Behavioral/Systems/Cognitive

Biochemical and Behavioral Evidence for Antidepressant-Like Effects of 5-HT_6 Receptor Stimulation

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The primary action of several antidepressant treatments used in the clinic raises extracellular concentrations of serotonin (5-HT), which subsequently act on multiple 5-HT receptors. The present study examined whether 5-HT_6 receptors might be involved in the antidepressant-like effects mediated by enhanced neurotransmission at 5-HT synapses. A selective 5-HT_6 receptor antagonist, SB271046, was evaluated for its ability to counteract fluoxetine-induced biochemical and behavioral responses in mice. In addition, biochemical and behavioral effects of the 5-HT_6 receptor agonist, 2-ethyl-5-methoxy-*N*,*N*-dimethyltryptamine (EMDT), were assessed in mice to ascertain whether enhancement of 5-HT_6 receptor-mediated neurotransmission engenders antidepressant-like effects. SB271046 significantly counteracted the stimulatory actions of fluoxetine on cortical c-*fos* mRNA, phospho-Ser845-GluR1, and in the tail suspension antidepressant assay, whereas it had no effect on these parameters by itself. EMDT increased the phosphorylation states of Thr^{34}-DARPP-32 and Ser^{845}-GluR1, both in brain slices and in the intact brain, which were effects also seen with the antidepressant fluoxetine; as with fluoxetine, these effects were demonstrated to be independent of D_1 receptor stimulation. Systemic administration of EMDT increased c-*fos* mRNA expression in the striatum and cerebral cortex and reduced immobility in the tail suspension test. The antidepressant-like effects of EMDT in the tail suspension test were prevented by SB271046. Our results indicate that 5-HT_6 receptor stimulation may be a mechanism initiating some of the biochemical and behavioral outcomes of 5-HT reuptake inhibitors, such as fluoxetine. These findings also indicate that selective 5-HT_6 receptor agonists may represent a novel antidepressant drug class.

Key words: **serotonin; antidepressants; fluoxetine; signal transduction; protein phosphorylation; tail suspension test**

Introduction

The serotonin (5-hydroxytryptamine; 5-HT) neurotransmitter system regulates complex sensory, motor, affective, and cognitive functions. Many of the current treatments for depression and anxiety act by increasing serotonergic neurotransmission (Barnes and Sharp, 1999), and such data form the basis for the monoamine hypothesis of affective disorders (Iversen, 2005). However, a causative role of perturbed 5-HT function in depression has been difficult to prove (Heninger et al., 1996), and the specific serotonergic receptor targets responsible for antidepressant efficacy are poorly defined. Fourteen 5-HT receptor subtypes have been identified (Hoyer et al., 1994; Barnes and Sharp, 1999). They are divided into seven different subclasses: 5-$HT_{1A–F}$, 5-$HT_{2A–C}$, 5-HT_3, 5-HT_4, 5-HT_5, 5-HT_6, and 5-HT_7 receptors. These receptors act primarily through the following second messenger transduction systems: 5-HT_1- and 5-HT_5-class receptors decrease cAMP formation; 5-HT_2-class receptors increase inositol triphosphate and diacylglycerol formation; 5-HT_3 receptors increase Na^+ and Ca^{2+} influx; and 5-HT_4, 5-HT_6, and 5-HT_7 receptors increase cAMP formation.

Dopamine- and cAMP-regulated phosphoprotein (DARPP-32) plays an important role in integrating signaling via multiple neurotransmitters in several brain regions (Svenningsson et al., 2004). When phosphorylated at Thr^{34}, DARPP-32 acts as an inhibitor of protein phosphatase-1 and thereby reduces the dephosphorylation and alters the function of multiple substrates, including glutamate receptor 1 (GluR1) subunits of AMPA receptors (Snyder et al., 2000). Systemic administration of fluoxetine increases the phosphorylation states of Thr^{34}-DARPP-32 and of Ser^{845}-GluR1 receptors, and DARPP-32 is involved in the fluoxetine-mediated decrease of immobility in the tail suspension test of antidepressant efficacy (Svenningsson et al., 2002a). Likewise, in brain slices, 5-HT activation of 5-HT_4 and 5-HT_6 receptors induces an increased phosphorylation state at Thr^{34}-DARPP-32, the protein kinase A site, and a decreased phosphorylation state at Thr^{75}-DARPP-32, the cyclin-dependent kinase 5 site (Svenningsson et al., 2002b). The ability of fluoxetine to modulate DARPP-32-mediated phosphorylation was, in turn,

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linked to phosphorylation of Ser^{831}- and Ser^{845}-GluR1 subunits of the AMPA receptor (Svenningsson et al., 2002a). Phosphorylation of these sites activates AMPA receptor conductance (Wang et al., 2005). Potentiation of AMPA receptors results in antidepressant-like effects in rodent models (Alt et al., 2006).

The present series of experiments was directed toward evaluating the role of the protein kinase A (PKA)-activating 5-HT$_6$ receptor subtype (Monsma et al., 1993; Ruat et al., 1993) in the antidepressant-like effects of 5-HT reuptake inhibitors. Accordingly, the present study examined the role of 5-HT$_6$ receptors in mediating biochemical and behavioral actions of fluoxetine indicative of its antidepressive properties. Specifically, we assessed the ability of the 5-HT$_6$ receptor antagonist, SB271046 (Bromidge et al., 1999), to modify fluoxetine-induced c-*fos* mRNA, phospho-Ser845 GluR1, and antidepressant-like behavioral effects in mice. In addition, we examined the effects of the 5-HT$_6$ receptor agonist 2-ethyl-5-methoxy-*N*,*N*-dimethyltryptamine (EMDT) (Glennon et al., 2000) on PKA-mediated signaling, c-*fos* mRNA expression, and antidepressant-like effects in mice. Collectively, the present biochemical and behavioral findings suggest that activation of 5-HT$_6$ receptors initiates a cascade of events that may be involved in the antidepressant-like effects of 5-HT reuptake inhibitors. As such, selective targeting of this 5-HT receptor subtype may provide an improvement in the therapeutic outcome of 5-HT-based antidepressants.

Materials and Methods

Animals. C57BL/6 male mice aged 2–4 months were used in all experiments in this study. Mice were bred at Rockefeller University or supplied by Harlan (Indianapolis, IN), Iffa Credo (Arbresle, France), or BK Universal (Sollentuna, Sweden) for United States and European locations, respectively, at 2–3 months of age. The mice were allowed to acclimatize to the colony for 2 weeks before they were used for experiments. All animals were group housed (four to six per cage).

