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Eileen K Sawyer Date

Neuroadaptive Changes in the Serotonin System Associated with Chronic SSRI Treatment in the Context of Cocaine Use

By

Eileen K Sawyer

Doctor of Philosophy

Graduate Division of Biological and Biomedical Sciences

Neuroscience

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Neuroadaptive Changes in the Serotonin System Associated with Chronic SSRI Treatment in the Context of Cocaine Use

By

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B.A., Smith College, 2007

Advisor: Leonard L. Howell, PhD

An abstract of

A dissertation submitted to the Faculty of the

James T. Laney School of Graduate Studies of Emory University

in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

in Graduate Division of Biological and Biomedical Sciences

Neuroscience

2011

Abstract

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By Eileen K Sawyer

Rationale: Acute administration of selective serotonin reuptake inhibitors (SSRIs) has been shown to attenuate the behavioral effects of cocaine in pre-clinical studies but SSRIs have not proven successful in human clinical trials, which may be due to differences in acute versus chronic dosing regimens. Since potential pharmacotherapies for cocaine abuse need to be administered chronically, and the mechanism for SSRIs' therapeutic effects is believed to be neurobiological changes that emerge during chronic, but not acute, administration, we first designed and then examined the effects of a clinically-relevant dosing regimen with fluoxetine on cocaine-related behavior and neurochemistry and potential underlying mechanisms in the serotonin system.

Methods: We designed a dosing regimen with fluoxetine in rhesus macaques that approximates human conditions over 4-6 weeks. As there is no clear serum concentration-therapeutic effect relationship for depressive symptoms and the mechanism for SSRIs' neurobiological changes are not well-understood, we matched the pharmacokinetic measures as closely as possible to those reported in human clinical studies. We then evaluated the effects of this chronic treatment (10 mg/kg/day, oral) in a repeated measures design on cocaine self-administration, reinstatement, and dopamine overflow in rhesus macaques trained to self-administer cocaine. In order to determine potential neurobiological mechanisms, we examined serotonin transporter and 2A receptor binding using positron emission tomography and serotonin overflow using *in vivo* microdialysis. Prolactin challenges served as a measure of integrated serotonin function.

Results: Rates of ongoing cocaine self-administration behavior were not affected while reinstatement to cocaine prime was attenuated during treatment and after washout. The dopamine response to cocaine was also attenuated during treatment and after a 6-week washout period. These effects were associated with a persistent increase in 5HT2A binding potential and decreased prolactin response, despite no changes in serotonin overflow or the transporter.

Conclusions: Together, the results suggest that chronic fluoxetine treatment is associated with a desensitization of the 5HT2A receptor which may lead to a decrease in cocaine-elicited dopamine overflow and thus cocaine-primed reinstatement. These results suggest that while fluoxetine may not be an effective intervention for ongoing cocaine abuse, it may be useful as a treatment to prevent relapse.

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Acknowledgements

I would like to thank my advisor, Dr. Leonard Howell, and the current and past member of his lab for their help, guidance, and support: Dr. Heather Kimmel, Dr. Kevin Murnane, Dr. Daniel Manvich, Lisa Neidert, Juliet Brown, Jessica McClung, Debra Cooper, and Dr. Monica Andersen. I also thank the members of my committee for their guidance and support: Dr. Michael Owens, Dr. James Rowlett, Dr. John Votaw, and Dr. Mark Wilson. I greatly appreciate the assistance of Dr. James Ritchie, Bailey Glover, and Catie Capello with the fluoxetine MS/MS analyses, and the assistance of Dr. Jonathon Nye, Paul Chen, and the Yerkes Imaging Core staff with the PET scans and analyses. Thanks also to the Yerkes National Primate Center veterinary, animal care, and facilities staff for their excellent care, and last but not least to the monkeys.

This work was funded by DA10344 (LLH), DA000517 (LLH), and RR00065 (YNPRC). EKS received support from T-32 DA015040 and T-32GM008605.

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1. GENERAL INTRODUCTION

Cocaine abuse is a serious problem, with 1.1 million people meeting the criteria for abuse in 2009 (Substance Abuse and Mental Health Services Administration, 2010) and an estimated societal cost of $46,000 million per year in health care, lost productivity, and crime (Cartwright, 2000). Thirty six percent of student in high school say cocaine or crack is easily obtainable (Johnston et al., 2011) and there were an estimated 722,000 new initiates to cocaine use in 2008 (Office of National Drug Control Policy, 2010). Cocaine was responsible for 24% of emergency department drug-related visits in 2008 and accounted for 13% of admissions to treatment programs (Office of National Drug Control Policy, 2010; Substance Abuse and Mental Health Services Administration, 2010), yet there is currently no FDA-approved effective pharmacotherapy for cocaine addiction (Volkow and Li, 2004; Vocci et al., 2005). While substitute agonist therapies are being considered (Howell and Wilcox, 2001a), these medications often have high abuse potential. Different pharmacological targets should be considered.

1.1 Cocaine

Cocaine is a nonselective monoamine reuptake inhibitor (Reith et al., 1986; Madras et al., 1989), binding to and blocking the action of the norepinephrine, serotonin (5HT), and dopamine (DA) transporters (NET, SERT, DAT, respectively), which causes acute increases in the extracellular levels of these neurotransmitters (Chen and Reith, 1994). However, cocaine's reinforcing and motor-stimulant effects are believed to rely primarily on DAT blockade (Ritz et al., 1987). Strong correlations between DAT binding and the behavioral effects of cocaine have been demonstrated using cocaine analogs in mice (Cline et al., 1992)

and nonhuman primates (Bergman et al., 1989; Spealman et al., 1989). DAT knockout mice fail to reliably self-administer cocaine (Thomsen et al., 2009), and in humans, DAT blockade is correlated with the intensity of the perceived "high" (Volkow et al., 1997). Furthermore, the effects of DAT blockade appear to be mediated by the resulting increase in DA neurotransmission (Woolverton and Johnson, 1992). Like DAT blockade, the amount of released DA in humans is correlated with the intensity of the perceived "high" (Volkow et al., 1999b).

The dopamine system has three major projections: the nigrostriatal system, originating in the substantia nigra and projecting to the striatum; the mesocortical system, originating in the ventral tegmental area (VTA) and projecting to the frontal and prefrontal cortex; and the mesolimbic system, also originating in the VTA in a distinct but overlapping population of cells, and projecting to the nucleus accumbens (Fallon, 1988; Alex and Pehek, 2007). 6-hydroxydopamine lesions in either the VTA or the nucleus accumbens markedly reduced cocaine self-administration in rats, indicating that the mesolimbic pathway is crucial to the process of drug reinforcement (Roberts et al., 1977; Roberts et al., 1980; Roberts and Koob, 1982; Caine and Koob, 1994). Contingent or non-contingent administration of systemic cocaine also results in dose-dependent increases in DA in the nucleus accumbens (Pettit and Justice, 1989; Pettit and Justice, 1991). Additionally, Goeders and colleagues demonstrated that rats will self-administer cocaine into the medial prefrontal cortex (mPFC) and that this is disrupted by 6-OHDA lesions in the mPFC, indicating a role for the mesocortical projection in reinforcement as well (Goeders et al., 1986; Goeders and Smith, 1986). This mesocorticolimbic pathway has been considered the "final common pathway"

through which drugs of abuse exert their reinforcing and subjective effects (Koob, 1992; Nestler, 2005; Pierce and Kumaresan, 2006).

5HT may also play a role in cocaine's effects, as acute cocaine administration results in an increase of extracellular 5HT, due to cocaine's blockade of the SERT, and a decrease in raphe cell firing partially due to auto-inhibition via 5HT1A receptors (Kirby et al., 2011). Repeated administration of cocaine results in sensitization of both DA and 5HT release in the nucleus accumbens in rats (Parsons and Justice, 1993b), and manipulations of the 5HT system can enhance the discriminative-stimulus effects of low doses of cocaine (Cunningham and Callahan, 1991; Schama et al., 1997).

However, 5HT appears to play an inhibitory role in modulating the abuse-related effects of cocaine. Manipulations of the 5HT system through selective reuptake inhibitors or direct agonists are able to attenuate the behavioral (Carroll et al., 1990; Richardson and Roberts, 1991; Spealman, 1993; Howell and Byrd, 1995; Czoty et al., 2002; Glatz et al., 2002; Ruedi-Bettschen et al., 2009) and neurochemical effects (Czoty et al., 2002) of cocaine in rodents and primates. In humans, pretreatment with a selective serotonin reuptake inhibitor (SSRI) decreases ratings of cocaine's positive effects (Walsh et al., 1994). Similarly, increasing the relative potency of a compound at the SERT compared to DAT decreases its abuse-related effects in rats (Baumann et al., 2011) and primates (Wee et al., 2005; Kimmel et al., 2009). Together, these studies suggest that 5HT is able to negatively modulate the abuse-related effects of psychostimulants.

1.2 Serotonin and dopamine

The 5HT system may be able to influence cocaine's effects through its direct and indirect interaction with the DA system. The raphe nucleus, which contains serotonergic cell bodies, projects to key brain areas involved in drug abuse, including the VTA, the nucleus accumbens, dorsal striatum, and the prefrontal and frontal cortex (Parent et al., 1981; Halliday and Tork, 1989; Di Matteo et al., 2008). Furthermore, serotonin neurons synapse directly onto both DA and non-DA neurons in the VTA (Herve et al., 1987), positioning them to influence the output of the VTA in a variety of ways.

The role of endogenous serotonin in regulating DA appears complex, with many varied reports in the literature. Microdialysis studies demonstrated that direct infusion of serotonin into the PFC (Iyer and Bradberry, 1996) or nucleus accumbens (Parsons and Justice, 1993a) can locally increase DA release; however, this effect desensitized following either continuous or repeated pulses of 5HT (West and Galloway, 1996). Dorsal raphe electrical stimulation, which increases 5HT transmission, increased DA release in the nucleus accumbens but reduced it in the striatum (De Deurwaerdere and Spampinato, 1999), while infusing 5HT directly into the dorsal raphe reduced both 5HT and DA in the nucleus accumbens (Yoshimoto and McBride, 1992). Lesioning the 5HT system enhanced the locomotor response to amphetamine, a DA releaser (Geyer et al., 1976). Together, the variety of data reported in the literature indicates that 5HT modulates DA in complex manner that depends on the brain region and receptor subtypes involved (Hayes and Greenshaw, 2011).

1.2.1 SERT and SSRIs

SERT functions to remove 5HT from the synapse and thus terminate 5HT signaling. SERT binding is detected widely throughout the human brain, with highest concentrations in the raphe nuclei, substantia nigra, caudate, putamen, hypothalamus, and hippocampus.

Moderate binding is found in the amygdala, thalamus, and most cortical areas as well (Cortes et al., 1988; Laruelle et al., 1988). Similar broad distribution of SERT is also reported in the rat brain, although relative concentrations between areas differ slightly, with highest binding in the midbrain nuclei and the brainstem (De Souza and Kuyatt, 1987). Subcellularly, SERT is not confined to synapses; a large population of transporters is present along axons, suggesting that SERT not only regulates synaptic 5HT transmission, but also extrasynaptic volumic transmission (Zhou et al., 1998).

SSRIs bind and block the SERT and are currently approved to treat depression and mood disorders. When given acutely, SSRIs cause an increase in 5HT in rats (Kreiss and Lucki, 1995; Wong et al., 1995; Clark et al., 1996; Marek et al., 2005; Qu et al., 2009) and may affect DA transmission, although the effects may vary depending on brain region and particular SSRI used. For example, local perfusion of alaproclate into the striatum increased local DA (Yadid et al., 1994), while acute and chronic systemic administration of a different SSRI, fluoxetine, decreased DA in the striatum (Perry and Fuller, 1992; Ichikawa and Meltzer, 1995) and nucleus accumbens (Ichikawa and Meltzer, 1995; Ichikawa et al., 1998) but increased DA in the PFC (Bymaster et al., 2002). Systemic administration of various other SSRIs had no effect on DA in the PFC (Bymaster et al., 2002). In addition, both acute (Di Mascio et al., 1998) and sustained (Dremencov et al., 2009) administration of SSRIs can actually reduce the firing and burst rate of DA cells in the VTA, which would predict reductions in DA in brain areas innervated by it.

Some of these varying effect may be due to the fact that, although all SSRIs bind to SERT with high affinity and selectivity compared to other monoamine transporters, they possess varying nonselective effects (Sanchez and Hyttel, 1999). For example, fluoxetine, the

prototypical SSRI, has moderate affinity for the 5HT2C receptor, which it antagonizes (Palvimaki et al., 1996; Sanchez and Hyttel, 1999).

Preclinically, SSRIs have shown promise as a potential treatment for cocaine addiction, having been reported to attenuate the reinforcing (Carroll et al., 1990; Richardson and Roberts, 1991; Czoty et al., 2002; Glatz et al., 2002), reinstating (Ruedi-Bettschen et al., 2009), and locomotor stimulant (Spealman, 1993; Howell and Byrd, 1995) effects of cocaine in rodents and nonhuman primates. Furthermore, alaproclate attenuated the cocaine-induced increase in DA in nonhuman primates (Czoty et al., 2002) while pretreatment with fluoxetine decreased ratings of cocaine's positive subjective effects in a human laboratory setting (Walsh et al., 1994).

However, despite the overwhelming evidence suggesting that SSRIs can reduce abuse-related effects of cocaine, SSRIs have repeatedly failed to show reductions in cocaine abuse in clinical trials (Grabowski et al., 1995; Batki et al., 1996; Cornelius et al., 1998; Schmitz et al., 2001; Lima et al., 2003; Winstanley et al., 2011). This may be due in part to the different dosing regimens employed by the preclinical and clinical studies; preclinical studies generally employ single dose treatments whereas clinical studies administer the drug chronically. Furthermore, it is well known that the therapeutic effects of SSRIs for depression and mood disorders do not emerge until 3-4 weeks after beginning treatment, an effect which is believed to rely on neurobiological changes (Wong et al., 1995; Thompson, 2002; Vaswani et al., 2003). For example, 5HT levels are not elevated during chronic treatment (Clark et al., 1996; Smith et al., 2000), which is currently thought to be due to down-regulation of the SERT (Benmansour et al., 1999; Horschitz et al., 2001; Benmansour et al., 2002; Kugaya et al., 2003; Iceta et al., 2007) and desensitization of the 5HT1A

autoreceptor (Ceglia et al., 2004; Riad et al., 2008) which do not occur during acute treatment. Neurobiological changes due to prolonged SSRI treatment may alter the effects of the SSRI on cocaine-related behavior and neurobiology. Additionally, the presence of cocaine-induced neurobiological changes, such as an increase in SERT (Mash et al., 2000; Banks et al., 2008), could alter the effects of prolonged SSRI treatment. In addition to changes in SERT, prolonged use of a reuptake inhibitor may also induce changes in the post-synaptic receptors. Therefore, in order to fully understand the effects of chronic administration of reuptake inhibitors, both the role of and the effects on post-synaptic receptors must be considered.

1.2.2 Serotonin 2A receptors

5HT receptors are widely expressed throughout the brain, and several different 5HT receptor types have been implicated in mediating the effects of endogenous 5HT on dopamine (Bubar and Cunningham, 2006; Alex and Pehek, 2007; Bubar and Cunningham, 2008; Di Matteo et al., 2008; Navailles and De Deurwaerdere, 2011) but the serotonin 2A (5HT2A) and 2C receptors in particular have been implicated as likely candidates for mediating the influence of 5HT in drug abuse (Bubar and Cunningham, 2006; Bubar and Cunningham, 2008). 5HT2A and 2C receptors are expressed throughout the brain with distinct but overlapping expression patterns (Pompeiano et al., 1994), and frequently functionally oppose each other in regulation of the DA system (Bubar and Cunningham, 2008). For the purposes of this study, we will focus on the 5HT2A receptor, which has been implicated in the therapeutic effects of SSRIs (Marek et al., 2005; Meyer, 2007), and its role in DA regulation and cocaine-related effects.

5HT2A receptors are seven transmembrane region metabotropic receptors and couple to Gq, resulting in stimulation of either phospholipase C (PLC) or A_2 (PLA_2) (Bubar and Cunningham, 2006). PLC cleaves membrane lipids to form diacylglycerol (DAG) and inositol triphosphate (IP_3), which active protein kinase C and release intracellular stores of Ca^{2+}, respectively, ultimately stimulating the cell as well as enabling other protein and signaling changes. PLA_2 releases archadonic acid, which can then be metabolized to form a variety of secondary effectors that can affect adenlyl cyclase, protein kinase C, or ion channel function (Kandel et al., 2000). 5HT2A receptors have a degree of constitutive activity *in vivo* (Bubar and Cunningham, 2006; Alex and Pehek, 2007) and possible homodimerization and functional selectivity among the different effector pathways (Raote et al., 2007; Brea et al., 2009).

High levels of 5HT2A receptors and mRNA have been reported in the rat, non-human primate and human cortex, particularly in layer V, as well as moderate levels in the nucleus accumbens and striatum (Pompeiano et al., 1994; Lopez-Gimenez et al., 1997; Cornea-Hebert et al., 1999; Lopez-Gimenez et al., 2001; Forutan et al., 2002; Varnas et al., 2004). 5HT2A receptors are also expressed throughout the VTA in rats (Nocjar et al., 2002) and humans (Ikemoto et al., 2000) on DA cells that project mesolimbically and mesocortically. Immunocytochemical studies suggest that the receptors are often localized to the dendrites of these cells (Doherty and Pickel, 2000). Similarly, in the cortex, 5HT2A are predominately localized post-synaptically on dendrites (Miner et al., 2003), although there is some support for a small population of receptors pre-synaptically located on monoaminergic fibers (Jakab and Goldman-Rakic, 1998; Miner et al., 2003). Electron and light microscopy studies demonstrate that these post-synaptic sites are largely on pyramidal neurons, likely in the

apical dendritic region, and that most pyramidal neurons express 5HT2A receptors (Jakab and Goldman-Rakic, 1998). Additionally, some large- and medium-size parvalbumin- and calbindin-containing GABAergic interneurons that synapse on pyramidal cell bodies also express 5HT2A (Jakab and Goldman-Rakic, 2000).

In the mesolimbic system, 5HT2A receptor activation facilitates DA cell activity and release. *In vitro* work demonstrated that 5HT increased the firing rate of VTA DA neurons, and that this effect was blocked by ketanserin, a preferential 2A antagonist (Pessia et al., 1994). Additionally, DOI, a 5HT2A/2C agonist, increased both VTA cell firing and DA release *in vivo*, which is reversed by pretreatment with M100907, a selective 5HT2A antagonist (Bortolozzi et al., 2005). Together, these data demonstrate that the 5HT2A receptor acts to facilitate VTA neuronal activity and DA release.

Similarly, in the nucleus accumbens, local perfusion of DOI results in increases in DA which can be blocked by 5HT2 antagonists (Bowers et al., 2000; Yan, 2000), although neither of these studies used compounds selective enough to distinguish between 5HT2A and 5HT2C receptors. However, 5HT2A antagonism is able to reduce the increase DA in the nucleus accumbens resulting from DRN stimulation (De Deurwaerdere and Spampinato, 1999) as well as increases resulting from D2 antagonism (Liegeois et al., 2002). Additionally, systemic DOI potentiated amphetamine-induced DA release in the nucleus accumbens, an effect which was blocked by M100907 (Kuroki et al., 2003). Together, these data support the idea that 5HT2A receptors in the nucleus accumbens modulate stimulated DA release, although the precise mechanism is unclear since the subcellular localization of these receptors is unknown.

5HT2A receptors may also indirectly influence DA release through modulation of excitatory inputs from the PFC. Systemic or direct infusion of DOI into the PFC results in increases in local DA which can be blocked by selective 5HT2A antagonists (Pehek et al., 2001; Bortolozzi et al., 2005; Pehek et al., 2006). Although some 5HT2A receptors have been reported on unidentified monoaminergic axons (Jakab and Goldman-Rakic, 1998; Miner et al., 2003), the most probable explanation for this is that activating 5HT2A receptors, on pyramidal neurons, has downstream polysynaptic effects. 5HT2A receptors mediate excitatory post-synaptic currents in pyramidal cells (Aghajanian and Marek, 1997) and thus may increase glutamate release. Furthermore, 5HT2A receptors are present on approximately 55% of pyramidal neurons that project to the VTA (Vazquez-Borsetti et al., 2009). Both systemic and direct administration of DOI into the PFC resulted in increased firing rates and DA release in the VTA, which were blocked by M100907 (Bortolozzi et al., 2005). Furthermore, systemic DOI increased glutamate release in the VTA, which was blocked by intracortical administration of M100907 (Pehek et al., 2006). Together, these data suggest that 5HT2A receptors increase pyramidal cell glutamate activity, thus increasing their stimulatory drive on the VTA and explaining the observed 5HT2A-stimulated increase in DA release in the PFC (Pehek et al., 2001; Bortolozzi et al., 2005; Pehek et al., 2006). However, this polysynaptic model may have additional effects, since approximately 50% of the 5HT2A-expressing pyramidal neurons that synapse in the VTA also synapse in the DRN (Vazquez-Borsetti et al., 2011), which could then change the serotonergic inputs to a broad spectrum of brain areas, including the PFC, nucleus accumbens, and VTA.

5HT2A receptor stimulation clearly facilitates DA release in the mesocorticolimbic system in areas key for addiction. However, 5HT2A antagonism has no effect on basal DA

release on its own in the PFC (Pehek et al., 2001; Bortolozzi et al., 2005; Pehek et al., 2006), VTA (Bortolozzi et al., 2005), or nucleus accumbens (Liegeois et al., 2002), suggesting that the 5HT2A receptor is involved primarily in control of phasic, not tonic, DA release.

The 5HT2A receptor has also been implicated in the behavioral effects of cocaine. 5HT2A antagonists are able to block the locomotor-stimulant effects in rats (McMahon et al., 2001; Fletcher et al., 2002; Filip et al., 2004) while DOI potentiates them (Filip et al., 2004). This effect is likely due to the influence of 5HT2A receptors at the level of the VTA, as microinjections of M100907 in the VTA are able to attenuate the locomotor-stimulant effects of systemic cocaine, while microinjections in the nucleus accumbens are not (McMahon et al., 2001). 5HT2A antagonists also attenuate the discriminative-stimulus effects of cocaine, shifting the dose-response curve to the right (Filip et al., 2006), and block both cocaine-associated cue-induced and cocaine-primed reinstatement in rats (Fletcher et al., 2002; Filip, 2005; Nic Dhonnchadha et al., 2009; Pockros et al., 2011) without attenuating sucrose-associated cue-induced reinstatement (Nic Dhonnchadha et al., 2009). Reinstatement may be in part mediated through 5HT2A activation in the PFC, as local microinjections of M100907 are able to specifically attenuate cocaine cue-induced reinstatement, but only slightly reduce the effects of a systemic cocaine prime (Pockros et al., 2011). However, 5HT2A antagonists have repeatedly failed to affect cocaine self-administration (Fletcher et al., 2002; Filip, 2005; Nic Dhonnchadha et al., 2009; Pockros et al., 2011). Furthermore, neither agonists nor antagonists had any effect on the reward-facilitating effects of cocaine in intracranial stimulation studies (Katsidoni et al., 2010).

1.3 Aims and choice of model

The goal of the present study was to examine the neurochemical and neurobiological changes and behavior associated with chronic SSRI treatment, at doses relevant to human doses, in the context of cocaine use. Accordingly, we first developed a clinically-relevant chronic treatment regimen with an SSRI and then applied it in a primate model of ongoing cocaine self-administration. Cocaine-related behavior and neurochemistry were examined repeatedly throughout the study to ascertain the effects of the treatment regimen. Additionally, we examined pre- and post-synaptic 5HT function to determine the effects of combined long-term SSRI and cocaine exposure and the relationship of any neurobiological changes to behavior.

