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Inhibition of the Serotonin Transporter Induces Microglial Activation and Downregulation of Dopaminergic Neurons in the Substantia Nigra

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KEY WORDS selective serotonin reuptake inhibitor; serotonin transporter; citalopram; fluoxetine; microglia; tyrosine hydroxylase

ABSTRACT Drugs that selectively inhibit the serotonin transporter (SERT) are widely used in the treatment of depression and anxiety disorders. These agents are associated with a range of extrapyramidal syndromes such as akathisia, dystonia, dyskinesia and parkinsonism, suggesting an effect on dopaminergic transmission. We studied the time course of changes in dopaminergic neurons in the substantia nigra (SN) after initiation of two different SERT inhibitors, citalopram and fluoxetine. In the first experiment, groups of Sprague-Dawley rats received daily meals of rice pudding either alone ($N = 9$) or mixed with citalopram 5 mg/kg/day ($N = 27$). Rats were sacrificed after 24 h, 7 days or 28 days of treatment. Sections of SN were processed for tyrosine hydroxylase (TH) immunohistochemistry. Citalopram induced a significant decrease in TH-positive cell counts at 24 h (44%), 7 days (38%) and 28 days (33%). No significant differences among the citalopram treatment groups were observed in the SN. To determine whether these changes would occur with other SERT inhibitors, we conducted a second experiment, this time with a 28 day course of fluoxetine. As was observed with citalopram, fluoxetine induced a significant 21% reduction of TH cell counts in the SN. Immunoblot analysis showed that fluoxetine also induced a 45% reduction of striatal TH. To investigate a possible role for the innate immune system in mediating these changes, we also studied the microglial marker OX42 after administration of fluoxetine and noted a significant 63% increase in the SN of fluoxtine-treated animals. These results indicate that SERT inhibition can activate microglia and alter the regulation of TH, the rate limiting enzyme for dopamine biosynthesis. These changes may play a role in mediating the extrapyramidal side effects associated with SERT inhibitors. **Synapse 65:1166–1172, 2011.**

INTRODUCTION

Selective serotonin reuptake inhibitors (SSRIs) are widely prescribed for depression and a range of anxiety disorders including panic and obsessive-compulsive disorder (Boyer, 1995; Pigott and Seay, 1999; Vaswani et al., 2003; Zohar and Westenberg, 2000). After the prototypical SSRI fluoxetine was introduced in the late 1970's, drugs that selectively block the serotonin transporter (SERT) largely supplanted the older tricyclic medications and monoamine oxidase inhibitors, primarily because they were considered less likely to cause adverse effects (Steffens et al., 1997). Over the years, however, it has become clear that SSRIs produce side effects of their own, including nausea, sleep disturbance, sexual dysfunction (Brambilla et al., 2005) and a range of extrapyramidal syndromes (EPS) including akathisia, dystonia,

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dyskinesia and parkinsonism (Caley, 1997; Gerber and Lynd, 1998; Leo, 1996).

The first report of SSRI-induced EPS was published in 1979 and described a fluoxetine-treated patient who developed dystonia, rigidity, increased serum prolactin and reduced cerebrospinal levels of the dopamine metabolite homovanillic acid (Meltzer et al., 1979). By the mid 1990s, nearly 3600 cases of EPS had been reported to the fluoxetine manufacturer Eli Lilly & Company (Gerber and Lynd, 1998), a number that is likely quite conservative considering the unsolicited nature of the reports. Though fluoxetine is the SSRI most commonly associated with EPS (Caley, 1997; Gerber and Lynd, 1998; Leo, 1996), this relationship may be an artifact of the drug's longevity and broad label use; citalopram (Najjar and Price, 2004; Parvin and Swartz, 2005), paroxetine (Baldassano et al., 1996), fluvoxamine (George and Trimble, 1993), and sertraline (Lambert et al., 1998) have all been reported to cause extrapyramidal side effects similar to those associated with fluoxetine.

