Research report

Differential effects of 5-HT₁₆ receptor deletion upon basal and fluoxetine-evoked 5-HT concentrations as revealed by in vivo microdialysis

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Abstract

An involvement of serotonin (5-HT) 1A receptors in the etiology of psychiatric disorders has been suggested. Hypo-responsiveness of the 5-HT₁₆ receptor is linked to anxiety and constitutive deletion of the 5-HT₁₆ receptor produces anxiety-like behaviors in the mouse. Evidence that 5-HT receptor inactivation increases the therapeutic effects of antidepressants has also been presented. The present studies used in vivo microdialysis and homologous recombination techniques to examine the contribution of 5-HT autoreceptors to these effects. Basal and fluoxetine-evoked extracellular concentrations of 5-HT were quantified in the striatum, a projection area of dorsal raphe neurons (DRN), of wild-type (WT) and 5-HT₁₆ receptor knock out (KO) mice. The density of 5-HT transporters was also determined. Basal 5-HT concentrations did not differ in WT and KO mice. Fluoxetine (10 mg/kg) increased 5-HT concentrations in both genotypes. This increase was, however, 2-fold greater in KO mice. In contrast, no differences in KCl-evoked 5-HT concentrations were seen. Similarly, neither basal nor stimulation-evoked DA differed across genotype. Autoradiography revealed no differences between genotype in the density of 5-HT transporters or post-synaptic 5-HT receptors, an index of 5-HT neuronal activity. These experiments demonstrate that, under basal and KCl stimulated conditions, adaptive mechanisms in the 5-HT system compensate for the lack of 5-HT₁₆ autoreceptor regulation of DRN. Furthermore, they suggest that the absence of release-regulating 5-HT₁₆ autoreceptors in the DRN cannot account for the anxiety phenotype of KO mice. The enhanced response to fluoxetine in KO mice is consistent with pharmacological studies and suggests that adaptive mechanisms that occur in response to 5-HT₁₆ receptor deletion are insufficient to oppose increases in 5-HT concentrations produced by acute inhibition of the 5-HT transporter. Published by Elsevier Science B.V.

Theme: Neurotransmitter, modulators, transporters and receptors

Topic: Serotonin

Keywords: 5-Hydroxytryptamine; 5-Hydroxytryptamine₁₆ receptor; Dialysis; Fluoxetine; Knockout

1. Introduction

Several laboratories [19,28,34,38] have shown that mice lacking the 5-HT₁₆ receptor exhibit anxiety-like behavior in different experimental models indicating that hypofunction of the 5-HT₁₆ receptor leads to anxiety. Indeed, hyporesponsiveness of the 5-HT₁₆ receptor is associated with panic disorder, one form of anxiety [25]. The role, however, of pre- versus post-synaptic 5-HT₁₆ receptors in the etiology of anxiety is unclear.

An involvement of 5-HT₁₆ receptors in the delayed onset of action of selective serotonin uptake inhibitors

Abbreviations: DA, dopamine; DRN, dorsal raphe nuclei; 5-HT, 5-hydroxytryptamine; KO, knockout; LSD, lysergic acid diethylamide; MRN, medial raphe nuclei; SSRI, selective serotonin reuptake inhibitor; WT, wild-type

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been attributed to the blockade of 5-HT autoreceptors were crossbred to produce homozygous F2 mutants [28].

Changes in 5-HT neurotransmission that occur following 1.2 mM MgCl₂, 5.4 mM D-glucose, and 0.25 mM ascorbic acid (pH 7.2±7.4) was perfused through the microdialysis probe. The in-flow tubing, ending at the swivel inside a mouse in the cage. The out-flow tubing followed the same path as the in-flow tubing, ending at the swivel inside a constant temperature (25°C).

Neurons in regulating mesostriatal dopamine (DA) concentrations were also monitored. Path as the in-flow tubing, ending at the swivel inside a mouse in the cage. The out-flow tubing followed the same path as the in-flow tubing, ending at the swivel inside a constant temperature (25°C).