1.3.1 Fluoxetine

For the current studies, we chose fluoxetine as the SSRI due to its widespread use in both clinical and preclinical studies. Fluoxetine is one of the oldest SSRIs, developed by Eli Lily and first described in the literature in 1974, and therefore is extremely well-characterized. It consists of two enantiomers, *R*- and *S*-fluoxetine, which inhibit 5HT uptake with similar potency. Its major metabolite, norfluoxetine, is also active and has *R*- and *S*-enatomiomers with respective K_i values of 20 and 268 nM (Wong et al., 1995) and is able to increase 5HT similarly to fluoxetine (Qu et al., 2009). Fluoxetine has 6.8, 5,000 and 370 nM affinities at SERT, DAT and NET (Sanchez and Hyttel, 1999), respectively, as well as a reported affinity of approximately 55-270 nM for the 5HT2C receptor (Wong et al., 1995; Palvimaki et al., 1996). This affinity is high enough that fluoxetine may bind to the 5HT2C receptors at therapeutic doses, especially since doses of fluoxetine that are active in preclinical models of depression also result in 25-43% occupancy of the 5HT2C receptor (Palvimaki et al., 1999). Although norfluoxetine has similar affinities at the transporters, it

does not share an appreciable affinity for the 5HT2C receptor (Sanchez and Hyttel, 1999), and neither fluoxetine nor norfluoxetine have high affinity for 5HT2A receptors (approximately 708 nM) (Palvimaki et al., 1996; Sanchez and Hyttel, 1999).

Fluoxetine is active in a range of animal models of depression and commonly prescribed clinically, although there is a delay to onset of therapeutic effects, as with many SSRIs (Wong et al., 1995). Furthermore, a variety of studies have shown that fluoxetine is capable of inducing neurobiological changes when administered chronically, ranging from decreases in SERT (Kugaya et al., 2003; Iceta et al., 2007) to desensitization of 5HT1A receptors (Riad et al., 2008) and alterations of its effect on VTA cell firing (Di Mascio et al., 1998). Fluoxetine has also been examined previously as a potential treatment for cocaine abuse, showing promise in preclinical models (Carroll et al., 1990; Richardson and Roberts, 1991; Walsh et al., 1994; Glatz et al., 2002; Ruedi-Bettschen et al., 2009) but failing in clinical trials (Grabowski et al., 1995; Batki et al., 1996; Cornelius et al., 1998; Schmitz et al., 2001; Lima et al., 2003; Winstanley et al., 2011). However, no studies have directly addressed the potential neurobiological changes induced by fluoxetine in the context of cocaine use or their behavioral consequences.

1.3.2 Self-administration and reinstatement

The techniques of drug self-administration and reinstatement in animals have long-demonstrated validity as models of human drug addiction and relapse (Griffiths et al., 1980; Stewart and de Wit, 1987; O'Brien and Gardner, 2005) and serve as excellent tools for the evaluation of both behavioral and neurobiological consequences of drug use as well as potential pharmacotherapies (Mello and Negus, 1996; O'Brien and Gardner, 2005). Furthermore, the consequences of non-contingent or experimenter-administered drugs can

differ from the consequences of self-administered drugs (Jacobs et al., 2003; Kimmel et al., 2005). Therefore, in order to provide a cocaine background against which to test the efficacy and determine the neurobiological consequences of fluoxetine treatment, we chose to use a cocaine self-administration model. Additionally, to provide a more complete picture of cocaine sensitivity, we also used a reinstatement model in the same animals.

In operant self-administration studies, the animal is trained to make a response in order to receive the reinforcer, for example, a drug infusion or a food pellet. The response is frequently a nose poke (for rodents) or a lever press. Reinforcer delivery is often also paired with conditioned stimuli, such as lights or sounds that then become associated with the reinforcer. There are multiple options for scheduling the relationship between responses and reinforcers, each of which is optimal for modeling different aspects of human cocaine abuse. For example, there are fixed ratio (FR) schedules, in which a certain number of lever presses results in a presentation of the reinforcer, providing a direct relationship between the two and thus response rate and number of reinforcers earned serve as an index of drug-taking behavior. FR requirements may range from a single lever press to 30 or more, depending on other factors such as mandatory timeout between reinforcers (to reduce drug accumulation and minimize motor effects) or the dose of drug available (Ator and Griffiths, 2003; O'Brien and Gardner, 2005). Progressive ratio schedules increase the response requirement following each presentation of the reinforcer; for example, the first reinforcer may take 1 lever press to earn, the second 2, the third 4, and so on. The point at which the animal stops responding is called the breakpoint and serves as an indicator of the strength of the reinforcer (Oleson and Roberts, 2008). A third commonly used schedule is the fixed-interval fixed-ratio (FI:FR) second order schedule, which uses fixed intervals in which responding results not in

presentation of the reinforcer, but in a presentation of the conditioned stimuli which have previously been associated with delivery of the reinforcer. At the end of the fixed interval, the first completion of the response requirement (fixed ratio) results in reinforcer delivery. Second order schedules are capable of maintaining high rates of behavior for few drug infusions and are remarkably stable (Everitt and Robbins, 2000). For the present study, we chose a fixed-ratio 20 schedule due to its simplicity, frequent use in drug studies, and sensitivity to experimental intervention (Ator and Griffiths, 2003; O'Brien and Gardner, 2005), since it was unclear how potent an influence our experimental fluoxetine treatment might be. Furthermore, fixed-ratio schedules have successfully detected reductions in reinforcing efficts in the past (O'Brien and Gardner, 2005).

In addition to evaluating self-administration, we also used a reinstatement model based on the fixed-ratio schedule we chose. Reinstatement relies on distinct but overlapping neurobiological substrates from self-administration (Gardner, 2000; O'Brien and Gardner, 2005; Koob and Volkow, 2010) and therefore assessing reinstatement in addition to self-administration provides a more complete evaluation of cocaine's effects. Briefly, responding under the FR schedule was extinguished by substituting saline for cocaine in the syringe and removing the conditioned stimuli associated with drug delivery. Animals were allowed to self-administer until their response rate met extinction criteria (20% of baseline). At this point, a non-contingent dose of the reinforcer or reintroduction of the conditioned stimuli may cause an increase in responding, despite no further reinforcer delivery, which is called reinstatement and serves as an indicator of relapse-inducing factors in humans (Stewart and de Wit, 1987; Gardner, 2000). Responding under these conditions is most robust in second

order schedules; however, reinstatement is still measurable under simple FR schedules and provides an additional assessment of cocaine's abuse-related effects.

1.3.3 Nonhuman primates

For our model, we chose nonhuman primates, for several reasons. Nonhuman primates are better able to learn the complex behavioral tasks necessary for drug abuse studies compared to other laboratory animals. Furthermore, monkeys offer the ability to do extended, repeated measure studies on a scale that rodents do not (Weerts et al., 2007; Moser et al., 2010), particularly for drug abuse studies that require long-term maintenance of catheter or cannula preparations (Weerts et al., 2007) such as the present one. Moreover, pharmacokinetics, which have been shown to greatly influence drug effects (Volkow et al., 1999a; Kimmel et al., 2007; Kimmel et al., 2008), are better predicted in humans by pharmacokinetics in monkeys (Weerts et al., 2007; Moser et al., 2010).

In addition to being well-suited to the behavioral tasks, nonhuman primates are well-suited for neuroimaging studies. Their brains are larger than rodents', thus offering better resolution for *in vivo* imaging, and their neuroanatomy closely resembles humans'. Nonhuman primates are more closely related genetically to humans than other laboratory species and show greater genetic homology to humans than rodents in their serotonin transporter (Weerts et al., 2007). Furthermore, particularly important for the present study, differences between the rodent and primate serotonin systems have been documented (Weerts et al., 2007). SERT are distributed more diffusely in rats (Owashi et al., 2004) and the distribution of 5HT2A receptors in the caudate, putamen, and nucleus accumbens is different in rats compared to monkeys and humans, which are similar (Lopez-Gimenez et al 2001). Additionally, the prefrontal cortex in monkeys is more similar to humans' than are

other laboratory species' (Fuster, 1997), particularly the rat (Preuss, 1995). Importantly, the organization of dopaminergic inputs to the PFC in nonhuman primates is also very close to humans'(Weerts et al., 2007).

Finally, drug effects are more easily translated from nonhuman primates to humans, and adverse events are predicted better from nonhuman primate models (Moser et al., 2010). The brain metabolic effects evoked by psychostimulants are greater in both humans and monkeys compared to rats (Lyons et al., 1996). Moreover, rodents exhibit sensitization to many different classes of abused drugs, while nonhuman primates generally do not, suggesting that monkeys may be a better model of the long-term and neurobiological effects of many drugs (Weerts et al., 2007).

1.4 Summary

Cocaine abuse is a significant problem in society, and there are currently no FDA-approved treatments. Serotonin negatively modulates the abuse-related effects of cocaine, yet SSRIs, which increase serotonin and show promise as pharmacotherapies for cocaine addiction in preclinical models, fail to reduce cocaine use when given chronically in the clinic, possibly due to the development of the neuroadaptive changes upon which their therapeutic effects are believed to rely. The effects of the 5HT system's neuroadaptive responses on the DA system and cocaine-related behavior are not well understood. Since a pharmacotherapy needs to act chronically in order to be successful in treating cocaine abuse, we examined the effects of chronic treatment with fluoxetine at clinically relevant serum concentrations on cocaine-related neurobiology and behavior. Additionally, since the potential neuroadaptive changes caused by chronic SSRI treatment have not been examined

in the context of cocaine abuse, we also examined proteins in the 5HT system that could be targets for neuroadaption that also influence the effects of cocaine.

These studies will provide an integrative analysis of the 5HT system's role in the underlying neurochemistry of cocaine abuse, characterizing the neurobiological changes in the 5HT system and how they influence the DA system and cocaine-taking behavior. They will also extend previous work on the mechanisms of SSRIs into primate models and into the context of cocaine abuse, as well as examine the potential of SSRIs as a treatment. This will enable better understanding of the neurobiology of cocaine abuse and lead to the development of better treatment strategies, of which SSRIs may constitute a part.

2. METHODS DEVELOPMENT

2.1 Introduction

Fluoxetine is a SSRI which is currently approved for treatment of depression and mood disorders. It is one of the oldest and most frequently prescribed SSRIs (Wong et al., 1995) and is selective for the serotonin transporter (SERT) over the other monoamine transporters (see Table 2-1 for structure and affinities). Fluoxetine blocks reuptake of serotonin into the presynaptic cell via its action at the SERT, which results in acute increases in levels of extracellular serotonin (Wong et al., 1995; Clark et al., 1996). However, the onset of therapeutic effects is delayed for 3-4 weeks, suggesting that there may be neurobiological adaptations involved in its action (Wong et al., 1995; Thompson, 2002; Vaswani et al., 2003).

Fluoxetine is metabolized by cytochrome P450 enzymes, with the CYP2CP and CYP2D6 enzymes playing a large role. The major metabolite is norfluoxetine, which is biologically active with equal affinity for SERT as fluoxetine (Preskorn, 1997; Hiemke and Hartter, 2000; Vaswani et al., 2003). Unlike the metabolites of most SSRIs, norfluoxetine contributes to the inhibition of serotonin reuptake in a functionally significant manner (Preskorn, 1997; Qu et al., 2009). In humans, fluoxetine has a half-life of 1-4 days while norfluoxetine has longer half-life of 7-15 days (Wong et al., 1995; Preskorn, 1997; Hiemke and Hartter, 2000; Vaswani et al., 2003). Furthermore, fluoxetine and norfluoxetine inhibit their own metabolism through interactions with the cytochrome P450 liver enzymes, particularly the CYP2D6 (Preskorn, 1997; Vaswani et al., 2003). Thus cumulative dosing, as is frequently used clinically, results in different blood concentrations and pharmacokinetics than acute dosing (Hiemke and Hartter, 2000). There is no clear serum concentration-

therapeutic effect relationship reported for fluoxetine (Baumann, 1996; Preskorn, 1997; Hiemke and Hartter, 2000), but serum concentrations from the human clinical setting are generally reported to be between 200 ng/ml and 1000 ng/ml, including norfluoxetine (Amsterdam et al., 1995; Preskorn, 1997; Brunswick et al., 2002; Baumann et al., 2004; Reis et al., 2009).

The distinctive pharmacokinetics of fluoxetine may contribute to its clinical profile, as may the action of norfluoxetine. Therefore, it is important to take this information into consideration when designing and performing preclinical or animal studies using fluoxetine. However, very little information exists on the pharmacokinetics of fluoxetine in species commonly used for preclinical studies, particularly nonhuman primates. It has been reported that chronic administration of fluoxetine can achieve serum concentrations comparable to human clinical concentrations in rhesus macaques (Fontenot et al., 2009), yet there is no information available regarding the sustainability of these concentrations or the pharmacokinetics. Since pharmacokinetics may differ among species, we sought to characterize the pharmacokinetics of fluoxetine and norfluoxetine in nonhuman primates, and develop a clinically-relevant dosing method for use in preclinical studies.

2.2 Methods

Subjects. Adult female rhesus macaques (*Macaca mulatta*) between 7-9 kg served as subjects. Animals were singly housed within a primate colony and fed Purina monkey chow, fruits, and vegetables. Water was continually available. All procedures and studies strictly followed the National Institutes of Health Guide for the Care and Use of Laboratory Animals (Publication no. 85-23, revised 1985) and were approved by the Institutional Animal Care

and Use Committee of Emory University. All subjects had been previously fitted with a collar and trained to sit in a primate chair (Primate Products, Woodside, CA) and had a history of exposure to psychostimulants.

Drugs. Fluoxetine HCL was purchased from Spectrum Chemicals and Laboratory Products (New Jersey, USA) and administered in a range of doses via different routes of administration. For intramuscular (i.m.), intravenous (i.v.), and subcutaneous (s.c.) administration, fluoxetine HCL was dissolved in sterile water at a concentration of 7 mg/ml. For oral (p.o.) administration, fluoxetine HCL was mixed with peanut butter or other palatable foods and spread on a piece of bread. All doses were calculated and measured as the salt. Compliance with oral dosing was assessed by visually inspecting the animal's cage 1-2 hours after the medicated treat had been given.

Acute studies. Female rhesus macaques (n = 4; 7-9kg) served as subjects. Animals were seated comfortably in primate chairs (Primate Products) with gentle arm restraint for the duration of blood sample collection. Each animal was prepared by placing a temporary intravenous catheter into the saphenous vein (BD Saf-T-Intima Closed Catheter System; BD, Franklin Lakes, NJ). Drug was administered either i.m. (1, 3, 5.6, 10 mg/kg) or i.v. (3 mg/kg; via the temporary catheter and followed by 3ml of saline). Blood samples were collected through the catheter at 5, 15, 30, 60 and 120 minutes after injection. The animals were then returned to their home cages. For all doses except 10 mg/kg, animals were re-chaired and samples collected at 8 and 12 hours post-injection, and again at 24 hours. Approximately 2 ml of blood were collected per sample and placed in a serum-separating collection tube (BD Vacutainer; BD Franklin, Lakes NJ, USA). Two animals were used per dose.

Chronic studies. Female rhesus macaques (n = 7; 7-9kg) served as subjects. Doses were chosen based on the results of the acute studies. Fluoxetine HCl (5.6 mg/kg or 10 mg/kg) was administered once daily in the cage for 7 (5.6 mg/kg, s.c. or p.o., n = 2) or 42 days (10 mg/kg, p.o., n = 5). Blood draws occurred once daily before drug administration for the 6-day administration and continued for a 6-day follow-up after ceasing drug administration. For the 42-day administration, blood draws occurred once weekly before daily drug administration. Approximately 2 ml of blood was collected from the saphenous vein via a 3ml luer-lock syringe and placed in a tube. 42-day administration was carried out twice, first in 5 subjects with no previous exposure to fluoxetine, and again in 3 of the same subjects. The 6th week blood draw was not performed during the second chronic administration session.

Sample analysis. Following collection, blood was centrifuged at 3000g for 20 minutes to isolate the serum, which was then collected in a 1.5 ml microcentrifuge tube and frozen at 0°C for later analysis using ultra-performance liquid chromatography (UPLC)-liquid chromatography/mass spectrometry/mass spectrometry assay procedures described in Ritchie et al, 2009 (Ritchie et al., 2009b). Briefly, fluoxetine and norfluoxetine were extracted by a protein crash method by adding mobile phase, internal standards, and methanol to the sample, which caused precipitation of the proteins. Samples were then centrifuged and the supernatant collected for analysis on an Acquity UPLC System with a Triple Quadrapole Detector (Waters Inc, Milford, MA, USA) with 2 mobile phases. Samples were quantified against internal standards and separate standard curves. Inter-assay agreement ranges between 87-97% (Ritchie et al., 2009b).

Half-life determination. Half-lives were calculated based on data from chronic studies. The following formula was used to calculate half-life:

$$k_{elimination} = (\ln C_2 - \ln C_1)/(t_2 - t_1)$$

$$t½ = 0.693/ k_{elimination}$$

C is concentration and t is time. Half-lives for fluoxetine and norfluoxetine were determined separately. For calculations for fluoxetine, t_1 was 24 hours following the final administration of fluoxetine, and t_2 was 96 hours post final administration. For norfluoxetine, t_1 was the time-point at which fluoxetine concentrations were less than 5 ng/ml to ensure that metabolism of fluoxetine to norfluoxetine would no longer be significantly contributing to its concentration. T_2 was 48 hours after t_1. Calculations were performed in Excel. Student's t-test was used to compare half-lives for fluoxetine and norfluoxetine and was calculated using GraphPad (GraphPad Prism version 5.01 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com.)

2.3 Results

Acute fluoxetine administration. 1.0, 3.0, 5.6, and 10 mg/kg fluoxetine all resulted in detectable serum concentrations of fluoxetine (Figure 2-1). Peak concentrations for fluoxetine, norfluoxetine, and total drug are reported in Table 2-2. Norfluoxetine first appeared at 120 minutes (Figure 2-2) and was still present at the 24 hour time-point for all doses (14, 61, 142.8 ng/ml, respectively; not evaluated for 10 mg/kg dose), forming the majority of active drug concentration at that time-point (see Table 2-2). Total active drug concentration generally peaked twice following i.m. administration, once within 60 minutes

following the injection and again around 8-12 hours after the injection. The second peak results from the conversion of fluoxetine to norfluoxetine (Figure 2-1; Table 2-2).

Intravenous administration of 3.0 mg/kg resulted in higher peak serum concentrations than observed after intramuscular injection (Figure 2-3; see Table 2-2). The concentration peaked 15 minutes post-injection and then rapidly declined. Norfluoxetine had a comparatively small contribution to overall drug concentrations when administered i.v., reaching levels <20 ng/ml up to 120 minutes, and peaking at 108 ng/ml at 8 hours post injection. At 24 hours, a combined concentration of 84.6 ng/ml remained.

Chronic fluoxetine administration. Oral administration of 5.6 mg/kg resulted in lower serum concentrations (fluoxetine: 43.5 ng/ml; norfluoxetine: 193.6 ng/ml) as compared to subcutaneous administration (fluoxetine: 328.7 ng/ml; norfluoxetine: 404.1 ng/ml) (Figure 2-4). However, s.c. administration resulted in skin sores on two separate occasions, thus it was decided to discontinue this route of administration for safety and ethical considerations. The half-lives of both fluoxetine and norfluoxetine were significantly longer following subcutaneous administration as compared to oral administration (16.16 (± 2.56) vs. 11.89(± 5.06) hours for fluoxetine ($t(2)=3.01$; $p<0.01$) and 29.72(± 2.56) vs. 21.29(± 0.08) hours for norfluoxetine ($t(2)=4.66$; $p<0.05$), respectively; see Table 2-3.)

Block design. Based on the serum concentrations achieved by 5.6 mg/kg/day oral administration, a 10 mg/kg/day dose was selected for a 6-week block design intended to approximate human clinical conditions (see Table 2-3). Daily administration of this dose successfully resulted in stable serum concentrations in the human clinical range (Reis et al., 2009) with concentrations between 250-450 ng/ml total active drug. These levels cleared in approximately 1 week (≤10 ng/ml) (Figure 2-5). During chronic administration,

norfluoxetine accounted for 70-80% of total active drug concentration (see Table 2-3 for comparative pharmacokinetics).

The 10 mg/kg/day dose was administered for 6 weeks in a block design for the purpose of investigating the effects of this treatment on the neurobiology and behavior associated with cocaine abuse as described in Chapters 3 and 4. The 6-week time period for treatment was chosen based on the time-to-onset of therapeutic effects, and therefore any putative neurobiological changes (3-4 weeks)(Wong et al., 1995; Thompson, 2002; Vaswani et al., 2003), with additional time to allow for different behavioral measures (taken during weeks 4-6 of treatment). Fluoxetine treatment was preceded by a 6-week baseline and followed by a 6-week washout period (see Figure 2-6).

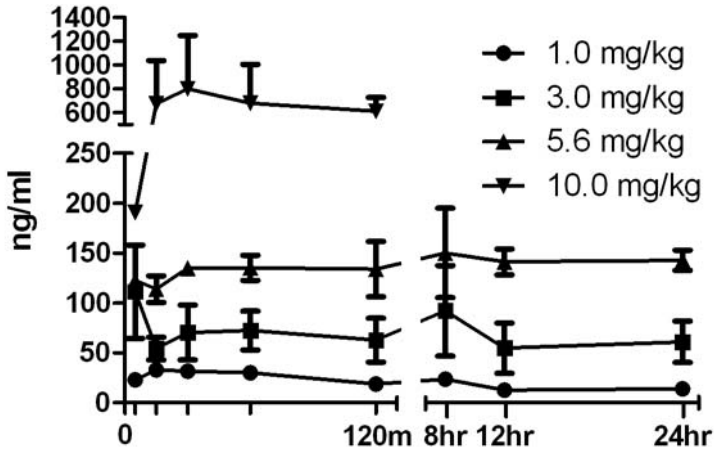


Figure 2-1: Combined serum concentrations of fluoxetine and norfluoxetine following i.m. injection (n = 2).

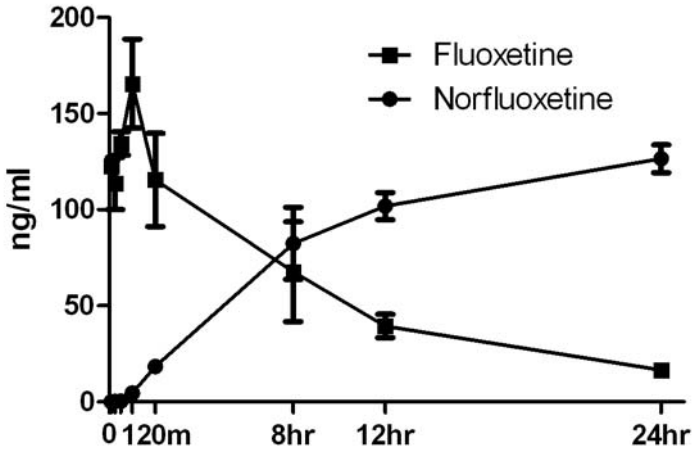


Figure 2-2: Representative data showing concentrations of fluoxetine and norfluoxetine resulting from a single i.m. injection of 5.6 mg/kg fluoxetine (n = 2).

