MODULATION OF BRAIN SEROTONERGIC AND DOPAMINERGIC NEURONS

REGULATION OF BRAIN SEROTONERGIC AND DOPAMINERGIC NEURONS: THE MODULATORY EFFECTS OF SELECTIVE SEROTONIN REUPTAKE INHIBITORS, ATYPICAL NEUROLEPTICS AND ENVIRONMENTAL ENRICHMENT

By

LINDSEY E.S. MACGILLIVRAY, B.Sc.

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AUTHOR:	Lindsey E.S. MacGillivray
SUPERVISOR:	Dr. Michael F. Mazurek
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ABSTRACT

The brain serotonergic and dopaminergic systems broadly influence our internal experience and the ways in which we interact with the outside environment, with crucial regulatory roles in mood, sleep, appetite and the control of voluntary movement. Serotonin and dopamine neurons are themselves influenced by a wide variety of internal and external factors, many of which remain poorly understood. The central aim of this thesis was to better characterize several of these modulatory influences via exploratory investigations involving pharmaceutical agents or environmental modification. Specifically, I examined the modulatory effects of selective serotonin reuptake inhibitors (SSRIs), atypical neuroleptics and environmental enrichment with exercise on the regulation of brain serotonin and dopamine neurons.

This thesis documents, for the first time, that (1) inhibition of the serotonin transporter (SERT) by SSRIs induces a rapid and region-selective reduction of tryptophan hydroxylase (TPH)-immunoreactive neurons in serotonergic brainstem nuclei that persists over a prolonged treatment course; that (2) selective blockade of SERT by SSRIs can rapidly induce a reduction of tyrosine hydroxylase (TH)-positive dopaminergic neurons in the substantia nigra (SN) and the ventral tegmental area (VTA) that, again, persists over a lengthy treatment course; that (3) environmental enrichment with exercise can potentiate the effect of SERT inhibition on SN dopaminergic neurons, but not the dorsal raphe nucleus (DRN) serotonergic neurons; that (4) that SSRI fluoxetine triggers a significant upregulation of microglia in the SN; that (5) environmental enrichment with exercise can reduce TPH immunoreactivity in the DRN

and TH immunoreactivity in the SN and VTA, even in the absence of any pharmacological intervention, and finally, that (6) the atypical neuroleptic risperidone significantly reduces TPH in the DRN of both young and aged animals and reduces DRN Nissl counts in aged animals. Taken together, the body of work included in this thesis suggests that SSRIs, atypical neuroleptics and environmental enrichment with exercise can have profound effects on brain serotonergic and dopaminergic neurons, possibly accounting for some of the side effects and therapeutic benefits associated with these interventions.

Dedication

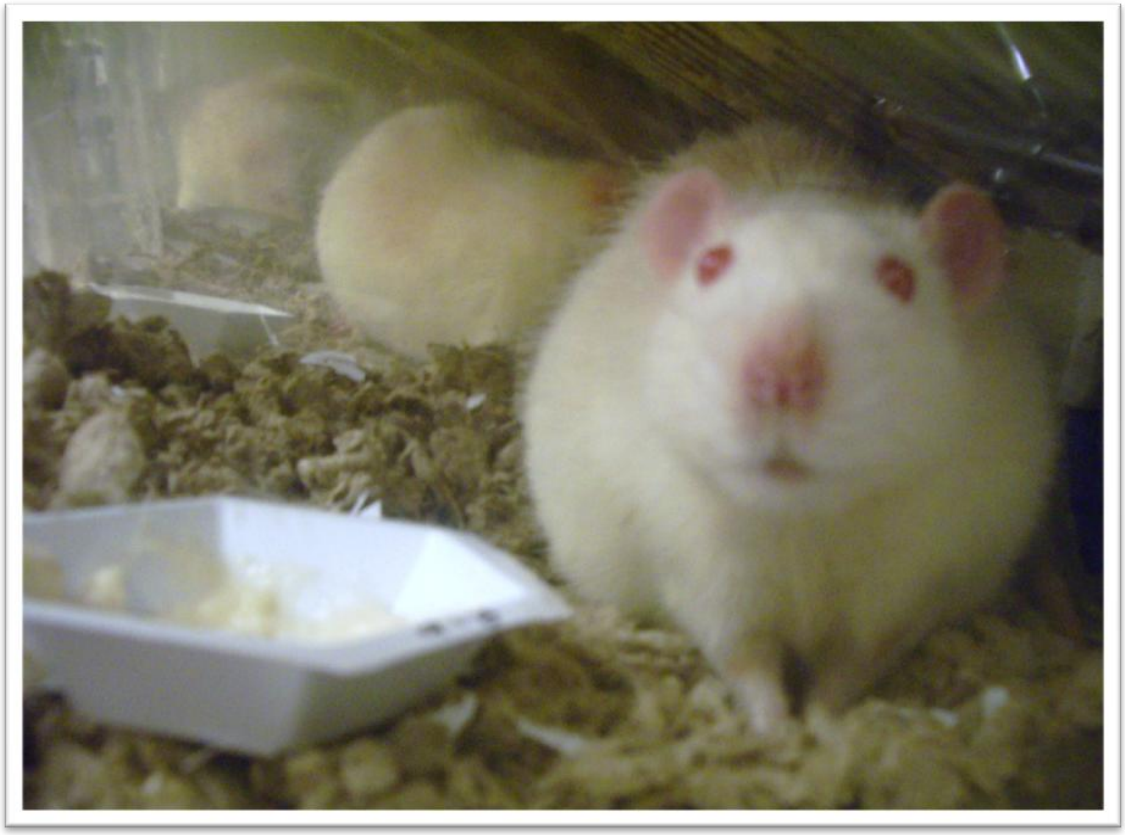
This thesis is dedicated to my mother, Terry Penny, who exemplifies the excellence I seek to achieve and who gave me the freedom and courage to follow my own path, and to my father, Ron MacGillivray, who reminded me to enjoy life and all of its absurdities. Thank you for your unconditional love, guidance and support. I love you both with all of my heart.

With Special Thanks:

To Benjamin Robinson – your encouragement and love has meant the world to me. Thank you for being in my corner, no matter what.

To Michael Mazurek – thank you for opening my eyes to life's opportunities. I submit this thesis with an unfettered love of learning and a yearning to know more, sentiments which speak to the superb mentorship I have received over the past seven years.

To Katelyn Reynolds, Manisha Sickand and Lisa Lagrou – you are remarkable women. I am so fortunate that, with each of you, lab collegiality evolved to true friendship. Thank you for the shared experiences and selflessly offered assistance.



To my animal friends - thank you.

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LIST OF ABBREVIATIONS

Abbreviation	Meaning
5-HIAA	5-hydroxyindole acetic acid
5-HT	5-hydroxytryptamine (serotonin)
5-HTP	5-hydroxytryptophan
6-OHDA	6-hydroxydopamine
ABC	avidin biotin complex
AF	alexa fluor
ANOVA	analysis of variance
ATP	adenosine triphosphate
BH_4	tetrahydropterin
cAMP	cyclic adenosine monophosphate
Cl^-	chloride ion
CLN	caudal linear nucleus
CNS	central nervous system
COMT	catechol-O-methyl transferase
DA	dopamine
DAB	diaminobenzidine tetrahydrochloride
DAT	dopamine transporter
DNA	deoxyribonucleic acid
DOPAC	3,4-dihydroxyphenylacetic acid
DRN	dorsal raphe nucleus
EE	enriched environment; environmental enrichment
EPS	extrapyramidal syndromes
G-protein	guanine nucleotide binding proteins
GABA	gamma-aminobutyric acid
HAL	haloperidol
HVA	homovanillic acid
IgG	immunoglobulin G
i.p.	intraperitoneal
K^+	potassium ion
L-DOPA	L-3,4-dihydroxyphenylalanine
MAO	monoamine oxidase
MAOIs	monoamine oxidase inhibitors
MDMA	methylenedioxymethamphetamine
MFB	medial forebrain bundle
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MRN	median raphe nucleus

mRNA	messenger ribonucleic acid
Na^+	sodium ion
NDS	normal donkey serum
NO	nitric oxide
PBS	phosphate buffered saline
PCA	para-chloroamphetamine
PD	Parkinson's disease
PFA	paraformaldehyde
PKA	protein kinase A
PLC	phospholipase C
p.o.	"*per os*", by mouth
RMN	raphe magnus nucleus
s.c.	subcutaneous
SCN	suprachiasmatic nucleus
SE	standard environment
SERT	serotonin transporter
SN	substantia nigra
SSRI	selective serotonin reuptake inhibitor
TCA	tricyclic antidepressant
TH	tyrosine hydroxylase
TPH	tryptophan hydroxylase
VTA	ventral tegmental area

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CHAPTER 1: Summary of Rationale and Objectives

1.1. Rationale

The brain serotonergic and dopaminergic output systems play crucial roles in the regulation of mood, sleep, appetite and the control of voluntary movement. Though most of the literature focuses on the function and regulation of these post-synaptic circuits, the serotonin and dopamine neurons are themselves influenced by a wide range of internal and external factors, many of which remain poorly understood. The central aim of this thesis was to better understand several of these modulatory influences via exploratory investigations involving pharmaceutical agents or environmental modification.

The widespread use of selective serotonin reuptake inhibitors (SSRIs) for the treatment of depression and anxiety disorders has focused attention on the importance of the serotonin transporter, a large presynaptic protein that mediates reuptake of extracellular serotonin. The focus in the literature has been on postsynaptic targets downstream of the serotonergic projection pathways. I was instead interested in the role of serotonin blockade by SSRIs in regulating the serotonergic neurons themselves. Accordingly, I studied the timecourse and regional specificity of SSRI-induced changes in brainstem raphe nuclei.

SSRI medications have largely supplanted the older tricyclic medications and monoamine oxidase inhibitors primarily because they are considered less likely to cause the anticholinergic and potentially dangerous cardiac effects that are associated with the older classes. Nevertheless, SSRIs produce side effects of their own, including nausea,

sleep disturbance, sexual dysfunction and a range of extrapyramidal syndromes including akathisia, dystonia, dyskinesia and parkinsonism. Because SSRIs are associated with extrapyramidal reactions similar to those induced by dopamine blocking drugs, it seemed reasonable to postulate that the inhibition of the serotonin transporter might alter dopaminergic transmission in the basal ganglia. To investigate this possibility, I examined the effects of SSRI medication on dopaminergic cells in the substantia nigra.

Environmental enrichment offers animals the opportunity to engage in exploration and physical activity and is associated with significant benefit in animal models of depression and Parkinson's disease. I postulated that environmental enrichment with exercise would modulate SSRI-induced changes in serotonin and dopamine neurons and accordingly, compared animals treated with an SSRI but housed in a standard environment with animals treated with an SSRI and housed in an enriched environment. I also examined the effect of environmental enrichment alone on numbers of midbrain dopamine and brainstem serotonin neurons.

Risperidone is an atypical neuroleptic that, because of its action on serotonin as well as dopamine neurotransmission, is touted to have a better side effect profile than those of the typical class. Reports that risperidone increases extracellular serotonin and decreases the firing rate of serotonin neurons in the dorsal raphe nucleus suggested to me that risperidone might have an effect on the biosynthetic enzyme for serotonin as well. Accordingly, I studied the influence of risperidone on tryptophan hydroxylase-positive cells in the rat dorsal raphe nucleus.

1.2. Objectives

- To study the role of serotonin transporter inhibition in the regulation of tryptophan hydroxylase in brainstem raphe nuclei (Chapter 3). Specifically, to:
  - investigate the time course and regional specificity of changes in tryptophan hydroxylase in four raphe nuclei after exposure to citalopram (Chapter 3)
  - investigate the effect of a second SERT inhibitor, fluoxetine, on changes in tryptophan hydroxylase immunoreactivity in the same four raphe nuclei

- To study the role of serotonin transporter inhibition in microglial recruitment and the regulation of tyrosine hydroxylase in substantia nigra dopamine neurons (Chapter 4). Specifically, to:
  - examine the time course of changes in tyrosine hydroxylase^{+} neurons in the substantia nigra after exposure to citalopram
  - investigate the effect of a second SERT inhibitor, fluoxetine, on changes in tyrosine hydroxylase immunoreactivity in the substantia nigra
  - explore the role of SERT inhibition by fluoxetine in recruitment of OX42+ cells to the substantia nigra

- To explore strategies for modifying drug-induced changes in serotonergic and dopaminergic neurons (Chapters 5 and 6). Specifically to:
  - characterize the influence of environmental enrichment with exercise on tryptophan hydroxylase^{+} cells in the dorsal raphe nucleus and tyrosine hydroxylase^{+} cells in the substantia nigra and ventral tegmental area in rats treated with fluoxetine
  - study the influence of enrichment alone on these same neurons

- examine the effect of SERT inhibition and environmental enrichment with exercise on microglial recruitment and activation in the substantia nigra and ventral tegmental area (Chapter 6).

❖ To investigate the influence of a second class of psychotropic medication on brainstem serotonergic neurons, in both young and aged animals (Chapter 7). Specifically, to:

- study the effect of atypical antipsychotic risperidone on the regulation of tryptophan hydroxylase^{+} neurons in the dorsal raphe nucleus
- use histological Nissl counts to investigate the influence of risperidone on total cell presence in the dorsal raphe nucleus

1.3. Thesis Structure

The reader should note that this thesis has been structured in a "sandwich thesis" format, consisting of five publication-ready chapters, bridged together by a common introduction and discussion. The five papers summarizing my work are at various stages of the publication process. The status and relevant peer-reviewed journal for each paper is indicated on the title page for each chapter. The reader should also be prepared for the inevitable overlap of introductory material and procedural detail that accompanies sequentially published work.

Lindsey MacGillivray is the primary author on all papers included within this thesis. Her contributions to each paper include experimental design, animal care and drug administration, tissue processing and immunohistochemistry, data collection and data analyses. She drafted and edited all five manuscripts, with much appreciated editorial assistance from Dr. Michael Mazurek.

CHAPTER 2: Introduction

2.1. The Neurobiology of the Serotonin System

A. Anatomy of the Serotonin System

Serotonin (5-hydroxytryptamine, 5-HT) was first isolated from beef serum in 1948 and was appropriately named for its source "sero" and vasoconstrictor activity "tonin" (Rapport et al., 1948). Broadly considered to be the most complex anatomic and neurochemical system in the mammalian central nervous system (Jacobs and Azmitia, 1992), the physiological influence of serotonin is extensive; mood, cardiovascular regulation, respiration, thermoregulation, circadian rhythm entrainment, sleep-wake cycle, appetite, aggression, sexual behaviour, sensorimotor reactivity, pain sensitivity and learning are all affected by serotonergic neurotransmission (Lucki, 1998).

i) Serotonergic Nuclei

Serotonin neural cell bodies are located almost exclusively in the brain stem and are commonly divided into superior and inferior subsets:

The superior subset of serotonin neurons is comprised of five primary nuclei: supralemniscal nucleus, raphe pontis oralis, the caudal linear nucleus, median raphe nucleus (previously referred to as the nucleus centralis), and the dorsal raphe nucleus. Conversely, the raphe magnus, raphe obscurus, raphe pallidus, ventral lateral medulla and the area postrema form the inferior subset of serotonergic cell bodies (Jacobs and Azmitia, 1992).

For the experiments described in this thesis, I studied tryptophan hydroxylase immunoreactivity in four functionally and anatomically distinct raphe nuclei: the dorsal raphe nucleus (DRN, Figure 1); median raphe nucleus (MRN, Figure 1); raphe magnus nucleus (RMN, Figure 2); and caudal linear nucleus (CLN, Figure 3). The rat DRN is the largest serotonergic nucleus and has widespread projection sites in the forebrain and the brainstem. Sites with particularly dense innervation from the DRN include the fronto-parietal cortex, ventral hippocampus, basolateral amygdala, lateral septum, nucleus accumbens shell, substantia nigra (SN), striatum and lateral hypothalamus (Vertes, 1991; Vertes and Kocsis, 1994; Lechin et al., 2006). The rat MRN is smaller, containing roughly 1100 serotonin neurons (Wiklund et al., 1981) and projects to the temporal cortex, dorsal hippocampus, central amygdala, medial septum, nucleus accumbens core, ventral tegmental area (VTA) and mesolimbic structures, as well as the mammillary and suprachiasmatic nuclei (SCN) of the hypothalamus (Vertes, 1991; Lechin et al., 2006; Pickard and Rea, 1997; Dudley et al., 1999; Vertes et al., 1999). The CLN is the most rostral of the raphe nuclei, located in the ventral tegmental area just dorsal to the interpeduncular nucleus (Tork, 1990; Hornung, 2003). Less is known about the CLN than either the DRN or MRN, but this nucleus is thought to share most efferent pathways of the DRN and not the MRN (Imai et al., 1986). The RMN, unlike the other nuclei that I studied, has projections to the spinal cord and is well known for its involvement in descending pain inhibition (Mason, 2001).

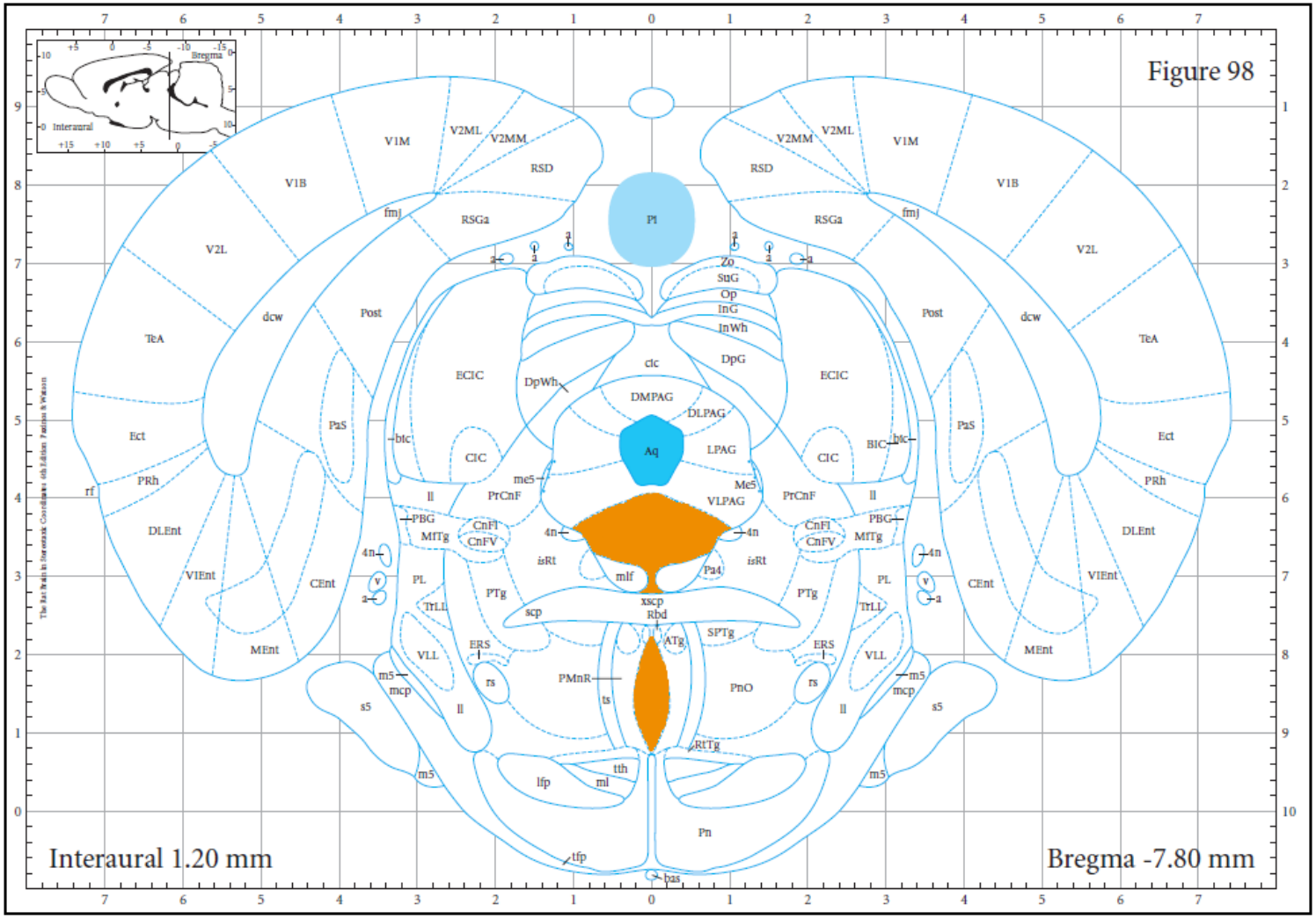

Figure 1. Dorsal raphe nucleus (DRN), highlighted (dorsal) and median raphe nucleus (MRN), highlighted (ventral) (Paxinos and Watson, 2006)

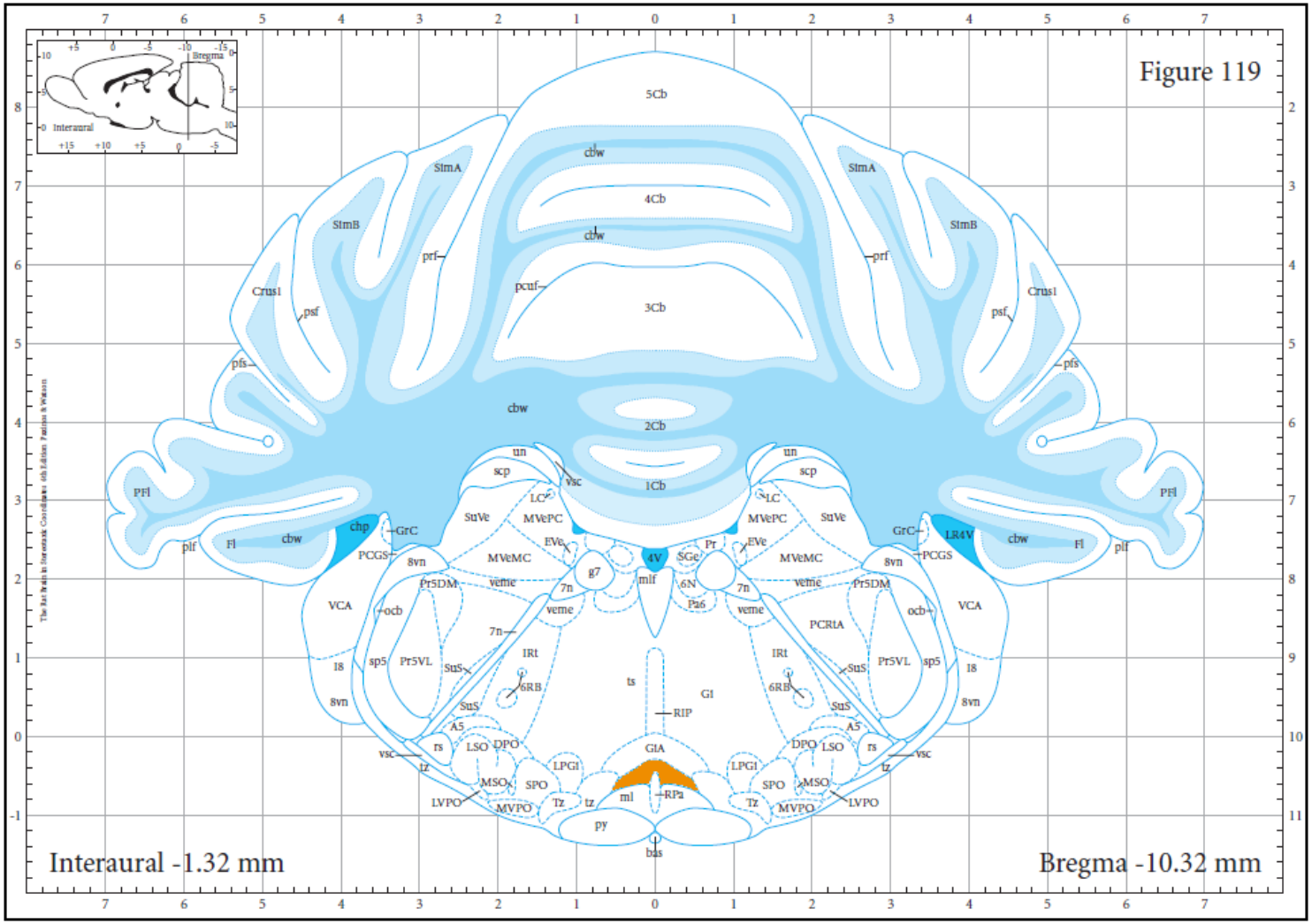

Figure 2. Raphe magnus nucleus (RMN), highlighted (Paxinos and Watson, 2006)

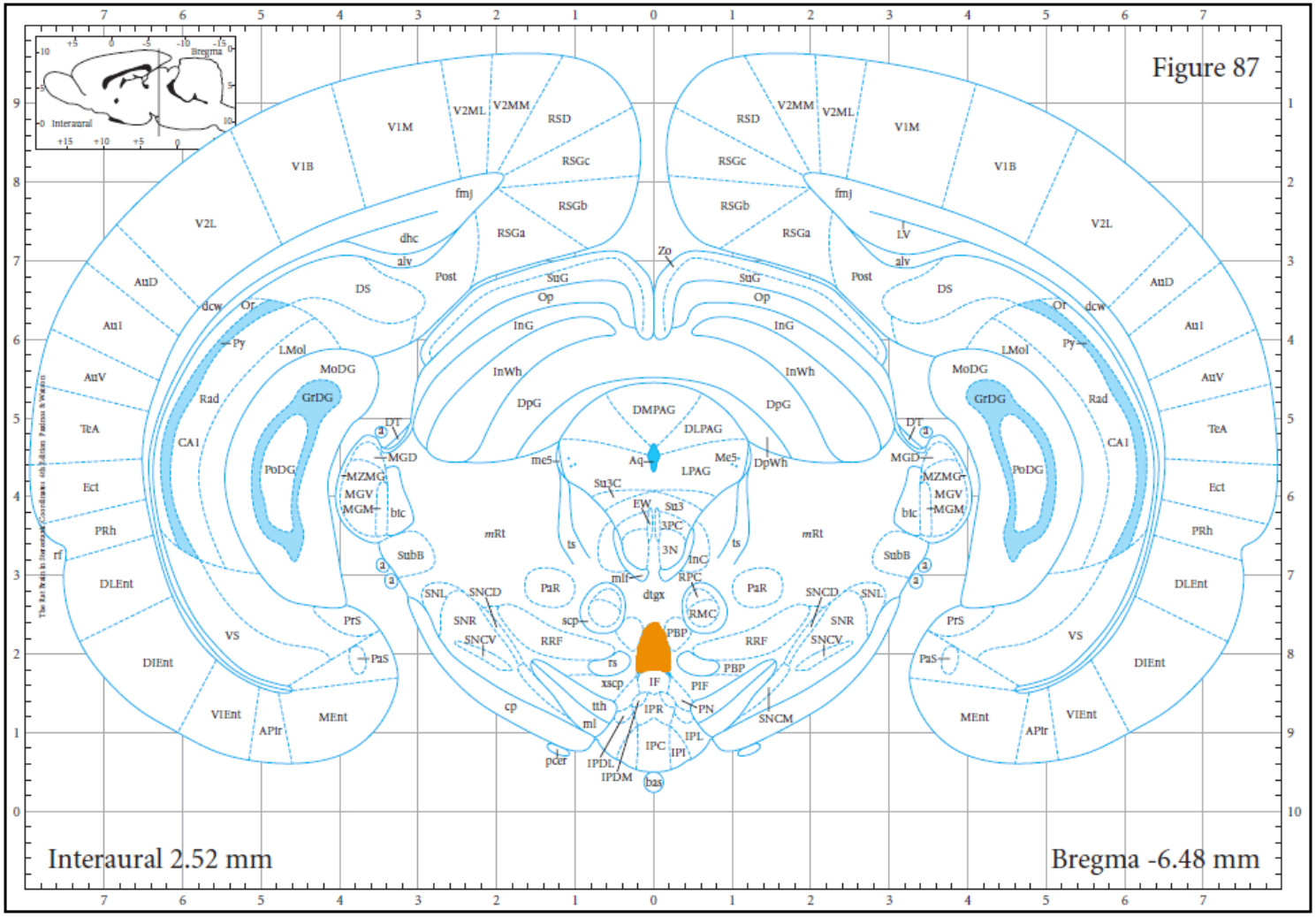

Figure 3. Caudal linear nucleus (CLN), highlighted (Paxinos and Watson, 2006)

ii) CNS Pathways & Terminals

More broadly, three major ascending pathways project from the raphe nuclei: the dorsal ascending pathway, the medial ascending pathway and the ventral ascending pathway. The dorsal ascending pathway stems from the medial and rostral regions of the DRN and terminates primarily in the caudate-putamen complex. Isolated fibers of this pathway extend to the nucleus accumbens and globus pallidus. The medial ascending pathway arises from the dorsal raphe nucleus and projects largely to the SN, though collaterals to the caudate-putamen complex are also present. The ventral ascending pathway projects from the DRN and MRN to the VTA and, later, the medial forebrain bundle (MFB) of the hypothalamus. The MFB distributes fibers to a wide range of forebrain sites some of which include the interpeduncular nucleus, VTA, thalamus, hypothalamus, lateral geniculate nucleus, amygdala, hippocampus and prefrontal cortex (Steinbusch, 1984).

Conversely, the two prominent descending pathways include: projections from the DRN to the locus coeruleus that in turn project the dorsal tegmental nucleus and the raphe pontis, and the bulbospinal pathway which consists of three subsystems: the dorsal, intermediate and ventral pathways. The dorsal bulbospinal pathway extends from the RMN to the superficial dorsal horn via the dorsolateral funiculus; the raphe obscurus and pallidus contribute to both the intermediate descending pathway and the ventral descending pathway that terminate in the intermediolateral cell column and somatic motor neurons in the ventral horn, respectively (Steinbusch, 1984).

iii) Serotonin Receptors

Serotonergic activity is mediated by 7 families of receptors, together totaling at least 14 genetically, pharmacologically and functionally distinct receptor subtypes that are located on both pre- and post-synaptic membranes (Roth, 1994; Hoyer et al., 2002; Hannon and Hoyer, 2008). All subtypes exist in a post-synaptic location but only members of the 5-HT_1 family have been identified pre-synaptically. With one exception, the serotonin receptors are G-protein coupled and function via second messenger mechanisms to either hyperpolarize (i.e. the 5-HT_1 family) or depolarize (i.e. 5-$HT_{2,4,5,6,7}$ families) their host neurons (Barnes and Sharp, 1999). The 5-HT_3 receptor is an ion channel coupled to cation influx; activation of this receptor produces depolarization of the host neuron (Yakel et al., 1990). Graphical representation of the current 5-HT receptor classification scheme is depicted in figure 4.

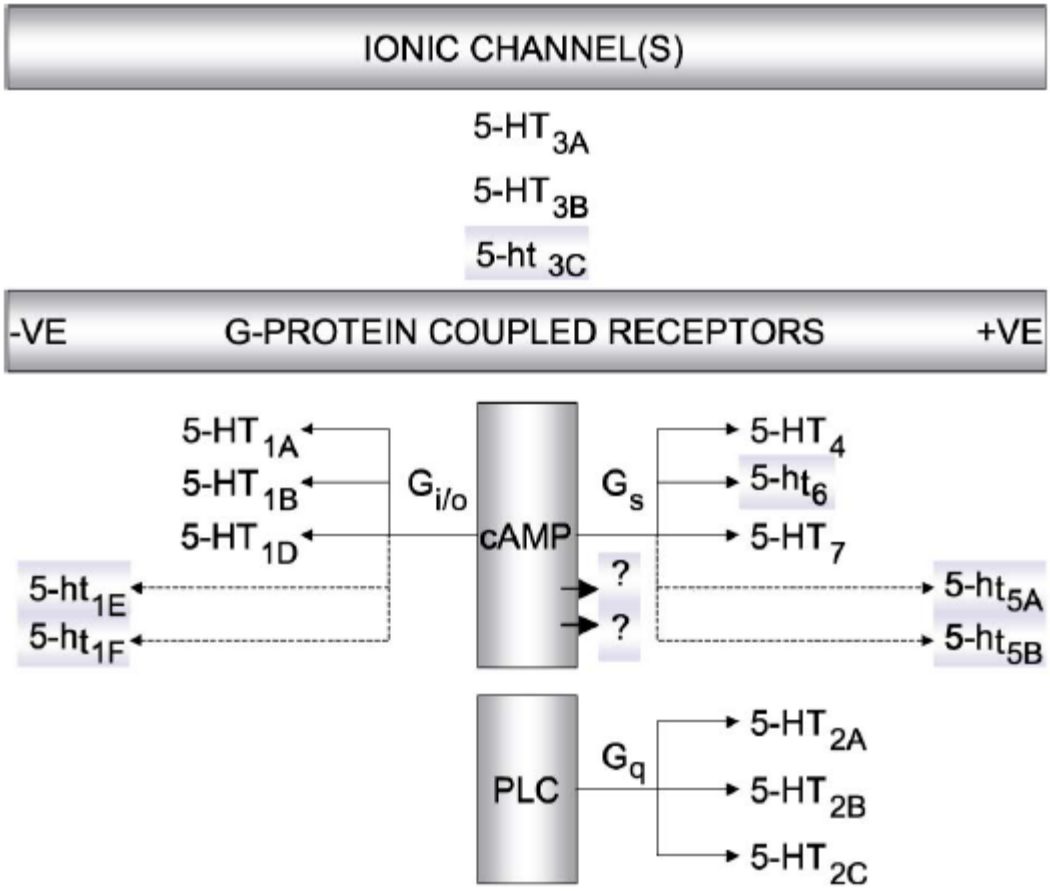

Figure 4. Graphical representation of the current classification of 5-HT receptors. Abbreviations: cyclic adenosine monophosphate (cAMP); phospholipase C (PLC) (Hoyer et al, 2002).

a) Focus on the $5HT_{1A}$ Autoreceptor

The $5\text{-}HT_{1A}$ autoreceptor is particularly relevant to my studies of brainstem serotonergic neurons because of its pre-synaptic location and regulatory influence on tryptophan hydroxylase (TPH). The $5\text{-}HT_{1A}$ receptor is a G-protein coupled somatodendritic autoreceptor that prevents calcium-calmodulin-dependent activation of TPH (Sawada and Nagatsu, 1986) and inhibits cell firing by mediation of K^+ conductance (Aghajanian and Lakoski, 1984; Williams et al., 1988). Variation of $5HT_{1A}$ receptor

densities might account for the differential susceptibility to $5HT_{1A}$ autoreceptor agonists that has been noted in the literature. For example, a higher susceptibility to $5HT_{1A}$ autoreceptor agonists in the DRN compared to the MRN is consistent with the higher density of $5HT_{1A}$ autoreceptors in the DRN compared to the MRN (Pazos and Palacios, 1985; Weissmann-Nanopoulos et al., 1985). The density of $5HT_{1A}$ autoreceptors in the CLN and RMN have not yet been elucidated, though these may be similarly variable.

Serotonin transporter (SERT) blockade acutely increases serotonin bioavailability in the synapse (Wong et al., 2005) and results in heightened activation of the somatodendritic $5HT_{1A}$ autoreceptor. This reportedly inhibits serotonin neuronal firing (Sprouse and Aghajanian, 1987), synthesis (Barton and Hutson, 1999) (Carlsson and Lindqvist, 1978) and release (Hjorth and Auerbach, 1994a). A striking temporal disjunction between SERT blockade and selective serotonin reuptake inhibitor (SSRI) efficacy is widely noted in the medical literature; while inhibition of the transporter occurs almost immediately, alleviation of depressive symptoms typically requires 2-4 weeks of treatment. This delay would suggest a drug mechanism more complex than simple inhibition of serotonin reuptake. Several authors have postulated that sizable enhancement of synaptic serotonin by SERT inhibition is dependent on $5HT_{1A}$ autoreceptor desensitization and, accordingly, that the time necessary for autoreceptor desensitization could account for the delay in SSRI-mediated improvement of depressive symptoms. Several electrophysiological studies support this notion: the firing rate of brainstem serotonergic neurons apparently recovers over time (de Montigny and Blier, 1984) while the sensitivity of $5HT_{1A}$ autoreceptors to $5HT_{1A}$ agonists reportedly

decreases during chronic treatment with SERT inhibitors (Blier and de Montigny, 1987). Conversely, Hjorth and Auerbach (1994b) found that the $5HT_{1A}$ autoreceptor did not desensitize following chronic SSRI treatment.

iv) The Serotonin Transporter (SERT)

The widespread use of pharmacological selective SERT inhibitors (i.e. SSRI-type medications) for the treatment of depression and anxiety disorders (Fava et al., 1993; Boyer, 1995; Pigott and Seay, 1999; Zohar and Westenberg, 2000; Vaswani et al., 2003) has focused attention on the importance of the serotonin transporter (Figure 5) Preferentially located in axonal varicosities and terminal boutons, the SERT is a large neurotransmitter transporter that mediates the Na^+/Cl^-–dependent reuptake of extracellular serotonin into the presynaptic neuron (Schloss and Williams, 1998).

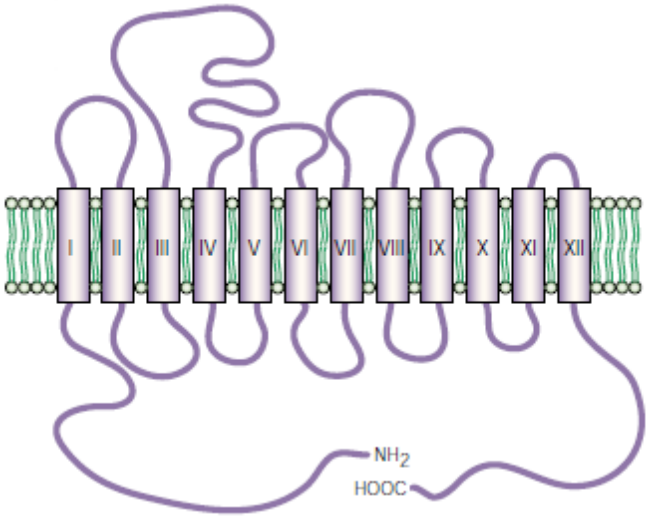

Figure 5. Structure of the serotonin transporter.

SERT protein is largely limited to raphe serotonergic cell bodies; expression is virtually absent from other brainstem nuclei or forebrain sites (Cooper et al., 2003). Activation of

SERT is remarkably fast, with uptake initiated less than one millisecond after transmitter release (Bruns et al., 1993).

