Robust presynaptic serotonin 5-HT1B receptor inhibition of the striatoni gral output and its sensitization by chronic fluoxetine treatment

Running title: Fluoxetine sensitizes 5-HT1B receptors

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Key words: Basal ganglia, medium spiny neuron, selective serotonin reuptake inhibitor, 5-HT1B receptor sensitization, substantia nigra pars reticulata
Abstract

The striatonigral projection is a striatal output pathway critical to motor control, cognition and emotion regulation. Its axon terminals in the substantia nigra pars reticulata (SNr) express a high level of serotonin (5-HT) 1B receptors (5-HT1BRs), while the SNr also receives an intense 5-HT innervation that expresses 5-HT transporters, providing an anatomical substrate for 5-HT and selective 5-HT reuptake inhibitor (SSRI)-based antidepressant treatment to regulate the striatonigral output. Here we show that 5-HT, via activating presynaptic 5-HT1BRs on the striatonigral axon terminals, potently inhibited the striatonigral GABA output, as reflected in the reduction of the striatonigral inhibitory postsynaptic currents (IPSCs) in SNr GABA neurons. Functionally, 5-HT1BR agonism reduced the striatonigral GABA output-induced pause of the spontaneous high frequency firing in SNr GABA neurons. Equally important, chronic SSRI treatment with fluoxetine enhanced this presynaptic 5-HT1BR-mediated pause reduction in SNr GABA neurons. Taken together, these results indicate that activation of the 5-HT1BRs on the striatonigral axon terminals can limit the motor-promoting GABA output. Furthermore, in contrast to the desensitization of 5-HT1 autoreceptors, chronic SSRI-based antidepressant treatment sensitizes this presynaptic 5-HT1BR-mediated effect in the SNr, a novel cellular mechanism that alters the striatonigral information transfer, potentially contributing to the behavioral effects of chronic SSRI treatment.
Introduction

The substantia nigra pars reticulata (SNr) is a critical node in the vast cortico-basal ganglia-thalamo-cortical (CBGTC) loop (Fig. 1A) (Haber 2003; Hikosaka 2007) important to motor control (Friend and Kravitz 2014) and also to cognition (Simpson et al. 2010), emotion (Marchand et al. 2012; Révy et al. 2014) and learning and habit formation (Sesack and Grace 2010; Graybiel and Smith 2014). A main characteristic of the SNr is that its GABA projection neurons fire autonomous high frequency spikes that are paused by the massive GABA input from the striatonigral projection (Schultz 1986; Hikosaka et al. 2000, 2014; Zhou and Lee 2011; Sano et al. 2013). Another prominent anatomical feature of the SNr is its dense 5-HT innervation originated in the dorsal raphe (Fig. 1B) (Moukhles et al., 1997; Parent et al. 2011). 5-HT neurons fire tonic spikes at 1-2 Hz, likely leading to spontaneous tonic 5-HT release and an ambient 5-HT level in the SNr (Jacobs and Azmitia 1992). Matching the heavy 5-HT innervation, the SNr has an expression of 5-HT1BRs on the striatonigral axon terminals that is the highest in the brain (Fig. 1C) (Voigt et al. 1991; Maroteaux et al. 1992; Boschert et al. 1994; Riad et al. 2000; Sari, 2004; Ding and Zhou 2014). 5-HT1BRs are commonly coupled to the Gi/o protein and inhibitory (Hannon and Hoyer 2008). Thus, there is an anatomical basis for 5-HT to inhibit striatonigral GABA output.

Another key feature of the SNr is the densely expressed 5-HT transporters (SERTs) on the 5-HT axons (Fig. 1B). SERTs are the target of SSRI-based antidepressant treatment (Wong et al. 2005). SSRIs block SERT-mediated 5-HT reuptake and increase the extracellular 5-HT level (Wong et al. 2005). Chronic increase in the extracellular 5-HT level can desensitize the 5-HT1A and 5-HT1B autoreceptors in 5-HT neurons and their axon terminals via diminished receptor-G-protein coupling (Hensler 2002; Castro et al. 2003; Cornelisse et al. 2007) or receptor internalization (Descarries and Riad 2012), thus contributing to their antidepressant effect (Blier and El Mansari 2013), although the precise neuronal mechanisms are not established for either depression or antidepressant treatment.
To our knowledge, although studies have examined the molecular and behavioral aspects of 5-HT1BRs in the brain (Zhuang et al. 1999; Woehrle et al. 2013), neurophysiological studies on chronic SSRI effects on presynaptic 5-HT1 heteroreceptors on non-5-HT axon terminals are lacking, and currently there is no data on the potential chronic SSRI treatment-induced alteration of the 5-HT1BRs on the striatonigral axon terminals. Given the importance of the nigral node in the emotion-regulating CBGTC loop (Marchand et al. 2012), it is critical to understand how chronic SSRI treatment affects these 5-HT1BRs and the functional consequences. Based on the prevailing idea that 5-HT1 autoreceptors are desensitized following chronic SSRI-type antidepressant (Newman et al. 2004; Descarries and Riad 2012; Blier and El Mansari 2013), we initially reasoned that chronic SSRI treatment may desensitize 5-HT1BRs on the striatonigral axon terminals; our data, however, indicate a substantial sensitization, a novel finding that is the opposite of the changes for 5-HT1 autoreceptors reported in the literature.

Material and Methods

Electrophysiology

Preparation of brain slices. Wild type, 25 to 35-day old male and female C57BL mice were used. Equal numbers of male and female mice were used; no difference in 5-HT effects was noticed and data were pooled. Young mice were used because brain slices from young animals survive tissue-sectioning better and thus facilitate recording. We have determined that the baseline 5-HT1B effect was stable during this period. Additionally, it has been reported that in rodents, the 5-HT system develops rapidly, reaching adult levels at postnatal day 21 (Murrin et al. 2007), and SSRIs produced antidepressant-like effects in 21-day old juvenile rats (Reed et al. 2008). Further, depression is a common psychiatric disorder in children and adolescents, and SSRIs are the common pharmacological treatment (Birmaher 2014; Kennard et al. 2014;
Kessler et al. 2001; March et al. 2004). Thus, data from juvenile and adolescent animals are important.

All procedures were carried out in accordance with Institutional Animal Care and Use Committee of The University of Tennessee Health Science Center (UTHSC) and followed the guidelines of the National Institutes of Health. The procedure to prepare 15° angular sagittal slices containing the SNr, the GPe and the striatum (Fig. 2A) has been described in detail (Connelly et al. 2010; Ding et al. 2013; Ding et al. 2015). Briefly, mice were killed by decapitation under deep anesthesia, and brains were quickly dissected out and immediately immersed in an oxygenated ice-cold cutting solution containing (in mM): 220 glycerol, 2.5 KCl, 1.25 NaH$_2$PO$_4$, 25 NaHCO$_3$, 0.5 CaCl$_2$, 7 MgCl$_2$ and 20 D-glucose. Three hundred (300) μm-thick parasagittal slices were cut on a Leica Zero Z VT1200S vibratome (Leica Microsystems, Wetzlar, Germany) at a 15° angle to the sagittal plane using a precision metal wedge. The brain slices were transferred to a holding chamber filled with a standard artificial cerebrospinal fluid (ACSF) (in mM: 125 NaCl, 2.5 KCl, 25 NaHCO$_3$, 1.25 NaH$_2$PO$_4$, 2.5 CaCl$_2$, 1.3 MgCl$_2$, and 10 D-glucose) that was kept at 34 °C and continuously bubbled with 95% O$_2$ and 5% CO$_2$. After an initial 30 min incubation at 34 °C, the brain slices were kept at room temperature (25 °C). Ascorbic acid (0.4 mM) was added to all brain slice-bathing solutions including the cutting solution to protect the tissue (Rice 1999).