The goals of the present studies were twofold. Since presynaptic 5-HT₁A receptors regulate 5-HT neurotransmission [12,21], we sought to determine whether the lack of presynaptic receptors in 5-HT₁A receptor knock out (KO) mice is associated with increases in the activity of 5-HT neurons. Secondly, we sought to determine whether the absence of these receptors results in an enhanced response of 5-HT neurons to the SSRI, fluoxetine. In vivo microdialysis was used to measure basal and stimulus-evoked 5-HT overflow in the dorsal striatum, a terminal projection area of the raphe that include the frontal cortex, hippocampus, and striatum [6,7,22–24]. Post-synaptic 5-HT₁A receptors are highly expressed in hippocampus, lateral septum, and the frontal and entorhinal cortex [30]. Lower concentrations are observed in amygdala, thalamic and hypothalamic nuclei, and the lateral striatum.

The coordinates (mm from bregma) were: AP: −1.8, ML: ±3.8, DV: 4.4 [40]. Four days after cannula implantation, the mice were gently restrained and a microdialysis probe (2 mm membrane, 6000 mol. wt. cutoff; CMA 11, CMA Inc.) was inserted into the guide cannula. The in-flow line to the probe was connected to a microinfusion pump (Harvard 22, Harvard Apparatus, South Natick, MA) via a liquid swivel (quartz lined) and wire tether. The tether was attached at one end to the liquid swivel and, at the other end, to a pedestal embedded in the cranial cement stage on the head. The swivel tether assembly (Instech Laboratories Inc., Plymouth Meeting, PA) was hung from the top of the cage by a balance arm (Instech Laboratories) to allow free movement of the mouse in the cage. The outflow tubing followed the same path as the inflow tubing, ending at the swivel inside a microcentrifuge tube. Artificial cerebral spinal fluid (aCSF) containing 145 mM NaCl, 2.8 mM KCl, 1.2 mM CaCl₂, 1.2 mM MgCl₂, 5.4 mM D-glucose, and 0.25 mM ascorbic acid (pH 7.2–7.4) was perfused through the microdialysis probe.
probe at a rate of 0.3 µl/min for an equilibration period of 8 h. The flow rate was increased to 0.6 µl/min, 2 h prior to the first sample collection, and remained at this rate for the experiment. Four consecutive 30 min dialysate samples were collected for determination of basal concentrations of 5-HT and DA. The aCSF was then replaced with that containing 60 mM KCl for 30 min. Three consecutive 30 min samples were then collected. Additional animals received an i.p. injection of fluoxetine (10.0 mg/kg) or saline and four 30-min dialysate samples were collected. Following completion of experiments, animals were euthanized and their brains removed for histological verification of probe placements. Only animals with probes confined to the dorsal striatum were used for subsequent data analysis.

2.3. Chromatographic analysis

The 5-HT and DA content of dialysate samples were analyzed using HPLC coupled to electrochemical detection. Chromatographic separations were performed with a microbore HPLC column (C18: i.d. 100×1 mm; 3 µm; BAS, West Lafayette, IN) in conjunction with a dual piston pump (PM 80; BAS, West Lafayette, IN). A six-port rotary valve (Model 7125, Rehodyne, Berkeley, CA) was used for sample injection. The mobile phase consisted of 25 mM NaH₂PO₄, 100 µM ethylenediaminetetraacetate, 3.9 mM sodium octyl sulfate, 8% methanol (v/v), 0.6% tetrahydrofuran (v/v), and an apparent pH of 5.0. The flow rate of the mobile phase was 100 µl/min. Electrochemical detection was accomplished using a BAS LC-4C amperometric detector. The working electrode was set at +650 mV versus Ag/AgCl. Output from the detector was recorded on a dual pen chart recorder. Standard curves were constructed for each analyte and used to quantify concentrations in dialysate samples. The detection limits for 5-HT and DA (signal/noise ratio: 3:1) were 0.25 and 0.5 nM, respectively.

