SEROTONIN 5-HT_{2C} RECEPTORS: ROLE IN (+)-MDMA SENSITIZATION AND DISTRIBUTION IN THE VENTRAL TEGMENTAL AREA

by

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To my parents, sisters, and brother;
Written in memory of “Nan”

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Serotonin (5-HT) released consequent to acute (+)-3,4-methylenedioxy-methamphetamine [(+)-MDMA; "ecstasy"] administration stimulates 5-HT_{2C} receptors (5-HT_{2C}R) to exert inhibitory influence on (+)-MDMA-induced behaviors. Thus, changes in 5-HT_{2C}R responsiveness upon repeated intermittent exposure to (+)-MDMA may contribute to the development and/or expression of behavioral sensitization. We tested the hypothesis that intermittent exposure to (+)-MDMA or the 5-HT_{2C}R agonist MK 212 results in enhanced (+)-MDMA-evoked locomotor activity ("behavioral sensitization") concurrent with decreased functional responsiveness of the 5-HT_{2C}R. Male Sprague-Dawley rats pretreated with saline, (+)-MDMA, or MK 212 for 7 days revealed that (+)-MDMA or MK 212 pretreatment results in transient tolerance to MK 212-induced hypomotility, indicating loss of 5-HT_{2C}R responsiveness, that coincides with enhanced (+)-MDMA-evoked hyperactivity at an early (24 h) withdrawal time-point. This suggests a role for 5-HT_{2C}R in the induction and early expression of (+)-MDMA sensitization. While behavioral sensitization in (+)-MDMA-pretreated rats was transient and paralleled the time-course of diminished 5-HT_{2C}R responsiveness, MK 212-pretreated rats displayed persistent ($\geq$ 2 wks) enhancement of (+)-MDMA-evoked hyperactivity despite recovery of 5-HT_{2C}R responsiveness. The loss of 5-HT_{2C}R responsiveness at 24h withdrawal was not linked to reduced 5-HT_{2C}R protein expression in the ventral tegmental area (VTA), nucleus accumbens (NAc), or prefrontal cortex in either (+)-MDMA- or MK 212-pretreated rats. However, an up-regulation of 5-HT_{2C}R protein expression was observed in the VTA at 2 wks withdrawal in MK 212-pretreated rats, which may contribute to the persistence of (+)-MDMA-evoked hyperactivity. The ability of 5-HT_{2C}R to limit the expression of (+)-MDMA-evoked hyperactivity is attributable to the inhibitory influence of 5-HT_{2C}R upon VTA dopamine (DA) neuron firing and DA release in the NAc. This effect may be mediated indirectly via depolarization of GABA

neurons. However, we revealed (via double-label immunofluorescence and retrograde tracing) that 5-HT_{2C}R are located on *both* GABA and DA neurons in the VTA, a subset of which project to the NAc. Thus, the potential for a direct stimulatory effect of 5-HT_{2C}R upon DA mesocorticoaccumbens pathway activation also exists. This may predominate under certain conditions, such as in response to repeated 5-HT_{2C}R stimulation, as a result of modifications in 5-HT_{2C}R responsiveness.

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CHAPTER 1: INTRODUCTION

The widely abused recreational drug 3,4-methylenedioxymethamphetamine (MDMA, "ecstasy") is hailed by its users for its ability to acutely produce mood elevation, mental stimulation, increased closeness/empathy for others, and decreased anxiety upon acute administration (Vollenweider et al., 1998). However, MDMA is reported to result in a number of negative effects including cognitive dysfunction, sleep disorders, increased anxiety, and depression upon withdrawal from repeated use (Morgan, 2000). These effects of MDMA are possibly related to its ability to alter serotonin (5-HT) neurotransmission, given serotonergic involvement in the regulation of these physiological and psychological processes (for review, see Dubovsky and Thomas, 1995). MDMA binds with high affinity (nM) to the 5-HT transporter (SERT) and induces a transporter-mediated exchange, resulting in the release of 5-HT into the synapse (Rudnick and Wall, 1992). In addition, MDMA binds with lesser affinity (μM) to, and reverses, the dopamine and norepinephine (NE) transporters, and also exhibits low affinity (μM) for the 5-HT_{1B} and 5-HT_{2A} receptors (Battaglia et al., 1988).

The release of 5-HT evoked by MDMA is believed to contribute to a unique behavioral profile (Bankson and Cunningham, 2001) observed in rodents characterized by increased forward locomotion in the periphery of the chamber, decreased investigatory behaviors such as holepokes and rearing activity (Gold et al., 1988; Callaway et al., 1990), and elicitation of the serotonin syndrome (Spanos and Yamamoto, 1989). This profile differs from the patterns elicited by its parent compound, amphetamine, which primarily consists of increased locomotion throughout the entire activity monitor and increased investigatory behaviors that are attributed to the selective release of DA by amphetamine (Geyer et al., 1987; Bankson and Cunningham, 2001). Serotonergic modulation of MDMA-evoked behaviors may be mediated through any number of the 16 known 5-HT receptor subtypes (Hoyer et al., 2002). Indeed, researchers have demonstrated prominent roles for the 5-HT_{1B} receptor (5-HT_{1B}R), 5-HT_{2A}R, and 5-

$HT_{2C}R$ in mediating the hypermotive and other behavioral effects of MDMA (Callaway et al., 1992; McCreary et al., 1999; Bankson and Cunningham, 2002; Fletcher et al., 2002; Herin et al., 2005). However, while the 5-$HT_{1B}R$ and 5-$HT_{2A}R$ are thought to play a facilitative role in MDMA-evoked hyperactivity (Callaway et al., 1992; McCreary et al., 1999; Herin et al., 2005), the 5-$HT_{2C}R$ appears to provide an inhibitory input that limits both the magnitude of hyperactivity evoked upon acute MDMA administration (Herin and Cunningham, 2001; Bankson and Cunningham, 2002; Fletcher et al., 2002), as well as the ability of MDMA to suppress food maintained responding (Fletcher et al., 2002).

Repeated administration of MDMA results in a progressive enhancement of MDMA-evoked locomotor activity, a phenomenon known as "behavioral sensitization" (Spanos and Yamamoto, 1989; Kalivas et al., 1998; McCreary et al., 1999; Itzhak et al., 2004). Behavioral sensitization is evoked by psychostimulants across chemical class and has been shown to endure for long periods of time following discontinuation of drug administration (Kalivas et al., 1993b; Vanderschuren and Kalivas, 2000). Thus, the adaptations which underlie sensitization may provide insight into the mechanisms underlying the behavioral sequleae observed during abstinence in chronic psychostimulant abusers (Sherer et al., 1988; Robinson, 1993). A variety of transient and enduring neuroadaptations encompassing several neurotransmitter systems have been reported in response to chronic psychostimulant administration (for reviews, see (Robinson and Becker, 1986; Kalivas et al., 1998; Vanderschuren and Kalivas, 2000), including MDMA (McNamara et al., 1995; Obradovic et al., 1998; Mayerhofer et al., 2001). However, enhancement of DA release in the nucleus accumbens (NAc) arising from adaptations within the DA mesocorticoaccumbens "reward" pathway [DA neurons which originate in the ventral tegmental area (VTA) and terminate in the NAc and prefrontal cortex (PFC)], appears to be a critical feature of behavioral sensitization to psychostimulants (Kalivas et al., 1993b; Vanderschuren and Kalivas, 2000).

Serotonin released by MDMA has been shown to contribute to the efflux of DA in the NAc evoked by MDMA (Koch and Galloway, 1997). As such, even though the primary mechanism of MDMA action is to reverse the SERT, the influence of 5-HT and the 5-HT receptors on the behavioral effects of MDMA may be due to the ability of 5-HT neurons from the dorsal raphe nucleus to innervate and modulate activity of the DA mesocorticoaccumbens pathway (Bankson and Cunningham, 2001). Indeed, DA release and stimulation of DA receptors has been shown to be integral in mediating the hyperlocomotive, discriminative stimulus, and reinforcing effects of MDMA (Callaway and Geyer, 1992a; Schechter, 1997; Bubar et al., 2004; Daniela et al., 2004), which are thought to be contingent upon activation of the DA mesocorticoaccumbens pathway (Pettit et al., 1984; Delfs et al., 1990; Callahan et al., 1997). Likewise, the 5-HT_{1B}R and 5-HT_{2A}R which facilitate MDMA-evoked hyperactivity (Bankson and Cunningham, 2002; Fletcher et al., 2002), have also been shown to enhance DA release in the NAc (Parsons et al., 1999; Lucas and Spampinato, 2000), while the 5-HT_{2C}R inhibits both the extent of hyperactivity (Bankson and Cunningham, 2001; Fletcher et al., 2002; Herin et al., 2005) and accumbal DA efflux induced by MDMA administration (Bankson and Yamamoto, 2004). Interestingly, the unique ability of the 5-HT_{2C}R to oppose the actions of other 5-HT receptors by limiting the amount of DA efflux and hyperactivity evoked by MDMA suggest that this receptor may be involved in the induction of the progressive enhancement of hyperactivity and increase in accumbal DA efflux associated with behavioral sensitization subsequent to repeated MDMA administration.

The 5-HT_{2C}R is widely expressed in the brain (Pompeiano et al., 1994; Abramowski et al., 1995; Eberle-Wang et al., 1997) and has been shown to undergo rapid desensitization and down-regulation in response to chronic agonist administration *in vitro* (Pranzatelli et al., 1993; Saucier et al., 1998; Berg et al., 2001b). Thus, the recurring enhancement of 5-HT efflux that occurs during repeated MDMA administration may induce similar methods of 5-HT_{2C}R regulation. Indeed, a reduction in the number of 5-HT_{2C}R binding sites has been reported in several brain regions following neurotoxic

regimens of MDMA (Scheffel et al., 1992; McGregor et al., 2003). In addition to these common forms of receptor regulation, the 5-HT_{2C}R has also been shown to possess a unique ability to undergo mRNA editing, resulting in the expression of several different 5-HT_{2C}R isoforms that differ in their agonist affinity, G-protein coupling, and functional efficacy (Herrick-Davis et al., 1999; Price et al., 2001). Although the effects of MDMA and other psychostimulants on the expression of the various 5-HT_{2C}R isoforms has not yet been investigated, studies have shown that changes in levels of 5-HT can induce alterations in the relative expression of these different isoforms (Gurevich et al., 2002a). As such, enhanced expression of edited 5-HT_{2C}R isoforms with lower agonist affinity and functional efficacy (Herrick-Davis et al., 1999; Price et al., 2001), would result in a reduced response of 5-HT_{2C}R to 5-HT released by MDMA and a diminished capacity to inhibit DA mesocorticoaccumbens pathway activation. Thus, the 5-HT_{2C}R has the potential to undergo various forms of regulation in response to repeated MDMA administration which may contribute to mechanisms involved in sensitization.

The 5-HT_{2C}R is a seven-transmembrane G-protein linked receptor that primarily couples to the $G\alpha_{q/11}$ G-protein, inducing phopholipase C-mediated inositol phosphate accumulation and enhancement of intracellular Ca^{++} (Conn and Sanders-Bush, 1987), and is thereby thought to induce neuronal depolarization upon exposure to endogenous or exogenous ligands (Sheldon and Aghajanian, 1991). Thus, the tonic and phasic inhibitory influence of the 5-HT_{2C}R upon DA mesocorticoaccumbens pathway activation and DA release in the NAc (for reviews, see Di Giovanni et al., 2002; Higgins and Fletcher, 2003) are thought to be mediated indirectly via depolarization of inhibitory γ-aminobutyric (GABA) neurons which synapse on VTA DA neurons (Johnson and North, 1992; Di Giovanni et al., 2001; Bankson and Yamamoto, 2004). The VTA, located in the ventral portion of the mesencephalon comprises the A10 DA population of mesencephalic catecholamine neurons (Dahlstrom and Fuxe, 1964). Efferent projections from the VTA are sent not only to the NAc and PFC, as mentioned above, but also to a wide variety of other brain regions, including components of the limbic system (for review, see

Domesick, 1988). Likewise, regulation of VTA neuronal activation is controlled by various afferents (Phillipson, 1979a), including 5-HT projections from the dorsal raphe nucleus (Herve et al., 1987; Van Bockstaele et al., 1993) and GABA projections from the NAc, PFC, and ventral pallidum (VP; Kalivas et al., 1993a). In addition, a population of GABA neurons is also present in the VTA appear to form synapses on local VTA DA neurons (Johnson and North, 1992) and to send projections outside of the VTA to regions such as the NAc (Van Bockstaele and Pickel, 1995) and PFC (Carr and Sesack, 2000a).

Although mRNA (Pompeiano et al., 1994; Eberle-Wang et al., 1997) and protein (Abramowski et al., 1995; Clemett et al., 2000) for the 5-HT_{2C}R have been shown be present in the VTA, distribution of 5-HT_{2C}R to specific neuronal subtypes has not been throroughly investigated. Transcript for the 5-HT_{2C}R was detected in VTA neurons adjacent to, but not within, neurons containing mRNA for the DA synthethic enzyme tyrosine hydroxylase (TH; Eberle-Wang et al., 1997). The presence of 5-HT_{2C}R mRNA was, however, detected in cells containing the mRNA for the rate limiting enzyme for GABA synthesis, glutamate decarboxylase (GAD) in the substantia nigra, which is adjacent to the VTA (Eberle-Wang et al., 1997). Thus this study suggested that the 5-HT_{2C}R is also likely to be localized on GABA neurons within the VTA, thereby supporting the hypothesis of indirect modulation of VTA DA neurons by 5-HT_{2C}R via inhibitory GABA neurons. However, no studies to date have been conducted to confirm the presence of 5-HT_{2C}R protein on GABA neurons within the VTA.

The following set of experiments was developed to gain further insight into the mechanisms and impact of 5-HT_{2C}R modulation of DA mesocorticoaccumbens pathway activation. These studies sought to examine the functional adaptations of 5-HT_{2C}R in response to repeated MDMA administration and whether these adaptations may contribute to behavioral sensitization to MDMA. Furthermore, experiments were conducted to examine the distribution of 5-HT_{2C}R on neurons within the VTA and the potential for these receptors to influence activity of DA mesoaccumbens neurons.

CHAPTER 2:

TRANSIENT CHANGES IN 5-HT$_{2C}$ RECEPTOR RESPONSIVENESS ACCOMPANY SHORT-TERM SENSITIZATION FOLLOWING REPEATED (+)-MDMA ADMINISTRATION

INTRODUCTION

Behavioral sensitization upon repeated intermittent administration is linked to adaptations within the dopamine (DA) mesocorticoaccumbens or "reward" pathway (Kalivas et al., 1993b; Vanderschuren and Kalivas, 2000). This pathway, consisting of DA neurons that originate in the ventral tegmental area (VTA) and terminate in the nucleus accumbens (NAc) and prefrontal cortex (PFC), appear to be critical for the initiation and expression of behavioral sensitization (Kalivas et al., 1993b; Vanderschuren and Kalivas, 2000). In particular, a hallmark feature of behavioral sensitization is the development of enhanced psychostimulant-induced DA release in the NAc (Robinson, 1993). Although less well studied than for cocaine or amphetamine (Kalivas et al., 1993b; Vanderschuren and Kalivas, 2000), behavioral sensitization to (+)- and (±)-MDMA has been demonstrated (Spanos and Yamamoto, 1989; Kalivas et al., 1998; McCreary et al., 1999; Itzhak et al., 2003) and expression of (±)-MDMA sensitization is associated with enhanced (±)-MDMA-evoked DA release (Kalivas et al., 1998). Because (±)-MDMA-evoked DA release is controlled via a serotonin (5-HT)-mediated mechanism (Gudelsky and Nash, 1996; Koch and Galloway, 1997), the 5-HT system may contribute to the development and expression of MDMA sensitization, although few studies have explored this hypothesis to date.

A primary mechanism of action for MDMA is the enhancement of synaptic 5-HT levels via reversal of the 5-HT transporter (Rudnick and Wall, 1992). Stimulated release of 5-HT would then be available to act at the 16 identified 5-HT receptors (Hoyer et al., 2002). The 5-HT$_{1A}$, 5-HT$_{1B}$, 5-HT$_{2A}$, and 5-HT$_{2C}$ receptors have been shown to mediate

some of the behavioral effects of MDMA, including hyperactivity evoked by acute (+)- or (±)-MDMA (Bankson and Cunningham, 2001; Fletcher et al., 2002). Of particular interest is the 5-HT_{2C} receptor (5-HT_{2C}R), which provides a unique inhibitory influence over MDMA-induced hyperactivity, as 5-HT_{2C}R antagonists greatly enhance hyperactivity evoked by acute administration of (+)-MDMA (Bankson and Cunningham, 2002) and (±)-MDMA-evoked hyperactivity (Fletcher et al., 2002). The influence of the 5-HT_{2C}R upon MDMA-evoked hyperactivity may be related to modulation of the DA mesocorticoaccumbens pathway (Di Matteo et al., 2001) via 5-HT_{2C}R localized within this pathway. The functional relevance of the 5-HT_{2C}R in nuclei of the mesocorticoaccumbens circuit in the behavioral effects of acute MDMA exposure is suggested by recent findings that 5-HT_{2C}R ligands microinfused into the subnuclei of this circuit control expression of hyperactivity evoked by another psychostimulant, cocaine (McMahon et al., 2001; Filip and Cunningham, 2002; Filip and Cunningham, 2003; Fletcher et al., 2004).

Considering the robust influence of the 5-HT_{2C}R over (+)- and (±)-MDMA-evoked hyperactivity (Bankson and Cunningham, 2002; Fletcher et al., 2002) and the regulatory influence of the 5-HT_{2C}R over function of the DA mesocorticoaccumbens circuitry (Di Giovanni et al., 1999; De Deurwaerdere et al., 2004), it is plausible that alterations in 5-HT_{2C}R function following repeated (+)- or (±)-MDMA exposure may contribute to the development and/or expression of sensitization. Thus, the present study was conducted to test the hypothesis that repeated (+)-MDMA administration results in decreased 5-HT_{2C}R responsiveness and protein expression and that the alterations in 5-HT_{2C}R responsiveness parallel the expression of sensitization. Although the majority of studies involving repeated MDMA treatment employ frequent administration of high doses of (±)-MDMA that result in long-term depletion of 5-HTergic markers (Kalivas et al., 1998; Itzhak et al., 2003) and degeneration of 5-HTergic axons and terminals (referred to as “neurotoxicity”; Gudelsky and Yamamoto, 2003), evidence suggests that the development of sensitization to (±)-MDMA is not dependent upon 5-HTergic

neurotoxicity (Kalivas et al., 1998; Itzhak et al., 2003). Thus, our goal was to employ a repeated (+)-MDMA dosing regimen that results in sensitization, but does not cause depletion of 5-HTergic or DAergic markers. We administered the (+)-isomer of MDMA as this isomer is more potent than (-)-MDMA at releasing DA (Hiramatsu and Cho, 1990) and evoking hyperactivity (Callaway et al., 1990). Following administration of the "sub-neurotoxic" sensitizing regimen of (+)-MDMA (4 mg/kg/day, 7 d), animals were challenged with either (+)-MDMA, the 5-HT_{2C}R agonist MK 212, or saline at 24 h, 72 h, or 2 wk withdrawal and locomotor activity was measured. The extent of (+)-MDMA-evoked hyperactivity revealed the presence or absence of behavioral sensitization, while the extent of MK 212-induced hypomotility was utilized to determine the responsiveness of the 5-HT_{2C}R. In addition, Western blot analysis was utilized to determine whether the changes in 5-HT_{2C}R responsivity were due to alterations in 5-HT_{2C}R protein expression in the VTA, NAc, or PFC. Due to the close homology between the 5-HT_{2C}R and the 5-HT_{2A}R (Baxter et al., 1995) and the modest affinity of (+)-MDMA for the 5-HT_{2A}R (Battaglia et al., 1988), we also established whether the repeated (+)-MDMA regimen altered 5-HT_{2A}R protein expression in these brain areas.