**Fluoxetine treatment.** Fluoxetine hydrochloride was dissolved in distilled and autoclaved water and administered subcutaneously with daily dose of 20 mg/kg. Briefly, starting on postnatal day (PN) 15, male and female C57BL mice received 2 subcutaneous doses of 10 mg/kg fluoxetine at 8:00 AM and 5:00 PM. Injection of 0.9% NaCl saline served as control. Mice were sacrificed at 10:00 AM on the appropriate treatment days. For 1 day treatment to test the acute effect of fluoxetine, mice received the first fluoxetine injection at 5:00 PM the day before and the second fluoxetine injection at 8:00 AM on the day of experiment and sacrificed at 2 hours later. To test
the effect of chronic fluoxetine treatment, mice received fluoxetine treatment for up to 20 days; the effects during 10-20 days were similar and the data from these treatment days were pooled. The daily 20 mg/kg fluoxetine dose was chosen because literature reports indicate that 5-30 mg/kg/day fluoxetine produces antidepressant-like effects (Cryan et al. 2005) and also biochemical changes in 5-HT1AR- and 5-HT1BR-signaling pathways (Hensler 2002; Le Poul et al. 2000; Pejchal et al. 2002; Shen et al. 2003). This dose did not affect the body weight of the mice. A two subcutaneous injections/day protocol was chosen because the blood half life of fluoxetine in rodents is only about 6 hours, whereas it is 12 hours in non-human primates and 2 days in humans (Ansorge et al. 2008; Czachura and Rasmussen 2000; Hirano et al. 2005; Sawyer and Howell 2011).

Whole cell patch clamp recording. Slices were placed in a recording chamber mounted on the microscope stage and continuously perfused at 2 ml/min with the standard ACSF saturated with 95% O₂ and 5% CO₂. Recordings were made at 30 °C (TC 324B temperature controller, Warner Instruments) under visual guidance of a video microscope (an Olympus BX51WI and a Zeiss Axiocam MRm digital camera) equipped with Nomarski optics and a 60X water immersion lens (Fig. 2B). Patch pipettes were pulled from borosilicate glass capillary tubing (KG-33, 1.1 mm i.d., 1.65 mm o.d., King Precision Glass, Claremont, CA) using a PC-10 puller (Narishige, Tokyo, Japan) and had resistances of 1.5-2.5 MΩ when filled with one of the following intracellular solutions. A KCl-based intracellular solution (in mM: 135 KCl, 0.5 EGTA, 10 HEPES, 2 Mg-ATP, 0.2 Na-GTP, and 4 Na₂-phosphocreatine, pH 7.25, 280-290 mOsm) was used to record IPSCs in voltage clamp recording mode. To record hyperpolarizing inhibitory postsynaptic potentials (IPSPs) in current clamp mode, we used a KMeSO₄-based intracellular solution (in mM: 130 KMeSO₄, 5 KCl, 0.5 EGTA, 10 HEPES, 2 Mg-ATP, 0.2 Na-GTP, and 4 Na₂-phosphocreatine, pH 7.25, 280-290 mOsm). When recording evoked IPSCs, the Na⁺ channel blocker lidocaine N-ethyl bromide (5 mM) was added to the intracellular solution to
block Na spikes. All recordings were made in the presence of 10 μM DNQX and 20 μM AP-5 to block ionotropic glutamate receptors.

**Electrical stimulation to evoke synaptic responses.** To evoke striatonigral IPSCs, a bipolar tungsten electrode (Microprobes, Gaithersburg, Maryland) was placed in the striatum. A paired-pulse with an inter-pulse interval of 50 ms was generated by a Master-8 pulse generator (AMPI, Jerusalem, Israel) and delivered at 0.05 Hz. The stimulation intensity was from 50 to 300 μA at a constant duration of 0.2 ms. To evoke pallidonigral IPSCs, a minimal stimulation method was used to minimize the activation of the passing striatonigral axons. Briefly, a theta capillary pipet with a tip diameter of 5 μm was placed in the GPe. Paired pulses with an inter-pulse interval of 50 ms were delivered at 0.05 Hz. The stimulation intensities ranging from 10 to 50 μA were first adjusted to elicit all-or-none IPSCs with 20-50% failure rate. Then the stimulation intensities were increased a few μA to decrease the failure rate to less than 10%.

**Data acquisition and analysis.** A Multiclamp 700B amplifier, pClamp 9.2 software and Digidata 1322A interface (Molecular Devices, Sunnyvale, CA) were used to acquire data. For voltage clamp recording, cells were held at –70 mV, leading to inward IPSCs with our KCl-based intracellular solution. Access resistance was monitored by a 10 mV, 50 ms pulse before every evoked IPSC. Cells in which the access resistance increased by >15% were discarded. Liquid junction potentials were not corrected because our experimental questions did not have any voltage dependence.

The peak IPSC amplitudes were measured using Clampfit 9.2 software. Averages of 10 consecutive IPSCs before or during drug administration were used to evaluate baseline and drug response, respectively. Paired pulse ratio (PPR) was calculated by dividing the peak amplitude of the second IPSCs by the peak amplitude of the first IPSCs. To analyze the effect of
a train of IPSPs on spontaneous firing of SNr GABA neuron, the spike number during the 300 ms immediately after the first stimulation artifact was measured.

To obtain the IC50, we fitted the averaged data points in the dose-response plot to the Hill equation: \( Y = A \cdot X^n \left[ 1/(K^n + X^n) \right] \), where \( A \) is the maximal inhibition, \( X \) is the 5-HT concentrations, \( K \) is the IC50.

**Drugs.** All drugs used in electrophysiology experiments were made into stock solutions in ddH2O or dimethyl sulfoxide. Stock solutions of drugs were diluted at least 1:1000 to the desired concentration in ACSF immediately prior to their application. APV, CNQX, lidocaine N-ethyl bromide (QX-314), 5-HT, picrotoxin, CP93129 and NAS-181 were purchased from either Tocris or Sigma-Aldrich. Drugs were bath-applied. Fluoxetine hydrochloride was also obtained from the National Institute of Mental Health Chemical Synthesis and Drug Supply Program.