While the precise pathophysiology of drug-induced EPS has not been elucidated, there is strong evidence pointing to disordered dopaminergic signaling. Drugs which alter dopaminergic signaling in the basal ganglia are associated with a high incidence of extrapyramidal disturbances (Mazurek and Rosebush, 1996; Rosebush and Mazurek, 1999). At the same time, extrapyramidal signs are features of Parkinson's disease (PD), a hallmark of which is the degeneration of dopaminergic neurons in the nigrostriatal pathway. Because SSRIs are associated with extrapyramidal reactions similar to those induced by dopamine D2 receptor antagonists, it seems reasonable to postulate that the inhibition of the serotonin transporter might alter dopaminergic transmission in the basal ganglia.

To investigate the role of SERT inhibition in the regulation of nigrostriatal dopamine neurons, we examined the effects of two different SSRI medications, citalopram and fluoxetine, on cells containing tyrosine hydroxylase (TH), the rate-limiting enzyme in the biosynthesis of dopamine. We also investigated whether SERT blockade can induce microglial activation, as has been observed in other models of dopaminergic dysfunction (Gerhard et al., 2006; Marinova-Mutafchieva et al., 2009; McGeer et al., 1988; Mirza et al., 2000; Sherer et al., 2003; Wu et al., 2002).

Abbreviations

DRN	dorsal raphe nucleus
EPS	extrapyramidal syndromes
MRN	median raphe nucleus
PD	Parkinson's disease
SERT	serotonin transporter
SN	substantia nigra
SSRI	selective serotonin reuptake inhibitor
TH	tyrosine hydroxylase

MATERIALS AND METHODS

Subjects

Male Sprague-Dawley rats (Charles River, PQ) weighing 250-275g were singly housed with free access to food and water on a 12 h:12 h light dark cycle throughout the experiment. Rat weights were recorded weekly and the experimental drug dosage was adjusted accordingly. Animal health was monitored daily. All experiments were conducted in accordance with the guidelines set forth by the Canadian Council on Animal Care and the McMaster University Animal Research Ethics Board.

Treatment protocol

Experiment 1

Rats were randomly assigned to one of four treatment groups: (1) control; (2) 24 h citalopram; (3) 7 day citalopram; or (4) 28 day citalopram. All animals received daily 20 g meals of rice pudding either alone ($N = 9$) or mixed with the SSRI citalopram (Apotex, McMaster University Hospital Pharmacy) 5 mg/kg/day ($N = 27$). We selected a dosage of citalopram which has been shown in rats to produce plasma concentrations that are clinically relevant in humans (Fredricson Overo, 1982a,b; Hyttel et al., 1984). A mortar and pestle was used to grind the citalopram tablets to a powder, which was then weighed and mixed with the rice pudding. The animals were monitored to ensure that they consumed pudding in a timely fashion.

Experiment 2

As in experiment 1, groups of Sprague-Dawley rats received daily meals of rice pudding either alone ($N = 9$) or mixed with the SSRI fluoxetine 5 mg/kg/day ($N = 9$). As for citalopram, we selected a dosage of fluoxetine which has been shown in rats to produce plasma concentrations that are clinically relevant in humans (Caccia et al., 1990). Rats were sacrificed after 28 days of treatment. Coronal sections (40 μm) of SN were processed for TH and OX42 immunohistochemistry. Striatal homogenates were prepared and processed for TH immunoblotting. All other experimental procedures were consistent with those described for experiment 1.

Immunohistochemistry

Citalopram-treated animals were sacrificed by decapitation after 24 h, 7 days or 28 days of citalopram treatment. Fluoxetine-treated animals were sacrificed after 28 days of fluoxetine. Controls were sacrificed with the 28 day treatment groups. Prior to decapitation all rats were deeply anaesthetized with 40 mg pentobarbitol i.p. Brains were removed, placed in 4% paraformaldehyde and refrigerated at 4°C for

4 to 7 days. Brains were cryoprotected in 15% sucrose solution for 24 h immediately prior to sectioning. Consecutive coronal sections (20 μm) were cut at −18°C with a Leica 1900 cryostat (Heidelberg, Germany) and placed from rostral to caudal in groups of 2 sections per well in a 24-well plate filled with 0.1 M phosphate buffered saline (PBS).