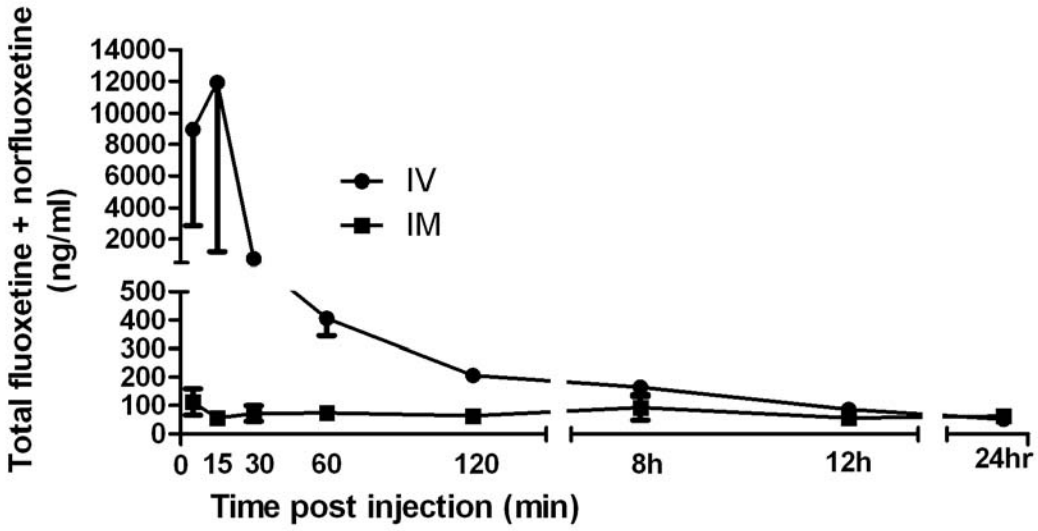


Figure 2-3: Intravenous administration of fluoxetine results in greater magnitude and more rapid changes in serum concentration of active drug than does intramuscular administration (n = 2).

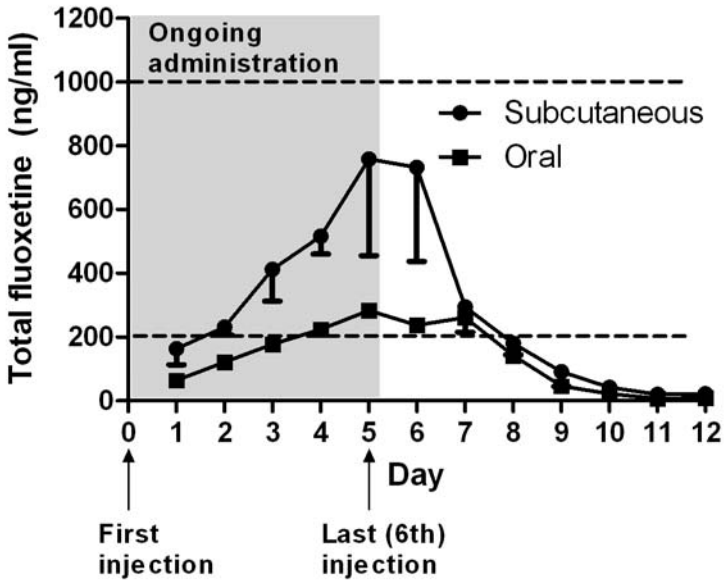


Figure 2-4: Total active drug concentrations during daily administration of 5.6 mg/kg s.c. or p.o. (n = 2).

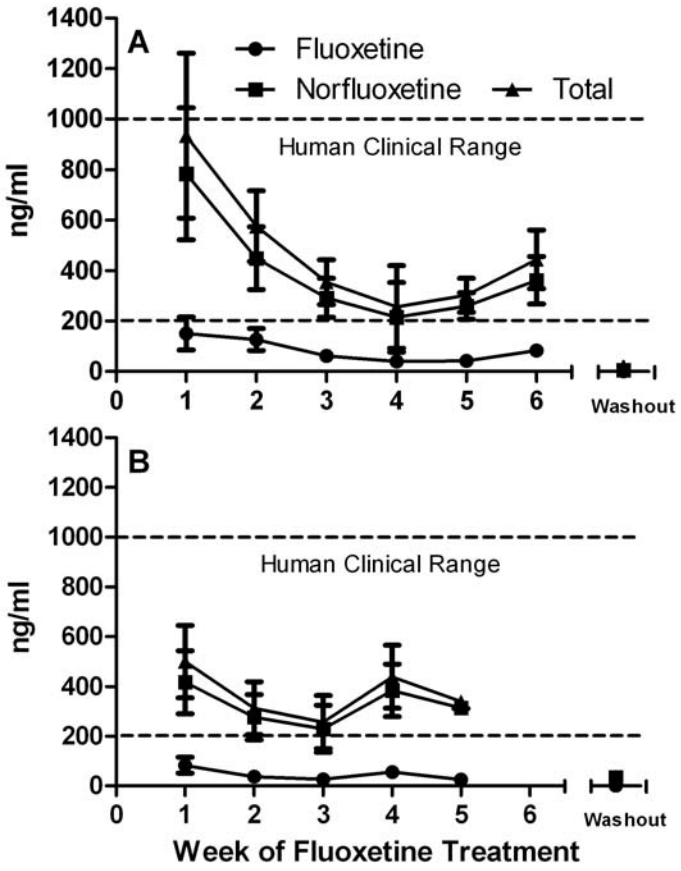


Figure 2-5: Fluoxetine, norfluoxetine, and total concentrations of active drug during chronic administration of 10.0 mg/kg fluoxetine (p.o.). (A) The initial 6-week administration period (n = 5). No animals had previously received fluoxetine. (B) A second 6-week administration (n = 3). Blood was not collected for week 6. All subjects had previously participated in (A).

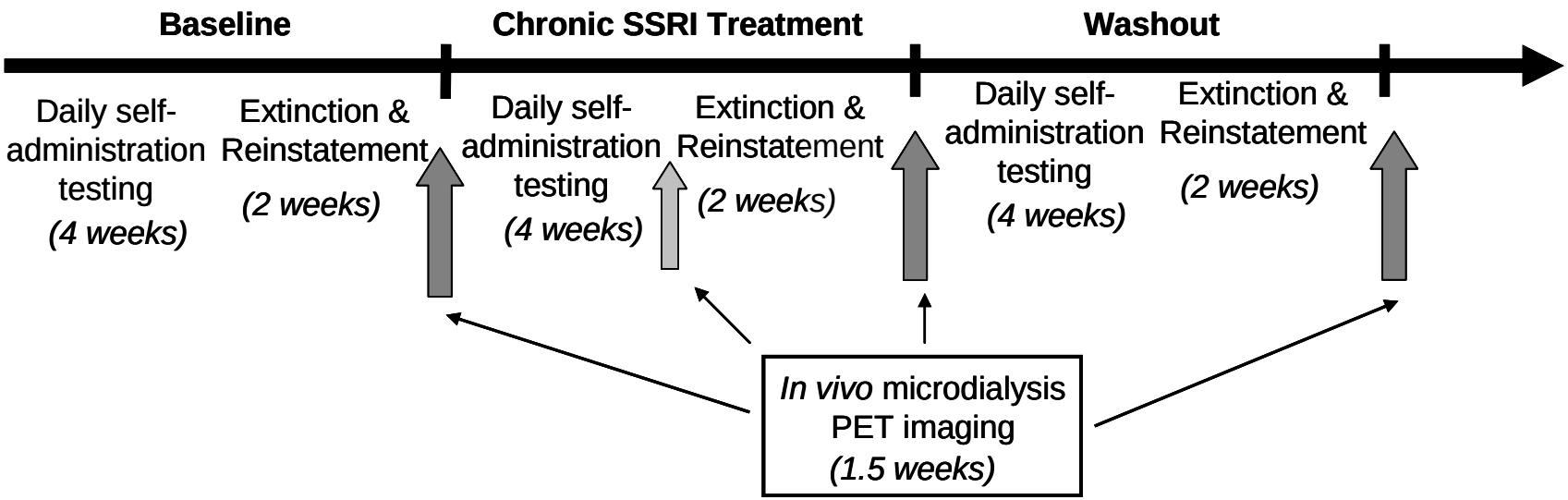
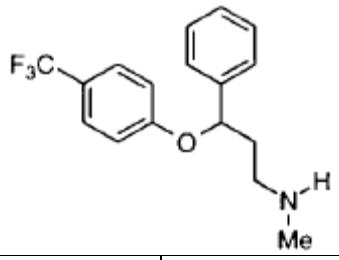
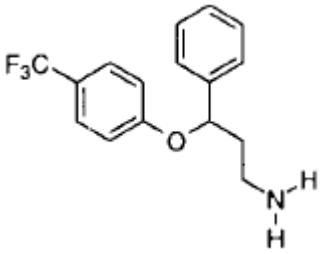


Figure 2-6: Experimental paradigm. Fluoxetine treatment (10mg/kg/day, p.o.) was administered in a block design consisting of three 6-week blocks of cocaine self-administration and reinstatement testing, followed by neurochemical and neurobiological assessments (dark gray arrows). The light gray arrow indicates a single *in vivo* microdialysis session with cocaine challenge during fluoxetine treatment; all other testing at dark gray arrows is timed such that no fluoxetine would be present in the system. Total time for all three blocks is 18 weeks.

Table 2-1: The binding affinities of fluoxetine and norfluoxetine for selected receptors and transporters.

	Fluoxetine		Norfluoxetine	
Chemical structure				
Receptor	Ki (nM)	Reference	Ki (nM)	Reference
SERT	0.008; 0.02; 1.96	2,3	0.045; 3.15	23
DAT	4.5; 2.88	3	2.19	3
NET	0.25; 1.23	3	2.4	3
5HT1A	11,000; 79,000	1, 3	79,000	1
5HT2A	708; 710; 1,800	1, 2, 3	>1000; 2500	1, 2
5HT2C	42; 160; 270	1, 2, 3	157; 20,000	1, 2
DA D1	10,000	1	22,000	1
DA D2-like	32,000	1	13,000	1
α_1	14,000	1	15,000	1
α_2	2,800	1		
B	18000	1	>1,000	1

Chemical structures adapted from (Sanchez and Hyttel, 1999)
1-(Sanchez and Hyttel, 1999)
2-(Palvimaki et al., 1996)
3-(Wong et al., 1995)

Table 2-2: Serum concentrations following acute dosing. Concentrations are presented as means ± SEM; times are relative to injection time 0. Two values for peak total concentration indicate two peaks, separated by a decrease in concentration. The first consists primarily of fluoxetine and the second of norfluoxetine.

Dose (mg/kg)	Fluoxetine		Norfluoxetine		Total active drug		
	Time of peak concentration	Peak concentration (ng/ml)	Time of peak concentration	Peak concentration (ng/ml)	Time of peak concentration	Peak concentration (ng/ml)	24 hrs post-injection
1.0 i.m.	15 m	32.7 ± 1.9	8 hr	13.3 ± 4.1	15 m 8 hr	32.9 ± 1.9 23.8 ± 4.8	12.7 ± 2.1
3.0 i.m.	30 m	70.1 ± 27.5	12 hr	58.2 ± 31.7	60 m 12 hr	72.5 ± 19.5 92.2 ± 45.3	54.8 ± 25.4
3.0 i.v.	15 m	11913.0 ± 10713.0	8 hr	108. 7 ± 23.3	15 m	11917.9 ± 10715.3	84.6 ± 3.3
5.6 i.m.	60 m	165.7 ± 23.1	24 hr ‡	101.9 ± 7.1	60 m 12 hr	135.3 ± 12.6 150.2 ± 44.8	141.3 ± 13.1
10.0 i.m.	30 m	785.0 ± 459.4	120m ‡	56.7 ± 7.7	30 m ‡‡	801.1 ± 449.0	ND

ND: not determined

‡ Last time point determined; may not be true peak since a decrease was not observed within the given timeframe

‡‡ Only peak observed due to truncated time course

Table 2-3. Comparative pharmacokinetics of fluoxetine and norfluoxetine between species. Plasma levels are reported from repeated dosing measures (minimum of 6 days).

Species	Dose	Route of administration	Plasma levels (ng/ml)		Half-life (hrs)		Fluoxetine/ Norfluoxetine ratio	Reference
			Fluoxetine	Norfluoxetine	Fluoxetine	Norfluoxetine		
Mice	5 mg/kg	Intraperitoneal	729	847	NR	NR	0.86‡	(Hodes et al., 2010)
	10 mg/kg		1835.5	1516.5	NR	NR	1.21‡	
Rats	3 mg/kg	Subcutaneous	N/A	N/A	2.08	6.51	NC	(Qu et al., 2009)
	10mg/kg		N/A	N/A	2.71	11.8	NC	
Nonhuman primate	5.6 mg/kg	Oral	63.9	193.6	11.89**	21.29*	0.33	Present study
	5.6 mg/kg	Subcutaneous	328.7	404.1	16.16	29.72	0.81	
	10 mg/kg	Oral	62-83	292-361	NC	NC	0.23	
Human	20 mg	Oral	73.62§, 98, 80, 89-100	108.39§, 127, 126, 114-200	1-4 days	7-14 days	0.68§	(Altamura et al., 1994; Wong et al., 1995; Preskorn, 1997; Hiemke and Hartter, 2000; Vaswani et al., 2003; Reis et al., 2009)
	40 mg		172.29§, 195	168.93§, 221			1.02§	

** = $p<0.01$; * = $p<0.05$ with respect to subcutaneous route of administration in nonhuman primates
‡ calculated from plasma levels reported in Hodes et al 2010, Table 1
§ calculated from data reported in Reis et al 2009, Table 3
NR: Not reported NC: Insufficient data; not calculated

2.4 Discussion

The present study characterized the pharmacokinetics of fluoxetine in rhesus macaques, a species frequently used for preclinical studies, following both acute and chronic administration. To achieve serum concentrations comparable to the human clinical range, it was necessary to administer higher doses and to do so chronically, as expected from a previously published study by Fontenot et al (Fontenot et al., 2009). Based on these studies, a dose of 10 mg/kg/day, p.o., was selected for use in a 6-week block design.

2.4.1 Acute pharmacokinetics

Acute administration of fluoxetine at all doses and routes of administration examined resulted in potentially functionally significant serum levels even 24 hours later. Although this may confound certain experimental designs, it demonstrates that fluoxetine is amenable to once-daily dosing for chronic designs in rhesus macaques that require cumulative dosing. Additionally, the conversion of fluoxetine to norfluoxetine resulted in a second peak of active drug concentration approximately 8-12 hours after administration. This peak was similar in magnitude to the initial peak following administration and should be considered when designing single dose experiments with multiple measures or time-courses.

2.4.2 Comparative pharmacokinetics

Half-life

Fluoxetine has a reported half-life of 1-4 days in humans while norfluoxetine has a half-life of 7-15 days (Altamura et al., 1994; Wong et al., 1995; Preskorn, 1997; Hiemke and Hartter, 2000; Vaswani et al., 2003). The pharmacokinetics of fluoxetine have also been described in mice (Hodes et al., 2010) and rats (Qu et al., 2009) (see Table 1-2), but have not been comprehensively described in nonhuman primates. In the present study, chronic

administration of 5.6 mg/kg fluoxetine orally or subcutaneously resulted in significantly different half-lives for both fluoxetine and norfluoxetine. Since both fluoxetine and norfluoxetine inhibit CYP450 enzymes, which are involved in their metabolism (Preskorn, 1997; Hiemke and Hartter, 2000; Vaswani et al., 2003), this difference in half-life is likely due to the difference in serum concentrations achieved by each mode of administration; the higher concentrations associated with subcutaneous administration resulted in longer half-lives, which is in concordance with previous reports of nonlinear pharmacokinetic parameters in humans (Preskorn, 1997; Hiemke and Hartter, 2000; Vaswani et al., 2003) and in rats (Qu et al., 2009). Our observed half-lives for fluoxetine and norfluoxetine are greater than that reported in rats (Qu et al., 2009) but still considerably less than that reported in humans (Altamura et al., 1994; Wong et al., 1995; Preskorn, 1997; Hiemke and Hartter, 2000) (see Table 1-2). As in rats, a single administration resulted in steady levels of active drug maintained through 24 hours (Qu et al., 2009). It is especially important to be aware of the possibility of functionally significant serum concentrations of drug remaining present past 24 hours when designing pre-clinical studies with fluoxetine.

Fluoxetine: Norfluoxetine ratio

Under steady state in humans norfluoxetine exceeds fluoxetine (Hiemke and Hartter, 2000); this finding was also observed in mice (Hodes et al., 2010) and monkeys (Fontenot et al., 2009) and now has been replicated in the present study (see Table 1-2). Unlike the data reported by Fontenot et al (Fontenot et al., 2009), fluoxetine: norfluoxetine ratios were not substantially higher in monkeys than those reported in humans (Amsterdam et al., 1995; Brunswick et al., 2002; Baumann et al., 2004; Reis et al., 2009). Norfluoxetine is as potent a reuptake inhibitor as fluoxetine (Wong et al., 1995; Preskorn, 1997) and it has been

suggested that it may play a significant role in the therapeutic effects (Preskorn, 1997; Qu et al., 2009). This is especially relevant because acute, single-administration experimental designs do not achieve significant levels of norfluoxetine until at least after 120 minutes have passed.

2.4.3 Development of dosing regimen & block design

There are varying reports of both observed and recommended serum concentrations in the clinic. In general, serum concentrations of combined fluoxetine and norfluoxetine are reported to be between 200 ng/ml and 1000 ng/ml (Amsterdam et al., 1995; Preskorn, 1997; Brunswick et al., 2002; Baumann et al., 2004; Reis et al., 2009). However, there is no clear relationship between clinical effect and serum concentration for the SSRIs, including fluoxetine. It has been hypothesized that there may be a curvilinear relationship such as the one described for tricyclic antidepressants (Reis et al., 2009), although generally dose-response curves appear to be flat (Vaswani et al., 2003). It has also been suggested that instead of a dose-effect curve, there is a threshold that must be exceeded before therapeutic effects emerge (Preskorn, 1997). With such a broad range observed clinically and no clear relationship to therapeutic effects, it is difficult to pinpoint optimal serum levels for preclinical studies. Nonetheless, it is important to consider the human clinical conditions.

The present study demonstrates that the pharmacokinetics of fluoxetine are more rapid in non-human primates, and that multiple dosing paradigms are best suited for replicating both human serum concentrations and ratios of fluoxetine to norfluoxetine. Specifically, 5.6 mg/kg administered daily either orally or subcutaneously results in combined serum levels with-in the reported human clinical range. However, on two occasions, sores developed following subcutaneous administration, thus oral administration

was chosen for longer studies (i.e. 4-6 weeks). In humans, fluoxetine is completely absorbed from the gut but has oral bioavailability less than 90%, (Hiemke and Hartter, 2000; Vaswani et al., 2003) so to ensure consistent concentrations in the human therapeutic range, we chose a higher dose (10mg/kg/day) for our 6-week study. This dose, mixed with a variety of palatable foods, reliably produced serum concentrations in the clinical range (>200ng/ml) over the 6-week period, although concentrations were considerably higher and more variable during the initial two weeks of the first treatment. These levels (250-450ng/ml) are comparable to those reported following 20 mg/daily and 40 mg/daily in humans (Amsterdam et al., 1995; Preskorn, 1997; Brunswick et al., 2002; Baumann et al., 2004; Reis et al., 2009). Serum concentrations were replicated in a second 6-week administration period, demonstrating the reliability of producing serum concentrations in the desired range.

There are two possible reasons for the reduction in serum concentrations of total fluoxetine seen after week 1 (Figure 2-5A). The first is enzyme induction, which has been reported following fluoxetine administration in rat liver (Daniel et al., 2006; Haduch et al., 2008), and the second is decreasing compliance in consuming the medicated treat, which would be consistent with the experience of Fontenot et al (Fontenot et al., 2009). There are no studies currently available on the effect of chronic fluoxetine administration on enzymes in the rhesus macaque. While we were not able to quantify compliance, daily visual inspection following treat administration did indicate that levels of compliance varied, suggesting that this is the more probable explanation. Additionally, three animals underwent a second 6-week administration period and did not show high initial levels. If enzyme induction had occurred, enzyme production might be expected to either normalize during the interval between treatments, and thus produce high initial levels again, or to reoccur, driving

serum concentrations further down regardless of initial levels. However, the lack of these effects in the second round of treatment does not rule out the possibility of enzyme induction and persistence of the increase in enzyme levels. Nonetheless, given that the concentrations maintained in later weeks of chronic treatment are closer to those of the lower doses (2-4 mg/kg/daily) tested by Fontenot and colleagues (Fontenot et al., 2009), it seems likely that the animals were not consuming the full dose. However, since serum concentrations remained in the clinical range, this is unlikely to pose a problem for translational studies, especially as the majority of clinical reports are also in this range (Amsterdam et al., 1995; Brunswick et al., 2002; Baumann et al., 2004).

2.4.4 Conclusions

This study describes the pharmacokinetics of fluoxetine and its active metabolite norfluoxetine in rhesus macaques. Based on these results, we developed a chronic dosing regimen able to approximate serum concentrations similar to those observed in humans. Additionally, as in humans under the chronic administration conditions, norfluoxetine concentrations exceeded fluoxetine concentrations in rhesus macaques. While the half-lives are shorter than those reported in humans, there is still a significant possibility that active concentrations will persist in the blood past the conclusion of treatment, which was taken into consideration when determining the timing of experimental measures. Use of comparable dosing regimens and conditions in preclinical studies are important considerations for translating results to the human condition.

3. COCAINE-RELATED BEHAVIOR AND NEUROCHEMISTRY

3.1 Introduction

Cocaine is a nonselective monoamine reuptake inhibitor that targets the DAT, SERT, and NET. The stimulant and abuse-related effects of cocaine are currently believed to rely upon an increase in extracellular DA resulting from blockade of the DAT (Woolverton and Johnson, 1992), particularly in the nucleus accumbens (Howell and Wilcox, 2002). Administration of cocaine or other DAT reuptake inhibitors results in measurable, dose-dependent increases in extracellular DA that are temporally correlated with drug infusion (Czoty et al., 2000; Kimmel et al., 2005). In animals, self-administration of a compound depends on both DA release and speed of onset (Kimmel et al., 2008), while in humans, the perceived "high" is strongly correlated with both factors (Volkow et al., 1999a; Volkow et al., 1999b).

The 5HT system modulates the DA system. The raphe nucleus, which contains serotonergic cell bodies, projects to key brain areas involved in drug abuse, including the VTA, the nucleus accumbens, and the prefrontal and frontal cortex, which express a range of 5HT receptors that modulate DA release and psychostimulant effects (Alex and Pehek, 2007; Di Matteo et al., 2008; Hayes and Greenshaw, 2011). SSRIs and direct serotonergic agonists do not support self-administration or induce behavioral-stimulant effects on their own, but were able to attenuate self-administration (Carroll et al., 1990; Richardson and Roberts, 1991; Czoty et al., 2002; Glatz et al., 2002), reinstatement (Ruedi-Bettschen et al., 2009), and the behavioral-stimulant effects of cocaine (Spealman, 1993; Howell and Byrd, 1995; Czoty et al., 2002). SSRIs are also able to reduce cocaine-induced DA overflow in squirrel monkeys (Czoty et al., 2002). Furthermore, these effects are not dependent on cocaine's

effects at the SERT, as both SSRIs and direct 5HT agonists are able to attenuate the behavioral-stimulant effects of a selective DAT inhibitor with no appreciable effects at SERT (Howell et al., 1997).