[^{125}I]-SB258585 autoradiography in mouse brain sections. [^{125}I]-SB258585, a selective antagonist radioligand at 5-HT$_6$ receptors (Hirst et al., 2000), was used to determine the distribution of 5-HT$_6$ receptors in the mouse forebrain. Coronal cryostat tissue sections (12 μm thick) from C57BL/6 mice were incubated in assay buffer consisting of 50 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 10 μM pargyline, 0.1% ascorbic acid, and 0.5 mM EDTA. The sections were incubated in this solution containing 1 nM [^{125}I]-SB258585 (specific activity, 2000 Ci mmol^{-1}) (GE Healthcare, Uppsala, Sweden) for 45 min at 37°C. Slides were then washed three times in ice-cold (4°C) 50 mM Tris-HCl buffer (pH 7.4) for 30 min each, then dipped in ice-cold water to remove buffer salts. Nonspecific binding was generated on sections adjacent to those used for total binding by the addition of 10 μM 5-HT. Displacement experiments were performed with increasing concentrations (0.001–10 μM) of EMDT or SB271046 (synthetized at Eli Lilly and Company). Sections were dried in a stream of cool air and then exposed to autoradiographic film (Biomax MR; Kodak, Upplands Vasby, Sweden) for 4–7 d. Radioactive iodine standards (GE Healthcare) were coexposed with the sections on the same x-ray films. Autoradiograms were quantified by densitometry using NIH Image 1.61 software.

In situ *hybridization.* Adult male C57BL/6 mice were injected intraperitoneally with saline, EMDT (5 or 15 mg/kg), fluoxetine (10 or 20 mg/kg), SB271046 (1 or 10 mg/kg), or SB271046 (1 or 10 mg/kg) together with fluoxetine (10 or 20 mg/kg) and killed 20 min after the injection by decapitation. Brains were rapidly dissected out and frozen at −80°C. Cryostat sections (12 μm thick) were prepared and hybridized with [α-^{35}S] UTP-labeled riboprobes prepared by *in vitro* transcription from a cDNA clone corresponding to c-*fos* mRNA as described previously (Svenningsson et al., 1997). After hybridization, the sections were exposed to Biomax MR film (Kodak) for 2–14 d and quantified by densitometry using NIH Image 1.61 software.

In vivo *whole animal studies to measure protein phosphorylation.* Adult male C57BL/6 mice were given intraperitoneal injections of saline, fluoxetine (20 mg/kg), SB271046 (10 mg/kg), or SB271046 (10 mg/kg) together with fluoxetine (20 mg/kg) and killed 30 min after the injection by focused microwave irradiation (4.5–5 kW for 1.4 s) using a small animal microwave (Muromachi Kikai, Tokyo, Japan). In a separate experiment, adult male C57BL/6 mice were given intraperitoneal injections of saline or EMDT (5 mg/kg) and killed 15 min after the injection. Frontal cortices and striata were rapidly dissected out and stored at −80°C until assayed.

In vitro *brain slice experiments to measure protein phosphorylation.* Striatal slices (300 μm) were prepared from adult male C57BL/6 wild-type or D_1 knock-out mice. The slices were preincubated in Krebs buffer at 30°C under constant oxygenation (95% O_2/5% CO_2) for 60 min, with a change of buffer after 30 min. The slices were then treated with EMDT (3–100 μM) for 5 min. After drug treatment, the buffer was removed and the slices were rapidly frozen on dry ice and stored at −80°C until assayed.

Immunoblotting. Frozen tissue samples from the *in vitro* and *in vivo* experiments were sonicated in 1% SDS and boiled for 10 min. Small aliquots of the homogenate were retained for protein determination by the bicinchoninic acid protein assay method (Pierce, Stockholm, Sweden). Equal amounts of protein were processed using 12% acrylamide gels as described previously (Svenningsson et al., 2003). Immunoblotting was performed with phosphorylation state-specific antibodies against phospho-Thr^{34}-DARPP-32 (Snyder et al., 1992), phospho-Thr^{75}-DARPP-32 (Bibb et al., 1999), phospho-Ser^{831}-GluR1 (Millipore, Bedford, MA), phospho-Ser^{845}-GluR1 (Millipore), or antibodies that are not phosphorylation state specific against total DARPP-32 (Hemmings and Greengard, 1986) or total GluR1 (Millipore). Antibody binding was detected by enhanced chemiluminescence (GE Healthcare) and quantified by densitometry using NIH Image 1.61 software. Data on protein phosphorylation are expressed as percentage of control.

Tail suspension test. The day of the tail suspension test, experimental mice were transferred to the experiment room and allowed to acclimatize for 3–4 h. Mice were injected intraperitoneally with saline, EMDT (1, 2.5, 5, 10, or 15 mg/kg), fluoxetine (20 mg/kg), SB271046 (1, 5, or 10 mg/kg), or SB271046 (1, 5, or 10 mg/kg) combined with fluoxetine (20 mg/kg) 30 min before the tail suspension test trial. In the tail suspension test paradigm, each mouse was tested in an individual cubicle while suspended from a tail hanger with adhesive tape wrapped around its tail (1.5–2 cm from tip) 80 cm above the floor. The trial was conducted for a period of 5 min, during which the duration of immobility was measured with the Porsolt program (Infallible Software, Rockville, MD) and manually by a blinded observer. Mice were considered immobile when they hung passively and motionless. In our hands, an extremely small number of animals (~1–2%) climbed their tails. These animals were removed from the study. Decreases in basal levels of immobility are highly predictive of antidepressant efficacy (Steru et al., 1985; Cryan et al., 2002).

Results

Autoradiographic determination of 5-HT$_6$ receptors in the mouse brain

[^{125}I]-SB258585 is an antagonist radioligand at 5-HT$_6$ receptors (Hirst et al., 2000) that can detect these receptors in the rat brain (Roberts et al., 2002). The level of 5-HT$_6$ receptors is lower and less concentrated in the forebrain of mice than rats (Hirst et al., 2003). Nevertheless, in an autoradiographic experiment, using [^{125}I]-SB258585 as a radioligand, we demonstrated specific binding in the striatum, nucleus accumbens, and cortex of mice (Fig. 1). Binding of [^{125}I]-SB258585 could be displaced by both the antagonist, SB271046, and the agonist, EMDT, with EC_{50} values of 4 and 15 nM, respectively (Fig. 1).