Coronal sections of SN (bregma −5.8 mm) were selected and processed for TH immunohistochemistry. Free-floating sections were incubated in 0.3% hydrogen peroxide in methanol for 30 min at room temperature, rinsed twice in 0.1 M PBS and incubated in 5% normal donkey serum (NDS) for 1 h. Sections were rinsed three times in 0.1 M PBS and then incubated in monoclonal mouse anti-TH primary antibody ((Millipore, Etobicoke, Canada; 1:1500) for 72 h at 4°C. After three additional washes in 0.1 M PBS, sections were incubated in biotinylated anti-mouse IgG secondary antibody (Millipore, Etobicoke, Canada; 1:200) for 1 h at room temperature, washed three more times in 0.1 M PBS, and then incubated in ABC solution (Vector Laboratories, Burlington, Canada) for 1 h at room temperature to form an avidin-biotin complex. After three final washes in 0.1 M PBS, the TH immunocomplex was visualized with the chromagen diaminobenzidine tetrahydrochloride (DAB, Vector Laboratories, Burlington, Canada). Sections were mounted on Aptex-coated slides (Sigma, Oakville, Canada), air dried, and coverslipped with DPX neutral mounting medium (Sigma, Oakville, Canada).

For TH and OX42 double immunofluorescent staining, free-floating sections were incubated in 0.3% hydrogen peroxide in methanol for 30 min at room temperature, rinsed twice in 0.1 M PBS and incubated in 5% NDS for 1 h. After 3 more rinses in 0.1 M PBS, sections were incubated in a mixture of rabbit anti-TH (Millipore, Etobicoke, Canada; 1:1500) and mouse anti-OX42 (Serotec, Raleigh, NC; 1:500) primary antibodies for 72 h at 4°C. At this stage, the section staining plates were covered in foil to prevent light-mediated decay in fluorescent intensity. Light was avoided for the remainder of the stain. Sections were washed and incubated in a blend of secondary antibody solution that contained donkey anti-rabbit conjugated with AF 568 (Millipore, Etobicoke, Canada; 1:200) and donkey anti-mouse conjugated with AF 488 (Millipore, Etobicoke, Canada; 1:200). After three final washes, sections were mounted on Aptex-coated slides (Sigma, Oakville, Canada), air dried in the dark, and coverslipped with Prolong Gold antifade medium (Invitrogen, Burlington, Canada).

Quantitative morphometry

Coronal sections corresponding to bregma −5.8, an area of SN known to be densely populated with dopamine neurons, were selected for analysis. Anatomical landmarks were used to ensure that comparable brain sections were analyzed in each animal. To minimize error, all sections from the various treatment cohorts were cut and stained at the same time. The histochemically stained sections were mounted on microscope slides, which were then coded and photographed at on a confocal microscope (CarlZeiss LSM 510). The TH- and OX42-immunoreactive cells in the SN were manually counted at 20× magnification by an observer who was strictly blinded to treatment status. All counted cells were clearly demarcated from background staining; this was assessed based on the judgment of the blinded observer. Anatomical boundaries for the SN were identified and every labeled cell within those boundaries was counted. For each animal, immunoreactive cell counts from multiple SN hemisections were averaged to generate a single value. After completion of manual counting, all images were decoded and arranged according to treatment group. For each SN hemisection, the average cross sectional area (μm^2) of TH-positive cell somata was determined using 10 randomly selected immunoreactive cell bodies per animal. The raw cell counts were then adjusted with a correction formula for cell size and section thickness according to the method of Abercrombie (Abercrombie, 1946).