**Immunohistochemistry**

**Serotonin transporter (SERT) immunostain:** Under overdose urethane anesthesia, 40-50 days old mice (n=3) were intracardially perfused with phosphate buffered saline (PBS) and then 4% paraformaldehyde dissolved in PBS. The brains were further post-fixed in the same 4% paraformaldehyde at 4 °C overnight. Coronal brain sections (50 μm in thickness) were cut on a Leica VT1200S vibratome. Free-floating sections were incubated with 2% fat-free milk, 1% bovine serum albumin (BSA), and 0.4% Triton X-100 in PBS for 1 hour at room temperature (RT) to block nonspecific binding and permeabilize the cell membrane, respectively. After thorough rinsing, the free-floating sections were incubated for 48 hours at 4 °C with the primary antibody (see below) and then rinsed in PBS three times for 5 min each, followed by incubating with the secondary antibody (see below) for 3 hours at RT in the dark. Both the primary and secondary antigen-antibody reactions occurred in PBS containing 3% normal donkey serum, 1% BSA, and 0.1% Triton X-100. The primary antibody was a polyclonal SERT antibody raised...
in goat (Santa Cruz Biotechnology; diluted at 1:800). The secondary antibody was a donkey anti-goat IgG antibody, conjugated with red Alexa Fluor 568 (diluted at 1:200). At the age of the mice used here, SERT immunostain reliably labels SERT-expressing 5-HT axon terminals (Nielsen et al. 2000).

5-HT1BR immunostain: Under overdose urethane anesthesia, mice were intracardially perfused with PBS. For this particular antigen and antibody pair, we used the following procedure to enhance the detection of 5-HT1BR protein. The brains were quickly dissected out and immediately frozen and stored at −80 ºC. Brains were sectioned on a Leica cryostat at 20 µm in thickness. The sections were collected onto SuperFrostPlus microscope slides (Fisher Scientific) and stored at −80 ºC until use. To process the tissue sections for 5-HT1BR immunostain, the slides were removed from −80 ºC, quickly thawed, air-dried and lightly fixed in 4% paraformaldehyde for 6 min and then in Bouin’s solution for 5 min and finally in 80% ethanol for 5 min. Then these sections were incubated in 2% fat-free milk, 1% BSA, and 0.4% Triton X-100 in PBS for 1 hour at RT to block nonspecific binding and permeabilize the cell membrane, respectively. After thorough rinsing, the sections were incubated for 48 hours at 4°C with the primary antibody (see below) and then rinsed in PBS three times for 5 min each, followed by incubating with the secondary antibody (see below) for 3 hours at RT in the dark. Both the primary and secondary antigen-antibody reactions occurred in PBS containing 3% normal donkey serum, 1% BSA, and 0.2% Triton X-100. The primary antibody was a polyclonal 5HT1BR antibody raised in rabbit (Santa Cruz Biotechnology; diluted at 1:1000). The secondary antibody was donkey anti-rabbit IgG antibody, conjugated with green Alexa Fluor 488 (diluted at 1:200; Invitrogen).

Fluorescence images were acquired on a Zeiss 710 laser scanning confocal microscope at the UTHSC Neuroscience Imaging Center in Memphis, Tennessee.
Statistics. Data are reported as mean ± standard error. The paired t test was used to make comparisons of evoked events before and during drug administration. Unpaired t-test, one-way ANOVA, and two-way ANOVA were also used when needed. When two-way ANOVA was used, drug treatment and dose of 5-HT were the independent variables. p value<0.05 was considered statistically significant.

Results

Independent elicitation of the striatonigral and pallidonigral IPSCs

The main neuron type in the SNr is the GABA projection neurons that fire spontaneously around 10 Hz under in vitro conditions (Atherton and Bevan 2005; Ding et al. 2011a,b; Zhou and Lee 2011). The SNr also contains sparse dopamine neurons that fire spontaneously around 1.5 Hz under similar in vitro conditions (Zhou et al. 2006; Connelly et al. 2010; Ding et al. 2011a,b). Thus, we first briefly recorded action potentials of neurons in SNr in a cell-attached mode in our angular sagittal brain slices (Fig. 2A, B, C). Neurons in the SNr with a spontaneous firing rate ≥ 5 Hz were presumed to be GABA neurons (Zhou et al. 2006; Connelly et al. 2010; Ding et al. 2011a,b). After cell identification, we proceeded to whole-cell recording mode and started striatal or GPe stimulation to activate striatonigral or pallidonigral GABAergic transmission, respectively. As shown in Fig. 2D, focal stimulation in the striatum consistently evoked IPSCs in SNr GABA neurons. These evoked IPSCs and also the spontaneous IPSCs were blocked by bath applied picrotoxin (100 µM), indicating that these IPSCs were mediated by GABA_A receptors, as expected. Focal GPe minimal stimulation also consistently evoked IPSCs in SNr GABA neurons that were blocked by bath applied 100 µM picrotoxin too.

We also used low intensity stimuli and a paired pulse protocol to ensure that our focal stimulation in the dorsal striatum and in the GPe evoked isolated striatonigral IPSCs and
pallidonigral IPSCs, respectively. We found that in our sampled cells, as shown in Fig. 2D, the latency was 7.8 ± 0.2 ms (n=23 cells) for the striatum-evoked IPSCs and 2.5 ± 0.2 ms (n=11) for GPe-evoked IPSCs (unpaired t-test, p<0.000 for the 2 latency values). The PPR was 1.6 ± 0.1 (n=23 cells) for the striatum-evoked IPSCs and 0.7 ± 0.1 (n=11 cells) for GPe-evoked IPSCs (unpaired t-test, p<0.000 for the 2 PPR values). These are consistent with data that the striatonigral IPSC has a long latency (~7 ms, due to the long distance pathway) and a facilitating paired pulse ratio (PPR= ~2), while pallidonigral IPSC had a short latency (~2 ms) and a depressing PPR (~0.7) (Connelly et al. 2010; Ding et al. 2015). Thus, under our experimental conditions, the striatum-evoked IPSCs in SNr GABA neurons were predominantly striatonigral IPSCs while the GPe-evoked IPSCs in SNr GABA neurons were predominantly pallidonigral IPSCs. This allowed us to study 5-HT effects on these relatively pure striatonigral IPSCs and pallidonigral IPSCs.

**5-HT inhibits striatonigral IPSCs via a presynaptic mechanism**

After obtaining a stable baseline recording of striatonigral IPSCs, we started to bath-apply 5-HT. We initially used 10 µM 5-HT and found that it reduced the striatonigral IPSC amplitude by a profound 94% on the average (Fig. 3A). By using 5-HT ranging 0.5 to 20 µM, we determined that 5-HT inhibited striatonigral IPSCs in a dose response manner with an IC₅₀ of 1.2 µM and a Hill coefficient of 1.3, based on fitting the data points to the Hill equation (Fig. 3A,C). In contrast, bath application of 0.5 to 20 µM 5-HT did not significantly affect pallidonigral IPSCs (Fig. 3B,C). These results indicate that 5-HT can potently inhibit the striatonigral GABAergic transmissions while having no effect on the pallidonigral GABAergic transmissions.