METHODS

Animals

Adult male Sprague-Dawley rats (N = 224; Harlan Sprague-Dawley, Inc., Indianapolis, IN) weighing 225-350g at the beginning of the experimental procedures were used. The animals were housed four to a cage in a temperature (21-23ºC) and humidity (40-50%) controlled environment and lighting was maintained under a 12-h light-dark cycle (lights on at 7:00 a.m.-7:00 p.m.). Food and water were available ad libitum (except during testing procedures). All experimental protocols were carried out in accordance with the *Guide for the Care and Use of Laboratory Animals* (National

Institutes of Health, 1986) and with the approval by the Institutional Animal Care and Use Committee.

Drugs

Chloral hydrate, (+)-MDMA (National Institutes on Drug Abuse; Research Triangle, NC) and MK 212 [6-chloro-2-(1-peperazinyl)pyrazine HCL; Tocris Cookson, Ellisville, MO] were utilized in the study. Drugs were dissolved in sterile saline (0.9% NaCl) and administered subcutaneously (SC) or intraperitoneally (IP); doses refer to the weight of the salt.

Locomotor Activity Experiments

Apparatus

Locomotor activity was monitored and quantified under low light conditions using a modified open field activity system (San Diego Instruments, San Diego, CA) housed within sound attenuating outer chambers. Each of the 8 chambers consisted of a clear Plexiglas open field (40 X 40 X 40 cm). A 4 X 4 photobeam matrix located 4 cm from the floor measured horizontal activity. Activity recorded in the inner 16 x 16 cm of the open field was counted as central activity while activity in the outer 12 cm registered peripheral activity. A second row of 16 photobeams placed 16 cm from the monitor floor allowed measurement of rearing. The control software (Photobeam Activity Software; San Diego Instruments) was used to count peripheral, central, and rearing activity and data was stored for subsequent statistical evaluation. Video cameras located above the chambers were used to monitor activity continuously without disruption of behavior.

Experimental Protocols

Establishment of (+)-MDMA Sensitization

Behavioral Protocol and Analyses. Rats (n=8/group) were habituated to the test chamber for 3 hrs/day on each of the 2 days prior to the start of the experiment. On each of 7 consecutive days between 12:00 and 15:00 h, the rats were habituated to the test chamber for 1 hr prior to the injection of saline (1 ml/kg, SC) or (+)-MDMA (4 mg/kg,

SC). Measurements of locomotor activity began immediately following the injection and continued for 90 min. Upon completion of the daily locomotor activity session, animals were immediately returned to their home cages.

Because similar trends in activity were observed in both fields of the activity monitor, peripheral and central activity counts were summed to provide a single measure of horizontal activity for each individual animal throughout the 90-min session. Behavioral data are presented as mean total horizontal activity counts (±SEM) for each 90 min session on days 1, 3, 5, and 7 of the repeated (+)-MDMA regimen. A two-way analysis of variance (ANOVA) was used to analyze the effects of (+)-MDMA pretreatment (Factor 1) and the day of injection (Factor 2) on horizontal activity with repeated measures on Factor 2 (day). Because group comparisons were specifically defined prior to the start of the experiment, *a priori* planned pairwise comparisons were then made with Dunnett's test (Keppel, 1973) with the experimentwise error rate (α) set at 0.05.

Monoamine Determinations. At 24 h withdrawal from the repeated (+)-MDMA regimen, the rats were decapitated, and the PFC, striatum (STR) and NAc were microdissected (Heffner et al., 1980) on a cool tray (4°C) and the tissue was immediately submerged in liquid nitrogen and transferred to storage at -80°C until analyses of monamine levels were conducted. Content of DA, 3,4-dihydrophenylacetic acid (DOPAC), 5-HT, 5-hydoxyindole-3-acetic acid (5-HIAA), norepinephrine (NE) and homovanillic acid (HVA) were analyzed in the laboratory of Richard De La Garza II (Albert Einstein College of Medicine, Bronx, NY). Each sample was transferred to 200 µl ice-cold 0.1 N perchloric acid containing N-methyl-5-HT and 3,4-dihydroxybenzylamine as internal standards (for indoleamines and catecholamines, respectively). The tissue was sonicated in this solution and centrifuged at 23,000 x g for 20 min at 4° C. A portion of the supernatant (20 µl) was removed and analyzed by HPLC to determine the concentration of DOPAC, DA, 5-HT and 5-HIAA. The column employed was from Bioanalytical Systems (BAS: West Lafayette, IN: Phase II ODS-

3μM, 100 mm x 3.2 mm). The on-line degassed mobile phase consisted of an 8% solution of acetonitrile containing 0.6% tetrahydrofuran, 0.1% diethylamine, 0.025 M Methylenediaminetetra acetic acid, 2.3 mM 1-octane-sulfonic acid, 30 mM sodium citrate and 13.7 mM sodium dihydrogen phosphate (final pH 3.1), and was delivered at 800 μl/min.

The tissue pellet was saved for protein determination using the Bio-Rad *DC* Protein Assay protocol (Hercules, CA). The tissue pellet was sonicated in a 0.6 ml tube containing 200μl 0.5N NaOH. Protein standards were based on 1.5 mg/ml protein (Fetal bovine serum, Sigma, St. Louis, MO). The microplate reader (Benchmark Plus microplate spectrophotometer, Bio-Rad, Hercules, CA) was set to read at 750 nm with a dilution factor of 1. In this system, optical densities are converted into μg units derived from the standard curve.

Chromatograms were recorded using a DA-5 data acquisition analog to digital interface module coupled to an LC-4C electrochemical detector (BAS). Post-separation signals were derived from a 2 mm glassy-carbon working electrode whose potential was set at 600 mV versus an Ag/Ag Cl reference. Peak height was established using Chromagraph® and Report® software (BAS) and quantification involved dividing the peak height of the unknown by that of the internal standard, and referring this ratio to external standards. Samples from all animals in the two test groups were processed in parallel on the same day for each brain region.

Data are presented as mean (± SEM) 5-HT, 5-HIAA, DA, DOPAC, NE, or HVA content in nanograms per milligram of tissue. Individual Student's *t*-tests were used to compare the content of each monoamine or metabolite in repeated saline vs. repeated (+)-MDMA treated rats for each brain area analyzed (PFC, NAc, and VTA); the experimentwise error rate (α) set at $p < 0.05$.

Challenge with (+)-MDMA or MK 212 during Withdrawal

Behavioral Protocol and Analyses. Rats (n=8/group) were removed from their home cage between 09:00 and 11:00h, weighed, and injected with either (+)-MDMA (4mg/kg, SC) or saline (1 ml/kg, SC) and immediately returned to their home cage each day for 7 consecutive days. At 24 h, 72 h, or 2 wks following the last repeated injection (referred to as withdrawal), rats were removed from their home cage between 07:30 and 10:30h, weighed and injected with a challenge injection of (+)-MDMA (4 mg/kg, SC), MK 212 (2 mg/kg, SC), or saline (1 ml/kg, SC) and immediately placed in the test chamber. Locomotor activity was recorded for 90 min in all animals, however, the ability of MK 212 to induce suppression of locomotor activity was most evident during the first 20 min of the test session (unpublished observations), thus data collected for this period of time was analyzed for the MK 212 challenge. Each rat underwent only one test session.

Peripheral and central activity counts were again summed to provide a single measure of horizontal activity for each individual animal throughout the 90-min [(+)-MDMA challenge] or 20 min (MK 212 challenge) session. Data are presented as mean total horizontal activity counts (±SEM) for the session length. A two-way ANOVA was used to analyze the effects of repeated (+)-MDMA pretreatment (Factor 1) and challenge (Factor 2) at 24 h, 72 h, or 2 wks of withdrawal in separate groups of rats. *A priori* planned pairwise comparisons were made with the Student Newman Keuls test (Keppel, 1973) with the experimentwise error rate (α) set at 0.05.

To confirm the results obtained upon (+)-MDMA challenge, an additional group of rats (n = 32) underwent a similar procedure as described above for administration of the repeated saline or (+)-MDMA pretreatment regimen. However, in this experiment, challenges at all three withdrawal time-points were assessed in the same group of animals (repeated measures). Thus at 24 h, 72h *and* 2 wks following the last repeated injection, rats were removed from their home cage between 0:7:30 and 10:30h, weighed and injected with a challenge injection of (+)-MDMA (4 mg/kg, SC) or saline (1 ml/kg, SC)

and were immediately placed in the test chamber; locomotor activity was recorded for 90 min. Each rat received the same drug challenge on all three challenge days.

Data are presented as mean horizontal activity (±SEM) across the 90-min test session expressed as a percentage of (+)-MDMA-evoked hyperactivity for each withdrawal time-point. A three-way ANOVA was used to analyze the effects of repeated (+)-MDMA pretreatment (Factor 1), withdrawal time-point (Factor 2), and challenge (Factor 3) on horizontal activity with repeated measures on Factor 2 (time-point). *A priori* planned pairwise comparisons were made with the Fisher's Least Significant Difference test (Keppel, 1973) with the experimentwise error rate (α) set at 0.05. This approach to statistical analysis is supported by a number of statisiticians (Keppel, 1973; Sheskin, 2000).

5-HT$_{2C}$R and 5-HT$_{2A}$R Protein Expression during Withdrawal

Pretreatment Protocol**.** Naive rats (n=8/group) were removed from their home cage, weighed, and injected with the identical regimen of either (+)-MDMA (4 mg/kg, SC) or saline (1 ml/kg, SC) and immediately returned to their home cage each day for 7 consecutive days.

Western Blot. At 24 h or 2 wks following the last repeated injection, rats were anesthetized using chloral hydrate (800 mg/kg, IP) and decapitated. The VTA, NAc, and PFC tissue were microdissected (Heffner et al., 1980) on a cool tray (4°C) and lysed in HEPES containing, EDTA, EGTA, dithiothreitol (DTT), and protease inhibitor cocktail. Insoluble matter was removed by microcentrifugation at 5000 x g at 4°C for 10 min. The resulting supernatant was centrifuged at 20000 x g for 30 min. This pellet, containing the membrane-bound proteins, was resuspended in 1% SDS and frozen at -80°C until protein analysis was conducted. Total membrane-bound protein concentration was determined using a BCA protein determination kit (Pierce, Rockford, IL). Total protein (20-30 μg)

was reduced with an appropriate volume of Laemmli sample buffer with DTT for 1 min/ 100°C and separated on a 10% Tris-glycine gradient gel (Novex, San Diego, CA) using SDS-PAGE. Following gel electrophoresis, proteins were transferred to a PVDF membrane (BIORAD, Hercules, CA) via semi-dry electroblotting (Alltech, Deerfield, IL). The membrane was blocked with 5% non-fat dry milk for 1h, followed by incubation in 1% non-fat dry milk with either the polyclonal goat anti-5-HT_{2C}R antibody (1:500, 4°C overnight; Santa Cruz Biotechnology, Santa Cruz, CA), the monoclonal mouse anti-5-HT_{2A}R antibody (1:5000; RT 1hr; BD PharMingen, San Diego, CA), or the monoclonal mouse anti-β-actin antibody (1:5000; RT 1hr; Chemicon International, Temecula, CA). After 3 x 20 min washes with TBS-T, the blot was incubated in horseradish peroxidase (HRP) conjugated sheep anti-mouse (1:5000-1:10000; Sigma, St. Louis, MO) or mouse anti-goat secondary antibody (1:2000; Santa Cruz) in 1% non-fat dry milk for 1h, RT. Following 3 x 10 min washes in TBS-T and an overnight wash in TBS (4°C), the membrane was incubated in ECL Plus (Amersham) for 5 min and then exposed to film (Kodak MXR hyperfilm) for 1 sec to 10 min, depending upon the antibody being detected, and developed. Each membrane was probed with all three antibodies; antibodies were stripped from the membrane via incubation in Re-blot Plus Mild (Chemicon) for 7-15 min prior to re-probing with each additional antibody according to the procedures described above.

Films were scanned and subjected to densitometric analysis using Scion Image Analysis Software (Scion Corporation, Fredrick, MD). The density of each band was calculated as the actual band density minus the background density of an adjacent area of the same size. Following background subtraction, the ratio of 5-HT_{2C}R or 5-HT_{2A}R band density to actin band density was determined for each sample to normalize for the amount of protein loaded. A Student's *t*-test was used to compare the resultant 5-HT_{2C}R or 5-HT_{2A}R band densities in saline-treated vs. (+)-MDMA-treated rats for each brain area (VTA, NAc, or PFC) at each withdrawal time-point (24 h or 2 wks) with an experimentwise error rate (α) set at $p < 0.05$. Data are presented as the mean (± SEM) 5-

$HT_{2C}R$ protein expression expressed as percent change from repeated saline-treated controls.

RESULTS

Establishment of (+)-MDMA Sensitization

The ability of (+)-MDMA to induce sensitization was determined via measurement of locomotor activity immediately following daily (+)-MDMA (4 mg/kg, SC) or saline injections. Mean total horizontal activity data from days 1, 3, 5, and 7 are presented in **Fig. 1**. A main effect of (+)-MDMA treatment ($F_{1,63}$ = 185.23; $p < 0.0001$) and day of injection ($F_{3,63}$ = 8.09; $p < 0.001$), and a treatment x day of injection interaction ($F_{3,63}$= 11.96; $p < 0.0001$) were observed for mean total horizontal activity summed across each 90 min test session during the repeated treatment regimen. As shown in **Fig. 1**, (+)-MDMA (4 mg/kg) induced significantly higher levels of horizontal activity compared to saline on days 1, 3, 5, and 7 ($p < 0.05$). Animals also displayed significantly higher levels of horizontal activity in response to (+)-MDMA on days 5 and 7 compared to day 1 of the repeated treatment ($p < 0.05$), suggesting that sensitization had indeed developed. Horizontal activity levels following saline administration did not differ between day 1 and any other test day.

Monoamine Determinations

There were no significant differences in levels of 5-HT, DA, NE, or their respective metabolites observed between the saline- and (+)-MDMA-treated rats in the FC, STR, or NAc when examined 24 h following the last repeated injection in the animals used to establish sensitization (**Table 1**), suggesting that our sensitization

regimen [(+)-MDMA (4 mg/kg/day, 7 d)] is sub-neurotoxic as measured by monoamine content at these time-points.

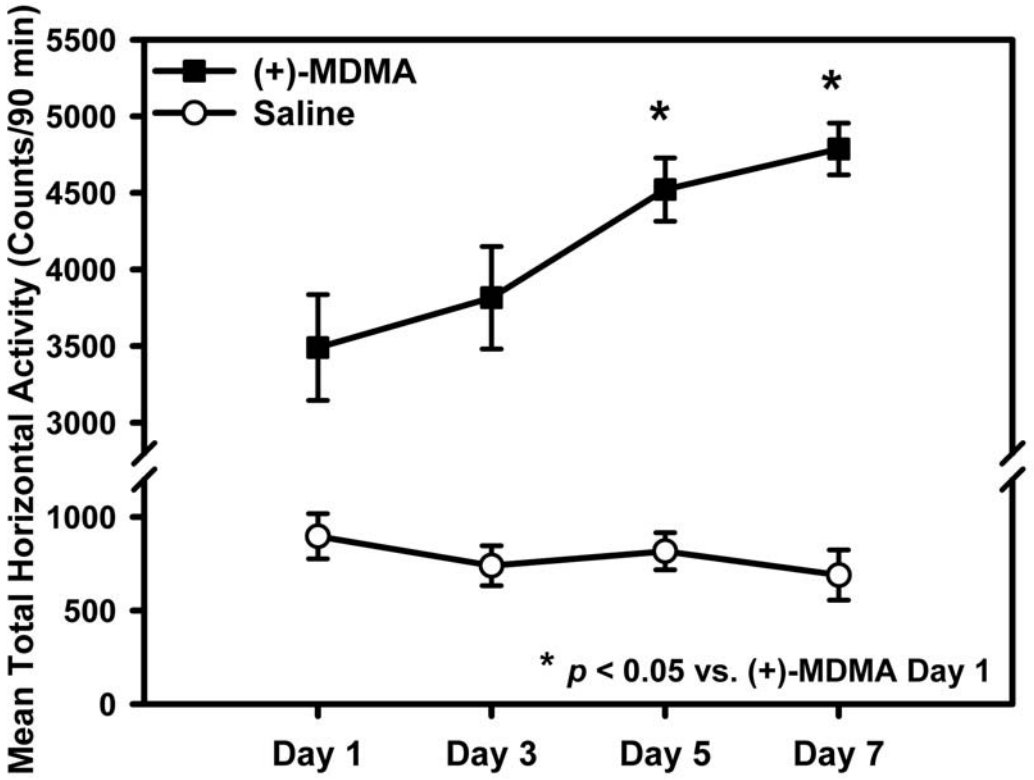

Figure 1. Sensitization to repeated (+)-MDMA administration. Data represent the mean horizontal activity counts (± SEM; n = 8/group) summed over the 90 min session following injection of saline (1 ml/kg, SC) or (+)-MDMA (4 mg/kg, SC) on days 1, 3, 5 and 7 of the 7-day repeated regimen. * $p < 0.05$ vs. (+)-MDMA on day 1.

TABLE 1: MONOAMINE AND METABOLITE LEVELS IN PREFRONTAL CORTEX, STRIATUM, AND NUCLEUS ACCUMBENS 24 H FOLLOWING REPEATED SALINE OR (+)-MDMA

Brain Area GROUP	DA (ng/mg prot ±SEM)	DOPAC (ng/mg prot ±SEM)	5-HT (ng/mg prot ±SEM)	5-HIAA (ng/mg prot ±SEM)	NE (ng/mg prot ±SEM)	HVA (ng/mg prot ±SEM)
Prefrontal Cortex						
Saline	0.42 ± 0.14	1.90 ± 0.21	4.54 ± 1.58	19.52 ± 0.55	11.16 ± 0.95	1.26 ± 0.16
(+)-MDMA	0.59 ± 0.16	2.23 ± 0.51	5.81 ± 1.00	16.22 ± 2.15	10.27 ± 0.668	1.21 ± 0.19
	$p = 0.43$	$p = 0.57$	$p = 0.29$	$p = 0.16$	$p = 0.45$	$p = 0.82$
Striatum						
Saline	919.72 ± 349.26	293.20 ± 92.08	8.28 ± 1.70	14.26 ± 1.17	54.25 ± 34.58	79.22 ± 25.04
(+)-MDMA	726.90 ± 180.37	223.23 ± 48.66	7.57 ± 1.14	12.86 ± 0.94	35.58 ± 11.99	58.70 ± 11.55
	$p = 0.83$	$p = 0.10$	$p = 0.73$	$p = 0.37$	$p = 0.66$	$p = 0.17$
Nucleus Accumbens						
Saline	214.87 ± 55.84	153.84 ± 33.86	7.82 ± 1.43	12.50 ± 0.68	70.76 ± 17.08	29.05 ± 6.37
(+)-MDMA	198.20 ± 51.17	123.71 ± 20.86	9.74 ± 4.45	12.33 ± 1.07	51.42 ± 15.60	24.90 ± 4.32
	$p = 0.83$	$p = 0.48$	$p = 0.40$	$p = 0.90$	$p = 0.42$	$p = 0.61$

Challenge with (+)-MDMA during Withdrawal

A main effect of pretreatment ($F_{1,31} = 11.44$, $p < 0.01$), challenge ($F_{1,31} = 146.41$, $p < 0.0001$), and a pretreatment x challenge interaction ($F_{1,31} = 19.24$; $p < 0.001$) were observed for mean horizontal activity across the 90-min session following drug challenge at 24 h withdrawal. As shown in **Fig. 2**, challenge with (+)-MDMA induced significant horizontal hyperactivity in both (+)-MDMA and saline pretreated rats at 24 h withdrawal compared to saline challenge. The hyperactivity induced by (+)-MDMA challenge in repeated (+)-MDMA-pretreated rats was significantly higher than (+)-MDMA-induced hyperactivity displayed by saline-pretreated rats at 24 h ($p < 0.05$). Activity levels seen upon challenge with saline in rats pretreated with (+)-MDMA did not significantly differ from saline-pretreated rats at 24 h withdrawal.

A main effect of pretreatment ($F_{1,30} = 6.64$; $p < 0.05$), challenge ($F_{1,30} = 118.53$; $p < 0.0001$), and a pretreatment x challenge interaction ($F_{1,30} = 8.35$; $p < 0.01$) were also observed for mean horizontal activity across the 90-min session following drug challenge at 72 h withdrawal. As was the case at the 24 h withdrawal time-point, (+)-MDMA-evoked hyperactivity was significantly greater in (+)-MDMA- vs. saline-pretreated rats ($p < 0.05$), while there were no significant differences in activity levels observed following saline challenge at 72 h withdrawal.