However, SSRIs have repeatedly failed to reduce cocaine abuse during clinical trials (Grabowski et al., 1995; Batki et al., 1996; Schmitz et al., 2001; Lima et al., 2003; Winstanley et al., 2011). This may be partially due to different dosing regimens; preclinical studies generally employ single dose treatments whereas clinical studies administer the drug chronically. It is well known that the therapeutic effects of SSRIs for depression and mood disorders do not emerge until 3-4 weeks after beginning treatment, an effect which is believed to rely on neurobiological changes (Wong et al., 1995; Thompson, 2002; Vaswani et al., 2003). Neurobiological changes due to prolonged SSRI treatment may alter the effects of the SSRI on cocaine-related behavior and neurobiology. Therefore, we examined the effects of a clinically-relevant dosing regimen with fluoxetine on cocaine-related behavior and neurochemistry.

3.2 Methods

Subjects. Adult female rhesus macaques (*macaca mulatta*; n=5) weighing between 6-9kg served as subjects in this study. All subjects were individually housed within a primate colony and fed with Purina monkey chow (Ralston Purina, St. Louis, MO, USA) supplemented with fruits and vegetables. Water was freely available. Each subject was fitted with a collar (Primate Products, Woodside, CA) and acclimated to being comfortably seated in a primate chair (Primate Products) for up to 4 hours. All procedures and studies strictly followed the National Institutes of Health Guide for the Care and Use of Laboratory Animals

(Publication No. 85-23, revised 1985), and were approved by the Institutional Animal Care and Use Committee of Emory University.

Drugs. Cocaine HCL (National Institute on Drug Abuse, Rockville, Md, USA) was dissolved in 0.9% saline and diluted to the desired unit dose according to each individual's body mass. Fluoxetine HCL was purchased from Spectrum Chemicals and Laboratory Products (New Jersey, USA) and administered orally (10 mg/kg/day) for 42 days. Each animal's dose was individually weighed and mixed with peanut butter or other palatable substances and the animals' cages visually inspected after administration to ensure consumption. Additionally, serum concentrations were monitored via weekly blood draws to ensure levels within the target range (Sawyer and Howell, 2011). Following each draw, the blood was centrifuged at 3000g for 20 minutes and serum was then collected and frozen at 0°C for later analysis. Samples were analyzed using mass spectrometry and quantified against both internal standards and separate standard curves (Ritchie et al., 2009a).

Catheter surgery and maintenance. Each animal was implanted with a chronic indwelling subcutaneous access port (Access Technologies, Skokie, IL, USA) and venous catheter according to procedures previously described (Howell and Wilcox, 2001b). Surgery was carried out under aseptic conditions. Prior to surgery, animals were sedated with Telazol (4-5mg/kg) and brought to the surgery prep room and prepared with an intravenous saline drip and intubated. Anesthesia was then maintained using isofluorane (1-2%). The site for surgery was shaved and scrubbed using alternating alcohol and betadine wipes. The animal was positioned on the surgery table and draped, and an incision made into either the leg or neck, for femoral or jugular catheters, respectively. The vein was then isolated and return blood flow tied off using 0-0 PDS (Ethicon, San Angelo, TX). A small incision was made

into the vein and a silicone catheter (Access Technologies) inserted. The catheter was then anchored to the vein using 3-0 Ethilon (Ethicon). A "pocket" was then created between muscle layers and a loop of catheter inserted and anchored with 3'0 Ethilon. A trephine was used to pass the free end of the catheter subcutaneously to the back, where the access port was attached and secured subcutaneously using 4-0 Vicryl (Ethicon). Both incisions were then closed using subcutaneous continuous sutures with 4'0 Vicryl. The animal received antibiotic (Rocephin) and analgesic (Banamine and buprenorphine) treatments according to direction by the veterinary staff and was permitted to recover for 2 weeks before beginning testing.

The subcutaneous port was accessed using Huber needles (Access Technologies), which are specially designed to minimize damage to both the skin and port membrane. Before inserting the needle, the area was first cleaned by shaving and cleaning with 3 sterile alcohol pads followed by a SeppStick (10% povidone iodine; CareFusion, Leawood, KS). Catheters were flushed a minimum of once per week with 1.5-2 ml of heparinized saline (100 USP, Hospira Inc, Lake Forest, IL) and daily during self-administration testing at the conclusion of the day's session, in order to maintain patency.

Behavioral paradigms. In order to assess the effects of chronic SSRI treatment on cocaine-related behavior, cocaine self-administration procedures were used to model both ongoing cocaine use and relapse (Griffiths et al., 1980; Stewart and de Wit, 1987; O'Brien and Gardner, 2005).

Self-administration. Subjects were trained to self-administer cocaine by responding on an operant panel equipped with 1 active lever and two stimulus lights (one white light to indicate cocaine availability and one red light paired with drug infusion). For each testing

session, animals were removed from the home cage and seated comfortably in a primate chair for transfer to the testing room. The operant panel was attached to the front of the chair and the chair placed in a sound-attenuating chamber equipped with a white noise generator and a fan to aid ventilation. An infusion set was placed in the port with a Huber needle and passed through a hole in the chamber wall to attach to a 35cc syringe on an automated syringe pump (Model PhD 2000, Harvard Apparatus, Holliston, MA). A computer program (MedPC, MedAssociates, St. Albans, VT) recorded data, controlled all experimental events during the session, and calculated session rate at the conclusion of each testing session.

All subjects self-administered cocaine on a fixed-ratio (FR) 20 response schedule. Sessions lasted until a maximum of 30 infusions were earned or 1 hour had elapsed, whichever occurred first. Each drug infusion was paired with a brief illumination of a red light and followed with a 30-second timeout. Prior to beginning the block design (Ch1), cocaine dose-response curves were determined in all subjects by substituting different doses of cocaine in the syringe until there were no discernable trends in responding and responding stabilized (<20% variability) for three days. The dose that engendered peak responding was chosen for subsequent experiments (0.01 mg/kg/inf for all subjects). Average response rate across a five-day period served as a measure of reinforcing effects.

Reinstatement. Subjects underwent extinction training in which saline was substituted for cocaine in the syringe and the brief paired stimuli (lights) omitted until responding fell below 20% of previous response rate. At this point, reinstatement sessions were conducted by restoring the brief paired stimuli and priming the animal with a non-contingent dose of cocaine immediately prior to beginning the day's testing session. Only saline was available during reinstatement sessions. Two doses of cocaine (0.01 mg/kg and 0.03 mg/kg, i.v.) and

saline were tested in reinstatement. Responding was re-extinguished between reinstatement sessions.

Microdialysis cannula surgery and maintenance. Subjects (n = 5) were surgically prepared with guide cannula targeting the caudate for *in vivo* microdialysis. Animals were sedated with Telazol (4-5 mg/kg) and brought to the surgery suite, where they were intubated, prepared with an intravenous saline drip, and maintained on isofluorane anesthesia for the duration of the surgery. Surgery was carried out under aseptic conditions. Animals were then placed in a stereotaxic frame and the top of the head shaved and scrubbed with alternating alcohol and betadine swabs. An incision was made using a cautery and the skull cleared of tissue and dried. Small burr holes were drilled directly above both caudate nuclei (approximate coordinates: AP 6.8; ML: 42.8, 34.8). Additional holes were drilled for screws to secure the implant. The guide cannulae were slowly lowered into the burr holes to minimize tissue damage. Once the cannulae were placed, a protective cap was placed over them. One titanium screw (Crist Instrument Co., Hagerstown, MD), 8-9 Teflon screws (PlasticsOne INC, Roanoke, VA), and cranioplastic cement (Lang Dental Manufacturing Co, Inc., Wheeling, IL) were used to secure the cannulae (CMA/12) and protective cap (Crist Instrument Co.), after which the area around the implant was sutured and the animal allowed to recover for 2-3 weeks before beginning experiments. Two of the 5 animals implanted lost the cannula preparation prior to starting experiments; thus only 3 animals participated in the microdialysis studies described. Stainless steel stylets (CMA) were placed in the cannulae when not in use. The stylets and protective cap were cleaned with alcohol following each microdialysis session.

In vivo *microdialysis and HPLC analysis.* To directly examine the effects of chronic SSRI treatment on the neurochemical effects of cocaine, *in vivo* microdialysis sessions were conducted using a cocaine challenge (1.0 mg/kg, i.v.) and the samples assessed for dopamine. On the day of the microdialysis experiment, animals were removed from the home cage and comfortably seated in a Primate Products chair and a Lexan neck plate placed at shoulder height prevent the animal from reaching the microdialysis probes. Commercially available microdialysis probes (CMA/12) were inserted into the guide cannula and attached to a microinjection pump (CMA/102) which continuously circulated artificial cerebrospinal fluid (Na2HPO4, 1.0 mM; NaCl, 150 mM; KCl, 3mM; CaCl, 1.3 mM; MgSO4, 1.0 mM; and ascorbic acid, 0.15 mM) via FEP Teflon tubing to the probe for perfusion at a flow rate of 2.0 µl/min. After insertion of the probe, a one-hour equilibrium period followed, after which sampling began. Samples were collected every 10 minutes for the duration of the experiment. A one-hour baseline sampling period was followed by administration of the drug challenge (1.0 mg/kg cocaine), after which sample collection continued for another two hours. After the final experimental sample was collected, the KCl concentration of the perfusate was increased to 100 mM for 10 minutes to induce voltage-dependent release of neurotransmitter to verify tissue integrity. Probes were tested in vitro to ensure probe efficiency and performance before and after in vivo experiments.

Small-bore, high-performance liquid chromatography (HPLC) and electrochemical detection were used to measure the extracellular levels of DA in each sample (Czoty et al., 2000; Murnane et al., 2010). The HPLC system consisted of a small bore column (3.2mm x 150mm x 3 µm), an ESA 582 model solvent delivery pump set to a flow rate of 0.6 ml/min, and an ESA model 542 autosampler (ESA, Inc., Chelmsford, MA). Electrochemical detection

was carried out with a guard cell (ESA model 5020; potential 350 mV), a dual-channel analytical cell (ESA model 5040; oxidative channel potential -150 mV, reductive channel 200 mV), and an ESA model Coulochem II detector. A commercially available mobile phase (MD-TM2; ESA, Inc.) composed of sodium dihydrogen phosphate (90 mM), octanesulfonic acid (1.7 mM), citric acid (50 mM), EDTA (50 μM), and acetonitrile (10%) was used. Phosphoric acid was added to bring the final pH to 3.

Chromatograms were generated by and analyzed using EZChrom Elite software (version 3.1, Scientific Software, Pleasanton, CA, USA). Each chromatograph was visually inspected to insure accurate peak selection and definition. Three sets of standards (25, 10, 5, 1, 0.5 nM) were run with each set of samples and used to construct a standard curve for concentration. Estimated concentrations based on area under the curve for each peak were output by EZChrom. Data were normalized to the average of the 6 baseline samples and presented as percent of baseline.

Experimental designs. *Experiment 1: Chronic fluoxetine treatment*. Subjects (n = 5; see Appendix Tables 1 and 2 for complete list of subjects participating in each measure) underwent the block design described in Chapter 2. No subjects had experience with any other drugs aside from cocaine beyond those necessary for animal care prior to beginning the study. Treatment was administered in a repeated-measures block design (Figure 2-6) and lasted for 6 weeks (42 days). Fluoxetine concentrations were monitored via weekly blood draws and are reported in Figure 2-5. Each 6-week block consisted of four weeks of cocaine self-administration sessions, followed by a two-week period of extinction and reinstatement testing (see Methods above). All behavioral measures were taken during weeks 4-6 of treatment, which was chosen based on the time to onset for therapeutic effects in depression

(3-4 weeks) (Wong et al., 1995; Thompson, 2002) to ensure that any putative neurobiological changes would be stable. Self-administration rates consisted of the average of the sessions from week 4 of treatment. Days to meet extinction criteria and reinstatement were measured during weeks 5-6. The DA response to cocaine was assessed using *in vivo* microdialysis at baseline, during week 4 of treatment to determine the direct effect of chronic fluoxetine treatment on cocaine's ability to increase DA, after the conclusion of treatment to capture the neurochemical effects of any neurobiological changes, and again after washout to determine if changes persist (Figure 2-6).

Experiment 2: Acute fluoxetine treatment. Subjects (n = 3) from Experiment 1 underwent acute fluoxetine (10 mg/kg; i.m.) pretreatment prior to self-administration sessions. Pretreatments were administered in the home cage 30 minutes prior to the start of the testing session and blood was collected immediately after the session to verify serum concentrations of fluoxetine. The effects of acute fluoxetine treatment on cocaine self-administration were determined 3 times and then averaged for each subject with a minimum of 1 week between determinations to avoid accumulation of fluoxetine. All sessions were conducted at least 5 months after the conclusion of chronic fluoxetine treatment and animals had no exposure to fluoxetine or other experimental drugs besides cocaine during that time.

Data analysis. Alpha was set at $p<0.05$. Data are presented as means and standard errors. Behavioral data were normalized to baseline (Experiment 1) or the average rate from 3 days preceding the treatment day (Experiment 2). The data were then analyzed using one-way repeated measures (RM) analysis of variance (ANOVA) tests with Boferroni's or Dunnet's post hoc tests where appropriate. Raw baseline values for microdialysis were compared using one-way RM ANOVA. Data were then normalized to the average of the 6

baseline samples and analyzed using a two-way RM ANOVA on both factors with post-hoc Bonferroni-corrected Tukey's critical difference tests. All analyses, except two-way RM ANOVA on both factors, were performed using GraphPad (GraphPad Prism version 5.01 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com.) RM ANOVA on both factors were conducted using SigmaStat for Windows (version 3; Systat Software, San Jose, CA).

3.3 Results

The effects of chronic fluoxetine treatment on maintenance and reinstatement of cocaine self-administration. Cocaine self-administration rates varied greatly between individuals (Figure 3-1), thus rate data were normalized to the stable baseline average for each subject. No significant differences were seen between baseline, chronic fluoxetine treatment, and washout conditions ($F(2,3) = 0.176$; $p = 0.84$; Figure 3-2), although chronic fluoxetine treatment caused a non-significant increase in cocaine self-administration rate. Acute fluoxetine pretreatment significantly attenuated self-administration rates ($t(2) = 4.04$; $p<0.05$; Figure 3-2).

The number of sessions required to meet extinction criteria did not differ between conditions ($F = 1.71$, $p = 0.24$; Figure 3-3). A dose of 0.01 mg/kg cocaine resulted in significant reinstatement of responding as compared to a saline prime ($F(2,3) = 4.94$, $p = 0.05$; Dunnet's multiple comparison $p<0.05$; Figure 3-4) and 0.03 mg/kg resulted in responding above extinction levels. Following chronic fluoxetine treatment, this significant reinstatement was no longer observed (main effect of dose: $F(2,3) = 1.84$, $p = 0.18$; main effect of treatment: $F(2,3) = 2.92$; $p = 0.07$; no interaction) and response rate remained at

extinction levels for all doses of cocaine tested (0.01 and 0.03 mg/kg). This effect persisted after the 6 week washout period.

The effects of chronic fluoxetine treatment on cocaine-induced dopamine overflow. There was no significant difference between baseline DA dialysate levels across the treatment time-points (baseline, mid-SSRI treatment, post-SSRI treatment, and post-washout) ($F(3, 6) = 0.3964$, $p > 0.05$; Table 3-1), thus each dataset was normalized to its respective baseline. Cocaine induced a significant increase in DA overflow in the caudate nucleus (main effect of time, $F(17,34) = 29.26$; $p < 0.001$). There was no main effect of fluoxetine treatment ($F(3,6) = 1.72$; $p > 0.05$) but there was a significant interaction between time and treatment ($F(51,102) = 1.65$, $p = 0.016$). Post hoc testing indicated that the DA overflow was significantly attenuated at 20 minutes post-injection (peak effect) during and immediately after fluoxetine treatment, as well as after the 6-week washout period ($p<0.05$; Figure 3-5).

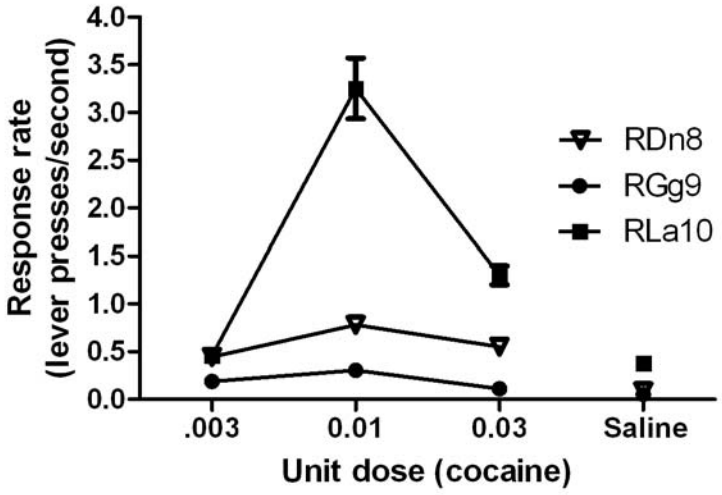


Figure 3-1: Representative dose-response curves from 3 subjects. Maximal response rates vary between individuals.

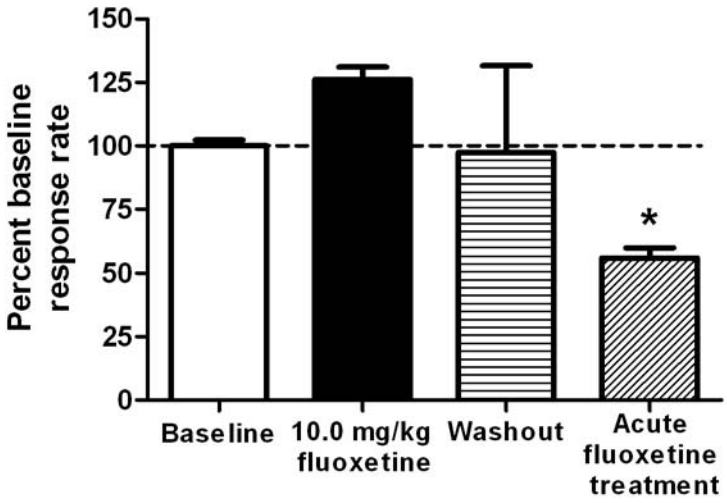


Figure 3-2: Chronic fluoxetine (n=4) has no effect on self-administration rates although acute pretreatment (n=3) with fluoxetine at matched serum concentrations attenuates rates.

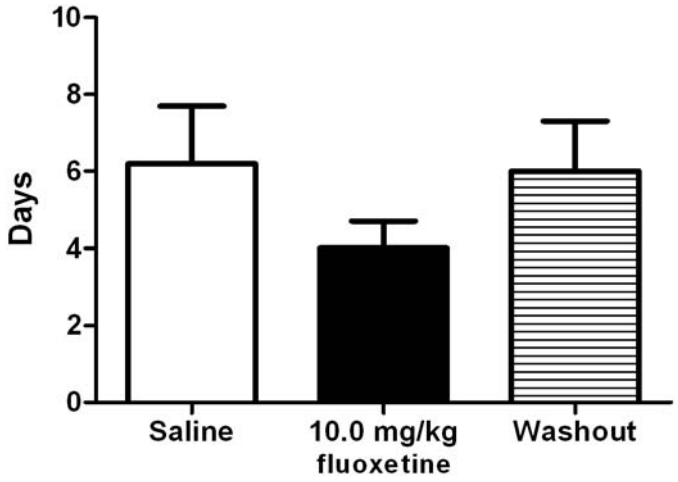


Figure 3-3: Chronic fluoxetine treatment had no effect on number of days required to meet extinction criteria (<20% baseline responding) (n=5).

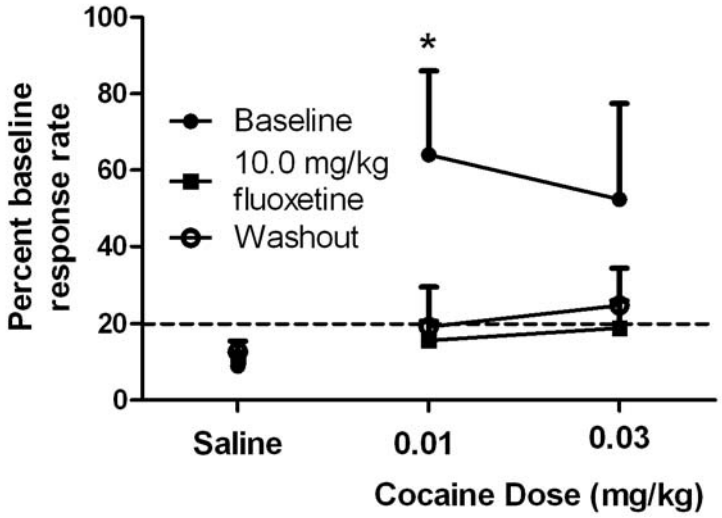


Figure 3-4: Cocaine-primed reinstatement is suppressed following chronic fluoxetine treatment and remains suppressed after a 6-week washout period (n=4).

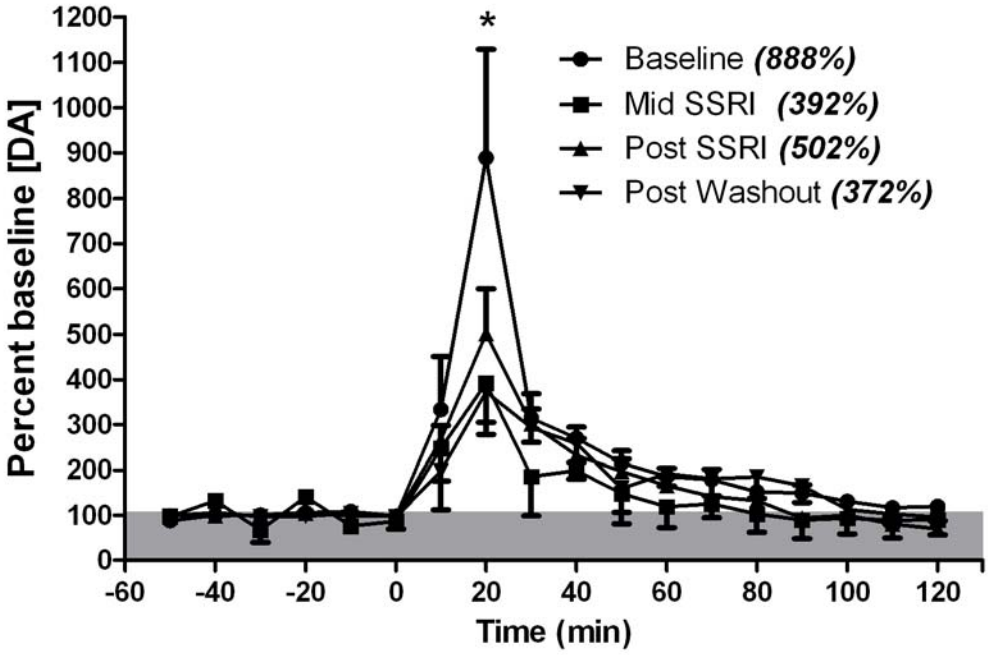


Figure 3-5: Cocaine-elicited (1.0 mg/kg, i.v.) dopamine overflow is significantly attenuated during and after chronic fluoxetine treatment and remains attenuated after a 6-week washout period (n = 3). Mean peak overflow values are reported in parentheses in the legend.