2.4. 5-HT transporter and 5-HT₂A receptor autoradiography

Brains were removed and kept at −80°C until assayed. Coronal sections were cut with a microtome cryostat. Consecutive superimposable sections were used to determine total and nonspecific binding. 5-HT transporter binding was measured by using 1.5 nM [³H]cyano-imipramine (American Radiolabeled Chemical Inc., St. Louis, MO). [³H]Cyano-imipramine rather than [³H]paroxetine was used in view of the higher specific activity of the former ligand [16,38]. Sections were incubated overnight at +4°C in a buffer containing 50 mM Tris–HCl and 150 mM NaCl (pH 7.4). Nonspecific binding was determined in the presence of 2 µM fluoxetine. Sections were exposed to Hyperfilm (Amersham Pharmacia, Piscataway, NJ) for 4 weeks. 5-HT₂A receptor binding was carried out according to a published procedure [16,38]. [¹²⁵I]Labeled lysergic acid diethylamide (LSD, 50 pM) (NEN, Boston, MA) was used in the presence of 0.2 nM haloperidol to reduce marginal LSD binding to dopamine D₂ receptors. Sections were incubated 1 h at room temperature in 50 mM Tris–HCl buffer (pH 7.4). Nonspecific binding was determined in the presence of 200 nM spiperone. Sections were exposed overnight. Computerized densitometry was performed with the NIH Image program. Quantification was based on a series of [³H] and [¹²⁵I] autoradiographic internal standards (Amersham Pharmacia, Piscataway, NJ). Seven mice were examined in each group.

2.5. Data analysis

The Student’s t-test was used to compare basal DA and 5-HT concentrations (nM) in WT and KO mice. A two-factor (genotype×K⁺) analysis of variance (ANOVA) was used to analyze the effects of K⁺. A two-factor (genotype×time) repeated-measures analysis of variance was used to analyze the effects of acute administration of fluoxetine and saline on dialysate concentrations. Simple effects tests or pairwise group comparisons were used to probe significant differences identified by ANOVA. The Student’s t-test was used to analyze autoradiographic data. Statistical significance was assumed at P<0.05.

2.6. Drugs

Fluoxetine hydrochloride was a gift from Lilly Pharmaceuticals (Indianapolis, IN). Haloperidol and spiperone were obtained from RBI–Sigma (Natick, MA). Drugs were dissolved in water (fluoxetine) or ethanol (haloperidol and spiperone, 5 mg/ml stock solution) and injected i.p. in sterile saline (0.9% NaCl).

3. Results

Dialysate concentrations of a neurotransmitter provide an estimate of extracellular concentrations [29]. Therefore, in vivo microdialysis was used to determine if loss of somatodendritic 5-HT₁A receptors alters basal 5-HT concentrations in the dorsal striatum, a terminal projection area of DRN neurons. In view of the previously documented interactions of 5-HT and DA neurons [14], DA concentrations were also quantified. Despite the lack of presynaptic 5-HT₁A receptors in KO mice, no difference between genotype in basal dialysate 5-HT concentrations was observed (t(1,27)=0.93; P=0.9). Similarly, basal DA concentrations did not differ in KO as compared to WT mice (t(1,28)=0.8; P=0.4).

KCl was perfused through the dialysis probe in order to determine whether the depolarization-evoked release of 5-HT or DA differs between genotypes (Fig. 1). The
addition of 60 mM KCl to the perfusate significantly increased 5-HT concentrations in both WT and KO mice \((F(1,25)=31.4; \ P=0.001)\). There was no difference between genotype in the magnitude of these effects \((F(1,25)=0.8; \ P=0.4)\). Similarly, K⁺-evoked DA concentrations were similar in the two genotypes. ANOVA revealed a significant effect of K⁺ \((F(1,8)=7.6; \ P=0.03)\) but no significant effect of genotype \((F(1,8)=2.9; \ P=0.1)\).

Pharmacological studies indicate an important role of presynaptic 5-HT₁₅ receptors in opposing SSRI-evoked increases in 5-HT concentrations [2]. Fig. 2 shows that acute administration of the prototypic SSRI, fluoxetine (10 mg/kg; i.p.), increased 5-HT concentrations in both KO and WT mice. However, the magnitude of this effect was significantly greater in KO mice. ANOVA revealed significant effects of genotype \((F(1,19)=4.4; \ P=0.05)\) and time \((F(4,19)=16.8; \ P=0.001)\) and a significant genotype×time interaction \((F(4,19)=3.9; \ P=0.05)\). In contrast to fluoxetine, the acute administration of saline did not modify 5-HT concentrations in either genotype (genotype: \(F(1,10)=0.7; \ P=0.8\); time: \(F(4,40)=0.6; \ P=0.7\) (data not shown).