A main effect of challenge ($F_{1,31} = 64.29$; $p < 0.0001$) for mean horizontal activity was observed at 2 wks withdrawal, however there was not a main effect of pretreatment ($F_{1,31} = 0.58$; $p = 0.452$) nor a pretreatment x challenge interaction ($F_{1,31} = 1.59$; $p = 0.218$) observed at this withdrawal time-point. As shown in **Fig. 2**, while (+)-MDMA challenge induced hyperactivity ($p < 0.05$), there was no longer a significant difference in the levels of hyperactivity induced by (+)-MDMA challenge between saline and (+)-MDMA-pretreated rats at the 2 wk withdrawal time-point.

To further validate that the (+)-MDMA pretreatment regimen employed resulted in a transient, but not sustained, expression of sensitization, an additional group of rats were pretreated with (+)-MDMA (4 mg/kg/day, SC, 7 d) or saline (1 ml/kg/day, 7 d) and

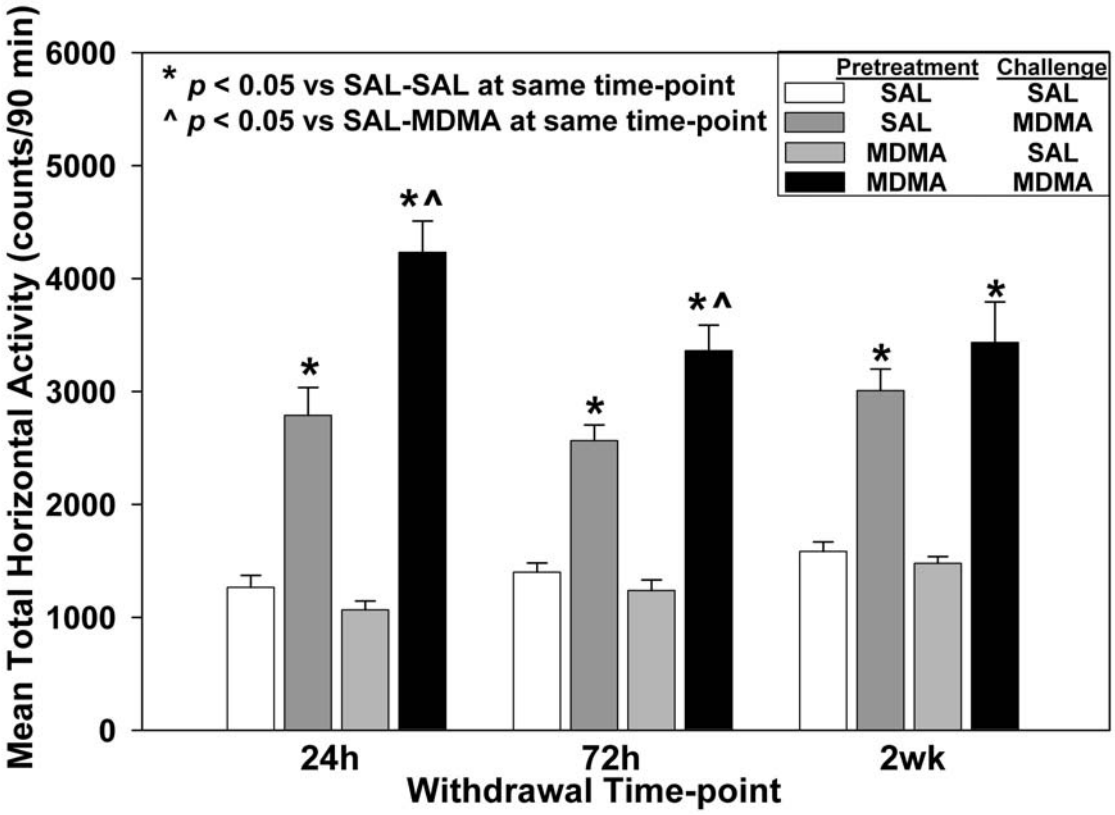

Figure 2. (+)-MDMA challenge during withdrawal from repeated (+)-MDMA administration. Data represent mean total horizontal activity counts (± SEM; n = 7-8/group) for the 90 min session following challenge with saline (SAL; 1 ml/kg, SC) or (+)-MDMA (MDMA; 4 mg/kg, SC) at 24 h, 72 h, or 2 wk withdrawal from the repeated saline (1 ml/kg/day, SC, 7 d) or (+)-MDMA (4 mg/kg/day, SC, 7 d) pretreatment regimen. * $p < 0.05$ vs. SAL-SAL at the same time-point; ^ $p = 0.05$ vs. SAL-MDMA at same time-point.

were challenged with either saline or (+)-MDMA (4 mg/kg, SC) at all three (24 h, 72 h, and 2 wk) withdrawal time-points. In the absence of a main effect of pretreatment ($F_{1,95}$ = 1.84; p = 0.185), withdrawal time-point ($F_{1,95}$ = 0.78; p = 0.46), or a pretreatment x time-point x challenge interaction ($F_{1,95}$ = 1.31; p = 0.278), a main effect of challenge ($F_{1,95}$ = 92.43; $p < 0.0001$) was observed for mean horizontal activity across the 90-min test session following (+)-MDMA challenge. As shown in **Fig. 3**, (+)-MDMA pretreatment significantly enhanced (+)-MDMA-evoked hyperactivity compared to saline pretreatment ($p < 0.05$) at the 24 h withdrawal time-point, while activity in response to saline challenge was similar between the saline and (+)-MDMA pretreatment groups (data not shown). There were no significant differences in (+)-MDMA-evoked hyperactivity between repeated saline- and (+)-MDMA-treated rats at either the 72 h or 2 wk withdrawal time-points.

Challenge with MK 212 during Withdrawal

A main effect of challenge ($F_{1,31}$ = 246.61; $p < 0.0001$), and a pretreatment x challenge interaction ($F_{1,31}$ = 13.70; $p < 0.001$), without a main effect of pretreatment ($F_{1,31}$ = 0.77; p = 0.387), were observed for mean horizontal activity across the first 20-min of the test session following MK 212 challenge at 24 h withdrawal. As shown in **Fig. 4**, MK 212 significantly suppressed locomotor activity in all groups tested. Rats that received repeated pretreatment with (+)-MDMA were tolerant to the suppressant effects of MK 212 challenge at 24 h withdrawal in that the (+)-MDMA-pretreated rats displayed significantly higher levels of activity following MK 212 challenge compared to saline-pretreated rats ($p < 0.05$), suggesting a decrease in responsiveness of 5-HT_{2C}R following the (+)-MDMA sensitization regimen.

While a main effect of challenge ($F_{1,29}$ = 118.53; $p < 0.0001$) was observed for mean horizontal activity at the 72 h withdrawal time-point, neither a main effect of pretreatment ($F_{1,29}$ = 6.64; $p < 0.05$) nor a pretreatment x challenge interaction ($F_{1,29}$ = 8.35; $p < 0.01$) were observed at 72 h withdrawal. As shown in **Fig. 4**, unlike at the 24 h withdrawal time-point, (+)-MDMA pretreatment did not significantly affect the ability of

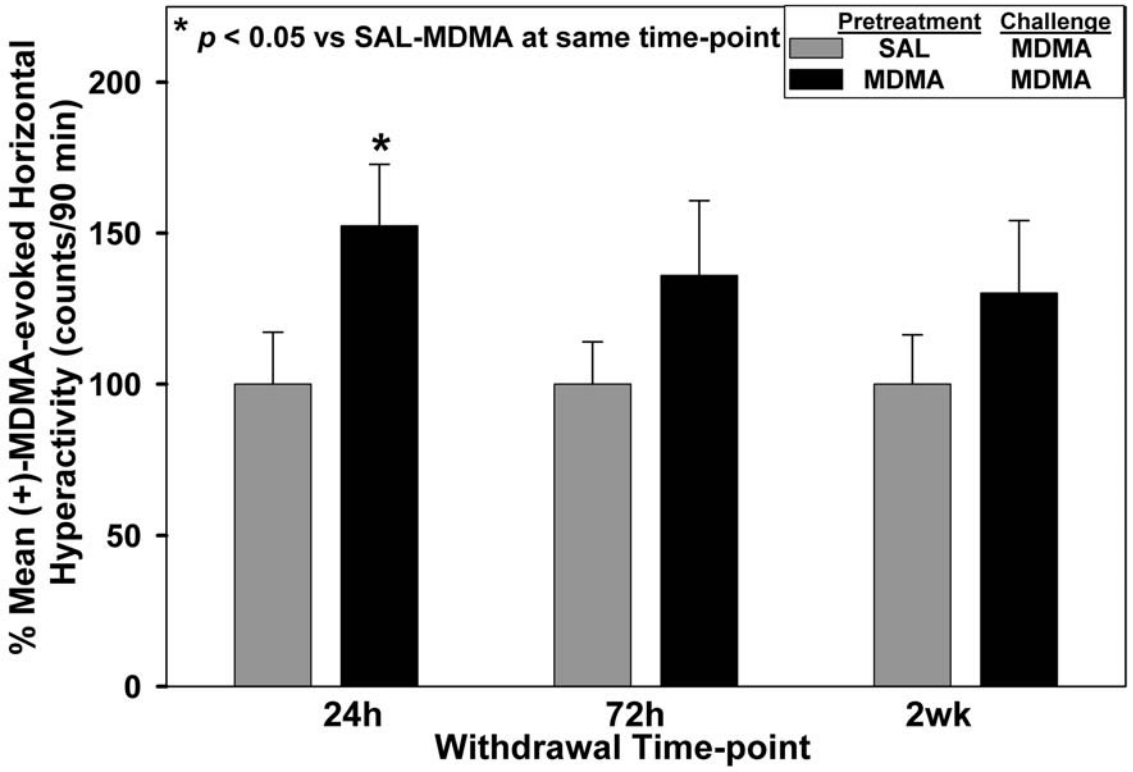

Figure 3. (+)-MDMA challenge during withdrawal from repeated (+)-MDMA administration; time-points assessed in the same group of rats. Data represent mean total horizontal activity counts (± SEM; n = 8/group) expressed as percentage of (+)-MDMA-evoked hyperactivity for the 90 min following challenge with saline (SAL; 1 ml/kg, SC) or (+)-MDMA (MDMA; 4 mg/kg, SC) at 24 h, 72 h, and 2 wk withdrawal from the repeated saline (1 ml/kg/day, SC, 7 d) or (+)-MDMA (4 mg/kg/day, SC, 7 d) pretreatment regimen. Each animal received the same challenge injection on all three challenge days. * $p < 0.05$ vs. SAL-MDMA at the same time-point.

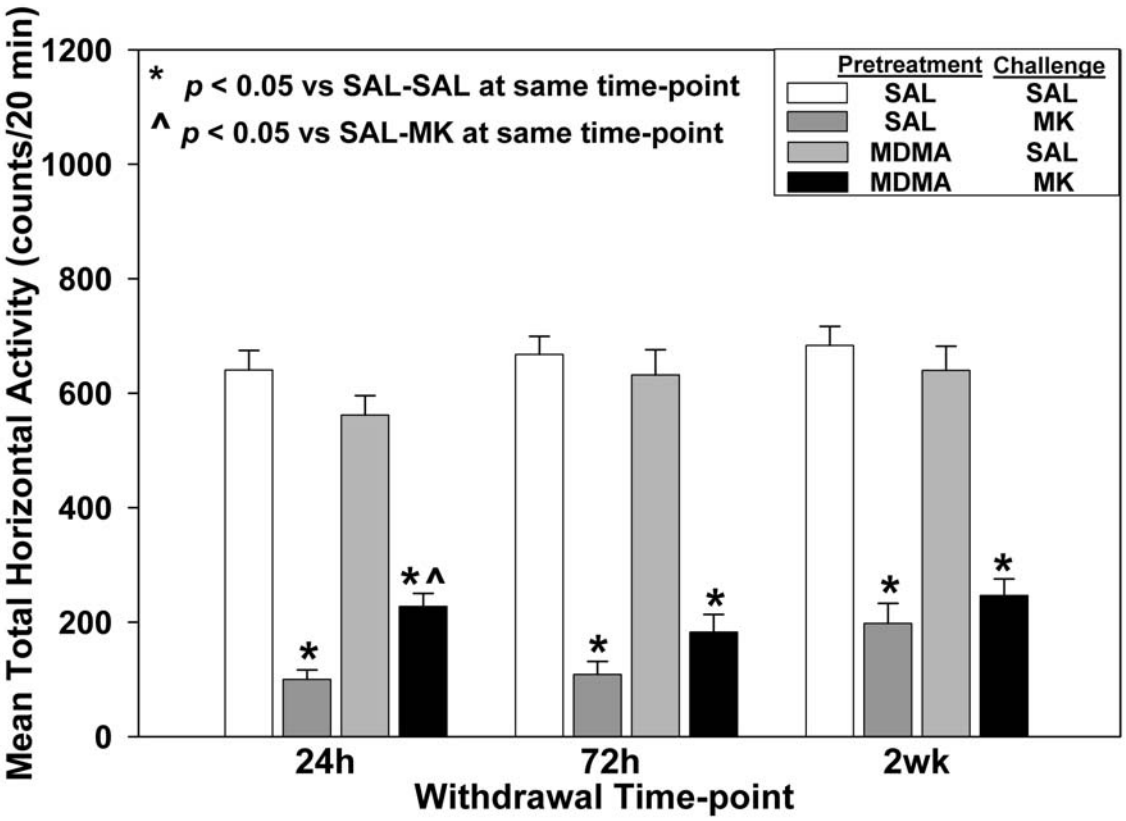

Figure 4. MK 212 challenge during withdrawal from repeated (+)-MDMA administration. Data represent mean total horizontal activity counts (± SEM; n = 7-8/group) for the first 20 min of the 90 min session following challenge with saline (SAL; 1 ml/kg, SC) or MK 212 (MK; 2 mg/kg, SC) at 24 h, 72 h, or 2 wk withdrawal from the repeated saline (1 ml/kg/day, SC, 7 d) or (+)-MDMA (4 mg/kg/day, SC, 7 d) pretreatment regimen. * $p < 0.05$ vs. SAL-SAL at the same time-point; ^ $p = 0.05$ vs. SAL-MK at same time-point.

MK 212 to induce hypomotility in rats at the 72 h time-point, suggesting that the responsiveness of the 5-HT_{2C}R was no longer compromised.

A similar result was observed at the 2 wk withdrawal time-point as at 72h withdrawal in that a main effect of challenge ($F_{1,31}$ = 155.57; $p < 0.0001$) was observed for mean horizontal activity, without a main effect of pretreatment ($F_{1,30}$ = 6.64; $p < 0.05$) or a pretreatment x challenge interaction ($F_{1,30}$ = 8.35; $p < 0.01$). Likewise, as shown in **Fig. 4**, repeated (+)-MDMA pretreatment did not alter the ability of MK 212 to induce hypomotility in rats suggesting that the responsiveness of the 5-HT_{2C}R had fully recovered by 2 wks.

5-HT_{2C}R and 5-HT_{2A}R Protein Expression

Analysis of membrane-bound protein levels revealed no significant differences in 5-HT_{2C}R protein expression between saline- and (+)-MDMA-treated rats at either 24 h or 2 wk withdrawal, respectively, in the VTA (p = 0.53 ; p = 0.80) or NAc (p = 0.73; p = 0.29), or in the PFC at the 2 wk withdrawal time-point (p = 0.61; **Fig. 5A**). However, a trend toward decreased 5-HT_{2C}R protein expression in the PFC in (+)-MDMA compared to saline-pretreated rats was observed at the 24 h withdrawal time-point (p = 0.054; **Fig. 5A**). A similar lack of significant differences in protein expression for the 5-HT_{2A}R between saline- and (+)-MDMA-pretreated rats were observed in the VTA (p = 0.79 ; p = 0.78), NAc (p = 0.77 ; p = 0.36) and PFC ($p < 0.88$; $p < 0.74$) at the 24h or 2 wk withdrawal time-points, respectively (**Fig. 5B**).

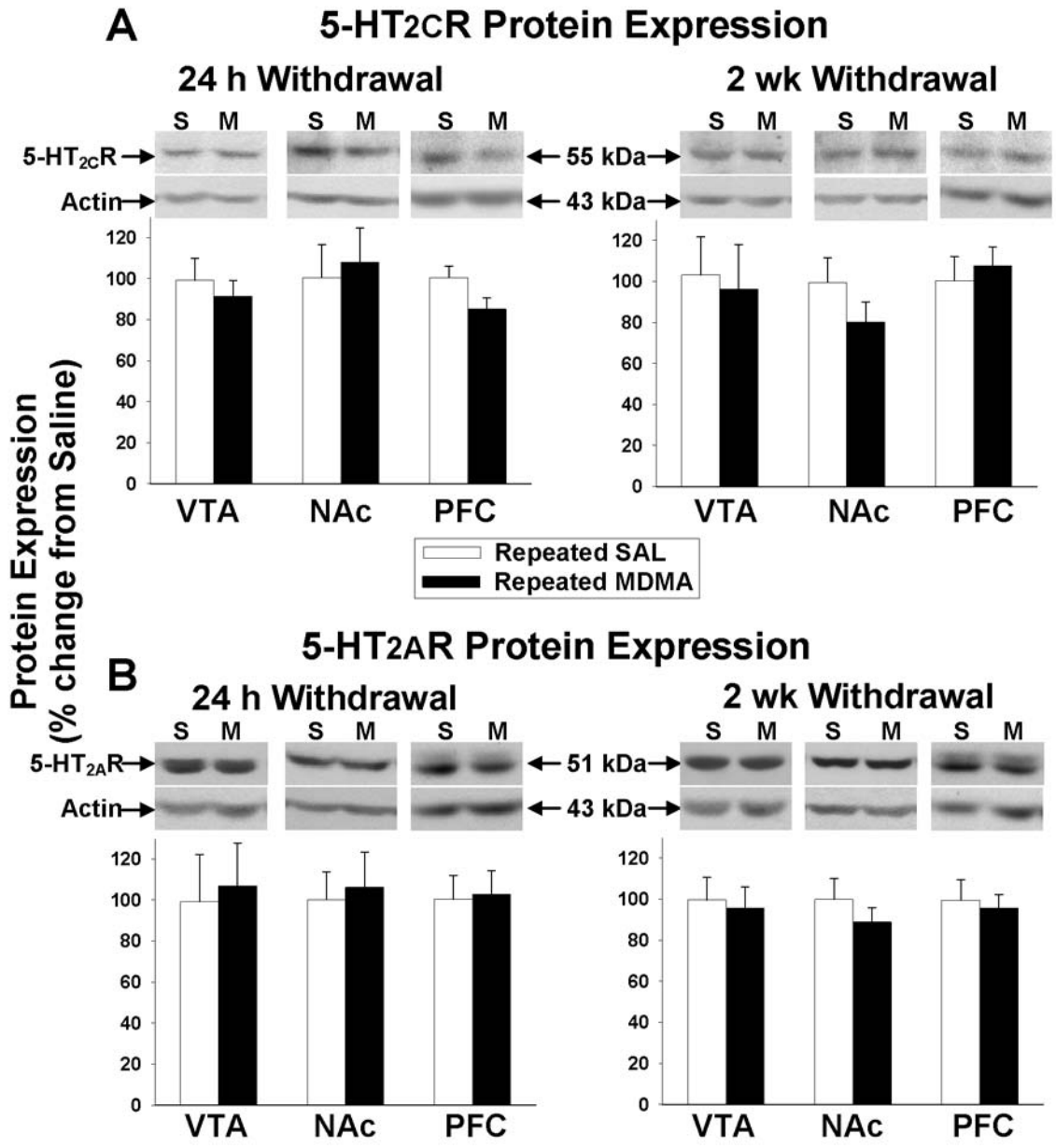

Figure 5. 5-HT$_{2C}$R and 5-HT$_{2A}$R protein expression during withdrawal from repeated (+)-MDMA administration. Representative Western blots of membrane-associated 5-HT$_{2C}$R protein [A] and 5-HT$_{2A}$R protein [B] in the VTA, NAc, and PFC at 24 h [left panel] or 2 wks [right panel] of withdrawal from repeated saline (S) or (+)MDMA (M; 4 mg/kg/day, SC, 7 d) pretreatment. Results of the densitometric analysis, expressed as percent change from repeated saline controls, represent the mean (± SEM; n = 6-8) 5-HT$_{2C}$R [A] and 5-HT$_{2A}$R [B] protein expression normalized to actin.