Immunoblotting

Striatal tissue was dissected from 2 mm coronal brain sections. Proteins were extracted via homogenization in lysis buffer (50 mM Tris, 1 mM EDTA, pH 7.4) and 20 s of sonication. The protein concentration for each lysate was determined with the BioRad Protein Assay (BioRad, Mississauga, Canada). An equal amount of protein (0.23 μg per 1 μL of 5× western loading buffer) from each sample was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane, and blocked in TBST (0.14 M NaCl, 2.7 mM KCl, 24.8 mM Tris, 0.1% Tween) with 5% skim milk overnight at 4°C. The blots were incubated with rabbit TH (AbCam, Cambridge, MA; 1:50,000) and mouse β-tubulin III (Sigma, Oakville, Canada; 1:20,000, T8660) primary antibodies in TBST and 3% milk for 1 h at room temperature. After subsequent washing in TBST, blots were incubated in anti-rabbit (GE Healthcare, Little Chalfont, UK; 1:5000) and anti mouse (GE Healthcare, Little Chalfont, UK; 1:5000) secondary antibodies in TBST and 3% milk for 1 h at room temperature. Blots were washed, processed with enzymatic chemiluminescence (ECL, Millipore, Etobicoke, Canada), and exposed to x-ray film (GE Healthcare, Little Chalfont, UK). The housekeeping protein β-tubulin-III was used as a control to ensure that an equal amount of protein was loaded from each sample, and for normalization purposes. Image J was used to quantify the optical density of the TH protein band.

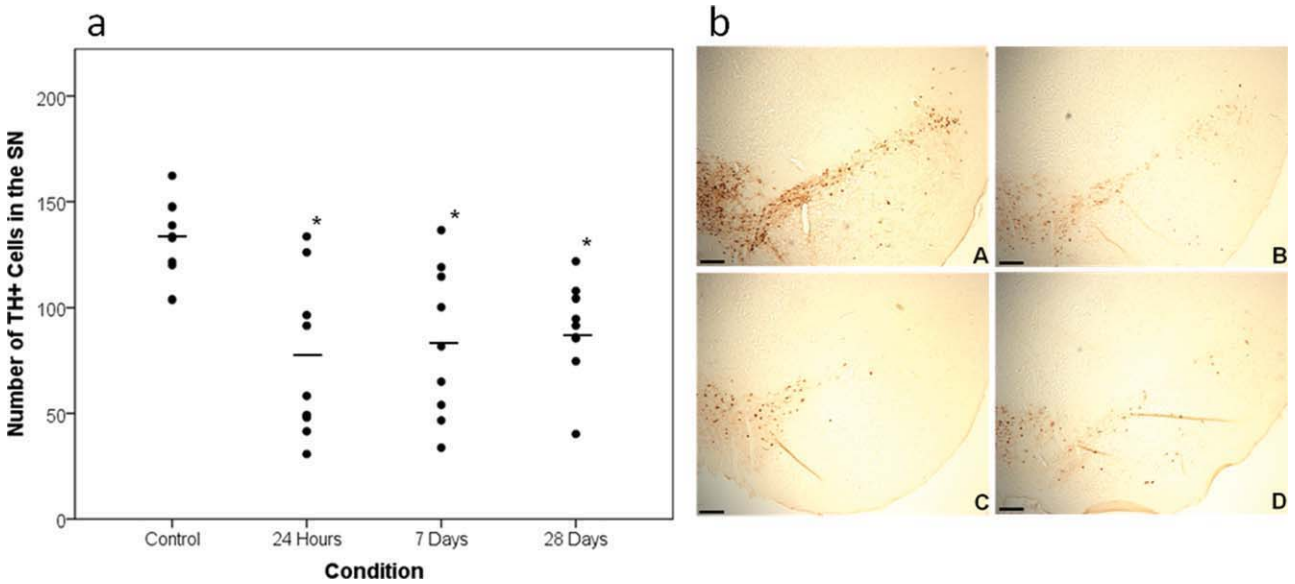

Fig. 1. (**a**) Number of TH-positive cells per 20 μm section of rat SN. The 24 h, 7 days and 28 days labels represent durations of citalopram treatment. Each dot represents the number of TH-positive cells for one animal; the horizontal line represents the group mean. Note: "*" indicates statistical significance; (**b**) Photomicrographs (5X magnification) of TH-positive cells in the rat SN. A: Control, B: 24 h citalopram, C: 7 day citalopram, D: 28 day citalopram. Scale Bar 300 μm [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com].

Statistical analysis

Cell count, cell size and immunoblot optical density data were analyzed by one way analysis of variance (ANOVA) followed by post hoc Tukey tests where appropriate. Statistical significance was defined as $P < 0.05$.