The 5-HT$_6$ receptor antagonist, SB271046, reverses biochemical and behavioral antidepressant-like effects of fluoxetine

Acute administration of antidepressant drugs increases expression of the immediate early gene c-*fos* mRNA in the brain (Beck 1995; Torres et al., 1998; Horowitz et al., 2003) and reduces immobility in behavioral tests of despair (see below). We examined

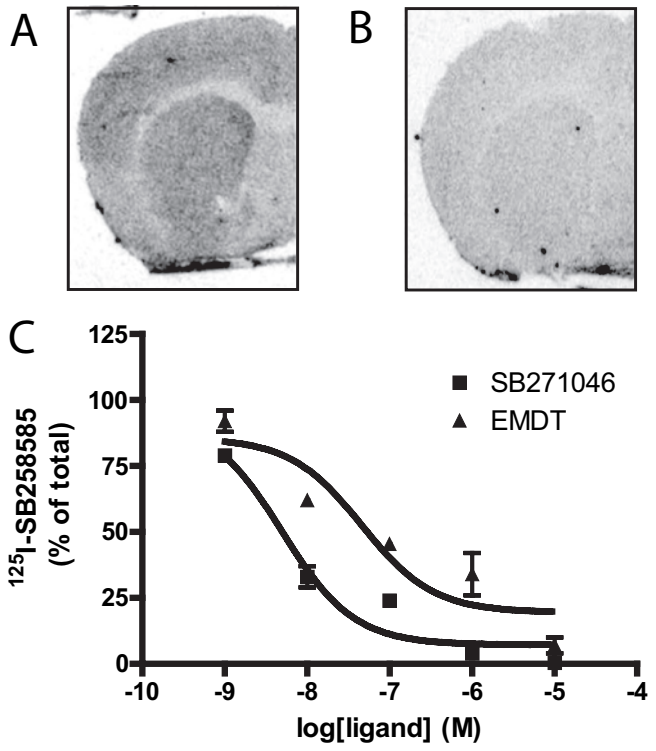

Figure 1. [^{125}I]-SB258585 binding in the mouse brain. ***A***, Autoradiogram showing total binding of [^{125}I]-SB258585 on a coronal section of a mouse brain, through the rostral part of the corpus striatum. ***B***, Autoradiogram generated over an adjacent section but incubated with [^{125}I]-SB258585 in the presence of 10 μM unlabeled serotonin to define nonspecific binding. ***C***, Displacement of [^{125}I]-SB258585 by increasing concentrations of EMDT and SB271046. Error bars indicate SEM.

the effects of the selective 5-HT$_6$ receptor antagonist, SB271046, on fluoxetine-induced c-*fos* mRNA expression. In agreement with previous studies (Torres et al., 1998; Horowitz et al., 2003), fluoxetine (10 or 20 mg/kg) increased the expression of c-*fos* mRNA in certain limbic regions of the frontal cerebral cortex, including the cingulate cortex and the endopiriform cortex (Fig. 2). Treatment with SB271046 (1 or 10 mg/kg) alone had no effect on c-*fos* mRNA expression in these regions. However, 10 mg/kg of SB271046, administered before fluoxetine (10 mg/kg, data not shown; or 20 mg/kg) (Fig. 2), significantly counteracted fluoxetine-induced c-*fos* mRNA expression in both the cingulate and the endopiriform cortex (Fig. 2).

In agreement with our previous study (Svenningsson et al., 2002), fluoxetine (20 mg/kg) increased the levels of phospho-Ser845-GluR1 in the frontal cortex (Fig. 3) and striatum (data not shown). Treatment with SB271046 (10 mg/kg) alone had no effect on phospho-Ser845-GluR1 in these regions but significantly counteracted fluoxetine-induced phospho-Ser845-GluR1 in the frontal cortex (Fig. 3).

SB271046 was also tested for its activity in the mouse tail suspension test. Learned-helplessness models, such as the tail suspension test, in which experimental animals are exposed to inescapable aversive situations, are of utility for predicting antidepressant efficacy. During these tests, mice show alternate periods of agitation and immobility (Steru et al., 1985). It is well established that acute treatment with various antidepressant drugs increases active attempts to escape and, thus, reduces immobility in these tests. In agreement with the biochemical data, SB271046 (1, 5, or 10 mg/kg) had no effect in the tail suspension test when administered alone. When SB271046 (5 or 10 mg/kg)