DISCUSSION

Behavioral sensitization in response to repeated, intermittent psychostimulant administration is a phenomenon that has captured the attention of researchers because knowledge of the neural adaptations that underlie sensitization may provide insight into the mechanisms underlying the behavioral sequelae (including anxiety, paranoia, craving, and relapse) observed in psychostimulant abusers (Sherer et al., 1988; Robinson, 1993). The present study reveals that a sub-neurotoxic regimen of (+)-MDMA (4 mg/kg/day, 7 d) does result in a short-term sensitization that is associated with a transient decrease in the functional responsiveness of the 5-HT_{2C}R in the absence of detectable changes in 5-HT_{2C}R protein expression in the VTA, NAc, or PFC. These results suggest that alterations in the responsivity of the 5-HT_{2C}R induced by repeated (+)-MDMA exposure are likely transduced by mechanisms more complex than simply decreased expression of the 5-HT_{2C}R protein, and this change in responsiveness may contribute to (+)-MDMA sensitization.

Our results support previous studies demonstrating sensitization to the locomotor activating effects of (+)-MDMA (McCreary et al., 1999) and (±)-MDMA (Spanos and Yamamoto, 1989; Kalivas et al., 1998), although some regimens of exposure either did not result in sensitization (Gold and Koob, 1989) or resulted in behavioral tolerance (Callaway and Geyer, 1992b). In the present study, we chose a (+)-MDMA dosing regimen (4 mg/kg/day, 7 d) that did not deplete 5-HT, DA, NE, or their primary metabolites. Thus, this regimen allowed us to focus on the neuroadaptations in response to repeated (+)-MDMA administration without the potential confounds associated with monoamine depletion (Heslop and Curzon, 1999). Consistent with previous studies of repeated administration of low doses of (±)-MDMA (Spanos and Yamamoto, 1989; Kalivas et al., 1998), the present sub-neurotoxic regimen resulted in robust sensitization evident *during* the repeated intermittent administration of (+)-MDMA as well as at short time-points (24 h and 72 h) after termination of (+)-MDMA pretreatment. By 2 wks withdrawal, the (+)-MDMA sensitization was no longer evident. A similar transient

expression of sensitization was also observed in animals pretreated with the same (+)-MDMA dosing regimen and assessed at all three withdrawal time-points, further supporting the suggestion that the regimen employed here [(+)-MDMA (4 mg/kg/day, 7 d)] results in a transient expression of behavioral sensitization.

While behavioral sensitization is more commonly reported to be an enduring phenomenon (Kalivas et al., 1993b; Kalivas et al., 1998), transient sensitization following certain repeated psychostimulant regimens has also been observed (Post et al., 1992; Henry and White, 1995). Some studies suggest that the degree of sensitization may depend on the environmental context in which animals receive injections (for review, see (Post et al., 1992). In the present study, we demonstrate for the first time that (+)-MDMA sensitization can be expressed in a context-independent manner [i.e., repeated injection of (+)-MDMA occurred in an environment (home cage) distinct from that in which expression of sensitization was measured (activity monitors)]. This suggests that an association between the injection of the drug and the environment in which the drug is experienced (i.e., test monitors) is not necessary for the development of short-term sensitization to (+)-MDMA. However, the lack of environmental association between the drug and the activity monitors may have contributed to the weakening of the sensitized response over time, as has been proposed for cocaine sensitization (Post et al., 1992; Henry and White, 1995).

The present study also reveals that the sub-neurotoxic (+)-MDMA regimen is associated with an initial tolerance to MK 212-induced hypomotility, suggesting decreased functional responsiveness of the 5-HT_{2C}R at the 24 h withdrawal time-point. The responsiveness of the 5-HT_{2C}R had begun to recover at 72 h withdrawal and was apparently fully recovered at the 2 wk withdrawal time-point as there was no longer a difference in the ability of MK 212 to evoke hypomotility between the saline- and (+)-MDMA-pretreated rats. This time-course of an early transient loss of 5-HT_{2C}R responsiveness that recovered over time resembles the early robust expression of (+)-MDMA sensitization that diminished across time from the last (+)-MDMA pretreatment

injection. The transient nature of the expression of (+)-MDMA sensitization and the functional responsiveness of the 5-HT_{2C}R suggests that changes in the influence of 5-HT_{2C}R may contribute to (+)-MDMA sensitization. In further support of this hypothesis, we also have evidence that repeated intermittent exposure to the 5-HT_{2C}R agonist MK 212 which results in a similar transient decrease in the functional responsiveness of the 5-HT_{2C}R also elicits a sensitization-like enhancement of (+)-MDMA-evoked hyperactivity (Bubar and Cunningham, 2003a). Together, these results suggest a possible role for 5-HT_{2C}R as a trigger for the initiation and/or early maintenance of expression of (+)-MDMA sensitization.

Down-regulation of the 5-HT_{2C}R by repeated agonist exposure has been reported (i.e., decreased B_{max}; Sanders-Bush and Breeding, 1990; Pranzatelli et al., 1993). Based upon this knowledge, one hypothesis to explain the loss of functional responsiveness of 5-HT_{2C}R following repeated (+)-MDMA administration is that the recurring exposure of 5-HT_{2C}R to 5-HT released by (+)-MDMA caused a down-regulation of 5-HT_{2C}R protein expression. In order to address this question, we examined 5-HT_{2C}R protein expression using within the mesocorticoaccumbens circuit, where a number of adapatations associated with development and expression of sensitization have been reported (Kalivas et al., 1993b; Vanderschuren and Kalivas, 2000). Western blot analysis of the membrane fractions of VTA, NAc, and PFC tissues taken at 24 h and 2 wk following the repeated (+)-MDMA regimen revealed no significant differences in 5-HT_{2C}R protein expression between repeated saline- and (+)-MDMA-pretreated rats in any of the brain areas examined. In support of these findings, 5-HT_{2C}R mRNA expression was also found to be similar between the saline- and (+)-MDMA-pretreated animals in the same brain areas when measured via DNA microarray analysis (Brooks, Bubar, and Cunningham, unpublished observations). Therefore, the decrease in 5-HT_{2C}R functional responsiveness following the present (+)-MDMA sensitization regimen does not appear to be due to gross changes in 5-HT_{2C}R protein expression within the DA mesocorticoaccumbens circuit.

Although overall changes in 5-HT_{2C}R protein expression were not detected via Western blot analysis, the nature of this technique (i.e., analysis of whole tissue homogenates) does not enable analysis of discrete changes in receptor protein expression, for example, within particular populations of neurons. As such, discrete differences in protein expression may be present in these nuclei that may alter the responsiveness of the 5-HT_{2C}R that were unable to be revealed using this technique. Previous studies have reported down-regulation of 5-HT_2R binding sites following repeated (±)-MDMA regimens (Scheffel et al., 1992; McGregor et al., 2003), however more rigorous, neurotoxic regimens were employed in these studies compared to the (+)-MDMA pretreatment regimen utilized in the present study. While the radioligands utilized to detect 5-HT_2R binding sites do not distinguish well between the 5-HT_{2C}R and 5-HT_{2A}R and therefore may reflect changes in one or both of the receptors in most instances, we also did not detect changes in protein expression for 5-HT_{2A}R following the current (+)-MDMA pretreatment. Disparities between ligand-induced alterations in protein expression detected via radioligand binding compared to Western blot techniques, which has previously been reported for other proteins (Salvatore et al., 2003; Yoburn et al., 2004), may reflect differences in subcellular localization of the proteins (Toda et al., 2003) that may only be detected using more selective cellular subfractionation techniques (Toda et al., 2003) than those employed in the present study. Thus, modifications in trafficking of the 5-HT_{2C}R (or 5-HT_{2A}R) to the plasma membrane, for example, which would have the potential to alter the functional response to 5-HT_{2C}R agonist administration (Becamel et al., 2002; Marion et al., 2004), may not have been detected in the present study.

In addition, to undergoing rapid desensitization (Stout et al., 2002) and internalization (Schlag et al., 2004), the 5-HT_{2C}R is thought to form homodimers (Herrick-Davis et al., 2004), and has been shown to couple to multiple G-proteins that appear to be differentially engaged by agonists ("agonist-directed trafficking of effector pathways;" Berg et al., 1998). Each of these methods of regulation have the potential to

modify 5-HT_{2C}R responsiveness without altering overall protein levels. Although little is known of the mechanisms or functional consequences of homodimerization of 5-HT_{2C}R (Herrick-Davis et al., 2004), the coupling of 5-HT_{2C}R to different G-protein effector pathways, desensitization, internalization, and eventual resensitization all appear to be linked to or dependent upon interactions with membrane trafficking proteins such as PDZ-interacting proteins and β-arrestin (Backstrom et al., 2000; Becamel et al., 2002; Xia et al., 2003). Recent DNA microanalyses suggest that several molecules involved in membrane trafficking may be substantially altered following the present (+)-MDMA regimen (Brooks et al., 2004). As such, alterations in the coupling of the 5-HT_{2C}R with G-proteins or the subcellular localization of 5-HT_{2C}R, which affect the function of 5-HT_{2C}R (Becamel et al., 2002; Xia et al., 2003), could contribute to depressed functional responsiveness of 5-HT_{2C}R after repeated (+)-MDMA exposure that would not be reflected in measurement of protein levels via Western blot.

In addition to these mechanisms, 5-HT_{2C}R also undergo a unique form of regulation through RNA editing. Editing of the 5-HT_{2C}R pre-mRNA results in the formation of several receptor isoforms, each of which appear to differ in their agonist binding affinities (Berg et al., 2001a), G-protein coupling (Price et al., 2001), and functional responses (Herrick-Davis et al., 1999; Price et al., 2001), including alterations in constitutive activity of the receptor (Herrick-Davis et al., 1999). As such, the relative expression of different 5-HT_{2C}R isoforms may greatly impact the functional responsiveness of 5-HT_{2C}R. Changes in synaptic 5-HT concentrations appear to regulate editing (Gurevich et al., 2002a) and repeated agonist stimulation of 5-HT_{2C}R seems to increase expression of edited 5-HT_{2C}R mRNA isoforms that ultimately result in expression of 5-HT_{2C}R protein with lower sensitivity to agonist stimulation and less efficiency in activation of linked G-proteins (Gurevich et al., 2002a). These studies suggest that repeated exposure of 5-HT_{2C}R to 5-HT released by (+)-MDMA during the sensitization regimen may have altered pre-mRNA editing of the 5-HT_{2C}R causing a loss of responsiveness to agonist stimulation and a reduction in the functional capabilities of

the 5-HT_{2C}R. Interestingly, differences in RNA editing of the 5-HT_{2C}R have been linked to certain psychiatric disorders including depression (for review, see Schmauss, 2003). Thus further examination of the potential for changes in 5-HT_{2C}R editing and the ultimate expression of non-edited and edited receptor isoforms associated with psychostimulant sensitization may provide insight into the mechanisms underlying depressive symptoms and related sequelae seen during early withdrawal from a (+)-MDMA binge (Verheyden et al., 2002).

Regardless of the mechanisms by which the functional responsiveness of the 5-HT_{2C}R is altered following repeated (+)-MDMA administration, changes in 5-HT_{2C}R responsiveness may affect the ability of the 5-HT_{2C}R to modulate DA mesocorticoaccumbens pathway activation. The 5-HT_{2C}R exerts a unique inhibitory influence upon DA neurotransmission. For example, systemic administration of 5-HT_{2C}R agonists reduce, while purported antagonists enhance, basal (Di Giovanni et al., 1999; De Deurwaerdere et al., 2004) as well as stimulated DA release (Bankson and Yamamoto, 2004), effects which may be related to a 5-HT_{2C}R-mediated inhibition of firing of DA neurons (Di Giovanni et al., 1999). The 5-HT_{2C}R is thought to exert its inhibitory influence upon DA neurons indirectly via depolarization of inhibitory γ-aminobutyric acid (GABA) neurons (Di Matteo et al., 2001). In support of this concept, administration of the 5-$HT_{2C/2B}$R antagonist SB 206553 directly into the VTA has recently been shown to depress (±)-MDMA-induced GABA release within the VTA and simultaneously enhance (±)-MDMA-evoked DA release in the NAc (Bankson and Yamamoto, 2004). Thus decreases in the functional responsiveness of the 5-HT_{2C}R in response to repeated (+)-MDMA administration as observed in the present study would likely produce an effect similar to 5-HT_{2C}R antagonist administration, i.e., disinhibition of DA release (Bankson and Yamamoto, 2004). The extent of DA released in the NAc is correlated with the expression of locomotor hyperactivity induced by psychostimulant challenge (Wise and Bozarth, 1987) and the development of enhanced DA release in the NAc appears to be a vital component of the process of behavioral sensitization, or enhanced locomotor

activation (Robinson, 1993) Taken together, the ability of the 5-HT_{2C}R to limit DA neurotransmission would likely contribute to the inhibitory influence of 5-HT_{2C}R upon basal and (+)-MDMA-evoked hyperactivity (Bankson and Cunningham, 2001; Bankson and Cunningham, 2002; Fletcher et al., 2002) and thus, the behavioral sensitization observed in the present study may be related to a reduced capacity of 5-HT_{2C}R to limit accumbal DA release following repeated (+)-MDMA administration.

In summary, the results from the present study suggest a potential role for 5-HT_{2C}R in the short-term sensitization that develops in response to a sub-neurotoxic regimen of (+)-MDMA, as expression of (+)-MDMA sensitization is associated with reduced 5-HT_{2C}R responsiveness. The alterations in 5-HT_{2C}R functional responsivity following exposure to the sub-neurotoxic regimen, which may alter the ability of the 5-HT_{2C}R to modulate DA neurotransmission, are not due to overall changes in 5-HT_{2C}R protein expression in the nuclei of the mesocorticoaccumbens circuits. Rather, reduced 5-HT_{2C}R responsiveness may be due to more complex mechanisms such as modifications in 5-HT_{2C}R G-protein coupling, trafficking or RNA editing. Further examination into the potential of 5-HT_{2C}R RNA editing in association with MDMA sensitization may be valuable for understanding the potential mechanisms underlying the sequelae (e.g., depression) observed following MDMA use.

CHAPTER 3:

TRANSIENT LOSS OF 5-HT$_{2C}$R RESPONSIVITY INDUCES PERSISTENT ENHANCEMENT OF (+)-MDMA-EVOKED HYPERACTIVITY

INTRODUCTION

The 5-HT$_{2C}$R has been implicated in a number of physiological and psychological conditions including schizophrenia, obsessive compulsive disorder, obesity, anxiety and depression (Dubovsky and Thomas, 1995). In addition, the 5-HT$_{2C}$R has recently been touted to potentially play an integral role in modulating the behavioral and rewarding effects of psychostimulants that are mediated through the DA mesocorticoaccumbens circuit (for review, see Higgins and Fletcher, 2003) upon which the 5-HT$_{2C}$R appears to exert a strong inhibitory influence. The impact of the 5-HT$_{2C}$R on hyperactivity (Herin and Cunningham, 2001; Bankson and Cunningham, 2002; Fletcher et al., 2002) and DA release evoked by MDMA (Bankson and Yamamoto, 2004) suggests that this receptor may also play an integral role in behavioral sensitization to MDMA, and likewise may be integral in mediating some of the behavioral sequelae, including depression and anxiety, observed during withdrawal from an MDMA binge (Morgan, 2000).

In the previous chapter, we demonstrated that the time-course for expression of (+)-MDMA behavioral sensitization parallels a transient decrease in 5-HT$_{2C}$R functional responsivity suggesting that the decrease in 5-HT$_{2C}$R functional responsiveness may contribute to the expression of (+)-MDMA sensitization. The present study was conducted to test the hypothesis that a selective decrease in functional responsiveness of the 5-HT$_{2C}$R via repeated pretreatment with the preferential 5-HT$_{2C}$R agonist MK 212 would result in a sensitization-like enhancement of (+)-MDMA-evoked hyperactivity. After pretreatment with MK 212 (2 mg/kg, 2x/day, 7 d), rats were challenged with either MK 212, (+)-MDMA, or saline at 24 h, 72 h, or 2 wks after the last pretreatment and locomotor activity was measured. The extent of MK 212-induced hypomotility was

utilized to determine the responsiveness of the 5-HT_{2C}R, while the extent of locomotor activity in response to (+)-MDMA challenge revealed the presence or absence of a sensitization-like enhancement of (+)-MDMA-evoked hyperactivity. In addition, Western blot analyses were utilized to determine whether the changes in 5-HT_{2C}R responsiveness were due to alterations in 5-HT_{2C}R protein expression in the VTA, NAc, or PFC. Two anti-5-HT_{2C}R antibodies that recognize distinct sites of the receptor (N-terminus vs. C-terminus) were utilized in these analyses to confirm that the changes in protein expression observed were 5-HT_{2C}R-related. Furthermore, given that a close homology exists between the 5-HT_{2C}R and the 5-HT_{2A}R (Baxter et al., 1995) and that MK 212 has modest affinity for the 5-HT_{2A}R (Forbes et al., 1993), we also established whether the repeated (+)-MDMA regimen altered 5-HT_{2A}R protein expression in these brain areas.

METHODS

Animals

Adult male Sprague-Dawley rats (N = 176; Harlan Sprague-Dawley, Inc., Indianapolis, IN) weighing 225-350g at the beginning of the experimental procedures were used. See Chapter 2 Methods for details.

Drugs

MK 212, (+)-MDMA, and Chloral hydrate were utilized in the study (See Chapter 2 Methods for details). Drugs were dissolved in sterile saline (0.9% NaCl) and administered subcutaneously (SC) or intraperitoneally (IP); doses refer to the weight of the salt.

Apparatus

Locomotor activity was monitored and quantified under low light conditions using a modified open field activity system (San Diego Instruments, San Diego, CA; see Chapter 2 Methods for details).

Experimental Protocols

Repeated MK 212 Pretreatment and Immobility Assessment

Twice a day (08:00h and 16:00h) on each of 7 days, rats (n=8/group) were removed from their home cage, weighed, and injected with either MK 212 (2 mg/kg, SC) or saline (1 ml/kg, SC) and immediately returned to their home cage. On days 1, 4, and 7 of the repeated treatment, rats were observed in their home cage for 15 min immediately following the morning injection. The amount of time during the 15 min period that each animal was active (i.e., ambulating, rearing, eating, drinking, grooming) vs. inactive (i.e., remained motionless) was recorded using a stop watch.

Data are presented as the mean amount of time (in min ±SEM) that the rats were active during the 15-min observation period in their home cage on days 1, 4, and 7 of the repeated treatment. A two-way analysis of variance (ANOVA) was used to analyze the effects of MK 212 pretreatment (Factor 1) and the day of injection (Factor 2) on time active with repeated measures on Factor 2 (day). Because group comparisons were specifically defined prior to the start of the experiment, *a priori* planned pairwise comparisons were then made with Dunnett's test (Keppel, 1973) with the experimentwise error rate (α) set at 0.05.

Challenge with MK 212 and (+)-MDMA during Withdrawal

At 24h, 72h, or 2 wks following the last saline or MK 212 pretreatment, rats (n=8/group)were injected with a challenge injection of MK 212 (2 mg/kg, SC), (+)-MDMA (3 mg/kg, SC), or saline (1 ml/kg, SC), and locomotor was activity was recorded

for 90 min as described previously (Chapter 2 Methods); only data collected from the first 20 min of the test session were analyzed for the MK 212 challenge.

Peripheral and central activity counts were summed to provide a single measure of horizontal activity for each individual animal throughout the 20-min (MK 212 challenge) or 90-min session [(+)-MDMA challenge]. Data are presented as mean total horizontal activity counts (±SEM) for the session length. A two-way ANOVA was used to analyze the effects of repeated MK 212 pretreatment (Factor 1) and challenge injection (Factor 2) at 24 h, 72 h, or 2 wks of withdrawal in separate groups of rats. *A priori* planned pairwise comparisons were made with the Fisher's least significant difference procedure (Keppel, 1973) with the experimentwise error rate (α) set at 0.05.