A host of radioligand binding and immunohistochemical investigations have examined regional variation in SERT densities throughout the brainstem raphe nuclei (Hrdina et al., 1985; Marcusson et al., 1988; Biegon and Mathis, 1993; Fujita et al., 1993; Austin et al., 1994). SERT concentrations are highest in the DRN, with lower concentrations in other raphe nuclei (Fujita et al., 1993). Specifically, SERT mRNA in the DRN is significantly higher than in the MRN, (Hoffman et al., 1998; Rattray et al., 1999; Clark et al., 2006) and the density of SSRI antidepressant binding sites within the DRN is nearly double that of the MRN (Hrdina et al., 1990). Unfortunately, the relative densities of SERT among raphe nuclei other than the DRN and MRN are less clear. Few studies have extensively analyzed the relative SERT densities of the individual raphe nuclei. Some authors simply group the raphe nuclei together as a single brainstem region rather than studying each nucleus individually, and typically note strong SERT labeling in this region (Lesch et al., 1996; Sur et al., 1996).

Proponents of the monoamine hypothesis of depression postulate that the putative deficiency of serotonin in depressed patients is corrected by agents that block the SERT-mediated reuptake of serotonin (White et al., 2005). Again, the timing discrepancy between SERT inhibition and clinical efficacy suggests that a more complex mechanism is at play and that longer term adaptive changes may underlie the therapeutic effects of SSRI antidepressants. Downregulation of SERT may be one such change: chronic SSRI treatment apparently reduces SERT density in the rat hippocampus and DRN (Pineyro et

al., 1994; Benmansour et al., 1999) and also downregulates SERT transport capacity in rats, possibly via a protein kinase A (PKA)-mediated internalization of the plasma membrane transporter (Horschitz et al., 2001; Torres et al., 2003).

B. Serotonin Biosynthesis and Catabolism

i) Serotonin Biosynthesis

Serotonin is synthesized from tryptophan in a two step reaction. The essential amino acid tryptophan is first hydroxylated by the rate-limiting enzyme tryptophan hydroxylase at the 5 carbon to form 5-hydroxytryptophan. Thereafter, the intermediate 5-hydroxytryptophan is rapidly decarboxylated to 5-hydroxytrytamine (serotonin) by aromatic amino acid decarboxylase (Cooper et al., 2003; Figure 6). Although serotonin is synthesized in high concentrations outside of the central nervous system (CNS), the brain requires its own internal supply because serotonin is unable to cross the blood-brain barrier. Synthesis of serotonin in the brain relies on tryptophan transport across the blood-brain barrier via the large neutral amino acid transporter, a process that is subject to competitive inhibition from other amino acids that share the same transport mechanism (Leathwood, 1987; Salter et al., 1989; Boadle-Biber, 1993). Neuronal uptake of plasma tryptophan also relies on the neutral amino acid transporter.

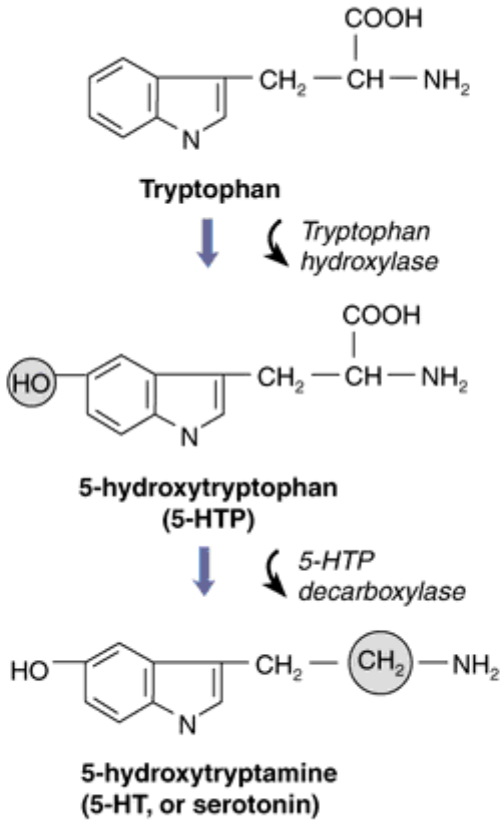

Figure 6. Serotonin biosynthetic pathway. Tryptophan is hydroxylated to form 5-hydroxytrptophan and subsequently decarboxylated to serotonin.

a) Tryptophan Hydroxylase

Structure and Function of TPH

Tryptophan hydroxylase is the catalyst for the rate-limiting conversion of tryptophan to 5-hydroxytryptophan (Figure 7).

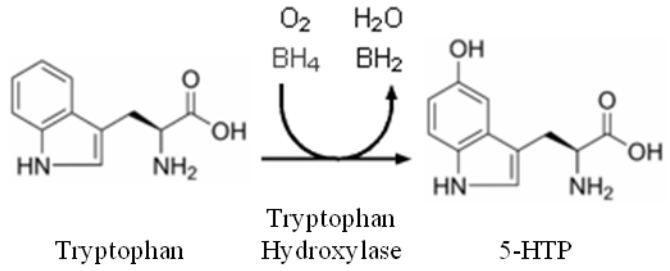

Figure 7. Conversion of tryptophan to 5-hydroxytryptophan (5-HTP) by tryptophan hydroxylase.

With a tetrameric structure and an estimated weight of 52 000-60 000 kDa (Tong and Kaufman, 1975), TPH belongs to a family of biopterin-dependant aromatic amino acid hydroxylases (Jiang et al., 2000). As is the case with other members of the enzyme family, functional TPH activity requires molecular oxygen substrate and cofactor (6R)-L-erythro-5,6,7,8-tetrahydrobiopterin (BH_4). An iron binding site on TPH allows ferrous iron to enhance the catalytic reaction velocity, though the exact nature of this relationship is not well understood (Hufton et al., 1995).

The molecular structure of TPH consists of a hydrophilic N-terminal regulatory domain and a hydrophobic carboxyl terminal catalytic domain (Figure 8) (Grenett et al., 1987; Darmon et al., 1988).

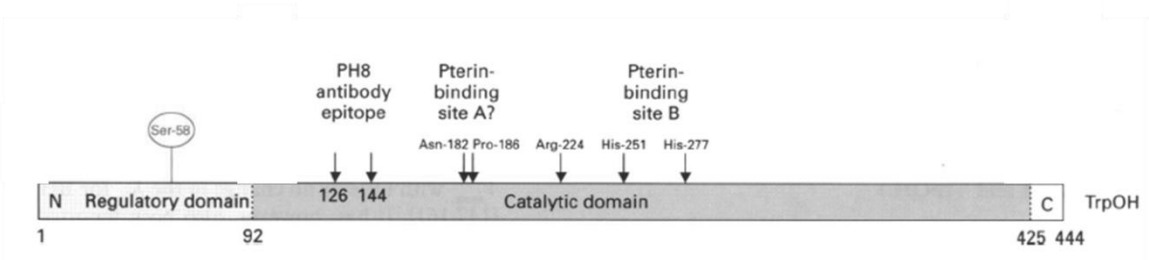

Figure 8. Regulatory and catalytic domains of TPH. Serine-58, a recognized phosphorylation site is located in the N-terminus regulatory domain (Hufton et al., 1995).

The enzymatic active site located within the catalytic domain mediates the hydroxylation of tryptophan via the oxidation of cofactor BH_4 and the reductive incorporation of molecular oxygen (Jiang et al., 2000; Moran et al., 1998) whereas the N-terminus includes regulatory phosphorylation sites that are activated by PKA and calcium calmodulin-dependent protein kinase (Yamauchi et al., 1981; Makita et al., 1990; Furukawa et al., 1993; Johansen et al., 1996). The structural locations of TPH phosphorylation sites are still being elucidated. Kuhn et al. (1997) demonstrated that the serine58 residue was a substrate site for PKA and serine260 and serine443 residues have been identified as putative calmodulin-dependent protein kinase sites (Darmon et al., 1988). Despite the lack of confirmed phosphorylation sites, the calcium-dependent activation of TPH has been the better studied of the regulatory mechanisms and it has emerged that the eventual phosphorylation is coupled with nerve depolarization and impulse flow (Hamon et al., 1977; Boadle-Biber, 1979; Boadle-Biber and Phan, 1987). This action may be a compensatory response to an increased demand for serotonin during peak activity (Cooper et al., 2003). Conversely, the $5\text{-}HT_{1A}$ somatodendritic autoreceptor prevents calcium-calmodulin-dependent activation of TPH (Sawada and Nagatsu, 1986).

Inactivation of TPH

Methylenedioxymethamphetamine (MDMA) and p-chloroamphetamine (PCA) rapidly inactivate TPH and cause extensive damage to serotonergic neurons (Richard et al., 1990; Sabol et al., 1996). The mechanism of inactivation is currently unknown, though recent reports point to drug-induced production of reactive oxygen species and nitric oxide (NO) (Kuhn and Arthur, 1997). Kuhn and Arthur (1996; 1997) demonstrated

that TPH is irreversibly inactivated by NO by selective action on sulfhydryl groups. Interestingly, TPH is also inactivated by dopamine, under mild oxidizing conditions or in the presence of tyrosinase (Kuhn and Arthur, 1998).

Localization of TPH

In the central nervous system, TPH is highly localized to brainstem serotonergic neurons (Weissmann et al., 1987) and is thus an appropriate immunohistochemical marker for serotonin cells. The exact cellular location of the enzyme is not clear. Cooper et al. (2003, p. 274) suggests that "tryptophan hydroxylase appears to be a soluble cytoplasmic enzyme" while Boadle-Biber (1993, p. 2), on the other hand reports that "it appears to be a membrane enzyme." The contradictory suggestions may stem from the inherent instability of the enzyme and the nature of procedural techniques; as Cooper et al. (2003) points out, the enzyme extraction process may alter the natural binding particle-capacity. Alternatively, TPH may shuttle back and forth between these forms.

ii) Serotonin Catabolism

The primary catabolic pathway for serotonin is oxidative deamination by cytoplasmic monoamine oxidase (MAO, Figure 9). The product of this initial deamination, 5-hydroxy-indol-acetaldehyde, is subsequently oxidized by aldehyde dehydrogenase to 5-hydroxy-indol-acetic acid (5-HIAA, Cooper et al., 2003). In humans, the biological half-life of serotonin ranges from 2 to 7 hours, and the plasma clearance of 5-HIAA ranges from 0.10 to 0.23 1/kg/hour (Westenberg et al., 1982).

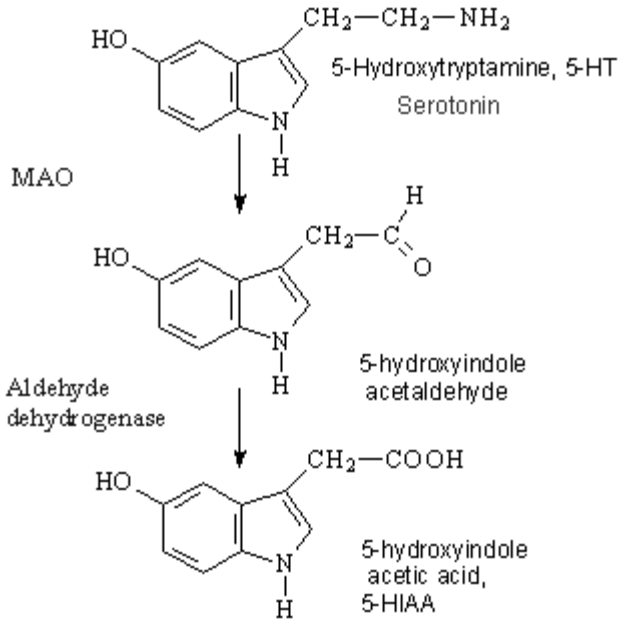

Figure 9. Serotonin catabolism.

2.2. Dopamine Neurobiology: A Brief Overview

The initial aim of my work was to explore the influence of SERT inhibition on brain serotonergic systems. With the realization that SSRIs can induce a variety of dopamine-dependent side effects such as extrapyramidal symptoms, sexual dysfunction and galactorrhea, however, I became interested in investigating the modulatory influence of SSRI medications on dopaminergic systems as well. I postulated that, in addition to altering serotonin transmission, SERT inhibition might have direct effects on dopaminergic neurons. Over the course of several experiments, we studied the effects of SERT inhibition and environmental enrichment on dopamine neurons in the SN (Figure 10) and VTA (Figure 10). Serotonin-dopamine interactions within these regions are described in sections 2.3 and 2.4 respectively. A brief overview of the neurobiology of midbrain dopaminergic systems in described here.

Dopamine (DA) is a catecholamine neurotransmitter with important roles in voluntary movement, cognition, motivation and reward. There are four major dopamine-containing pathways in the brain, described below.

- The nigrostriatal pathway originates in the SN and projects mainly to the dorsal striatum where the caudate and putamen are housed (Cooper et al., 2003). This pathway is important for voluntary movement and has been a central focus for investigations of Parkinson's disease and drug-induced extrapyramidal syndromes (EPS) since both of these conditions are characterized by a loss of neurons in the substantia nigra (Kitt et al., 1986; Damier et al., 1999).

- The mesolimbic pathway originates in the VTA and projects to areas of the limbic system including the nucleus accumbens, amygdala, septum and the olfactory tubercle. This pathway is important for emotional processing (Cooper et al., 2003).
- The mesocortical pathway also originates in the VTA, but projects to areas within the limbic cortex, i.e. the medial prefrontal, cingulated and entorhinal areas (Cooper et al., 2003).
- The tuberoinfundibular hypothalamic neuroendocrine system projects from dopamine neurons in the arcuate and periventricular nuclei to the pituitary median eminence (Cooper et al., 2003). Dopamine released at this target site inhibits the secretion of prolactin from the anterior pituitary gland.

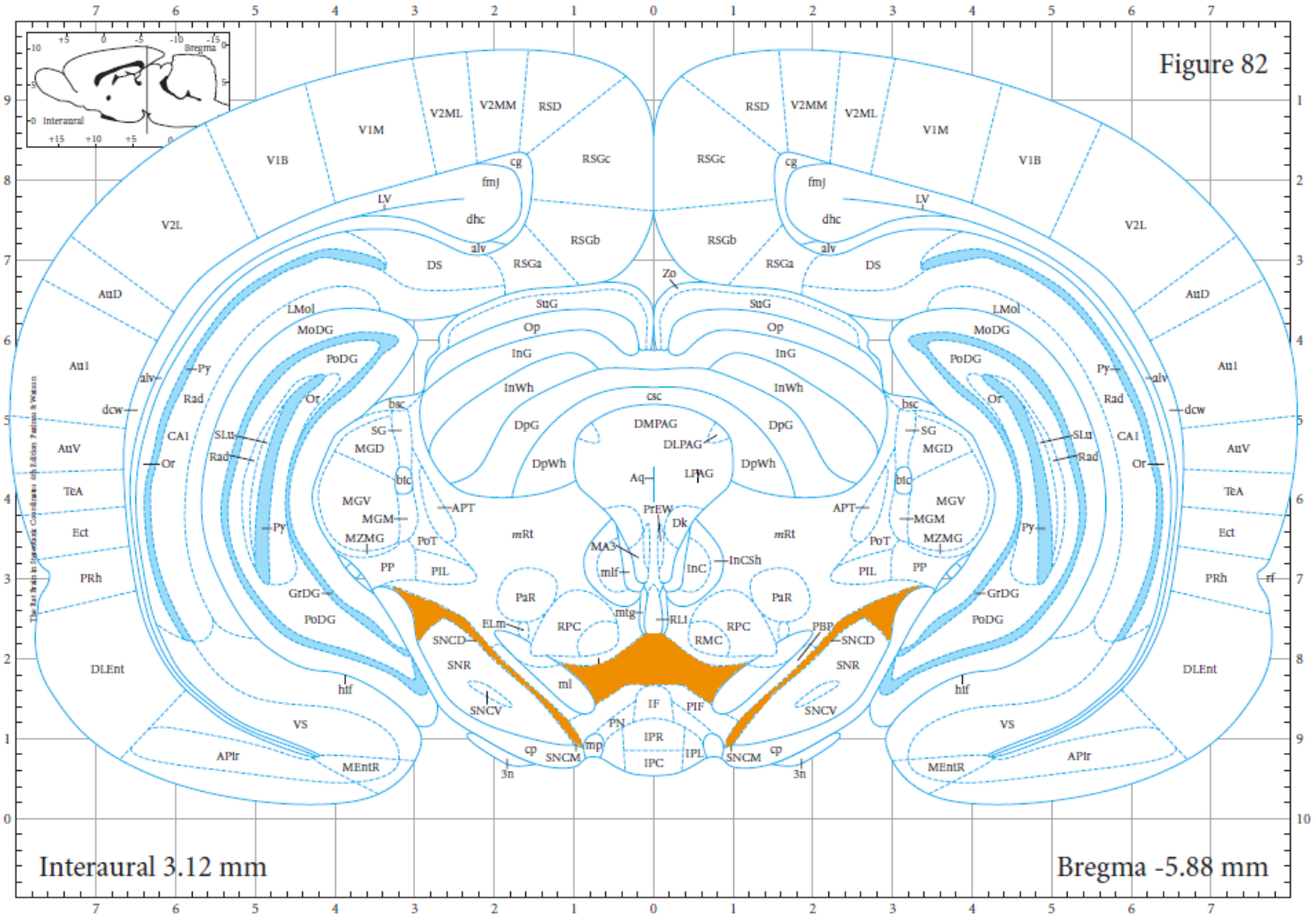

Figure 10. Substantia nigra (SN), highlighted (lateral structures) and ventral tegmental area (VTA), highlighted (medial structure) (Paxinos and Watson, 2006)

Dopamine synthesis originates with the nonessential amino acid tyrosine which is converted to L-dihydroxyphenylalanine (L-DOPA) by the rate-limiting enzyme tyrosine hydroxylase (TH, Cooper et al., 2003, Figure 11). L-DOPA is rapidly converted to dopamine by L-aromatic amino acid decarboxylase. As was the case for TPH and brainstem serotonin neurons, TH is highly specific for dopaminergic neurons within the SN and VTA and is thus an appropriate marker for dopamine neurons within these regions.

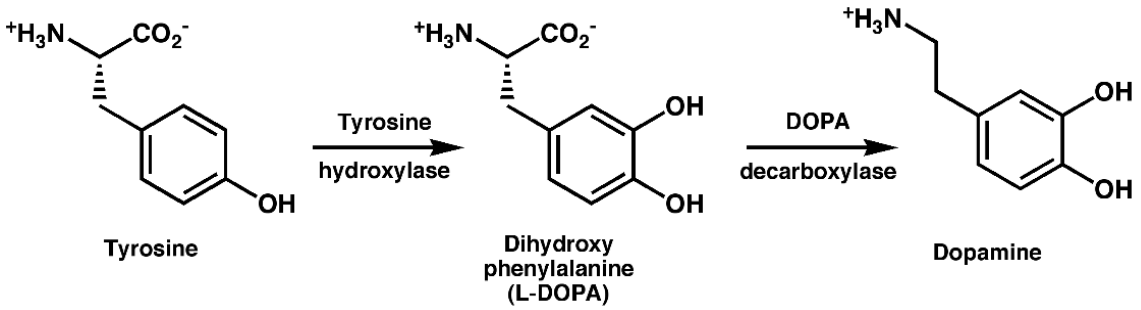

Figure 11. Dopamine biosynthetic pathway

The rate of dopamine synthesis in dopamine neurons is dependent on TH activity, which is in turn modulated by a number of modulatory influences which include (1) end-product inhibition by released dopamine, since dopamine competes with a tetrahydrobiopterin (BH_4) cofactor for a binding site on the TH enzyme; (2) the availability of the BH_4 cofactor; (3) autoreceptor-mediated feedback inhibition of dopamine synthesis by TH; (4) impulse flow, whereby increased impulse flow increases the rate of tyrosine hydroxylation, probably via enzyme phosphorylation (Cooper et al., 2003). The dopamine transporter (DAT) mediates the Na^+/K^-–dependent reuptake of extracellular dopamine into the terminal; recaptured dopamine is subsequently

metabolized to dihydroxyphenylacetic acid (DOPAC) by intraneuronal MAO. Alternatively, released dopamine can be converted at an extraneuronal site to homovanillic acid (HVA) via a series of enzymatic reactions involving catechol-O-methyltransferase (COMT) and MAO.

The five known subtypes of dopamine receptors (D1, D2, D3, D4 and D5) that exist in humans are typically divided into two families. The D1-like family includes subtypes D1 and D5, while the D2-like family includes subtypes D2, D3 and D4. All dopamine receptors are metabotropic G protein-coupled receptors capable of activating second messenger pathways. Dopamine activation of D1-like receptors is coupled to increases in adenylate cyclase, which subsequently increases the intracellular concentration of the second messenger cyclic adenosine monophosphate (cAMP). Conversely, dopamine activation of D2-like receptors is negatively coupled to adenylate cyclase, directly inhibiting the formation of cAMP (Cooper et al., 2003). Postsynaptic dopamine receptors include both the D1-like and D2-like families; autoreceptors are generally believed to be members of the D2 family only. Autoreceptors, present on both the somatodendritic and terminal regions of dopamine neurons, regulate nerve firing as well as dopamine synthesis and release. Specifically, activation of autoreceptors located in the somatodendritic region reduces the firing rate of dopamine neurons, while activation of those in the terminal region reduces dopamine synthesis and release (Cooper et al., 2003).

2.3. Serotonin-Dopamine Interactions in the Nigrostriatal Pathway

The traditional understanding of neurotransmitter interaction in the striatum envisioned a balance between dopamine and acetylcholine tone which, if disrupted, could produce extrapyramidal reactions (Calabresi et al., 1989). This notion is supported by the amelioration of drug-induced EPS by drugs which block the acetylcholine receptor (Arana et al., 1988). With the discovery of major glutamatergic and serotonergic inputs to the striatum (Bobillier et al., 1976; Geyer et al., 1976; Fonnum et al., 1981), however, it became clear that the dopamine/acetylcholine model was overly simplified. More recent models postulate that the serotonergic, glutamatergic and cholinergic systems in the striatum act in a complex way to modify the striatal gamma-aminobutyric acid (GABA) output neurons (Hamilton and Opler, 1992; Di Chiara and Morelli, 1993). According to one proposed model, raphe-striatal projections inhibit striatal dopamine release which might subsequently inhibit basal ganglia-mediated movement (Hamilton and Opler, 1992). It is conceivable that the putative inhibition of dopamine transmission by SSRIs is mediated at the receptor level in the striatum. However, a host of anatomical and physiological evidence suggests that, in addition to its action in the striatum, serotonin may also directly act at the dopamine cell bodies in the SN (Dray et al., 1976; Fibiger and Miller, 1977; Dray et al., 1978; Nedergaard et al., 1988). This lends credence to the notion that SSRIs may exert their influence on dopaminergic transmission by direct effects on the dopamine cell body.

The serotonin-synthesizing cell bodies in the brainstem raphe nuclei have widespread terminal projection sites in the CNS (Jacobs and Azmitia, 1992). It is

noteworthy, however, that these serotonin terminals preferentially target motor regions of the CNS, including all components of the basal ganglia circuitry (Steinbusch, 1981). In fact, the SN of various species, including rats (Moukhles et al., 1997), monkeys (Lavoie and Parent, 1990) and humans (Mackay et al., 1978) receives the greatest density of central serotonergic innervation. Fuxe (1965) was the first to identify serotonin containing nerve terminals in the SN with subsequent studies both validating and expanding on his finding. It is now well-established that serotonergic cell bodies located in the brainstem raphe nuclei project to the SN and the striatum, where serotonin axon terminals are in close proximity to dopamine cell bodies and axon terminals, respectively (Dray et al., 1976; Fibiger and Miller, 1977; Dray et al., 1978; Soghomonian et al., 1987). The majority of these serotonergic projections derive from the dorsal (DRN) and median (MRN) raphe nuclei of the brainstem, with the DRN providing the majority of input (Geyer et al., 1976; Conrad et al., 1974; Miller et al., 1975). The assertion that serotonin has an inhibitory influence on dopaminergic neurons in the SN was first corroborated by late 1970s reports demonstrating that electrical stimulation of the MRN or DRN depressed activity of neurons in the SN and that serotonin, microiontophoretically applied to the SN, produced inhibition of neuronal activity (Dray et al., 1976; Dray et al., 1978). In those same experiments, discrete electrolytic lesions of either the MRN or DRN decreased serotonin concentrations in the SN and increased striatal dopamine concentrations, suggesting that an inhibitory pathway from the raphe to the SN contributes to the regulation of nigrostriatal dopaminergic transmission. This inhibitory action appears to be modulated by 5-HT_{2A} heteroreceptors located on the somatodendritic

surface of the dopamine neurons and on dopamine terminals in the striatum (Pazos et al., 1987; Ugedo et al., 1989). Lesions that interrupt the raphe-nigral pathway (Giambalvo and Snodgrass, 1978; James and Starr, 1980), functional inhibition of raphe-nigral neurons by 5-HT_{1A} agonists (Arborelius et al., 1993) or 5-HT_2 antagonists (Ugedo et al., 1989) all lead to functional disinhibition of the nigrostriatal dopamine system.

Increased availability of serotonin induced by SSRI administration might exacerbate the already present tonic inhibition of nigrostriatal dopamine by serotonin by amplified action at 5-HT_{2A} heteroreceptors. It is conceivable, then, that the increased availability of serotonin induced by SSRIs might reduce striatal dopamine and induce extrapyramidal side effects similar to the D2 receptor blocking neuroleptics. In fact, Baldessarini and Marsh (1990) have reported that high doses of fluoxetine significantly inhibit dopamine synthesis in the dorsal striatum.

2.4. Serotonin-Dopamine Interactions in the Ventral Tegmental Area

The VTA is innervated by serotonergic axon terminals that originate in brainstem raphe nuclei (Azmitia and Segal, 1978; Phillipson, 1979). Similar to those in the SN, these serotonergic projections to the VTA appears to have an inhibitory influence since selective lesions of brainstem serotonin neurons enhance the firing activity of VTA dopamine neurons (Guiard et al., 2008). A substantial body of literature points to a role for the serotonin $5HT_{2C}$ receptor subtype in mediating the phasic and tonic inhibitory control of the mesolimbic dopamine system (Prisco and Esposito, 1995; Di Matteo et al., 1999; Di Matteo et al., 2000; Di Giovanni et al., 2000; Di Matteo et al., 2002).

The proposed existence of a serotonergic-dopaminergic interaction in the VTA has been substantiated by a series of publications noting that SSRI medications can inhibit the firing rate (Prisco and Esposito, 1995; Di Mascio et al., 1998; Dremencov et al., 2009) and spontaneous activity (Sekine et al., 2007) of dopamine neurons in the VTA. Prisco and Esposito (1995) reported that two different SERT inhibitors, citalopram and fluoxetine, both inhibited the firing rate of dopamine neurons in the VTA and that selective lesions of serotonin neurons with the neurotoxin 5,7-dihydroxytryptamine could eliminate this inhibition. Pretreatment with a $5\text{-}HT_{2C/2B}$ antagonist prevented the decrease in firing rate as well, corroborating further the important role for the $5HT_{2C/2B}$ receptor subtype in regulating serotonin's inhibitory influence on the mesolimbic dopamine system. The SSRI-induced reduction of TH-positive cell counts in the VTA might account for the limited efficacy of these medications in some patients, since hedonia,

motivation and reward are mediated by the mesolimbic dopamine system (Dremencov et al., 2009; Nestler and Carlezon, 2006).

2.5. Pharmacological Manipulation of the Serotonin System

The experiments outlined in this thesis examine the role of common psychotropic medications in the regulation of central serotonergic and dopaminergic neurons. These pharmaceutical agents were selected for their preferential action on the brain serotonin system and for their propensity to cause clinical side-effects. Of the three medications that I studied, two (citalopram and fluoxetine) selectively inhibit the serotonin transporter while a third (risperidone) is a serotonin 5-HT_2 receptor antagonist.

A. Selective Serotonin Reuptake Inhibitors (SSRIs)

The selective serotonin reuptake inhibitors (SSRIs) are potent blockers of the SERT (Figure 12).

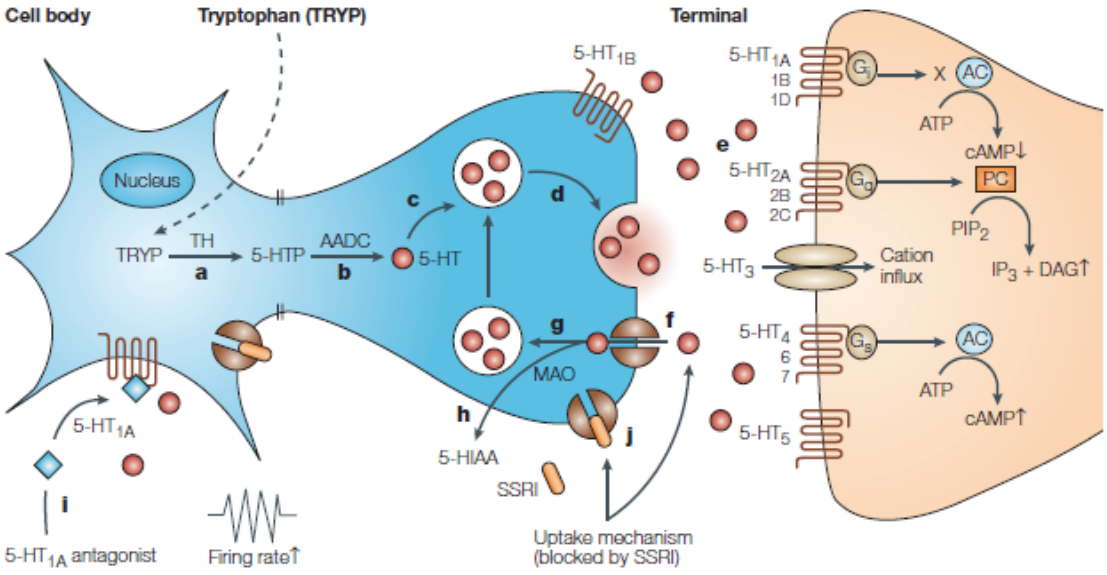

Figure 12. Serotonergic neurotransmission. (j) SSRI antidepressants inhibit the serotonin transporter and increase synaptic serotonin (Wong et al., 2005).

The prototypical SSRI fluoxetine emerged in the late 1970's from a rational design process aimed at the development of a medication that would selectively target serotonin reuptake and lack secondary actions at the histamine, acetylcholine, and α-adrenaline receptors responsible for the pervasive side-effects associated with the older tricyclic antidepressants (TCAs) and MAO inhibitors (MAOIs, Katsung, 2009). Once approved for clinical use, SSRIs largely supplanted largely supplanted the older TCA medications and MAOIs, primarily because they were considered less likely to cause adverse effects (Goldstein and Goodnick, 1998; Steffens et al., 1997). Today, they are among the most commonly prescribed pharmaceutical agents in medical practice.

The primary indication for SSRIs is major depressive disorder, though they are increasingly prescribed for a range of anxiety disorders (Boyer, 1995; Pigott and Seay, 1999; Zohar and Westenberg, 2000; Vaswani et al., 2003), premenstrual dysphoric disorder (Shah et al., 2008) and bulimia (Kaye et al., 1998; Milano et al., 2005). Six SSRIs medications are broadly available and although each of these preferentially inhibits serotonin reuptake, they are structurally different and are characterized by varying degrees of potency and selectivity. As part of this thesis, I studied changes in brainstem serotonergic neurons after initiation of two different SERT inhibitors, citalopram and fluoxetine. The structure and properties of each are briefly described below.

i) ***Citalopram***

Citalopram is a bicyclic phthalane derivative (Figure 13) known for its highly selective and potent action on SERT (Compendium of Pharmaceuticals and Specialties,

2009). Considered the 'cleanest' of the SSRI medications, citalopram has negligible binding affinities for the serotonin 5-HT_{1A}, 5-HT_2, dopamine D_1 and D_2, α_1-, α_2-, β-adrenergic, histamine H_1, muscarinic cholinergic, benzodiazepine, GABA and opioid receptors (Compendium of Pharmaceuticals and Specialties, 2009) and does not significantly inhibit reuptake of norepinephrine or dopamine (Sanchez and Hyttel, 1999).

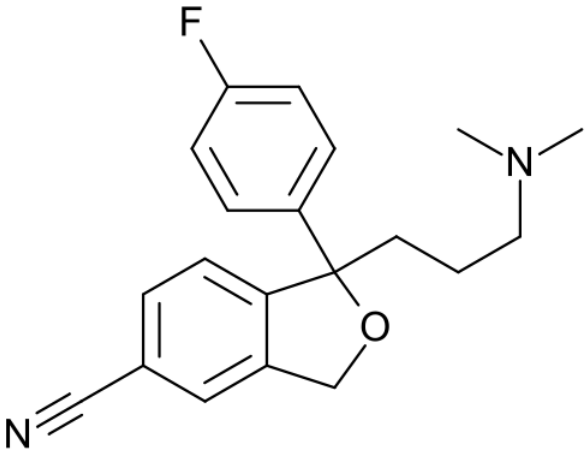

Figure 13. Chemical structure of citalopram

The kinetics of citalopram in laboratory animals as well as humans have been extensively studied, particularly during the early 1980s when citalopram safety studies were being conducted (Fredricson Overo, 1982a; Fredricson Overo, 1982b; Hyttel et al., 1984). It was noted by Fredricson Overø (1982a) that, for a 40 mg daily dosage, the steady stage plasma citalopram level in humans averages 78.4 ng/mL. A variety of citalopram dosages and dosing regimens have been tested in rats. A review of that literature is beyond the scope of this thesis, but for example, a single 8 mg/kg oral dose of citalopram given to a rat produces an average 200 nM plasma drug level (Fredricson

Overo, 1982b), roughly comparable to the plasma drug level noted with a 40 mg/day human dosage. Since the typical daily dosage for depressed patients ranges from 20-60 mg of citalopram per day, the 5 mg/kg/day dose chosen for our experiments should produce a plasma drug level in the rat that is comparable to a patient taking a relatively low dose of citalopram.

Citalopram is metabolized in the liver by N-demethylation to primary metabolite demethylcitalopram and secondary metabolites didemethylcitalopram, citalopram-N-oxide, and a deaminated propionic acid derivative (Hyttel et al., 1984, Compendium of Pharmaceuticals and Specialties, 2009). Citalopram metabolites inhibit SERT to a small degree but are much less selective and potent than citalopram itself (Compendium of Pharmaceuticals and Specialties, 2009). The plasma elimination half-life of citalopram is approximately 1.5 days in humans (Fredricson Overo, 1982a) and 3 hours in rats (Fredricson Overo, 1982b).

ii) Fluoxetine

Fluoxetine is a bicyclic antidepressant (Figure 14) and is the least selective of the SSRI-class, with detectable binding affinities for serotonin 5-HT_{1A}, 5-HT_2, dopamine D_1 and D_2, α_1-, α_2-, β-adrenergic, histamine H_1, muscarinic cholinergic, benzodiazepine, gamma aminobutyric acid (GABA) and opioid receptors (Compendium of Pharmaceuticals and Specialties, 2009). Fluoxetine does not appreciably inhibit the reuptake of dopamine or norepinephrine (Hyttel, 1994).

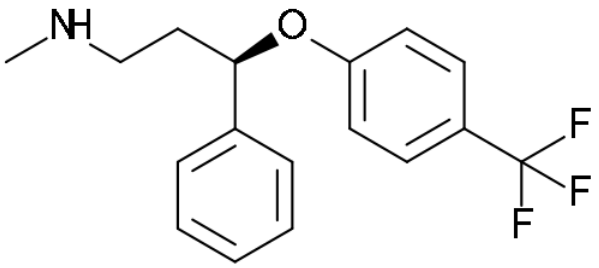

Figure 14. Chemical structure of fluoxetine.

Like for citalopram, the pharmacokinetics of fluoxetine is well studied. In humans a single 40 mg dose produces a peak plasma concentration of 15 – 55 ng/mL in 6-8 hours (Compendium of Pharmaceuticals and Specialties, 2009), a range that is roughly comparable to the peak plasma concentration of approximately 30 ng/mL that is produced in a rat after a 5 mg/kg/day oral dose of the same drug (Caccia et al., 1990). The therapeutic human dosage ranges from 20 to 80 mg of fluoxetine per day and so again, the 5 mg/kg/day dose chosen for our experiments should produce a plasma drug level in the rat that is comparable to a patient taking a relatively low dose of the drug.

Fluoxetine is metabolized in the liver to the primary metabolite norfluoxetine. Plasma concentrations of fluoxetine and norfluoxetine equalize after repeated dosing and the pharmacological action of the metabolite is similar, or perhaps even more potent and selective, to that of the parent compound (Sanchez and Hyttel, 1999). The half-life of the metabolite is considerably longer than of fluoxetine itself; 1-3 days for fluoxetine versus 7-15 days for norfluoxetine (Lemberger et al., 1985).

B. Atypical Neuroleptics

i) Risperidone

Risperidone is a benzisoxazole derivative (Figure 15) that is classified as an atypical neuroleptic, or likewise, an atypical antipsychotic. This medication was among the first new generation antipsychotic agents and, because of its action on serotonin as well as dopamine neurotransmission, is reported to have fewer side effects than those of the typical class (Borison et al., 1992; Chouinard et al., 1993; Janicak et al., 2001). Like the earlier generation antipsychotics, risperidone's ability to treat the positive symptoms of schizophrenia is attributed to dopamine D_2 receptor antagonism. The supposed improved side-effect profile, on the other hand, is attributed to its potent antagonism of serotonin 5-HT_2 receptors (Leysen et al., 1988; Schotte et al., 1996).

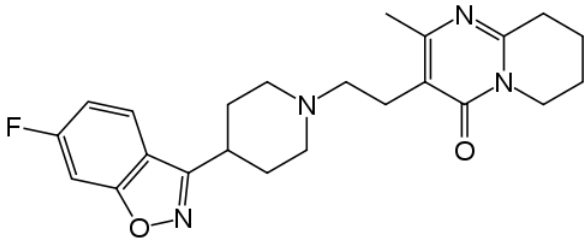

Figure 15. Chemical structure of risperidone

In humans, average peak plasma concentrations of risperidone and it's biologically active metabolite, 9-OH-risperidone are reached at approximately 1 hour and 3 hours, respectively (Compendium of Pharmaceuticals and Specialties, 2009). Riedel et al. (2005) reported that, in human patients, a mean 4.3 mg daily dose of risperidone yielded mean plasma concentrations of 13.1 ng/mL for risperidone and 32.8 ng/mL for 9-

OH-risperidone. In rats, a 6 mg/kg oral dose of risperidone has reportedly produced mean plasma concentrations of 288.7 ng/mL and 1308.5 ng/mL for 9-OH-risperidone (Aravagiri and Marder, 2002). It would appear, then, that the typical dose we administer to rats (~ 1 mg/kg) would still produce a plasma level higher than what is typically achieved with a therapeutic dose in humans. Nevertheless, the dose we chose is well within the typical range of dosages used in the animal literature and in fact, the dosages used by other laboratory groups are often much higher.