Next, we determined if 5-HT was reducing the striatonigral IPSC amplitude by a presynaptic mechanism. To answer this question, we used a paired pulse protocol with a pair of stimulating pulses separated by 50 ms (20 Hz). This frequency was chosen because 20 Hz is a common firing frequency when the striatonigral neurons are phasically active in freely moving
mice (Miller et al. 2008; unpublished data of Ben Sagot & Fu-Ming Zhou). Using this paired pulse protocol, we found that under control conditions, striatonigral IPSCs exhibited a paired pulse facilitation with a PPR value of 1.66 ± 0.22 (Fig. 3D1,D4). During bath application of 5 µM 5-HT, the peak amplitude of the first of the paired striatonigral IPSCs was reduced to a larger extent than that of the second IPSC, thus increasing the PPR from 1.66 ± 0.22 under control to 4.10 ± 0.67 during 5-HT application (p<0.05, paired t-test, n=6) (Fig. 3D2-D4). Because an increased PPR is an indication of inhibition of presynaptic vesicular release (Fioravante and Regehr 2011), these results suggest that 5-HT inhibits striatonigral IPSCs by acting presynaptically to inhibit transmitter release. To further support our conclusion of presynaptic 5-HT inhibition of GABA release of the striatonigral axon terminals, we calculated the coefficient of variation (CV) of the striatonigral IPSCs by dividing the standard deviation by the mean of the 10 or more individual IPSCs, as described in Michaeli and Yaka (2010). Under control condition, the baseline CV was 0.43±0.08 (n=6 cells); during 5 µM 5-HT, the CV was increased to 0.78±0.05. The increase in CV was significant with p<0.05 (paired t-test), indicating a decreased vesicular release probability during 5 µM 5-HT.

**5-HT1B agonism mimics and 5-HT1B antagonism blocks 5-HT effects on the striatonigral IPSCs**

Next, we asked this question: Are presynaptic 5-HT1BRs mediating 5-HT’s inhibitory effect on striatonigral IPSCs? To answer this question, we evoked striatonigral IPSCs with the same paired pulse protocol used above and bath applied 1 µM CP93129, a 5-HT1BR agonist. As shown in Fig. 4A, after bath application of 1 µM CP93129, the peak amplitude of the first of the paired striatonigral IPSCs was reduced to 11.0 ± 3.0 % of control, a 89% reduction (p<0.000, paired t-test, n=6). Similar to the effect of 5-HT, 1 µM CP93129 significantly increased the PPR from 1.67 ± 0.21 to 4.08 ± 0.72 (p<0.05, paired t-test, n=6), indicating a presynaptic 5-HT1BR-
mediated inhibition of vesicular GABA release. This is consistent with the literature that 5-HT1BR activation decreases vesicular transmitter release (Mizutani et al. 2006).

Furthermore, we tested if NAS181, a 5-HT1BR antagonist, can block 5-HT’s effect on striatonigral IPSCs. As shown in Fig. 4E,F, bath application of 5 µM 5-HT reduced the striatonigral IPSCs to 18.0 ± 3.0% of control, a 82% reduction (p<0.0001, paired t-test, n=6). Bath application of 10 µM NAS181 almost completely blocked the inhibitory effect of 5 µM 5-HT, restoring the striatonigral IPSC amplitude to 87.5 ± 10.1% of control (p<0.005, paired t-test, n=6) (Fig. 4E,F). Taken together, these results indicate that 5-HT potently inhibits the striatonigral IPSCs through the presynaptic 5-HT1BRs on striatonigral axon terminals.

5-HT and 5-HT1B receptor agonist CP93129 reduce the striatonigral inhibition of SNr GABA neuron firing

Next, we examined the functional consequences of the robust presynaptic 5-HT1BR inhibition of the GABA release from striatonigral axon terminals, a critical issue that had never been studied. We predicted that 5-HT may reduce striatonigral IPSP’s inhibitory effect on the spontaneous firing of SNr GABA neurons. To test this idea, we evoked a train of 5 striatonigral IPSPs by a train of 5 stimuli at 20 Hz, mimicking a common firing pattern of the striatonigral neurons in freely moving mice (Miller et al. 2008; unpublished data of Ben Sagot and Fu-Ming Zhou). As shown in Fig. 5A, B, under the control condition, the striatonigral IPSP train strongly inhibited or paused the spontaneous firing of SNr GABA neurons. After bath application of 5 µM 5-HT, the amplitude of the striatonigral IPSPs and hence the effect of the striatonigral IPSPs on the spontaneous firing of SNr GABA neurons were reduced substantially. To quantify 5-HT’s effect on the striatonigral IPSP-induced pause in SNr GABA neurons, we calculated the spike number in the 300-ms window that starts at the first stimulus, as illustrated in Fig. 5. The spike number in the 300-ms window was only 0.23 ± 0.14 (spike firing was almost completely inhibited or paused) under control condition, but it was increased to 2.20 ± 0.43 under 5 µM 5-HT (the
pause was substantially reduced) (p<0.005, paired t-test, n=6). This effect is likely mediated by presynaptic 5-HT1BRs that inhibit the striatonigral IPSPs. At the same time, bath application of 5 µM 5-HT significantly increased the baseline spontaneous firing of SNr GABA neurons from 8.4 ± 1.7 Hz to 16.7 ± 1.8 Hz (p<0.001, paired t-test, n=6), probably through activating postsynaptic 5-HT2C receptors known to express in SNr GABA neurons (Clemett et al. 2000).

To further test our idea, we studied the effect of the 5-HT1B agonist CP93129 on striatonigral IPSP-induced pause in the spontaneous firing of SNr GABA neurons with the same stimulation protocol. As shown in Fig. 5A4-5,B4-5, the spike number in the 300-ms window was 0.23 ± 0.13 under control condition and increased to 2.58 ± 0.38 during bath application of 5 µM CP93129 (p<0.001, paired t-test, n=8). The effect was recovered upon washing out 5 µM CP93129. Unlike 5-HT, 5 µM CP93129 had no significant effect on the baseline spontaneous firing of SNr GABA neurons (10.4 ± 1.4 Hz under control vs. 11.2 ± 1.6 Hz under CP93129, p>0.05, paired t-test, n=8), since SNr GABA neurons are not known to express postsynaptic 5-HT1BRs (Sari 2004). Together, these results suggest that 5-HT can reduce striatonigral IPSPs by activating the presynaptic 5-HT1BRs and soften the pausing effect of the striatonigral output on the spontaneous high frequency firing in SNr GABA neurons.