5-HT$_{2C}$R and 5-HT$_{2A}$R Protein Expression during Withdrawal

Pretreatment Protocol. Naive rats (n=8/group) were removed from their home cage, weighed, and injected with the same pretreatment regimen of either MK 212 (2 mg/kg, IP) or saline (1 ml/kg, IP) and immediately returned to their home cage each day for 7 consecutive days.

Western Blot. At 24 h or 2 wks following the last repeated injection, rats were anesthetized using chloral hydrate (800 mg/kg, IP) and decapitated. The VTA, NAc, and PFC tissue were microdissected (Heffner et al., 1980) on a cool tray (4°C) and total membrane-bound protein was isolated, protein concentration was measured, and gel-electrophoresis were conducted as described in Chapter 2 Methods. Following gel electrophoresis, proteins were transferred to a PVDF membrane (BIORAD, Hercules, CA) via semi-dry electroblotting (Alltech, Deerfield, IL). The membrane was blocked with 5% non-fat dry milk for 1h, followed by incubation in 1% non-fat dry milk with either the polyclonal goat anti-5-HT$_{2C}$R antibody (1:500, 4°C overnight; Santa Cruz Biotechnology, Santa Cruz, CA), the monoclonal mouse anti-5-HT$_{2C}$R antibody (1:500; 4°C overnight; BD PharMingen, San Diego, CA), the monoclonal mouse anti-5-HT$_{2A}$R antibody (1:5000; 1hr RT; BD PharMingen), or the monoclonal mouse anti-β-actin

antibody (1:5000; 1hr RT; Chemicon International, Temecula, CA). After washes with TBS-T, the membrane was incubated in horseradish peroxidase (HRP) conjugated sheep anti-mouse (1:5000-1:10000; Sigma, St. Louis, MO) or mouse anti-goat secondary antibody (1:2000; Santa Cruz) in 1% non-fat dry milk for 1h, RT. Following washes in TBS-T and an overnight wash in TBS (4°C), the membrane was incubated in ECL Plus (Amersham) and then exposed to film (Kodak MXR hyperfilm) and developed. Each membrane was probed with all four antibodies; antibodies were stripped from the membrane via incubation in Re-blot Plus Mild (Chemicon) prior to re-probing with each additional antibody according to the procedures described above and in Chapter 2 Methods.

Films were scanned and subjected to densitometric analysis using Scion Image Analysis Software (Scion Corporation, Fredrick, MD) as described in Chapter 2 Methods. A Student's T-test was used to compare the resultant 5-HT_{2C}R or 5-HT_{2A}R band densities in saline-treated vs. MK 212-treated rats for each brain area (VTA, NAc, or PFC) at each withdrawal time-point (24 h or 2 wks) with an experimentwise error rate (α) set at $p < 0.05$. Data are presented as the mean (± SEM) 5-HT_{2C}R or 5-HT_{2A}R protein expression expressed as percent change from repeated saline-treated controls.

RESULTS

Repeated MK 212 Pretreatment: Immobility Assessment

A significant main effect of pretreatment ($F_{1,425} = 389.90$; $p < 0.0001$), day of injection ($F_{2,425} = 79.16$; $p < 0.0001$) and a pretreatment x day interaction ($F_{2,425} = 54.74$; $p < 0.0001$) were observed for amount of time active during the 15-min observation period on days 1, 4, and 7 of the pretreatment regimen. As shown in **Fig. 6**, MK 212 (2 mg/kg, IP) significantly reduced the amount of time that each rat was active compared to saline-treated rats on all three pretreatment days. However, as the rats continued to be

exposed to MK 212 they grew tolerant to the MK 212-induced locomotor suppression, such that activity following MK 212 pretreatment on days 4 and 7 were significantly higher than that recorded on day 1 ($p < 0.05$). Activity levels in the saline-treated rats did not differ on days 4 or 7 compared to day 1.

Challenge with MK 212 during Withdrawal

A main effect of challenge ($F_{1,29} = 82.13$, $p < 0.0001$), but not of pretreatment ($F_{1,29} = 3.57$, $p = 0.07$) or a pretreatment x challenge interaction ($F_{1,29} = 3.93$; $p = 0.058$) were observed for mean horizontal activity across the first 20-min of the test session following drug challenge at 24 h withdrawal. As shown in **Fig. 7**, MK 212 significantly suppressed locomotor activity in all rats tested. Rats that were pretreated with MK 212 were tolerant to the suppressant effects of MK 212 challenge at 24 h withdrawal in that the MK 212-pretreated rats displayed significantly higher levels of activity following MK 212 challenge compared to saline-pretreated rats challenged with MK 212 ($p < 0.05$). These results suggest a decrease in responsiveness of 5-HT_{2C}R following the 5-HT_{2C}R agonist pretreatment. Levels of activity upon saline challenge were not altered in MK 212-pretreated rats.

While a main effect of MK 212 challenge ($F_{1,31} = 79.17$; $p < 0.0001$) was observed for mean horizontal activity at the 72 h withdrawal time-point, neither a main effect of MK 212 pretreatment ($F_{1,31} = 0.82$; $p = 0.37$) nor a pretreatment x challenge interaction ($F_{1,31} = 0.66$; $p = 0.42$) were observed. As shown in **Fig. 7**, in contrast to the 24 h withdrawal time-point, MK 212 pretreatment did not significantly affect the ability of MK 212 to induce hypomotility in rats at the 72 h time-point, suggesting that the responsiveness of the 5-HT_{2C}R was no longer compromised.

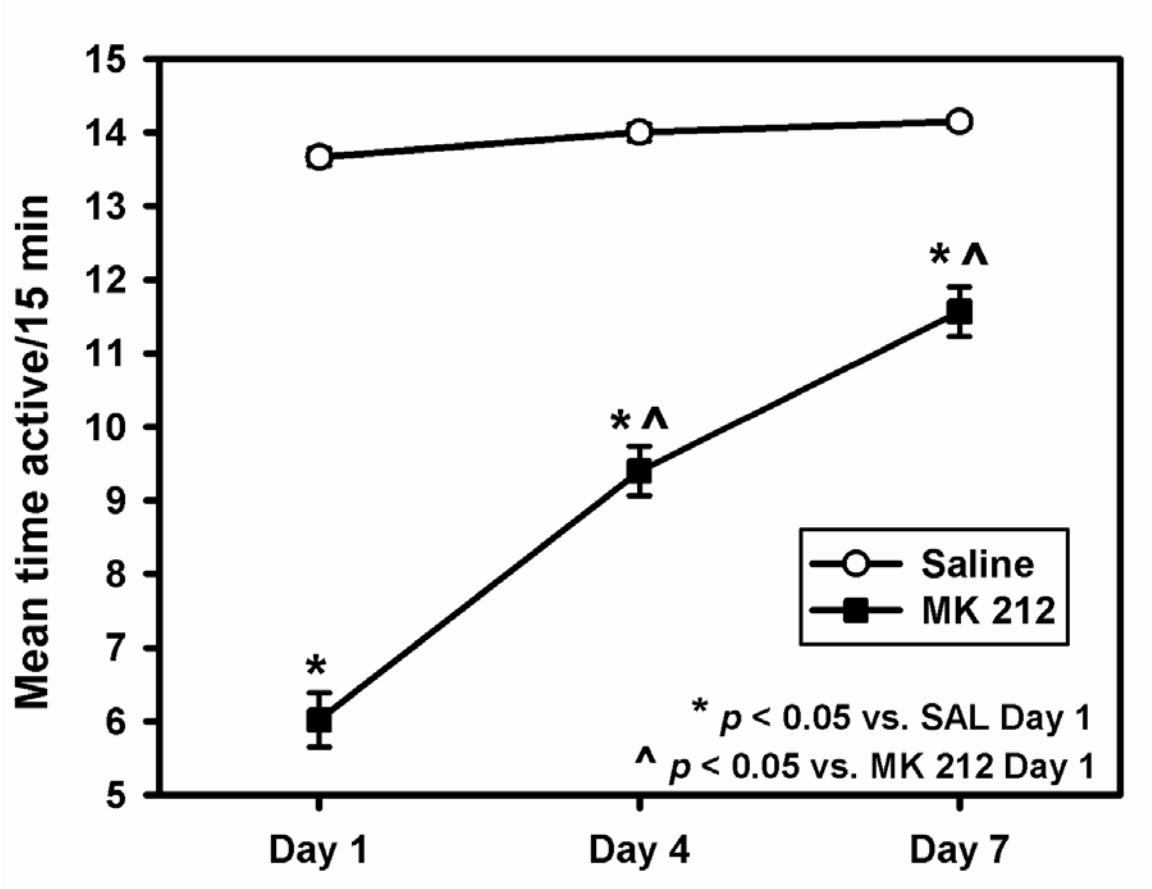

Figure 6. Immobility assessment during repeated MK 212 pretreatment. Data represent the average time (min ± SEM; n = 8/group) that the rats were active during the 15-min observation period in their home cage immediately following the morning injection of MK 212 (2 mg/kg, IP) on days 1, 4, and 7 of the 7-day repeated regimen. * $p < 0.05$ vs. SAL day 1; ^ $p = 0.05$ vs MK 212 Day 1.

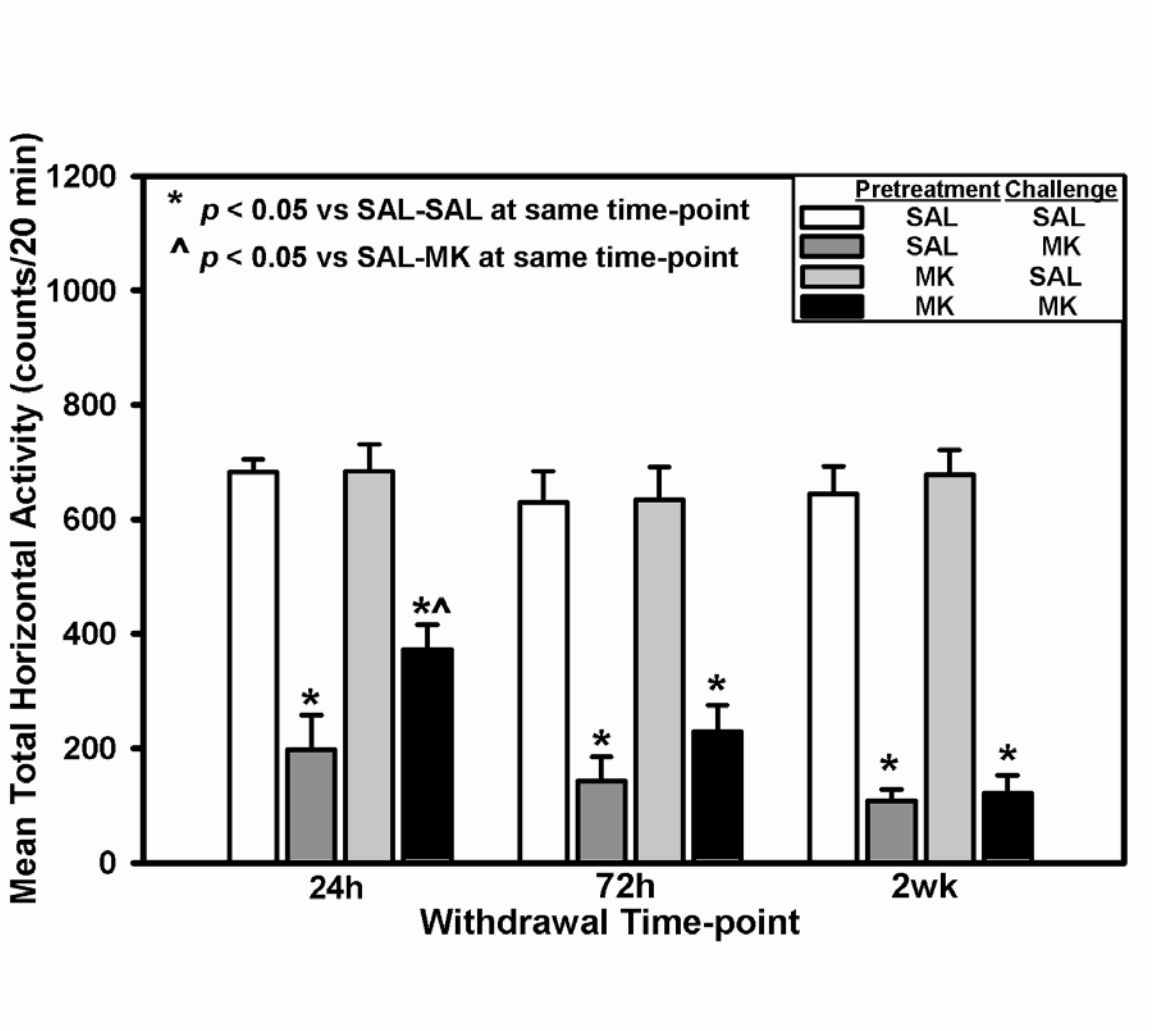

Figure 7. MK 212 challenge during withdrawal from repeated MK 212 administration. Data represent mean total horizontal activity counts (± SEM; n = 8/group) for the first 20 min of the 90 min session following challenge with saline (SAL; 1 ml/kg, SC) or MK 212 (MK; 2 mg/kg, SC) at 24 h, 72 h, or 2 wk withdrawal from the repeated saline (1 ml/kg, 2x/day, IP, 7 d) or MK 212 (2 mg/kg, 2x/day, IP, 7 d) pretreatment regimen. * $p < 0.05$ vs. SAL-SAL at the same time-point; ^ $p = 0.05$ vs. SAL-MK at same time-point.

A similar result was observed at the 2 wk withdrawal time-point as at 72h withdrawal in that a main effect of MK 212 challenge ($F_{1,31} = 221.46$; $p < 0.0001$) was observed for mean horizontal activity, without a main effect of MK 212 pretreatment ($F_{1,31} = 0.41$; $p = 0.53$) or a pretreatment x challenge interaction ($F_{1,31} = 0.07$; $p = 0.79$). Likewise, as shown in **Fig. 7**, MK 212 pretreatment did not alter the ability of MK 212 to induce hypomotility in rats suggesting that the responsiveness of the 5-HT_{2C}R had fully recovered.

Challenge with (+)-MDMA during Withdrawal

A main effect of pretreatment ($F_{1,31} = 5.81$; $p < 0.05$), challenge ($F_{1,31} = 38.47$; $p < 0.0001$), and a pretreatment x challenge interaction ($F_{1,31} = 4.62$; $p < 0.05$) were observed for mean horizontal activity following drug challenge at 24 h withdrawal. As shown in **Fig. 8**, (+)-MDMA challenge induced significant horizontal hyperactivity in both MK 212- and saline-pretreated rats at 24 h withdrawal compared to saline challenge. The hyperactivity induced by (+)-MDMA challenge in MK 212-pretreated rats was significantly higher than (+)-MDMA-induced hyperactivity displayed by saline-pretreated rats at 24 h ($p < 0.05$). MK 212 pretreatment did not significantly alter activity levels evoked by saline challenge at 24 h withdrawal.

A main effect of pretreatment ($F_{1,31} = 6.73$; $p < 0.05$), challenge ($F_{1,31} = 34.48$; $p < 0.0001$), and a pretreatment x challenge interaction ($F_{1,31} = 6.88$; $p < 0.05$) were also observed for mean horizontal activity following drug challenge at 72 h withdrawal. As at the 24 h withdrawal time-point, MK 212 pretreatment significantly enhanced (+)-MDMA-evoked hyperactivity compared to saline pretreatment ($p < 0.05$), without altering levels of activity evoked by saline challenge.

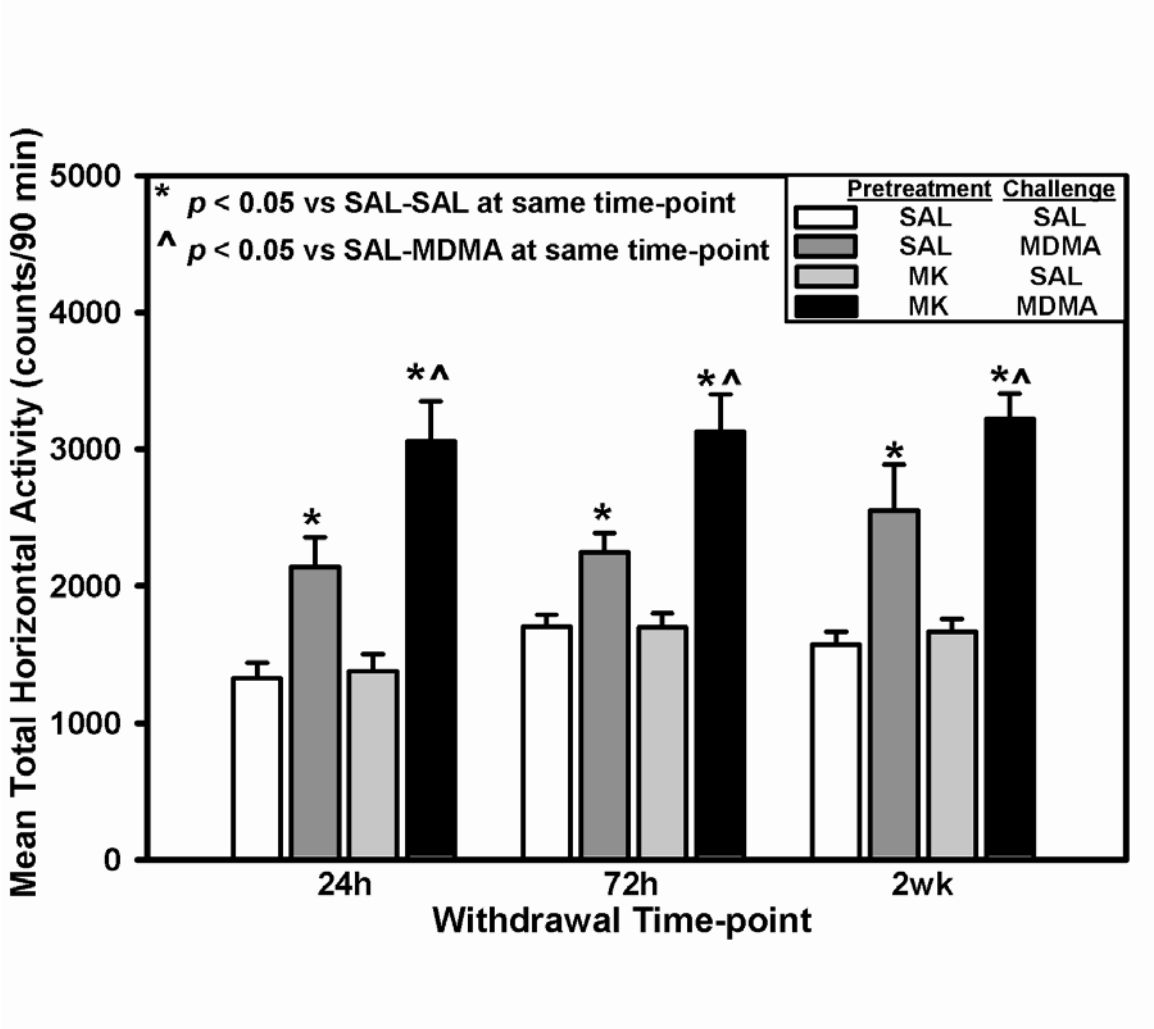

Figure 8. (+)-MDMA challenge during withdrawal from repeated MK 212 administration. Data represent mean total horizontal activity counts (± SEM; n = 7-8/group) for the 90 min session following challenge with saline (SAL; 1 ml/kg, SC) or (+)-MDMA (MDMA; 3 mg/kg, SC) at 24 h, 72 h, or 2 wk withdrawal from the repeated saline (1 ml/kg, 2x/day, IP, 7 d) or MK 212 (2 mg/kg, 2x/day, IP, 7 d) pretreatment regimen. * $p < 0.05$ vs. SAL-SAL at the same time-point; ^ $p = 0.05$ vs. SAL-MDMA at same time-point.