Table 3-1: Raw baseline values for dopamine (mean ± SEM)

Time point	Dopamine (nM)
Baseline	2.63 ± 1.01
Mid-SSRI	4.76 ± 3.10
Post-SSRI	4.74 ± 1.13
Post-Washout	4.13 ± 1.52

3.4 Discussion

The present study demonstrates that chronic fluoxetine treatment at clinically-relevant serum concentrations is able to attenuate cocaine-primed reinstatement and cocaine-induced DA overflow, and that these effects persist up to 6 weeks following the conclusion of fluoxetine treatment. However, chronic fluoxetine treatment did not affect cocaine self-administration, in contrast to a serum concentration-matched acute injection and previous preclinical reports (Carroll et al., 1990; Richardson and Roberts, 1991; Czoty et al., 2002; Glatz et al., 2002). Together, these results suggest that chronic SSRI treatment results in neurobiological changes that influence specific aspects of cocaine-related behavior and neurobiology.

3.4.1 Cocaine-related behavior and neurochemistry

Previous preclinical work has demonstrated that acute administration of SSRIs attenuates cocaine self-administration (Carroll et al., 1990; Richardson and Roberts, 1991; Czoty et al., 2002; Glatz et al., 2002), reinstatement (Ruedi-Bettschen et al., 2009), locomotor-stimulant (Spealman, 1993; Howell and Byrd, 1995) and subjective (Walsh et al., 1994) effects. During chronic fluoxetine treatment, cocaine-primed reinstatement was significantly suppressed, in agreement with the results of previous studies using acute pretreatments (Ruedi-Bettschen et al., 2009). Cocaine-induced DA overflow was also significantly attenuated during chronic treatment, suggesting that the presence of fluoxetine in the system partially blocked cocaine's effects. However, this attenuation of cocaine's neurochemical effects did not affect ongoing cocaine self-administration during treatment, which is in contrast to previous preclinical studies (Carroll et al., 1990; Richardson and Roberts, 1991; Czoty et al., 2002; Glatz et al., 2002), but in concordance with clinical studies

reporting no effect of fluoxetine treatment in human cocaine users (Grabowski et al., 1995; Batki et al., 1996; Schmitz et al., 2001; Winstanley et al., 2011). The difference in effects between the current study and previous preclinical studies is unlikely to be due to a difference in serum concentrations and pharmacokinetics achieved by acute versus chronic dosing, since when serum concentrations achieved by chronic treatment were matched using a single injection, fluoxetine significantly attenuated cocaine self-administration. Rather, it suggests the effects of chronic SSRI treatment in altering cocaine use may be mediated in part by neurobiological changes, as is hypothesized for the therapeutic effects of SSRIs in depression (Wong et al., 1995; Thompson, 2002; Vaswani et al., 2003). This is further supported by the observation that these effects persisted for 6 weeks following the conclusion of fluoxetine treatment.

A previous study has shown that chronic cocaine self-administration blunts the amount of dopamine overflow induced by a non-contingent cocaine challenge (Kirkland Henry et al., 2009), and this phenomenon could also explain the reduction in cocaine-induced DA overflow. However, Kirkland Henry and colleagues compared the naïve state to 10 weeks of a "short access" schedule of cocaine self-administration, followed by a transition to 10 weeks of a "long access" schedule, and only observed a decrease in cocaine-elicited DA between the naïve state and either cocaine-exposed state, but no differences between cocaine access conditions. This suggests that the initiation of cocaine self-administration alters the neurochemical response to cocaine, but that it is not progressive. Prior to beginning the baseline measures, our subjects self-administered cocaine for a minimum of one year, and thus any neurochemical changes in the DA response to cocaine would have already developed and stabilized. Thus, we do not believe this to underlie the observed decrease in

cocaine-elicited DA. Another possible explanation could be that the microdialysis sites deteriorated over time, resulting in fewer synapses near the probe and thus less collected DA. However, this seems unlikely, as previous studies have demonstrated that the tissue around remains stable even after multiple accesses, yielding consistent responses over time, and that no glial scarring is induced by repeated probe insertion (Czoty et al., 2000).

Given the observed reductions in cocaine-induced DA overflow, it is surprising that cocaine self-administration behavior was not affected. However, self-administration involves multiple, repeated exposures to the reinforcer during a single session and therefore it may be possible to overcome a decrease in the reinforcing potency of a single injection, making self-administration more robust and difficult to disrupt than reinstatement, in which a single, non-contingent dose is given (O'Brien and Gardner, 2005). An increase in self-administration rates would be expected if the subjects were compensating for decreased potency; no significant increases in self-administration rates were seen, but, since the number of infusions available per session was limited to 30, the sensitivity of our schedule may have been limited in its ability to detect compensation. This question could be further addressed through concurrent microdialysis and cocaine self-administration studies, to directly compare the DA overflow achieved during the self-administration session prior to and during chronic fluoxetine treatment (e.g. Kimmel et al., 2005), or use of a different self-administration schedule such as the progressive ratio schedule.

3.4.2 Methodological considerations

Validity of animal models

Animal self-administration and drug-induced reinstatement models are well established and used to model both human drug-taking and drug-seeking behaviors. These

models have strong face and predictive validity, in that drugs that humans abuse are self-administered by animals, and pharmacotherapies that reduce drug-taking in animals often possess a degree of efficacy in humans (Mello and Negus, 1996; Ator and Griffiths, 2003; O'Brien and Gardner, 2005). Additionally, conditions which trigger relapse in humans stimulate reinstatement in animals and vice versa (Stewart and de Wit, 1987; Gardner, 2000). However, there are some notable exceptions to the success of these models, such as the lack of animal self-administration of many hallucinogens (Fantegrossi et al., 2008). Furthermore, animal models are often able to only model one or two aspects of the complexity of the human situation; for example, self-administration procedures describe different aspects of the reinforcing effects while drug discrimination procedures compare the internal stimuli associated with each drug (Ator and Griffiths, 2003). Additionally, animal models often do not account for possible drug history, early life stress, variation in drug quality and dose, polydrug use, social influences, and other factors that all affect human drug taking. However, while it is important to keep these factors in mind when translating results from animal models, these models still provide useful tools for studying the difference aspects of human drug-taking behavior.

Choice of schedule

There are several different procedures widely used for drug self-administration procedures in primates, ranging from fixed-ratio schedules, in which a certain number of responses (in the present study, lever presses) results in delivery of the reinforcer, to more complex second order schedules, in which additional requirements such as a waiting period are built in, and progressive ratio schedules which increase the response requirement for each successive presentation of the reinforcer (Everitt and Robbins, 2000; O'Brien and Gardner,

2005; Oleson and Roberts, 2008). We chose the fixed ratio schedule due to its simplicity, sensitivity to experimental intervention, and past success in detecting reductions in reinforcing effectiveness (O'Brien and Gardner, 2005), although the interpretation of the behavioral results of the schedule do have limits (see above for discussion).

Appropriate controls

Another important consideration in drug self-administration studies is the use of a control group, such as animals working under a food or non-drug maintained schedule, in order to evaluate the specificity of the intervention's effects. Manipulations that reduce all appetitive behavior may have unwanted side effects in humans. In the present study, we were unable to have a non-drug maintained group. Previous studies have demonstrated that acute administration of fluoxetine can reduce non-drug appetitive behavior in addition to drug-maintained behavior (Carroll et al., 1990; Kleven and Woolverton, 1993). However, we did not see any reduction in self-administration rates over the course of our chronic study, and thus do not believe this to be an influencing factor in our results.

In order to provide a control for fluoxetine treatment, animals underwent the 6 week behavioral block of testing and neurochemical assessment before receiving treatment and thus served as their own control in the present study, increasing the power and reducing the number of animals required. No saline or placebo treatment was administered; however, the animals all receive treats on a daily basis and thus the administration of a medicated treat is not disruptive to the animals' usual routine.

3.4.3 Clinical implications

The lack of effect on ongoing self-administration behavior aligns with the results of clinical, rather than preclinical, studies, suggesting that the discrepancy in the results of these

previous studies is due to differences in SSRI administration, underscoring the need to appropriately design preclinical studies to mirror the potential clinical usage of a treatment. Furthermore, the present data suggest that there may be potential for increased cocaine consumption during chronic fluoxetine treatment to compensate for decreased potency, a risk that needs further investigation. Importantly, cocaine-primed reinstatement was suppressed, suggesting that while fluoxetine may not be effective as an intervention in ongoing cocaine abuse, it may be useful as an adjunct treatment to prevent relapse during initial or continuing abstinence, especially given the striking persistence of the effects after the conclusion of treatment.

4. SEROTONERGIC NEUROBIOLOGY AND NEUROCHEMISTRY

4.1 Introduction

It is well established that the therapeutic effects of SSRIs for depression do not emerge until 3-4 weeks after starting treatment (Wong et al., 1995; Thompson, 2002; Vaswani et al., 2003). This lag in onset is believed to be the amount of time necessary for neurobiological changes, which are then responsible for the clinical improvements, to develop and stabilize. These neurobiological changes are varied and affect many aspects of the serotonin system's function. The SERT, responsible for reuptake of 5HT and termination of 5HT signaling, down-regulates following chronic treatment with SSRIs *in vitro* (Horschitz et al., 2001; Iceta et al., 2007), in rats (Benmansour et al., 1999; Benmansour et al., 2002), and in humans (Kugaya et al., 2003). During chronic SSRI treatment, serotonin levels, which increase following acute administration of an SSRI (Kreiss and Lucki, 1995; Clark et al., 1996), are no longer elevated (Clark et al., 1996; Smith et al., 2000), and SSRIs do not induce acute increases in 5HT (Benmansour et al., 1999). However, serotonin clearance is markedly slower following chronic treatment, an effect with correlates with the time-course of SERT down-regulation, which takes 10-15 days (Benmansour et al., 2002). Recently, it has been shown that this down-regulation may occur through SSRI-mediated up-regulation of microRNA-16, which interferes with SERT expression (Baudry et al., 2010).

The lack of increased basal 5HT levels during chronic SSRI treatment may be due to changes in regulation of 5HT system activity. Acute SSRI treatment causes internalization of 5HT1A autoreceptors, which provide inhibitory feedback to the 5HT cell bodies in the raphe (Aznavour et al., 2006; Riad et al., 2008); however 5HT1A binding is unchanged during chronic treatment in mice (Gunther et al., 2008), cats (Aznavour et al., 2006), and humans

(Moses-Kolko et al., 2007; Moulin-Sallanon et al., 2009). Additionally, no 5HT1A receptor internalization occurs following agonist challenge (Riad et al., 2008), and the response to a 5HT1A agonist is attenuated (Cremers et al., 2000; Ceglia et al., 2004), suggesting that the receptors exist in a desensitized state. This desensitization is believed to contribute to the antidepressant effects of SSRIs, as it removes the auto-inhibitory counteraction of their transmission-facilitating effects (Blier et al., 1998). The mechanism for desensitization is unclear, as reductions in GTPγS-binding were not seen (Moulin-Sallanon et al., 2009), and other mechanisms may compensate for the reduced auto-inhibitory feedback (Cremers et al., 2000). Furthermore, post-synaptic 5HT1A receptors in the frontal cortex may actually sensitize following chronic treatment (Moulin-Sallanon et al., 2009; Carr and Lucki, 2011).

Many post-synaptic serotonin receptors have been implicated in the antidepressant effect (Carr and Lucki, 2011) and may also be targets for regulatory changes resulting from chronic SSRI treatment. In particular, the post-synaptic 5HT1A (Gunther et al., 2008; Moulin-Sallanon et al., 2009), 5HT2A (Massou et al., 1997; Meyer et al., 2001; Gunther et al., 2008) and 5HT2C (Yamauchi et al., 2004) receptors may be affected, while the 5HT3 and 5HT4 receptors do not appear to undergo regulatory changes (Le Poul et al., 1995; Gobbi et al., 1997). Additionally, recruitment and production of new neurons may result from chronic treatment with SSRIs (Navailles et al., 2008; Perera et al., 2011).

Chronic exposure to cocaine also induces changes in the serotonin system. Increases in the SERT following chronic cocaine have been reported in cells (Kittler et al., 2010), rodents (Cunningham et al., 1992), primates (Banks et al., 2008; Gould et al., 2011), and humans (Jacobsen et al., 2000; Mash et al., 2000). Cocaine exposure has also been reported to affect a number of post-synaptic 5HT receptors, ranging from decreases in 5HT3 receptors

in the nucleus accumbens shell of rats sensitized to cocaine (Ricci et al., 2004) to reduced sensitivity of 5HT1A receptors (Baumann and Rothman, 1998). Cocaine exposure and withdrawal conversely regulate 5HT1B receptors (Hoplight et al., 2007; Neumaier et al., 2009) and have been reported to affect 5HT2A receptor expression and function, although no consensus has been reached as to the exact nature of these effects (Carrasco et al., 2006; Carrasco and Battaglia, 2007; Huang et al., 2009). However, several studies indicate that 5HT2A receptors may sensitize during withdrawal (Baumann and Rothman, 1996; Baumann and Rothman, 1998; Carrasco et al., 2006). Overall, chronic cocaine clearly results in an altered state of the serotonin system, which could affect how SSRIs are able to exert their effects. Therefore, we examined potential targets for neurobiological change in order to determine the effects of SSRIs in the context of cocaine abuse.

We selected two proteins in the 5HT system: one presynaptic protein, the SERT, and one post-synaptic protein, the 5HT2A receptor, in order to capture both pre- and post-synaptic changes. The SERT transporter was chosen for several reasons. It is the target for SSRIs and their effects upon it are well understood. Additionally, the SERT is a target for cocaine, which also exerts effects on its regulation. Thus, by examining the SERT, we may determine whether the changes in its regulation are typical of chronic SSRI treatment or whether chronic cocaine exposure has influenced them. Furthermore, changes in SERT regulation are believed to be important to the therapeutic effects of SSRIs for depression (Benmansour et al., 2002), and the presence or lack thereof may aid interpretation of behavioral studies (Ch 3). Finally, the SERT plays a major role in regulating 5HT signaling and thus changes in SERT may have far-reaching consequences.

The 5HT2A receptor was chosen as the post-synaptic protein due to its extensive implication in the control of DA and the behavioral effects of cocaine (Alex and Pehek, 2007; Bubar and Cunningham, 2008; Di Matteo et al., 2008; Filip et al., 2010). In addition to drug abuse, it has also been implicated in depression and anti-depressant effects (Marek et al., 2005; Meyer, 2007; Carr and Lucki, 2011), suicidality (Audenaert et al., 2006), and schizophrenia (Leuner and Muller, 2007; Meltzer and Massey, 2011). Importantly for drug abuse research, the 5HT2A receptor has also been linked to measures of impulsivity (Fletcher et al., 2007; Anastasio et al., 2011; Kirby et al., 2011), which may influence drug taking and addiction. Furthermore, recent studies have demonstrated that 5HT2A receptor regulation is more complex than previously understood (Aloyo et al., 2001; Van Oekelen et al., 2003; Brea et al., 2009; Yadav et al., 2011), and therefore additional studies are needed to fully elucidate it.

In order to capture the functional consequences of any neurobiological changes, we also directly assessed 5HT overflow and prolactin release. Prolactin response has traditionally been used as a measure of central serotonergic function (O'Keane and Dinan, 1991; Newman et al., 1998) and is correlated with serotonin release (Murnane et al., 2010). It is also correlated with other measures of 5HT function; for example, chronic MDMA users display blunted prolactin responses to drug challenge along with decreased SERT density, decreased 5-HIAA in the cerebrospinal fluid, and cognitive and mood alterations (Gerra et al., 1998; Gerra et al., 2000; Parrott, 2002). The prolactin response is reliable across time (Flory et al., 2002) and thus able to detect differences. Along with the SERT, multiple 5HT receptor subtypes are involved in stimulating prolactin release (Jorgensen, 2007), including the 5HT2A receptor (Aulakh et al., 1994; Jorgensen, 2007; Chaiseha et al., 2010). Thus,

combined with direct measures of 5HT release, the prolactin response can provide a measure of integrated post-synaptic response.

4.2 Methods

Subjects. Adult rhesus macaques (*macaca mulatta*) weighing between 6-14kg served as subjects in this study. All subjects were individually housed within a primate colony and fed with Purina monkey chow (Ralston Purina, St. Louis, MO, USA) supplemented with fruits and vegetables. Water was freely available. Each subject was fitted with a collar (Primate Products, Woodside, CA) and acclimated to being comfortably seated in primate chair (Primate Products) for up to 4 hours. All subjects had been previously prepared with a chronic indwelling catheter and access port and trained to self-administer cocaine using operant procedures (see Chapter 3, Methods, for details.) All procedures and studies strictly followed the National Institutes of Health Guide for the Care and Use of Laboratory Animals (Publication No. 85-23, revised 1985), and were approved by the Institutional Animal Care and Use Committee of Emory University.

Drugs. Cocaine HCL (National Institute on Drug Abuse, Rockville, MD, USA) was dissolved in 0.9% saline and diluted to the desired unit dose according to each individual's body mass. Fenfluramine, a selective serotonin releaser (Invernizzi et al., 1986; Garattini et al., 1987), was purchased from Sigma-Aldrich (St. Louis, MO, USA) and dissolved in 0.9% saline. Fluoxetine HCL was purchased from Spectrum Chemicals and Laboratory Products (New Jersey, USA) and administered orally (10 mg/kg/day) for 42 days. Each animal's dose was individually weighed and mixed with peanut butter or other palatable substances and the animals' cages visually inspected after administration to ensure consumption. Additionally,

serum concentrations were monitored via weekly blood draws to ensure levels within the target range (see Chapter 2)(Sawyer and Howell, 2011).

Positron emission tomography imaging. Subjects underwent separate positron emission tomography (PET) scans to determine 5HT2A and SERT density, using [^{11}C]M100907 (Ito et al., 1998) and [^{18}F]FEmZIENT (Stehouwer et al., 2008), respectively. All scans were performed using a MicroPET Focus 220 scanner in the Yerkes Imaging Center, which has reconstructed resolution of 2 mm in all directions. Each scan was reconstructed and attenuation-corrected, then inspected for movement and registered to a standard adult rhesus template according to Emory University's standard protocol, which ensures accurate location of regions of interest (ROIs). Three-dimensional ROIs for the frontal cortex, midbrain/brainstem, caudate, and cerebellum (see Figure 4-1) were drawn on the baseline scan; subsequent scans for each individual were aligned to their respective baseline scan and the ROIs re-sliced into the new scan's space in order to ensure that the ROI volume and dimensions were consistent within individual across scans. Each ROI was visually inspected following re-slicing to ensure accurate placement. A computer program was used to apply the Logan method (Logan et al., 1990) to generate the time-activity curve for each region, which quantifies the amount of activity per time point in that region. The cerebellum served as the reference region to account for non-specific binding (Kish et al., 2005; Hinz et al., 2007; Meyer et al., 2010), although it is important to note that it does contain a low level of 5HT2A expression and thus low binding potentials reflect similar levels, as opposed to the absence of 5HT2A binding. Non-displaceable binding potentials, BP_{ND}, were calculated using the generalized reference tissue model. Four rate constants, *R,*

k_2, k_3, and k_4, were used to fit a curve to the time-activity curve for each ROI (Votaw et al., 2002). Binding potential (k_3/k_4) served as an estimate of B_{max}.

On the day of the scan, the subject was sedated with 4-5 mg/kg of Telazol and transferred to the Yerkes Imaging Center, where the animal was intubated and prepared with an i.v. line. The animal was maintained on isofluorane anesthesia throughout the scan and pulse, body temperature, and breathing continually monitored. Following the attenuation scan, the animal was then injected with the radiotracer. Each scan lasted 90 ([^{11}C]M100907) or 120 ([^{18}F]FEmZIENT) minutes to ensure binding equilibrium; comparative analysis was performed between scans at equilibrium. At the end of the scan, the subject was returned to the animal rooms, and radiation safety precautions instated for 24 hours to allow for sufficient decay of the radioligand.

In vivo *microdialysis.* Subjects (n = 3) were prepared with guide cannula and *in vivo* microdialysis sessions conducted as described in Chapter 3; however, to assess the function of the serotonin system, the drug challenge consisted of a selective serotonin releaser, fenfluramine (3.0 mg/kg, i.v.), instead of cocaine. Sample collection remained the same as described in Chapter 3, although sessions were conducted concurrently with blood draws for prolactin response (see below). Samples were analyzed for 5HT content using HPLC and electrochemical detection as described in Chapter 3 (Czoty et al., 2000; Murnane et al., 2010). Chromatograms were generated and analyzed using EZChrom Elite software as described in Chapter 3. Data were normalized to the average of the 6 baseline samples and presented as percent of baseline.

Prolactin response. In order to assess the functional output of the 5HT system (Newman et al., 1998), prolactin time courses were conducted either concurrently with

microdialysis sessions (n =2) or independently (n = 1; animal lacked guide cannula) using methods similar to those described previously (Murnane et al., 2010). Prior to beginning, a temporary intravenous catheter was placed in the saphenous vein (BD Saf-T-Intima Closed Catheter System; BD, Franklin Lakes, NJ). Sample collection began 20 minutes before the administration of the fenfluramine challenge and continued at 10 minute intervals until 60 minutes post-injection. Two additional samples were collected at 90 and 120 minutes post-injection, after which the temporary catheter was removed. The samples were centrifuged for 20 minutes at 3000g, and the serum collected and frozen at 0°C for later analysis. A fluorescence-based enzyme-linked immunosorbent assay was used to quantify prolactin, and was carried out as described previously by the Yerkes National Primate Research Center's Biomarkers Core Laboratory (Mook et al., 2005).

Experimental designs. *Experiment 1: Chronic fluoxetine treatment.* Female subjects (n = 5; see Appendix Tables 1 and 2 for complete list of subjects participating in each measure) from Chapter 3, Experiment 1, underwent the block design described in Chapter 2. All procedures were carried out concurrently with those described in Chapter 3, Experiment 1. No subjects had experience with any other drugs aside from cocaine beyond those necessary for animal care prior to beginning the study. Treatment was administered in a repeated-measures block design (Figure 2-6) and lasted for 6 weeks (42 days). Length of treatment was chosen based on the time to onset for therapeutic effects in depression (3-4 weeks) (Wong et al., 1995; Thompson, 2002) to ensure that any putative neurobiological changes would be stable. Fluoxetine concentrations were monitored via weekly blood draws and are reported in Figure 2-5. Each 6-week block consisted of four weeks of cocaine self-administration sessions, followed by a two-week period of extinction and reinstatement

testing (see Chapter 3, Methods). Neurobiological and neurochemical measures were collected at three time points: *baseline*, before beginning chronic fluoxetine treatment; *post-SSRI treatment,* immediately following the conclusion of chronic fluoxetine treatment; and *post-washout*, immediately following the conclusion of a 6-week washout period following chronic fluoxetine treatment (Figure 2-6). All post-SSRI treatment measures were taken a minimum of 3-4 days following the conclusion of the 6-week block, to ensure that, based on the half-life, fluoxetine would no longer be present in the system (Sawyer and Howell, 2011), and thus that any effects observed would reflect the presence of the putative neurobiological changes as opposed to the direct effects of fluoxetine's presence. At each time point, subjects underwent a concurrent *in vivo* microdialysis/prolactin session with a fenfluramine challenge (n = 3) and 2 PET scans (n = 5); the first PET scan in the sequence was [^{11}C]M100907, to determine 5HT2A receptor binding potential, and the second was [^{18}F]FEmZIENT. Scans were separated by a minimum of 1 week for safety reasons and all procedures were separated by a minimum of 24 hours. On non-testing days, animals were allowed to self-administer cocaine under the usual schedule and conditions (see Chapter 3).