Risperidone is metabolized in the liver by CYP 2D6 to primary metabolite 9-hydroxyrisperidone (9-OH-risperidone). Since 9-OH-risperidone is biologically active, apparently to a similar or greater degree than its parent compound, the clinical effects of the drug are attributed to the combined concentrations (i.e. the active moiety) of risperidone and its main metabolite. These plasma concentrations vary considerably according to the genetically-determined metabolic capacity of an individual; 'extensive metabolizers' produce more debrisoquine 4-hydroxylase, the enzyme responsible for risperidone hydroxylation, and subsequent metabolize risperidone at a faster rate than 'poor metabolizers.'

2.6. The Side Effects of SSRI Medication: Evidence for Changes in the Brain Serotonin and Dopamine Monoaminergic Systems

The introduction of the SSRI-class of medications in the late 1970s was met with considerable enthusiasm largely because these drugs were considered less likely to cause adverse outcomes than the older TCAs and MAOIs (Goldstein and Goodnick, 1998; Steffens et al., 1997). Over time, however, it has become clear that patients taking SSRI medications continue to experience significant side effects, albeit of a different nature than those taking TCAs or MAOIs. A Canadian meta-analysis that compared the adverse effects associated with SSRIs and TCAs suggested that nausea, anorexia, diarrhea, insomnia, nervousness, anxiety and agitation all occurred more frequently with SSRIs than with TCAs and, conversely, that TCAs were more likely to cause anticholinergic side effects such as dry mouth and constipation (Trindade et al., 1998). As with the TCAs, the adverse effects associated SSRIs have a significant impact on treatment outcomes and patient compliance; conservative reports suggest that nearly 30% of patients discontinue their SSRI medication within 3 months, with adverse effects the most frequently cited reason for discontinuation (Bull et al., 2002).

A common side effect associated with SSRIs is gastrointestinal upset (Bergeron and Blier, 1994). The nausea and vomiting evoked by SSRI medication is generally attributed to hyperstimulation of peripheral 5-HT_3 receptors located on visceral fibres projecting to the area postrema (Bergeron and Blier, 1994) and it is noteworthy that antagonists for this receptor reduce the gastrointestinal side effects (Bailey et al., 1995). SSRI-induced dysfunction of the serotonergic cell bodies in the area postrema (Jacobs

and Azmitia, 1992; Lidov and Molliver, 1982) might also contribute to the gastrointestinal side effects associated with SSRI medications. Serotonin neurons in the area postrema project largely to the parabrachial nucleus and have been postulated to play an important role in the ascending neurotransmission of gastrointestinal malaise (Lanca and van der Kooy, 1985; Miceli et al., 1987). Sleep disturbance is another frequent complication of SSRI medication (Settle, 1998; Yang et al., 2005), most likely as the result of drug-induced modifications of the dense serotonergic projections from the DRN and MRN to the SCN of the hypothalamus, locus of the mammalian circadian clock (Rusak and Zucker, 1979; Moore, 1983; Pickard and Rea, 1997; Dudley et al., 1999; Morin, 1999; Glass et al., 2000; Hay-Schmidt et al., 2003). For patients, one of the most bothersome SSRI-induced side effects is the high incidence of sexual dysfunction, characterized by disturbances in desire, arousal and orgasm (Clayton and Montejo, 2006). The pathobiology of drug-induced sexual dysfunction is poorly understood. Orgasm and ejaculation are autonomic functions that are partly regulated by dopamine so, given that serotonin is commonly believed to inhibit dopaminergic neurotransmission (Dray et al., 1976; Dray et al., 1978), SSRIs might augment this inhibition, negatively affecting sexual function (Zajecka, 2001).

Suffice it to say, side effects associated with SSRIs are common and can be persistent. Data on the incidence and duration of side effects associated with SSRI treatment for depression was collected by Hu and colleagues in 2004 and is presented below in tabular format (Figure 16).

Table 2. Incidence of All Side Effects and Bothersome Side Effects During the First 3 Months of SSRI Treatment for Depression (N = 401)[a]

	Frequency of Side Effects		Frequency of Bothersome Side Effects[b]	
Side Effect	N	%	N	%
Drowsiness	154	38.4	66	16.5
Sexual dysfunction	136	33.9	67	16.7
Dry mouth	136	33.9	26	6.5
Headache	94	23.4	40	10.0
Dizziness	92	22.9	43	10.7
Insomnia	90	22.4	45	11.2
Anxiety	77	19.2	44	11.0
Nausea	70	17.5	23	5.7
Weight gain	69	17.2	46	11.5
Tremors	62	15.5	19	4.7
Diarrhea	60	15.0	10	2.5
Constipation	50	12.5	19	4.7
Rash or itching	46	11.5	24	6.0
Weight loss	45	11.2	5	1.2
Stomach upset	44	11.0	13	3.2
Blurred vision	44	11.0	22	5.5
Swelling	22	5.5	6	1.5

[a]Only those side effects that patients attributed to the antidepressant initially prescribed are included.
[b]Defined as "a lot bothersome" or "extremely bothersome."
Abbreviation: SSRI = selective serotonin reuptake inhibitor.

Figure 16. Incidence of side effects associated with SSRIs prescribed for depression (Hu et al., 2004)

In addition to the treatment-related side effects, SSRI medications induce a clinically relevant discontinuation syndrome that may persist over time (Coupland et al., 1996; Lejoyeux and Ades, 1997; Zajecka et al., 1997; Black et al., 2000). Withdrawal symptoms include dizziness, light-headedness, vertigo, insomnia, fatigue, anxiety, agitation, nausea, headache, paresthesia, diarrhea, gait instability, tremor and sensory disturbances (Coupland et al., 1996; Zajecka et al., 1997; Black et al., 2000). Sexual dysfunction can continue long after the drug has been withdrawn (Csoka and Shipko, 2006). These clinical observations imply that chronic blockade of the SERT may induce changes in serotonergic function that persist after the blockade has been lifted.

Altered serotonergic modulation of dopamine signaling may underlie the extrapyramidal syndromes (EPS) such as akathisia, dystonia, dyskinesia and parkinsonism that are experienced by some patients taking SSRI medication (Leo, 1996; Caley, 1997; Gerber and Lynd, 1998; Mazurek and Rosebush, 2005). Though the precise pathophysiology of drug-induced EPS has not been elucidated, it is clear that drugs which alter dopaminergic signaling in the basal ganglia are associated with a high incidence of extrapyramidal disturbances. Agents which block the D2 dopamine receptor (i.e. the neuroleptics), for instance, have long been recognized to cause extrapyramidal side effects. At this point in time, the most plausible notion is that EPS reflects impaired dopaminergic transmission in the nigrostriatal dopamine pathway. Because SSRIs are associated with extrapyramidal reactions similar to those induced by neuroleptics, it is reasonable to postulate that SSRIs themselves might alter dopaminergic transmission in the basal ganglia.

2.7. The Neural Consequences of Exercise & Enriched Environments

The neural circuitry of the mammalian brain is remarkably plastic, demonstrating a now widely accepted capacity to be shaped and modified by a complex interplay of genetic and environmental influences. Formal environmental enrichment laboratory protocols were first introduced in the late 1960s by Rosenzweig and colleagues (1966; 1969) as a means to study the contributions of external stimuli and life experience to brain structure and function.

Environmental enrichment (EE) is classically defined as "a combination of complex inanimate and social stimulation" (Rosenzweig et al., 1978). Environmentally enriched animals are typically group-housed in a large 'play-pen' style cage that is equipped with a variety of toys, climbing structures and nesting materials designed to provide cognitive, sensory and motor stimulation (Figure 17). Running wheels allow animals to frequently engage in voluntary exercise and are a critical component of most enriched environment designs. This is in stark contrast to standard animal housing in laboratory settings, where animals are typically singly-housed in small cages with little to no opportunities for exploration or exercise. Enrichment objects in standard cages, if provided at all, are limited to one plastic tube.

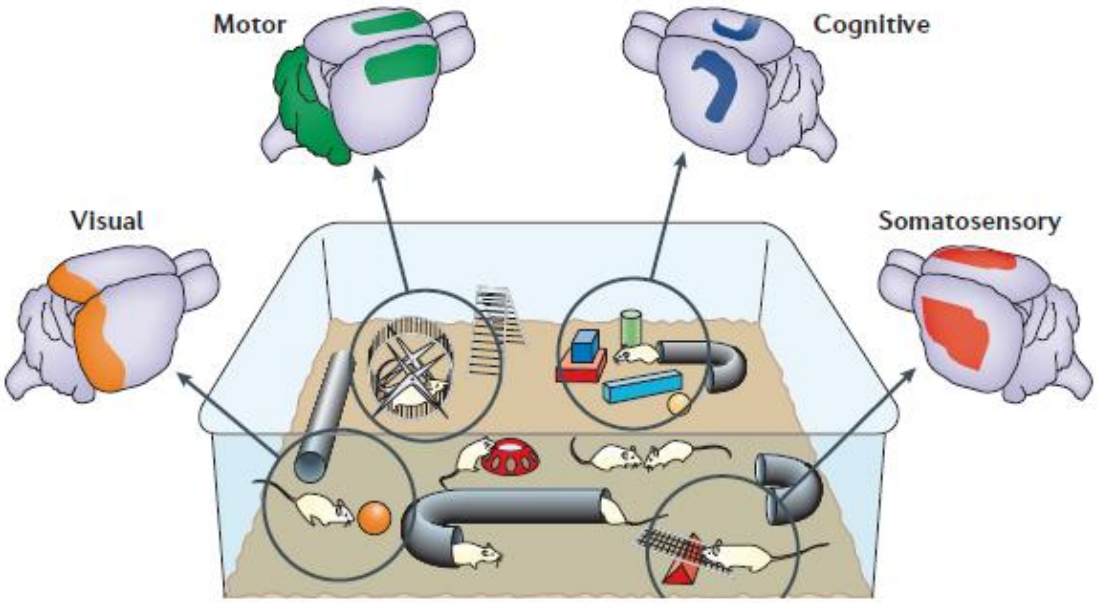

Figure 17. Enriched environments provide cognitive, sensory and motor stimulation (Nithianantharajah and Hannan, 2006)

The combination of physical exercise, exploration, cognitive stimulation and social interaction provided by an enriched environment has been reported to dramatically alter brain structure and function (van Praag et al., 2000). In general, no single variable has demonstrably accounted for the brain changes associated with environment enrichment and it seems likely that, instead, it is the complex interaction of enriching factors that has the greatest capacity to effect change. With that said, nearly all enriched environments provide animals with the opportunity to engage in voluntary exercise and there has been an increasing tendency in the literature to highlight physical exercise as a potentially crucial factor for the behavioural and structural consequences of an enriched environment.

Animals housed in an enriched environment demonstrate improved learning and memory (van Praag et al., 2000; Pacteau et al., 1989; Wainwright et al., 1993; van Praag et al., 1999) and show a lesser degree of cognitive decline with aging (Bennett et al., 2006). Reported brain changes in enriched animals include increased cortical thickness and weight (Beaulieu and Colonnier, 1987), dendritic arborisation (Greenough et al., 1973; Kozorovitskiy et al., 2005) and amplification of synaptic size and number (Turner and Greenough, 1985). The exciting reports that enrichment and exercise can promote cell survival and neurogenesis in the hippocampus have been of particular interest in recent years. Kemperman et al. first reported in 1997 that brains of mice exposed to an enriched environment had significantly more new neurons in the dentate gyrus than control mice housed in standard cages (Kempermann et al., 1997). Van Praag et al. (1999) followed with a related study, noting that running alone increased cell proliferation and neurogenesis in the adult mouse dentate gyrus. Van Praag, Kempermann and Gage (2000) have suggested that the mechanism by which enrichment and exercise generate new cells may differ since enrichment without running enhances cell survival but not proliferation whereas running increases cell division and neuronal survival. They postulated that the effects of running and enrichment might be additive, in that access to both an enriched environment and a running wheel could possibly enhance the number of new cells to a greater degree than either condition alone.

The potential benefits of an enriched environment and exercise for sufferers of central nervous system disorders have been well studied, with neuroprotective effects noted in animal models of Parkinson's disease (Bezard et al., 2003; Faherty et al., 2005),

depression (Chapillon et al., 1999; Larsson et al., 2002; Fox et al., 2006; Pollak et al., 2008), Huntington's disease (van Dellen et al., 2000; Spires et al., 2004), Alzheimer's disease (Jankowsky et al., 2003; Lazarov et al., 2005), epilepsy (Young et al., 1999), stroke (Ohlsson and Johansson, 1995), amyotrophic lateral sclerosis (Kirkinezos et al., 2003) and traumatic brain injury (Chen et al., 2005; Gaulke et al., 2005). The consequences of environmental enrichment and exercise for depression and Parkinson's disease are of particular relevance for this thesis, given their relationship to the serotonergic and dopaminergic systems, respectively. Environmental enrichment enhances stress resilience (Chapillon et al., 1999; Larsson et al., 2002; Pollak et al., 2008; Mohammed et al., 1993) and reduces depression-like behaviours (Fox et al., 2006) in animal models of depression. Running has a similar effect in these models, promoting antidepressant and anxiolytic effects (Dishman et al., 1997; Solberg et al., 1999; Moraska and Fleshner, 2001; Greenwood et al., 2003). In the nigrostriatal dopamine system, environmental enrichment attenuates the behavioural, structural and molecular consequences of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and 6-hydroxydopamine (6-OHDA), dopamine-targeting toxins used in animal models of Parkinson's disease. Specifically, environmental enrichment and exercise enables animals to move more easily and reduces the loss of TH-immunoreactivity in the substantia nigra and striatum (Bezard et al., 2003; Faherty et al., 2005; Tillerson et al., 2003; Steiner et al., 2006; Urakawa et al., 2007).

2.8. The Potential Role of Microglia-Mediated Inflammation

Microglia are the resident innate immune cells in the central nervous system. Derived from the monocyte lineage, these innate immune cells have a remarkable capacity to phenotypically change in response to small alterations in their microenvironment. In the absence of brain pathology, microglia are typically found in a resting state, morphologically characterized by a small cell body, ramified processes and low expression of surface antigens (Garden and Moller, 2006). Resting microglial function as microenvironment surveyors, on perpetual alert and continuously reorganizing their ramified process for the purposes of detecting signals indicative of nervous system pathology (Davalos et al., 2005; Fetler and Amigorena, 2005; Nimmerjahn et al., 2005). Recognition of aberrant signals and subsequent initiation of an immune response is mediated by a vary array of membrane and nuclear receptors (Hanisch, 2002; van Rossum and Hanisch, 2004).

Neuronal injury, ischemia and immunological stimuli can activate and trigger a rapid morphological change in resting microglia. Activated microglia, characterized by retracted processes and enlarged cell bodies can proliferate and migrate to the site of injury where they are able to phagocytose cellular debris. Alternatively, stimulus-mediated activation of microglia can provoke the release of a variety of noxious compounds such as cytokines or nitric oxide that are capable of directly inducing apoptosis (Benn and Woolf, 2004). While this process is often beneficial and indeed

necessary for the maintenance of normal brain function, microglial dysregulation or excess activation can have harmful and potentially neurotoxic consequences.

Demonstrations that the blockade of microglial activation is neuroprotective in the MPTP mouse model of Parkinson's disease (Du et al., 2001; Wu et al., 2002) suggests that microglia can play a significant role in mediating neurotoxic events. Minocycline, a tetracycline derivative known to inhibit microglia, has been shown to prevent the release of noxious apoptosis-inducing compounds from microglia and to ameliorate the MPTP-mediated damage to nigrostriatal dopamine neurons (Wu et al., 2002). In our own work with haloperidol (HAL), a neuroleptic medication that damages dopamine neurons in a manner similar to MPTP, we found that minocycline could prevent the toxic effects of HAL on nigrostriatal dopamine neurons (Bell et al., 2004). We later noted that HAL administration induced a rapid and significant increase in microglia presence. This upregulation of microglia occurred in advance of observable changes in the nearby dopamine neurons, suggesting that microglial activation and release of noxious compounds might be an instigating factor in neuroleptic-induced dopamine neurotoxicity.

In light of my recent finding that SSRI medications can significantly downregulate nigrostriatal dopamine neurons (see Chapter 3), I have devoted a portion of my thesis to the exploration of whether SSRIs might have an activating influence on microglia, similar to that of MPTP or haloperidol.

Chapter 3: Role of serotonin transporter inhibition in the regulation of tryptophan hydroxylase in brainstem raphe nuclei: time course and regional specificity

MacGillivray L[1,2], Lagrou LM[1,2], Reynolds, KB[1,2], Rosebush PI[2], Mazurek MF[1,2]

Departments of Medicine[1] (Neurology) and Psychiatry and Behavioural Neurosciences[2], McMaster University Medical Centre, Hamilton, Ontario, Canada

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Abstract

Drugs that selectively inhibit the serotonin transporter (SERT) are widely prescribed for treatment of depression and a range of anxiety disorders. We studied the time course of changes in tryptophan hydroxylase (TPH) in four raphe nuclei 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 hours, 7 days or 28 days of treatment. Sections of dorsal raphe nucleus (DRN), median raphe nucleus (MRN), raphe magnus nucleus (RMN) and caudal linear nucleus (CLN) were processed for TPH immunohistochemistry. Citalopram induced a significant reduction in DRN TPH-positive cell counts at 24 hours (41%), 7 days (38%) and 28 days (52%). Similar reductions in TPH-positive cell counts were also observed at each timepoint in the MRN and in the RMN. In the MRN, citalopram resulted in significant reductions at 24 hours (26%), 7 days (16%) and 28 days (23%). In the RMN, citalopram induced significant reductions of TPH-positive cell counts at 24 hours (45%), 7 days (34%) and 28 days (43%). By contrast, no significant differences between control and treatment groups were observed in the CLN at any of the time points that we studied. To investigate 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 significant reductions of TPH cell counts in the DRN (39%), MRN (38%) and RMN (41%), with no significant differences in the CLN. These results indicate that SERT inhibition can alter the regulation of TPH, the rate limiting

enzyme for serotonin biosynthesis. This persistent and regionally specific downregulation of serotonin biosynthesis may account for some of the clinical withdrawal symptoms associated with drugs that inhibit SERT.

Keywords:

selective serotonin reuptake inhibitor

serotonin transporter

dorsal raphe nucleus

median raphe nucleus

raphe magnus nucleus

caudal linear nucleus

The serotonin transporter (SERT) is a large neurotransmitter transporter that mediates the Na^+/Cl^-–dependent reuptake of extracellular serotonin (Schloss and Williams, 1998). The widespread use of selective serotonin reuptake inhibitors (SSRIs) for the treatment of depression and anxiety disorders (Fava et al., 1993; Boyer, 1995; Pigott and Seay, 1999; Zohar and Westenberg, 2000; Vaswani et al., 2003) has focused attention on the importance of SERT. To date, most of the literature has concentrated on the effects of SERT inhibition on postsynaptic targets that are 'downstream' of the serotonergic projection pathways. We were interested in the role of SERT blockade in regulating the serotonergic neurons themselves.

The original classification by Dahlstroem and Fuxe (1964) identified nine clusters of serotonin cell bodies in the brainstem, designated areas B1-B9. These areas roughly correspond to the familiar descriptive names for the raphe nuclei; B7, for example, is the equivalent of the dorsal raphe nucleus (DRN). Brainstem serotonin neurons vary considerably in their size and shape and differentially respond to physiological and pharmacological stimuli (Jacobs and Azmitia, 1992). Moreover, each raphe nucleus innervates a distinct set of projection areas (Imai et al., 1986; Mason, 2001; Hornung, 2003; Lechin et al., 2006). Given the morphological and functional differences among brainstem serotonin neurons and associated clusters, it is not clear whether SSRI antidepressants affect all raphe nuclei equally. In fact, the differential vulnerability of serotonergic nuclei to transporter-targeting compounds such as 3-4-methylenedioxymethamphetamine (MDMA) and p-chloroamphetamine (PCA) (Mamounas and Molliver, 1988; O'Hearn et al., 1988; Battaglia et al., 1991; Mamounas

et al., 1991) suggest that the influence of SSRIs on raphe nuclei might be regionally specific.

To investigate the role of SERT inhibition in the regulation of brainstem serotonin nuclei, we examined the effects of two different SSRI medications, citalopram and fluoxetine, on cells containing tryptophan hydroxylase (TPH), the rate-limiting enzyme in the biosynthesis of serotonin. To examine the regional specificity and timecourse of these effects, we studied TPH immunoreactivity in four functionally and anatomically distinct raphe nuclei: the DRN; median raphe nucleus (MRN); raphe magnus nucleus (RMN); and caudal linear nucleus (CLN), following one-day, one-week, or four-weeks of treatment with a SERT inhibitor.

Experimental Procedures

Subjects

Fifty-four male Sprague-Dawley rats (Charles River, PQ) weighing 250-275g were singly housed with free access to food and water on a 12h:12h light dark cycle throughout the experiments. 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:

For 7 days prior to initiation of the experimental protocol, all rats received a daily 20 g meal of rice pudding. The animals were monitored over the course of the week to ensure that they consumed the pudding in a timely fashion and hence that the drug would reliably be delivered. The animals were randomly assigned to one of four treatment groups: (1) control; (2) 24 hour citalopram; (3) 7 day citalopram; or (4) 28 day citalopram. Groups of rats 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; Fredricson Overo, 1982b; Hyttel et al., 1984). A mortar and pestle was used to grind the citalopram tablets to a powder. The powder was weighed and the appropriate dose mixed with rice pudding.

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 (20µm) of DRN, MRN, RMN and CLN were processed for TPH immunohistochemistry. All other experimental procedures were consistent with those described for experiment 1.

Immunohistochemistry

Citalopram-treated animals were sacrificed by decapitation after 24 hours, 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 hours 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 (one per region for each animal) of CLN (bregma -6.3 mm), DRN (bregma -7.8 mm), MRN (bregma -7.8 mm) and RMN (bregma -10.3 mm) were processed for TPH immunohistochemistry. Free-floating sections were incubated in 0.3% hydrogen peroxide in methanol for 30 minutes at room temperature, rinsed three times in

0.1 M PBS and incubated in 5% normal rabbit serum for 1 hour. Sections were again rinsed three times in 0.1 M PBS and then incubated in sheep anti-TPH primary antibody (Millipore, CA, USA, 1:500) for 24 hours at 4°C. After three additional washes in 0.1 M PBS, sections were incubated in biotinylated anti-sheep IgG secondary antibody (Millipore, CA, USA) for 1 hour at room temperature, washed three more times in 0.1 M PBS, and then incubated in ABC solution (Vectastain ABC Systems, Vector Labs, ON, CA) for 1 hour at room temperature to form an avidin-biotin complex. After three final washes in 0.1 M PBS, the TPH immunocomplex was visualized with the chromagen diaminobenzidine tetrahydrochloride (DAB, Vector Labs, ON, CA). Sections were mounted on Aptex-coated slides (Sigma, St. Louis, MO, USA), air dried, and coverslipped with DPX neutral mounting medium (Sigma, St. Louis, MO, USA).

Quantitative Morphometry

The selected coronal brain sections permitted evaluation of raphe regions known to be heavily populated with serotonergic neurons. Anatomical landmarks were used to ensure that comparable brain sections were analyzed for each region in each animal. To minimize error, all sections from a particular brain region were cut and stained at the same time. DAB-stained raphe sections were mounted on microscope slides. The slides were coded and photographed at 5X on a confocal microscope (CarlZeiss LSM 510). The TPH-immunoreactive cells in the CLN, DRN, MRN and RMN were manually counted at 20X 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 were established for

each nucleus of interest and every labeled cell within those boundaries was counted. TPH-positive neurons on both sides of the midline were included in the total count. After completion of manual counting, all images were decoded and arranged according to treatment group. The mean number of DAB-stained TPH-positive cells in each of four brainstem raphe nuclei (CLN, DRN, MRN and RMN) was determined for each group and a standard error of the mean was calculated. For each raphe section, average cell diameter was determined for TPH-positive cell somata using 10 randomly selected immunoreactive cell bodies per animal. All raw cell counts were adjusted with a correction formula for cell size and section thickness according to the method of Abercrombie (1946).

Statistical Analysis

Cell count and cell size data were analyzed by one-way analysis of variance (ANOVA) followed by *post-hoc* Tukey tests where appropriate. Statistical outliers (~5% of sections) were identified by the boxplot method (Tukey, 1977) and were not included in analysis. Statistical significance was defined as $p < 0.05$.

Results

Experiment 1:

Citalopram treatment resulted in reductions of TPH-positive cells in three of the raphe nuclei that we studied: the DRN ($F_{3,31}$ = 11.322, p <0.001), MRN ($F_{3,31}$ = 5.805, p=0.003) and RMN ($F_{3,27}$ = 6.289, p = 0.002). In contrast to the reduction of TPH immunoreactivity observed in the DRN, MRN and RMN, the CLN cell counts were unaffected by citalopram treatment ($F_{3,32}$ = 0.379, p = 0.769). This regionally specific reduction of TPH-positive cells in the DRN, MRN and RMN occurred within 24 hours of treatment initiation. Average cell counts in all three nuclei remained reduced after 7 and 28 days of daily drug administration. Cell sizes were not significantly different among any of the groups (Table 1).

Dorsal Raphe Nucleus

Citalopram induced a significant 41% reduction (p=0.001) in the number of TPH-immunoreactive cells in the DRN at 24 hours (figure 1A). This reduction persisted at 7 days (38%, p=0.003) and 28 days (52%, p< 0.001). There was no significant difference in the number of TPH-positive cells among the 24 hour, 7 day and 28 day treatment groups. Representative photomicrographs are shown in figures 1B and 1C.

Median Raphe Nucleus

Citalopram resulted in significant reductions in the number of TPH-positive cells in the MRN at 24 hours (26%, p=0.004), 7 days (16%, p=0.034) and 28 days (23%, p=0.012) (figure 2A). There was no significant difference in the number of TPH-stained

cells among the 24 hour, 7 day and 28 day treatment groups. Representative photomicrographs are shown in figures 2B and 2C.

Raphe Magnus Nucleus

The citalopram-treated group had significant reductions in the number of TPH-positive cells in the RMN at 24 hours (45%, p=0.009), 7 days (34%, p=0.009) and 28 days (43%, p=0.006) (figure 3A). There was no significant difference in the number of TPH-stained cells among the 24 hour, 7 day and 28 day treatment groups. Representative photomicrographs are shown in figures 3B and 3C.

Caudal Linear Nucleus

No significant differences in TPH-positive cells counts were observed in the CLN between rats treated with citalopram and control rats at any of the time points that we studied (figure 4A). Representative photomicrographs are shown in figures 4B and 4C.

Experiment 2:

Fluoxetine induced changes comparable to those observed with citalopram, with significant reductions of TPH in the DRN (39%, p=0.003), MRN (38%, p<0.001) and RMN (41%, p=0.036) (figures 5a-c), with no significant differences in the CLN (figure 5d). Representative photomicrographs are shown in Figure 6.

Discussion

These results indicate that inhibition of SERT can induce a regionally specific reduction of TPH-positive cell counts in brainstem serotonergic nuclei within 24 hours 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 in the number of TPH-immunoreactive neurons in the DRN, MRN and RMN, with no effect on TPH cell counts in the CLN. These reductions ranged from 16% to 26% in the MRN, from 38% to 52% in the DRN and from 35% to 45% in the RMN. Similar observations were noted with a 28 day course of fluoxetine, indicating that the regionally specific reduction of TPH immunoreactivity may be an effect common to all agents that inhibit SERT.

This study is the first to demonstrate a rapid and selective reduction of TPH-immunoreactive neurons in serotonergic nuclei following SSRI administration. Several previous studies have examined the impact of SSRI medications on TPH markers, with conflicting results. Lapierre et al. (1983) found that 25 mg/kg/day of the SSRI fluvoxamine injected (i.p.) into rats once daily for 7 days led to a significant 32% decrease in midbrain homogenate TPH compared to controls. Maciag et al. (2006) observed similar changes, noting a persistent decrease of TPH-positive cells in both the dorsal and median raphe in rats that had been exposed to 10 mg/kg/day citalopram (s.c.) during postnatal days 8 to 21. Abumaria et al. (2007) and Dygalo et al. (2006) both found significantly reduced TPH mRNA expression in the DRN after 4 weeks of citalopram or 2 week fluoxetine treatments, respectively. Our finding that two different SERT

inhibitors decreased TPH-positive cell counts in raphe nuclei is consistent with the observations of these previous studies. Kim et al. (2002), on the other hand, reported apparent upregulation of TPH in the DRN after treating rats with sertraline for 14 days. At present, there is no clear way to reconcile the apparent discrepancies between the groups noting reduced markers of TPH expression after SSRI administration (Lapierre et al., 1983; Dygalo et al., 2006; Maciag et al., 2006; Abumaria et al., 2007) and those who found the opposite (Kim et al., 2002).

The mechanism by which SERT inhibition leads to decreased TPH-immunoreactive cell counts is not entirely clear. Blockade of the transporter will, at least in the short run, lead to increased bioavailability of serotonin in the synapse (Wong et al., 2005), and augmented stimulation of the 5HT1a autoreceptor. The persistence of the TPH effect would suggest that the 5HT1a receptor, despite literature suggesting the contrary (Chaput et al., 1986), may not desensitize after 28 days of apparent hyperstimulation by the elevated concentrations of synaptic serotonin. Alternatively, the SSRI-type medications might themselves be having some direct effect on intracellular processes, such as altering levels of BH4 (Miura et al., 2004; 2005; 2007).

Regional Specificity

The rapid and persistent reduction of TPH-immunoreactivity that we observed in this study was regionally specific. Both citalopram and fluoxetine led to a reduced number of TPH-positive cells in the DRN, MRN and RMN, while the number of TPH-positive cells in the CLN was not affected by either agent. 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 TPH changes may be more robust in some subparts of the nuclei than others.

The anatomical characteristics of the DRN and MRN, which account for the majority of serotonergic CNS innervation, have been extensively studied (Jacobs and Azmitia, 1992; Hajos et al., 1995). With approximately 11, 500 serotonin neurons, (Wiklund et al., 1981) the rat DRN is the largest serotonergic nucleus and has widespread terminal projection sites in the forebrain and the brainstem (Vertes, 1991; Vertes and Kocsis, 1994; Lechin et al., 2006). The rat MRN is smaller, containing roughly 1100 serotonin neurons (Wiklund et al., 1981) and, likewise, projects to a broad range of sites in the forebrain and brainstem, though the targets of the MRN pathways tend to be non-overlapping with those of the DRN (Vertes, 1991; Pickard and Rea, 1997; Dudley et al., 1999; Vertes et al., 1999; Lechin et al., 2006). The CLN is the most rostral of the raphe nuclei, located in the ventral tegmental area just dorsal to the interpeduncular nucleus (Tork, 1990; Hornung, 2003). Less is known about the CLN than either the DRN or MRN, but this nucleus is thought to share most efferent pathways of the DRN (Imai et al., 1986). This would mean that properties of the target regions cannot explain the differential responses of the DRN and CLN to SERT inhibition. The RMN, unlike the other nuclei that we studied, has projections to the spinal cord and is well known for its involvement in descending pain inhibition (Mason, 2001).

The localization and regional density of SERT in the central nervous system have been studied using a range of radioligand and immunohistochemical techniques (Hrdina et al., 1985; Marcusson et al., 1988; Biegon and Mathis, 1993; Fujita et al., 1993; Austin

et al., 1994). SERT concentrations appear to be highest in the DRN, with lower concentrations in other raphe nuclei (Hrdina et al., 1990; Fujita et al., 1993; Hoffman et al., 1998; Rattray et al., 1999; Clark et al., 2006). The density of 5HT1a autoreceptor binding is likewise higher in the DRN than in other serotonergic structures (Pazos and Palacios, 1985; Weissmann-Nanopoulos et al., 1985). The regional variability in the effects of SERT inhibition on TPH-immunopositive cell counts observed in our study may reflect differential concentrations of SERT and the 5HT1a autoreceptor among the various raphe nuclei (Hrdina et al., 1990).

Timecourse & Functional Implications

The rapid and persistent reduction of TPH immunoreactivity following the inhibition of SERT indicates that production of serotonin itself may be reduced in those taking SSRI-type medications. The persistently reduced biosynthesis of serotonin could lead to a dramatic reduction of serotonergic transmission if the SERT inhibitor were to be suddenly discontinued. This might account for the clinical withdrawal syndromes that have been described in patients who stop taking SSRI medications (Coupland et al., 1996; Lejoyeux and Ades, 1997; Zajecka et al., 1997; Black et al., 2000). The persistence of some withdrawal syndromes (Gerber and Lynd, 1998; Csoka and Shipko, 2006) suggests that the downregulation of TPH may continue after SERT blockade has been lifted, perhaps reflecting cell toxicity. We are conducting studies to investigate these issues.

Conclusions

The regionally specific reduction of TPH immunoreactivity by SERT antagonists citalopram and fluoxetine suggests that SERT is important for regulation of TPH, the rate-limiting enzyme for serotonin biosynthesis, in some, but not all, serotonergic nuclei. The effect of SERT inhibition on TPH occurs within 24 hours and does not habituate after 28 days. These results indicate that SERT inhibition can alter the regulation of TPH, the rate limiting enzyme for serotonin biosynthesis. This persistent and regionally specific downregulation of serotonin biosynthesis may account for some of the clinical withdrawal symptoms associated with drugs that inhibit SERT.

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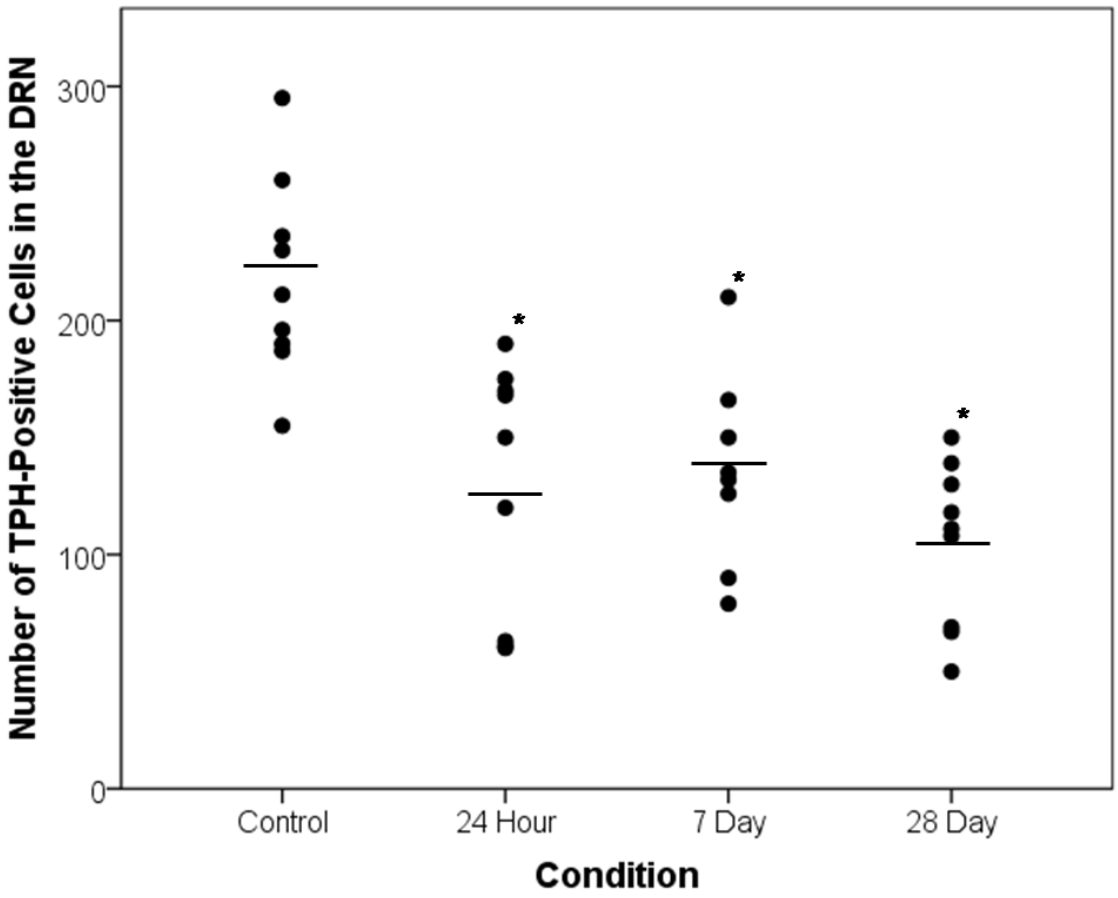

Figure 1A. Number of TPH-positive cells per 20 µm section of rat DRN. Each dot represents the DRN TPH-positive cell count for one animal; the horizontal line represents the group mean. The 24 hour, 7 day and 28 day labels represent groups of animals that were treated with citalopram. Note: '*' indicates statistical significance.