Fig. 3 shows the influence of acute fluoxetine administration upon DA concentrations in the striatum. In contrast to 5-HT, fluoxetine failed to modify DA concentrations in KO or WT mice. ANOVA revealed no significant effect of genotype \((F(1,21)=0.05; \ P=0.9)\) or time \((F(4,84)=0.5; \ P=0.8)\) on DA concentrations.

To determine whether the loss of presynaptic 5-HT₁₅ receptors is associated with an alteration in the 5-HT transporter, \(^{3}H\)cyano-imipramine binding was quantified in the striatum. No difference between KO and WT mice in the binding of \(^{3}H\)cyano-imipramine to the 5-HT transporter was seen \((P=0.10)\). Similarly, no difference in 5-HT transporter binding was observed in the DRN, MRN or terminal projection areas of these nuclei (Table 1). Table 2 shows that the binding of \(^{125}I\)LSD to 5-HT₂₅ receptors is unaltered in either the striatum or cortex of KO mice.
did not differ between WT and KO mice. The failure of lack of change of DA concentrations in KO mice is a

Table 1

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>KO</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prefrontal cortex</td>
<td>12.46±0.55</td>
<td>12.46±0.71</td>
<td>0.5</td>
</tr>
<tr>
<td>Frontal cortex</td>
<td>20.13±1.21</td>
<td>21.01±0.78</td>
<td>0.5</td>
</tr>
<tr>
<td>Hypothalamus</td>
<td>48.11±0.52</td>
<td>45.34±1.01</td>
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<tr>
<td>Amygdala (BLA)</td>
<td>33.05±1.21</td>
<td>33.95±0.85</td>
<td>0.5</td>
</tr>
<tr>
<td>Hippocampus (CA3)</td>
<td>19.92±0.91</td>
<td>18.40±1.02</td>
<td>0.3</td>
</tr>
<tr>
<td>Dorsal raphe</td>
<td>61.04±1.78</td>
<td>57.73±2.81</td>
<td>0.2</td>
</tr>
<tr>
<td>Median raphe</td>
<td>87.45±4.68</td>
<td>87.14±5.80</td>
<td>0.6</td>
</tr>
<tr>
<td>Central gray</td>
<td>32.34±0.82</td>
<td>30.37±0.63</td>
<td>0.1</td>
</tr>
<tr>
<td>Striatum</td>
<td>25.84±1.09</td>
<td>25.02±1.52</td>
<td>0.1</td>
</tr>
</tbody>
</table>

Values are in fmol/mg wet tissue.

4. Discussion

These studies demonstrate that the ability of the SSRI, fluoxetine, to increase 5-HT concentrations in the dorsal striatum is enhanced following constitutive deletion of the 5-HT1A receptor. In contrast, the absence of 5-HT1A receptors does not modify basal or depolarization-evoked 5-HT concentrations, or the density of 5-HT transporters. Similarly, basal and stimulus-evoked DA concentrations are unaltered in the striatum following deletion of this receptor type.

Targeted inactivation of the 5-HT1A receptor in mice results in anxiety [19,28,34]. Since many pharmacological agents that increase 5-HT neurotransmission produce anxiety-like behavior in different experimental models [15,17], it has been hypothesized that the anxiety phenotype of KO mice is due to the absence of 5-HT1A autoreceptors and the resulting increase in 5-HT neurotransmission in terminal projection areas of raphe neurons. Indeed, evidence that 5-HT1A receptors inhibit the firing of DRN 5-HT neurons in the mouse has recently been presented [11]. If, in fact, this is the case, then extracellular concentrations of 5-HT as well as the depolarization-evoked release of 5-HT should be increased in KO as compared to WT mice.