Experiment 2: The effects of chronic cocaine on 5HT2A receptor binding potential. Male subjects (n = 4) underwent cocaine self-administration following procedures and schedule as described in Chapter 3. All subjects had previous experience self-administering cocaine (Henry and Howell, 2009) and 1 animal had extensive exposure to psychostimulants. All subjects had been abstinent from any drugs for greater than 10 months. Each subject underwent a baseline PET scan with [^{11}C]M100907 to determine 5HT2A binding potential and then was retrained to self-administer cocaine (0.03 mg/kg/infusion) on an FR20 schedule of i.v. drug self-administration (see Chapter 3, Methods). Each animal could earn up to 60

infusions per hour-long session to maximize exposure to cocaine. Daily self-administration testing continued for 3.5 months, at which point the animals were scanned again. All scans occurred approximately 24 hours after the subject's last self-administration session.

Data analysis. Alpha was set at 0.05. Data were normalized to baseline values for microdialysis and prolactin time-courses following one-way ANOVA to ensure that baseline raw values did not significantly differ. Data were analyzed with two-way ANOVA with RM on both factors using SigmaStat for Windows (version 3). For time-course data, one factor was time and one was treatment stage. For PET binding potentials, the factors were ROI and treatment stage. Post-hoc testing consisted of Boferroni-corrected Tukey's critical difference tests. Graphical depictions were creating using GraphPad Prism (version 5.01 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com.) Data are plotted as mean with error bars representing standard error of the mean.

4.3 Results

Fenfluramine-induced serotonin overflow and prolactin release during and after chronic fluoxetine treatment. To determine the effects of chronic SSRI treatment on the serotonin system in the context of cocaine use, we examined the function of the 5HT system using a fenfluramine challenge. There was no difference between baseline 5HT dialysate levels between testing days ($F(2,5) = 4.02$, $p > 0.05$; Table 4-1), thus each dataset was normalized to its respective baseline. Fenfluramine induced significant increases in 5HT at all treatment stages as indicated by a main effect of time ($F(17,85) = 7.39$, $p < 0.0001$; Figure 4-2); however, chronic fluoxetine treatment had no effect on fenfluramine-induced 5HT

overflow as measured by treatment main effect ($F(2,5) = 0.93$, $p > 0.05$), peak effects ($F(2, 5) = 0.61$; $p > 0.05$), or area under the curve ($F(2, 5) = 0.80$, $p > 0.05$).

Prolactin levels, collected concurrently, were used to assess post-synaptic function and integrity of the 5HT system. Baseline levels of prolactin did not differ between testing days ($F(2, 4) = 1.31$, $p > 0.05$; Table 4-1), thus each dataset was normalized to its respective baseline. Fenfluramine administration resulted in significant increases in serum prolactin levels at all treatment stages with a main effect for time ($F(10,20) = 19.36$, $p < 0.001$; see Figure 4-3), a significant interaction with treatment ($F(20,40) = 2.29$, $p = 0.013$), and a trend towards a main effect of treatment ($F(2,4) = 4.563$; $p = 0.09$), indicating that prolactin release was blunted following fluoxetine treatment. Poc hoc tests did not reveal significant pair-wise comparisons at any time point. However, this lack of a statistically strong treatment effect may be due to high variability in the washout condition, during which one animal's prolactin response recovered (RLa10) and the other two did not (RGg9 and RZq8; Figure 4-4).

Effects of chronic cocaine alone and in combination with chronic fluoxetine treatment on 5HT2A and SERT binding potential. To further investigate the mechanism for the observed effects of chronic fluoxetine treatment, the density of 5HT2A, a potential mediator of the 5HT/DA interaction in the brain, and SERT, the target for fluoxetine, were assessed using [^{11}C]M100907 and [^{18}F]FEmZIENT, respectively. [^{11}C]M100907 demonstrated uptake in all regions of interest (see Figure 4-5). [^{11}C]M100907 uptake values in the cerebellum, the reference region, did not significantly vary between scans in either Experiment 1 (main effect of treatment; $F(2,8) = 0.27$, $p = 0.77$) or Experiment 2 (main effect of condition; $F(1,3) = 1.61$; $p = 0.29$). Binding potentials for [^{11}C]M100907 are reported in Table 4-2.

Since the chronic fluoxetine treatment was done against a background of ongoing cocaine self-administration, the effect of cocaine self-administration on 5HT2A binding was determined since it has not been previously reported. All of the subjects in Experiment 2 robustly self-administered cocaine (Table 4-3). Cocaine self-administration significantly increased 5HT2A availability in the frontal cortex (main effect of treatment, $F(1,3) = 15.46$; $p = 0.029$; main effect of ROI, $F(2,6) = 101.72$, $p < 0.001$; interaction effect, $F(2,6) = 12.18$, $p = 0.008$; Tukey's critical difference $p < 0.05$; Figure 4-6). No significant effect of cocaine history was seen on binding in the midbrain/brainstem or caudate. Following chronic fluoxetine treatment, 5HT2A binding was unchanged in the caudate and midbrain/brainstem, but significantly increased in the frontal cortex (main effect for treatment, $F(2,8) = 6.50$, $p = 0.021$; ROI, $F(2,8) = 41.49$, $p < 0.001$; interaction effect, $F(4,16) = 6.95$, $p = 0.002$; Tukey's critical difference $p < 0.05$; Figure 3-7). To determine if sex had an effect on 5HT2A binding, the baseline group from Experiment 1 and the post-cocaine group from Experiment 2, to control for the effects of cocaine self-administration, were compared. Males had significantly higher binding in the frontal cortex than did females (main effect of sex ($F(1,7) = 23.45$, $p = 0.002$; main effect of ROI, $F(2,14) = 57.35$, $p < 0.001$; sex by ROI interaction effect, $F(2,14) = 16.71$, $p < 0.001$; Bonferroni's post hoc $p < 0.001$; Table 4-2).

[^{18}F]FEmZIENT demonstrated uptake in all regions of interest (see Figure 4-8). Cerebellar uptake significantly differed across treatments ($F(2,8) = 5.501$, $p = 0.031$). Post hoc testing revealed that mean uptake in the post-SSRI condition significantly differed from post-washout, but additional post hoc testing revealed no significant differences during quasi-equilibrium, thus this difference is unlikely to affect calculations of binding potential. Chronic fluoxetine treatment had no effect on SERT binding potentials in the frontal cortex,

midbrain/brainstem, or caudate (significant main effect for ROI, $F(2,8) = 21.01$, $p < 0.001$; no significant effect of treatment, $F(2,8) = 1.76$, $p = 0.23$, or interaction, $F(4,16) = 0.23$, $p = 0.92$; Figure 4-9.) Binding potentials for [^{18}F]FEmZIENT are reported in Table 4-4.

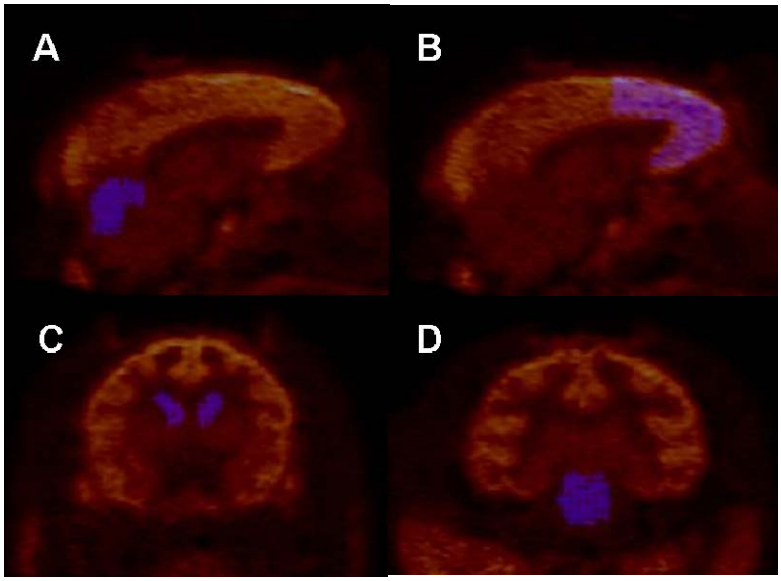


Figure 4-1: Regions of interest drawn on a [11C]M100907 scan. (A) Cerebellum, (B) Frontal cortex, (C) Caudate, (D) Midbrain/brainstem.

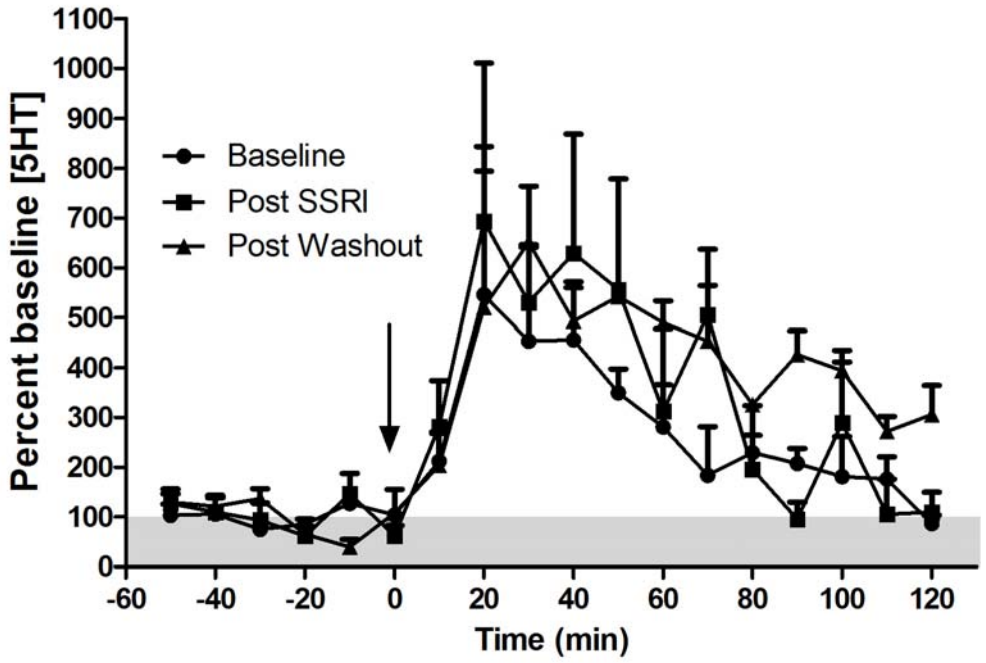


Figure 4-2: Serotonin levels did not differ from baseline in response to a fenfluramine challenge (3.0 mg/kg, i.v.; indicated by arrow) following chronic SSRI treatment or washout (n = 3).

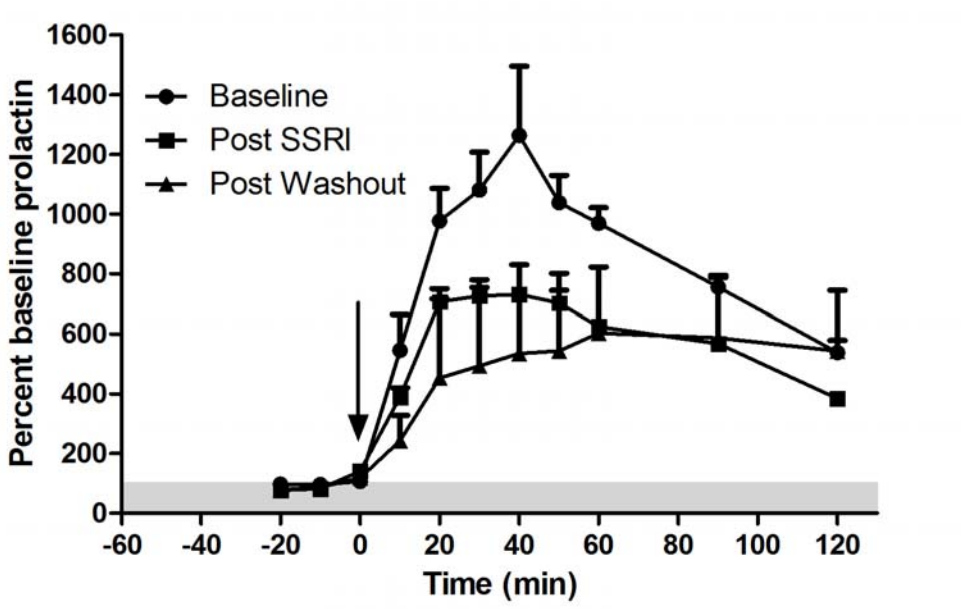


Figure 4-3: Prolactin response to fenfluramine challenge (3.0 mg/kg, i.v.; indicated by arrow) was significantly blunted following SSRI treatment and remained blunted after washout (n = 3).

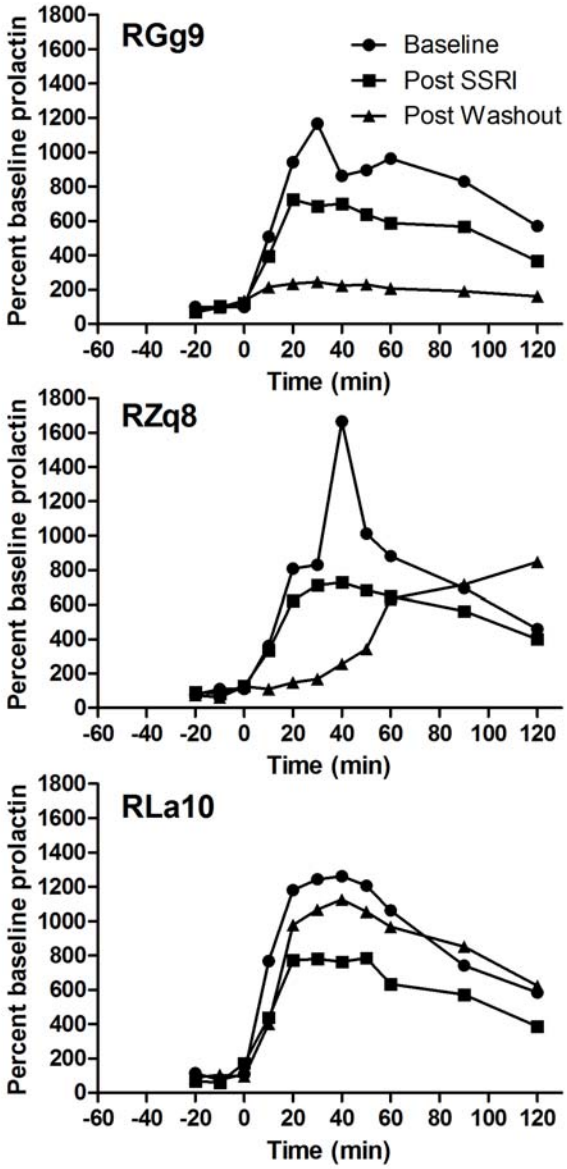


Figure 4-4: Individual prolactin responses to fenfluramine challenge (3.0 mg/kg, i.v.). All three animals showed blunting of the response at the post-SSRI time-point, but only two, (A) and (B), showed blunting after washout, while the third (C) recovered to near-baseline levels.

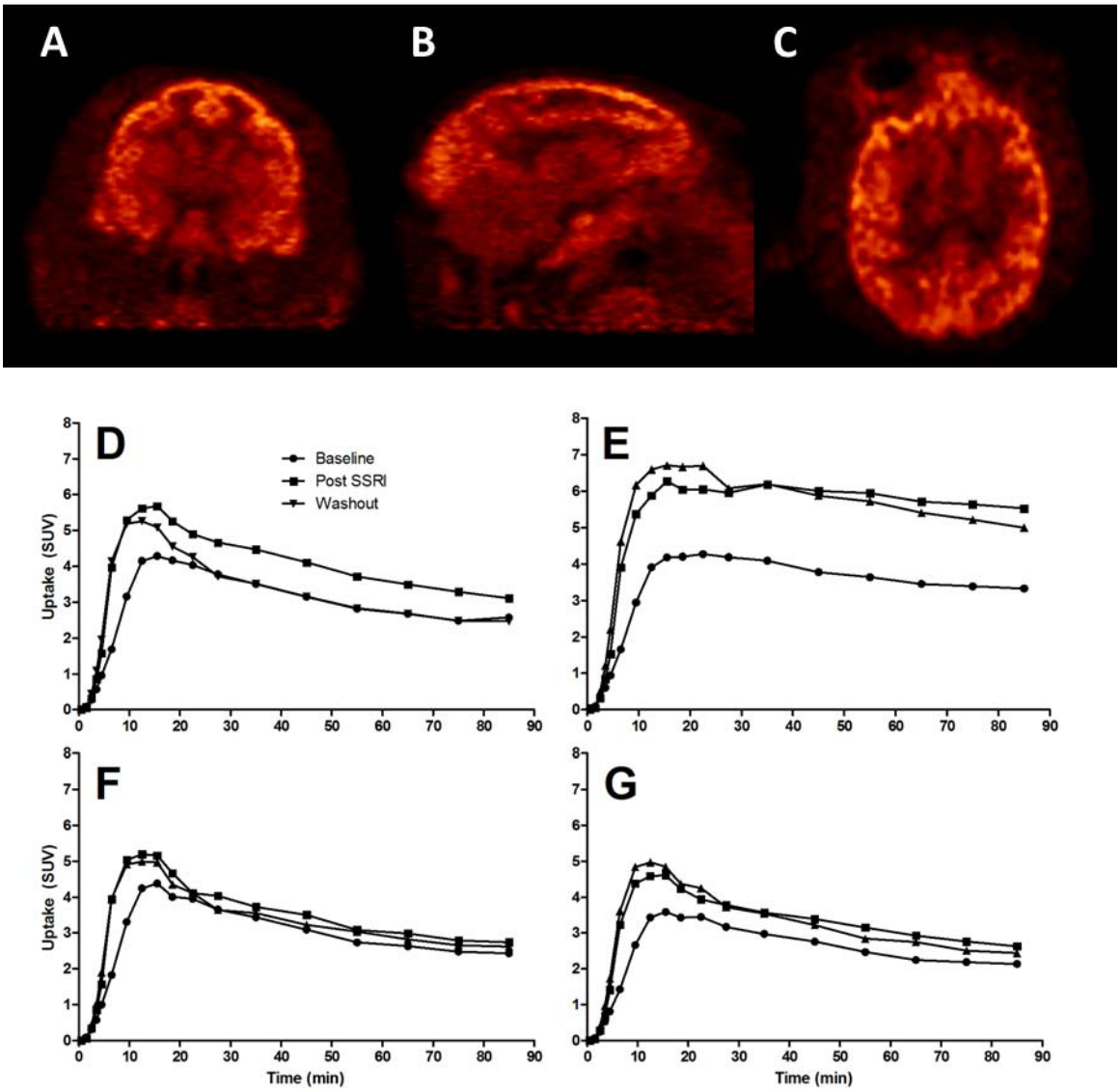


Figure 4-5: [^{11}C]M100907 uptake. A-C: Representative A) coronal, B) sagittal, and C) transaxial slices showing [^{11}C]M100907 uptake. D-G: Representative TAC curves showing the timecourse of [^{11}C]M100907 uptake in the D) cerebellum, E) frontal cortex, F) midbrain/brainstem, and G) caudate.

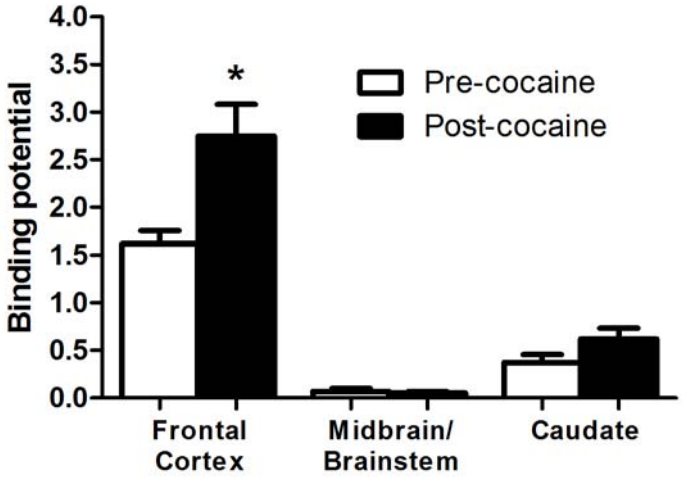


Figure 4-6: 5HT2A binding potential is significantly increased in the frontal cortex following 3.5 months of cocaine self-administration (n = 4).

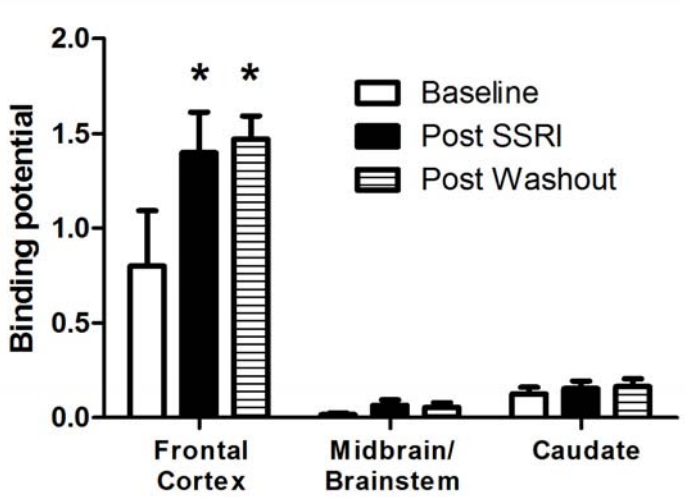


Figure 4-7: 5HT2A binding potential is significantly increased in the frontal cortex following chronic fluoxetine treatment and remains elevated following the washout period (n = 5).

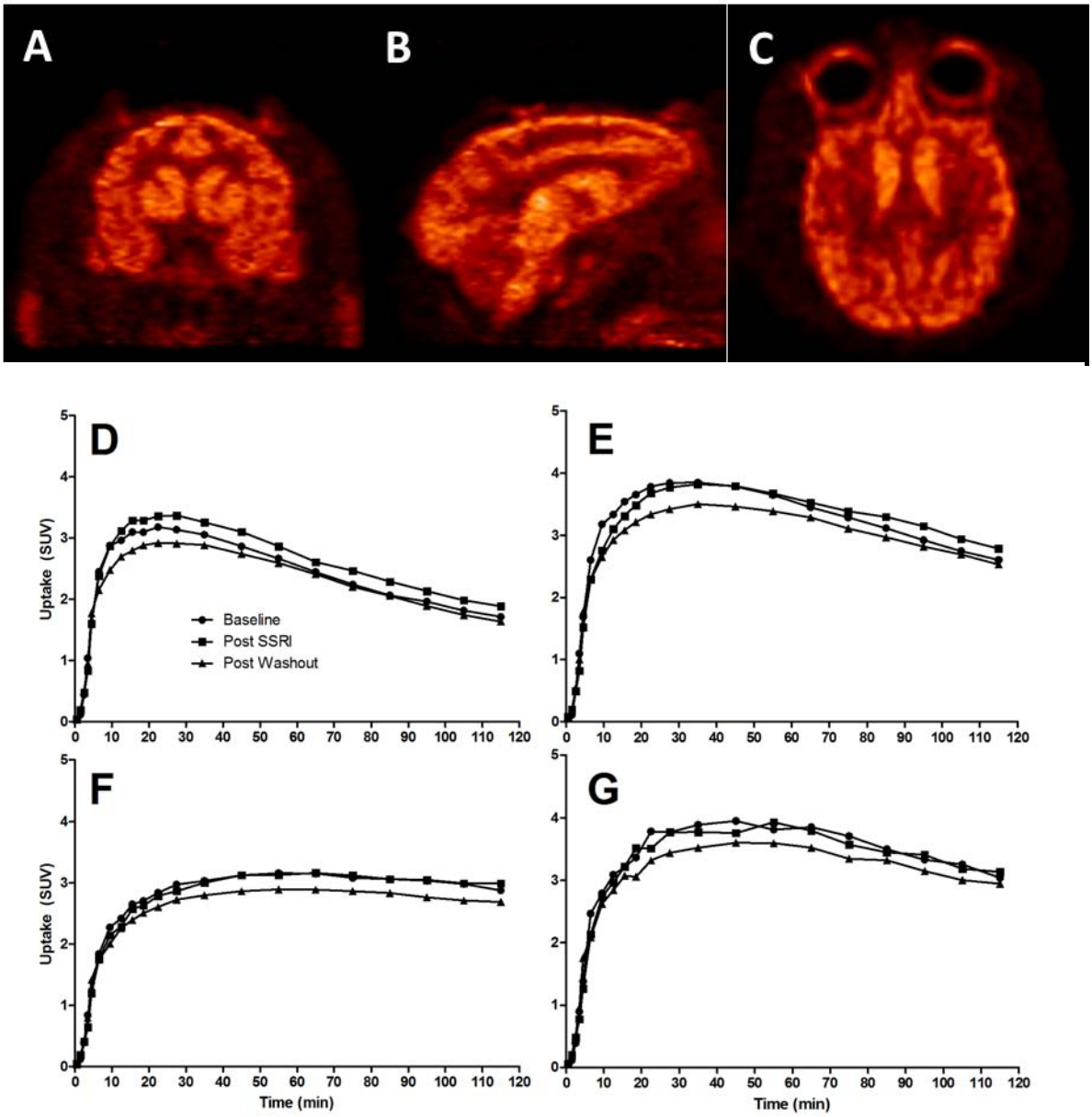


Figure 4-8: [^{18}F]FEmZIENT uptake. A-C: Representative A) coronal, B) sagittal, and C) transaxial slices showing [^{18}F]FEmZIENT uptake. D-G: Representative TAC curves showing the timecourse of [^{18}F]FEmZIENT uptake in the D) cerebellum, E) frontal cortex, F) midbrain/brainstem, and G) caudate.