RESULTS

Experiment 1

Citalopram reduced the number of TH-positive cells in the SN for all three of the time points that we studied (Fig. 1a; $F_{3,32} = 7.023$, $P = 0.001$): by 44% at 24 h ($P = 0.001$); by 38% at 7 days ($P = 0.006$); and by 33% at 28 days ($P = 0.017$). There were no significant differences among the citalopram treatment groups. Representative photomicrographs are shown in Figure 1b. Cell sizes were not significantly different among any of the groups.

Experiment 2

TH immunohistochemistry in the substantia nigra

A 28-day course of fluoxetine induced a significant 21% reduction of TH cell counts in the SN ($P = 0.005$, Fig. 2a). Representative photomicrographs are shown in Figure 2b. Cell sizes were not significantly different among any of the groups.

TH immunblotting in the striatum

Striatal TH was decreased by 45% ($P = 0.001$, Fig. 2c). A representative immunoblot is shown in Figure 2d.

OX42 immunohistochemistry

Fluoxetine treatment for 28 days resulted in a significant 64% increase in the number of OX42-positive cells in the SN ($P < 0.001$) as shown in Figures 3a and 3b.

DISCUSSION

These results indicate that selective blockade of SERT can induce a significant reduction of TH-positive dopaminergic neurons in the SN within 24 h and that this reduction persists throughout a treatment course of at least 28 days. Citalopram administration for 1, 7, or 28 days resulted in a significant decrease of 33 to 44 percent in the number of TH-immunoreactive neurons in the SN. Similar observations were noted with a 28 day course of fluoxetine, indicating that the reduction of TH immunoreactivity may be an effect common to all agents that inhibit SERT. Downregulation of nigral TH in fluoxetine-treated animals was accompanied by a significant decrease of striatal TH and a dramatic increase of microglia cells in the SN, suggesting activation of the innate immune system in the brain. Because our use of manual cell counts from carefully matched coronal sections did not allow us to assess the entire rostral-caudal dimension of each nucleus, we cannot completely exclude the possibility that TH and OX42 changes may be more robust in some subparts of the SN than others.

Tyrosine hydroxylase immunohistochemistry and immunoblotting

To the best of our knowledge, this is the first direct evidence that SSRI medication can affect the biosyn-