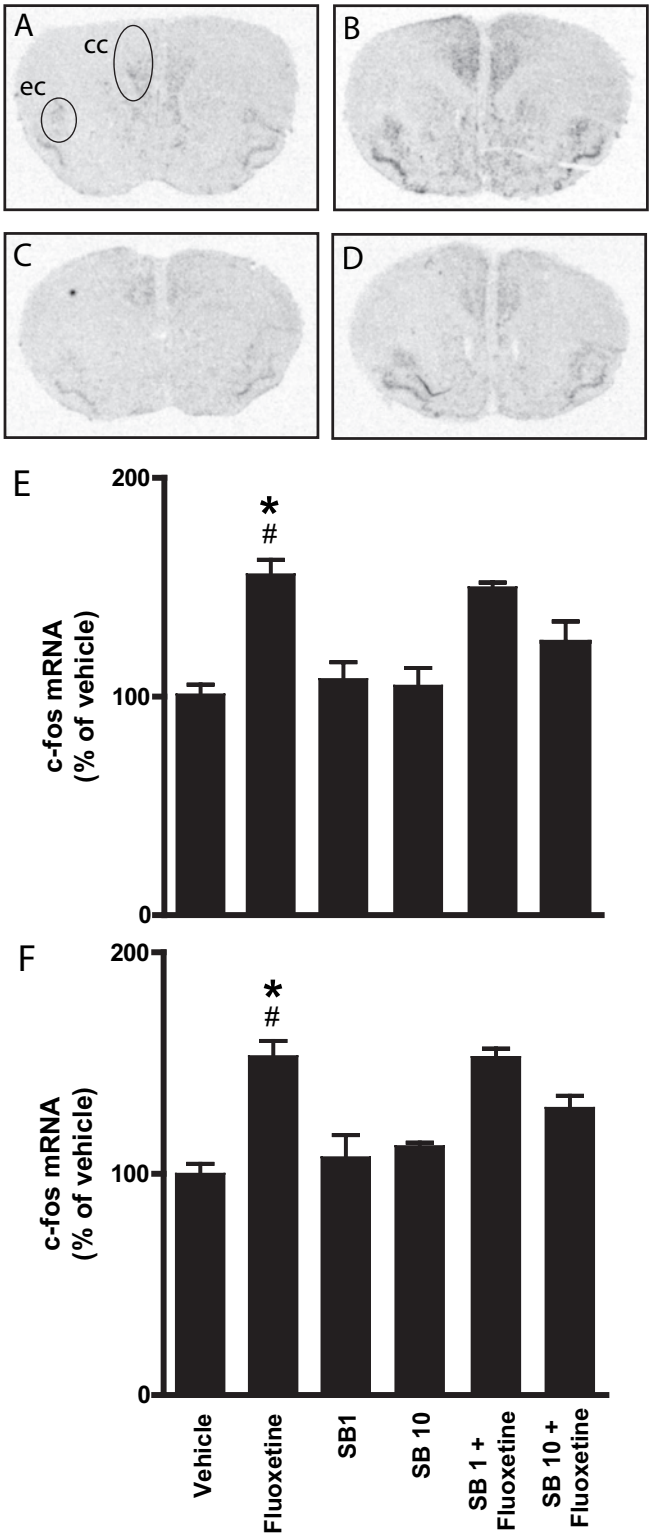

Figure 2. Regulation by fluoxetine and SB271046 of c-*fos* mRNA expression in the cerebral cortex of intact mice. ***A–D***, Bright-field autoradiograms showing the expression of c-*fos* mRNA 20 min after intraperitoneal administration of saline (***A***), fluoxetine (20 mg/kg) (***B***), SB271046 (10 mg/kg) (***C***), or SB271046 (10 mg/kg) together with fluoxetine (20 mg/kg) (***D***) in mice (magnification, 5×). ec, Endopiriform cortex; cc, cingulate cortex. ***E***, ***F***, Histograms show quantification of the expression of c-*fos* mRNA in the cingulate cortex (***E***) and dorsal endopiriform cortex (***F***) after the indicated treatments. Data represent means ± SEM for four to six mice per group. $^*p < 0.05$ compared with saline-treated mice; $^\#p < 0.05$ compared with SB271046 (10 mg/kg) plus fluoxetine-cotreated mice; one-way ANOVA followed by Newman–Keuls test.

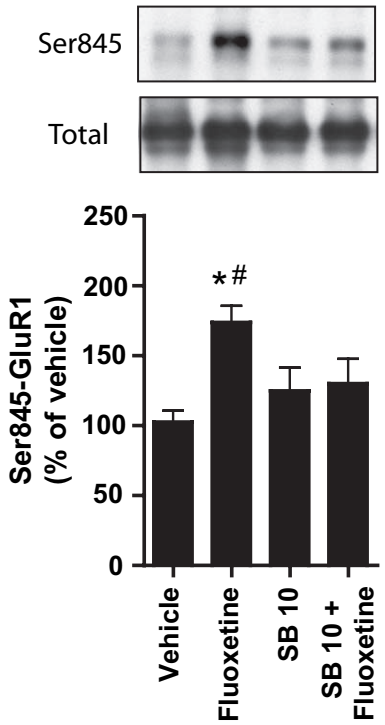

Figure 3. Regulation by fluoxetine and SB271046 of phospho-Ser845-GluR1 in the frontal cortex in intact mice. Top, Immunoblots showing the levels of phospho-Ser845-GluR1 and total GluR1 in the frontal cortex 30 min after intraperitoneal administration of saline, fluoxetine (20 mg/kg), SB271046 (10 mg/kg), or SB271046 (10 mg/kg) together with fluoxetine (20 mg/kg) in mice. Bottom, The histogram shows the quantification of phospho-Ser845-GluR1 in the frontal cortex after the indicated treatments. Data represent means ± SEM for five to six mice per group. *$p < 0.05$ compared with saline-treated mice; #$p < 0.05$ compared with SB271046 (10 mg/kg) plus fluoxetine-cotreated mice; one-way ANOVA followed by Newman–Keuls test for pairwise comparisons.

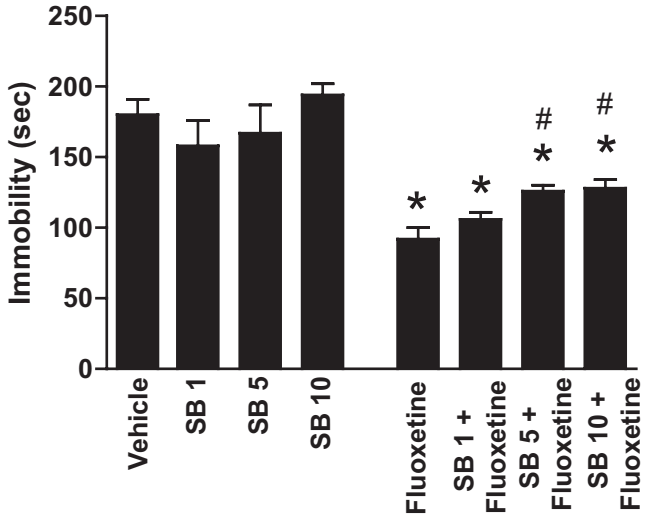

Figure 4. Effects of SB271046 on antidepressant-like effects of fluoxetine in the tail suspension test. Saline, fluoxetine (20 mg/kg), SB271046 (1,5, or 10 mg/kg), or SB271046 (1,5, or 10 mg/kg) combined with fluoxetine (20 mg/kg), 30 min before the tail-suspension test trial. The trial was conducted for a period of 5 min, during which the duration of immobility was recorded. Data represent means ± SEM for eight mice per group. *$p < 0.05$ compared with saline; #$p < 0.05$ compared with fluoxetine; one-way ANOVA followed by Duncan's test.

was given in conjunction with an antidepressant-like dose of fluoxetine (20 mg/kg), however, there was a partial reversal of its anti-immobility effect in this test (Fig. 4). It can be concluded from these studies that specific biochemical and behavioral actions of fluoxetine that are associated with its antidepressant effects may involve activation of 5-HT$_6$ receptors.

Antidepressant effects of the 5-HT$_6$ receptor agonist EMDT

We next assessed the ability of the 5-HT$_6$ receptor agonist EMDT to mimic some of the antidepressant-like biochemical and behavioral effects of fluoxetine. First, we measured its ability to regulate the phosphorylation state of two PKA phosphosubstrates, Thr^{34}-DARPP-32 and Ser^{845}-GluR1, in striatal slices. EMDT increased the phosphorylation states of Thr^{34}-DARPP-32 and Ser^{845}-GluR1 in a dose-dependent manner (Fig. 5). The phosphorylation of Thr^{34}-DARPP-32 and Ser^{845}-GluR1 in striatum is potently regulated by D$_1$ receptor stimulation (Snyder et al. 2000). To determine whether the effect of EMDT on phospho-Thr^{34}-DARPP-32 and phospho-Ser^{845}-GluR1 involved D$_1$ receptor activation, we compared the effect of EMDT (100 μM) on these phosphosubstrates in wild-type and D$_1$ receptor knock-out mice. As shown in Figure 6, EMDT significantly increased phospho-Thr^{34}-DARPP-32 and phospho-Ser^{845}-GluR1 not only in slices from wild-type mice but also in slices from D$_1$ receptor knock-out mice. It can be concluded that the stimulatory effect of EMDT on phospho-Thr^{34}-DARPP-32 and phospho-Ser^{845}-GluR1 is independent of D$_1$ receptor activation.