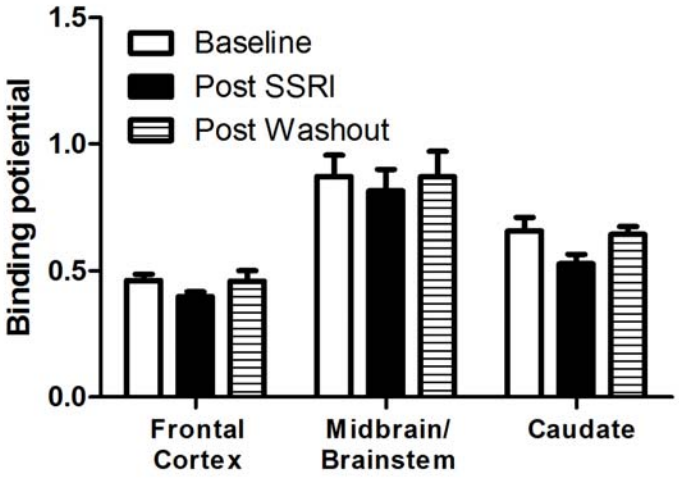


Figure 4-9: Chronic fluoxetine treatment has no effect on SERT binding potential (n = 5).

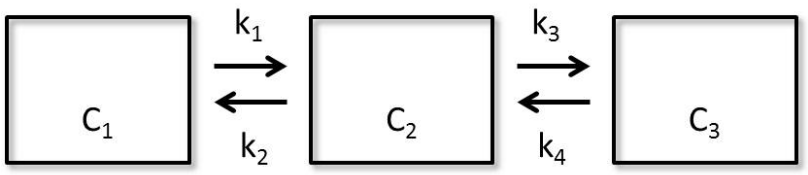


Figure 4-10: The three-compartment model for tracer kinetics, where C_1 is the blood, C_2 is unbound in the tissue, and C_3 is bound in the tissue. The rate constants $k_{1\text{-}4}$ describe the rates of transfer between each compartment.

Table 4-1: Raw baseline values for serotonin, and prolactin (mean ± SEM)

Time point	Serotonin (nM)	Prolactin (ng/ml)
Baseline	0.40 ± 0.14	8.17 ± 1.87
Post-SSRI	0.11 ± 0.03	7.41 ± 1.84
Post-Washout	0.46 ± 0.2	17.62 ± 10.45

Table 4-2. 5HT2A binding potentials (n = 4 for Experiment 2, n = 5 for Experiment 1)

ROI	5HT2A binding potential (mean ± SEM)				
	Cocaine (Experiment 2)		Chronic fluoxetine (Experiment 1)		
	Baseline	Post cocaine	Baseline	Post-treatment	Post washout
Frontal Cortex	1.618 ± 0.14	2.748 ± 0.33 *§	0.802 ± 0.29	1.399 ± 0.21 *	1.470 ± 0.12 *
Midbrain/Brainstem	0.073 ± 0.03	0.055 ± 0.02	0.016 ± 0.01	0.066 ± 0.03	0.055 ± 0.02
Caudate	0.375 ± 0.08	0.623 ± 0.11	0.126 ± 0.035	0.154 ± 0.04	0.164 ± 0.04

* indicates significance within ROI and experiment with respect to baseline
§ indicates significance within ROI with respect to chronic fluoxetine baseline

Table 4-3. Cocaine intake over 3.5 months between [^{11}C]M100907 scans for Experiment 2.

Monkey	Total Cocaine Intake (mg)	Total Cocaine intake (mg/kg)
ROf8	856.32	55.00
RJl8	699.5	50.91
RYj8	1106.09	79.51
RLk4	1089.77	77.64

Table 4-4. SERT binding potentials (n = 5)

ROI	SERT binding potential (mean ± SEM)		
	Baseline	Post-treatment	Post washout
Frontal Cortex	0.462 ± 0.02	0.398 ± 0.02	0.460 ± 0.04
Midbrain/Brainstem	0.872 ± 0.08	0.814 ± 0.85	0.870 ± 0.10
Caudate	0.656 ± 0.05	0.528 ± 0.04	0.642 ± 0.03

4.4 Discussion

Given the prolonged changes in cocaine-related neurochemistry and behavior following chronic fluoxetine administration, we evaluated potential mechanisms underlying these changes by examining pre- and post-synaptic markers and function. There was no change in SERT density in any of the brain areas examined immediately following chronic treatment or after the 6-week washout period, and there was a corresponding lack of change in the magnitude and time-course of 5HT overflow elicited by fenfluramine. However, fenfluramine-induced prolactin release, measured concurrently, was blunted following chronic treatment. Together, these data indicate that, while there was no change in the function of the SERT protein, there were altered downstream, post-synaptic effects.

Increased 5HT2A availability was observed in the frontal cortex following chronic treatment and 6 weeks after the conclusion of treatment with fluoxetine. The 5HT2A receptor contributes to the prolactin response (Jorgensen, 2007; Chaiseha et al., 2010) and therefore an increase in the prolactin response would be expected following an increase in receptor availability. However, the prolactin response decreased with no change in the amount of 5HT overflow. Agonist treatment is known to cause desensitization of 5HT2A receptors (Van Oekelen et al., 2003), and this is further supported by data demonstrating reduced g-protein signaling in cells (Brink et al., 2004), reductions in DOI-stimulated wet dog shakes in antidepressant-sensitive cataleptic mice (Tikhonova et al., 2010), and reductions in 5HT2A-mediated corticosterone release in rats (Yamauchi et al., 2006) following chronic SSRI treatment. Therefore, it seems plausible that the decrease in prolactin response may be due to desensitization of the 5HT2A receptor.

4.4.1 SERT regulation and presynaptic effects

It is well accepted that chronic administration of SSRIs induces neurobiological changes (Wong et al., 1995; Thompson, 2002; Vaswani et al., 2003) but these have not been previously evaluated in the context of cocaine abuse, which also induces neurobiological changes (Koob and Volkow, 2010). Our data demonstrate that, in contrast to previous reports of SERT downregulation by SSRIs (Benmansour et al., 1999; Benmansour et al., 2002; Kugaya et al., 2003; Iceta et al., 2007), prolonged fluoxetine treatment has no effect on SERT binding potentials in the context of ongoing cocaine use. Chronic exposure to cocaine has previously been shown to induce increases in SERT in monkeys and humans (Mash et al., 2000; Banks et al., 2008; Gould et al., 2011), and thus may oppose the effects of fluoxetine on SERT regulation. Since both compounds are reuptake inhibitors, it is not clear why they affect SERT regulation differently, but it may be due to different binding kinetics or actions at other serotonergic targets. Future experiments should address this question.

In concordance with the lack of changes in SERT, 5HT overflow induced by a selective serotonin releaser, fenfluramine, was also unchanged by chronic fluoxetine treatment. Fenfluramine releases 5HT via a SERT-dependent mechanism (Garattini et al., 1986; Garattini et al., 1987; Cinquanta et al., 1997) and therefore 5HT release is affected by the availability of the transporters. Additionally, this suggests that there was no change in the presynaptic synthesis or availability of 5HT, since the pool of available serotonin also affects the magnitude of elicited release; for example, reserpine, which irreversibly blocks vesicular monoamine transporters (VMAT), is able to reduce fenfluramine's ability to release serotonin (Garattini et al., 1986). Therefore, it seems unlikely that chronic fluoxetine treatment in the context of cocaine use altered presynaptic serotonin function at the level of the terminal.

Prolactin response is used as a measure of integrated 5HT function (O'Keane and Dinan, 1991; Newman et al., 1998) and is generally correlated with 5HT release (Murnane et al., 2010) and reliable over time (Flory et al., 2002). However, despite the lack of change in 5HT release, prolactin response was blunted following chronic SSRI treatment and remained blunted. Together, these data strongly suggest that the reason for the blunted prolactin response resides in the post-synaptic mechanisms downstream from 5HT release.

4.4.2 5HT2A regulation and post-synaptic effects

There is less consensus on regulation of the 5HT2A receptor, which has been reported to down-regulate in response to direct agonists or antagonists (Gray and Roth, 2001; Van Oekelen et al., 2003; Bubar and Cunningham, 2008), while other studies have described up-regulation in response to antagonists (Aloyo et al., 2001; Dave et al., 2007). Recently, antagonist-specific regulation has been proposed following demonstration of up-, down- or no change in regulation of the 5HT2A receptor in response to different antagonists in the same study (Dave et al., 2007; Yadav et al., 2011). These antagonist-specific effects may be due to 5HT2A receptor homodimerization (Brea et al., 2009). Furthermore, repeated agonist treatment has been reported to desensitize 5HT2A receptors as measured by 5HT2A-mediated head bobs in rabbits or hormone release in rats (Aloyo et al., 2001; Damjanoska et al., 2004; Dave et al., 2007; Shi et al., 2007; Shi et al., 2008). In several studies, changes in 5HT2A-mediated response correlated with changes in receptor protein where measured (Aloyo et al., 2001; Dave et al., 2007; Yadav et al., 2011). However, the functional status of the 5HT2A receptors may be modulated independently of the protein expression level (Roth et al., 1995). For example, Yadav et al. found decreases in 5HT2A receptor protein with no effect on PCP-stimulated locomotion in mice (Yadav et al., 2011), and Shi et al. found

decreased 5HT2A-mediated oxytocin release in rats following repeated DOI-treatment but increased protein expression and reduced g-protein coupling, suggesting that not all the receptors were functional (Shi et al., 2007; Shi et al., 2008). Thus, although agonists appear to generally down-regulate or desensitize receptors, regulation of 5HT2A receptor expression and functional status is complex, and its underlying mechanisms, particularly following antagonism, are not well understood.

SSRIs increase endogenous 5HT and thus act as indirect agonists; however, when given over time the effects may be more complex due to the alterations in SERT and extracellular 5HT levels that occur during chronic treatment. Conflicting evidence regarding the effect of SSRIs on 5HT2A receptors has been reported. For example, paroxetine decreased cortical 5HT2A, but only in the younger patients (Meyer et al., 2001), while increased 5HT2A has been reported in patients currently undergoing chronic SSRI treatment (Massou et al., 1997) as well as recovered patients formerly treated with SSRIs for depression (Bhagwagar et al., 2006) after age correction. Audenaert et al reported that both decreases and increases in 5HT2A seen in depressed and impulsive (respectively) suicidal patients appear to normalize following SSRI treatment (Audenaert et al., 2006), suggesting that the effect of the SSRI may depend on the initial state of the system. It is possible, particularly given the findings of Audenaert and colleagues, that the increase in 5HT2A binding potential seen in the present study could be correcting a decrease caused by chronic cocaine exposure. Since the effects of cocaine on 5HT2A binding *in vivo* have not been previously reported, we examined 5HT2A binding in monkeys that had been abstinent from cocaine before and after resuming cocaine use, and found that 5HT2A binding potential

increased in the frontal cortex as well. This removes the possibility that fluoxetine treatment is normalizing cocaine-induced changes in 5HT2A expression.

Similar to direct agonists, chronic SSRI treatment appears to also modulate the functional status of 5HT2A receptors. In the present study, there was a decrease in fenfluramine-induced prolactin release despite no decrease in concurrent 5HT overflow and an increase in 5HT2A receptor binding. Given that 5HT2A normally plays a stimulatory role in prolactin release (Jorgensen, 2007), this points to desensitization of the receptor, particularly since it has been known to desensitize under similar conditions. Chronic treatment with two different SSRIs reduced 5HT2A-mediated corticosterone release in rats (Yamauchi et al., 2006), and chronic treatment with fluoxetine reduced DOI-stimulated wet dog shakes in antidepressant-sensitive cataleptic mice (Tikhonova et al., 2010) and g-protein signaling in cells (Brink et al., 2004). Additionally, three weeks of SSRI treatment attenuated self-reported subjective effects of LSD, a 5HT2A agonist, in humans (Bonson et al., 1996). Conversely, increases in PLC activity have been reported in tissue take from rats chronically treated with fluoxetine (Damjanoska et al., 2003), but this study did not rule out other possible sources of increased PLC activity, such as the 5HT2C receptor, which is also Gq-coupled. However, it does raise the possibility that the 5HT2A receptors are functional and that other neurobiological changes, such as in the 5HT2C receptor, could be compensating for it and producing the blunted prolactin response. Future experiments that use an additional measure, such as DOI-stimulated wet dog shakes (Sawyer, Howell, *unpublished results*), should further address the question of whether chronic fluoxetine treatment desensitizes 5HT2A receptors in the context of cocaine use in order to obtain a decisive answer. Additionally, one animal showed recovery of the prolactin response after the 6-week washout

period while the other two did not; future studies should examine multiple time-points after washout to determine when responsiveness re-emerges and whether 5HT2A binding potential changes correlate with it temporally.

Chronic cocaine self-administration also induced an up-regulation of 5HT2A binding in the frontal cortex. Previous experiments have reported either no change (Carrasco and Battaglia, 2007; Huang et al., 2009) or an increase (Carrasco et al., 2006) in receptor levels; however, all three of these experiments used experimenter-administered cocaine, which may affect its neurobiological consequences (Jacobs et al., 2003), and shorter periods of exposure, and two specifically looked at withdrawal time-points. Thus, this is the first report of the effects of self-administered, current cocaine use on 5HT2A binding potentials. The observed increase is surprising since SSRIs also produced the same result. However, the effects of chronic cocaine and chronic SSRI treatment were not examined in the same animals, so it is not possible to say whether the increases may be additive. Furthermore, the functional status of the receptors after chronic cocaine exposure was not examined. Previous studies have suggested that withdrawal from repeated or even a single administration of cocaine can cause sensitization of 5HT2A-mediated endocrine responses within 12-24 hours in rats (Baumann and Rothman, 1996; Baumann and Rothman, 1998; Carrasco et al., 2006; Carrasco and Battaglia, 2007) and so it may be possible that the changes in regulation we observed have functionally different consequences. However, a different study showed that repeated cocaine administration reduced the ability of 5HT to enhance small excitatory post-synaptic currents via 5HT2A receptors in pyramidal cells in rats (Huang et al., 2009), suggesting that in the PFC, cocaine desensitizes 5HT2A receptors. It is possible that current cocaine use causes desensitization of 5HT2A receptors, which rebounds into sensitization following cessation of

cocaine use, and that chronic fluoxetine treatment may prevent this. Additional experiments examining 5HT2A binding potential and functional status repeatedly in the same animals before and after acquiring cocaine self-administration and then after chronic SSRI treatment are needed to address this question.

5HT2A binding potential in females with cocaine history differed from that in males with a recent cocaine history, despite increased variability in the female sample, indicating that there may be sex differences in 5HT2A receptor expression. The increased variability in the females may be due to the menstrual cycle, as these subjects were intact and freely cycling, and there may be a relationship between estrogen and 5HT2A binding (Frokjaer et al., 2010). However, these results are in contrast to a recent, large study in healthy adults demonstrating no sex differences in 5HT2A (Moses-Kolko et al., 2011). Our analysis was done post-hoc, not *a priori*, and as such is not decisive. Furthermore, cocaine history was not matched between the two groups, and our results clearly show that cocaine history affects 5HT2A binding, but do not address the question of how long those changes might persist or what the influence of a past cocaine history might be; thus differences between them could be due to differences in cocaine history. 5HT2A binding potential declines with age (Moses-Kolko et al., 2011), but the two groups of animals were similar in age and thus this is unlikely to be the explaining factor. Further, well-controlled studies of drug-naïve, age matched males and females before, during, and after extended cocaine exposure are required to adequately address this issue.

4.4.3. Methodological considerations

Prolactin studies

An additional consideration with respect to prolactin is the influence of menstrual cycle. Plasma estrogen increases the prolactin response and basal and stimulated prolactin can vary across cycle in humans (O'Keane et al., 1991; Tanner et al., 2011). All subjects were female and intact, and menstrual cycle was not monitored, and thus may introduce a confound. However, variability was low during the baseline and post-SSRI time-points and examination of individual data shows an orderly decrease in prolactin across all subjects. Therefore we feel that menstrual cycle is unlikely to be a biasing factor at these points. However, the variation seen at the post-washout time point could be in part due to differing stages of the menstrual cycle and thus be obscuring treatment effects.

PET imaging

PET imaging and mathematical modeling are subject to a number of assumptions. In order to estimate the number of available receptors (B_{max}), the binding potential is used (Mintun et al., 1984). This is derived using a three-compartment model (Figure 3-10) and four rate constants (Lammertsma and Hume, 1996; Votaw et al., 2002). In order to model the kinetics for C_2, a reference region is used. Ideally, a reference region should be devoid of specific binding, so as to model the kinetics of the portion of tracer that is free and nonspecifically bound in the tissue compartment (Meyer et al., 2007). Binding potential, which correlates with B_{max}, is equal to B_{max}/K_d, where K_d is the equilibrium dissociation constant, also known as the affinity. K_d is equal to k_{off}/k_{on}, and the ratio B_{max}/K_d is the same as k_4/k_3 when only a trace amount of radioligand is used, such as in the present study. Therefore, binding potential is $(B_{max} * k_3)/ k_4$, which can be determined more reliably than its individual factors due to covariance (Votaw et al., 2002). Assuming that K_d remains constant, variations in the binding potential reflect changes in B_{max}. However, it is possible that

changes in affinity may occur (e.g. D2High receptors, see Seeman, 2010) and thus be responsible for the observed changes in binding potential. However, there are currently no reports in the literature suggesting a high vs. low affinity state for either the SERT or 5HT2A receptor. Other possible influences on binding potential that could vary include the amount of endogenous neurotransmitter competing for binding and the depth of anesthesia used during the scan. However, we saw no changes in 5HT function during microdialysis drug challenges, and the depth of anesthesia was kept as constant as possible across scans. Furthermore, [^{18}F]FEmZIENT binding is not affected by anesthesia (Stehouwer et al., 2008).

We report a marked and sustained increase in 5HT2A binding potential in the frontal cortex, but not the caudate or the midbrain/brainstem, following chronic fluoxetine treatment. Binding potentials in the caudate and midbrain/brainstem areas are similar to those previously reported (Lopez-Gimenez et al., 2001; Meyer et al., 2010). It is important to note that although the binding potentials are low, this does not mean the areas are devoid of receptors. Rather, since a reference region, the cerebellum, was used, it means that the receptor binding is similar to that in the reference region. Although the cerebellum is frequently used (e.g. Lundkvist et al., 1996; Talvik-Lotfi et al., 2000; Bhagwagar et al., 2006) and has been validated as a reference region for 5HT2A receptors (Hinz et al., 2007; Meyer et al., 2010), it does express a low level of receptors and thus is not free of specific binding (Ito et al., 1998). Furthermore, in the context of the present study, that means that the level of specific binding could possibly increase. However, cerebellar uptake did not significantly vary, so this is unlikely to strongly influence the findings, especially given the relative magnitude of the increase in the frontal cortex. This could also be a concern for the SERT studies, since the cerebellum also expresses a very low level of SERT (Kish et al., 2005).

However, the cerebellum has been validated as a reference region for SERT studies using tracers with high free and nonspecific binding such as the one used in the present study (Kish et al., 2005; Stehouwer et al., 2008).

The presence of a low level of specific binding for [^{11}C]M100907 in the reference region violates one of the assumptions regarding the reference region in the generalized reference tissue model used to analyze the data. Previous studies have examined the best method to match the results of analysis using arterial blood-derived input functions and found that noninvasive graphical methods such as Logan plots provide the best agreement and reliability, but may have a large bias under high noise conditions (Watabe et al., 2000; Meyer et al., 2010). Simplified reference tissue models such as the one used in the present study also provide good correlation, although they present a larger negative bias in estimates of binding potential (Meyer et al., 2010); thus it is important to note that the binding potential estimates in the present study may be low, although the relative magnitudes are likely correct.

4.4.4 Clinical implications

This study is the first to evaluate the effects of long-term, clinically-relevant serum concentrations of fluoxetine on serotonergic neurobiology in the context of cocaine use and highlights the importance of evaluating potential treatments in the context of the target population. Although previous studies have demonstrated down-regulation of SERT availability in non-cocaine using populations (Kugaya et al., 2003), the results of our study suggest that this may not be the case for cocaine users and caution against the use of non-drug-using populations to predict results in drug-using populations. Furthermore, as down-regulation of SERT and alteration of presynaptic 5HT signaling may be integral to the

therapeutic effects of SSRIs for depression (Blier et al., 1998; Benmansour et al., 2002), our results indicate that SSRIs may be less effective in alleviating depression in current cocaine abusers. Again, this is supported by reports of no change in depressive symptoms in cocaine abusers during clinical trials with SSRIs (Batki et al., 1996; Cornelius et al., 1998), further emphasizing the need to examine potential treatments in the target population.

5. GENERAL DISSCUSSION

In this study, we examined the effects of a clinically-relevant dosing regimen with fluoxetine, an SSRI, on cocaine-related behavior and neurochemistry and potential underlying mechanisms in the function and regulation of the serotonin system and two key serotonergic proteins. Previous studies showed a discrepancy between preclinical reports of SSRIs' effectiveness in reducing cocaine's effects (e.g. Czoty et al., 2002) and clinical reports of no effect (e.g. Grabowski et al., 1995). Since potential pharmacotherapies for cocaine abuse need to be administered chronically, and the mechanism for SSRIs' therapeutic effects is believed to be neurobiological changes that emerge during chronic, but not acute, administration (Wong et al., 1995; Vaswani et al., 2003), we designed a dosing regimen with fluoxetine in rhesus macaques that approximates human conditions over 4-6 weeks. As there is no clear serum concentration-therapeutic effect relationship for depressive symptoms (Baumann, 1996) and the mechanism for SSRIs' neurobiological changes are not well-understood, we matched the route of administration, length of treatment before beginning experimental measures, serum concentrations, and fluoxetine: norfluoxetine ratios as closely as possible to those reported in human clinical studies in order to minimize the possible confounds introduced by interspecies dose-scaling and maximize translational quality of the results. We found that under these clinically-relevant conditions, fluoxetine was able to attenuate cocaine-primed reinstatement and cocaine-induced dopamine overflow but not ongoing self-administration behavior in animals. This aligns closely with the results of clinical studies reporting no effect as an intervention (Grabowski et al., 1995; Batki et al., 1996; Winstanley et al., 2011); however, no clinical studies have looked at fluoxetine's effectiveness in preventing relapse. Our results suggest that such a study would be warranted.