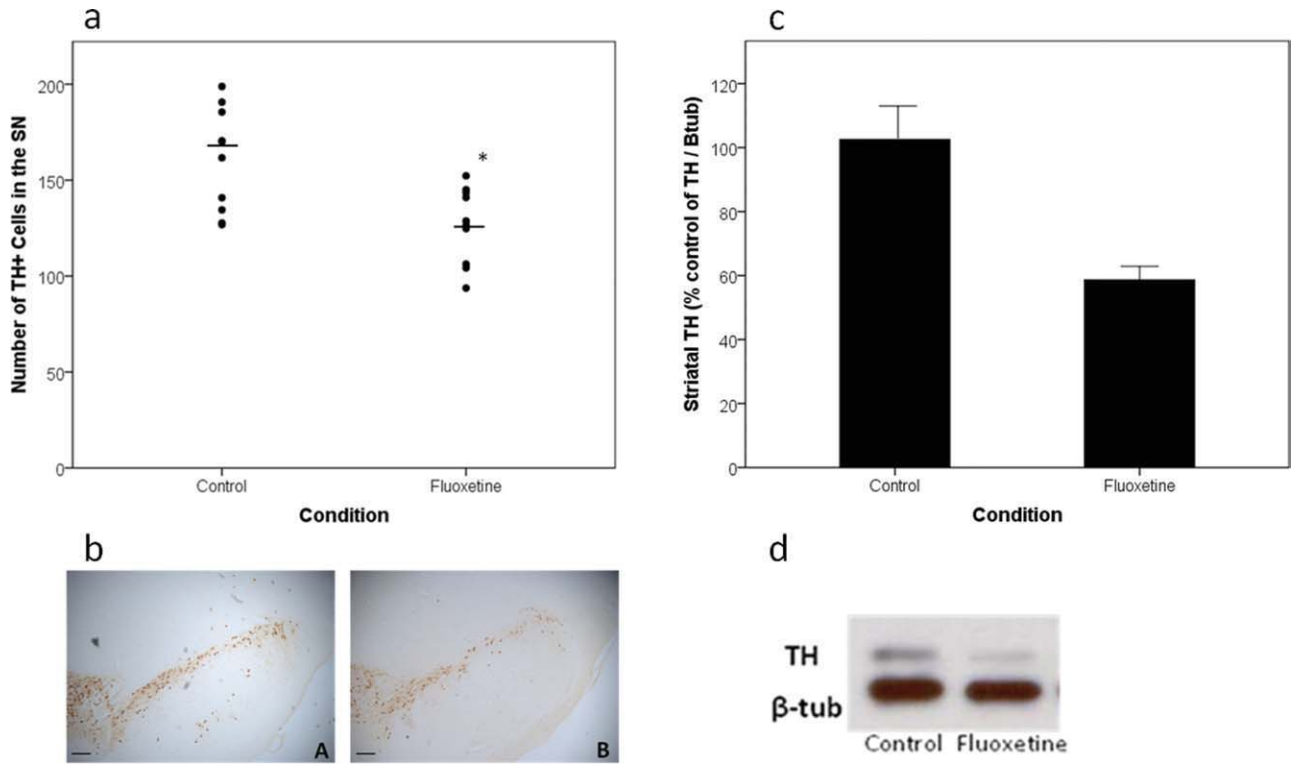

Fig. 2. (**a**) Number of TH-positive cells per 40 μm section of rat SN. Each dot represents the number of TH-positive cells for one animal; the horizontal line represents the group mean. Note: "*" indicates statistical significance. (**b**) Photomicrographs (5× magnification) of TH-positive cells in the rat SN. A: Control, B: 28 Day Fluoxetine. Scale Bar 200 μm. (**c**) Relative TH signal in the rat striatum, expressed as a percent control of TH/β tubulin III. (**d**) Immunblot of TH signal in the striatum [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com].

thetic enzyme for dopamine in the nigrostriatal system. The rapid and persistent reduction of TH-positive cell counts in the SN and TH signal in the striatum is reminiscent of the changes observed with agents that block the D2 dopamine receptor (Levinson et al., 1998) and is consistent with clinical reports that SSRIs can cause side effects similar to those induced by dopamine antagonists (Caley, 1997; Gerber and Lynd, 1998; Leo, 1996).

These findings draw attention to the effects of serotonergic pathways on dopaminergic cell function. Anatomical and electrophysiological investigations have demonstrated that serotonergic neurons in the brainstem project to the SN (Dray et al., 1976; Dray et al., 1978; Fibiger and Miller, 1977). The majority of these serotonergic projections derive from the dorsal (DRN) raphe nuclei, with a lesser contribution from the median (MRN) raphe nuclei (Conrad et al., 1974). Electrical stimulation of the MRN or DRN depresses neuronal activity in the SN (Dray et al., 1976), as does microiontophoretic application of serotonin onto the SN (Dray et al., 1976). Conversely, discrete electrolytic lesions of either the MRN or DRN, producing decreased concentrations of SN serotonin, result in elevated dopamine concentrations in the striatum (Dray et al., 1978), while pharmacological inhibition of raphe neurons leads to functional disinhibition of the nigrostriatal dopamine system (James and Starr, 1980; Ugedo et al., 1989). Taken together, these findings point to an inhibitory pathway from the raphe to the SN that contributes to the regulation of nigrostriatal dopaminergic transmission. This inhibitory action appears to be modulated, at least in part, by 5-HT_{2A} heteroreceptors located on the somatodendritic surface of the dopamine neurons (Ugedo et al., 1989; Pazos et al., 1987). The resulting reduction of TH activity in nigrostriatal dopamine neurons can lead to a reduction of striatal dopamine release, such as was reported by Baldessarini and Marsh (1990) following high doses of fluoxetine.