We next examined the effect of systemic administration of

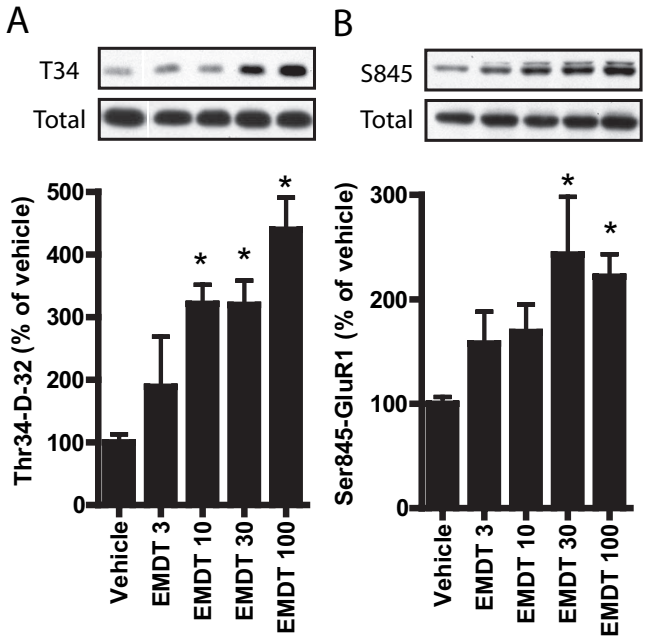

Figure 5. Regulation by EMDT of the phosphorylation states of DARPP-32 and GluR1 in slices of neostriatum. Dose-response experiments of *in vitro* regulation by EMDT of phosphorylation of Thr^{34}-DARPP-32 (***A***) and Ser^{845}-GluR1 (***B***) in striatal slices. Slices were incubated with EMDT (3, 10, 30, and 100 μM) for 5 min. Data represent means ± SEM ($n = 6–10$). *$p < 0.05$ compared with vehicle; one-way ANOVA followed by Newman–Keuls test.

EMDT on the PKA sites, phospho-Thr^{34}-DARPP-32 and phospho-Ser^{845}-GluR1. It was found that 5 mg/kg of EMDT increased the phosphorylation states of both Thr^{34}-DARPP-32 and Ser^{845}-GluR1 in striatal extracts (Fig. 7). No significant alterations of phospho-Thr^{75}-DARPP-32 or phospho-Ser^{831}-GluR1 were found in the same extracts. Treatment with EMDT also increased phospho-Ser^{845}-GluR1, but not phospho-Ser^{831}-GluR1, in the frontal cortex (Fig. 7). These data indicate that the effects of EMDT on phosphorylation of PKA phosphosubstrates

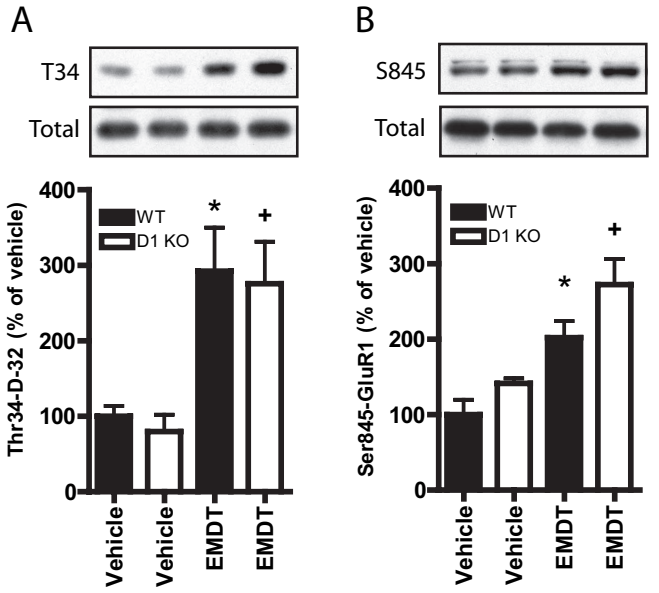

Figure 6. Comparison of the regulation by EMDT of the phosphorylation states of DARPP-32 and GluR1 in striatal slices from wild-type (WT) and D_1 receptor knock-out (D1 KO) mice. *In vitro* regulation of Thr^{34}-DARPP-32 (***A***) and Ser^{845}-GluR1 (***B***) phosphorylation by EMDT (100 μM) in slices of neostriatum from wild-type and D_1 knock-out mice. The amounts of phospho-Thr^{34}-DARPP-32 and phospho-Ser^{845}-GluR1 in extracts of slices were quantified by densitometry. Data represent means ± SEM ($n = 6–12$). *$p < 0.05$ compared with wild-type control; $^{+}p < 0.05$ compared with D_1 knock-out control; unpaired two-tailed Student's *t* test.

in brain slices can be reproduced by its systemic administration to intact animals.

To further examine the ability of EMDT to regulate signal transduction in the intact brain, we studied its effect on c-*fos* mRNA expression. EMDT was found to significantly induce c-*fos* mRNA expression in striatum as well as subregions of the cerebral cortex, including the cingulate cortex (Fig. 8). This effect of EMDT was observed after its systemic administration at 5 and 15 mg/kg but not at 1 mg/kg. To investigate the involvement of 5-HT$_6$ receptors in engendering antidepressant-like activity, we assessed the effects of acute EMDT administration in the tail suspension test in mice. It was found that EMDT dose-dependently decreased immobility in the tail suspension test (Fig. 9). This effect was abolished by pretreatment with the 5-HT$_6$ receptor antagonist SB271046 (Fig. 9), demonstrating the specificity of the antidepressant-like profile of this compound for 5-HT$_6$ receptors.