5.1 Importance of the model

The concordance between clinical results and our results suggest that the model of fluoxetine administration developed and used in the present study is able to predict the consequences of SSRIs more effectively than the more commonly used acute dosing methods. Furthermore, it suggests that the previous discrepancy between preclinical and clinical results may not be due to species differences or a failure of the self-administration model to capture the complexity of the human situation, but rather a lack of the preclinical model to model the long-term consequences of SSRIs: neurobiological changes. Our model presents the opportunity to study these changes and determine their relationship to cocaine-related behavior and neurochemistry.

In order to determine the mechanism for the reductions in cocaine-primed reinstatement and cocaine-induced dopamine overflow, we examined the function of the serotonin system and changes in key proteins implicated in serotonergic regulation of dopamine. Although many studies have previously examined neurobiological changes induced by SSRIs, no studies have examined these changes in the context of cocaine abuse, which also alters brain function. The alterations induced by SSRIs may depend on the state of the serotonin system (Audenaert et al., 2006), and therefore effects described in depressed patients may not generalize to cocaine-addicted patients. Before beginning fluoxetine treatment, our subjects were trained to self-administer cocaine and allowed to do so for a minimum of a year to allow both behavior and neurobiological changes to stabilize. This provided them with a cocaine history against which to examine the effects of fluoxetine treatment.

5.2 Serotonin transporter changes and cocaine

Prolonged cocaine use can influence SERT regulation (Mash et al., 2000; Banks et al., 2008; Gould et al., 2011) in the opposite direction of SSRIs (Kugaya et al., 2003). It is not clear why the two drugs, both serotonin reuptake inhibitors, cause different changes in SERT expression, although it may be due to different kinetics at the level of the transporter. Additionally, it may also relate to the selectivity of the compound; changes that cocaine evokes due to its actions at DAT or NET could affect the upstream inputs to the serotonin system and thus induce changes to compensate. In the present study, we found that SSRIs induced no changes in SERT in subjects currently using cocaine. It seems likely that the effects of the SSRI were offset by the effects of cocaine, resulting in no change. However, we did not examine SERT binding potential in the animals prior to beginning cocaine use or at multiple time-points during cocaine use without fluoxetine; therefore it is possible that continuing cocaine use, such as in these subjects, would continue to up-regulate SERT expression and that exposure to fluoxetine prevented this continuing increase. To the best of our knowledge, the effects of short vs. long-term cocaine use on SERT have not been examined. However, regardless of this issue, fluoxetine did not induce SERT down-regulation in 4-6 weeks, which should be a sufficient amount of time (Benmansour et al., 2002), in the context of cocaine use. Similarly, there was no change in the amount or clearance of 5HT elicited by a serotonin releaser, suggesting that there were no changes in transporter function either. Thus, changes in the presynaptic function of the serotonin system are unlikely to be responsible for the observed changes in cocaine-primed reinstatement and dopamine overflow.

5.3 Serotonin 2A receptors

5.3.1 Serotonin 2A receptors and cocaine

The 5HT2A receptor has been extensively implicated in cocaine's reinstating and discriminative-stimulus effects (Fletcher et al., 2002; Filip, 2005; Bubar and Cunningham, 2006; Filip et al., 2006) as well as in the antidepressant effects of SSRIs (Carr and Lucki, 2011). In the present study, a sustained increase in 5HT2A availability was observed in the frontal cortex following chronic fluoxetine treatment with a concomitant decrease in the prolactin response elicited by a serotonin release, which we suggest represents desensitization of 5HT2A receptors in the presence of fluoxetine (Ch 3). In addition to contributing to the prolactin response (Jorgensen, 2007; Chaiseha et al., 2010), the 5HT2A receptor is involved in facilitating DA release (Bubar and Cunningham, 2008; Navailles and De Deurwaerdere, 2011); therefore, due to the increase in 5HT2A binding, an increase in DA overflow in addition to an increase in prolactin would be expected. However, similar to the prolactin response, cocaine-induced DA overflow decreased despite the lack of change in the amount of 5HT overflow, further suggesting that these receptors may be desensitized. This possibility is supported by previous studies that reported 5HT2A desensitization following fluoxetine treatment (Brink et al., 2004; Yamauchi et al., 2006; Tikhonova et al., 2010) or agonist treatment (Van Oekelen et al., 2003). Furthermore, functional desensitization in the presence of increased 5HT2A protein has also been reported (Shi et al., 2008). Together, these data strongly point to desensitization of the 5HT2A receptor as the underlying mechanism for the observed decrease in prolactin response, cocaine-induced DA overflow, and thus, suppressed cocaine-induced reinstatement.

Similar paradoxical regulation of function and protein levels have also been reported for nicotinic receptors (Marks et al., 1993; Fenster et al., 1999; Gentry and Lukas, 2002). Nicotinic receptors desensitize following chronic exposure to agonists at concentrations that also induce upregulation of cell surface receptor expression (Fenster et al., 1999), raising the possibility that the increase in receptor expression is a consequence of and possible attempt to overcome the decrease in function. A similar mechanism may underlie the increased 5HT2A binding potential and decreased functional measures observed in both the present study and the studies by Shi and colleagues (Shi et al., 2008). However, the nature of the relationship between the changes in nicotinic receptor expression and functional status is not fully understood (Gentry and Lukas, 2002). Additionally, nicotinic receptors are ionotropic and may desensitize by moving into a higher affinity state following phosphorylation (Gentry and Lukas, 2002) whereas 5HT2A receptors are metabotropic and their desensitization is most likely a result of decoupling from the associated g-protein following phosphorylation (Brink et al., 2004; Shi et al., 2007). Further studies are necessary to elucidate the connection between regulatory changes in function and expression for both receptor types.

Interestingly, this hypothesized desensitization of the 5HT2A receptor affected cocaine-primed reinstatement but not cocaine self-administration, which depends on many of the same neurobiological substrates (Gardner, 2000; O'Brien and Gardner, 2005). However, manipulations of the 5HT2A receptor have been unable to affect cocaine self-administration while attenuating both cue- and cocaine-primed reinstatement (Fletcher et al., 2002; Filip, 2005; Nic Dhonnchadha et al., 2009). Furthermore, site-specific injections of M100907 in the prefrontal cortex in particular have been shown to attenuate reinstatement but not self-administration or cocaine-induced locomotion (Pockros et al., 2011). Thus it appears that

5HT2A receptors in the frontal cortex are not necessary for the direct reinforcing effects of cocaine, but that they are involved in mediating other aspects of its effects. Drug-primed reinstatement in particular involves more than just the reinforcing effects of a drug, since no further access beyond the prime is available. Rather, it may depend more on interoceptive or discriminative stimuli (Stewart and de Wit, 1987), only part of which may depend on dopamine release, and in which 5HT2A receptors are believed to play a role for cocaine (Filip et al., 2006).

Similarly to fluoxetine, chronic self-administration of cocaine resulted in an up-regulation of 5HT2A receptors in the frontal cortex. Previous studies have suggested that withdrawal from cocaine sensitizes 5HT2A receptors (Baumann and Rothman, 1996; Baumann and Rothman, 1998; Carrasco et al., 2006; Carrasco and Battaglia, 2007), which may contribute to relapse, particularly as antagonists at 5HT2A receptors are able to block cue- and cocaine-primed reinstatement (Fletcher et al., 2002; Filip, 2005; Nic Dhonnchadha et al., 2009). Furthermore, 5HT2A agonists are able to both induce dopamine release (Bowers et al., 2000; Bortolozzi et al., 2005; Huang et al., 2011) and potentiate drug effects (Kuroki et al., 2003; Filip et al., 2004), and thus sensitized 5HT2A receptors may make individuals more vulnerable to drug-induced relapse. In fact, one study reported sensitization within 12-24 hours of cocaine withdrawal (Carrasco et al., 2006) and our neurochemical and endocrine studies were conducted at least 12 hours after the animal's previous self-administration session and thus may capture this sensitization. Our studies suggest that 5HT2A receptors are desensitized following fluoxetine treatment in the context of cocaine abuse; therefore it is possible that the presence of fluoxetine prevents sensitization and thus reduces vulnerability to relapse. However, since the functional status of 5HT2A receptors in

animals without a recent cocaine history was not examined, it is unclear whether fluoxetine actually results in desensitization of the receptors or merely prevents or reverses the sensitization induced by cocaine.

5.3.2 5HT2A and dopamine regulation

The 5HT2A receptor is believed to modulate dopamine both directly, via direct expression on dopaminergic VTA cells, and indirectly, via modulation of excitatory inputs to the VTA (Alex and Pehek, 2007; Bubar and Cunningham, 2008). In the present study, we saw no change in midbrain/brainstem binding potential, which would include the VTA. However, the ROI was large compared to the size of the VTA, and resolution was limited due to the nature of the PET scanner, preventing the inclusion of a VTA-only ROI. For these reasons, possible changes in binding potential within the VTA may not be detected and so we cannot say definitively that chronic fluoxetine treatment did not influence the 5HT2A receptor availability in the VTA. Furthermore, our functional assay (prolactin) did not distinguish among receptor populations, and therefore it is possible that any changes in 5HT2A receptor functional status were global and not region-specific. Thus, it is possible that alterations in 5HT2A receptors in the VTA may contribute to the decreases in evoked DA release we observed.

A significant increase in 5HT2A binding potential was observed in the frontal cortex. The 5HT2A receptor in the frontal cortex has previously been suggested to modulate DA levels in the nucleus accumbens and prefrontal cortex via modulation of glutamatergic pyramidal cell input to the VTA (Pehek et al., 2006). Based solely on binding potential, our data would not support this model, as an increase in 5HT2A receptors would be expected to increase DA release. However, the results of our concurrent *in vivo* microdialysis and

prolactin studies suggest that these receptors may be desensitized as previously discussed; if this is the case, then our data support the model, with decreased 5HT2A function resulting in decreased excitatory pyramidal cell input to the VTA leading to reduced DA release in the caudate and nucleus accumbens.

5HT2A receptors are also expressed on GABAergic interneurons in the frontal cortex (Jakab and Goldman-Rakic, 2000). PET imaging cannot distinguish cellular localization, and thus it is possible that the affected 5HT2A receptors are located on GABAergic interneurons. Selective upregulation of 5HT2A on GABAergic interneurons could also result in decreased DA release. Many of the 5HT2A-expressing interneurons synapse onto pyramidal cells (Jakab and Goldman-Rakic, 2000); therefore, increasing 5HT2A-mediated stimulation would increase inhibitory input onto these cells, decreasing their glutamatergic drive on the VTA. This possibility is consistent with our microdialysis data but does not explain the blunted prolactin response, since dopamine inhibits prolactin release (Fitzgerald and Dinan, 2008) and this model would predict no change or increased prolactin.

Additionally, a small subpopulation of 5HT2A receptors are expressed on unidentified monoaminergic fibers in the frontal cortex (Jakab and Goldman-Rakic, 1998; Miner et al., 2003). It is possible that the increased 5HT2A binding potential could be derived in part from increases in this population of receptors; however, since the identity and target of the fibers are not known, it is difficult to say whether or not this possibility is likely.

5.3.3 5HT2A receptors and impulsivity in drug abuse

5HT2A antagonists are able to attenuate many abuse-related effects of cocaine, although not self-administration (Fletcher et al., 2002; Nic Dhonnchadha et al., 2009; Zayara et al., 2011). Thus it appears that the 5HT2A receptor is not involved in modulating the

effects of cocaine self-administration but is involved in mediating some other aspect of drug abuse. One such possibility is impulsivity, a trait that is believed to contribute to both the predisposition to develop drug abuse and to relapse in humans (de Wit, 2009; Winstanley et al., 2010; Kirby et al., 2011). Higher scores on impulsivity measures are correlated with more severe cocaine use and decreased treatment retention (Moeller et al., 2001). Additionally, cocaine increases measures of impulsivity in animal models using delay discounting tasks when given acutely (Dandy and Gatch, 2009; Anastasio et al., 2011) or repeatedly (Setlow et al., 2009). Furthermore, rats with a cocaine history had greater rates of impulsive choice when tested 3 months after cocaine exposure (Simon et al., 2007), demonstrating that the effects can be long lasting. These effects are not specific to experimenter-administered cocaine, as self-administered cocaine also increases preference for immediate rewards in delay discounting tasks (Mendez et al., 2010), although the exact nature of the effects may depend on task as Winstanley et al (2009) reported tolerance to the disrupting effects of cocaine self-administration on the 5-choice serial reaction time task (5CSRT) in rats over time. Moreover, withdrawal from cocaine resulted in immediate and marked disruptions in 5CSRT performance (Winstanley et al., 2009). Therefore, trait impulsivity may contribute to the initiation of cocaine use and the cocaine use may then increase impulsivity, thus contributing to the development and maintenance of cocaine abuse and dependence as well as the risk for relapse (de Wit, 2009; Kirby et al., 2011).

The 5HT2A receptor has been linked to measures of impulsivity (Kirby et al., 2011). M100907, a selective 5HT2A antagonist, was able to reduce premature responding on the 5-choice (Winstanley et al., 2003; Fletcher et al., 2007) and one-choice serial reaction time test (Anastasio et al., 2011) in rats. Site-specific infusions of M100907 into the PFC or nucleus

accumbens but not limbic cortex also reduce premature responding (Winstanley et al., 2003; Robinson et al., 2008). M100907 similarly reduced responding on a differential reinforcement of low rate (DRL) task (Anastasio et al., 2011). Importantly, M100907 was able to reverse cocaine-induced deficits in both the 1CSRT and DRL tasks (Anastasio et al., 2011). Together, these studies suggest that 5HT2A receptor function is important for both non-drug and drug-induced impulsive choice, and that decreasing 5HT2A function may decrease impulsivity.

Cocaine withdrawal has been suggested to sensitize 5HT2A receptors (Carrasco et al., 2006; Carrasco and Battaglia, 2007). In light of the studies demonstrating that 5HT2A antagonists reduce impulsivity, sensitization would predict an increase in impulsivity during withdrawal, which is in concordance with the results of Winstanley and colleagues (2009), who reported decreases in performance on the 5CSRT in rats during the withdrawal but not maintenance phase of cocaine self-administration. Impulsivity additionally predicts decreased treatment retention (Moeller et al., 2001) and increases the likelihood of relapse (de Wit, 2009). 5HT2A receptors are also implicated in relapse, as selective antagonists can reduce both cue- and drug-primed reinstatement (Fletcher et al., 2002; Nic Dhonnchadha et al., 2009; Pockros et al., 2011). It is plausible that 5HT2A receptors in the frontal cortex may contribute to relapse in part through a role in mediating impulsivity. Therefore, through increasing the sensitivity of 5HT2A receptors, withdrawal may also increase impulsivity, thereby increasing the chances of relapse. This may provide a possible mechanism through which chronic drug use can contribute to the addiction cycle (Koob and Volkow, 2010; Winstanley et al., 2010).

In the present study, 5HT2A receptors appeared to be desensitized, along with a concomitant suppression of reinstatement. Previous studies have shown that SSRI treatment is able to reduce measures of impulsivity in animals and humans, and that 5HT2A blockade can increase these anti-impulsivity effects (Wolff and Leander, 2002; Marek et al., 2005; Ardayfio et al., 2008; Butler et al., 2010). Moreover, only chronic and not acute treatment with three different SSRIs was able to reduce impulsive behavior in animals (Wolff and Leander, 2002). Thus, it is possible that chronic fluoxetine treatment, by preventing or reducing the possible sensitization of 5HT2A receptors following cocaine use (see above), also prevented increases in impulsivity which ordinarily contribute to reinstatement. This question could be addressed through assessment of behavioral measures of impulsivity throughout chronic fluoxetine treatment and washout.

5.4 Clinical implications

Together, the results of the present study indicate that SSRIs may be effective in preventing relapse in abstinent or treatment-seeking individuals. For instance, approximately 25% of individuals completing residential treatment programs relapse within 1-5 years and an additional 18% return to a second treatment programs within one year (Simpson et al., 1999; Simpson et al., 2002). SSRI treatment, if begun during the residential phase, may aid in maintaining abstinence and preventing relapse following release, particularly if a patient is exposed to the drug or drug-associated cues. Indeed, to date only one study has examined the effects of SSRIs in preventing relapse in abstinent patients and found that treatment with sertraline delayed time to relapse in depressed cocaine-dependent patients (Oliveto et al., 2011). Our results strongly suggest additional studies would be warranted. Additionally,

compliance with treatment is a substantial challenge in treating cocaine abuse and dependence. The effects of fluoxetine in suppressing reinstatement were long lasting in our study, which may increase the likelihood of successful recovery in spite of lapsed compliance.

However, these studies do not suggest that SSRIs would be effective as an intervention for current, ongoing cocaine abuse, since no significant effect on cocaine self-administration was seen. Notably, a small but non-significant increase in self-administration rates was seen during treatment, which may represent an attempt to surmount the effects of treatment and could increase risk of overdose in a clinical setting. These results are in concordance with the results of clinical studies examining the effects of SSRI treatment for ongoing cocaine abuse (e.g.Grabowski et al., 1995; Batki et al., 1996).

The current study also suggests that desensitization of 5HT2A receptors may contribute to the behavioral effects observed in cocaine-primed reinstatement. In this case, the addition of a 5HT2A selective antagonist to SSRI treatment may increase or enhance the beneficial effects. The time-course of 5HT2A receptor changes was not examined in the present study, but previous studies have demonstrated that SSRIs frequently require longer (14-21 day) dosing periods in order to induce neurobiological changes (Wong et al., 1995; Edwards and Anderson, 1999; Benmansour et al., 2002; Thompson, 2002; Vaswani et al., 2003). Co-treatment with a 5HT2A antagonist might reduce this delay in onset.

5.5 Future directions

Fluoxetine is a SSRI but also possesses affinity for the 5HT2C receptor that may be relevant at therapeutic doses (Palvimaki et al., 1999; Sanchez and Hyttel, 1999). Similar to

the 5HT2A receptor, the 5HT2C receptor has been implicated in mediating both the effects of psychostimulants and endogenous serotonin on dopamine, although the effects are generally in opposition to the effects of the 5HT2A receptor (Alex and Pehek, 2007; Bubar and Cunningham, 2008). Notably, fluoxetine functions as an antagonist at the 5HT2C receptor (Palvimaki et al., 1996) and other 5HT2C receptor antagonists have been shown to potentiate the effects of cocaine (Fletcher et al., 2002; Filip et al., 2006; Manvich and Howell, *unpublished data*). However, we did not observe any potentiation in our current study; therefore, experiments examining the effect of chronic cocaine and chronic fluoxetine on the function and regulation of the 5HT2C receptor would be informative, particularly as fluoxetine's antagonist actions may be counter to its beneficial effects for cocaine use. Determining the role of 5HT2C in fluoxetine's effects on cocaine use may lead to a better understanding of its role in neurobiology of cocaine abuse as well as the dynamic interaction between the serotonin and dopamine systems and provide additional information regarding its regulation. Together, this knowledge could aid in assessment and development of therapeutic compounds targeting 5HT2C receptors.

Similarly, other SSRIs have differing nonselective effects (Sanchez and Hyttel, 1999). Comparison of several of these compounds would determine whether the effects observed in the present study generalize to other SSRIs or whether they are specific to fluoxetine and thus may depend more heavily on its particular binding profile. Additionally, the different SSRIs are associated with different types and rates of side effects (Edwards and Anderson, 1999), and thus the range of choices allows for greater therapeutic application. If the cocaine-related effects generalize to the drug class as whole, this may improve the clinical utility of the findings.

In the current study, we demonstrated an upregulation of 5HT2A receptors with concomitant decreases in 5HT2A-related function. However, many factors influence both prolactin and DA release, and therefore more direct assessments of 5HT2A function would further enhance our understanding. For example, unconditioned behaviors such as head twitches and bobs are used in rodents and rabbits to measure 5HT2A activation (Aloyo et al., 2001; Dave et al., 2002); preliminary data from our own lab indicates that the wet dog shake may serve a similar function in nonhuman primates (Sawyer and Howell, *unpublished data*). Additionally, *in vivo* microdialysis in the prefrontal cortex and nucleus accumbens would further illuminate the question of how site-specific desensitization might affect dopaminergic function. As a corollary, similar studies systematically examining the effects of chronic, self-administered cocaine on 5HT2A receptor function would greatly enhance our understanding of the neurobiology of cocaine abuse and the potential of 5HT2A receptors as targets for pharmacotherapy.

Strikingly, the neurobiological and behavioral effects of fluoxetine persisted for 6 weeks following the conclusion of fluoxetine treatment. However, we did not examine additional time-points; studies demonstrating the time-course for both induction of and recovery from these effects would allow a better understanding of the mechanisms and of the potential therapeutic utility of these effects. As noted previously, persistence of effects beyond the treatment period is a desirable characteristic for pharmacotherapies in populations prone to compliance issues.

5.6 Conclusions

In the present study, we examined the effects of chronic fluoxetine treatment at clinically-relevant doses on cocaine-related behavior and neurochemistry as well as serotonergic neurobiology in rhesus macaques. Chronic fluoxetine treatment suppressed cocaine-primed reinstatement and dopamine overflow, likely through desensitization of 5HT2A receptors. However, there was no effect on ongoing cocaine self-administration or presynaptic serotonin function. Together, these studies provide additional knowledge regarding the function of SSRIs and the role of 5HT2A in cocaine-related neurobiology, and suggest that SSRIs and co-treatment with 5HT2A antagonists may be effective treatments to promote abstinence and prevent relapse.

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APPENDIX

Table A-1: Treatments by individual

Animal	Guide cannula	Fluoxetine treatment # 1				Fluoxetine treatment # 2		
		Behavior	Microdialysis-cocaine	Microdialysis-fenfluramine	PET imaging	Microdialysis-cocaine	Microdialysis-fenfluramine	Prolactin
RGg9	Y	Y	Y	X	Y		Y	Y
RZs9	*	Y			Y			
RLa10	*	Y			Y			Y
RZq8	Y	X	X	X	Y	Y	Y	Y
RDn8	Y	Y	Y	Y	Y			

Y = yes
* = Implanted but lost before beginning experiments (failed prep)
X = Participated but data excluded due to unreliability or equipment failure
Blank = Did not participate
Note: Fluoxetine treatments were always done concurrent with ongoing self-administration regardless of behavioral data collection

Table A-2: Participants in each measure

Behavior		Microdialysis			PET imaging	
Self-administration	*Reinstatement*	*Cocaine*	*Fenfluramine*	*Prolactin*	*M100907*	*FEmZIENT*
RGg9	RGg9	RGg9	RGg9	RGg9	RGg9	RGg9
RZs9	RZs9	RZq8	RZq8	RZq8	RZs9	RZs9
RLa10	RLa10	RDn8	RDn8	RLa10	RLa10	RLa10
RDn8	RDn8				RZq8	RZq8
					RDn8	RDn8