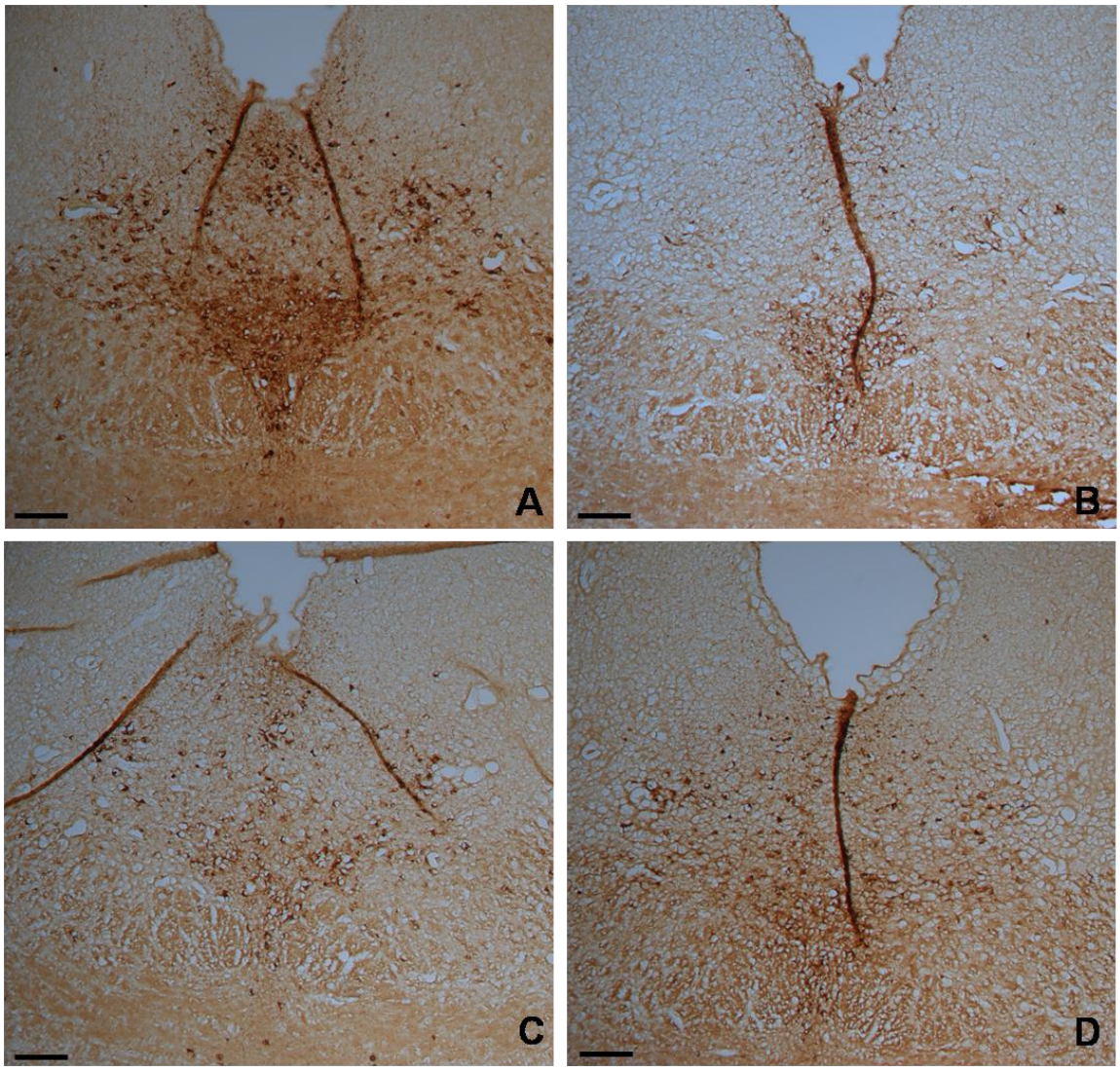

Figure 1B. Photomicrographs (5x magnification) of TPH-immunoreactive cells in 20 µm sections of rat DRN (bregma – 7.8mm). A, control; B, 24 hour citalopram; C, 7 day citalopram; D, 28 day citalopram. Scale bar 200 µm.

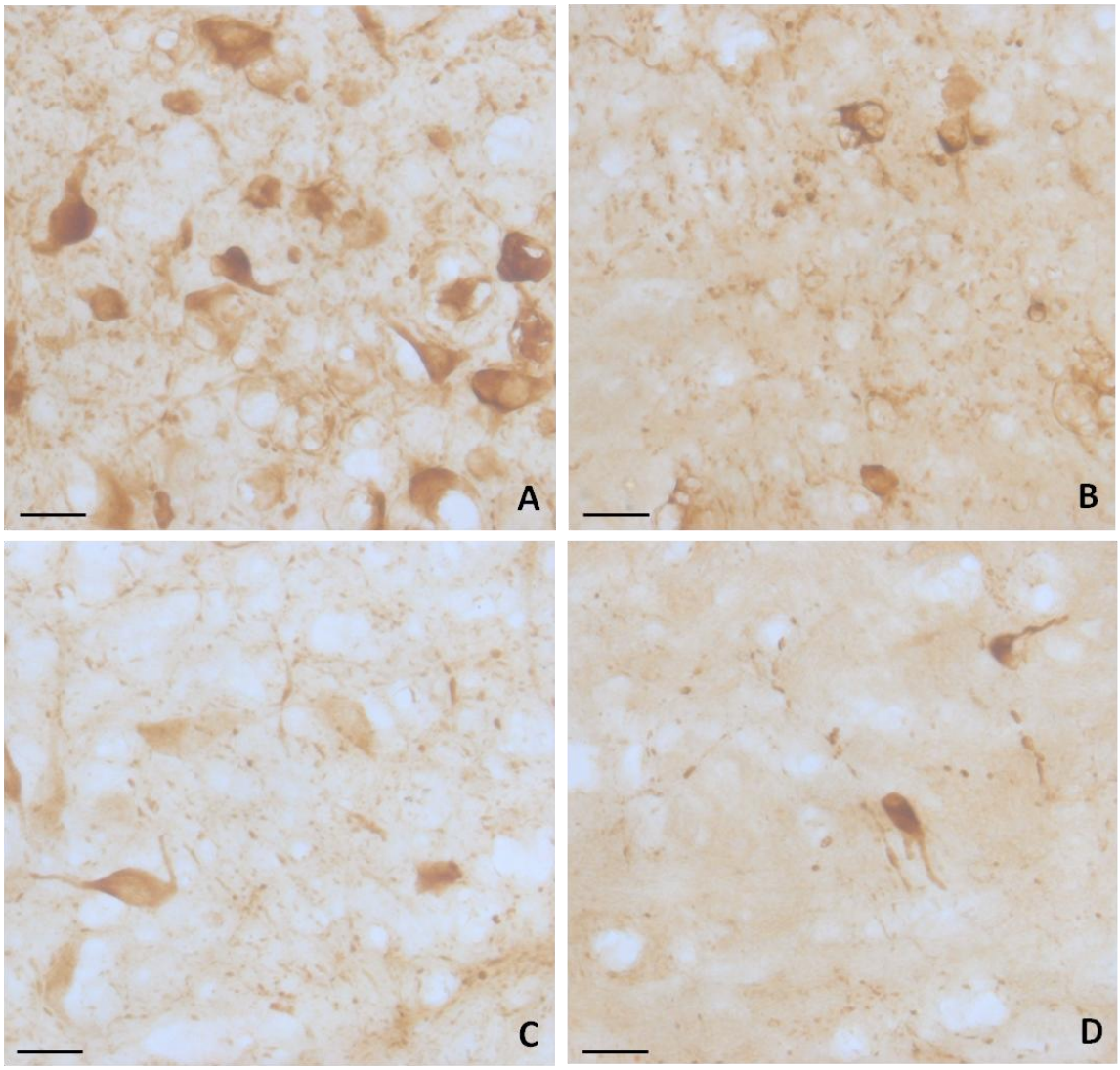

Figure 1C. Photomicrographs (63x magnification) of TPH-immunoreactive cells in 20 µm sections of rat DRN (bregma – 7.8mm). A, control; B, 24 hour citalopram; C, 7 day citalopram; D, 28 day citalopram. Scale bar 25 µm.

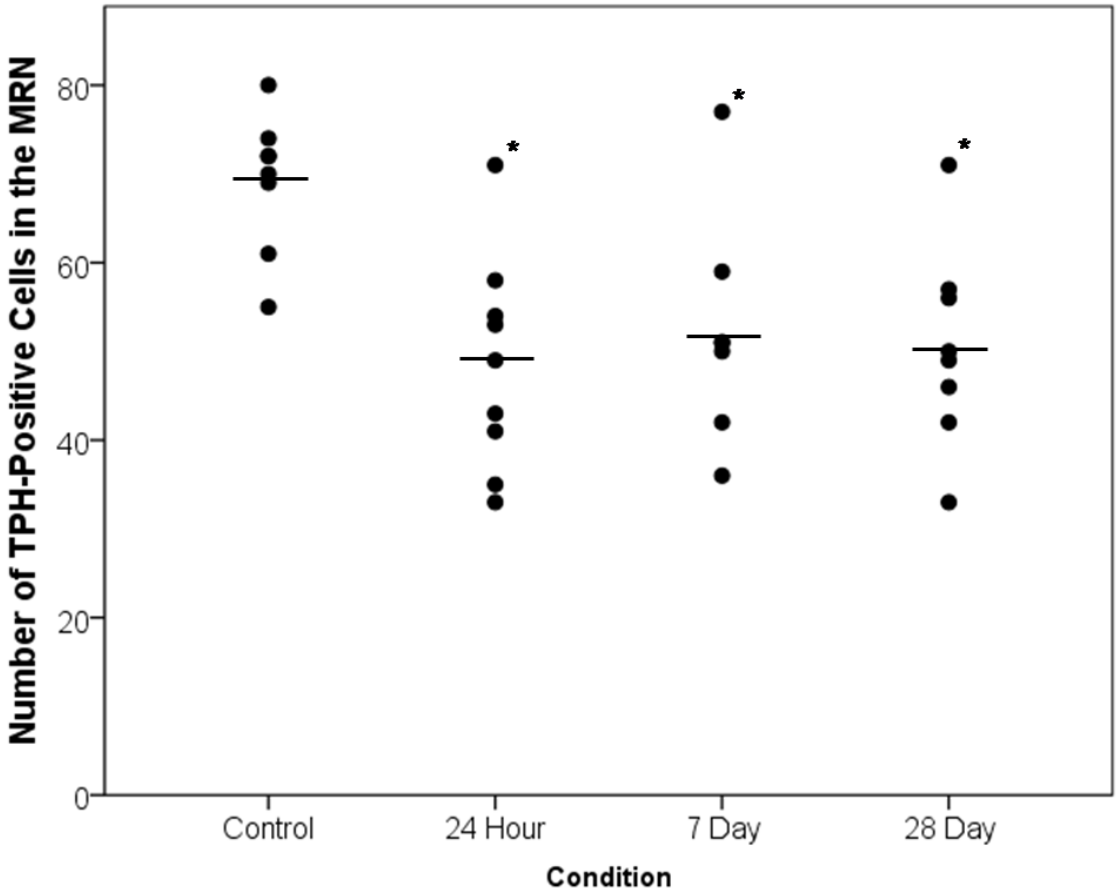

Figure 2A. Number of TPH-positive cells per 20 µm section of rat MRN. Each dot represents the MRN TPH-positive cell count for one animal; the horizontal line represents the group mean. The 24 hour, 7 day and 28 day labels represent groups of animals that were treated with citalopram. Note: '*' indicates statistical significance.

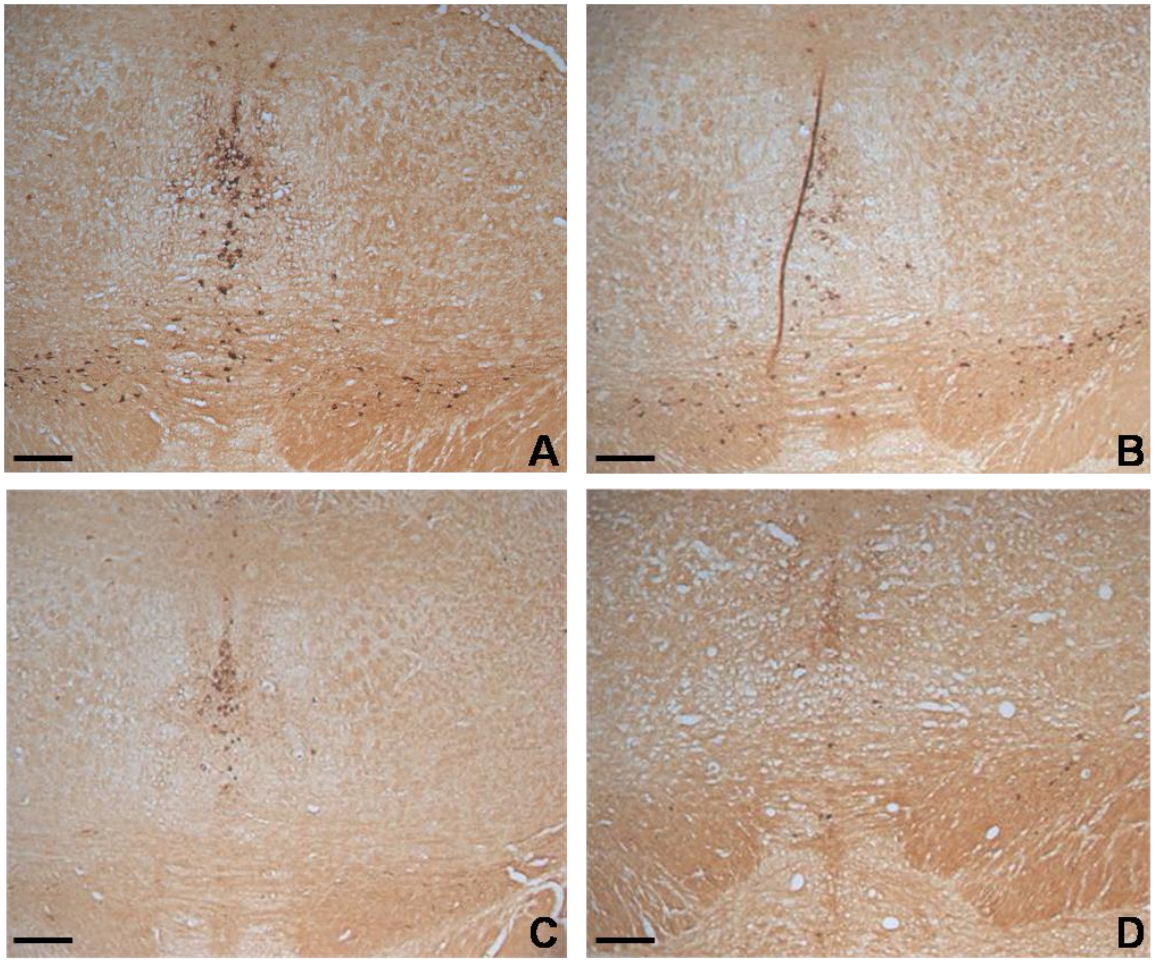

Figure 2B. Photomicrographs (5x magnification) of TPH-immunoreactive cells in 20 µm sections of rat MRN (bregma – 7.8mm). A, control; B, 24 hour citalopram; C, 7 day citalopram; D, 28 day citalopram. Scale bar 200 µm.

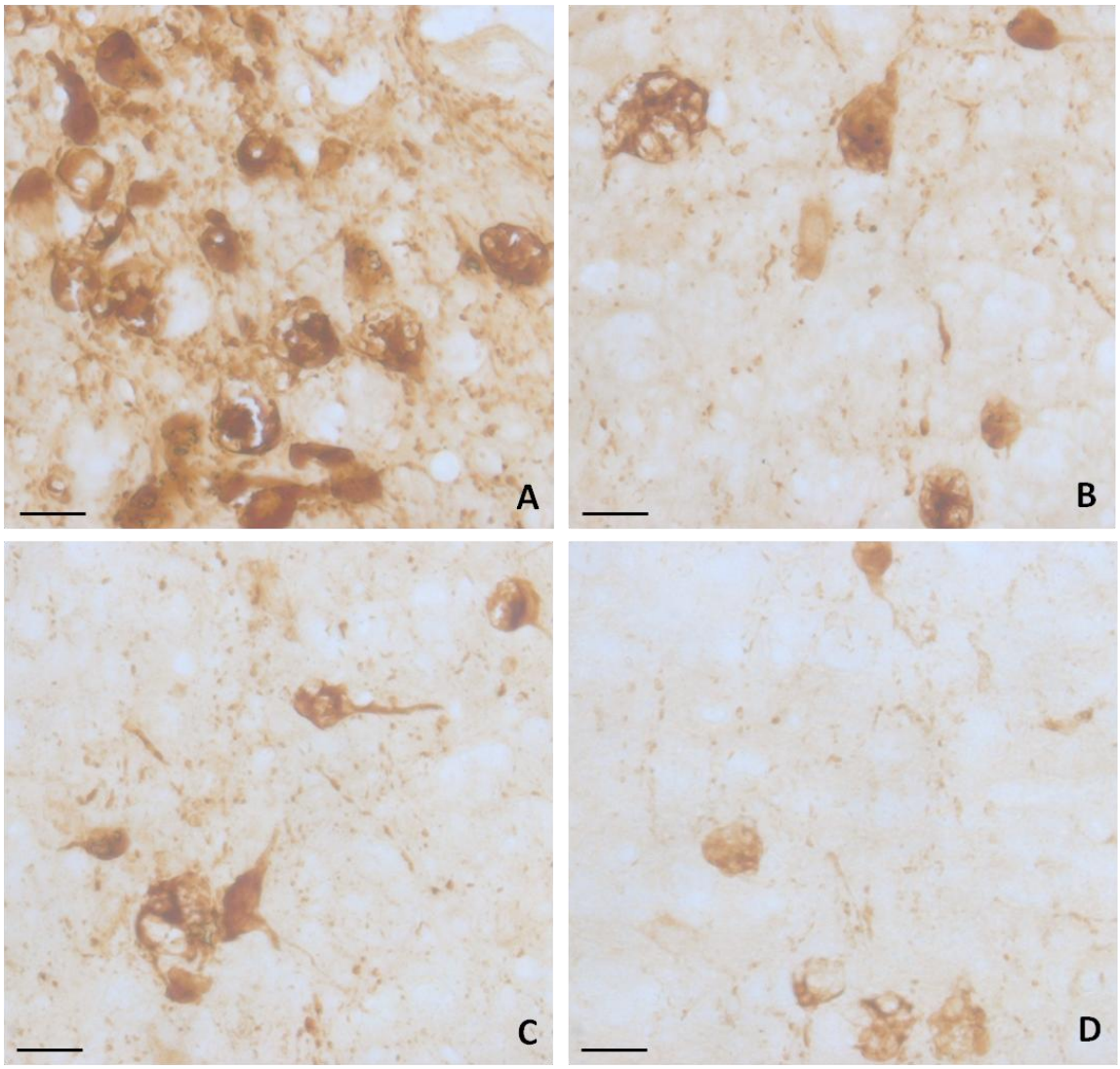

Figure 2C. Photomicrographs (63x magnification) of TPH-immunoreactive cells in 20 µm sections of rat MRN (bregma – 7.8mm). A, control; B, 24 hour citalopram; C, 7 day citalopram; D, 28 day citalopram. Scale bar 25 µm.

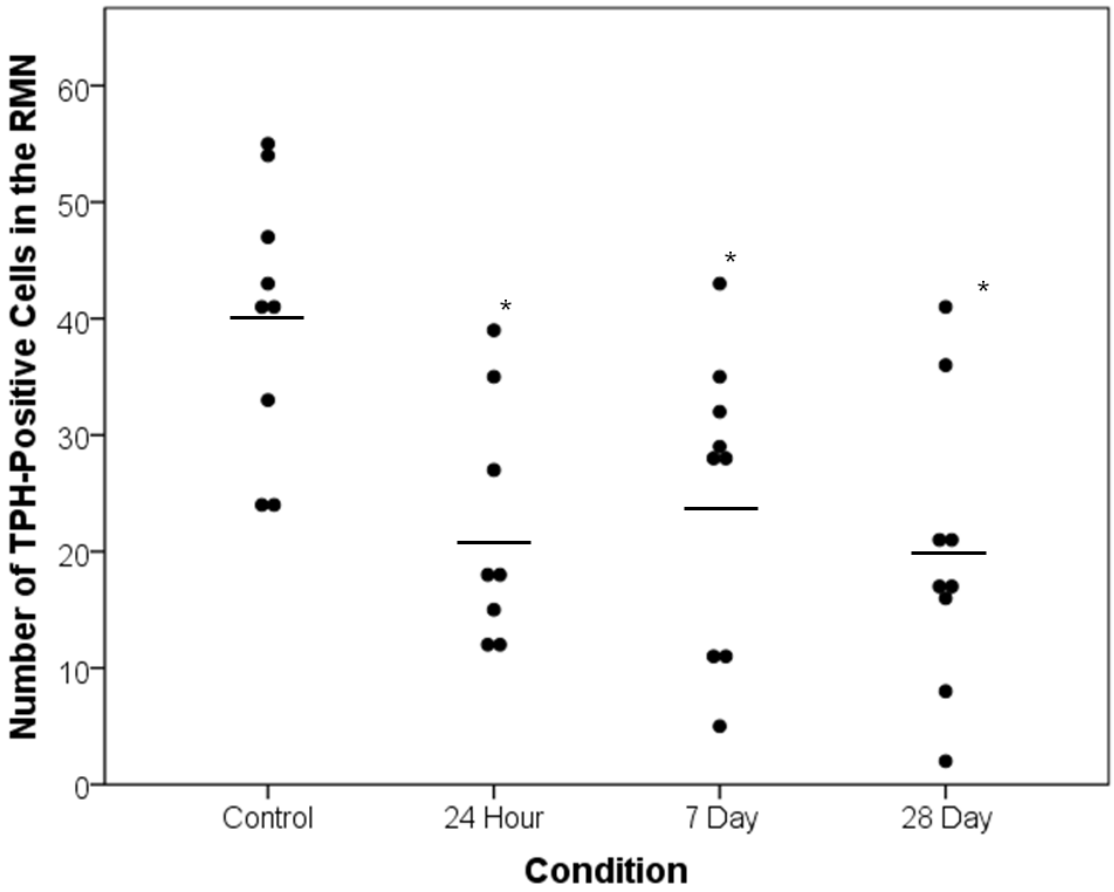

Figure 3A. Number of TPH-positive cells per 20 µm section of rat RMN. Each dot represents the RMN TPH-positive cell count for one animal; the horizontal line represents the group mean. The 24 hour, 7 day and 28 day labels represent groups of animals that were treated with citalopram. Note: '*' indicates statistical significance.

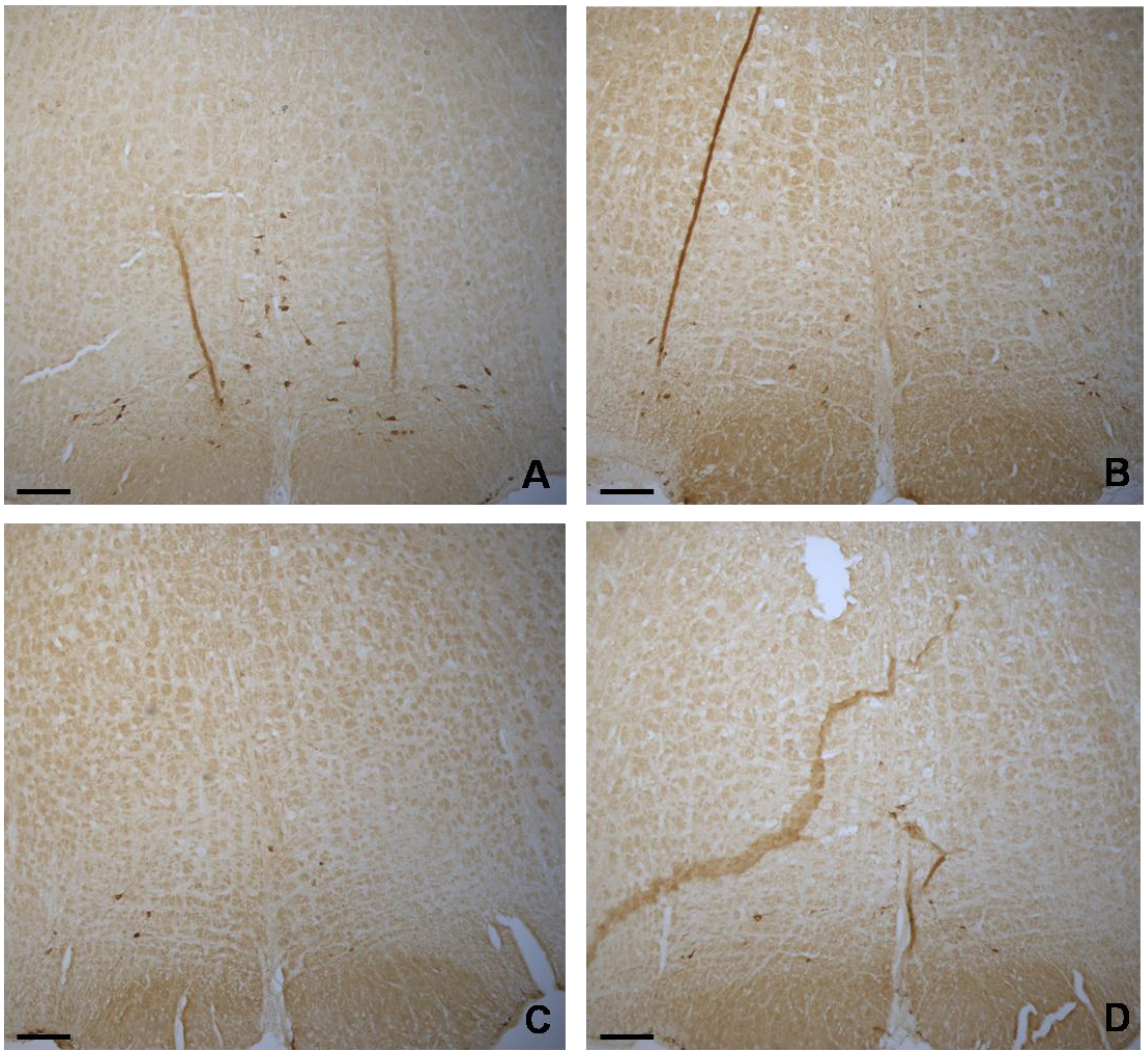

Figure 3B. Photomicrographs (5x magnification) of TPH-immunoreactive cells in 20 µm sections of rat RMN (bregma – 9.8mm). A, control; B, 24 hour citalopram; C, 7 day citalopram; D, 28 day citalopram. Scale bar 200 µm.

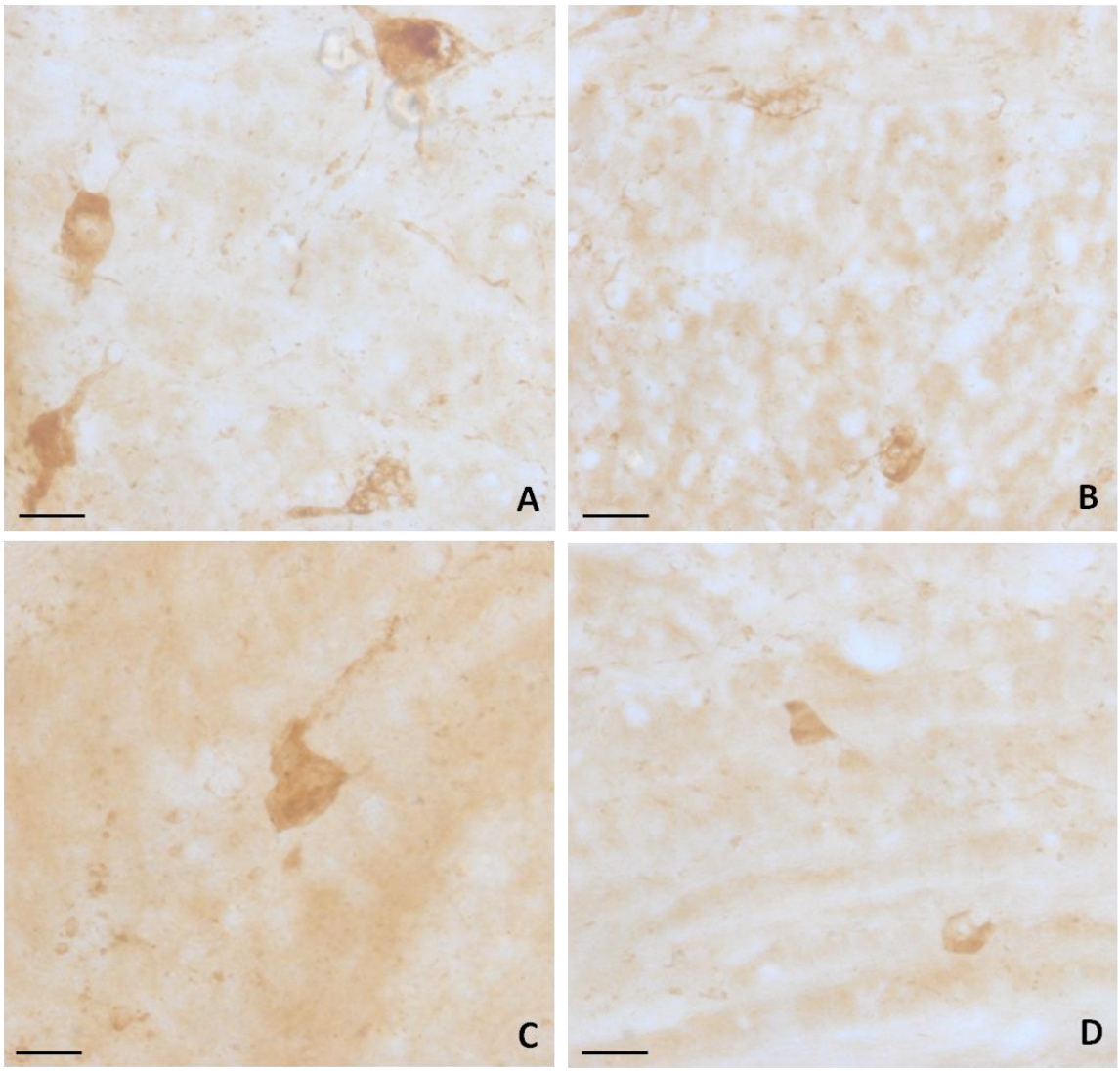

Figure 3C. Photomicrographs (63x magnification) of TPH-immunoreactive cells in 20 µm sections of rat RMN (bregma – 9.8mm). A, control; B, 24 hour citalopram; C, 7 day citalopram; D, 28 day citalopram. Scale bar 25 µm.

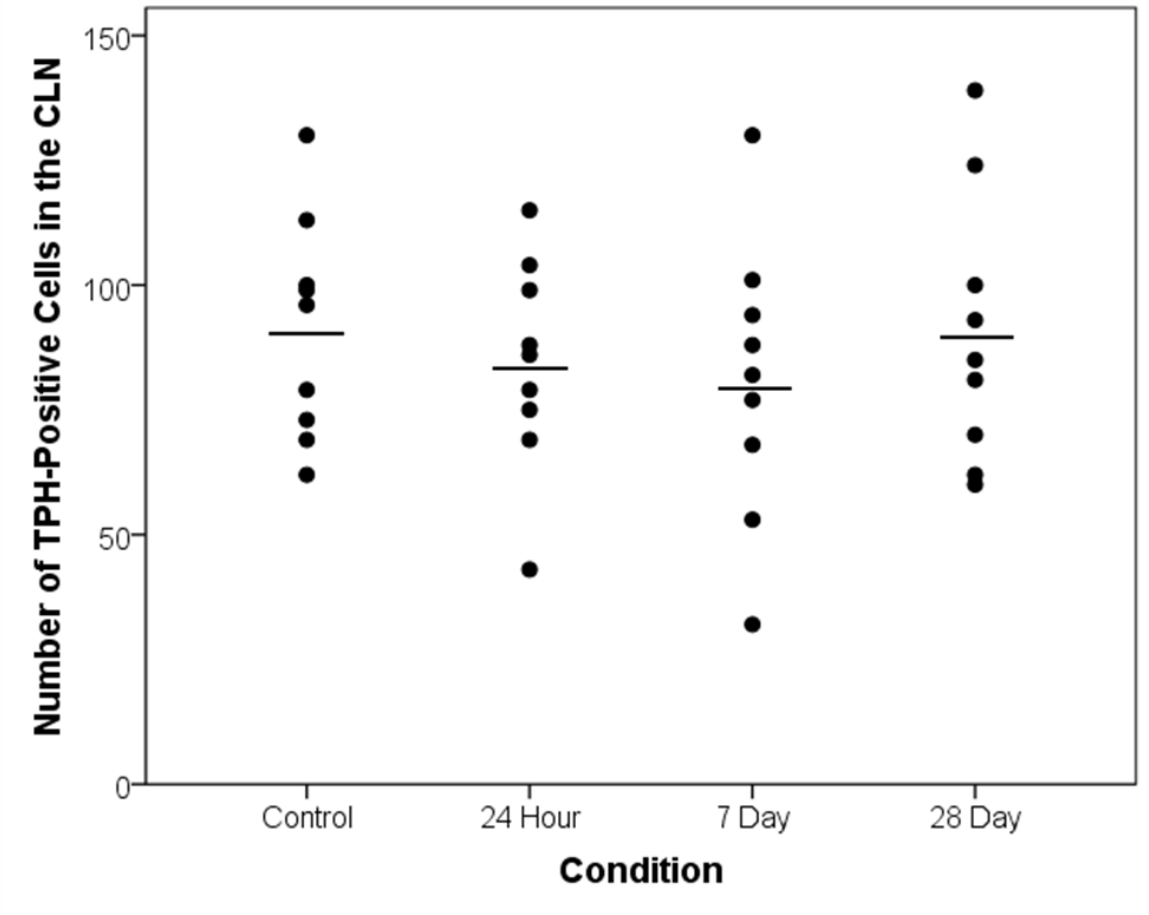

Figure 4A. Number of TPH-positive cells per 20 µm section of rat CLN. Each dot represents the CLN TPH-positive cell count for one animal; the horizontal line represents the group mean. The 24 hour, 7 day and 28 day labels represent groups of animals that were treated with citalopram.

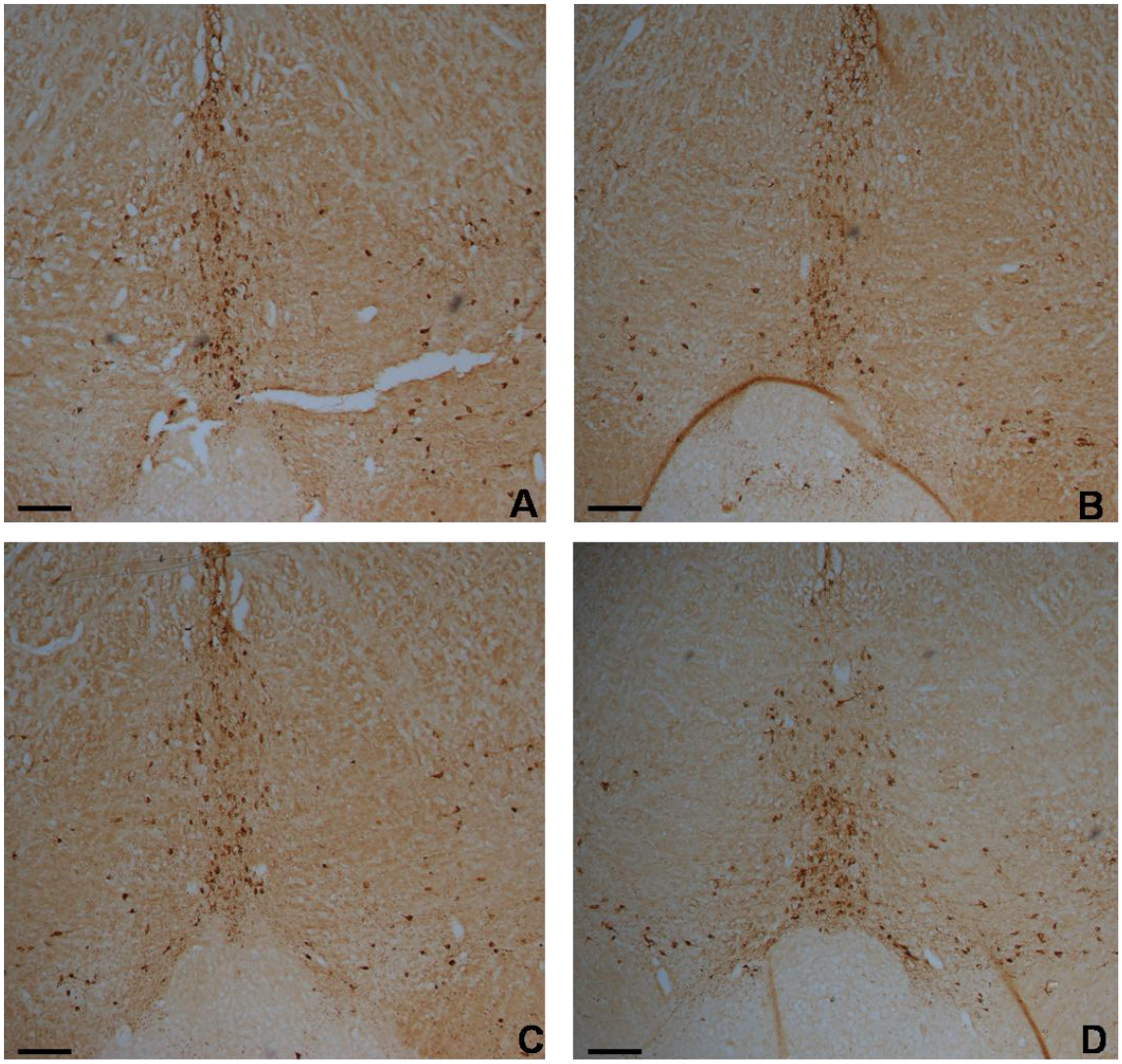

Figure 4B. Photomicrographs (5x magnification) of TPH-immunoreactive cells in 20 µm sections of rat CLN (bregma – 6.3mm). A, control; B, 24 hour citalopram; C, 7 day citalopram; D, 28 day citalopram. Scale bar 200 µm.