Discussion

We have previously reported that fluoxetine increases the phosphorylation states of Thr^{34}-DARPP-32 and of Ser^{845}-GluR1 receptors and that DARPP-32 is involved in the fluoxetine-mediated decrease of immobility in the tail-suspension test of antidepressant efficacy (Svenningsson et al., 2002b), in agreement with the idea that activation of cAMP/PKA signaling is associated with antidepressant effects (Duman et al., 1997). These findings indicated that at least one of the 5-HT receptors that stimulate PKA activity (i.e., 5-HT$_4$, 5-HT$_6$, and/or 5-HT$_7$ receptors) is involved in mediating the actions of fluoxetine.

In the present study, we examined biochemical and behavioral effects exerted via 5-HT$_6$ receptors with a special emphasis on the potential role of this receptor subtype in antidepressant actions. For this purpose, we used selective pharmacological tools, namely the selective 5-HT$_6$ receptor antagonist, SB271046 (Bro-

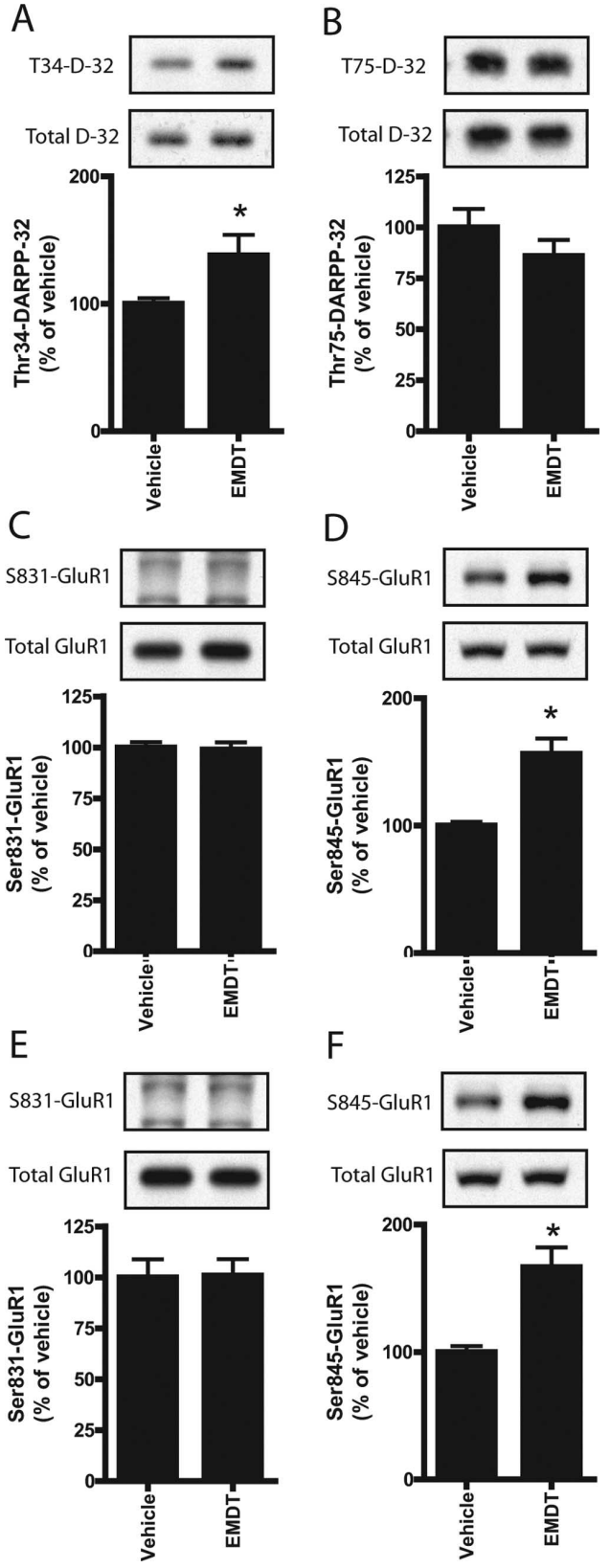

Figure 7. Regulation by EMDT of the phosphorylation states of DARPP-32 and GluR1 in striatal and cortical extracts from intact mice. Regulation of Thr^{34}- and Thr^{75}-DARPP-32 and Ser^{831}- and Ser^{845}-GluR1 phosphorylation *in vivo* in the striatum (***A–D***) and frontal cortex (***E***, ***F***) by EMDT. Mice were injected intraperitoneally with saline or EMDT (5 mg/kg). Fifteen minutes later, mice were killed by focused microwave irradiation. Data represent means ± SE for 5–10 mice per group. *$p < 0.05$ compared with saline-treated mice; one-way ANOVA followed by Newman–Keuls test.

midge et al., 1999), and the 5-HT$_6$ receptor agonist, EMDT (Glennon et al., 2000). We confirmed that both ligands have high affinities for 5-HT$_6$ receptors in the mouse brain by showing that they displace specific [^{125}I]-SB258585 binding at nanomolar concentrations in the forebrain. Our results are consistent with previous studies showing that 5-HT$_6$ receptors appear restricted to the brain with high levels in the caudate–putamen, nucleus accumbens, and olfactory tubercle and moderate levels in the cerebral cortex hippocampus and amygdala (Monsma et al., 1993; Ruat et al., 1993; Ward et al., 1995; Gerard et al., 1997; Hamon et al., 1999; Roberts et al., 2002).

Despite their well characterized anatomical distribution, the functional importance of 5-HT$_6$ receptors in brain pathophysiology is only emerging. In accordance with a predominant corticolimbic localization of 5-HT$_6$ receptors, their blockade modulates responses to the psychostimulant amphetamine (Frantz et al., 2002), as well as motor, emotional, and cognitive functions. Thus, administration of either 5-HT$_6$ receptor antagonists or antisense oligonucleotides toward 5-HT$_6$ receptors decreases locomotion, induces chewing, yawning, and stretching, and improves performance in learning and memory tasks (Bourson et al., 1995; Sleight et al., 1996; Sleight et al., 1998; Yoshioka et al., 1998; Bentley et al., 1999; Rogers and Hagan, 2001; Woolley et al., 2001; Lindner et al., 2003; Riemer et al., 2003; Hatcher et al., 2005), while increasing anxiety (Hamon et al., 1999; Otano et al., 1999). 5-HT$_6$ receptor knock-out mice, however, perform normally in a wide variety of behavioral assays that assess cognition and anxiety (Bonasera et al., 2006). To our knowledge, the present study is the first to examine 5-HT$_6$ receptor function in relation to antidepressant-like activity and to indicate that 5-HT$_6$ receptor stimulation causes antidepressant-like behavioral and biochemical alterations.