A main effect of challenge injection ($F_{1,29}$ = 34.50; $p < 0.0001$) for mean horizontal activity was observed at 2 wks withdrawal, however neither a main effect of MK 212 treatment ($F_{1,29}$ = 2.82; $p = 0.105$) nor a pretreatment x challenge interaction ($F_{1,29}$ = 1.74; $p = 0.199$) were observed at this withdrawal time-point. As shown in **Fig. 8**, in addition to the significant hyperactivity produced by (+)-MDMA challenge in both MK 212- and saline-pretreated rats ($p < 0.05$), *a priori* analyses revealed that (+)-MDMA-evoked hyperactivity was enhanced in the MK 212-pretreated compared to saline-pretreated rats, suggesting that the sensitization-like enhancement of (+)-MDMA-evoked hyperactivity persisted throughout the 2 wk withdrawal period.

5-HT_{2C}R and 5-HT_{2A}R protein expression

Analysis of membrane-bound protein levels revealed a significant enhancement of 5-HT_{2C}R protein expression (**Fig. 9**) in the VTA of MK 212-pretreated rats compared to saline-pretreated rats at the 2 wk withdrawal time-point using the Santa Cruz anti-5-HT_{2C}R antibody ($p < 0.05$; **Fig. 9A**) that was not present at the 24 h withdrawal time-point ($p = 0.56$). These results were confirmed using the PharMingen anti-5-HT_{2C}R antibody (**Fig. 9B**), which recognizes the opposite end of the 5-HT_{2C}R (C-terminal binding site vs. N-terminal binding site for the Santa Cruz antibody), revealing a similar 2-fold increase in 5-HT_{2C}R protein expression in the VTA of MK 212-pretreated rats at the 2 wk withdrawal time-point ($p < 0.05$), with no significant difference in 5-HT_{2C}R protein expression observed in the VTA at the 24 h withdrawal time-point ($p = 0.30$).

No significant differences in 5-HT_{2C}R protein expression levels were observed using either the Santa Cruz (**Fig. 9A**) or PharMingen anti-5-HT_{2C}R antibody (**Fig. 9B**) at the 24 h or 2 wk withdrawal time-points, in the NAc or PFC ($p < 0.05$). In addition, we did not observe differences in 5-HT_{2A}R protein levels between the saline- or MK 212-pretreated rats at either time-point in the VTA or NAc (data not shown).

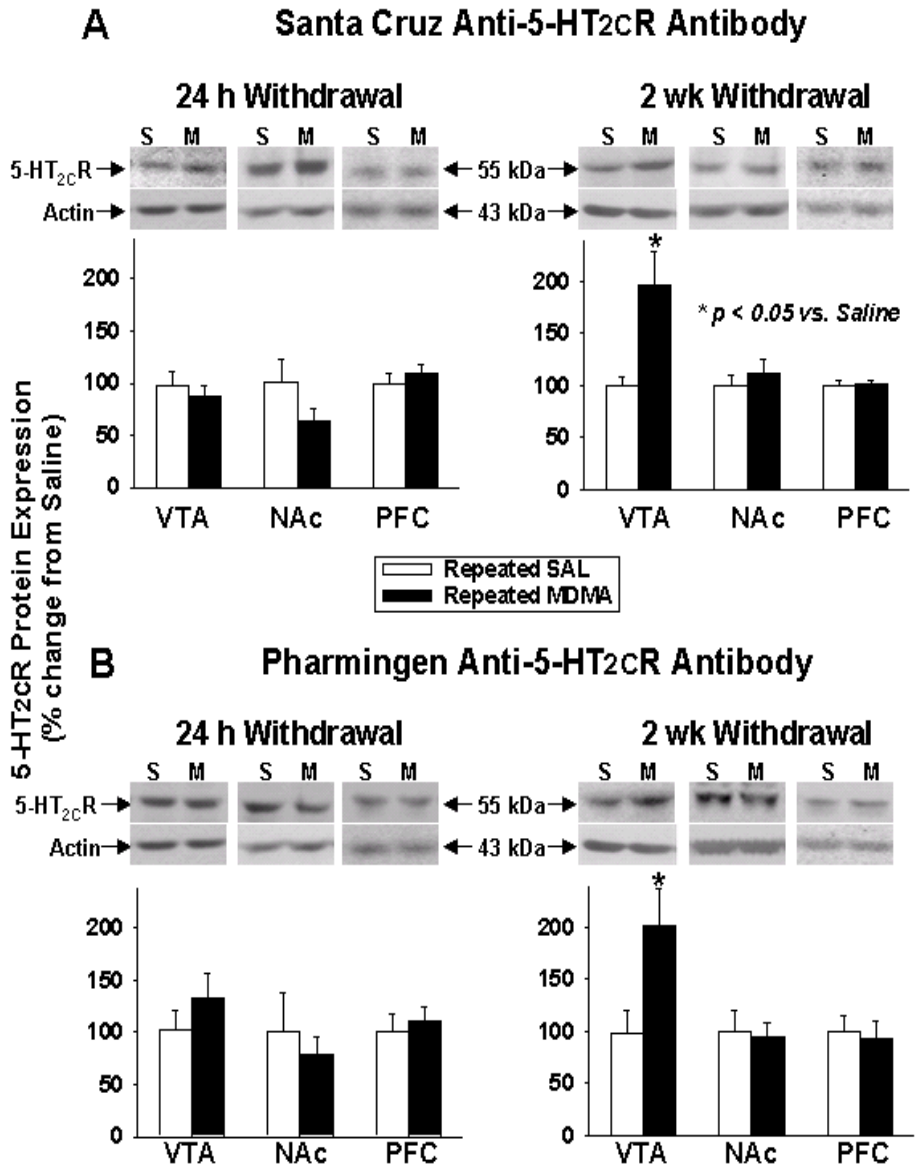

Figure 9. 5-HT_{2C}R protein expression during withdrawal from repeated (+)-MDMA administration. Representative Western blots of membrane-associated 5-HT_{2C}R protein using [A] the Santa Cruz anti-5-HT_{2C}R antibody and [B] the PharMingen anti-5-HT_{2C}R antibody in the VTA, NAc, and PFC at 24 h [left panel] or 2 wks [right panel] of withdrawal from repeated saline (S; 1 ml/kg, 2x/day, IP, 7 d) or MK 212 (M; 2 mg/kg, 2x/day, IP, 7 d) pretreatment. Results of the densitometric analysis, expressed as percent change from repeated saline controls, represent the mean (± SEM; n = 6-8) 5-HT_{2C}R protein expression normalized to actin.

DISCUSSION

The present study reveals that repeated 5-HT_{2C}R agonist pretreatment, which induced a transient decrease in 5-HT_{2C}R function, resulted in a sensitization-like enhancement of (+)-MDMA-evoked hyperactivity that persisted for at least 2 wks. These results suggest that decreased 5-HT_{2C}R responsiveness, which was not associated with a detectable decrease in 5-HT_{2C}R protein expression in the mesocorticoaccumbens circuit, may be integral in the initiation and early expression of sensitization to (+)-MDMA. However, the ability of the repeated 5-HT_{2C}R agonist pretreatment to induce sensitization that perists beyond the timeframe of diminished 5-HT_{2C}R function suggests that the repeated 5-HT_{2C}R stimulation must also induce additional adaptations which are involved in the maintenance of sensitization. One potential adaptation is an up-regulation of 5-HT_{2C}R protein expression in the VTA which was observed in the present study at 2wk, but not 24h, following the repeated 5-HT_{2C}R agonist pretreatment.

The results from the present study demonstrate that repeated exposure to a dose of MK 212 (2 mg/kg) that causes significant hypomotility in rats, results in the development of tolerance to MK 212-induced behavioral suppression. This tolerance is suggestive of a reduction, although not complete abolishment, of functional responsiveness of the 5-HT_{2C}R, since hypomotility was still evident in MK 212-pretreated rats, albeit to a lesser degree, both during the repeated MK 212 pretreatment and at 24 h withdrawal. These results are consistent with studies demonstrating that chronic administration of 5-HT_{2C}R agonists *in vitro* results in a decrease in 5-HT_{2C}R-evoked phosphoinosotide hydrolysis (Berg et al., 2001b) and Ca^{++} release (Saucier et al., 1998). Studies also suggest that this decrease in functional outcomes is partially due to a "down-regulation" or decrease in 5-HT_{2C}R protein levels evidenced by a decrease in Bmax in receptor binding assays (Sanders-Bush and Breeding, 1990; Pranzatelli et al., 1993; Saucier et al., 1998). However, the decrease in 5-HT_{2C}R functional responsiveness observed at the 24 h withdrawal time-point in the present study was not associated with a significant decrease

in 5-HT_{2C}R (or 5-HT_{2A}R) protein levels in the nuclei of the mesocorticoaccumbens circuit, which are thought to be critical sites for 5-HT_{2C}R modulation of psychostimulant-evoked locomotor activation (McMahon et al., 2001; Filip and Cunningham, 2002; Filip and Cunningham, 2003; Fletcher et al., 2004). These findings are consistent with the results presented in Chapter 2 which demonstrated a loss in 5-HT_{2C}R function without detectable changes in 5-HT_{2C}R protein at 24h withdrawal from repeated (+)-MDMA pretreatment.

As noted in Chapter 2, due to the limitations of the Western blot technique, discrete differences in 5-HT_{2C}R protein expression may be present in the VTA and NAc at the 24 h withdrawal time-point that were not detected in the present study. Alternatively, other regulatory mechanisms, which serve to reduce the ability of 5-HT_{2C}R to function, without necessarily changing overall protein levels, may be responsible for the loss of 5-HT_{2C}R functional responsiveness seen during and shortly after repeated MK 212 pretreatment. These alternative mechanisms may include agonist directed trafficking of the 5-HT_{2C}R effector pathways (Berg et al., 1998), rapid desensitization of the receptor via uncoupling of the receptors from their G-proteins (Saucier et al., 1998; Berg et al., 2001b), and/or receptor internalization (Saucier et al., 1998; Schlag et al., 2004) Interestingly, each of these mechanisms can in turn be regulated by mRNA editing of the 5-HT_{2C}R. This suggests that editing of 5-HT_{2C}R pre-mRNA, which results in the production of several different isoforms of the 5-HT_{2C}R which differ in agonist affinity, G-protein coupling, and functional efficacy (Herrick-Davis et al., 1999; Price et al., 2001), may be the ultimate method of regulation of 5-HT_{2C}R function. Since repeated agonist treatment has been shown to increase expression of the edited isoforms that display decreased agonist affinity and potency and decreased efficiency for activation of linked G-proteins (Gurevich et al., 2002a), it is plausible that the repeated MK 212 pretreatment in the present study also enhanced expression of these edited 5-HT_{2C}R isoforms resulting in a reduced ability of the 5-HT_{2C}R agonist MK 212 to induce hypomotility. The recovery of 5-HT_{2C}R functional responsiveness over time during

withdrawal from either the MK 212 (present study) or (+)-MDMA (Chapter 2) pretreatment suggests that regulation of 5-HT_{2C}R function through mechanisms such as mRNA editing, which appear to predominate in response to conditions such as over-stimulation of the receptor, are likely to be transient, reversing over time upon removal of the stimulus (i.e., withdrawal).

Previous studies have demonstrated that blockade of 5-HT_{2C}R with the 5-$HT_{2C/2B}$R antagonist SB 206553 (Bankson and Cunningham, 2002) or the selective 5-HT_{2C}R antagonist SB 242084 (Herin and Cunningham, 2001; Fletcher et al., 2002) enhanced acute (+)-MDMA-evoked hyperactivity, suggesting that the 5-HT_{2C}R limits the amount of locomotor activity evoked by (+)-MDMA challenge. This effect is thought to be related to the ability of 5-HT_{2C}R to inhibit firing of DA neurons in the VTA and DA release in the NAc (Di Matteo et al., 1998; Bankson and Cunningham, 2001). Thus, the present observation that decreased 5-HT_{2C}R function induced by repeated 5-HT_{2C}R agonist pretreatment is associated with enhanced hyperactivity evoked by (+)-MDMA challenge, suggests that the repeated 5-HT_{2C}R agonist pretreatment reduces the inhibitory actions exerted by the 5-HT_{2C}R upon the DA mesocorticoaccumbens pathway. The enhanced locomotor response to (+)-MDMA after MK 212 pretreatment is similar to the enhancement of (+)-MDMA-evoked hyperactivity, or "behavioral sensitization," that is observed following repeated (+)-MDMA administration (see Chapter 2; McCreary et al., 1999). In Chapter 2 we demonstrated that a sensitizing regimen of (+)-MDMA resulted in a similar transient decrease in 5-HT_{2C}R function and that the time-course for expression of (+)-MDMA sensitization paralleled the changes in 5-HT_{2C}R functional responsiveness. Thus, repeated treatment with MK 212 and (+)-MDMA may trigger a cascade of neural events that include reduced responsiveness of 5-HT_{2C}R as a component important in sensitization-like outcomes. Furthermore this evidence indicates that a decrease in 5-HT_{2C}R functional responsiveness, which would reduce the inhibitory actions of 5-HT_{2C}R in the DA mesocorticoaccumbens pathway activation, may be integral in the mechanisms involved in how sensitization develops to (+)-MDMA.

Interestingly, as opposed to the transient sensitization observed following repeated (+)-MDMA pretreatment detailed in the previous Chapter, the sensitization-like enhancement of (+)-MDMA-evoked hyperactivity observed following the repeated MK 212 pretreatment was not only present at times when there was an observable decrease in 5-HT_{2C}R functional responsiveness (24h withdrawal), but also persisted well beyond the time frame in which 5-HT_{2C}R functional responsiveness had recovered (72 h and 2 wks withdrawal). Thus, these data suggest that the ability of the repeated 5-HT_{2C}R agonist pretreatment to enhance (+)-MDMA-evoked hyperactivity is not limited to the decrease in functional responsiveness of the 5-HT_{2C}R. Rather, the 5-HT_{2C}R agonist pretreatment must trigger adaptations in addition to or in conjunction with the loss of 5-HT_{2C}R functional responsiveness that maintain the sensitization-like response.

The disparity between the time-course of enhanced (+)-MDMA evoked hyperactivity during withdrawal from repeated MK 212 versus (+)-MDMA pretreatment cannot be readily explained at present. MK 212 acts a full agonist at 5-HT_{2C}R, having similar efficacy as 5-HT for inducing 5-HT_{2C}R-mediated inositol phosphate hydrolysis in vitro (Conn and Sanders-Bush, 1987; Cussac et al., 2002b). On the other hand, MK 212 has lower affinity than 5-HT for both edited and unedited isoforms of the 5-HT_{2C}R (Quirk et al., 2001). Furthermore, agonists for the 5-HT_{2C}R have been shown to differentially engage specific 5-HT_{2C}R effector pathways (Berg et al., 1998) and differentially induce desensitization (Stout et al., 2002) and internalization (Schlag et al., 2004) of the 5-HT_{2C}R. Thus, since the mechanisms that underlie the diminution of 5-HT_{2C}R function following each pretreatment regimen are also unknown, it is plausible that variations in the nature of 5-HT_{2C}R regulation that occurred in response to each pretreatment may have resulted in differing abilities of the 5-HT_{2C}R to induce downstream effects. It should also be noted that the repeated MK 212 pretreatment induced a greater loss of 5-HT_{2C}R function than that induced by (+)-MDMA [i.e., MK 212-induced hypomotility was reduced by 50% in MK 212-pretreated rats versus a 25% reduction in (+)-MDMA-treated rats]. Thus, since the maintenance of sensitized-like

response was observed in the group of rats with greater loss of 5-HT_{2C}R responsiveness, this could indicate that a threshold of diminished 5-HT_{2C}R responsivity must be reached in order for the downstream adaptations to be initiated. Regardless of the mechanisms that underlie this discrepancy, the fact that the persistent enhancement of (+)-MDMA-evoked hyperactivity was induced by pretreatment with a preferential 5-HT_{2C}R agonist (Forbes et al., 1993) suggests that the mechanisms involved in the maintenance of the sensitization-like response in MK 212-pretreated rats must in some way be linked to the 5-HT_{2C}R.

An unexpected observation of the present study is the presence of increased 5-HT_{2C}R protein expression in the VTA at the 2 wk withdrawal time-point. Current evidence suggests that the 5-HT_{2C}R acts to decrease activation of DA mesocorticoaccumbens neurons and decrease DA release in the NAc (Higgins and Fletcher, 2003). Thus an up-regulation of 5-HT_{2C}R in the VTA would be expected to enhance the inhibitory influence of 5-HT_{2C}R upon DA mesocorticoaccumbens neurotransmission thereby further inhibiting (+)-MDMA-evoked hyperactivity. In contrast, the present study revealed an enhancement of (+)-MDMA-evoked hyperactivity in conjunction with an up-regulation of 5-HT_{2C}R. Although the nature of this discrepancy is not understood at this time, some evidence suggests that it may be related to the distribution of 5-HT_{2C}R within the VTA. The currently accepted hypothesis, based upon electrophysiology (Di Giovanni et al., 1999; Di Matteo et al., 2001) and *in situ* hybridization studies (Eberle-Wang et al., 1997), suggests that the inhibitory influence of 5-HT_{2C}R upon VTA DA neuron firing is mediated indirectly via 5-HT_{2C}R located on GABA neurons within (and/or outside of) the VTA. Thus, stimulation of 5-HT_{2C}R causes a depolarization of inhibitory GABA neurons (Di Giovanni et al., 2001) that synapse on DA neurons in the VTA (Johnson and North, 1992), reducing the firing rate of VTA DA neurons (Di Matteo et al., 1998). This hypothesis is also supported by recent evidence that infusion of the 5-$HT_{2C/2B}$R antagonist SB 206553 into the VTA simultaneously reduced (±)-MDMA-evoked GABA release in the VTA and enhanced (±)-MDMA-

evoked DA release in the NAc (Bankson and Yamamoto, 2004). However, a recent study from our laboratory suggests that 5-HT_{2C}R may be located on DA neurons in the VTA in addition to GABA neurons (See Chapters 4 and 5; Bubar and Cunningham, 2003b), implying that the 5-HT_{2C}R may also exert direct influence upon VTA DA neurons. In support of this evidence, systemic administration of a 5-HT_{2C}R antagonist has also been reported to reduce the firing rate of spontaneously active VTA DA neurons (Blackburn et al., 2002). Therefore, although purely speculative, it is plausible that the repeated 5-HT_{2C}R agonist pretreatment caused a change in distribution of 5-HT_{2C}R in the VTA, inducing a greater increase in expression of 5-HT_{2C}R located on DA neurons than on GABA neurons in the VTA. Under these circumstances, stimulation of 5-HT_{2C}R located directly on DA neurons by 5-HT release via (+)-MDMA challenge may prevail over the indirect inhibitory effects of 5-HT_{2C}R located on GABA neurons, resulting in an enhancement of (+)-MDMA-evoked DA release and, likewise, (+)-MDMA-evoked hyperactivity.

If these mechanisms are operational, then stimulation of the 5-HT_{2C}R following administration of an agonist might be expected to induce locomotor hyperactivity, an effect that is not observed with MK 212 or other 5-HT_{2C}R agonists (present study; Fone et al., 1998; Vickers et al., 2000). Thus, 5-HT_{2C}R stimulation alone is not sufficient to induce hyperactivity. Rather, it may be that, under conditions of repeated stimulation, the inhibitory actions of 5-HT_{2C}R on VTA DA neurons play a less dominant modulatory role, enabling an "un-masking" of the stimulatory role for 5-HT_{1B}R and 5-HT_{2A}R in (+)-MDMA-evoked DA release and locomotor hyperactivity. Such un-masking of the 5-HT_{1B}R and 5-HT_{2A}R influence on (+)-MDMA-evoked hyperactivity has previously been demonstrated following blockade of 5-HT_{2C}R with SB 206553 (Bankson and Cunningham, 2002). Thus, it is plausible that the up-regulation of the 5-HT_{2C}R in the VTA induced during withdrawal from MK 212 pretreatment altered the influence of the 5-HT_{2C}R upon VTA DA neurons such that the 5-HT_{2C}R began to work cooperatively with 5-HT_{1B}R and 5-HT_{2A}R to stimulate locomotor activity, rather than oppositionally as

typically observed (Bankson and Cunningham, 2001). Further examination, however, is necessary to fully understand the nature and outcomes of the up-regulation of 5-HT_{2C}R expression in the VTA observed in the present study following MK 212 pretreatment.