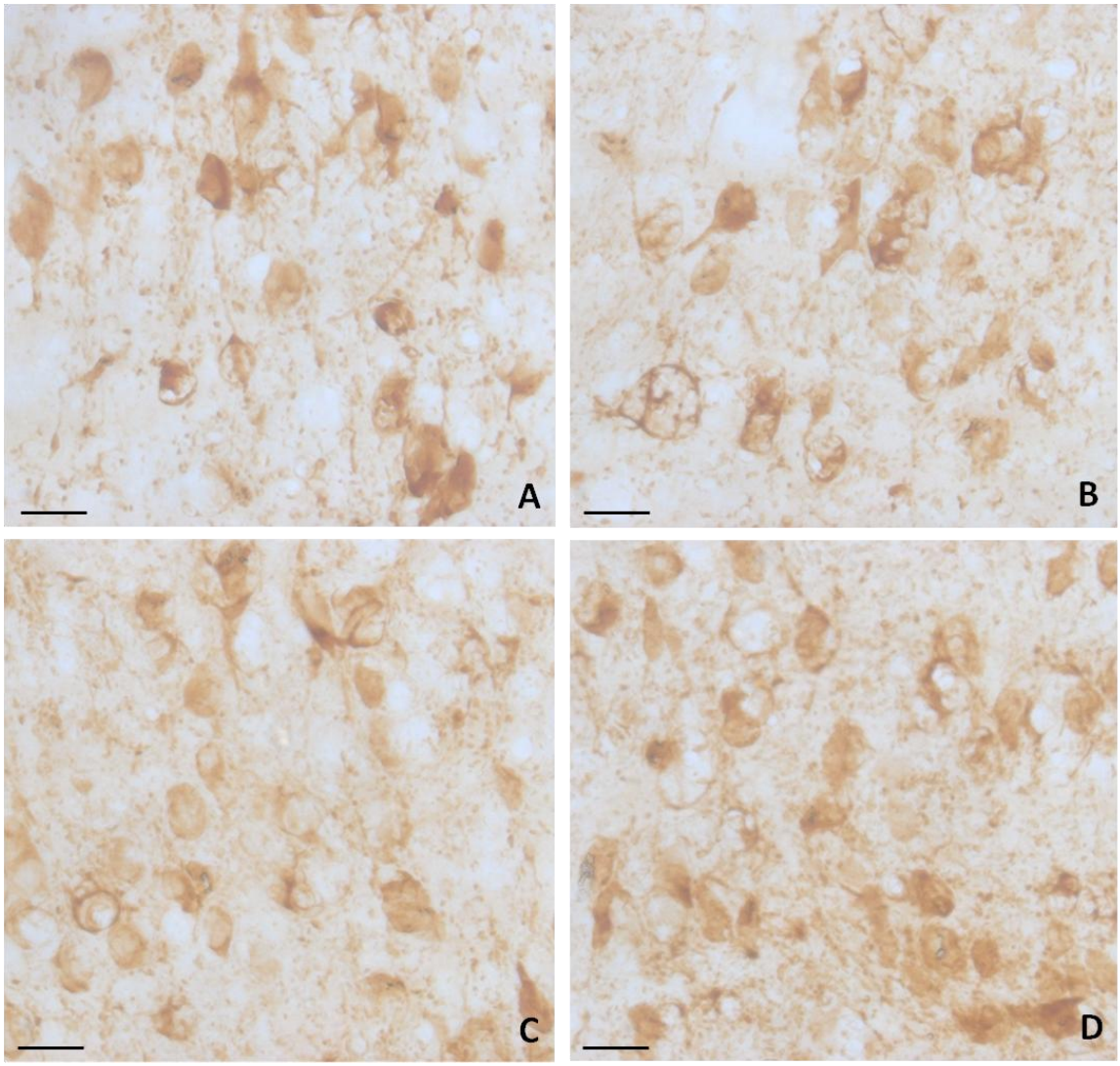

Figure 4C. Photomicrographs (63x magnification) of TPH-immunoreactive cells in 20 µm sections of rat CLN (bregma – 6.3mm). A, control; B, 24 hour citalopram; C, 7 day citalopram; D, 28 day citalopram. Scale bar 25 µm.

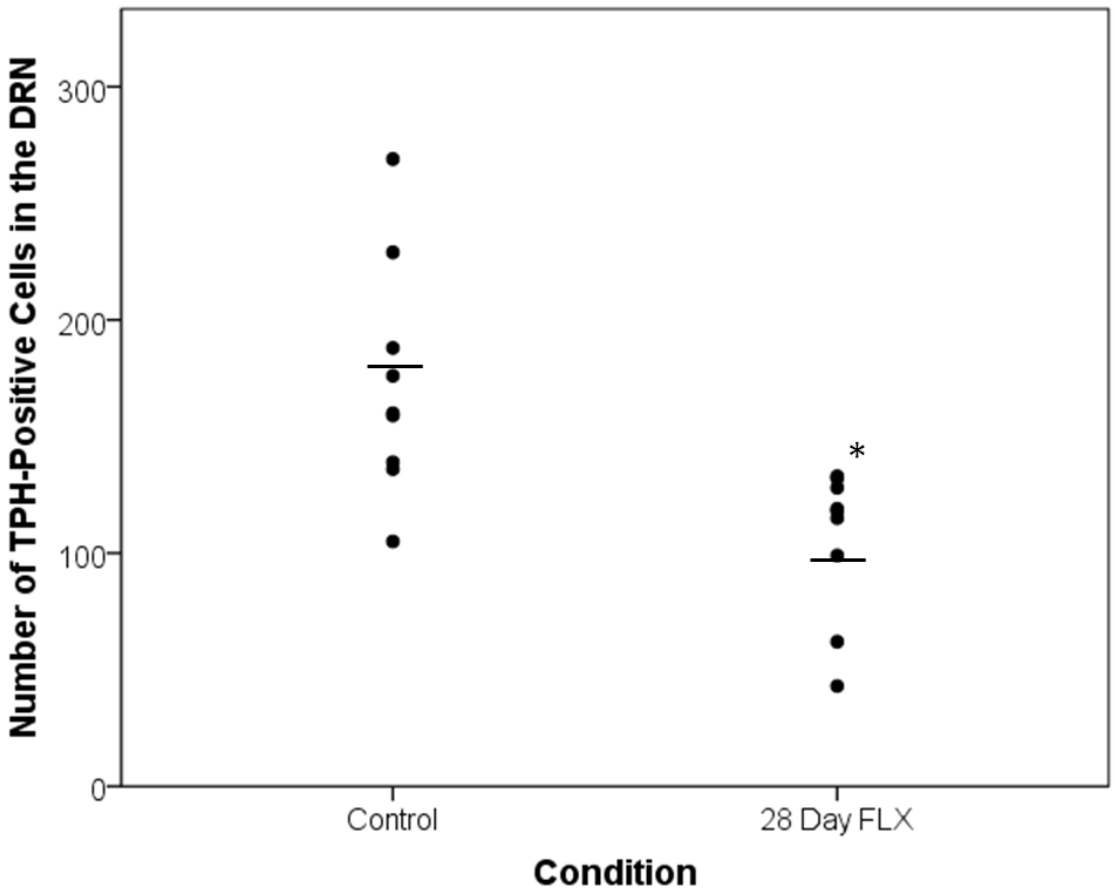

Figure 5A. Number of TPH-positive cells per 20 µm section of rat DRN. Each dot represents the DRN TPH-positive cell count for one animal; the horizontal line represents the group mean. Note: '*' indicates statistical significance. FLX = fluoxetine.

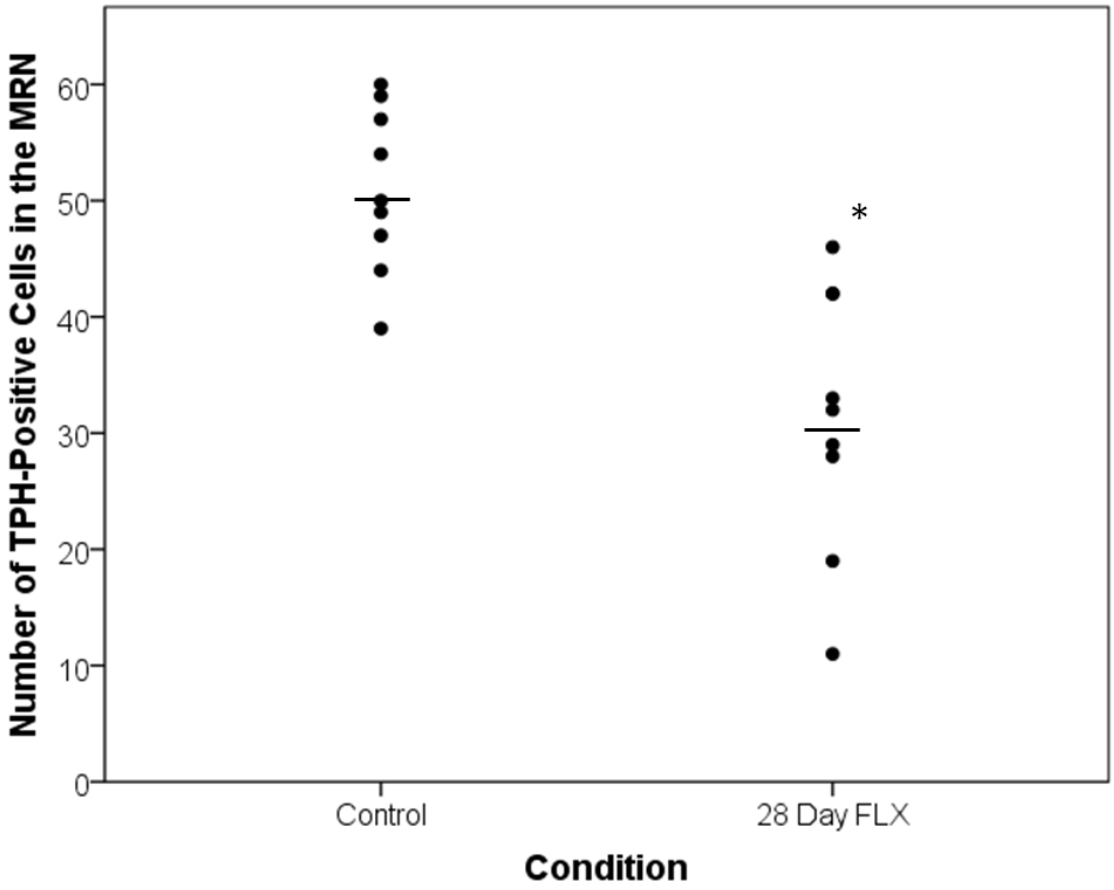

Figure 5B. Number of TPH-positive cells per 20 µm section of rat MRN. Each dot represents the MRN TPH-positive cell count for one animal; the horizontal line represents the group mean. Note: '*' indicates statistical significance. FLX = fluoxetine.

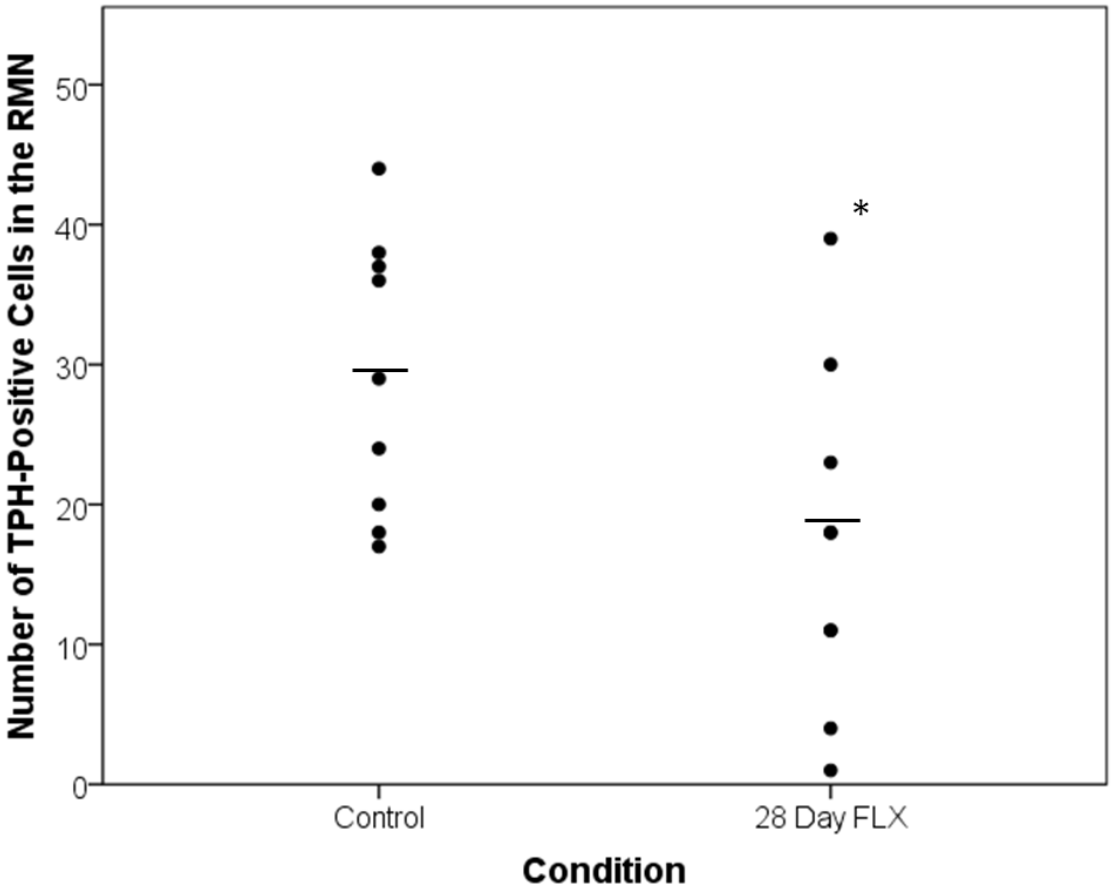

Figure 5C. Number of TPH-positive cells per 20 µm section of rat RMN. Each dot represents the RMN TPH-positive cell count for one animal; the horizontal line represents the group mean. Note: '*' indicates statistical significance. FLX = fluoxetine.

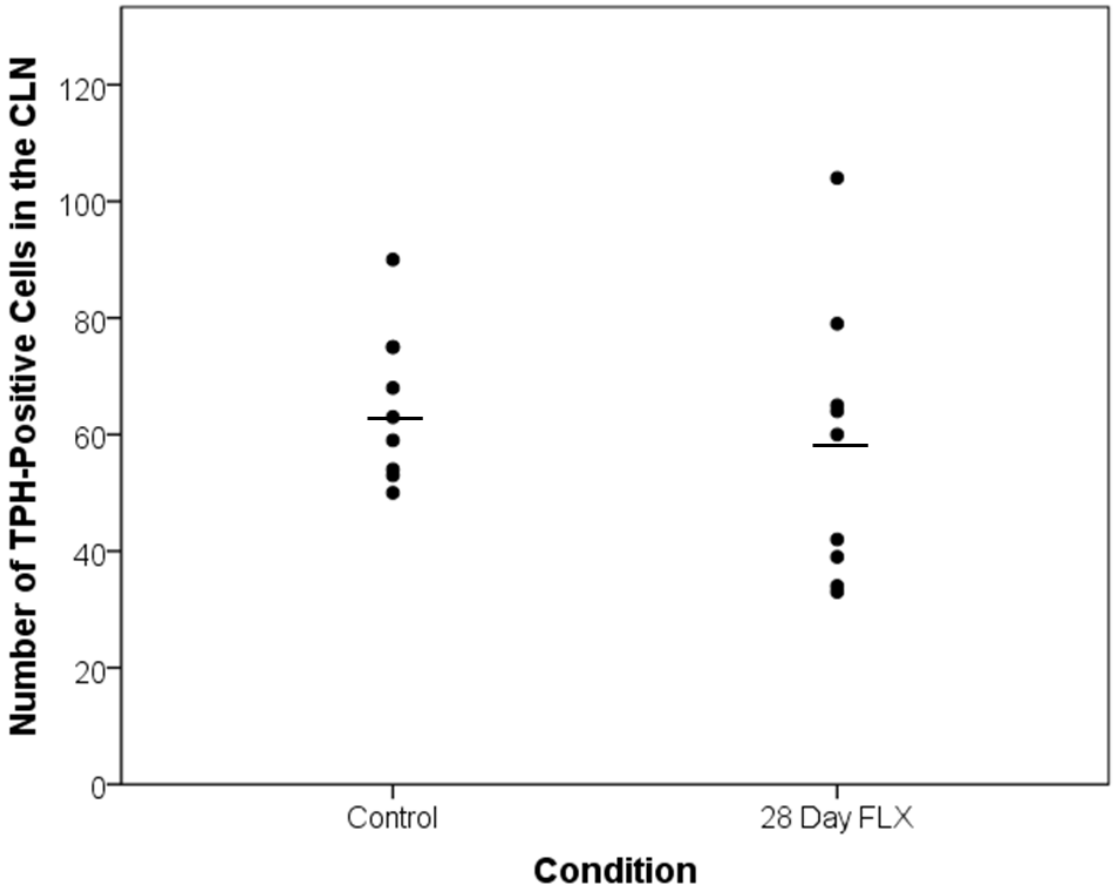

Figure 5D. Number of TPH-positive cells per 20 µm section of rat CLN. Each dot represents the CLN TPH-positive cell count for one animal; the horizontal line represents the group mean. Note: '*' indicates statistical significance. FLX = fluoxetine.

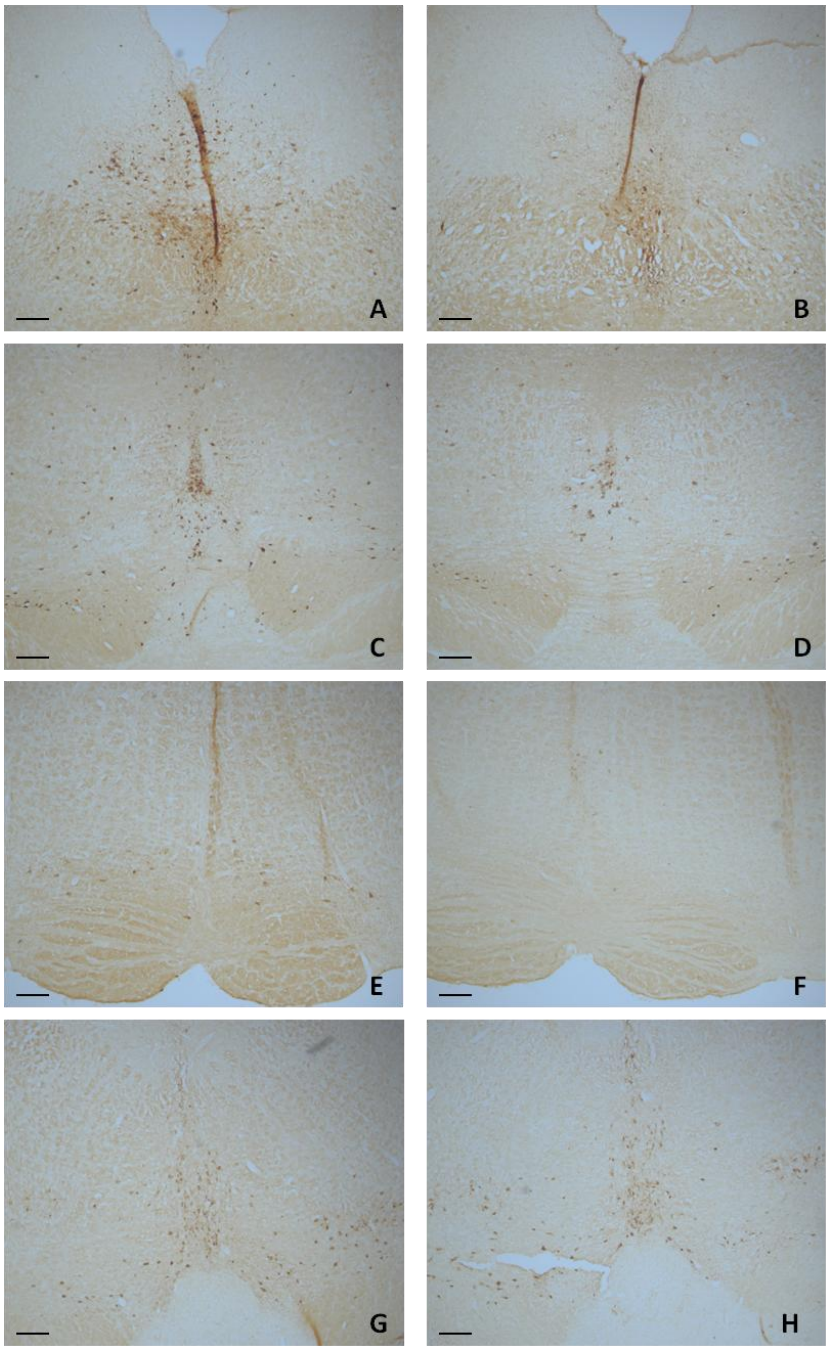

Figure 6. Photomicrographs (5x magnification) of TPH-immunoreactive cells in 20 µm sections of rat DRN (bregma – 7.8mm), MRN (bregma – 7.8mm), RMN (bregma – 9.8mm) and CLN (bregma – 6.3mm). A, Control - DRN; B, Fluoxetine - DRN; C, Control – MRN; D, Fluoxetine – MRN; E, Control – RMN; F, Fluoxetine – RMN; G, Control – CLN; H, Fluoxetine – CLN. Scale bar 200 µm.

Average Length of Nuclei (μm)

	CON	24 HR	7 DAY	28 DAY	p value
DRN	18.44	17.64	18.10	17.91	0.264
MRN	18.07	18.22	18.01	18.63	0.418
RMN	22.224	22.221	22.221	22.153	0.998
CLN	17.66	17.63	17.55	18.12	0.674

Table 1. Average cell diameter (μm) for each group and nucleus of interest. The 24 hour, 7 day and 28 day labels represent groups of animals that were treated with citalopram. One-way ANOVA *p*-values are indicated.

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Chapter 4: Inhibition of the serotonin transporter induces microglial activation and downregulation of dopaminergic neurons in the substantia nigra

MacGillivray L[1,2], Reynolds KB[1,2], Sickand, M[1,2], Rosebush PI[2], Mazurek MF[1,2]

Departments of Medicine[1] (Neurology) and Psychiatry and Behavioural Neurosciences[2], 4U, McMaster University Medical Centre, 1200 Main Street W, Hamilton, Ontario, L8N3Z5

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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 hours, 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 hours (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 fluoxetine-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.

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; Zohar and Westenberg, 2000; Vaswani et al., 2003). 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, dyskinesia and parkinsonism (Leo, 1996; Caley, 1997; Gerber and Lynd, 1998).

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 (Leo, 1996; Caley, 1997; Gerber and Lynd, 1998), 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, 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 (McGeer et al., 1988; Mirza et al., 2000; Gerhard et al., 2006; Wu et al., 2002; Sherer et al., 2003; Marinova-Mutafchieva et al., 2009).

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 12h:12h 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 hour 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; Fredricson Overo, 1982b; 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 (40µm) 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 hours, 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 hours 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 minutes at room temperature, rinsed twice in 0.1 M PBS and incubated in 5% normal donkey serum (NDS) for 1 hour. 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 hours 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 hour at room temperature, washed three more times in 0.1 M PBS, and then incubated in ABC solution (Vector Laboratories, Burlington, Canada) for 1 hour 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, 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 minutes at room temperature, rinsed twice in 0.1 M PBS and incubated in 5% NDS for 1 hour. 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 hours 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 20X 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 (50mM Tris, 1mM EDTA, pH 7.4) and 20 seconds of sonication. The protein concentration for each lysate was determined with the

BioRad Protein Assay (BioRad, Mississauga, Canada). An equal amount of protein (0.23µg per 1 µL of 5x western loading buffer) from each sample was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane, and blocked in TBST (0.14M NaCl, 2.7mM KCl, 24.8mM 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) primary antibodies in TBST and 3% milk for 1 hour 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 hour 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.

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 (figure 1a; $F_{3,32} = 7.023$, $p = 0.001$): by 44% at 24 hours (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, figure 2a). Representative photomicrographs are shown in figure 2b. Cell sizes were not significantly different among any of the groups.

TH Immunoblotting in the Striatum

Striatal TH was decreased by 45% (p=0.001, figure 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 figure 3a-b.

Discussion

These results indicate that selective blockade of SERT can induce a significant reduction of TH-positive dopaminergic neurons in the SN within 24 hours 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 & Immunoblotting

To the best of our knowledge, this is the first direct evidence that SSRI medication can affect the biosynthetic 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 (Leo, 1996; Caley, 1997; Gerber and Lynd, 1998).

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; Fibiger and Miller, 1977; Dray et al., 1978). 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 following high doses of fluoxetine (Baldessarini and Marsh, 1990).

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 (Souza et al., 1994; Curti et al., 1999). Reduced complex I activity has been associated with compromised dopaminergic function, and may have contributed to the effects we observed on TH-positive cell counts (Przedborski et al., 2004; Parker et al., 2008; 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 Parkinson's disease (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 (Wu et al., 2002; Du et al., 2001). These observations suggest that SSRI-induced microglial activation may mediate the downregulation of TH immunohistochemistry that we found in the present 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 hours 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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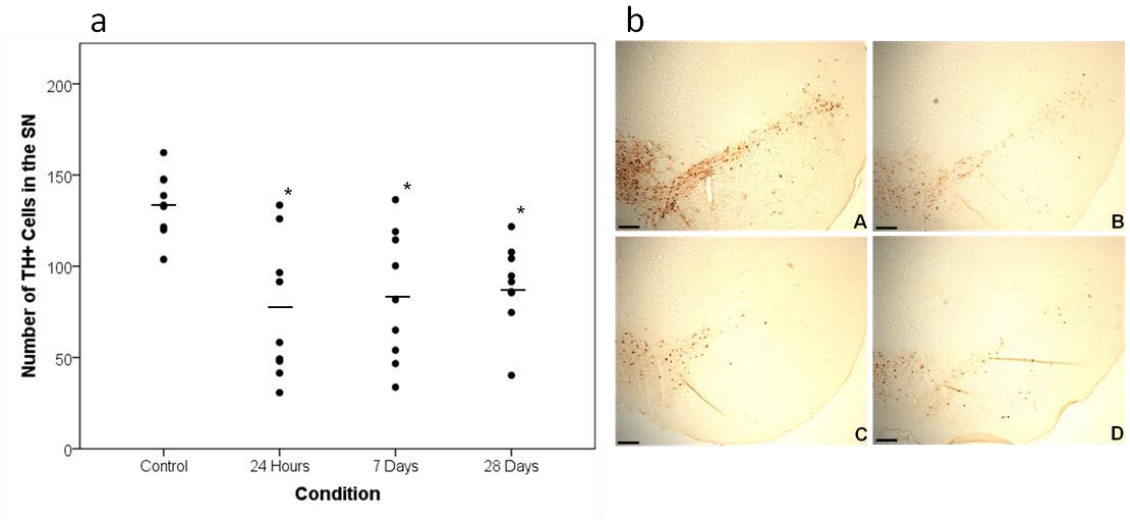

Figure 1. a) Number of TH-positive cells per 20 µm section of rat SN. The 24 hours, 7 days and 28 days labels represent durations of citalopram treatment. Each dot represents the number of TH-positive cells for one animal; the horizontal line represents the group mean. Note: '*' indicates statistical significance; b) Photomicrographs (5X magnification) of TH-positive cells in the rat SN. A: Control, B: 24 HR citalopram, C: 7 Day citalopram, D: 28 day citalopram. Scale Bar 300 µm.

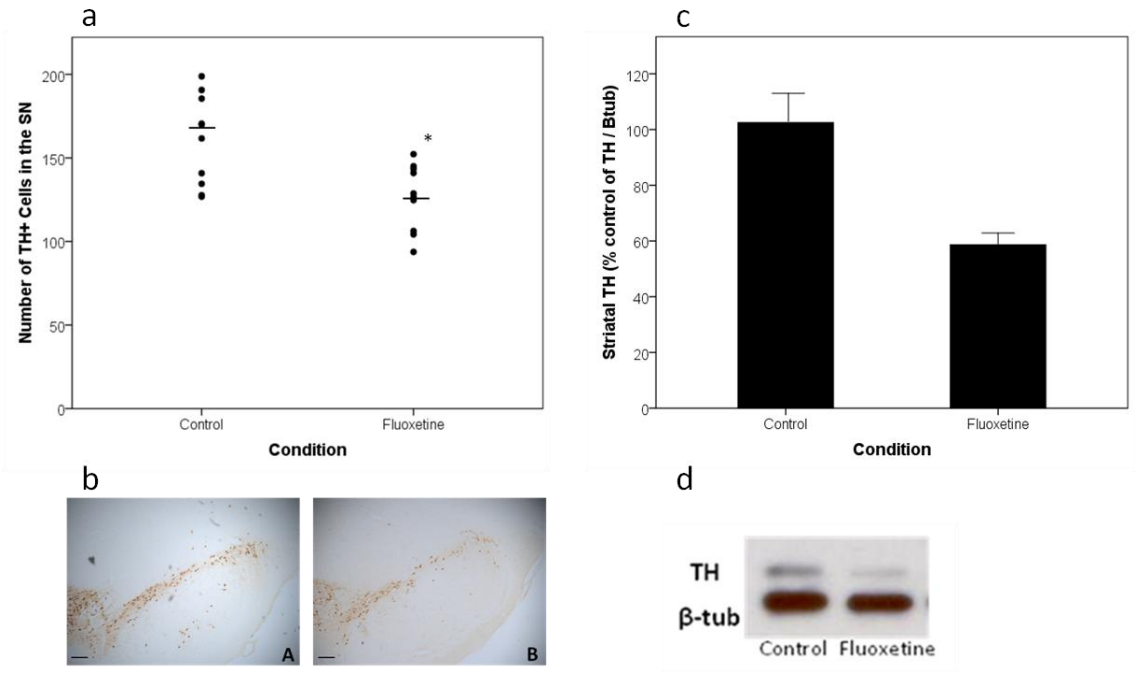

Figure 2. a) Number of TH-positive cells per 40 µm section of rat SN. Each dot represents the number of TH-positive cells for one animal; the horizontal line represents the group mean. Note: '*' indicates statistical significance. b) Photomicrographs (5X magnification) of TH-positive cells in the rat 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) Immunoblot of TH signal in the striatum.

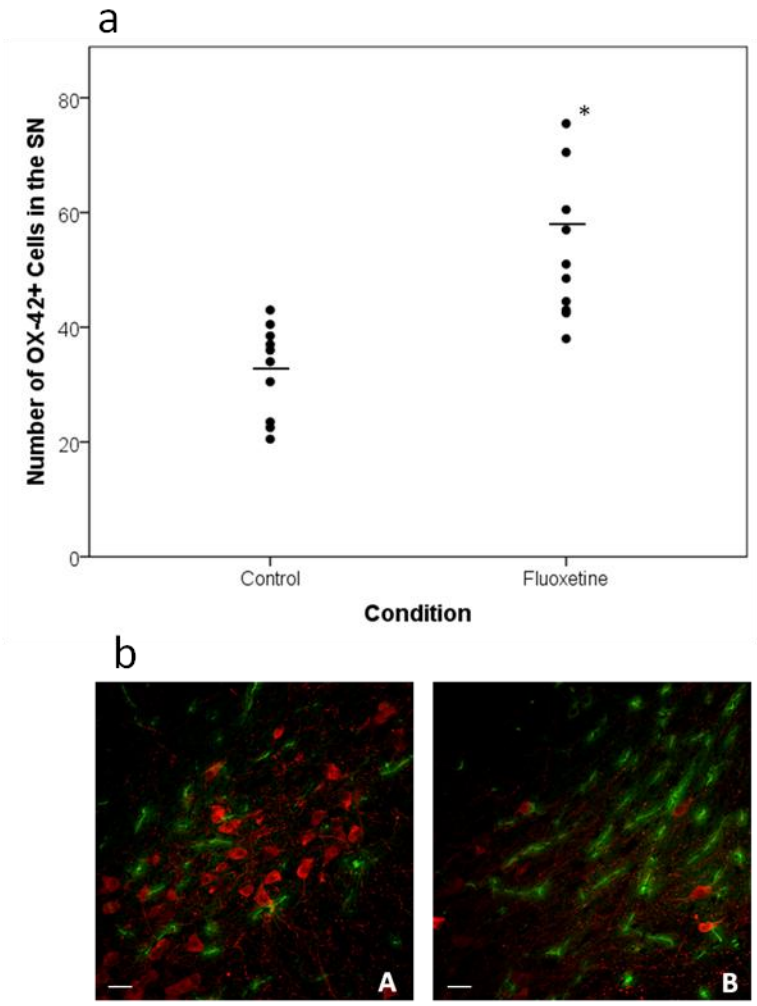

Figure 3. a) Number of OX42-stained cells per 40 µm section of rat SN. Each dot represents the number of OX42-stained cells for one animal; the horizontal line represents the group mean. Note: '*' indicates statistical significance; b) Photomicrographs (20x magnification) of TH(red)- and OX42 (green)-immunoreactive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

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Chapter 5: The Comparative Effects of Environmental Enrichment with Exercise and Serotonin Transporter Blockade on Serotonergic Neurons in the Dorsal Raphe Nucleus

MacGillivray L[a,b], Reynolds KB[a,b], Rosebush PI[b], Mazurek MF[a,b]

Departments of Medicine[1] (Neurology) and Psychiatry and Behavioural Neurosciences[2], 4U, McMaster University Medical Centre, 1200 Main Street W, Hamilton, Ontario, L8N3Z5

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ABSTRACT

We have previously reported that inhibition of the serotonin transporter (SERT) by selective serotonin reuptake inhibitor (SSRI) fluoxetine significantly reduces the number of tryptophan hydroxylase (TPH)-positive cells in the dorsal raphe nucleus. We have been interested in exploring whether this SSRI-induced change in TPH might be modified by housing in an enriched environment. Like SSRI antidepressants, environmental enrichment (EE) and physical exercise have been found to have efficacy in the prevention and alleviation of depression. We postulated that EE with exercise and SERT inhibition would similarly affect TPH regulation and that EE with exercise might modify the effect of fluoxetine on TPH. Three week old male Sprague-Dawley rats were housed in either a standard cage (SE) or an enriched environment (EE). SE animals were singly housed with no access to enrichment objects. EE animals were group housed and were provided with various enrichment objects (e.g. running wheel) that were changed and rearranged regularly. Nine weeks after the experiment began, the rats were randomly assigned to one of four treatment groups: (1) SE control; (2) SE fluoxetine; (3) EE control; or (4) EE fluoxetine. Fluoxetine (5 mg/kg/day) was placed in the drinking water. Sections of dorsal raphe (DRN) were processed for TPH immunohistochemistry. The number of TPH-positive cells was determined by blinded, manual counting. Results were analyzed by analysis of variance (ANOVA) followed by *post-hoc* Tukey tests. Significance was set at $p<0.05$. For animals housed in a standard environment, fluoxetine induced a significant 27% reduction in the number of TPH-immunoreactive cells in the

DRN. A similar reduction in TPH immunoreactivity was observed in animals that were housed in an enriched environment but not exposed to fluoxetine (39%). The number of TPH-positive cells in the DRN for animals housed in an enriched environment and exposed to fluoxetine was not significantly different than animals housed in an enriched environment and not exposed to fluoxetine. The reduction of TPH immunoreactivity in the DRN by EE with exercise suggests that a modified housing environment and voluntary exercise affects regulation of TPH, either via a mechanism similar to that of SERT inhibitors or through an independent pathway entirely. This downregulation of serotonin biosynthesis by fluoxetine and EE with exercise may ultimately play a role in the therapeutic action of both interventions.

Keywords:

environmental enrichment

exercise

serotonin transporter

fluoxetine

dorsal raphe nucleus

1. INTRODUCTION

The serotonin transporter (SERT) is a large integral membrane protein that mediates the Na^+/Cl^-–dependent reuptake of extracellular serotonin into presynaptic neurons (Schloss and Williams, 1998). Agents which inhibit SERT, including selective serotonin reuptake inhibitors (SSRIs), have gained widespread acceptance in the treatment of depression and anxiety disorders (Fava et al., 1993; Boyer, 1995; Pigott and Seay, 1999; Zohar and Westenberg, 2000; Vaswani et al., 2003). The mechanism of the antidepressant action remains unclear. While inhibition of the transporter occurs almost immediately, alleviation of depressive symptoms typically requires 2-4 weeks of treatment. This delay would suggest a mechanism more complex than simple inhibition of serotonin reuptake.

Environmental enrichment (EE) and physical exercise have also been found to have efficacy in the prevention and alleviation of depression (Morgan, 1985; Dunn and Dishman, 1991; Scully et al., 1998; Dunn et al., 2001; Salmon, 2001; Brosse et al., 2002; Mead et al., 2009). In the laboratory, EE can be provided through group housing in large cages equipped with toys, climbing structures and exercise apparatus. In animal models of depression, EE promotes resilience to stress (Mohammed et al., 1993; Chapillon et al., 1999; Larsson et al., 2002; Pollak et al., 2008) and can ameliorate the depression-like behaviours that are induced by stress (Fox et al., 2006). Voluntary wheel running may on its own have antidepressant and anxiolytic effects in some animal models of depression and anxiety (Dishman et al., 1997; Solberg et al., 1999; Moraska and Fleshner, 2001; Greenwood et al., 2003). The reported efficacy of environmental enrichment with

exercise and SSRI antidepressants in treating depression suggests that these interventions may share common cellular effects.

We previously reported that inhibition of SERT by SSRI medication can induce a regionally specific reduction of tryptophan hydroxylase (TPH)-positive cell counts in brainstem serotonergic nuclei within 24 hours and that this reduction persists throughout a treatment course of at least 28 days (MacGillivray et al., 2010). In the present study, we examined whether EE might have a similar effect on TPH-containing neurons in the dorsal raphe nucleus. We were also interested in the possibility that EE might be able to potentiate the changes induced by the SERT inhibitor fluoxetine.

2. MATERIAL AND METHODS

Subjects

Forty male Sprague-Dawley rats (Charles-River, PQ) were housed with free access to food and water on a 12h:12h light dark cycle throughout the experiment. Rat weights and water consumption were recorded every two days 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

At three weeks of age, the animals were housed in either a standard cage (SE, n=20) or an enriched environment (EE, n=20). SE animals were singly housed with no access to enrichment objects. EE animals were group-housed (10 per cage) and were provided with various enrichment objects (e.g. running wheel, hiding house, tubing, climbing objects, chew toys, bells, boxes, and nesting materials) that were changed and rearranged frequently. To promote exploration for the EE animals, food treats were hidden in the EE cage three times per week. Approximating the period of enrichment most often reported in the literature, the rats were randomly assigned to one of four treatment groups nine weeks after the experiment began: (1) SE control (n=10); (2) SE fluoxetine (n=10); (3) EE control (n=10); or (4) EE fluoxetine (n=10). Fluoxetine (5 mg/kg/day) was placed in the drinking water for 7 days. Groups of animals were maintained in their assigned environment for the duration of the experiment.