Microdialysis studies revealed no alteration in basal 5-HT concentrations in the striatum of KO mice. Since dialysate concentrations of a neurotransmitter provide an estimate of extracellular concentrations [9,29,42], these findings indicate that extracellular concentrations of 5-HT are unaltered following constitutive deletion of the 5-HT1A receptor. The ability of K+ to increase dialysate 5-HT concentrations also did not differ between genotype, indicating that the depolarization-evoked release of 5-HT did not differ between WT and KO mice. The failure of constitutive deletion of the 5-HT1A receptor to alter basal or depolarization-evoked dialysate 5-HT concentrations suggest that the absence of 5-HT1A autoreceptors and disinhibition of 5-HT neurons projecting to the striatum can not account for the anxiety phenotype of KO mice. In this regard, however, it is important to note that dialysis studies were conducted in the dorsal striatum, a region innervated by DRN neurons [30]. Therefore, increases in 5-HT neurotransmission may occur in projection regions (e.g. hippocampus, amygdala) of MRN and these may underlie the increased anxiety of KO mice. Alternatively, genotype-dependent difference in 5-HT neurotransmission may only become apparent following exposure of animals to stressful stimuli.

Recent studies have shown that alterations in 5-HT uptake by the 5-HT transporter can modify the extraction fraction of 5-HT from the dialysis probe leading to over or under estimations of true extracellular concentrations [9,29,42]. Pharmacological manipulations that increase uptake increase the extraction fraction of the probe leading to an increase in dialysate concentrations. In contrast decreases in uptake decrease these parameters. Therefore, the possibility arises that alterations in 5-HT uptake may occur as a consequence of 5-HT1A receptor deletion and these changes may mask increases in extracellular concentrations that occur in response to 5-HT1A receptor deletion. Although this hypothesis was not evaluated in the present study, autoradiography revealed no alterations in the density of 5-HT transporters in the striatum or other projection areas of raphe nuclei. These findings indicate that 5-HT transport capacity does not differ in KO mice.

Previous studies have shown that 5-HT2A receptors are down-regulated following pharmacological treatments that increase 5-HT release [5,32]. Therefore, differences in the density of these receptors provide an assessment of post-synaptic receptor function as well as an indirect measure of changes in 5-HT neuronal activity. The finding that 5-HT2A receptor density was unchanged in projection regions of DRN neurons is consistent with a lack of change of presynaptic 5-HT neurotransmission in the dorsal striatum of KO mice. Furthermore, they indicate that the function of post-synaptic 5-HT2A receptors is also unaltered following 5-HT1A receptor deletion.

Both in vitro and in vivo studies have shown that 5-HT facilitates DA release in the striatum [4,10,43]. No significant differences in basal or depolarization-evoked DA concentrations were observed in KO and WT mice. The lack of change of DA concentrations in KO mice is important in that it suggest that mesostriatal DA neurotransmission in unaltered following 5-HT1A receptor deletion. Furthermore, it provides additional, albeit indirect, evidence that presynaptic 5-HT function is unaltered in the striatum of KO mice.

Table 2

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>KO</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Frontal cortex</td>
<td>49.4±5.7</td>
<td>47.8±1.6</td>
<td>0.8</td>
</tr>
<tr>
<td>Parietal cortex</td>
<td>38.9±2.7</td>
<td>36.5±3.7</td>
<td>0.6</td>
</tr>
<tr>
<td>Striatum</td>
<td>68.8±1.3</td>
<td>66.2±1.1</td>
<td>0.2</td>
</tr>
</tbody>
</table>