**Chronic fluoxetine treatment sensitizes 5-HT1BRs on the striatonigral axon terminals**

To determine the potential effects of chronic SSRI treatment on the functional status of the 5-HT1BRs on striatonigral axon terminals, we injected mice with either saline or fluoxetine using the protocol described in the methods section. The effects during 10-20 days were similar and thus data from these treatment days were pooled. As shown in Fig. 6A-C, 5-HT inhibited the striatonigral IPSCs in a dose dependent manner in both chronically fluoxetine-treated and saline treated mice [2-way ANOVA, F(7) = 38.826, p=0.000]. However, at 0.5 µM, 5-HT reduced the striatonigral IPSC by 79.3 ± 5.2 % (n=6) in chronically fluoxetine-treated mice and only 16.1 ± 9.9% (n=6) in saline-treated mice [2-way ANOVA, F(1,71) = 66.56, p=0.000]. Similarly, at 1 µM, 5-
HT inhibited the striatonigral IPSC to a larger extent in chronically fluoxetine-treated mice than in saline-treated mice (85.4 ± 5.3 % for fluoxetine-treated mice, n=6 and 40.9 ± 4.7% for saline-treated mice, n=7, 2-way ANOVA, F(1,71) = 35.65, p=0.000). At 3 µM, 5-HT also had larger inhibitory effects on the striatonigral IPSCs in chronically fluoxetine-treated mice than in age matched saline-treated mice (89.8 ± 3.9% and 66.3 ± 7.3% for fluoxetine-treated mice and for saline-treated mice respectively, n=6 in each group, 2-way ANOVA, F(1,71) = 9.21, p=0.0034).

However, at 5, 10 and 20 µM, the 5-HT inhibition of the striatonigral IPSCs was a similarly total or near-total inhibition in both chronically fluoxetine- and saline-treated mice [2-way ANOVA, F(1,71) = 2.01, 0.07 and 0.11, p=0.16, 0.80 and 0.74 for 5,10 and 20 µM 5-HT, respectively]. When we fitted these data points to the Hill equation, we found that the IC_{50} was 0.24 ± 0.01 µM and 1.4 ± 0.2 µM for fluoxetine-treated mice and saline-treated mice, respectively (Fig. 6C).

There was a clear leftward shift in the dose-response curve of 5-HT inhibition of the striatonigral IPSCs in chronic fluoxetine-treated mice, compared with that of the age-matched saline-treated mice, indicating that 5-HT1BRs on the striatonigral axon terminals became more sensitive to 5-HT after chronic fluoxetine treatment.

We have also tested the potential effects of 1-day and 7-day fluoxetine treatment (see Method for dosing details). As shown in Fig. 7, after 1 day treatment with fluoxetine, 1 µM 5-HT inhibited striatonigral IPSC by 44.4 ± 4.5% (n=6 cells) in mice and by 40.9 ± 4.7% (n=7 cells) in saline-treated control mice (one-way ANOVA, p>0.05). In mice treated with fluoxetine for 7 days, 1 µM 5-HT inhibited striatonigral IPSC by 68 ± 4.1% (n=7), significantly larger than that in saline-treated control mice and also 1-day fluoxetine treatment mice (Fig. 7B, one-way ANOVA, p<0.05). However, compared to that in mice treated with fluoxetine for 10-20 days (chronic fluoxetine treatment), the inhibitory effect of 1 µM 5-HT on striatonigral IPSCs in 7-day fluoxetine treatment group is significantly smaller (one-way ANOVA, p<0.05). Starting on fluoxetine treatment day 10, the 1 µM 5-HT-induced inhibition of the striatonigral IPSCs is at 85%, near the maximal 94% inhibition induced by 10 µM 5-HT. Thus, 10-day fluoxetine treatment-induced
sensitization was near the saturating level, and thus data from 10-20 days’ treatment were justified to be pooled. These results indicated that fluoxetine treatment-induced sensitization of 5-HT1BRs on the striatonigral axon terminals is a gradual process and the timeline is similar to that of 5-HT1A autoreceptor desensitization in 5-HT neurons in intact animals (Blier and De Montigny 1983).

**Chronic fluoxetine treatment upregulates presynaptic 5-HT1BR-mediated reduction of the pause in SNr GABA neuron firing**

Since our data showed that chronic fluoxetine treatment sensitizes 5-HT1BRs on the striatonigral axon terminals (Fig. 6), we reasoned that the same treatment may upregulate 5-HT1BR-mediated reduction in the striatonigral IPSP-induced pause of SNr GABA neuron firing. To test our hypothesis, we treated mice with saline or fluoxetine using the same protocol described above. In brain slices from these treated mice, we tested the effects of the 5-HT1BR agonist CP93129 at a low 0.1 μM on the striatonigral IPSP-induced pause of SNr GABA neuron firing; 5-HT was not used to avoid 5-HT activation of somatic 5-HT2C receptors that would increase SNr GABA neuron firing and complicate data interpretation. To quantify CP93129’s effect on the pause, we measured the pause duration induced by single striatonigral IPSPs. Here the pause duration was defined as the time window between the artifact of the stimulus that evoked the striatonigral IPSP and the first spike after the stimulus artifact (Fig. 8A1, B1). Since the pausing effect of the striatonigral IPSP on SNr GABA neuron firing was also dependent on where the IPSP was located on the membrane potential trajectory, besides its amplitude, we selected sweeps in which the IPSPs were evoked at the same position on the rising part of inter-spike membrane potential trajectory before, during and after drug application; in these sweeps, we examined how the drug-induced changes in IPSP amplitudes affected the pausing of SNr GABA neuron firing.
We found that bath application of 0.1 μM CP93129 reduced the pause duration to 79.6 ± 6.1% of control condition in saline-treated mice (F(1,5) = 43.678, p<0.001, One-way repeated measures ANOVA) [from control pause duration of 79.3 ± 13.7 ms] (Fig. 8A, C). In chronic fluoxetine-treated mice, 0.1 μM CP93129 reduced the pause duration to 57.6 ± 4.6% of control condition (F(1,5) = 52.281, p<0.001, One-way repeated measures ANOVA) [from control pause duration of 93.6 ± 13.7 ms] (Fig. 8B, C). The effect of 0.1 μM CP93129 on the pause was larger in chronic fluoxetine-treated mice than in saline-treated mice (n=6 for each group, p<0.05, unpaired t-test, (Fig. 8C). Also, as expected, bath application of 0.1 μM CP93129 reduced the peak amplitude of striatonigral IPSPs to 67.5 ± 10.3% of control in saline treated mice (F(1,5) = 32.497, p<0.001, One-way repeated measures ANOVA) (from control IPSP amplitude of 8.8 ± 1.4 mV) and to 27.7 ± 10.3% of control in chronic fluoxetine treated mice (F(1,5) = 5.83, p<0.05, One-way repeated measures ANOVA) (from control IPSP amplitude of 9.7 ± 2.4 mV) (Fig. 8D); and this effect was also larger in chronic fluoxetine-treated mice than in saline-treated mice (n=6 for each group, p<0.05, unpaired t-test). Additionally, saline or fluoxetine treatment did not alter the basal firing frequency of SNr GABA neurons recorded in our brain slices; CP93129 also did not affect the spontaneous firing in these neurons: in saline-treated mice, the spontaneous firing rate was 9.95 ± 1.1 Hz under control, 10.9 ± 1.6 Hz under 0.1 μM CP93129, and 10.5 ± 1.4 Hz after wash; in fluoxetine-treated mice, the spontaneous firing rate was 11.1 ± 1.5 Hz under control, 12.1 ± 2.0 Hz under 0.1 μM CP93129, and 12.1 ± 2.2 Hz after wash. Together, these data clearly indicate that 5-HT1BR-mediated reduction of SNr GABA neuron firing pause was enhanced after chronic fluoxetine treatment.