Immunohistochemistry

All animals were sacrificed by decapitation 24 hours after the final treatment. 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 hours immediately prior to sectioning. Consecutive coronal sections (40 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 dorsal raphe nucleus (DRN, bregma -7.8 mm, one section per animal) were selected and processed for TPH immunohistochemistry. Free-floating sections were incubated in 0.3% hydrogen peroxide in methanol for 30 minutes at room temperature, rinsed twice in 0.1 M PBS and incubated in 5% normal donkey serum (NDS) for 1 hour. Sections were again rinsed three times in 0.1 M PBS and then incubated in sheep anti-TPH primary antibody (Millipore, CA, 1:500) for 24 hours at 4°C. After three additional washes in 0.1 M PBS, DRN sections were incubated in biotinylated anti-sheep IgG secondary antibody (Millipore, CA, 1:200) for 1 hour at room temperature, washed three more times in 0.1 M PBS, and then incubated in ABC solution (Vectastain ABC Systems, Vector Labs, CA) for 1 hour at room temperature to form an avidin-biotin complex. After three final washes in 0.1 M PBS, the TPH immunocomplex was visualized with the chromagen diaminobenzidine tetrahydrochloride (DAB, Vector Labs, CA). Sections were mounted on Aptex-coated slides (Sigma, St. Louis, MO), air dried, and coverslipped with DPX neutral mounting medium (Sigma, St. Louis, MO).

Quantitative Morphometry

The selected coronal brain sections permitted evaluation of the DRN, a region known to be heavily populated with serotonergic neurons. Anatomical landmarks were used to ensure that comparable brain sections were analyzed for each region in each animal. To minimize error, all sections were cut and stained at the same time. DAB-stained DRN sections were mounted on microscope slides. The slides were coded and photographed at 5X on a confocal microscope (CarlZeiss LSM 510). The TPH-immunoreactive cells in the DRN were manually counted at 20X 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 were established for the nucleus of interest and every labeled cell within those boundaries was counted. TPH-positive neurons on both sides of the midline were included in the total count. After completion of manual counting, all images were decoded and arranged according to treatment group. The mean number of DAB-stained TPH-positive cells in the DRN was determined for each group and a standard error of the mean was calculated.

Statistical Analysis

Cell count data were analyzed by analysis of variance (ANOVA) followed by *post-hoc* Tukey tests where appropriate. Statistical significance was defined as $p < 0.05$.

3. RESULTS

Of the forty animal subjects, one animal from the standard environment control group was excluded from analyses because the brain tissue was poorly preserved.

Two-factor analysis of variance for the number of TPH-positive cells in the rat DRN showed a main drug effect ($F_{1,37} = 9.759$, $p = 0.002$) but no statistically significant environment effect ($F_{1,37} = 1.684$, $p = 0.203$). Neglecting the influence of environment and considering all treated animals, fluoxetine reduced the number of TPH-positive cells in the DRN by 23%. A significant interaction effect between the drug and environment factors was noted ($F_{1,37} = 8.327$, $p = 0.007$).

One-way analysis of variance for the number of TPH-positive cell counts in the rat DRN showed a significant effect for group ($F_{3,35} = 6.304$, $p = 0.002$). For animals housed in a standard environment, a 7 day course of fluoxetine significantly reduced the number of TPH-positive cells by 29% (p=0.030) (figure 1). Similarly, a reduction of 39% in TPH immunoreactivity was observed in animals that were housed in an enriched environment but not exposed to fluoxetine (p=0.001) (figure 1). The number of TPH-positive cells in the DRN for animals exposed to both the enriched environment and fluoxetine was not significantly different than for the groups exposed to either the enriched environment alone or to fluoxetine alone (p=0.669, figure 1). Representative photomicrographs are shown in figure 2.

4. DISCUSSION

These results confirm our previously reported observation that selective inhibition of SERT can induce a significant reduction of TPH-positive serotonergic neurons in the brainstem (MacGillivray et al., 2010). Administration of fluoxetine for 7 days significantly decreased the number of TPH-immunoreactive neurons in the rat DRN. A similar reduction of TPH immunoreactivity was observed in our enriched environment control group, even in the absence of any pharmacological intervention. To the best of our knowledge, this study is the first to demonstrate that environmental enrichment with exercise, like SERT inhibition, results in reduction of the number of TPH-positive cells in the DRN.

We recently reported that two SERT inhibitors, citalopram and fluoxetine each decreased numbers of TPH-positive neurons in the DRN within 24 hours and that this reduction persisted throughout a treatment course of at least 28 days (MacGillivray et al., 2010). This finding was consistent with a number of earlier publications, including those by Lapierre et al. (1983) who found that 25 mg/kg/day of the SSRI fluvoxamine injected (i.p.) into rats once daily for 7 days led to a significant 32% decrease in midbrain homogenate TPH and Maciag et al. (2006) who observed a persistent decrease of TPH-positive cells in both the dorsal and median raphe of rats that had been exposed to 10 mg/kg/day citalopram (s.c.) during postnatal days 8 to 21. Our work is also in line with publications by Abumaria et al. (2007) and Dygalo et al. (2006), where significantly reduced TPH mRNA expression in the DRN was noted after 4 weeks of citalopram or 2 weeks of fluoxetine, respectively.

SERT blockade will, at least in the short run, produce increased bioavailability of serotonin in the synapse (Wong et al., 2005). Extracellular serotonin might also be increased by a direct effect of SSRIs on SERT expression, since fluoxetine reportedly up-regulates levels of the negative SERT regulator, miR-16, in serotonergic raphe nuclei by antagonizing WnT signaling (Baudry et al., 2010). Regardless of the mechanism, raised levels of synaptic serotonin would result in augmented stimulation of the $5HT1_A$ autoreceptor, and since $5\text{-}HT1_A$ binding reportedly prevents calcium-calmodulin-dependent activation of TPH (Sawada and Nagatsu, 1986), heightened activation of the somatodendritic autoreceptor may be one possible mechanism for the reduction of TPH immunoreactivity by SERT inhibitors. We are not aware of any investigations that directly show an autoreceptor-mediated decrease in TPH protein expression, but selective 5HT-1 autoreceptor agonists have been demonstrated to decrease TPH mRNA levels and repress TPH promoter activity (Wood & Russo, 2001). Alternatively, SSRI-type medications may also have a direct effect on intracellular processes. Miura et al. (2004; 2005; 2007) have reported that SSRI medications suppress (6R)-5,6,7,8-tetrahydrobiopterin (BH_4) , a coenzyme of TPH. Reduced BH_4 would attenuate TPH activity and may in part explain the SSRI-induced suppression.

The reduction of TPH-immunoreactive neurons in animals housed in the enriched environment was comparable in magnitude to what we observed in the fluoxetine-treated group. There was no additive or synergistic effect when we combined the environmental enrichment with fluoxetine treatment. This would suggest that the two experimental interventions may be acting through a common mechanism. Previous studies have found

that 3-6 weeks of voluntary wheel running will result in increased 5-$HT1_A$ inhibitory autoreceptor mRNA, decreased c-Fos, and reduced SERT mRNA in the rat DRN (Greenwood et al., 2005). Reduced SERT mRNA would presumably lead to reduced capacity for serotonin reuptake and a subsequent rise in extracellular serotonin, as happens when SERT blockade is produced by pharmacological agents. Along with increased expression of mRNA for the $5HT1_A$ autoreceptor, EE-induced SERT inhibition could explain the reduced TPH-positive counts that we observed in the EE animals. This might also explain why the effects of EE were not augmented by the addition of pharmacological SERT inhibition with fluoxetine.

Our finding that environmental enrichment with exercise can downregulate TPH-immunopositive cell counts is consistent with observations suggesting a relationship between exercise and serotonin metabolism. In the course of investigating the effect of endurance training on serotonin metabolism in rats, Langfort et al. (2006) noted that TPH protein levels were decreased by 15-25% in rat midbrain and cortex after a six-week non-exhaustive exercise program. Conversely, Chamas et al. (1999; 2004) reported that immobilization elevated TPH mRNA and protein levels in rat raphe nuclei. These findings also suggest that physical activity alone, even without environmental enrichment may be sufficient to produce the changes that we observed.

Conclusions

Environmental enrichment and fluoxetine each produced comparable reductions of TPH-immunoreactive neurons in the rat dorsal raphe. The significance of these findings is not entirely clear and an important future direction will be to determine the

functional state of these neurons with Nissl staining and quantification of apoptotic markers. No additive or synergistic effect was observed when we combined the environmental enrichment with fluoxetine treatment. This would suggest that the two experimental interventions might be acting through a common mechanism, possibly via increased expression of mRNA for the $5HT1_A$ autoreceptor or via direct inhibition of SERT. The downregulation of TPH protein expression by fluoxetine and EE with exercise may ultimately play a role in the therapeutic action of both interventions.

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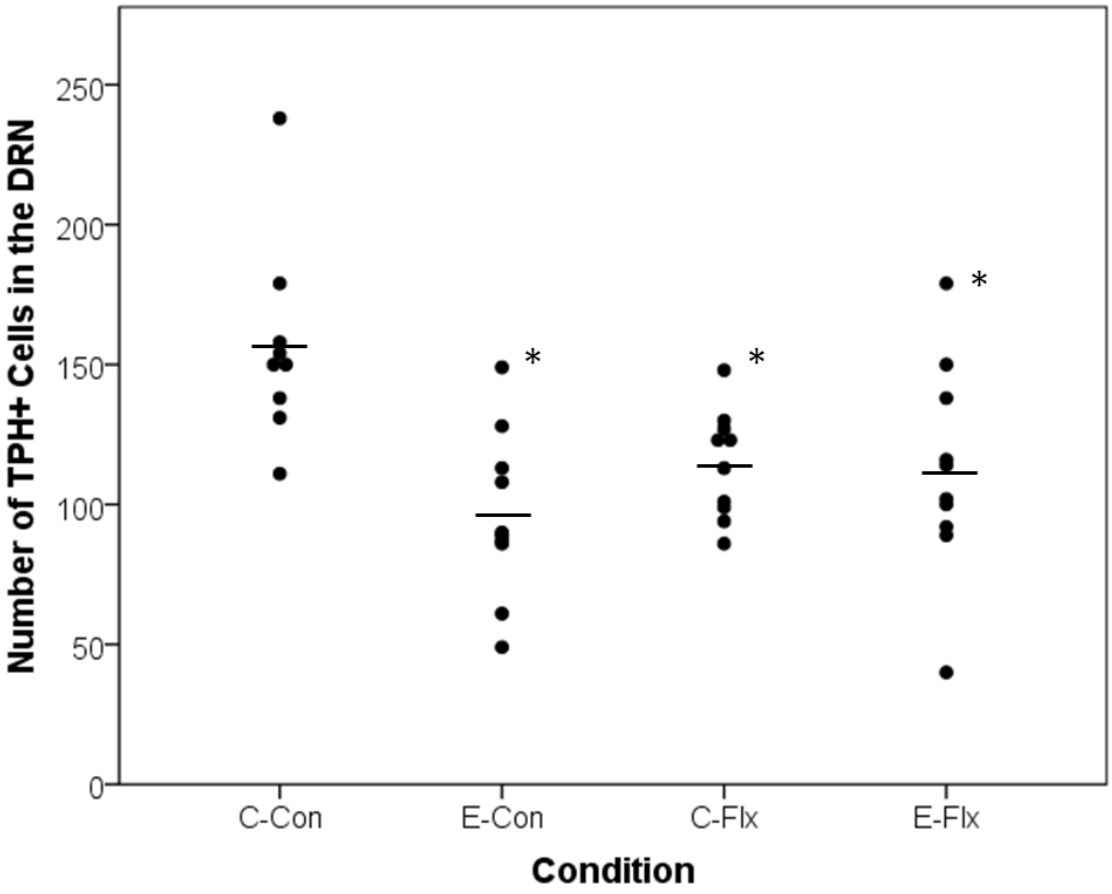

Figure 1. Number of TPH-positive cells per 40 µm section of rat DRN. Each dot represents the number of TPH-positive cells for one animal; the horizontal line represents the group mean. Note: '*' indicates statistical significance. C-Con = standard environment control; E-Con = enriched environment control; C-Flx = standard environment fluoxetine; E-Flx = enriched environment fluoxetine.

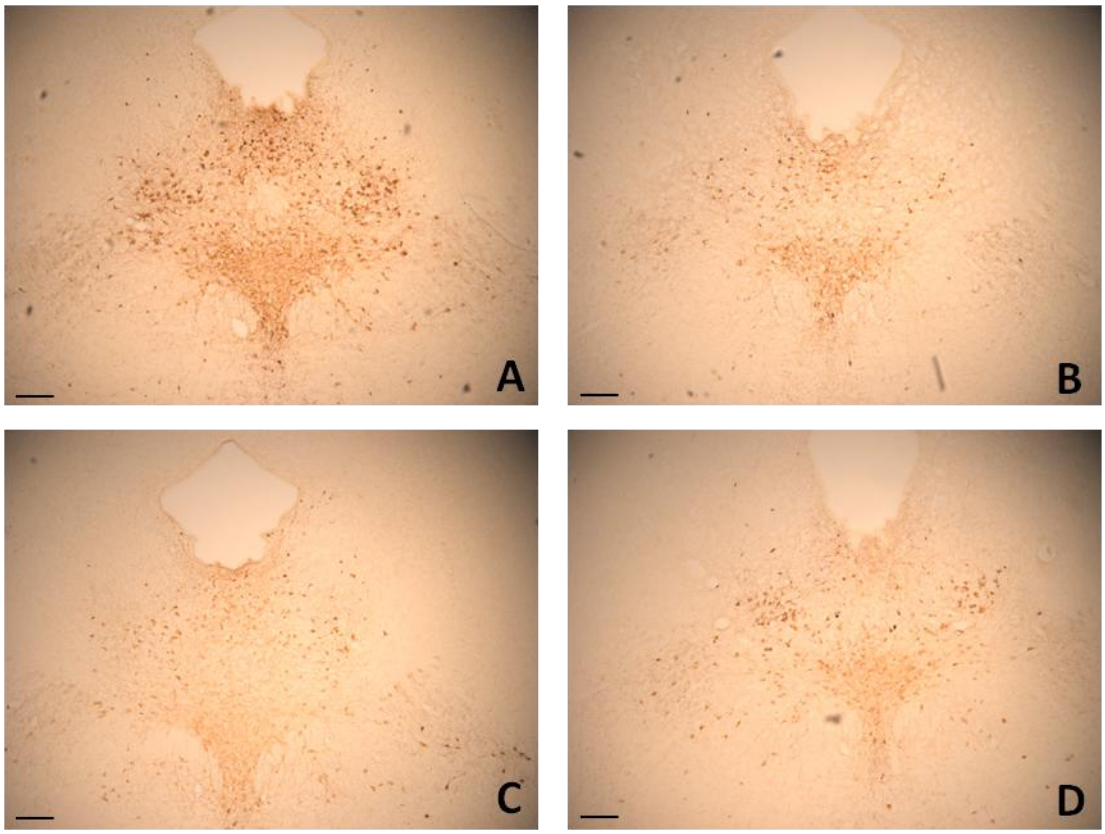

Figure 2. Photomicrographs (5x magnification) of TPH-immunoreactive cells in 40 µm sections of rat DRN (bregma – 7.8mm). A, Standard Environment Control; B, Enriched Environment Control; C, Standard Environment Fluoxetine; D, Enriched Environment Fluoxetine. Scale bar 200 µm.

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Chapter 6: The comparative effects of environmental enrichment and serotonin transporter blockade on dopaminergic neurons and microglia in the substantia nigra, ventral tegmental area and striatum

MacGillivray L[a,b], Reynolds KB[a,b], Sickand, M[a,b], Rosebush PI[b], Mazurek MF[a,b]

Departments of Medicine[1] (Neurology) and Psychiatry and Behavioural Neurosciences[2], 4U, McMaster University Medical Centre, 1200 Main Street W, Hamilton, Ontario, L8N3Z5

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ABSTRACT

Drugs which selectively inhibit the serotonin transporter (SERT) are widely used for the treatment for the treatment of depression and anxiety disorders. These selective serotonin reuptake inhibitors (SSRIs) produce extrapyramidal syndromes, including dystonia, akathisia, dyskinesia and parkinsonism, which are more typically associated with drugs that alter dopaminergic transmission in the basal ganglia. We were interested in examining the effects of the SERT inhibitor fluoxetine (FLX) on dopaminergic neurons in the SN, striatum and VTA, and the ability of environmental enrichment with exercise to attenuate these effects. Because the innate immune system has been implicated in other models of dopaminergic dysfunction we also studied the effects of SERT inhibition and EE with exercise on microglial activation.

Three week old male Sprague-Dawley rats were housed in either a standard cage (SE) or an enriched environment (EE). SE animals were singly housed with no access to enrichment objects. EE animals were group housed and were provided with various enrichment objects (e.g. running wheel) that were changed and rearranged regularly. Nine weeks after the experiment began, the rats were randomly assigned to one of four treatment groups: (1) SE control; (2) SE FLX; (3) EE control; or (4) EE FLX. FLX (5 mg/kg/day) was administered in the drinking water. Sections of SN and VTA were stained immunohistochemically for tyrosine hydroxylase (TH) and the microglial marker OX42. Striatal homogenates were processed for TH by immunoblotting. The numbers of TH- and OX42-positive cells were determined by blinded, manual counting. Optical

density measurements were used to quantify the TH immunoblots. Results were analyzed by analysis of variance (ANOVA) followed by *post-hoc* Tukey tests. Significance was set at $p<0.05$. FLX significantly reduced the number of TH-positive cells in the SN of SE animals (25%). A similar reduction in TH immunoreactivity was observed in animals that were housed in an EE but not exposed to FLX (29%). Animals housed in an EE *and* treated with 7 days of FLX showed a 54% reduction in TH-positive cell counts in the SN ($p<0.001$). For the VTA, TH-positive cell counts were reduced by 28% in SE-FLX animals, by 28% in EE-CON animals and by 36% in EE-FLX animals. Fluoxetine significantly reduced TH protein expression in the striatum, regardless of whether the animals were housed in a standard (44%) or enriched (32%) environment. TH protein expression was also reduced in animals that were housed in an enriched environment but not exposed to fluoxetine (28%). Compared to SE control animals, fluoxetine increased OX42 immunoreactivity in the SN of SE animals (53%) and EE animals (48%) and not at all in the VTA.

Keywords:

environmental enrichment

exercise

fluoxetine

serotonin transporter

substantia nigra

ventral tegmental area

1. INTRODUCTION

Drugs which selectively inhibit the serotonin transporter (SERT) are widely used for the treatment of depression and anxiety disorders (Fava et al., 1993; Boyer, 1995; Pigott and Seay, 1999; Zohar and Westenberg, 2000; Vaswani et al., 2003). These medications, known as selective serotonin reuptake inhibitors (SSRIs) produce a number of side effects, such as nausea, sleep disturbance and headache, which are plausibly attributable to alterations in serotonergic function (Brambilla et al., 2005). Other side effects, however, are more suggestive of disordered dopaminergic signaling. In particular, SSRI-type agents produce extrapyramidal syndromes, including dystonia, akathisia, dyskinesia, and parkinsonism, which are more typically associated with drugs that alter dopaminergic transmission in the basal ganglia (Rosebush and Mazurek, 1995; Mazurek and Rosebush, 1996; Mazurek and Rosebush, 2005; Leo, 1996; Caley, 1997; Gerber and Lynd, 1998; Damsa et al., 2004). Extrapyramidal side effects have been reported with fluoxetine (Leo, 1996; Caley, 1997; Gerber and Lynd, 1998), citalopram (Najjar and Price, 2004; Parvin and Swartz, 2005; Thwaites et al., 2006), paroxetine (Adler and Angrist, 1995; Baldassano et al., 1996), fluvoxamine (Wils, 1992; George and Trimble, 1993), and sertraline (Lambert et al., 1998); suggesting that these 'anti-dopaminergic' actions may be a consequence of SERT inhibition itself, rather than being specific to any particular pharmacological agent.

Environmental enrichment (EE) and exercise have been proposed as strategies for ameliorating dysfunction in the nigrostriatal dopamine system. EE has been reported to improve motor behaviour and to attenuate the reduction of TH-immunoreactivity in the

nigrostriatal pathway following striatal lesions or exposure to neurotoxins such as MPTP and 6-OHDA (Bezard et al., 2003; Tillerson et al., 2003; Faherty et al., 2005; Steiner et al., 2006; Urakawa et al., 2007). Exercise has been found to afford similar protection from the effects of 6-OHDA or MPTP (Tillerson et al., 2003; ODell et al., 2007; Yoon et al., 2007).

In the present study we examined the effects of the SERT inhibitor fluoxetine on dopaminergic neurons in the SN, striatum and VTA, and the ability of EE with exercise to attenuate these effects. Because the innate immune system has been implicated in other models of dopaminergic dysfunction (McGeer et al., 1988; Mirza et al., 2000; Gerhard et al., 2006; Gao et al., 2002; Wu et al., 2002; Depino et al., 2003; Sherer et al., 2003; Marinova-Mutafchieva et al., 2009) we also studied the effects of SERT inhibition and EE with exercise on microglial activation.

2. MATERIAL AND METHODS

2.1 Subjects

Forty male Sprague Dawley rats (Charles-River, PQ) were housed with free access to food and water on a 12h:12h light dark cycle throughout the experiment. Rat weights and water consumption were recorded every two days 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.

2.2. Treatment protocol

At three weeks of age, the animals were housed in either a standard cage (SE, n=20) or an enriched environment (EE, n=20). SE animals were singly housed with no access to enrichment objects. EE animals were group-housed and were provided with various enrichment objects (e.g. running wheel, hiding house, tubing, climbing objects, chew toys, bells, boxes, and nesting materials) that were changed and rearranged frequently. To promote exploration for the EE animals, food treats were hidden in the EE cage three times per week. Nine weeks after the experiment began, the rats were randomly assigned to one of four treatment groups: (1) SE control; (2) SE fluoxetine; (3) EE control; or (4) EE fluoxetine. Fluoxetine (5 mg/kg/day) was placed in the drinking water for 7 days.

2.3 Immunohistochemistry

All animals were sacrificed by decapitation 24 hours after the final treatment. 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 hours immediately prior to sectioning. Consecutive coronal sections (40 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 and VTA (bregma -5.8 mm) were selected and processed for TH and OX42 immunohistochemistry. To label TH with diaminobenzidine tetrahydrochloride (DAB), free-floating sections were incubated in 0.3% hydrogen peroxide in methanol for 30 minutes at room temperature, rinsed twice in 0.1 M PBS and incubated in 5% normal donkey serum (NDS) for 1 hour. Sections were again rinsed three times in 0.1 M PBS and then incubated in mouse anti-TH primary antibody (Millipore, Etobicoke, Canada; 1:1500) for 72 hours at 4°C. After three additional washes in 0.1 M PBS, SN and VTA sections were incubated in biotinylated anti-mouse IgG secondary antibody (Millipore, Etobicoke, Canada; 1:200) for 1 hour at room temperature, washed three more times in 0.1 M PBS, and then incubated in ABC solution (Vector Laboratories, Burlington, Canada) for 1 hour 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 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 SN and VTA sections were incubated in 0.3% hydrogen peroxide in methanol for 30 minutes at room

temperature, rinsed twice in 0.1 M PBS and incubated in 5% normal donkey serum (NDS) for 1 hour. 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 hours 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 brain sections corresponding to bregma -5.8 mm, an area known to be heavily populated with dopaminergic neurons, were selected for analysis. Use of anatomical landmarks permitted analysis of comparable brain sections. To minimize error, tissue from the different groups was cut and stained at the same time. Coded DAB-stained SN and VTA were photographed at 5X by light microscopy (CarlZeiss LSM 510). Coded fluorescently labeled sections of SN and VTA were imaged at 20x by confocal microscopy (CarlZeiss LSM 510).

The TH- and OX42-immunoreactive cells in the SN and VTA were manually counted at 20X 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 were established and every labeled cell within those boundaries was counted. Left and right hemisections were counted for each SN section. The VTA is a midline structure and was not divided into left and right subsections. After completion of manual counting, all images were decoded and arranged according to treatment group. The mean numbers of TH- and OX42-positive cells in the SN and VTA were determined for each animal.

For each SN and VTA section, 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 (50mM Tris, 1mM EDTA, pH 7.4) and 20 seconds of sonication. The protein concentration for each lysate was determined with the BioRad Protein Assay (BioRad, Mississauga, Canada). An equal amount of protein (0.23µg per 1 µL of 5x western loading buffer) from each sample was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane, and blocked in TBST (0.14M NaCl, 2.7mM KCl, 24.8mM 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)

primary antibodies in TBST and 3% milk for 1 hour 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 hour 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.

Statistical Analysis

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

3. RESULTS

A. Tyrosine Hydroxylase

Substantia Nigra

Fluoxetine treatment and EE with exercise both affected the number of TH-positive cells in the rat SN ($F_{3,35} = 11.828$, $p < 0.001$). For animals housed in a standard environment, a 7 day course of fluoxetine significantly reduced the number of TH-positive cells in the SN (25%, p=0.034) (figure 1). A similar reduction in TH immunoreactivity was observed in animals that were housed in an enriched environment but not exposed to fluoxetine (29%, p=0.012) (figure 1). EE with exercise did not attenuate the effect of fluoxetine on TH-immunoreactive cell counts in the SN; compared to control animals housed in a standard environment, animals housed in an enriched environment *and* treated with 7 days of fluoxetine showed a 54% reduction in TH-positive cell counts in the SN (p<0.001). Representative photomicrographs are shown in figure 2.

Striatum

Immunoblot analysis of TH protein expression in striatal tissues yielded results similar to those observed with TH cell counts in the SN. Fluoxetine treatment and EE with exercise both affected the number of TH-positive cells in the rat striatum ($F_{3,35} = 6.303$, $p = 0.002$). Fluoxetine significantly reduced TH protein expression in the striatum of animals housed in a standard environment (44%, p=0.001, figure 3a). TH protein expression was also reduced in animals that were housed in an enriched environment but not exposed to fluoxetine (28%, p=0.049, figure 3a). The fluoxetine-induced reduction of

striatal TH expression was neither potentiated nor attenuated by EE with exercise (p=0.699). Representative immunoblots are shown in figure 3b.

Ventral Tegmental Area

As was observed in the SN and striatum, there was a significant effect for group in the VTA ($F_{3,34}$ = 11.990, p < 0.001, figure 4). Compared to control animals housed in a standard environment, the number of TH-positive cells was reduced by 28% in fluoxetine-treated animals housed in a standard environment (p=0.001), by 28% among those housed in an enriched environment but not exposed to fluoxetine (p=0.001) and by 36% when the enriched environment was combined with fluoxetine treatment (p<0.001). The fluoxetine-induced reduction of TH-positive cell counts in the VTA was neither potentiated nor attenuated by EE with exercise (p=0.609). Representative photomicrographs are shown in figure 5.

B. OX42 Cell Counts

Substantia Nigra

Fluoxetine boosted OX42 immunoreactivity regardless of whether the animals were housed in a standard or an enriched environment (figure 6, $F_{3,35}$ = 12.427 p< 0.001). Compared to control animals housed in a standard environment, fluoxetine increased OX42- immunoreactive cell counts by 53% in the SN of animals housed in a standard environment (p=0.003) and by 48% in animals housed in an enriched environment (p=0.012). The numbers of OX42-positive cells in the SN of control animals housed in an

enriched environment were comparable to those observed in a standard environment (p=0.544). Representative photomicrographs are shown in figure 7.

Ventral Tegmental Area

As was observed with OX42-stained cells in the SN, there was a significant effect for group in the VTA ($F_{3,34}$ = 4.646, p = 0.008, figure 8). Compared to standard-housed control animals, numbers of OX42-positive cells in the VTA were not significantly different in fluoxetine-treated groups, regardless of whether the treated animals were housed in a standard (p = 0.983) or enriched (p = 0.473) environment. Although the numbers of OX42-positive cells in the VTA of control animals housed in an enriched environment and control animals housed in a standard environment were not significantly different (p=0.141), there was a trend toward reduced OX42 cell counts in the enrichment alone group. OX42 immunoreactivity was significantly greater in fluoxetine treated animals housed in an enriched environment than animals housed in an enriched environment but not exposed to fluoxetine (p = 0.005) Representative photomicrographs are shown in figure 9.

4. DISCUSSION

In this study, a 7 day course of the SERT inhibitor fluoxetine induced a significant reduction in the number of TH-immunoreactive neurons in the SN and the VTA and a comparable decrease of TH protein in the striatum. A non-pharmacological regimen of EE with exercise produced a similar reduction of TH-positive cell counts in the SN and VTA and of TH protein expression in the SN. When SERT inhibition was combined with the environmental enrichment, there was no attenuation of the fluoxetine effects in the SN, VTA or striatum.

Whereas SERT inhibition and environmental enrichment had similar effects on dopaminergic neurons, their actions on microglial cells were quite different. Fluoxetine induced a robust microglial activation in the SN, both with and without environmental enrichment and exercise. The environmental enrichment, on the other hand, had no independent effect on microglia and did not attenuate or potentiate the microglial response to fluoxetine.

The role of SERT inhibition

The fluoxetine-induced reduction of TH-positive cell counts in the SN is consistent with clinical reports that SERT inhibitors can cause side effects similar to those induced by drugs which block the D2 dopamine receptor (Leo, 1996; Caley, 1997; Gerber and Lynd, 1998). We have previously found that dopamine D2 receptor antagonists (Levinson et al., 1998; Mazurek et al., 1998) and SERT inhibitors (MacGillivray et al., 2011) decrease TH immunoreactivity in the SN. Both SERT inhibitors and D2 antagonists interfere with mitochondrial function (Burkhardt et al.,

1993; Souza et al., 1994; Maurer and Moller, 1997; Curti et al., 1999) and reduced complex I activity has been implicated in the pathobiology of dopaminergic cell death (Burkhardt et al., 1993; Maurer and Moller, 1997). Alternatively or additionally, SERT inhibition might reduce TH immunoreactivity through an inhibitory pathway between serotonergic cell groups in the raphe and dopaminergic neurons in the SN and VTA (Dray et al., 1976; Fibiger and Miller, 1977; Dray et al., 1978). Electrical stimulation of brainstem serotonin neurons is known to depress neuronal activity in the SN, as does microiontophoretic application of serotonin onto the SN (Dray et al., 1976), while lesions or pharmacological inhibition of the same neurons have the opposite effect (Dray et al., 1978; Giambalvo and Snodgrass, 1978; James and Starr, 1980; Ugedo et al., 1989; Arborelius et al., 1993).

The VTA appears equally susceptible to the inhibitory influence of serotonin since selective lesions of brainstem serotonin neurons enhance the firing activity of VTA DA neurons (Guiard et al., 2008). Our observation that fluoxetine reduces TH-positive cell counts in the VTA is in line with numerous reports of SSRI-mediated inhibition of the firing rate (Prisco and Esposito, 1995; Di Mascio et al., 1998; Dremencov et al., 2009) and spontaneous activity (Sekine et al., 2007) of dopamine neurons in the VTA. A substantial body of literature points to a role for the serotonin 5HT2C receptor subtype in mediating the phasic and tonic inhibitory control of the mesolimbic dopamine system (Prisco and Esposito, 1995; Di Matteo et al., 1999; Di Matteo et al., 2000; Di Giovanni et al., 2000; Di Matteo et al., 2002).

The Role of Environmental Enrichment with Exercise

Environmental enrichment and exercise have been proposed as neuroprotective strategies for reducing damage to nigrostriatal dopamine neurons, based on the ability of EE to attenuate the deleterious cellular and behavioural consequences of neurotoxins such as 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and 6-hydroxydopamine (6-OHDA) (Bezard et al., 2003; Tillerson et al., 2003; Faherty et al., 2005; Steiner et al., 2006; Urakawa et al., 2007). In the present study, however, EE with exercise did not attenuate the suppressive effects of fluoxetine on markers of TH in the SN, VTA and striatum. On the contrary, EE with exercise induces changes very similar to those observed with fluoxetine along, including reduction of TH-immunopositive cell counts in both the SN and VTA and a parallel decrease of TH protein in the striatum. TH suppression in the SN of animals housed in an enriched environment was also noted by Bezard et al. (2003) who found approximately 10% fewer dopaminergic neurons in the substantia nigra pars compacta of enriched mice compared with mice raised in a standard environment.

In animal models of depression, EE promotes resilience to stress (Mohammed et al., 1993; Chapillon et al., 1999; Larsson et al., 2002; Pollak et al., 2008) and can ameliorate depression-like behaviours induced by stress (Fox et al., 2006). Environmental enrichment has also been reported to decrease the stress-induced release of dopamine in the prefrontal cortex (Segovia et al., 2008), consistent with our finding of reduced TH cell counts in the VTA of EE animals.

Microglial Activation

Although SERT inhibition and EE with exercise produced comparable reductions of TH immunoreactivity in nigrostriatal dopamine neurons, their actions on microglial cells were quite different. Fluoxetine induced a robust microglial activation in the SN, both with and without environmental enrichment and exercise. EE, on the other hand, did not boost microglial immunoreactivity in the SN and instead tended to decrease OX42 cell counts in the SN, though this was a non-significant finding. Given the propensity for fluoxetine and not EE to cause extrapyramidal side effects, microglial activation may well be linked to dopamine cell dysfunction. Since there is nothing to suggest that environmental enrichment or exercise, either separately or together, produce impaired dopaminergic activity, these results indicate that the reduction of TH staining does not, by itself, indicate a reduction in dopaminergic transmission. Given that the neuroscience literature often associates decreased TH immunoreactivity with toxicity, our finding that EE can decrease TH without any evidence of dopaminergic dysfunction invites review of this common assumption.

Functional Significance

This study demonstrates that environmental enrichment with exercise and SERT inhibition by fluoxetine produce comparable reductions of TH immunoreactivity in the nigrostriatal and mesolimbic dopaminergic systems, and for the first time, that fluoxetine treatment potentiates the effect of EE with exercise on dopaminergic neurons in the SN but not in the VTA. The ability of fluoxetine and not environmental enrichment to induce microglial activation suggests that microglia are probably recruited via a mechanism

separate from SERT inhibition and, moreover, that microglial activation itself may be related to the propensity of SSRI medications to induce extrapyramidal side effects.

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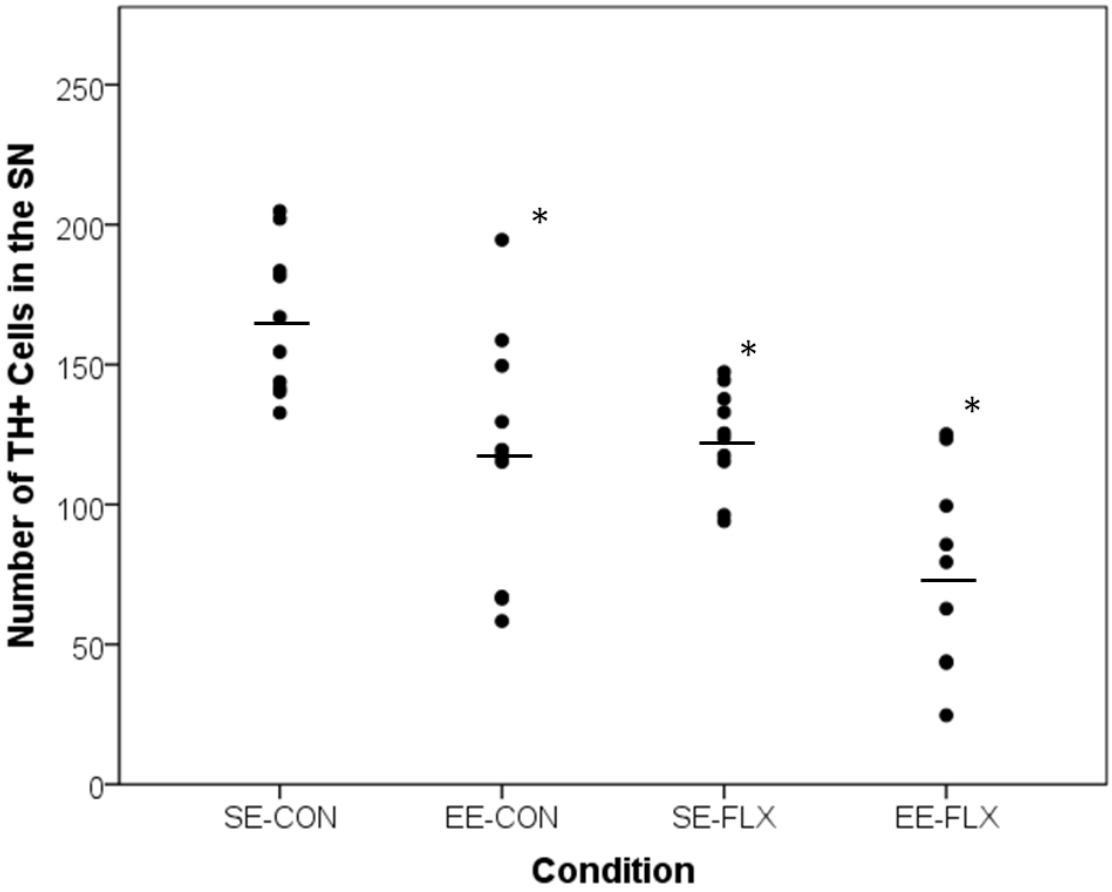

Figure 1. Number of TH-positive cells per 40 µm section of rat SN. Each dot represents the number of TH-positive cells for one animal; the horizontal line represents the group mean. Note: '*' indicates statistical significance. SE-CON = Standard Environment Control; EE-CON = Enriched Environment Control; SE-FLX = Standard Environment Fluoxetine; EE-FLX = Enriched Environment Fluoxetine.