Values are in fmol/mg of wet tissue.
5-HT\textsubscript{1A} receptor antagonists prevent decreases in extracellular 5-HT concentrations produced by acute agonist administration [24]. However, they do not, by themselves, modify 5-HT concentrations [1,13,37]. Therefore, alterations in 5-HT or DA neurotransmission that occur as a consequence of 5-HT\textsubscript{1A} deletion may only be detectable under conditions of increased receptor activation. Studies, however, comparing depolarization-evoked 5-HT concentrations in WT and KO mice revealed no differences between genotype. Similarly, no difference in DA concentrations were seen. These data strongly suggest that the depolarization-induced release of 5-HT as well as DA is unaffected by 5-HT\textsubscript{1A} receptor depletion. They are also in line with a recent study which showed no effect of 5-HT\textsubscript{1A} receptor inactivation on the electrically-evoked release of \([\text{H}]5\text{-HT}\) from mesencephalic slides [34]. Taken together, these findings strongly suggest that the lack of 5-HT\textsubscript{1A} autoreceptors regulating the activity of DRN neurons is not the critical abnormality underlying the increased anxiety observed in KO mice. Rather they suggest that the loss of 5-HT\textsubscript{1A} receptor-mediated feedback control of MRN neurons or the deletion of post-synaptic 5-HT\textsubscript{1A} receptors in terminal regions of DRN or MRN mediate the anxiety phenotype of KO mice. Evidence in support of the latter hypothesis has recently been presented [38].

An explanation for the lack of change of presynaptic 5-HT function in KO mice is lacking. In contrast to receptor antagonists, gene knockout techniques inactivate receptors from embryonic life. Therefore, adaptive changes most likely underlie the apparently normal presynaptic 5-HT function of KO mice. In this regard, it is important to note that compensatory increases in terminal 5-HT\textsubscript{1B} autoreceptor activity or sensitivity would oppose increases in the firing of raphe neurons resulting in no net change in release. Interestingly, Ramboz et al. [34] have reported that the inhibition of \([\text{H}]5\text{-HT}\) release produced by a 5-HT\textsubscript{1B} receptor agonist is more pronounced in mesencephalic slices of 5-HT\textsubscript{1A} receptor KO mice than in slices of WT animals. Therefore, sensitization of 5-HT\textsubscript{1B} receptors in KO mice may, at least partly, compensate for 5-HT\textsubscript{1A} autoreceptor loss. Alternatively, presynaptic 5-HT\textsubscript{1A} receptors may not be tonically active. If such was the case, their absence in KO mice would cause no change in 5-HT release. Although neurochemical data provide support for this hypothesis, several studies have shown that the firing rate of raphe neurons in increased in response to the selective 5-HT\textsubscript{1A} receptor antagonist WAY 100,635 [12,13,27].

An involvement of 5-HT\textsubscript{1A} autoreceptors in delaying the therapeutic effects of SSRIs and other antidepressants has previously been suggested [2]. Elimination of 5-HT\textsubscript{1A} receptor-mediated feedback inhibition increases SSRI-induced increases in 5-HT concentrations [2]. It has been hypothesized that 5-HT\textsubscript{1A} autoreceptor desensitization and the resulting enhancement of SSRI-evoked 5-HT concentrations are required for the antidepressant action of SSRIs [2,20]. The present microdialysis studies provide additional evidence that the ability of SSRIs to increase extracellular 5-HT concentrations is increased following 5-HT\textsubscript{1A} receptor inactivation. In agreement with previous studies rat [23,36], the acute administration of fluoxetine to WT mice significantly increased 5-HT concentrations relative to basal values. An increase in 5-HT concentrations was also observed in KO mice. However, the magnitude of this effect was over 2-fold greater than that in WT mice. These data are noteworthy. They demonstrate that the adaptive mechanisms that maintain normal 5-HT neurotransmission under physiological conditions are insufficient to oppose the increase in 5-HT concentrations caused by transporter inhibition [2]. In addition, they indicate that compensatory mechanisms that occur following prolonged 5-HT\textsubscript{1A} receptor inactivation (e.g. 5-HT\textsubscript{1B} autoreceptor up-regulation) will be insufficient to oppose the increase in 5-HT concentrations produced by combined SSRI and 5-HT\textsubscript{1A} receptor antagonist treatment. In contrast to 5-HT, the systemic administration of fluoxetine did not modify DA concentrations in the dorsal striatum of WT mice. This finding is in line with previous data obtained in the rat [8,26,33]. Fluoxetine was also ineffective in modifying DA concentrations in KO mice, suggesting that the enhanced response of DR neurons to 5-HT\textsubscript{1A} receptor deletion is selective.

References