Discussion

The main findings of this study are that 5-HT potently inhibits the striatonigral GABA output via the 5-HT1BRs on the striatonigral axon terminals. Consequently, 5-HT reduces the striatonigral...
IPSP-induced pause of the high frequency spontaneous firing in SNr GABA neurons. Moreover, after chronic fluoxetine treatment, these 5-HT1BRs became more sensitive to 5-HT, in contrast to the common, chronic SSRI-induced desensitization of 5-HT1 autoreceptors. Thus, our present study has revealed a novel aspect of chronic SSRI-based antidepressant treatment.

Presynaptic 5-HT1B heteroreceptors potently reduce striatonigral GABA output

We found that 5-HT can completely inhibit the striatonigral IPSCs while not affecting the GPe-evoked pallidonigral IPSCs; 5-HT also increased the PPR. Additionally, the 5-HT effects were mimicked by the 5-HT1BR agonist CP93129 and blocked by the 5-HT1BR antagonist NAS181, clearly indicating that presynaptic 5-HT1BRs on striatonigral axon terminals were mediating these effects. Our data are consistent with the anatomical fact that 5-HT1BRs are expressed at a high level on the striatonigral axon terminals while the 5-HT1BR gene expression is absent or very low in pallidal neurons (Voigt et al. 1991; Maroteaux et al. 1992; Boschert et al. 1994; Sari et al. 1999; Riad et al. 2000; Sari 2004). Our results also substantially expand the study of Stanford and Lacey (1996) that reported that 10 μM 5-HT induced a 60% inhibition of locally evoked IPSCs of unknown origin. In our present study, 10 μM 5-HT induced a virtually complete (94%) inhibition of the striatum-evoked, relatively pure striatonigral IPSCs, while having no effect on the pallidonigral IPSCs. The smaller 5-HT effect of Stanford & Lacey (1996) (60% vs. our 94% inhibition with 10 μM 5-HT) is probably due to their activation of mixed striatonigral and pallidonigral IPSCs, because pallidonigral IPSCs are insensitive to the presynaptic 5-HT inhibition. We also provided more compelling pharmacological evidence for the mediation of 5-HT1BRs by using the specific 5-HT1BR agonist CP93129 and antagonist NAS181.

Presynaptic 5-HT1B heteroreceptors reduce the pause in the high frequency firing in nigral GABA neurons
Our present study explored the functional importance of the potent presynaptic 5-HT1BR inhibition of the GABA release from striatonigral axon terminals, a critical issue that had never been studied until now. We found that striatonigral IPSPs can inhibit or pause the endogenously generated high frequency firing in SNr GABA neurons, consistent with previous studies (Hikosaka et al. 2000, 2014; Connelly et al. 2010; Kravitz at al. 2010). We further demonstrated that 5-HT and 5-HT1BR agonist CP93129 reduced striatonigral IPSP-induced pause of the spontaneous high frequency firing of SNr GABA neurons. This is important because the pause in the high frequency firing of SNr GABA neurons releases the downstream targets such as the thalamus and brainstem motor nuclei (Hikosaka et al. 2000, 2014; Kaneda et al. 2008; Kravitz at al. 2010; Basso and Sommer 2011; Sano et al. 2013), and consequently, a reduction in the pause is likely to affect the activity of these downstream nuclei.

We need to note here that 5-HT has other effects on the SNr. For example, 5-HT increases the basal spontaneous firing in SNr GABA neurons via activating 5-HT2C receptors in these neurons (Stanford and Lacey 1996; Zhou and Lee 2011). Additionally, presynaptic 5-HT1BRs reduce subthalamic glutamatergic input-induced burst firing in SNr GABA neurons (Ding et al. 2013). Clearly, 5-HT exerts different effects on different targets in the SNr but with an apparently common goal: to keep SNr GABA neurons in their default autonomous high frequency firing state. In other words, 5-HT exerts a homeostatic effect here: the 5-HT2CRs help SNr GABA neurons maintain their basal high frequency firing and prevent them from firing too slowly; the 5-HT1BRs on the subthalamonigral axon terminals prevent the glutamatergic input from triggering too much firing in SNr GABA neurons; and finally, the 5-HT1BRs on the striatonigral axon terminals prevent the striatal GABA input from inhibiting SNr GABA neuron firing too strongly.
Chronic fluoxetine treatment sensitizes 5-HT1BRs on the striatonigral axon terminals.

Our results showed that following chronic fluoxetine treatment, the sensitivity of the presynaptic 5-HT1BRs to 5-HT increased substantially, enabling a low dose of 5-HT to more strongly reduce the GABA release from the striatonigral axon terminals. This is a novel cellular mechanism for chronic SSRI treatment that has never been reported before, based on our knowledge. The prevailing idea is that SSRIs block SERT-mediated 5-HT reuptake and increase the extracellular 5-HT level that desensitizes the 5-HT1A and 5-HT1B inhibitory autoreceptors in 5-HT neurons and their axon terminals via diminished receptor-G-protein coupling (Hensler 2002; Castro et al. 2003; Cornelisse et al. 2007) or receptor internalization (Descarries and Riad 2012), this in turn leads to sustained increase in extracellular 5-HT and thus contributes to the antidepressant effect of SSRI treatment (Wong et al. 2005; Blier and El Mansari 2013). Reports indicate that postsynaptic 5-HT1ARs may also be desensitized via similar mechanisms (Li et al. 1997; Hensler 2002).

Thus, we were initially surprised by our results that chronic fluoxetine treatment sensitized the 5-HT1BRs on the striatonigral axon terminals. However, we quickly realized that although there is no published study on the potential effects of chronic SSRI treatment on presynaptic 5-HT1 heteroreceptors (presynaptic 5-HT1 receptors on non-5-HT axons), there are reports on the potential effects of chronic SSRI treatment on postsynaptic 5-HT1 heteroreceptors (5-HT1 receptors on the somata and dendrites of non-5-HT neurons). Particularly, an electrophysiological study indicated that postsynaptic 5-HT1A receptors in the hippocampal neurons in rats were sensitized by chronic fluoxetine treatment (Beck et al. 1997). Biochemical studies indicate that chronic SSRI treatment may sensitize postsynaptic 5-HT1A receptors by increasing the receptor-G protein coupling (Shen et al. 2002; Castro et al. 2003; Zanoveli et al. 2007), providing a possible molecular basis for our unexpected observation.