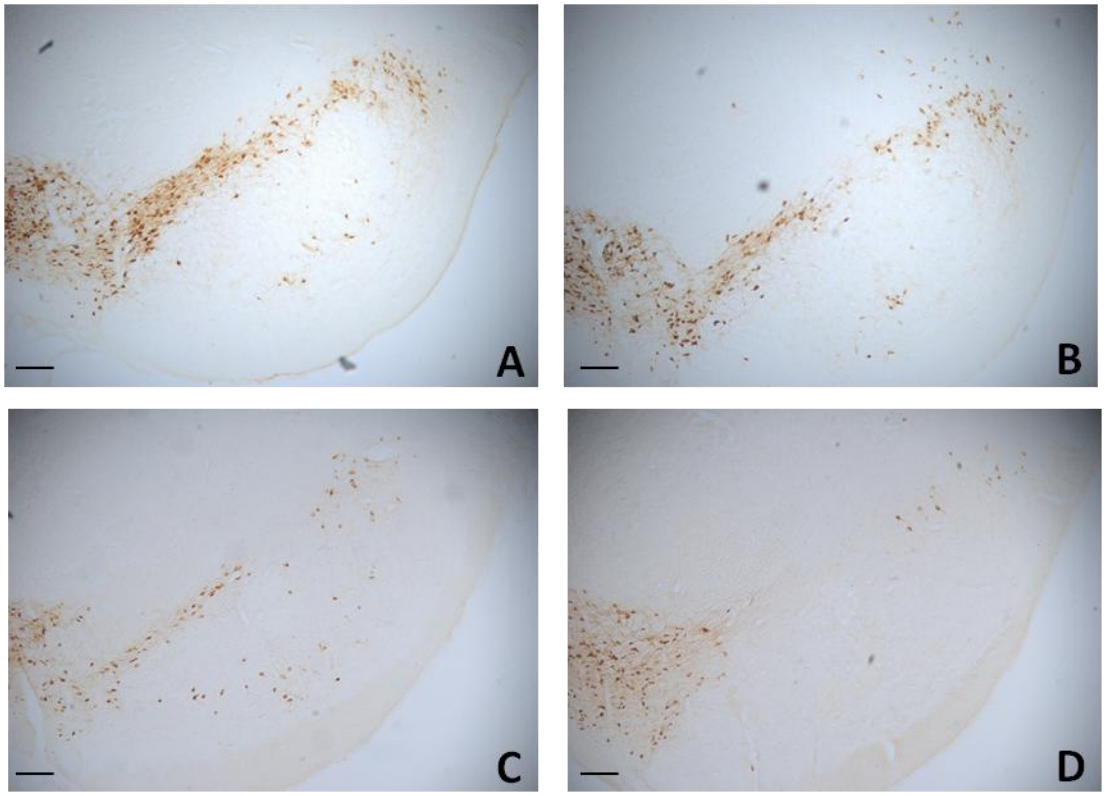

Figure 2. Photomicrographs (5x magnification) of TH-immunoreactive cells in 40 µm sections of rat SN (bregma – 5.8mm). A, Standard Environment Control; B, Standard Environment Fluoxetine; C, Enriched Environment Control; D, Enriched Environment Fluoxetine. Scale bar 200 µm.

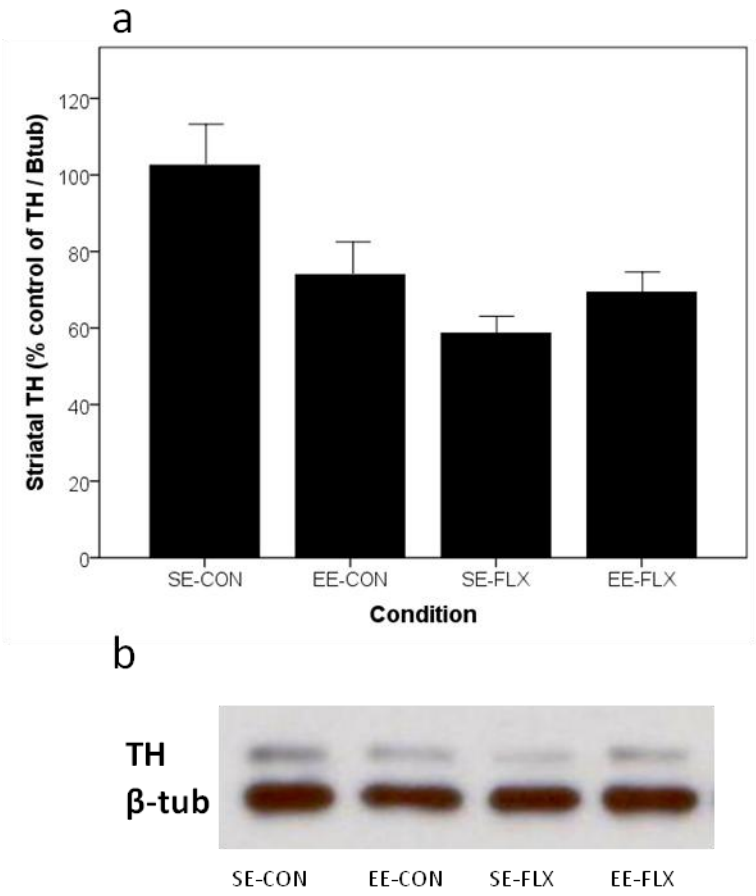

Figure 3. a) Relative TH signal in the rat striatum, expressed as a percent control of TH / β tubulin III. b) Immunoblot of TH signal in the striatum. SE-CON = Standard Environment Control; EE-CON = Enriched Environment Control; SE-FLX = Standard Environment Fluoxetine; EE-FLX = Enriched Environment Fluoxetine.

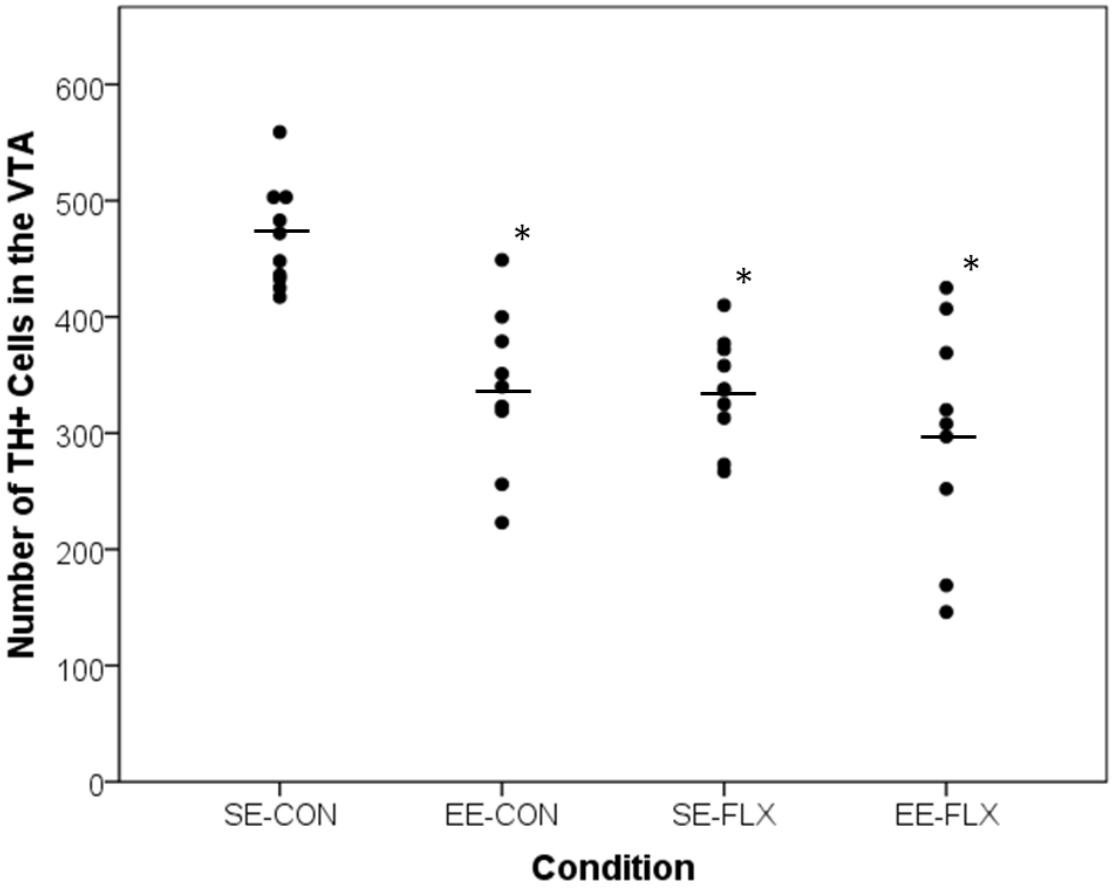

Figure 4. Number of TH-positive cells per 40 µm section of rat VTA. Each dot represents the number of TH-positive cells for one animal; the horizontal line represents the group mean. Note: '*' indicates statistical significance. SE CON = Standard Environment Control; EE CON = Enriched Environment Control; SE-FLX = Standard Environment Fluoxetine; EE-FLX = Enriched Environment Fluoxetine.

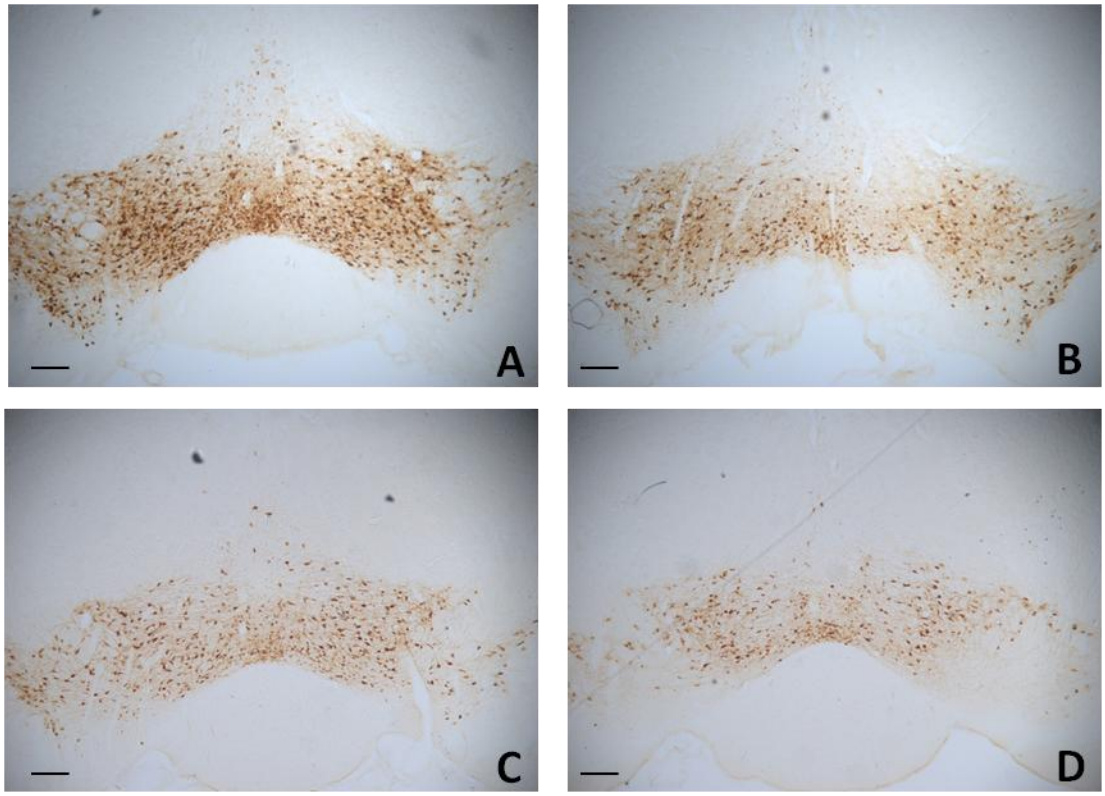

Figure 5. Photomicrographs (5x magnification) of TH-immunoreactive cells in 40 µm sections of rat VTA (bregma – 5.8mm). A, Standard Environment Control; B, Standard Environment Fluoxetine; C, Enriched Environment Control; D, Enriched Environment Fluoxetine. Scale bar 200 µm.

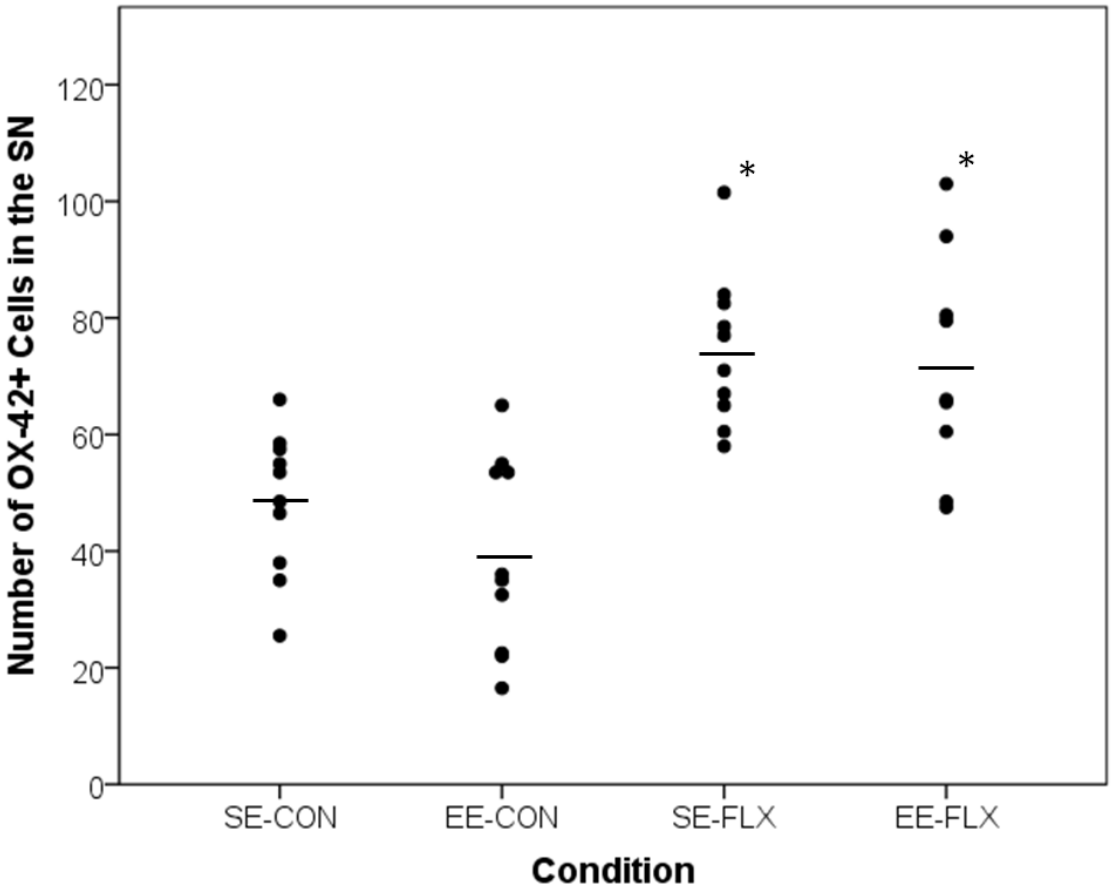

Figure 6. Number of OX42 stained cells per 40 µm section of rat SN. Each dot represents the number of OX42 stained cells for one animal; the horizontal line represents the group mean. Note: '*' indicates statistical significance. SE-CON = Standard Environment Control; EE-CON = Enriched Environment Control; SE-FLX = Standard Environment Control; EE-FLX = Enriched Environment Fluoxetine.

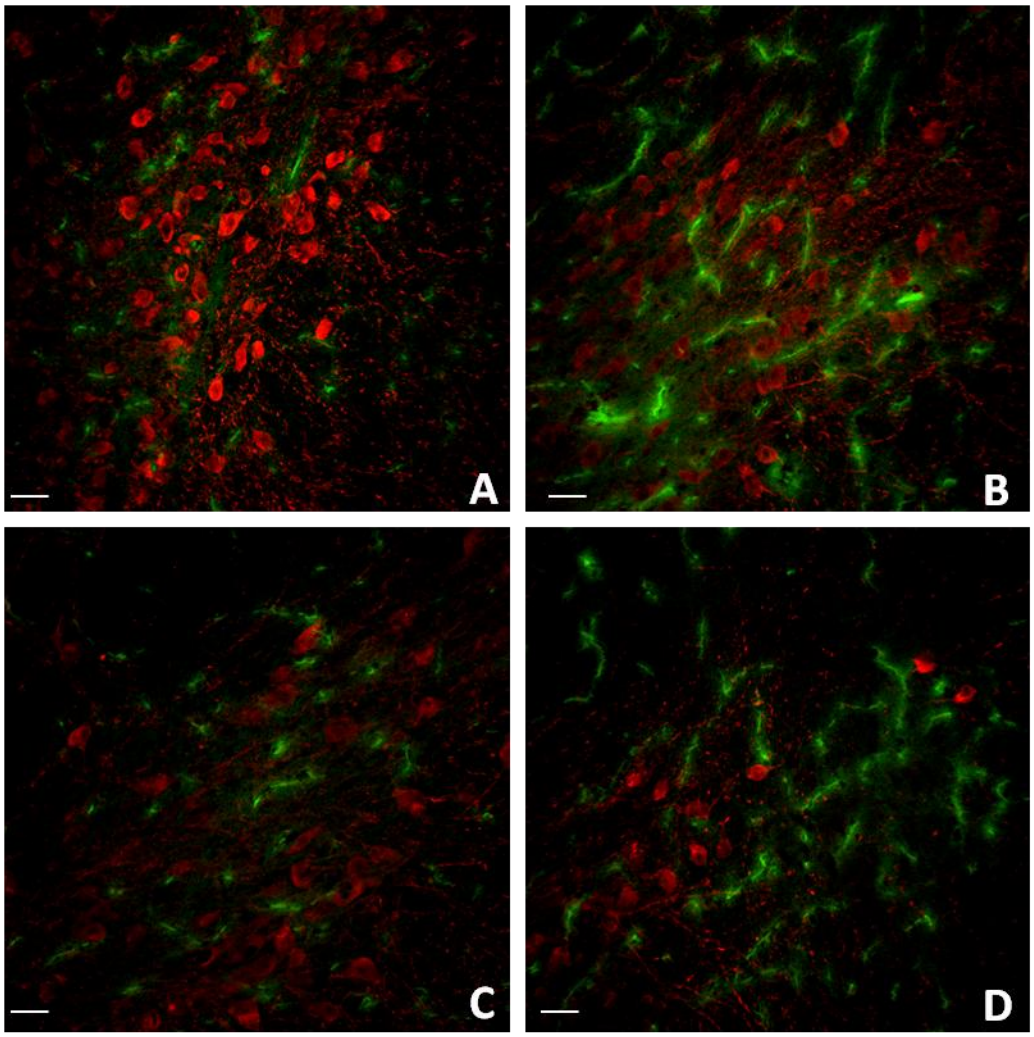

Figure 7. Photomicrographs (20x magnification) of TH(red)- and OX42(green)-immunoreactive cells in 40 µm sections of rat SN (bregma – 5.8mm). A, Standard Environment Control; B, Standard Environment Fluoxetine; C, Enriched Environment Control; D, Enriched Environment Fluoxetine. Scale bar 200 µm.

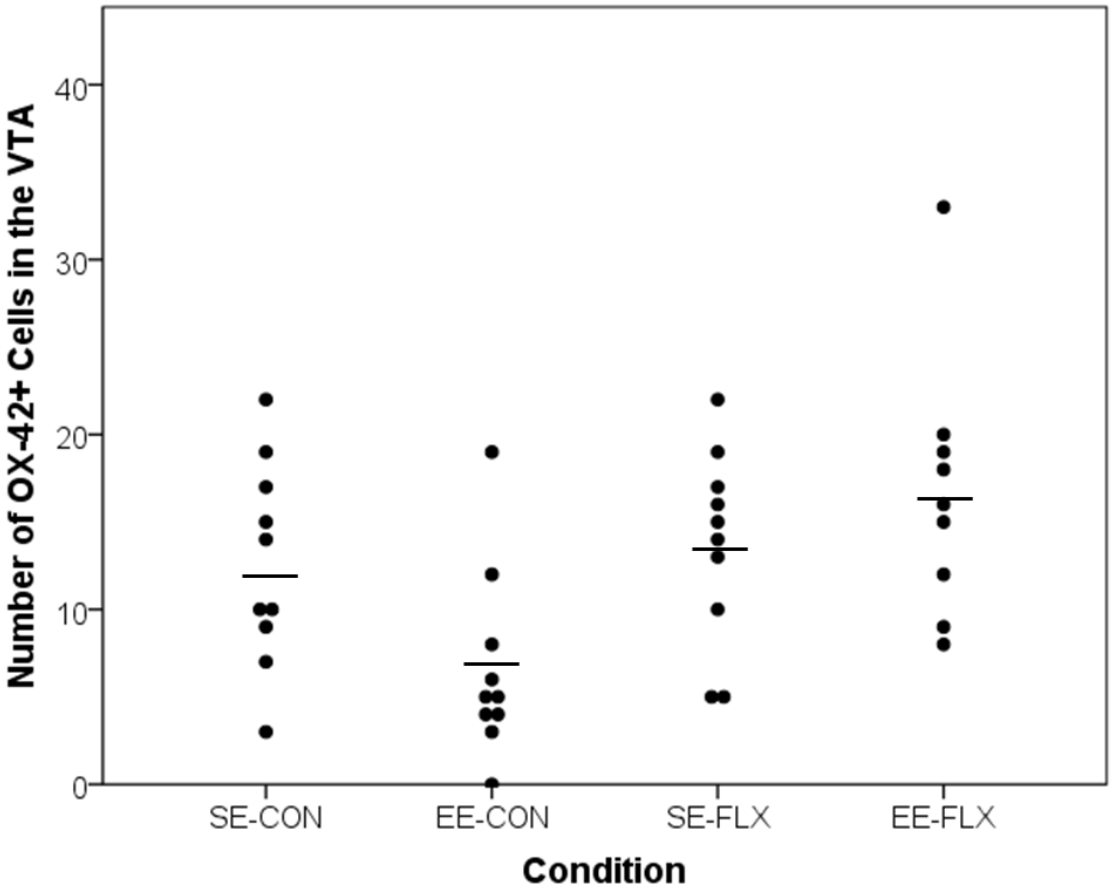

Figure 8. Number of OX42 stained cells per 40 µm section of rat VTA. Each dot represents the number of OX42 stained cells for one animal; the horizontal line represents the group mean. Note: '*' indicates statistical significance. SE-CON = Standard Environment Control; EE-CON = Enriched Environment Control; SE-FLX = Standard Environment Control; EE-FLX = Enriched Environment Fluoxetine.

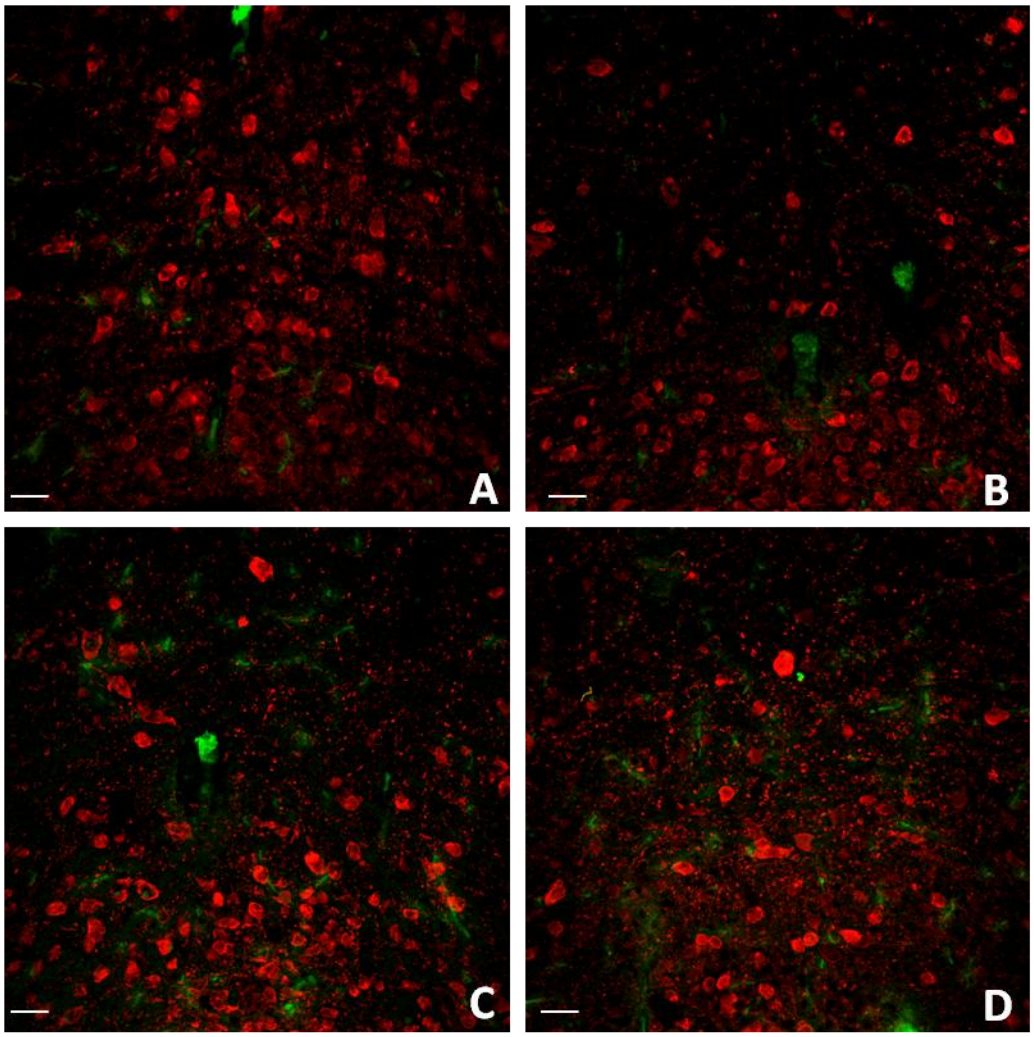

Figure 9. Photomicrographs (20x magnification) of TH(red)- and OX42(green)-immunoreactive cells in 40 µm sections of rat VTA (bregma – 5.8mm). A, Standard Environment Control; B, Standard Environment Fluoxetine; C, Enriched Environment Control; D, Enriched Environment Fluoxetine. Scale bar 200 µm.

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Chapter 7: Risperidone Affects Regulation of Tryptophan Hydroxylase in the Dorsal Raphe and Induces Nissl Cell Loss in Aged Rats

MacGillivray L[a,b], Zettler, M, Rosebush PI[b], Mazurek MF[a,b]

Departments of Medicine[1] (Neurology) and Psychiatry and Behavioural Neurosciences[2], 4U, McMaster University Medical Centre, 1200 Main Street W, Hamilton, Ontario, L8N3Z5

ABSTRACT

Risperidone is an atypical antipsychotic drug (APD) indicated for treatment of schizophrenia and related psychotic disorders. It was among the first new generation APDs and, because of its action on serotonin as well as dopamine neurotransmission, is reported to have a better side effect profile than those of the typical class. The clinical effects of risperidone are understood in terms of the drug's ability to antagonize both the serotonin 5-HT_2 receptor and dopamine D2 receptor. Little or no attention, however, has been given to risperidone's effects on presynaptic cell bodies. We studied changes in the biosynthetic enzyme for serotonin and in Nissl-stained cells in both young and aged animals. In the first experiment, groups of 8 week-old Sprague-Dawley rats received daily sub-cutaneous injections of either saline (N=9) or risperidone 1.5 mg/kg/day (N=9) for three days, after which time they were sacrificed. Sections of dorsal raphe nucleus (DRN) were processed for tryptophan hydroxylase (TPH) immunohistochemistry and Nissl staining. The numbers of TPH- and Nissl-counts were determined by blinded, manual counting and ImageJ particle analysis, respectively. Results were analyzed by Students t-test. In young animals, risperidone induced a significant decrease in DRN TPH-positive cell counts (55%, p=0.008). To investigate whether these changes would occur in aged animals, we conducted a second experiment, this time with a 28-day course of oral risperidone. As was observed in young animals, risperidone induced a significant reduction of TPH cell counts in the DRN (26%, p=0.038). In aged animals, risperidone also significantly reduced the number of nissl-stained cells in the DRN. *Conclusions*: These results indicate that risperidone induces downregulation of serotonergic neurons in

both young and aged animals and, in the more vulnerable aged animals, reduces the number of Nissl bodies. These reductions in TPH- and Nissl counts may play a role in mediating the therapeutic and toxic effects of atypical antipsychotic medications.

1. INTRODUCTION

The atypical antipsychotic drug risperidone is indicated for treatment of schizophrenia and related psychotic disorders. It was among the first new generation antipsychotic drugs and, because of its action on serotonin as well as dopamine neurotransmission, is reported to have a better side effect profile than those of the typical class (Borison et al., 1992; Chouinard et al., 1993; Janicak et al., 2001). Risperidone is a potent antagonist of the serotonin $5HT_{2A}$ and $5HT_{2C}$ receptors, dopamine D2 receptors, α1 and α2 adrenoreceptors and the histamine H1 receptor (Richelson and Souder, 2000), and unlike the prototypical first generation antipsychotic haloperidol, has a higher binding affinity for $5HT_{2A}$ receptors than for D2 receptors (Leysen et al., 1988; Schotte et al., 1996).

We previously reported that the selective serotonin reuptake inhibitor (SSRI) class of psychotropic medication affects the biosynthetic enzyme for serotonin, tryptophan hydroxylase (TPH), in brainstem raphe nuclei (MacGillivray et al., 2010). SSRIs citalopram and fluoxetine significantly decreased the number of TPH-immunoreactive neurons in the DRN, suggesting that serotonin transporter (SERT) inhibition can alter the regulation of TPH. We postulated that the downregulation of TPH by SERT inhibitors was related to augmented stimulation of the $5HT_{1A}$ autoreceptor by elevated extracellular serotonin. Since atypical antipsychotics have also been noted to increase extracellular serotonin in the rat DRN (Hertel et al., 1997), we hypothesized that risperidone might reduce TPH immunoreactivity in a manner similar to SSRI mediations. The ability of atypical antipsychotics to augment the clinical efficacy of SSRI medications (Tohen et

al., 2003; Papakostas et al., 2005; Simon and Nemeroff, 2005; Rapaport et al., 2006) and to decrease the firing rate of brainstem serotonin neurons (Hertel et al., 2001; Dremencov et al., 2007) is further evidence pointing to a possible role for risperidone in the regulation of TPH.

To investigate a role for atypical antipsychotic medications in the regulation of serotonin neurons in the DRN, we examined the effects of risperidone on cells containing TPH. We studied TPH immunoreactivity in the DRN, first in young animals and later in aged animals, following three days and 4 weeks of treatment, respectively. To assess for cell loss, we processed sections of young and aged animal DRN with a standard histological Nissl stain.

2. MATERIAL AND METHODS

2.1 Subjects

Thirty-six male Sprague Dawley rats (Charles-River, PQ) were singly housed with free access to food and water on a 12h:12h light dark cycle throughout the experiments. 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.

2.2. Treatment protocol

Experiment 1:

After one week of habituation to the animal care facility, the animals were randomly assigned to one of two treatment groups: (1) control or (2) risperidone. Groups of 8 week-old Sprague-Dawley rats received daily sub-cutaneous injections of either saline (N=9) or risperidone (Apotex, McMaster University Hospital Pharmacy) 1.5 mg/kg/day (N=9) for three days.

Experiment 2:

Male retired breeder rats were housed in the McMaster animal care facility from aged 6 months to approximately 2 years. At 2 years of age, the animals were randomly assigned to one of two treatment groups: (1) control or (2) risperidone. For 7 days prior to initiation of the experimental protocol, all rats received a daily 20 g meal of rice pudding. The animals were monitored over the course of the week to ensure that they consumed the pudding in a timely fashion and hence that the drug would reliably be delivered.

Groups of aged rats received daily 20 g meals of rice pudding either alone (N=9) or mixed with risperidone (Apotex, McMaster University Hospital Pharmacy) 1 mg/kg/day (N=9).

2.3 Immunohistochemistry

Twenty-four hours after the final treatment, all animals were deeply anaesthetized with 40 mg pentobarbitol i.p. and sacrificed. Young animals were sacrificed by decapitation; aged animals were transcardially perfused with 4% paraformaldehyde (PFA) before subsequent decapitation. Brains were removed, placed in 4% PFA and refrigerated at 4°C for 4 to 7 days. Brains were cryoprotected in 15% sucrose solution for 24 hours immediately prior to sectioning. Consecutive coronal sections (40 µm for young animals, 20 µm for aged animals) 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).

TPH Immunohistochemistry

Coronal sections of DRN (bregma -7.8 mm) were selected and processed for TPH immunohistochemistry. Free-floating sections were incubated in 0.3% hydrogen peroxide in methanol for 30 minutes at room temperature, rinsed twice in 0.1 M PBS and incubated in 5% normal donkey serum (NDS) for 1 hour. Sections were again rinsed three times in 0.1 M PBS and then incubated in sheep anti-TPH primary antibody (Millipore, CA, USA, 1:500) for 24 hours at 4°C. After three additional washes in 0.1 M PBS, young animal DRN sections were removed from light and incubated in donkey anti-sheep secondary antibody conjugated with AF488 (Millipore, CA, USA, 1:200), while aged animals DRN

sections were incubated in biotinylated anti-sheep IgG secondary antibody (Millipore, CA, USA, 1:200). Incubations in secondary antibody were for 1 hour at room temperature. After three washes, young animal sections were mounted and coverslipped with Prolong Gold mounting medium (Invitrogen, CA, USA). Aged animal sections were washed three more times in 0.1 M PBS, and then incubated in ABC solution (Vectastain ABC Systems, Vector Labs, ON, CA) for 1 hour at room temperature to form an avidin-biotin complex. After three final washes in 0.1 M PBS, the TPH immunocomplex was visualized with the chromagen diaminobenzidine tetrahydrochloride (DAB, Vector Labs, ON, CA). Aged animal sections were mounted on Aptex-coated slides (Sigma, St. Louis, MO, USA), air dried, and coverslipped with DPX neutral mounting medium (Sigma, St. Louis, MO, USA).

Histology: Cresyl Violet Staining

For both young and aged animals, coronal sections of DRN (bregma -7.8 mm) were selected, mounted onto Aptex-coated slides and allowed to air dry before processing with a standard cresyl violet procedure. Briefly, sections were immersed in 100% ethanol, followed by xylene and then rehydrated with descending concentrations of ethanol prior to immersion in cresyl violet acetate (Sigma, St. Louis, MO, USA; 6.25 mg/mL) for 2 minutes. After a brief rinse in distilled water, sections were dehydrated with ascending concentrations of ethanol, differentiated with subsequent immersions in 70 and 100% acid ethanols, and completely dehydrated with 100% ethanol. After a final clearing with xylene, sections were immediately coverslipped with DPX neutral mounting medium (Sigma, St. Louis, MO, USA).

Quantitative Morphometry

The selected coronal brain sections permitted evaluation of the DRN, a region known to be heavily populated with serotonergic neurons. Anatomical landmarks were used to ensure that comparable brain sections were analyzed for each region in each animal. To minimize error, all sections were cut and stained at the same time.

The microscope slides were coded and photographed at 5X on a confocal microscope (CarlZeiss LSM 510). The TPH-immunoreactive cells in the DRN were manually counted at 20X 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 were established for each nucleus of interest and every labeled cell within those boundaries was counted. After completion of manual counting, all images were decoded and arranged according to treatment group. The mean number of immunostained TPH-positive cells was determined for each group and a standard error of the mean was calculated.

For sections stained with cresyl violet, the microscope slides were coded and photographed with the 5x and 20x objectives on a confocal microscope (CarlZeiss LSM 510). The Nissl cells in the DRN were counted using the particle analysis feature of ImageJ software (version 1.43). A minimum enumerable cell size was preset on the ImageJ so that only large cells, with an area in the range of typical DRN serotonin cells, were counted.

Statistical Analysis

TPH and Nissl cell counts were analyzed by student's t-test. Statistical significance was defined as $p < 0.05$.

3. RESULTS

Experiment 1:

TPH

Risperidone, injected subcutaneously for 3 days, induced a significant 55% reduction (p=0.008) in the number of TPH-positive cells in the DRN of 8 week old rats (figure 1). Representative photomicrographs are shown in figure 2.

Nissl Counts

The 3 day course of risperidone treatment had no effect on the number of large Nissl-stained cells in the DRN of young rats (figure 3, p=0.900). Representative photomicrographs are shown in figures 4 and 5.

Experiment 2:

TPH

A 28 day course of oral risperidone significantly reduced the number of TPH-positive cells in the DRN of 2 year old rats (26%, p=0.038, figure 6). Representative photomicrographs are shown in figure 7.

Nissl Counts

Oral risperidone treatment for 28 days induced a significant 25% reduction in the number of large Nissl-stained cells in the DRN of aged rats (p=0.003, figure 8). Representative photomicrographs are shown in figures 9 and 10.

4. DISCUSSION

This study demonstrates that risperidone can induce a significant reduction of TPH-positive cell counts in serotonergic brainstem neurons within three days. Risperidone administration for 3 days or 28 days resulted in a significant decrease in the number of TPH-immunoreactive neurons in the DRN of young and aged animals, respectively. Histological examination of DRN sections showed that Nissl counts were reduced in aged animals but not in young animals, suggesting a possible aged-related vulnerability to risperidone toxicity.

To the best of our knowledge, this is the first direct evidence that risperidone can affect the biosynthetic enzyme for serotonin in brainstem serotonergic neurons. Literature reports have continued to focus on the influence of atypical antipsychotics on dopaminergic systems, with relatively little attention to their effects on serotonin synthesis and transmission, save for their purported role in reducing extrapyramidal side effects. This deficiency in the literature is surprising, especially given the high ratio of serotonin $5HT_{2A}$ to dopamine D2 binding that is characteristic of atypical antipsychotics (Svartengren and Simonsson, 1990; Axelsson et al., 1991; Schotte et al., 1996). Nonetheless, the literature published to date does suggest that risperidone increases serotonin efflux in the DRN and forebrain target regions (Hertel et al., 1996, Hertel et al., 1997a,b; Ichikawa et al., 1998; Hertel et al., 2001), and decreases the spontaneous firing of serotonin cells in the DRN (Hertel et al., 1997b; Dremencov et al., 2007).

The risperidone-mediated increases in DRN and cortical serotonin output have been attributed to antagonist activity at $5HT_{1D}$ receptors and α_2 noradrenergic receptors,

respectively (Hertel et al., 1997a; Hertel et al., 1999; Hertel et al., 2001). Serotonergic 5HT1D receptors reportedly inhibit serotonin release in the DRN (Davidson and Stamford, 1995; Pineyro et al., 1995) and it is well established that noradrenergic inputs to the serotonin terminal limit serotonin release via an inhibitory α_2 noradrenergic receptor (Gothert et al., 1981; Maura et al., 1982 Starke et al., 1989); antagonism at these sites by risperidone would produce a disinhibitory release of serotonin. The dose-dependent inhibition of serotonin cell firing in the DRN by risperidone is most likely mediated by enhanced $5HT_{1A}$ autoreceptor activation, since this effect is largely blocked by administration of the $5HT_{1A}$ antagonist, WAY 100,635 (Hertel et al., 1997a; Hertel et al., 1997b; Dremencov et al., 2007). The enhanced autoreceptor activation itself is presumably secondary to increased bioavailability of extracellular serotonin which would in turn inhibit impulse flow in serotonin neurons (Aghajanian, 1982).