Indeed, we show that blockade of the 5-HT$_6$ receptor with the antagonist SB271046 counteracts the stimulatory actions of fluoxetine on cortical c-*fos* mRNA and phospho-Ser845-GluR1 and reduces the antidepressant-like action of fluoxetine in the tail suspension test. Previous work demonstrated that several classes of atypical antipsychotics and tricyclic antidepressants, such as clozapine, mianserin, and amytryptiline, bind with high affinity to 5-HT$_6$ receptors (Monsma et al., 1993). However, fluoxetine has only low-to-moderate affinity for 5-HT$_6$ receptors (Monsma et al., 1993). It is therefore unlikely that the inhibitory action of SB271046 on fluoxetine-mediated actions depends on direct competition at 5-HT$_6$ receptors, but rather involves blockade of 5-HT$_6$ receptor activation elicited by the fluoxetine-induced elevations of extracellular 5-HT levels.

Furthermore, we show that the 5-HT$_6$ receptor agonist EMDT mimics antidepressant-like behavioral and biochemical effects of fluoxetine. Like fluoxetine and 5-HT (Svenningsson et al., 2002a,b), EMDT increases the phosphorylation state of Thr^{34}-DARPP-32 both in brain slices and in the intact brain. The fact that micromolar concentrations of EMDT are needed to cause a significant increase in P-Thr^{34}-DARPP-32, despite the fact that this compound has nanomolar affinity for 5-HT$_6$ receptors, is consistent with previous studies using other ligands at monoamine receptors (Svenningsson et al., 2002b). This discrepancy between the binding affinity and functional protein phosphorylation response may be because of the dilution of the compound when diffusing throughout the slices and to difficulties in accessing receptors in the synaptic cleft. Acute systemic administration of EMDT also increases phospho-Ser^{845}-GluR1 but not phospho-Ser^{831}-GluR1. Systemic administration of EMDT also leads to an increase in the expression of c-*fos* mRNA expression throughout the striatum and cerebral cortex, similar to that observed with fluoxetine. In the same dose range, EMDT leads to a significant antidepressant-like effect in the tail suspension test. These data indicate that stimulation of the positively cAMP/PKA

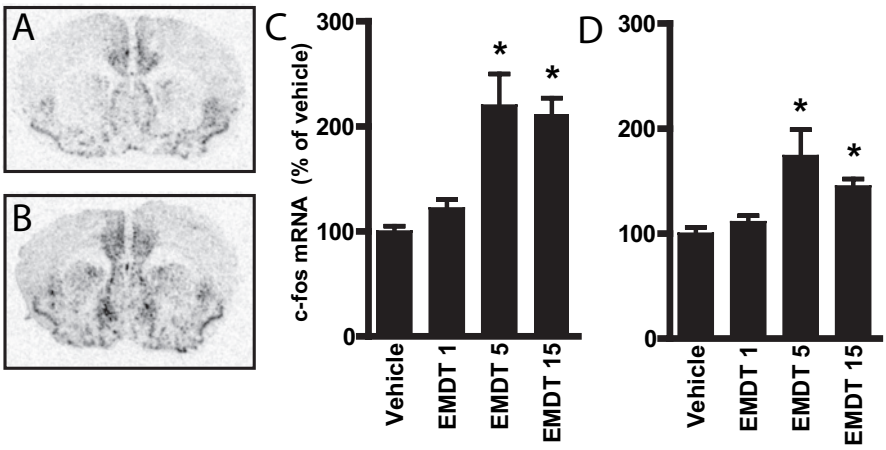

Figure 8. Regulation by EMDT of c-*fos* mRNA expression in the striatum and cerebral cortex in intact mice. ***A***, ***B***, Bright-field autoradiograms showing the expression of c-*fos* mRNA 20 min after treatment with saline (***A***) or EMDT (5 mg/kg) (***B***) in mice (magnification, 5×). ***C***, ***D***, Histograms show quantification of the expression of c-*fos* mRNA in the periventricular area of the striatum (***C***) and cingulate cortex (***D***) after each treatment. Data represent means ± SEM for four to six mice per group. *$p < 0.05$ compared with saline-treated mice; one-way ANOVA followed by Newman–Keuls test.

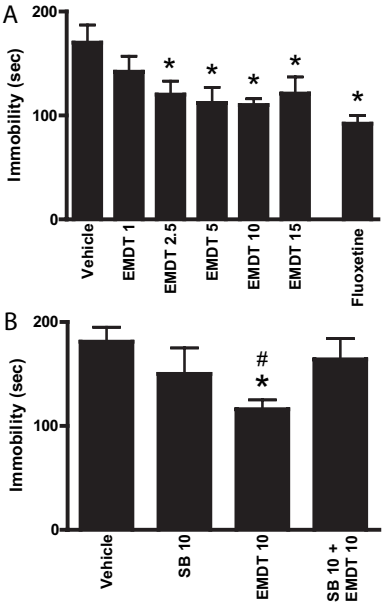

Figure 9. Antidepressant-like effects of EMDT in the tail suspension test. ***A***, Mice were injected with EMDT (1, 2.5, 5, 10, or 15 mg/kg) 30 min before the trial. ***B***, Saline, SB271046 (10 mg/kg), EMDT (10 mg/kg), or SB271046 (10 mg/kg) combined with EMDT (10 mg/kg), 30 min before the tail suspension test trial. The trial was conducted for a period of 5 min, during which the duration of immobility was recorded. Data represent means ± SEM for eight mice per group. *$p < 0.05$ compared with saline; #$p < 0.05$ compared with SB271046 (10 mg/kg) plus EMDT; one-way ANOVA followed by Duncan's test.

coupled 5-HT$_6$ receptors may be involved in antidepressant-like actions of 5-HT reuptake inhibitors. This is in accordance with our previous findings that 5-HT-induced phosphorylation of DARPP-32 at Thr^{34}, a key molecular element in antidepressant action, is mediated through activation of the cAMP pathway and primarily depends on 5-HT receptors positively coupled to adenylyl cyclase (Svenningsson et al., 2002b). The framework that our previous and present results provide agrees with the evidence for the participation of the cAMP cascade, particularly in the frontal cortex, in short- and long-term effects of antidepressants. However, it remains to be established which neuronal populations mediate the antidepressant-like actions of 5-HT$_6$ receptor stimulation. In this context, it should be noted that activation of cAMP/PKA/P-Ser^{133}-calcium/cAMP response element-binding protein (CREB) signaling in the nucleus accumbens and striatum actually appears to counteract antidepressant actions. Indeed, previous studies have demonstrated that overexpression of CREB in the nucleus accumbens increases, and expression of a dominant negative mutant of CREB decreases, immobility in the forced swim test (Carlezon et al., 2005). It is possible that additional signaling pathways mediate important actions of 5-HT$_6$ receptors. It has, for example, recently been demonstrated that 5-HT$_6$ receptors stimulate ERK1/2 (extracellular signal-regulated kinase 1/2) signaling via a Fyn tyrosine kinase-dependent mechanism (Yun et al., 2007).