Although the mechanisms underlying the sensitization of the presynaptic 5-HT1BRs on the striatonigral axon terminals was beyond the scope of our current study, the substantial
leftward shift in the dose-response curve (IC$_{50}$ changed from 1.4 μM to 0.2 μM, Fig. 6C) indicates an increased receptor binding affinity or more efficient downstream signaling mechanisms. Since the maximal effect here is a total inhibition under both conditions, this parameter is less informative. An alternative and non-exclusive possibility is that the 5-HT1BR expression at the striatonigral axon terminals is upregulated, also leading to a leftward shift in the dose-response curve. This possibility is consistent with a previous study reporting a 127% increase over the baseline in the 5-HT1B mRNA level following chronic fluoxetine-treated in rats, although the host cell was not identified (Le Poul et al. 2000). Thus, de novo increase in receptor expression may be another factor that allows lower concentrations of 5-HT to achieve the maximal and total inhibition of the striatonigral IPSC. A third possible mechanism for the increased 5-HT sensitivity or the leftward shift of the 5-HT dose-response curve (Fig. 6C) is an increased plasma membrane insertion on the striatonigral axon terminals after chronic fluoxetine treatment, a process that is opposite to the 5-HT1AR internalization underlying 5-HT1AR desensitization (Descarries and Riad 2012).

**Functional implications**

We found that 5-HT can almost completely inhibit the GABA release from the striatonigral axon terminal with an IC$_{50}$ at 1 μM. Although the endogenous 5-HT is unlikely to fully activate these 5-HT1BRs, this remarkable capacity, coupled with the intense 5-HT innervation in the SNr, still gives these receptors the power to regulate the striatonigral GABA output in both the normal brain and pathological conditions and thus has important implications.

An obvious implication is in motor control. It is now established that striatonigral GABA output promotes motor activity by inhibiting the SNr GABA neurons (Hikosaka et al. 2000, 2014; Kravitz et al. 2010; Friend and Kravtiz 2014). Our data show that the presynaptic 5-HT1BRs serve to reduce this motor-promoting striatonigral GABA output. We speculate that since too much motor activity can be harmful to the animal, these presynaptic 5-HT1BRs are expressed
there as a safety device to limit the striatonigral output. This may also be an evolutionary purpose for the exceptionally dense 5-HT innervation in the SNr. It will be interesting to determine the functional status of these presynaptic 5-HT1Rs in Parkinson's disease when there is a deficient or abnormal striatonigral output and in dyskinetic disorders when there is too much motor activity.

Another implication is in the regulation of emotion and antidepressant treatment. The precise anatomical location of emotional regulation is not known, but a proper function of the cortico-basal ganglia-thalamo-cortical (CBGTC) loop is essential to the emotional well-being, and dysfunction of the CBGTC loop is involved in depression pathogenesis (Marchand and Yurgelun-Todd 2010; Hamilton et al. 2012; Price and Drevets 2012; Hamon and Blier 2013). Aberrant functional connectivity in this loop has been indicated to be a primary pathology in depression (Marchand et al. 2012). Since the SNr is a narrow gate in the CBGTC loop, the information transfer in the SNr may critically affect the emotional regulation aspect of the loop. When the 5-HT1BRs on striatonigral axon terminals are sensitized during chronic SSRI treatment, these 5-HT1BRs can reduce GABA release and the striatonigral information transfer in a heightened manner, altering the information processing in the CBGTC loop and potentially contributing to the antidepressant effect of chronic fluoxetine treatment.
References


PMID: 24717335.


**Figure legends**

**Figure 1.** 5-HT innervation and 5-HT1BR expression in the SNr. **A,** A circuit diagram showing the position of the striatonigral projection in the cortico-basal ganglia-thalamo-cortical loop. **B,** A strikingly dense 5-HT innervation in the SNr revealed by SERT immunostaining in coronal brain sections. **C,** Relatively dense 5-HT1BR expression in the SNr revealed by 5-HT1BR immunostaining in coronal brain sections. As described in the methods section, **B** and **C** were obtained with different staining methods and 2 different tissue sections from 2 50-day old male mice. These two images illustrate the point that the SNr has an exceptionally dense 5-HT innervation and a relatively heavy expression of 5-HT1BRs in our experimental mice. Hippo, hippocampus; SNc, substantia nigra pars compacta; VTA, ventral tegmental area.

**Figure 2.** Focal stimulation in the striatum or globus pallidus evokes relatively pure striatonigral and pallidonigral IPSCs in SNr GABA neurons. **A,** A picture of a live, 15° angular sagittal brain slice taken with a 1X objective. The SNr, globus pallidus (GP) and striatum are clearly identifiable. Other structures such as the thalamus (Thal) and the zona incerta (ZI) are also clearly visible and marked. **B,** A picture, taken under a 60X objective, shows a typical SNr GABA neuron being patch clamped in a cell-attached mode. **C,** Typical spontaneous spikes (action potentials, ~ 17 Hz) in a presumed SNr GABA neuron recorded in cell-attached mode. **D,** Independent elicitation of striatonigral IPSCs and pallidonigral IPSCs. **D1** and **D2,** Example traces of striatum-evoked typical striatonigral facilitating IPSCs and GP-evoked depressing pallidonigral IPSCs, respectively. Time scale applies to both striatonigral IPSCs and pallidonigral IPSC. **D3,** Pooled data about paired pulse ratios (PPR) and latencies of the first evoked IPSCs induced by a paired pulse protocol with an interval of 50 ms of striatonigral IPSCs and pallidonigral IPSCs. For striatonigral IPSCs data, 26 cells from 26 brain slices of 22 mice; For pallidonigral data, 11 cells from 11 brain slices of 11 mice.
Figure 3. 5-HT strongly inhibits striatonigral IPSCs but does not affect pallidonigral IPSCs. A, A1, A2, Example traces of the striatonigral IPSCs by bath application of 3 and 10 μM 5-HT. B, B1, B2, Example traces of pallidonigral IPSCs not affected by bath application of 3 and 10 μM 5-HT. C, Dose-response relationship of the 5-HT inhibition of striatonigral IPSCs and pallidonigral IPSCs. The continuous line is the Hill equation fit. Each data point represents 6-8 experiments. For striatonigral IPSCs data, n=27 neurons, from 27 brain slices of 25 mice; For pallidonigral IPSCs data, n=15 neurons, from 15 brain slices of 14 mice. D, 5-HT increases paired pulse ratios of striatonigral IPSCs. D1, D2, Striatonigral paired IPSCs (evoked by 2 stimuli 50 ms apart) before and during bath application of 5 μM 5-HT. D3, These two traces were then scaled to the peak of their 1st IPSC to show the relative increase of the 2nd IPSC peak. D4, Summary showing a clear 5-HT-induced increase in PPR of striatonigral paired IPSCs.