Alternatively, blockade of α1 adrenoreceptors in the DRN by risperidone might partly explain the reduction in cell firing, since α1 heteroreceptors on serotonin cell bodies facilitate neuronal activity (Svensson et al., 1975; Baraban and Aghajanian, 1980), and the selective α1 antagonist prazosin inhibits neuronal activity (Hertel et al., 1997b). Attempts to prevent prazosin-induced inhibition of serotonin cell firing with the $5HT_{1A}$ receptor WAY 100,635, however, have not proved successful (Hertel et al., 1997a) and since WAY 100,635 has been previously shown to effectively attenuate the risperidone-mediated reduction of DRN cell firing (Hertel et al., 1997b), this suggests that α1 adrenoreceptor antagonism is not solely responsible for the regulatory influence of risperidone on serotonergic activity.

Tryptophan Hydroxylase+ Cell Counts in the DRN:

Our observation that risperidone reduces TPH immunoreactivity in the DRN is in line with risperidone's apparent ability to reduce serotonergic nerve firing. The underlying neurobiological mechanism for risperidone's influence on the biosynthetic enzyme for serotonin is not entirely clear, but it is reasonable to speculate that the $5HT_{1A}$ autoreceptor might again be involved. Augmented activation of the somatodendritic autoreceptor by raised extracellular serotonin levels reportedly prevents calcium-calmodulin-dependent activation of TPH (Sawada and Nagatsu, 1986); this might be one possible mechanism for the reduction of TPH immunoreactivity by risperidone.

The action of risperidone on brainstem serotonin neurons parallels that of the SSRI class of psychotropic medications. We previously reported that inhibition of SERT by SSRI medications can induce a regionally specific reduction of TPH-positive cell counts in brainstem serotonergic nuclei within 24 hours and that this reduction persists throughout a treatment course of at least 28 days (MacGillivray et al., 2010). Similar to risperidone, inhibition of the SERT by SSRIs also increases serotonin release and reduces neuronal firing in the DRN (Blier et al., 1987; Invernizzi et al., 1992; Arborelius et al., 1995). Nevertheless, since risperidone has relatively low affinities for the SERT and $5HT_{1A}$ autoreceptor (Leysen et al., 1992), it seems unlikely that risperidone's action on TPH is mediated by either of these receptor subtypes.

The functional significance of changes in TPH regulation in brainstem serotonin neurons is not immediately apparent. Atypical antipsychotics are touted for their reduced incidence of extrapyramidal side effects compared with typical antipsychotic medications

(Borison et al., 1992; Chouinard et al., 1993; Janicak et al., 2001), although this assertion has been contested, even by our own laboratory group (Knable et al., 1997; Rosebush and Mazurek, 1999a; Rosebush and Mazurek, 1999b). Nevertheless, attenuated serotonergic activity in the DRN might conceivably contribute to risperidone's allegedly improved extrapyramidal side effect profile since (1) serotonin has a well established inhibitory influence on nigrostriatal dopamine neurons (Dray et al., 1976; Fibiger and Miller, 1977; Dray et al., 1978) and (2) while the precise pathophysiology of drug-induced EPS has not been elucidated, there is strong evidence pointing to reduced dopaminergic signaling in the nigrostriatal dopamine system (Rosebush and Mazurek, 1995; Mazurek and Rosebush, 1996; Rosebush and Mazurek, 1999a; Rosebush and Mazurek, 1999b; Mazurek and Rosebush, 2005). Alternatively, risperidone-induced changes in TPH could actually contribute to a separate subset of side effects associated with the medication. Several of the common side effects of risperidone, including weight gain (Bobes et al., 2003b) and sexual dysfunction (Bobes et al., 2003a) are often linked with disordered serotonin functioning.

Nissl Counts in the DRN

The striking reduction of Nissl counts in aged animals is evidence of possible toxicity in the DRN. An obvious limitation of cresyl violet staining is its inability to differentiate among cell subtypes and as such, we are unable to conclude with certainty that Nissl cell loss in the DRN reflects loss of serotonin neurons. A high percentage of neurons in the DRN, however, are serotonergic (Baker et al., 1991) and the enumerated cells were restricted to those with an area in the range of a typical DRN serotonin neuron.

While the ability of antipsychotics to induce long lasting changes in the nigrostriatal dopamine system is well recognized (Melamed et al., 1991; Mazurek et al., 1998), we are not aware of any previous studies that have examined the potential for antipsychotics to cause toxic changes to serotonin cells. Our observation of reduced Nissl counts in the DRN in aged animals, but not young animals suggests that age-related decline in neuronal functioning might render aged animals more vulnerable to the toxic effects of psychotropic medications. Older animals are especially vulnerable to agents that interfere with mitochondrial function, possibly due to a loss of mitochondrial functional reserve. Antipsychotic medications, including risperidone, inhibit complex 1 of the mitochondrial electron transport chain (Maurer and Moller, 1997; Casademont et al., 2007) as does 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a neurotoxic agent used in animal models of Parkinson's disease. MPTP given to mice, for example, produces a much more profound degeneration of dopamine neurons in the substantia nigra of aged animals than young animals (Ricaurte et al., 1987). A similar process might be occurring in the DRN of aged animals. An alternative possibility is that the Nissl cell loss in the DRN is not related to vulnerability of aged animals, but instead to the length of risperidone administration. The two experiments described in this paper are not directly comparable. Young rats were given subcutaneous injections of risperidone for 3 days while aged animals were treated with oral risperidone for 28 days. Young animals might also have demonstrated Nissl cell loss in the DRN had we prolonged the duration of treatment, though our previous finding that a 21 day course of typical antipsychotic haloperidol produces reversible suppression of TH-positive cell counts in the substantia nigra of

young rats but induces apoptotic changes in dopamine neurons in the substantia nigra of aged rats (Krasnik, 2003) argues against this possibility.

Conclusions

The reduction of TPH immunoreactivity in the DRN by risperidone suggests that, in addition to their recognized affects on dopamine cells, atypical antipsychotics can also affect regulation of brainstem serotonergic neurons. The significant decrease of large Nissl counts in the DRN in aged animals but not young, suggests that risperidone may be toxic to vulnerable individuals. These reductions in TPH- and Nissl counts may play a role in mediating the therapeutic and toxic effects of risperidone.

ACKNOWLEDGEMENTS

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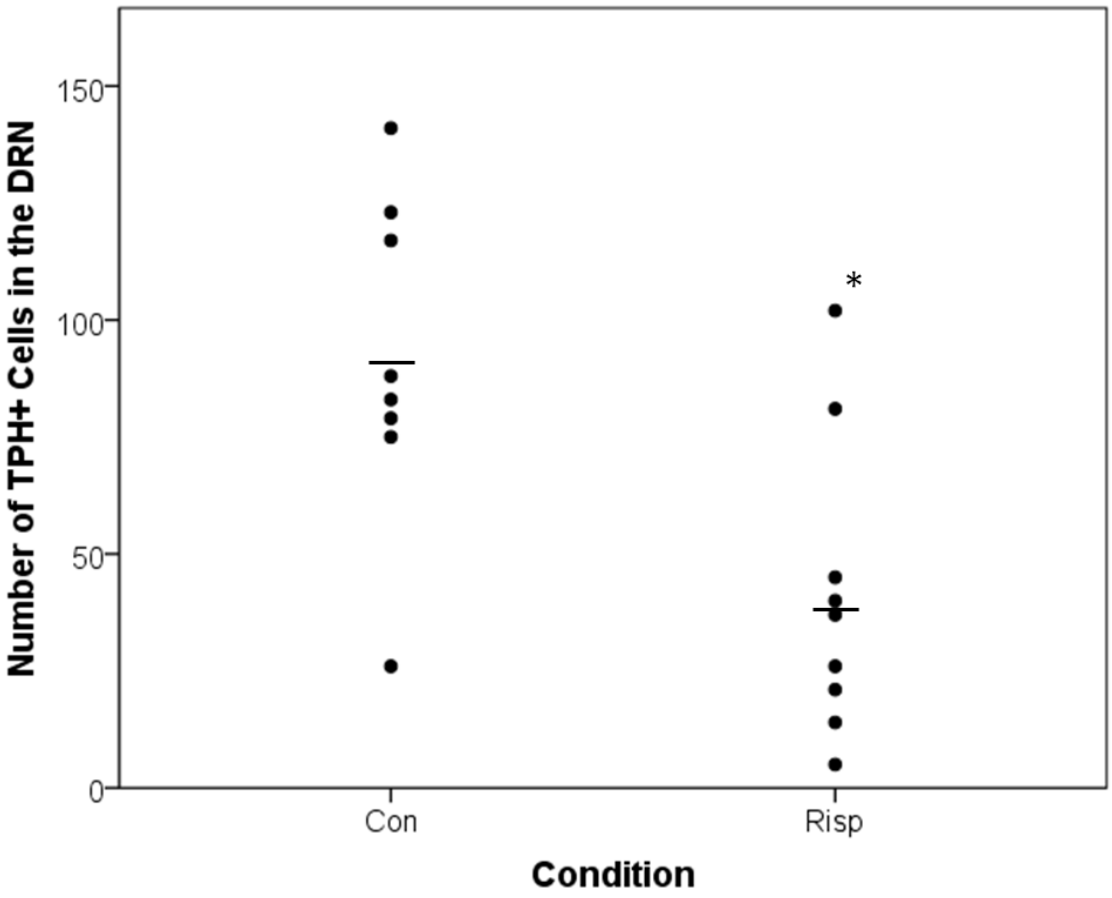

Figure 1. Number of TPH-positive cells per 40 µm section of young rat DRN. Each dot represents the number of TPH-positive cells for one animal; the horizontal line represents the group mean. Note: '*' indicates statistical significance (p<0.05 compared to controls). Con = Control; Risp = Risperidone.

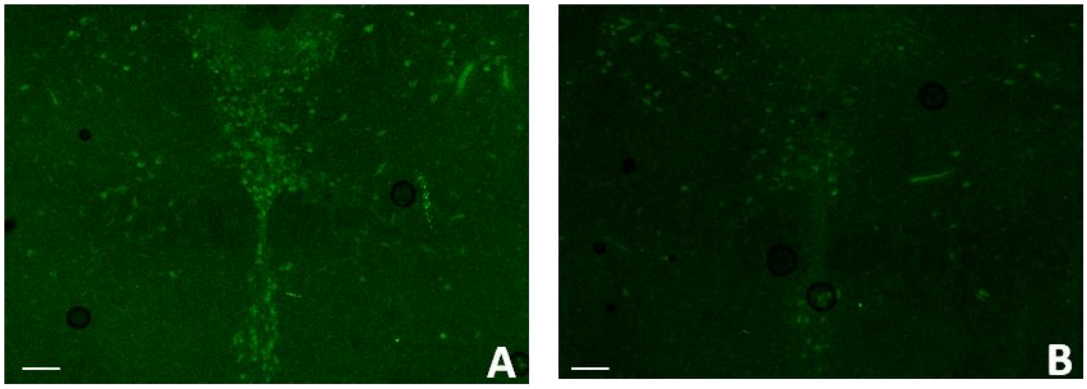

Figure 2. Photomicrographs (5x magnification) of TPH-immunoreactive cells in 40 µm sections of young rat DRN (bregma – 7.8mm). A, Control; B, Risperidone. Scale bar 200 µm.

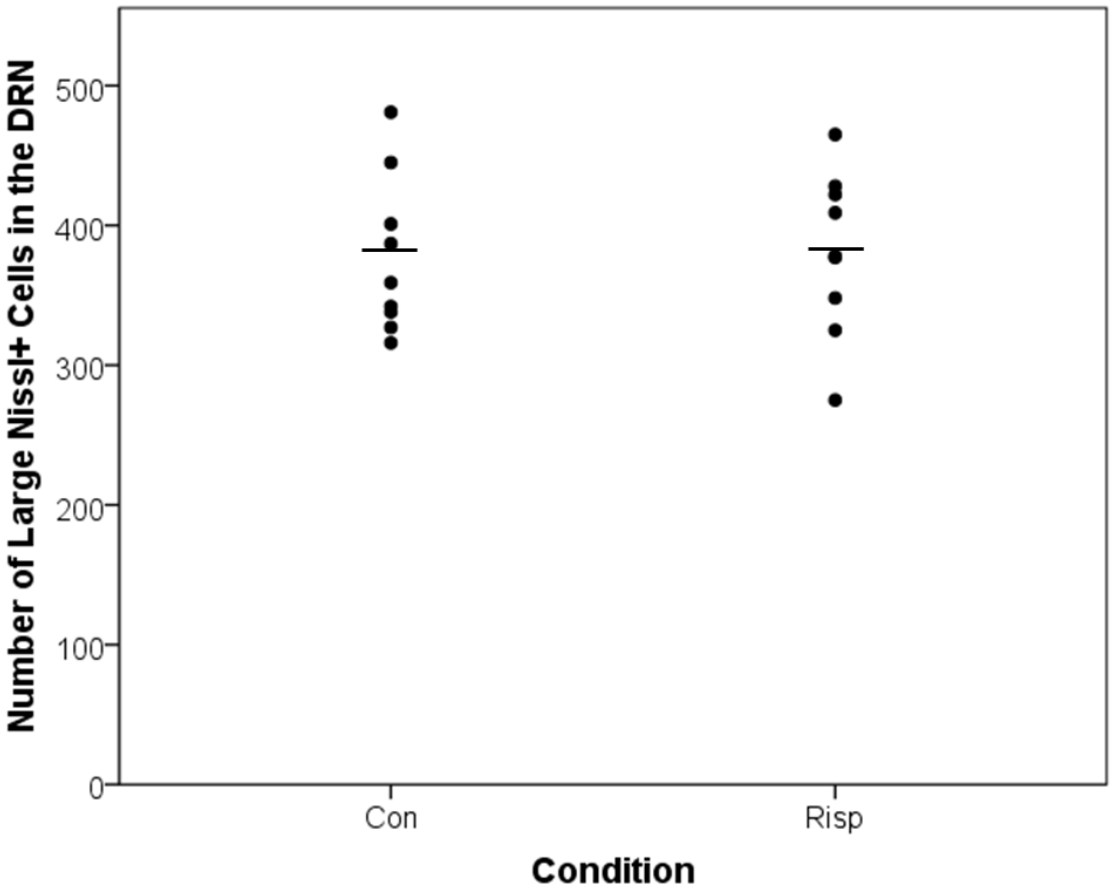

Figure 3. Number of Large Nissl-Stained cells per 40 µm section of young rat DRN. Each dot represents the number of Nissl-stained cells for one animal; the horizontal line represents the group mean. Note: '*' indicates statistical significance ($p<0.05$ compared to controls). Con = Control; Risp = Risperidone.

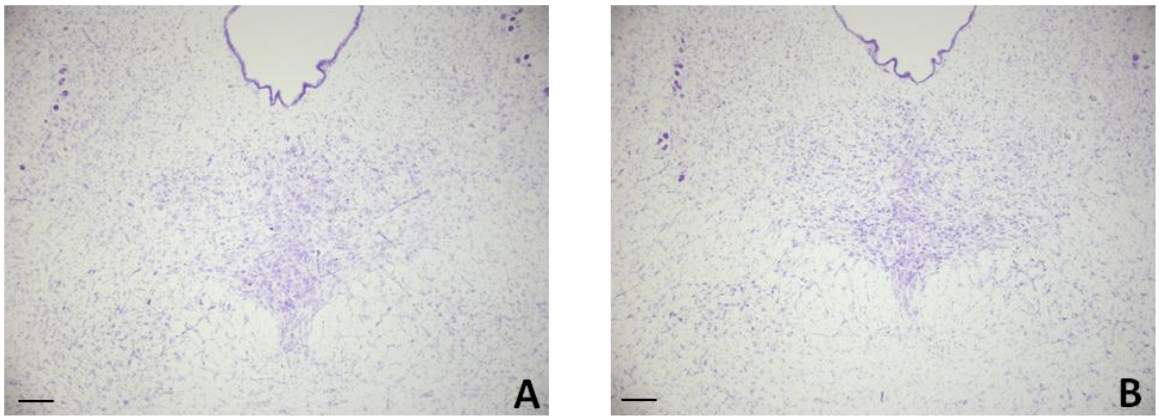

Figure 4. Photomicrographs (5x magnification) of Nissl-stained cells in 40 µm sections of young rat DRN (bregma – 7.8mm). A, Control; B, Risperidone. Scale bar 200 µm.

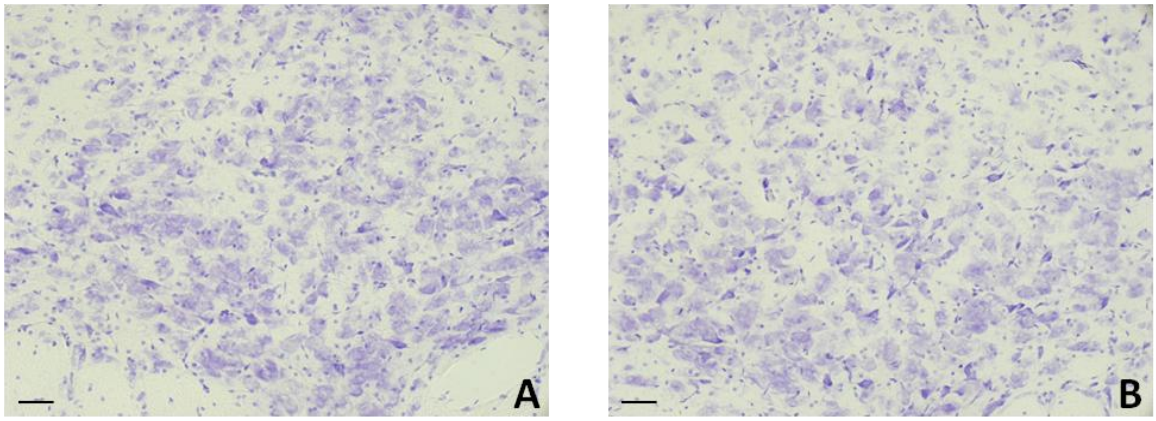

Figure 5. Photomicrographs (20x magnification) of Nissl-stained cells in 40 µm sections of young rat DRN (bregma – 7.8mm). A, Control; B, Risperidone. Scale bar 60 µm.

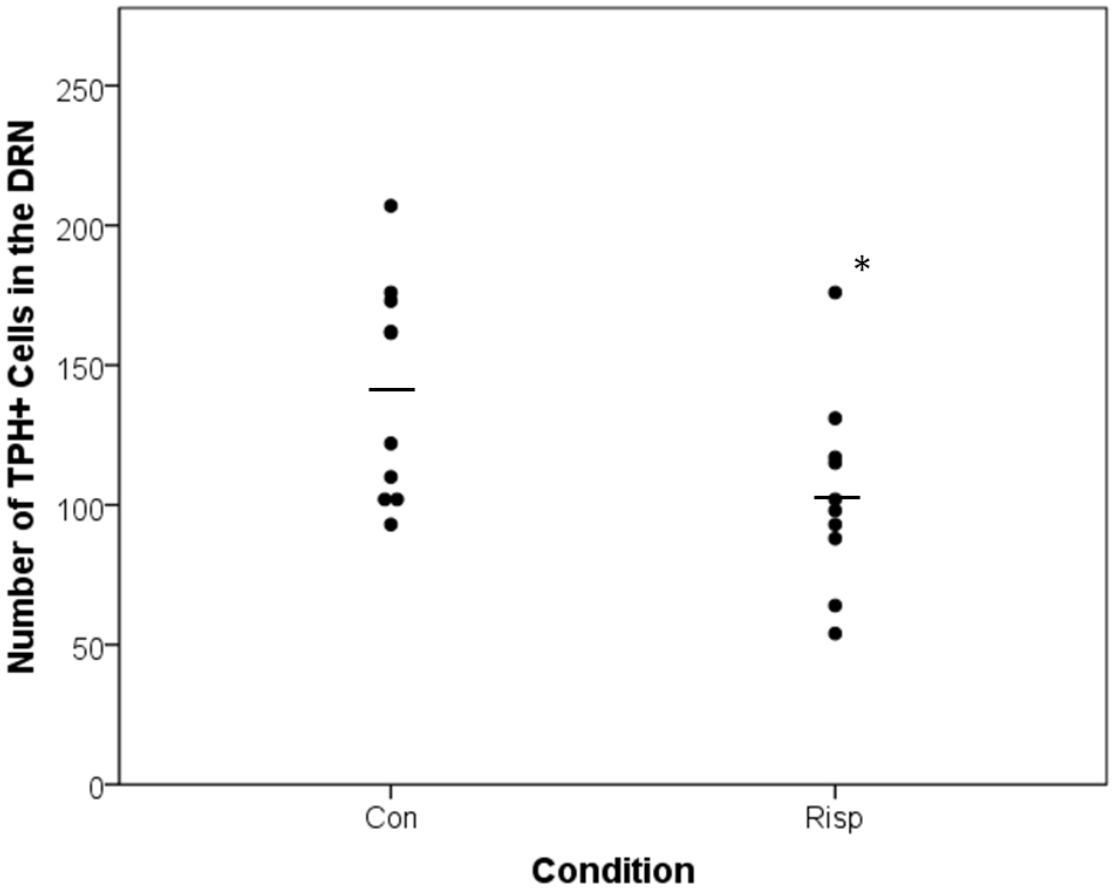

Figure 6. Number of TPH-positive cells per 20 µm section of aged rat DRN. Each dot represents the number of TPH-positive cells for one animal; the horizontal line represents the group mean. Note: '*' indicates statistical significance (p<0.05 compared to controls). Con = Control; Risp = Risperidone.

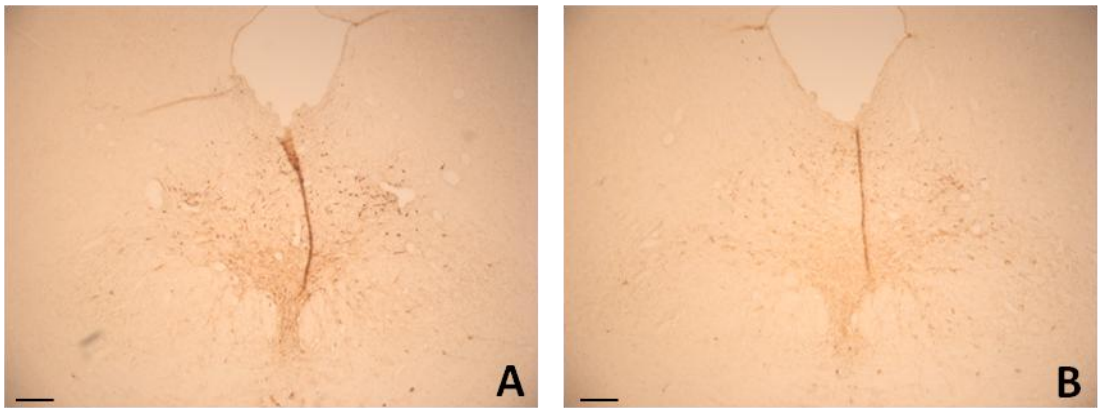

Figure 7. Photomicrographs (5x magnification) of TPH-immunoreactive cells in 20 µm sections of aged rat DRN (bregma – 7.8mm). A, Control; B, Risperidone. Scale bar 200 µm.

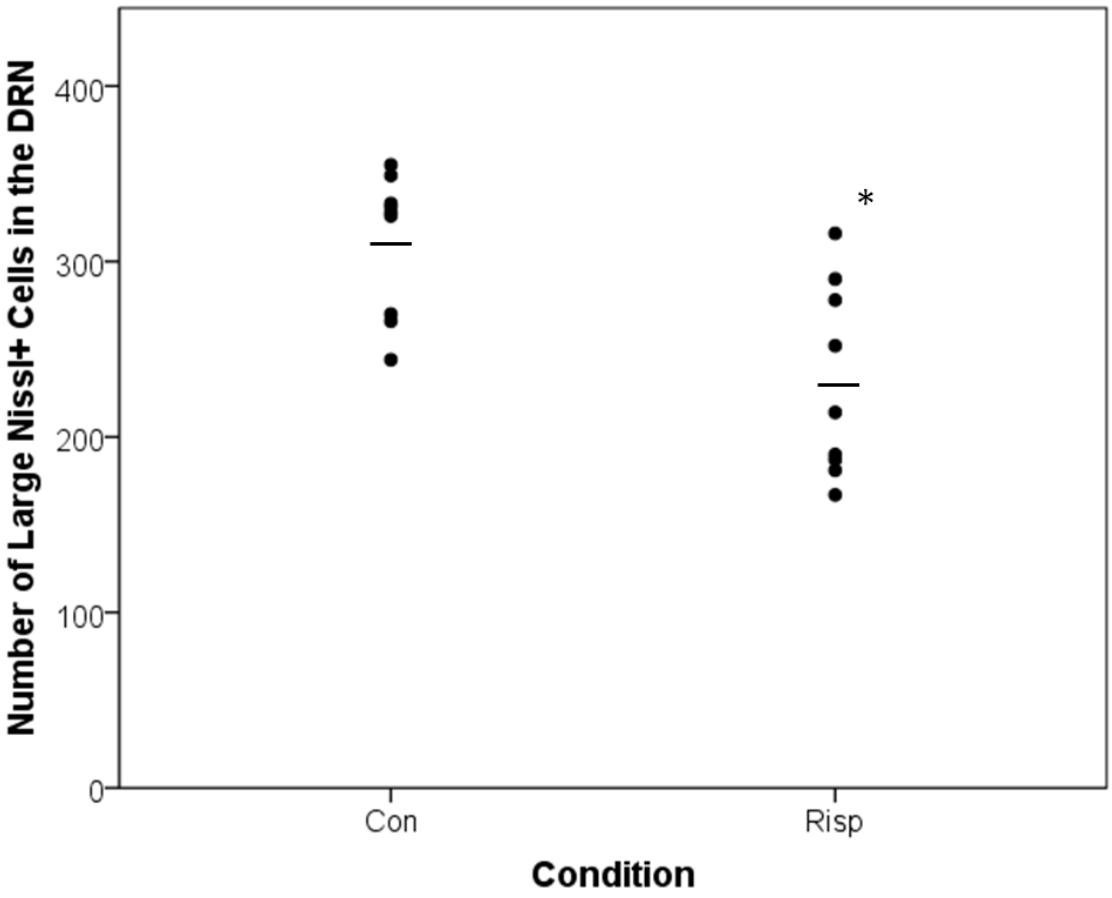

Figure 8. Number of Large Nissl-Stained cells per 20 µm section of aged rat DRN. Each dot represents the number of Nissl-stained cells for one animal; the horizontal line represents the group mean. Note: '*' indicates statistical significance (p<0.05 compared to controls). Con = Control; Risp = Risperidone.

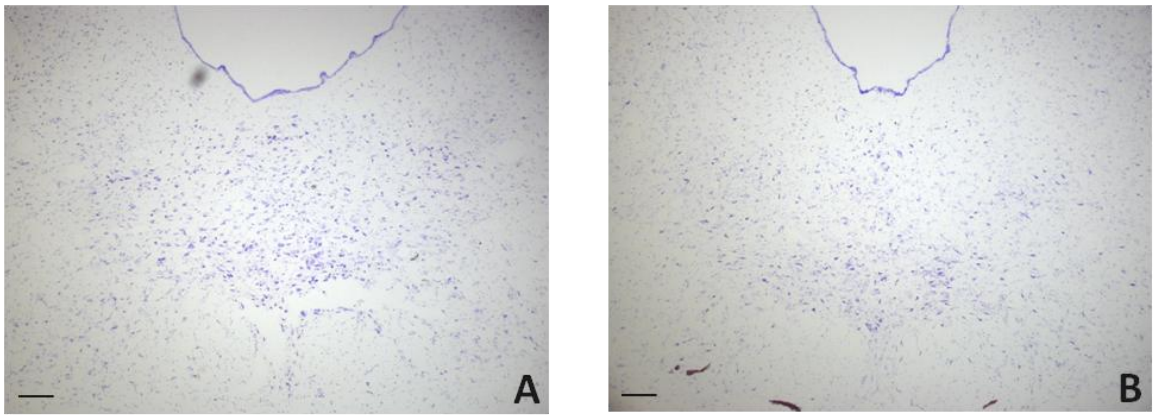

Figure 9. Photomicrographs (5x magnification) of Nissl-stained cells in 20 µm sections of aged rat DRN (bregma – 7.8mm). A, Control; B, Risperidone. Scale bar 200 µm.

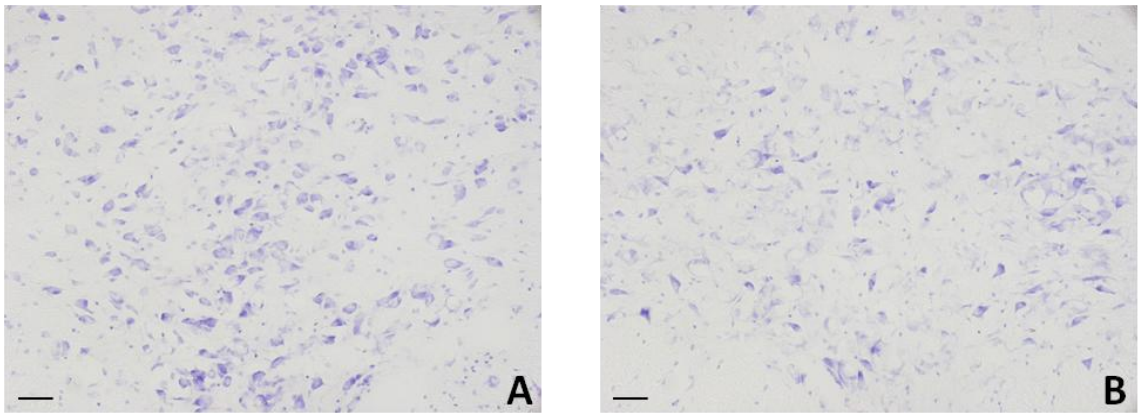

Figure 10. Photomicrographs (20x magnification) of Nissl-stained cells in 20 µm sections of aged rat DRN (bregma – 7.8mm). A, Control; B, Risperidone. Scale bar 60 µm.

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CHAPTER 8: Discussion

The brain serotonergic and dopaminergic systems broadly influence our internal experience and the ways in which we interact with the outside environment, with crucial regulatory roles in mood, sleep, appetite and the control of voluntary movement. Serotonin and dopamine neurons are themselves influenced by a wide variety of internal and external factors, many of which remain poorly understood. The central aim of this thesis was to better characterize several of these modulatory influences via exploratory investigations involving pharmaceutical agents or environmental modification. Specifically, I examined the modulatory effects of SSRIs, atypical neuroleptics and environmental enrichment with exercise on the regulation of brain serotonin and dopamine neurons.

This thesis documents, for the first time, that (1) inhibition of SERT by SSRIs induces a rapid and region-selective reduction of TPH-immunoreactive neurons in serotonergic brainstem nuclei that persists over a prolonged treatment course; that (2) selective blockade of SERT by SSRIs can rapidly induce a reduction of TH-positive dopaminergic neurons in the SN and the VTA that, again, persists over a lengthy treatment course; that (3) environmental enrichment with exercise can potentiate the effect of SERT inhibition on SN dopaminergic neurons, but not on the DRN serotonergic neurons; that (4) that SSRI fluoxetine triggers a significant upregulation of microglia in the SN; that (5) environmental enrichment with exercise can reduce TPH immunoreactivity in the DRN and TH immunoreactivity in the SN and VTA, even in the

absence of any pharmacological intervention, and finally, that (6) the atypical neuroleptic risperidone significantly reduces TPH in the DRN of both young and aged animals, but reduces DRN Nissl counts only in aged animals.

The ability of two different SERT inhibitors to downregulate serotonergic neurons in select raphe nuclei is outlined in Chapter 3. Specifically, I found that SSRIs citalopram and fluoxetine both reduced the number of TPH-positive cells in three of the raphe nuclei that we studied: the DRN, MRN and RMN. These reductions were present 24 hours after a single dose was administered and were still present after 28 days of treatment. By contrast, neither citalopram nor fluoxetine affected TPH-positive cell counts in the CLN. SSRI-induced reductions in TPH immunoreactivity may be the consequence of enhanced stimulation of the $5HT_{1A}$ autoreceptor by increased extracellular concentrations of serotonin. One could speculate that a relative paucity of $5HT_{1A}$ receptors in the CLN might render this region less susceptible to SSRI-mediated reduction of intracellular TPH. Decreased SERT density in the CLN compared to the DRN, MRN and RMN might also or alternatively account for the inability of SSRIs to affect TPH in this nucleus. Clinically, the persistent reduction of TPH might partly account for the common and often severe withdrawal syndrome associated with SSRI medications. Reduced TPH suggests that serotonin production is reduced, and sudden discontinuation of SSRI medication would presumably exacerbate the already reduced serotonin neurotransmission.

In chapter 4, my focus shifts to the influence of SERT inhibition on dopamine neurons in the SN. Our novel observation that both fluoxetine and citalopram reduced the

number of TH-positive cells in the rat SN indicates that SSRIs may compromise the presynaptic integrity of nigrostriatal DA neurons - a phenomenon that could conceivably explain the tendency of SSRI medications to cause extrapyramidal side effects in humans. Our assertion that SSRIs compromise nigrostriatal DA function may be relevant to the clinical efficacy of these drugs. Numerous authors have suggested that dopaminergic neurotransmission is reduced in major depression (Bowers et al., 1969; Mendels et al., 1972; Roy et al., 1989; Martinot et al., 2001). If depressed patients tend already to be DA deficient, SSRI treatment may actually worsen this deficiency, a possibility that could in part explain the drugs' marginal therapeutic effects.

The ability of SSRI fluoxetine to dramatically upregulate microglial recruitment in the SN is also highlighted in Chapter 4. To the best of our knowledge, the influence of SERT inhibition on microglia recruitment and activation has not previously been studied. Members of our laboratory group, however, have several times observed that the antipsychotic drug haloperidol reduces TH immunoreactivity and upregulates microglia in the SN. Since antipsychotic drugs and SSRI medications both appear to downregulate TH, it is worth considering that the abilities of these drugs to increase microglial recruitment might have similar downstream consequences. Demonstrations that the blockade of microglial activation is neuroprotective in the MPTP mouse model of Parkinson's disease (Du et al., 2001; Wu et al., 2002) and in our own investigations with haloperidol suggests that microglial activation might be a significant instigating factor in neuroleptic-induced neurotoxicity. Translated to the SSRI model, microglial activation

and subsequent dopaminergic toxicity might be one way in which fluoxetine compromises the integrity of nigrostriatal dopamine cells.

Chapters 5 and 6 focus on the modulatory influence of an enriched environment with exercise on the SSRI-induced downregulation of TPH and TH immunoreactivity in the DRN and SN, respectively. We were astonished by the observation that environmental enrichment with exercise profoundly downregulated serotonergic and dopaminergic neurons, even in the absence of any drug treatment. The functional significance of this reduction by environmental enrichment is not yet clear, but at the very least, it highlights the ability of non-pharmacological experience to affect brain chemistry. The ability of environmental enrichment with exercise to independently reduce TPH immunoreactivity in the DRN is particularly interesting because it might possibly account, or partly account, for the shared abilities of SSRIs and exercise to treat depression. Housing animals in an enriched environment exacerbated the effect of SSRI medication on TH immunoreactivity in the SN, but had no potentiating impact on the SSRI-induced reductions of TH-positive cell counts in the VTA or on the TPH-positive cell counts in the DRN. The interpretation of these findings is not straightforward; it is possible that SSRIs and environmental enrichment have an additive effect on TH immunoreactivity in the SN and not in the DRN or VTA because the two share common modulatory mechanisms in two nuclei and but act via independent mechanisms in the other.

The fifth and final paper of this thesis (Chapter 7) addresses the regulation of TPH in the DRN by the atypical neuroleptic risperidone. Risperidone is potent antagonist of

the serotonin $5HT_{2A}$ and $5HT_{2C}$ and D2 dopamine receptors, and unlike most typical antipsychotics, binds with higher affinity for $5HT_{2A}$ receptors than for D2 receptors (Leysen et al., 1988; Schotte et al., 1996). Because of its action on serotonin as well as dopamine neurotransmission, risperidone is reported to have a better side effect profile than those of the typical class. Reports that risperidone increases extracellular serotonin (Hertel et al., 1997) and decreases the firing rate (Hertel et al., 2001; Dremencov et al., 2007) of serotonin neurons in the DNR suggested to me that risperidone might downregulate TPH as well. Indeed, risperidone significantly reduced the number of TPH-positive cells in both young and aged rats. Remarkably, risperidone also resulted in a loss of Nissl bodies in the DRN of aged rats, suggesting that risperidone might produce toxic changes in serotonin neurons of vulnerable individuals.

Taken together, the body of work included in this thesis suggests that SSRIs, atypical neuroleptics and environmental enrichment with exercise can have profound effects on brain serotonergic and dopaminergic neurons. In some cases, reduced staining for the dopamine and serotonin biosynthetic enzymes might be indicative of compromised neuronal integrity, as appears to be the case with risperidone in which reduction of TH-positive cell counts in aged animals was associated with a loss of Nissl bodies as well. These changes might possibly account for some of the side effects associated with the SSRI and atypical neuroleptic classes of psychotropic medication. Alternatively, changes in the biosynthetic enzymes for dopamine and serotonin might by related to the therapeutic action of a drug or environmental intervention, as was raised by

the observation that SSRIs and environmental enrichment both reduce TPH immunoreactivity in the DRN.

The findings from this thesis provide a clear direction for future work. An obvious priority is a withdrawal experiment in which an SSRI or atypical neuroleptic is administered for a specified period of time and then withdrawn. Examination of TPH- and TH-positive cell counts in these animals after variable periods of withdrawal would help clarify whether the biosynthetic enzymes for serotonin and dopamine are permanently reduced. Should the TPH- and TH-positive cell counts remain reduced after a significant withdrawal period, immunohistochemistry for markers of apoptosis would be a useful next step for clarifying the fate of the affected cells. Since the effects of SSRIs, atypical neuroleptics and possibly environmental enrichment on TPH and TH immunoreactivity might possibly be mediated by the $5HT_{1A}$ autoreceptor on serotonin neurons, a reasonable approach would be to pharmacologically block the autoreceptor and reassess TPH and TH immunoreactivity; one would expect this blockade to prevent the downregulation of the biosynthetic enzymes.

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