In summary, the results from the present study reveal that a repeated 5-HT_{2C}R agonist pretreatment, which is associated with a transient loss in 5-HT_{2C}R functional responsiveness, induces a persistent sensitization-like enhancement of (+)-MDMA-evoked hyperactivity. Although the transient loss in 5-HT_{2C}R responsiveness was not associated with reduced 5-HT_{2C}R protein expression within the DA mesocorticoaccumbens circuit, an unexpected up-regulation of 5-HT_{2C}R protein expression developed over time during withdrawal which could potentially contribute to the persistence of enhanced hyperactivity in response to (+)-MDMA challenge. Further examination into the association of 5-HT_{2C}R up-regulation in the VTA and enhancement of (+)-MDMA-evoked hyperactivity will not only improve our understanding of 5-HT_{2C}R mediation of DA neurotransmission and locomotor activity, but may also provide insight into potential mechanisms involved in sensitization to MDMA.

CHAPTER 4:

DISTRIBUTION OF 5-HT_{2C} RECEPTORS IN THE VENTRAL TEGMENTAL AREA

INTRODUCTION

The 5-HT_{2C}R is known to exert inhibitory influence upon DA neurotransmission (Di Giovanni et al., 2002), and likewise, stimulation of the 5-HT_{2C}R inhibits psychostimulant-evoked behaviors thought to be mediated through the DA system (Bankson and Cunningham, 2002; Fletcher et al., 2002; Filip et al., 2004; Fletcher et al., 2004). Conversely, the closely related 5-HT_{2A}R seems to play a stimulatory role in DA neurotransmission (Lucas and Spampinato, 2000) and psychostimulant-evoked behaviors (Bankson and Cunningham, 2002; Fletcher et al., 2002; Filip et al., 2004), thereby opposing the effects of 5-HT_{2C}R. The 5-HT_{2A}R and 5-HT_{2C}R are highly homologous (Julius et al., 1990), and both receptors primarily couple to the $G\alpha_{q/11}$-protein resulting in stimulation of phospholipase C and intracellular Ca^{++} release (Hoyer et al., 2002), although the ability of these receptors to couple to other G-proteins has been reported (Berg et al., 1998; Cussac et al., 2002a). In addition, both receptors are prominently expressed in the nuclei associated with the DA mesocorticoaccumbens pathways, including the VTA, NAc, and PFC (Pompeiano et al., 1994). Thus, the mechanisms by which these receptors exert their oppositional effects are not readily apparent.

Studies employing microinfusion of 5-HT_{2C}R or 5-HT_{2A}R ligands into specific brain areas have reported that 5-HT_{2C}R in the VTA (Fletcher et al., 2004, but see McMahon et al., 2001), NAc (McMahon et al., 2001; Filip and Cunningham, 2002), and PFC (Filip and Cunningham, 2003), and 5-HT_{2A}R in the VTA (McMahon et al., 2001) all influence cocaine-evoked locomotor activity, without significantly influencing spontaneous activity. In particular, microinfusion of the selective 5-HT_{2C}R agonist RO

60,0195 (Fletcher et al., 2004) or the selective 5-HT_{2A}R antagonist M100907 (McMahon et al., 2001) into the VTA were both shown to decrease cocaine-evoked hyperactivity, although no effect of 5-HT_{2C}R antagonist microinfusion into the VTA was also reported (McMahon et al., 2001). These studies indicate that oppositional effects of 5-HT_{2A}R and 5-HT_{2C}R on DA mesocorticoaccumbens pathway activation and psychostimulant-evoked behaviors may be mediated, in part, through 5-HT_{2A}R and 5-HT_{2C}R located in the VTA.

The VTA is structurally defined by the distribution of DA cell bodies within the region as part of the A10 mesencephalic catecholamine cell population (Dahlstrom and Fuxe, 1964). A population of GABA neurons is also present within this region, although there have been conflicting reports on the relative size of GABA neuron population in the VTA (Yim and Mogenson, 1980; Johnson and North, 1992). In addition, VTA GABA neurons appear to contain collaterals which synapse on DA neurons within the VTA as well as projections that terminate in both the NAc (Van Bockstaele and Pickel, 1995) and/or PFC (Steffensen et al., 1998; Carr and Sesack, 2000a). The VTA extends from the level of the supramammillary bodies caudally to the most caudal pole of the caudal linear raphe nucleus at the level of the pontine nucleus (Swanson, 1982; German and Manaye, 1993) and has been divided, based upon cytoarchitecture, into five subnuclei (see **Fig. 10**): the paranigral nucleus (PN), the parabrachial pigmented nucleus (PBP), the intrafascicular nucleus (IF), the rostral linear raphe nucleus (Rli), and the caudal linear raphe nucleus (CLi). The cell morphology of the DA neurons and their projections to forebrain sites have been shown to differ among the subnuclei (Phillipson, 1979b; Swanson, 1982) thereby adding to the complexity of the VTA. Unfortunately, to our knowledge, there are no reports describing the characteristics of GABA cell populations within the different subnuclei.

Thus, although the 5-HT_{2A}R and 5-HT_{2C}R are both localized within the VTA, the particular distribution of these receptors within different subnuclei and on different subpopulations of neurons may greatly impact the influence of these receptors on the output of VTA DA neurons. Several researchers have examined the localization of 5-

$HT_{2A}R$ in the VTA, revealing that these receptors are localized on subsets of DA (and non-DA) neurons within this region (Ikemoto et al., 2000; Doherty and Pickel, 2000; Nocjar et al., 2002). A recent study by Nocjar and colleagues (2002) demonstrated that the greatest co-localization of 5-$HT_{2A}R$ within DA neurons occurred in the PN, PBP, and IF, subnuclei which comprise the largest population of VTA neuron efferents that innervate the NAc and PFC (Swanson, 1982). Thus stimulation of 5-$HT_{2A}R$ receptors on these neurons would likely induce depolarization of DA neurons and likewise enhance DA release in the NAc. On the other hand, researchers have hypothesized that 5-$HT_{2C}R$ are localized on GABA inhibitory neurons in the VTA, rather than DA neurons thereby indirectly influencing activity of DA neurons. In this circumstance, stimulation of 5-$HT_{2C}R$ would induce depolarization of GABA neurons that synapse on DA neurons which, in turn, act to hyperpolarize DA neurons and reduce DA release. Indeed, Eberle-Wang and colleagues (Eberle-Wang et al., 1997) reported that although 5-$HT_{2C}R$ mRNA was detected in the VTA, they did not observe co-localization of 5-$HT_{2C}R$ mRNA with mRNA for tyrosine hydroxylase (TH), the rate limiting enzyme for DA synthesis, and thus a marker for DA neurons. However, to our knowledge, there are no studies demonstrating 5-$HT_{2C}R$ mRNA or protein in GABA neurons in the VTA, or confirming that 5-$HT_{2C}R$ protein is not co-localized within DA neurons in the VTA.

Thus, the goal of the present set of experiments was to examine the distribution of 5-$HT_{2C}R$ within VTA neurons, with the hypothesis that 5-$HT_{2C}R$ would be located on GABA neurons, but not DA neurons, in this region. To accomplish this goal, we sought to perform a detailed analysis of double-label fluorescence immunohistochemistry for 5-$HT_{2C}R$ and glutamic acid decarboxylase (GAD), the rate limiting enzyme for GABA synthesis and a marker for GABA neurons, as well as for 5-$HT_{2C}R$ and TH in VTA tissue sections obtained from naive male Sprague-Dawley rats. Unfortunately, none of the commercially available anti-5-$HT_{2C}R$ antibodies have been extensively utilized in standard immunohistochemical procedures, thus the effectiveness and specificity of these antibodies for 5-$HT_{2C}R$ is unknown. As such, prior to initiating the detailed VTA

localization studies, we analyzed the specificity of two different commercially available anti-5-HT_{2C}R antibodies: a mouse monoclonal anti-5-HT_{2C}R antibody that is directed toward amino acids 384-459 of the C-terminal end of the 5-HT_{2C}R (PH 5-HT_{2C}R; BD PharMingen, San Diego, CA), and a goat polyclonal anti-5-HT_{2C}R antibody that is directed toward a 19 amino acid sequence at the N-terminus of the receptor (SC 5-HT_{2C}R; Santa Cruz Biotechnology, Santa Cruz, CA). While both of these antibodies have demonstrated ability to detect selective changes in 5-HT_{2C}R protein expression in the VTA to a similar extent via Western blot analysis (see Chapter 3), to our knowledge, only the PH 5-HT_{2C}R antibody been exploited for immunohistochemical analysis in the brain (Li et al., 2004). To assess the specificity of the 5-HT_{2C}R antibodies, we first conducted a double-label immunohistochemistry experiment combining the two 5-HT_{2C}R antibodies and compared their patterns of immunoreactivity (IR). Second, we compared 5-HT_{2C}R and 5-HT_{2A}R immunoreactivity in 5-HT_{2C}R knockout (KO) mice and their wildtype (WT) littermates to show that 5-HT_{2C}R immunoreactivity is eliminated in 5-HT_{2C}R KO mice, while 5-HT_{2A}R immunoreactivity is unaltered. Finally, in order to show that the 5-HT_{2C}R antibody does not bind to the 5-HT_{2A}R, we examined 5-HT_{2C}R and 5-HT_{2A}R immunoreactivity in Chinese hamster ovary (CHO) cells, which do not normally contain 5-HT_{2C}R or 5-HT_{2A}R, and CHO cells transfected with the 5-HT_{2A}R.

METHODS

Tissue Preparation

Naïve male Sprague-Dawley rats (N = 6; virus antibody-free; Harlan Sprague-Dawley, Inc., Houston, TX) were used. Rats were deeply anesthetized with pentobarbital (100 mg/kg, IP, Sigma) and perfused transcardially with phosphate

buffered saline (PBS) followed by 3% paraformaldehyde in PBS. Brains were removed, blocked at mid-pons, post-fixed for 2 h at room temperature (RT). Brains were then cryoprotected in 30% sucrose for 48 h at 4^{o}C, rapidly frozen on crushed dry ice, and stored at –80^{o}C until sectioning. All experiments conformed to the *NIH Guide for the Care and Use of Laboratory Animals* (National Institutes of Health, 1986) and were approved by the UTMB Institutional Animal Care and Use Committee.

In addition, frozen brains from the 5-HT$_{2C}$R knockout mouse (KO) on a C57B1/6J background and wild-type (WT) littermates (n = 2/genotype) that had been previously fixed (4% paraformaldehyde/PBS) and cryoprotected (30% sucrose) were obtained from the laboratory of Dr. Lawerence Tecott at the University of California at San Francisco. Details on the production of these mutant mice can be found elsewhere (Tecott et al., 1995).

Coronal sections (20 μm) containing the VTA (-4.8 through -6.5 from Bregma) were taken from all brains using a cryostat (Leica CM 1850 at 20 ^{o}C) according to the atlas of Paxinos and Watson (Paxinos and Watson, 1998). Free floating sections were processed as described below.

Cell Culture and Transfection

Chinese hamster ovary (CHO) K1 cells (obtained from the laboratories of Drs. Kelly Berg and William Clarke at the University of Texas Health Science Center, San Antonio, TX) were plated onto 4-well Lab-Tek chamber slides (Nalge Nunc, Naperville, IL) and maintained in α-MEM medium (supplemented with 5% fetal bovine cells and antibiotics). Cells were transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions with a plasmid containing the entire coding region of the rat 5-HT$_{2A}$R linked to a 24-base sequence coding for a synthetic marker, Flag peptide (CHO/5-HT$_{2A}$R). Cells were incubated at 37°C for 48 hours, then rinsed with PBS and fixed with 4% paraformaldehyde in PBS. Immunocytochemistry was performed as described below.

Immunohistochemistry

The primary and secondary antibodies utilized in the present studies are described in **Table 2**. Single- and double-label immunohistochemistry experiments employing several different antibody combinations were performed as described in **Table 3** to determine: **(1).** 5-HT_{2C}R antibody specificity employing rat brains, 5-HT_{2C}R KO and WT mice brains, and the CHO and CHO/5-HT_{2A}R transfected cells, and **(2).** the distribution of 5-HT_{2C}R on different subtypes of cells in the VTA from rat brains.

Free floating rat or mouse brain sections were washed using an orbital shaker in PBS (2 x 10 min), then incubated in 20 mM sodium acetate (15 min), and washed again (3 x 10 min) with PBS. The sections were then incubated in a blocking serum [1.5% normal donkey serum (ImmunoResearch Laboratories, Inc., West Grove, PA) or goat serum (Vector Laboratories, Burlingame, CA) in PBS] for one hour at room temperature (RT). The blocking serum was aspirated, and the sections were then incubated on an orbital shaker with primary antibodies (see **Tables 2, 3**) diluted in 1.5% normal donkey or goat serum for 44h at 4°C. The sections were then washed with PBS and incubated with the secondary antibodies (see **Tables 2, 3**) diluted in 1.5% normal donkey or goat serum for 1h RT, protected from light. Sections were washed with PBS (3 x 10 min) and mounted using a 0.1% Dreft® solution onto gelatin chrom alum-coated slides. The slides were then coverslipped using Vectashield® fluorescent mounting medium with DAPI (4',6-diamidoino-2-phenylindole; Vector Laboratories), and stored protected from light at 4°C until viewing.

CHO cells previously plated onto Lab-Tek chamber slides were treated analogously with the exception of a shorter primary antibody incubation time (2 h, RT). Following the final PBS washes, chambers were detached from the slides, and the slides were coverslipped using the Vectashield® fluorescent mounting medium with DAPI.

Table 2: List of antibodies employed in the experiments					
Primary Antibody	**Dilution**	**Species**	**Catalog #**	**Supplier**	**Location**
anti-SC 5-HT $_{2C}$ R	1:100	goat	sc-15081	Santa Cruz Biotechnology	Santa Cruz, CA
anti-PH 5-HT $_{2C}$ R	1:300	mouse	556335	BD PharMingen	San Diego, CA
anti-5-HT $_{2A}$ R	1:500	rabbit	----	Dr. Bryan Roth, Case Western Reserve University	Cleveland, Ohio
anti-Flag	1:1000	mouse	F-3165	Sigma	St. Louis, MO
anti-Flag	1:1000	rabbit	F-7425	Sigma	St. Louis, MO
anti-TH	1:3000	mouse	22941	Immunostar	Hudson, WI
anti-GAD 67	1:150	mouse	sc-5602	Santa Cruz Biotechnology	Santa Cruz, CA
Secondary Antibody	**Dilution**	**Fluorescent Dye[a]**	**Catalog #**	**Supplier**	**Location**
donkey anti-goat	1:2000	Alexa Fluor 488	A-11055	Molecular Probes	Eugene, OR
donkey anti-mouse	1:2000	Alexa Fluor 555	A-31570	Molecular Probes	Eugene, OR
donkey anti-rabbit	1:2000	Alexa Fluor 555	A-31572	Molecular Probes	Eugene, OR
goat anti-mouse	1:2000	Alexa Fluor 488	A-11029	Molecular Probes	Eugene, OR
goat anti-rabbit	1:2000	Alexa Fluor 555	A-21429	Molecular Probes	Eugene, OR
[a] Alexa Fluor 488 dye appears green; Alexa Fluor 555 dye appears red					

Table 3: Experimental design[a]			
Experiment Description	**Primary Antibody**	**Secondary Antibody[b]**	**Cell/tissue Tested**
1. 5-HT $_{2C}$ R Antibody specificity	SC 5-HT$_{2C}$R + PH 5-HT$_{2C}$R	donkey anti-goat 488 + donkey anti-mouse 555	rat
	SC 5-HT$_{2C}$R	donkey anti-goat 488	WT & 5-HT$_{2C}$R KO mice
	5-HT$_{2A}$R	donkey anti-rabbit 488	WT & 5-HT$_{2C}$R KO mice
	SC 5-HT$_{2C}$R + 5-HT$_{2A}$R	donkey anti-goat 488 + donkey anti-rabbit 488	CHO & CHO/5-HT$_{2A}$R cells
	SC 5-HT$_{2C}$R + Flag	donkey anti-goat 488 + donkey anti-rabbit 488	CHO & CHO/5-HT$_{2A}$R cells
	anti-5-HT$_{2A}$R + Flag	goat anti-rabbit + goat anti-mouse	CHO & CHO/5-HT$_{2A}$R cells
2. 5-HT $_{2C}$ R distribution in the VTA	SC 5-HT$_{2C}$R + TH	donkey anti-goat 488 + donkey anti-mouse 555	rat
	SC 5-HT$_{2C}$R + GAD	donkey anti-goat 488 + donkey anti-rabbit 488	rat

[a] No antibody, single antibody, and mis-matched primary and secondary controls were also run for each antibody combination

[b] 488 = Alexa Fluor 488, appears green; 555 = Alexa Fluor 555, appears red

Image Analysis

Digital images were captured from brain sections and CHO cells using an Olympus BX51 fluorescent microscope (Olympus, Melville, NY) equipped with a Hamamatsu camera (Hamamatsu, Bridgewater, NJ) interfaced to a personal computer and were analyzed using Simple PCI software (version 5.1, Compix Inc., Imaging Systems, Cranberry Township, PA). A 20x or 40x objective was used to capture all photomicrographs for final magnification of 400 and 800, respectively. Green fluorescence emitted by the Alexa Fluor 488 antibodies was visualized using a yellow GFP filter set (# 41017; Chroma Technology Corporation, Rockingham, VT), while the red fluorescence emitted by the Alexa Fluor 555 antibodies was visualized using a narrow band green excitation filter set (U-MNG2, Olympus). In addition, DAPI staining was visualized using a Blue GFP II filter set (#31041, Chroma). For each section three images of the same viewing area were captured, one for each filter set detecting immunoreactivity (IR) for each antibody and DAPI, and then resultant images were overlaid.

Rostral-caudal patterns of 5-HT_{2C}R distribution on TH-immuoreactive (IR) and GAD-IR cells in the VTA were analyzed for the five subnuclei of the VTA: paranigral nucleus (PN), parabrachial pigmented nucleus (PBP), intrafascicular nucleus (IF), rostral linear raphe nucleus (RLi), and caudal linear raphe nucleus (CLi; Swanson, 1982), according to Bregma locations described in **Fig. 10** and **Table 2** (Paxinos and Watson, 1998). Two to five 5-HT_{2C}R/TH-labeled sections and adjacent (when possible) 5-HT_{2C}R/GAD-labeled sections per rostral/caudal level were examined from each rat (n = 3). For each section, a composite photomicrograph comprised of 20-30 individual images captured using the 20x objective was assembled to visualize the entire VTA. In an effort to minimize the differences in the sizes and shapes of the subnuclei throughout the rostral/caudal levels, rectangular areas of a fixed size were utilized to count cells within each subnucleus for all sections at the same magnification, as described in **Fig. 10**. Cells contained in each rectangular area were counted using the "Region of Interest" function

of the Simple PCI software, which enabled selection of immunoreactive cells according to the intensity and area of each signal. First, the number of single-labeled cells was counted for each different color label (red and green), then the same parameters used for each single label were applied to the overlay image to determine the number of double-labeled cells. The total number of 5-HT_{2C}R-IR, TH- IR, and GAD- IR cells was averaged (mean ± SEM) for rostral, middle, and caudal levels of each subnucleus. Additionally, for each section, the percentage of 5-HT_{2C}R/TH-IR or 5-HT_{2C}R/GAD-IR co-labeled cells was determined by averaging the number of 5-HT_{2C}R/TH-IR or 5-HT_{2C}R/GAD-IR-co-labeled cells divided by the total number of TH-IR or GAD-IR cells, respectively. The resultant values were averaged (mean ± SEM) for rostral, middle, and caudal levels of each subnucleus. Individual one way analysis of variance (ANOVA) tests were utilized to examine differences in the percentages of total TH- or GAD-IR cells co-labeled with 5-HT_{2C}R-IR within: 1. the different subnuclei in each rostral-caudal level, and 2. The different levels of each subnucleus. Significant effects were followed with post hoc analysis using the Student Newman Keuls procedure (Keppel, 1973). Unpaired 2-tailed T-tests were utilized to examine the rostral-caudal differences in IF, RLi, and CLi, since these subnuclei each only contained two levels.