Figure 4. 5-HT1BR agonist CP93129 mimics and 5-HT1BR antagonist NAS181 inhibits the 5-HT effects on striatonigral IPSCs. A, A1, Superimposed averaged striatonigral IPSCs evoked by the paired pulse protocol before (black), during (red) and after (blue) 1 μM CP93129 application. A2, Current traces in A1 are normalized to the peak of the first IPSC in each pair to show clearly that the second IPSC is increased during 1 μM CP93129 application, leading to increased PPR. A3, Pooled data showing the inhibitory effect of 1 μM CP93129 on the peak amplitude of the 1st of the paired IPSCs in 6 SNr GABA neurons. A4, Pooled data showing the increased PPR during 1 μM CP93129 in the 6 SNr GABA neurons. B, 5-HT1BR antagonist NAS181 blocked the effect of 5-HT on striatonigral IPSCs. B1, Superimposed averaged IPSCs evoked by striatum stimulation before (black), during 5 μM 5-HT application (red) and 10 μM NAS181 + 5 μM 5-HT application (blue) are shown. B2, Pooled data showing the blockade effect of NAS181 on the effect of 5-HT on striatonigral IPSCs (n=6 neurons, from 6 brain slices of 5 mice).
Figure 5. 5-HT reduces the pausing effect of striatonigral IPSPs on the spontaneous firing of SNr GABA neurons. A, A1-A3, A train of five striatonigral IPSPs evoked by stimulating striatum with a train of 5-pulses with an interval of 50 ms paused the spontaneous firing in SNr GABA neuron, that was strongly reduced by bath application of 5 μM 5-HT. A4, Summary showing the effect of 5 μM 5-HT on baseline spontaneous firing frequency, and the spike number in the 300-ms period immediately after the first stimulus artifact. A5, Summary showing the effect of 5 μM 5-HT on baseline spontaneous firing frequency, and the spike number in the 300-ms period immediately after the first stimulus artifact (n=5 neurons, from 5 brain slices of 5 mice). B, B1-B3, 5-HT1BR agonist CP93129 reduces the pausing effect of striatonigral IPSPs on spontaneous firing of SNr GABA neuron. A train of five striatonigral IPSPs evoked by stimulating striatum with a train of 5-pulses with an interval of 50 ms paused the spontaneous firing in SNr GABA neuron, that was strongly reduced by bath application of 5 μM CP93129. B4, Summary showing the effect of 5 μM CP93129 on baseline spontaneous firing frequency. B5, Summary showing the effect of 5 μM CP93129 on the spike number in the 300-ms period immediately after the first stimulus artifact (n=9 neurons, from 9 brain slices of 8 mice).

Figure 6. Chronic fluoxetine treatment sensitizes 5-HT1BRs on the striatonigral axon terminals. A1-A3, Example traces of striatonigral IPSCs in saline-treated mice before, during and after bath application of 1 μM 5-HT, 3 μM 5-HT and 10 μM 5-HT. B1-B3, Example traces of striatonigral IPSCs in chronically fluoxetine-treated mice before, during and after bath application of 1 μM 5-HT, 3 μM 5-HT and 10 μM 5-HT. C, Dose-response relationship of the 5-HT inhibition of striatonigral IPSCs in saline-treated mice and in chronically fluoxetine-treated mice. The continuous line is the Hill equation fit. Each data point represents 6-8 experiments (n=28 neurons, from 28 brain slices of 23 chronically fluoxetine-treated mice; n=24 neurons, from 24 brain slices of 17 saline-treated mice).
Figure 7. Time course of fluoxetine treatment-induced sensitization of 5-HT1BRs on the striatonigral axon terminals. A, Example traces of 1 μM 5-HT effects on striatonigral IPSCs in mice treated with saline or fluoxetine for 1 day. B, Pooled data showing that the inhibitory effect of 1 μM 5-HT on striatonigral IPSCs in untreated mice, saline control mice and mice treated with fluoxetine for 1 day (n=7 neurons, from 7 brain slices of 6 acute fluoxetine-treated mice), 7 days (n=7, from 7 brain slices of 6 fluoxetine-treated mice for 1 week), and 10-20 days. Arrow indicates treatment day 1.

Fig. 8. Chronic fluoxetine treatment enhances presynaptic 5-HT1BR-mediated reduction of the pause of the spontaneous firing in SNr GABA neurons. A, Example traces showing the pausing effect of striatonigral IPSPs in a SNr GABA neuron before (A1), during (A2), and after (A3) bath application of 0.1 μM CP93129, respectively, in a saline-treated control mouse. B, Example traces showing the pausing effect of striatonigral IPSPs on the firing of a SNr GABA neuron before (B1), during (B2), and after (B3) bath application of 0.1 μM CP93129 respectively, in a chronic fluoxetine-treated mouse. The bars in A1 and B1 indicate the pause duration: the time window between the stimulus artifact and the first spike after the stimulus artifact in each sweep. C, Pooled data showing that 0.1 μM CP93129 reduced the pause duration to a larger extent in chronic fluoxetine-treated mice than in saline-treated mice. D, Pooled data showing that 0.1 μM CP93129 reduced the peak amplitude of the striatonigral IPSPs more strongly in chronic fluoxetine-treated mice than in saline-treated mice. N = 6 neurons from 6 slices of 5 fluoxetine-treated mice; n = 7 neurons from 7 slices of 6 saline-treated mice.
A  Circuit diagram

Circuit diagram:
- Cortex
- Striatal GABA neuron
- Striatonigral projection
- SNr
- SNr GABA neuron
- 5-HT1B
- SERT
- Raphe 5-HT neuron

The CBGTC loop:
- Thalamus
- Striatal GABA neuron
- Cortex

B  SERT-expressing 5-HT axons

C  5-HT1B receptors

Updated Fig 1
A live angular sagittal brain slice

B Patch clamping a SNr GABA neuron

C 1-s cell-attached recording of a presumed SNr GABA neuron

D Selective elicitation of striatonigral and pallidonigral IPSCs

D1 Striatonigral IPSCs

D2 Pallidonigral IPSCs

D3 Pooled data

Fig. 2
Fig. 3

**A** Striatonigral IPSC
- A1 3 μM 5-HT
- Red: 5-HT
- Black: control
- Blue: wash
- 10 ms
- 100 pA

**B** Pallidonigral IPSC
- B1 3 μM 5-HT
- Red: 5-HT
- Black: control
- Blue: wash
- 1000 pA

**C** Pooled data
- Striatonigral IPSC
- Pallidonigral IPSC
- 0%
- 50%
- 100%
- 5-HT, μM

**D** Striatonigral IPSC: paired-pulse protocol
- D1 Control
- D2 5 μM 5-HT
- 25 ms
- 200 pA

**D3** Scaled

**D4** Summary
- PPR (P2/P1)
- Control
- 5 μM 5-HT
- Wash
A  Paired-pulse protocol and 5-HT1B agonist CP93129 effect

A1 Original

A2 Scaled to 1st peak

Black: control
Red: CP93129
Blue: wash

200 pA
25 ms

A3

Striatal
IPSC amplitude, %

Control
μM CP93129
Wash

0 50 100

A4

PPR (P2/P1)

Control
1μM CP93129
Wash

0 2 4

B  5-HT1B antagonist NAS181 blocks 5-HT effect

B1

Black: control
Red: 5-HT
Blue: NAS181+5-HT

100 pA
10 ms

B2

Striatal
IPSC amplitude, %

Control
5 μM 5-HT
10 μM NAS181 + 5 μM 5-HT

0 50 100

Fig. 4
Fig. 5
**New Fig. 7. Time course**
A Chronic saline-treated mice

A1 Control

A2 0.1 μM CP93129

A3 Wash

B Chronic fluoxetine-treated mice

B1 Control

B2 0.1 μM CP93129

B3 Wash

C

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Fig. 8