While SSRI medications are generally known for their action on SERT inhibition, there is also evidence that they interfere with mitochondrial function, an action that has also been described with other types of psychotropic medication (Burkhardt et al., 1993; Maurer and Moller, 1997). Specifically, SSRIs have been reported to inhibit electron transport and the F1-F0 ATP synthase by altering the lipid bilayer of the inner mitochondrial membrane (Curti et al., 1999; Souza et al., 1994). Reduced complex I activity has been associated with compromised dopaminergic function and may have contributed to the effects we

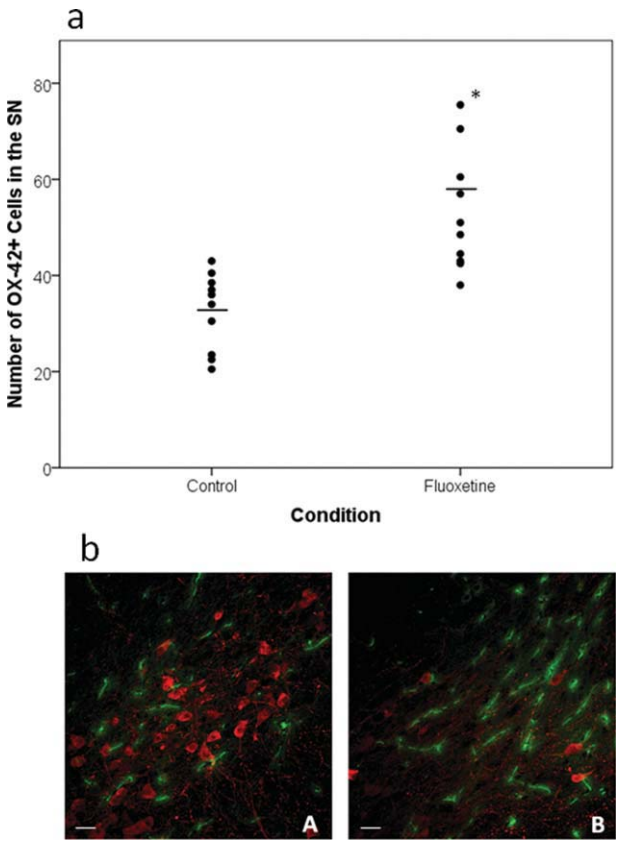

Fig. 3. (**a**) Number of OX42-stained cells per 40 μm section of rat SN. Each dot represents the number of OX42-stained cells for one animal; the horizontal line represents the group mean. Note: "*" indicates statistical significance; (**b**) Photomicrographs (20× magnification) of TH- and OX42-immunoreactive cells in 40 μm sections of rat SN (bregma – 5.8mm). A, Control; B, 28 Day Fluoxetine. Scale bar 200 μm.

observed on TH-positive cell counts (Parker et al., 2008; Przedborski et al., 2004; Schapira, 2010).

Microglial recruitment

Fluoxetine induced a clear amplification of microglia activation in the SN. This finding is consistent with the report that fluoxetine can stimulate nitric oxide release in vitro from murine microglial cells (Ha et al., 2006). Microglial cells are upregulated in PD (McGeer et al., 1988; Mirza et al., 2000), the essential feature of which is degeneration of dopaminergic neurons in the SN. Conversely, inhibition of microglial function with minocycline has a neuroprotective effect on TH-positive neurons in animal models of PD (Du et al., 2001; Wu et al., 2002). These observations suggest that SSRI-induced microglial activation may mediate the downregulation of TH immunohistochemistry that we found in this study. It is unclear at this point whether the microglial activation is a consequence of the SERT blockade or some as-yet-unidentified property of SSRI-type agents.

CONCLUSIONS

These results indicate that SERT inhibition can alter the regulation of TH, the rate limiting enzyme for dopamine biosynthesis. The effect of SERT inhibition on TH occurred within 24 h and did not habituate after 28 days, an observation that might help explain the tendency of SSRI medications to cause extrapyramidal side effects in humans. The fluoxetine-induced increase of microglial immunoreactivity in the SN points to a possible role for the innate immune system in mediating this change in the dopamine neurons, and may suggest a longer-term toxic potential for SSRI-type agents in susceptible individuals.

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