Reports on the role of the other two cAMP/PKA coupled 5-HT receptors, 5-HT$_4$ and 5-HT$_7$ receptors, in relation to depression and to antidepressant-like activity are scarce. Overall, there is little evidence for an involvement of 5-HT$_4$ receptors in mediating antidepressant effects. Thus, stimulation of 5-HT$_4$ receptors does not mediate behavioral antidepressant-like actions of tricyclic antidepressants or fluoxetine (Cryan and Lucki, 2000). However, stimulation of 5-HT$_4$ receptors exerts a facilitatory response on dorsal raphe 5-HT neurons (Lucas et al., 2005) and might be implicated in some behavioral effects of chronically administered fluoxetine (Holick et al., 2005). Surprisingly, it was shown that 5-HT$_7$ receptor antagonists could have antidepressant properties and that 5-HT$_7$ receptor knock-out mice exhibited an antidepressant-like phenotype (Guscott et al., 2005; Hedlund et al., 2005). This suggests that 5-HT$_4$, 5-HT$_6$, and 5-HT$_7$ receptors, despite their common effects on signal transduction, could have markedly different, and even opposing, effects in tests of antidepressant-like activity. It should, however, be noted that the antidepressant-like effects of 5-HT$_7$ receptor antagonists could be linked to the 5-HT$_7$ receptor-dependent regulation of the light/dark cycle (Guscott et al., 2005). An alternative possible explanation for the discrepancies in the actions of 5-HT$_4$, 5-HT$_6$, and 5-HT$_7$ receptors in antidepressant effects may reside in their different anatomical localization, in which case activation of each of these receptors would stimulate distinct and independent neuronal circuitries. In accordance with the latter possibility, a review of the literature on the effects of selective 5-HT receptor ligands on behavioral despair in mice (Table 1) indicates that selective 5-HT receptor stimulation can yield stimulatory or inhibitory behavioral responses depending on the receptor subtype. Similar results on behavioral despair have been described previously in rats (Cryan et al., 2005).

Our data are consistent with the notion that fluoxetine exerts its antidepressant actions by stimulating multiple 5-HT receptors. The distribution of 5-HT$_6$ receptors in the mouse brain is uniform throughout the cortex and striatum. However, it appears that certain regions of the cortex, including the cingulate cortex, are particularly sensitive in terms of fluoxetine-mediated

Table 1. Effects of selective 5-HT receptor ligands alone or in combination with an SSRI on immobility in the tail suspension test or forced swimming test in mice

Class of 5-HT receptor	Compound	Alone	Plus SSRI
5-HT$_{1A/B}$ agonist			
	RU24969[a,b]	↓	↓
5-HT$_{1A}$ agonists			
	8-OH-DPAT[c,d,e]	↓	
	LB50016[f]	↓	
	MKC-242[g]	↓	
5-HT$_{1A}$ antagonists			
	WAY-100635[a,d,h]	⇆	↑ [d,h]; ⇆ [a]
	NAN190[e]	⇆	
5-HT$_{1B}$ agonists			
	Anpirtoline[b,i]	↓	
	GP94253[j]	↓	
5-HT$_{1B}$ antagonist			
	GR125743[a,h,k]	⇆	↑ [a,k]; ↓ [h]
5-HT$_{2A/C}$ agonist			
	DOI[l]	⇆	
5-HT$_{2A/C}$ antagonists			
	LY53857[d]		↑
	Ritanserin[l]	↓	
5-HT$_{2A}$ antagonist/SSRI			
	YM992[m]		↓
5-HT$_{2C}$ agonists			
	WAY 161503[n]	↓	
	RO 60-0175[n]	↓	
	RO 60-0332[n]	↓	
5-HT$_{2C}$ antagonist			
	SB206553[n,o]	⇆	↓ [o]; ↑ [n]
5-HT$_3$ antagonists			
	Ondansetron[l]	↓	
	MDL72222[p]	↓	
5-HT$_4$ antagonist			
	SB 204070A[q]	⇆	⇆
5-HT$_6$ agonist			
	EMDT	↓	
5-HT$_6$ antagonist			
	SB271046		↑
5-HT$_7$ antagonists			
	SB-258719[r]	↓	
	SB-269970[s]	↓	

↓, Decreased immobility; ↑, increased/potentiated immobility; ⇆, no effect on immobility. References: [a]O´Neill et al., 1996; [b]Redrobe and Bourin, 1999; [c]Biala, 1998; [d]Miyata et al., 2004; [e]Redrobe et al., 1996; [f]Lee et al., 1999; [g]Matsuda et al., 1995; [h]Mayorga et al., 2001; [i]O´Neill and Conway, 2001; [j]Tatarczynska et al., 2005; [k]Gardier et al., 2001; [l]Redobe and Bourin, 1997; [m]Takeuchi et al., 1997; [n]Cryan and Lucki, 2000b; [o]Cremers et al., 2004; [p]Kos et al., 2006; [q]Cryan and Lucki, 2000a; [r]Guscott et al., 2005; [s]Hedlund et al., 2005. [n, q] Studies were performed on rats.

c-*fos* mRNA induction. Because SB271046 only partially blocks the actions of fluoxetine on c-*fos* mRNA and phospho-Ser845-GluR1, it is very likely that concomitant activation of several 5-HT receptors is required for the biochemical actions of fluoxetine in the cortex. Similarly, in the tail suspension test, the maximal antidepressant-like effects of EMDT are less pronounced than those of fluoxetine. It is likely that, in addition to 5-HT$_6$ receptors, other 5-HT receptors also contribute to the effects of fluoxetine, as evidenced by the fact that the 5-HT$_6$ antagonist SB271046 only partially blocks the antidepressant-like effects of fluoxetine in the tail suspension test when administered at 10 mg/kg, whereas at the same dose it completely blocks the antidepressant-like effects of the selective 5-HT$_6$ agonist EMDT.

In conclusion, the results from the present study suggest that stimulation of 5-HT$_6$ receptors causes antidepressant-like behavioral and biochemical effects and provide additional support to the idea that 5-HT$_6$ receptors may contribute to serotonergic

modulation of clinically relevant psychopharmacological processes.

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