et al., 2001c). In the present study, SSRIs were administered locally into the hippocampus and therefore it is unlikely that somatodendritic 5-HT autoreceptor changes account for the present findings. Recently, regional changes in 5-HT transporters have been reported for 5-HT$_{1B}$ KO mice. Binding densities of 5-HT transporters were increased in the ventral hippocampus and amygdalo-hippocampal nucleus of 5-HT$_{1B}$ KO mice (Ase et al., 2001). A higher density of 5-HT reuptake sites has also been demonstrated for the raphe nuclei of 5-HT$_{1B}$ KO mice (Evrard et al., 1999). In support of the explanation that 5-HT$_{1B}$ KO mice have changes in 5-HT
transporters, an interesting interaction between the 5-HT transporter and the 5-HT$_{1B}$ receptor has been reported. The clearance of 5-HT by the 5-HT transporter was prolonged after blockade of 5-HT$_{1B}$ receptors in the hippocampus (Daws et al., 2000). This finding suggests plasticity between the 5-HT transporter and the 5-HT$_{1B}$ autoreceptor. Taken together, these findings support compensatory changes of 5-HT transporters in mice lacking 5-HT$_{1B}$ receptors.

In conclusion, the differences in hippocampal 5-HT output between wildtype and 5-HT$_{1B}$ KO mice after local or systemic SSRI administration show that 5-HT$_{1B}$ autoreceptors play a significant role in the inhibition of 5-HT release at serotonergic nerve terminals. In addition, the results also suggest that 5-HT$_{1B}$ KO mice have possible adaptations of 5-HT transporters in order to compensate for the loss of the terminal 5-HT$_{1B}$ autoreceptor.