To verify the observation of co-localization of both 5-HT_{2C}R/TH and 5-HT_{2C}R/GAD, double-labeled sections were also viewed using a Zeiss LSM 510 META UV laser scanning confocal microscope in the UTMB Infectious Disease and Toxicology Optical Imaging Core.

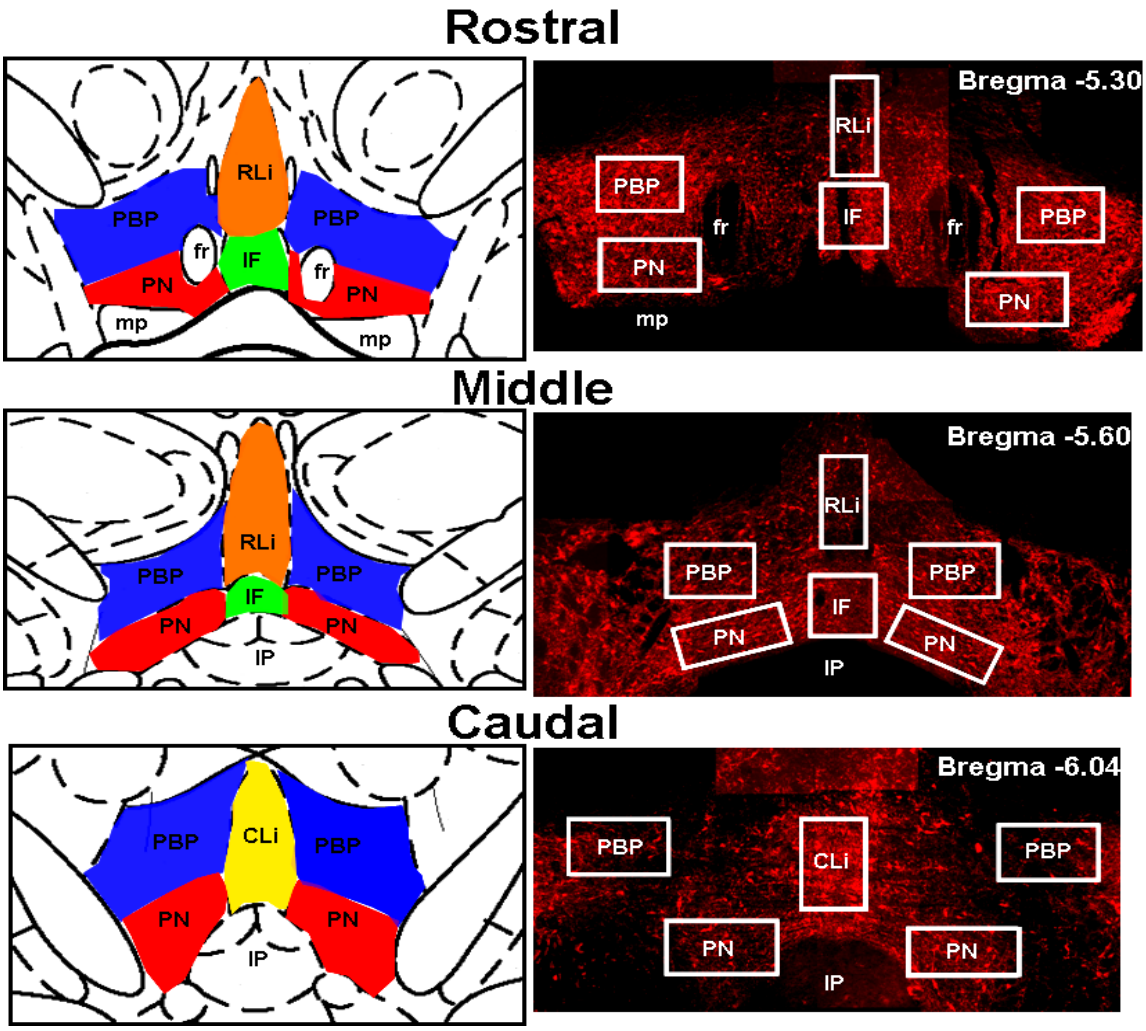

Figure 10. Delineation of the VTA subnuclei. [A,C,E] Schematic diagrams of the VTA subnuclei at rostral [Bregma -5.30 mm; A], middle [Bregma -5.60 mm; C], and caudal [Bregma -6.04 mm; C] levels of the VTA. [B,D,F] Representative composite photomicrographs displaying TH immunoreactivity (red) in rostral [B], middle [D] and caudal [F] levels of the VTA. White boxes represent the fixed rectangular areas used for counting immunoreactive cells. Two separate regions of similar size (64.52 cm2 each) were used in all levels for the paranigral nucleus (PN) and parabrachial nucleus (PBP), while a single region was used for the intrafascicular nucleus (IF; 58.06 cm2), rostral linear raphe nucleus (RLi; 58.06 cm2), and caudal linear raphe nucleus (CLi; 64.52 cm2). The fascicular retroflexus (fr), mammillary peduncle (mp), and interpeduncular nucleus (IP) are also labeled for orientation purposes.

RESULTS

1. 5-HT_{2C}R Antibody Specificity

SC 5-HT_{2C}R vs. PH 5-HT_{2C}R immunoreactivity in Rat VTA Sections

Immunoreactivity for the SC 5-HT_{2C}R antibody in the VTA appeared punctate and was widely distributed throughout the region. The 5-HT_{2C}R- immunoreactivity (IR) tended to form clusters that appeared similar in shape to neuronal cell bodies **(Fig. 11A,E)**. These clusters of immunoreactivity were typically associated with DAPI-labeled nuclei **(Fig. 11D,H)**, suggesting that the 5-HT_{2C}R-IR is localized to cell bodies. In addition, strings of immunoreactivity that took on the appearance of neuronal processes were also present (arrowheads, **Fig. 11A,C**); however this type of IR was not as prominent as the cell body clusters of IR staining.

Immunoreactivity for the PH 5-HT_{2C}R antibody **(Fig. 11B,F)** was also punctate, but much more diffuse than SC 5-HT_{2C}R immunoreactivity. Although similar clusters of IR were evident and found to be associated with DAPI-labeled nuclei, the amount of IR within these clusters appeared much less concentrated than that observed for the SC 5-HT_{2C}R antibody. In addition, double-label experiments conducted with both 5-HT_{2C}R antibodies demonstrated that the majority of the cell body clusters contained IR for both the SC 5-HT_{2C}R antibody and the PH 5-HT_{2C}R antibody **(Fig. 11C,G)**, however labeling for the PH 5-HT_{2C}R was less concentrated and less prominent in within the clusters.

Single-label experiments conducted for each 5-HT_{2C}R antibody demonstrated that the simultaneous application of the two antibodies in double-label experiments did not alter the pattern of staining of either antibody (data not shown). Experiments conducted in the absence of primary or secondary antibody revealed the presence of minimal auto-fluorescence and non-specific secondary antibody labeling (data not shown). This staining was minimal compared to the intense IR that was detected following either anti-5-HT_{2C}R antibody/secondary antibody combination.

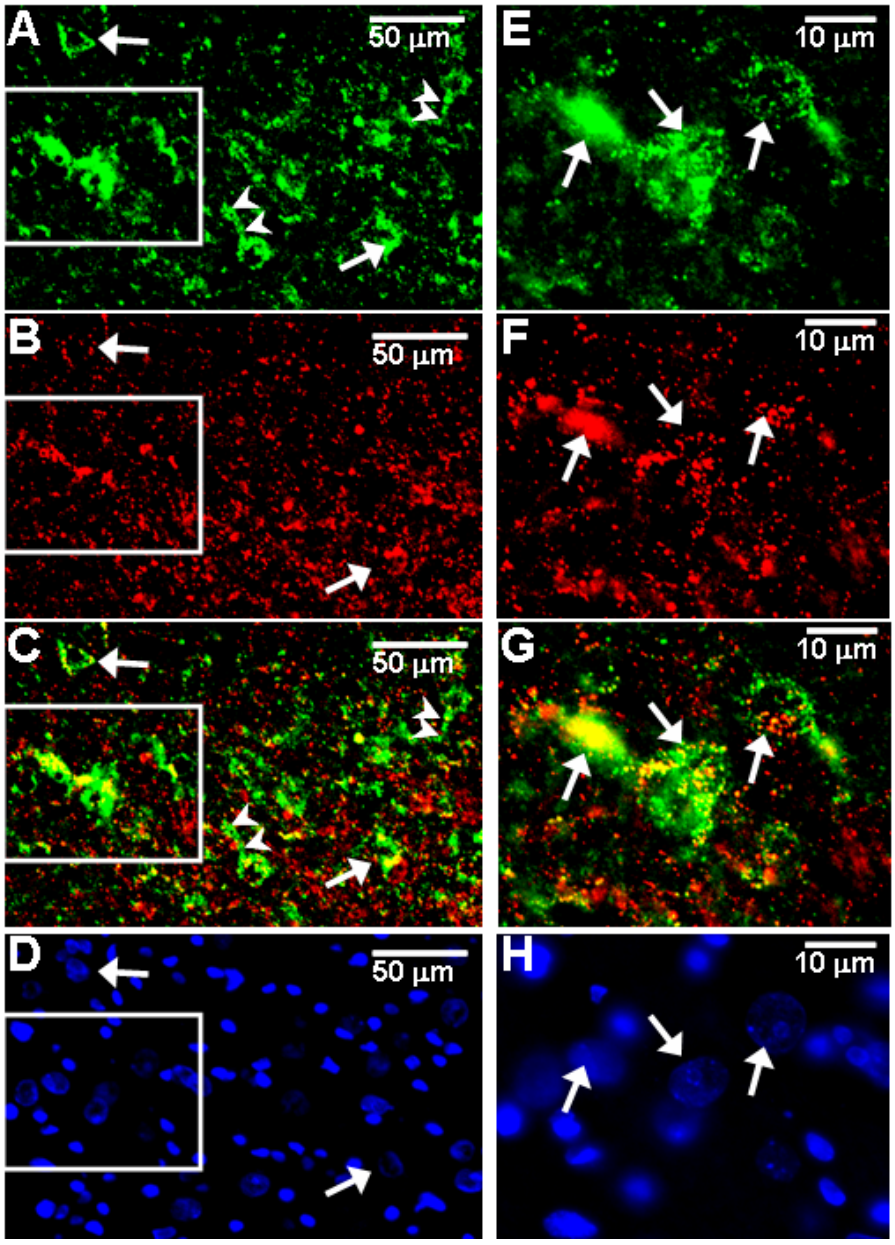

Figure 11. Co-labeling for the SC 5-HT$_{2C}$R and the PH 5-HT$_{2C}$R. Representative photomicrographs displaying immunoreactivity for the SC 5-HT$_{2C}$R antibody [A,E; green], the PH 5-HT$_{2C}$R antibody [B,F; red], the overlay of SC 5-HT$_{2C}$R and PH 5-HT$_{2C}$R images [C,G], and DAPI [D,H; blue] in a 20 µm section of the VTA. Double-labeling in the overlaid image appears yellow. [E-H] High magnification photomicrographs of the boxed area in A-D, respectively. ➞ 5-HT$_{2C}$R-IR cell bodies; ➢ neuronal processes. Scale bars = 50µm [A-D]; 10 µm [E-H].

Similar patterns of staining for each 5-HT_{2C}R antibody were also observed in other brain areas examined [NAc, PFC, septum, striatum (STR), substantia nigra (SN), ventral pallidum (VP); data not shown], however the patterns of IR observed with the SC 5-HT_{2C}R antibody were much more analogous to the type of staining previously described by immunohistochemical analyses conducted using a 5-HT_{2C}R antibody which is not commercially available (Clemett et al., 2000). Thus, since the IR for the SC 5-HT_{2C}R antibody was more concentrated than PH 5-HT_{2C}R-IR and SC 5-HT_{2C}R-IR was more comparable to previous reports of 5-HT_{2C}R antibody staining (Clemett et al., 2000), subsequent studies were conducted using the SC 5-HT_{2C}R antibody.

SC 5-HT_{2C}R and 5-HT_{2A}R Immunoreactivity in the VTA of 5-HT_{2C}R KO and WT Mice

Application of the SC 5-HT_{2C}R resulted in intense IR-staining throughout the rostral/caudal extent of the VTA in WT C57B1/6J mice **(Fig. 12A,C)**. This staining was similar to that observed in rat VTA tissue in that the IR occurred in clusters associated with DAPI-labeled nuclei (**Fig. 12C**), suggesting the presence of 5-HT_{2C}R-IR on cell bodies. Similar patterns of intense staining were also observed throughout other brain regions [NAc, PFC, septum, STR, SN; data not shown].

Immunoreactivity for the 5-HT_{2A}R in the WT C57B1/6J mice also produced intense staining throughout the VTA (**Fig. 12E**). 5-HT_{2A}R-IR was comparable in appearance to IR produced by the SC 5-HT_{2C}R antibody, forming clusters of immunoreactivity surrounding DAPI nuclei (data not show), and was analagous to the 5-HT_{2A}R-IR described for this antibody in the rat VTA (Nocjar et al., 2002).

In the 5-HT_{2C}R KO mice, SC 5-HT_{2C}R antibody IR was virtually undetectable in the VTA of 5-HT_{2C}R KO mice **(Fig. 12B,D)**, with only minor residual IR remaining, despite the detection of similar numbers of DAPI-stained nuclei as observed in WT littermates (**Fig. 12C,D**). A similar lack of 5-HT_{2C}R-IR was also observed in other brain areas examined (NAc, PFC, septum, STR, SN; data not shown). In contrast, the 5-HT_{2A}R antibody produced intense IR in the VTA of 5-HT_{2C}R KO mice **(Fig. 12F)** similar to the

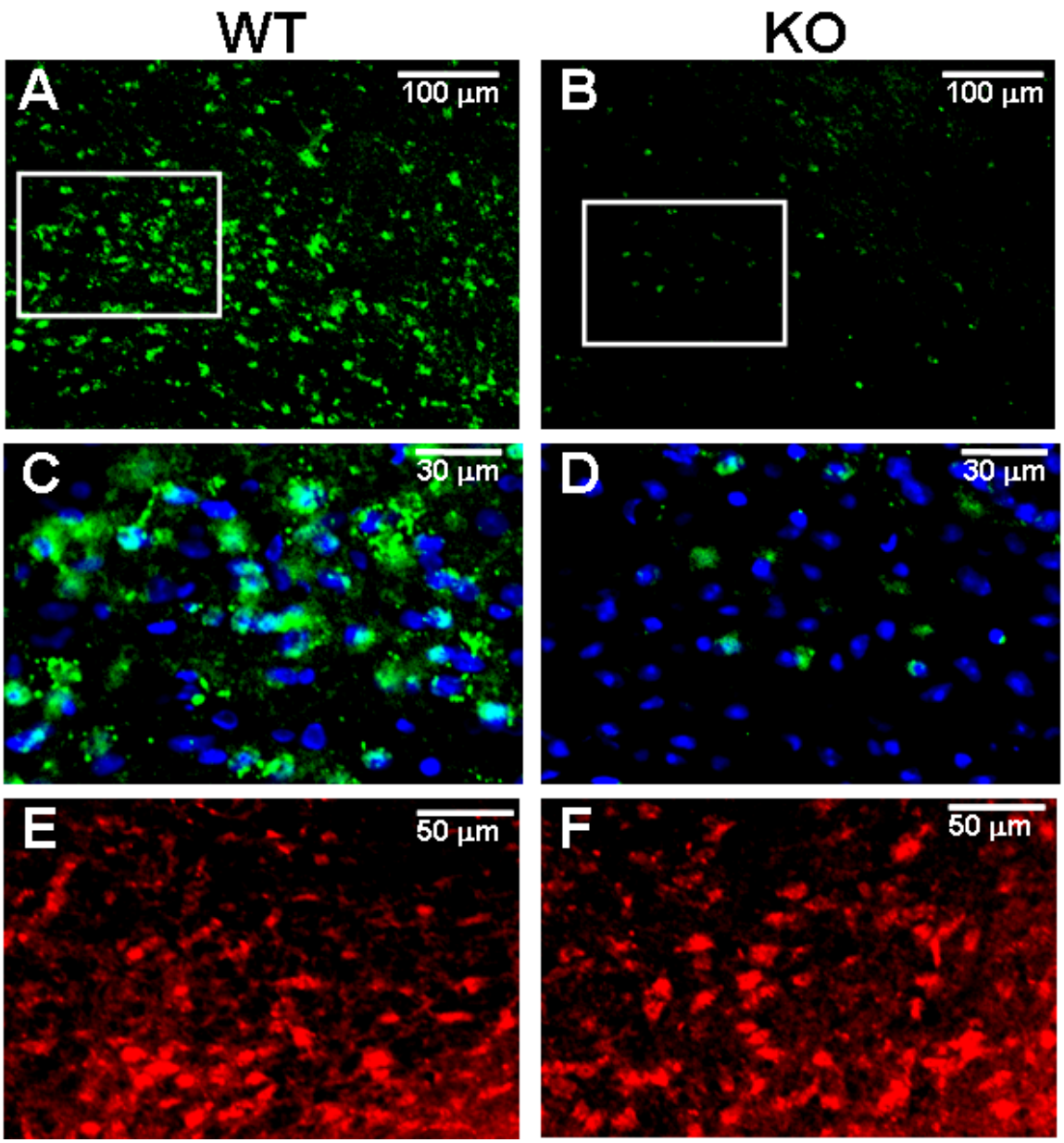

Figure 12. SC 5-HT$_{2C}$R and 5-HT$_{2A}$R immunoreactivity in WT and 5-HT$_{2C}$R KO mice. Representative photomicrographs of SC 5-HT$_{2C}$R [A-D; green] and 5-HT$_{2A}$R immunoreactivity in a 20 µm sections of the VTA from a wildtype mouse [WT; A,C,E] or a 5-HT$_{2C}$R knockout mouse [KO; B,D,F]. [C,D] High magnification photomicrographs of boxed regions in A and B, respectively. DAPI-labeled nuclei [C,D; blue] show the presence of cells, despite the lack of 5-HT$_{2C}$R staining in the 5-HT$_{2C}$R KO mouse. Scale bars = 100 µm [A-B], 30 µm [C-D], and 50 µm [E,F].

levels of IR detected in the VTA (and other brain regions; data not shown) of the WT littermates (**Fig. 12E**).

Immunohistochemical experiments conducted in the absence of primary or secondary antibodies revealed the presence of some auto-fluorescence and non-specific secondary antibody labeling (data not shown), however, this staining was minimal compared to the intense IR that was detected following either anti-5-HT_{2C}R or anti-5-HT_{2A}R antibody/secondary antibody combinations.

SC 5-HT_{2C}R, 5-HT_{2A}R, and Flag Immunoreactivity in CHO and CHO/5-HT_{2A}R cells

In order to determine whether the SC 5-HT_{2C}R antibody also exhibits cross-reactivity for the closely related 5-HT_{2A}R, IR for the SC 5-HT_{2C}R antibody was examined in CHO cells that were either untransfected or transfected with the 5-HT_{2A}R containing a Flag tag at the C-terminal end of the receptor. In the untransfected CHO cells, there was little to no staining observed following administration of the SC 5-HT_{2C}R (**Fig. 13A**), 5-HT_{2A}R (**Fig. 13B**), or Flag (**Fig. 13C**) antibodies tested separately or in double-label combinations (data not shown). The weak fluorescence that was detected in the untransfected CHO cells following SC 5-HT_{2C}R **(Fig. 13A)** and 5-HT_{2A}R **(Fig. 13B)** antibody incubation was also observed in the absence of the primary antibodies suggesting that the sparse fluorescence detected in the non-transfected CHO cells was due to auto-fluorescence or non-specific binding of the secondary antibodies (data not shown). In addition, this also suggests that, as previously reported, the untransfected CHO cells are devoid of 5-HT_{2C}R or 5-HT_{2A}R (Berg et al., 1994). Conversely, the fluorescence detected in the untransfected CHO cells incubated with the Flag antibody **(Fig. 13C)** was slightly more intense than that observed in the absence of the Flag antibody (data not shown), suggesting that some non-specific binding of the anti-Flag antibodies was evident.

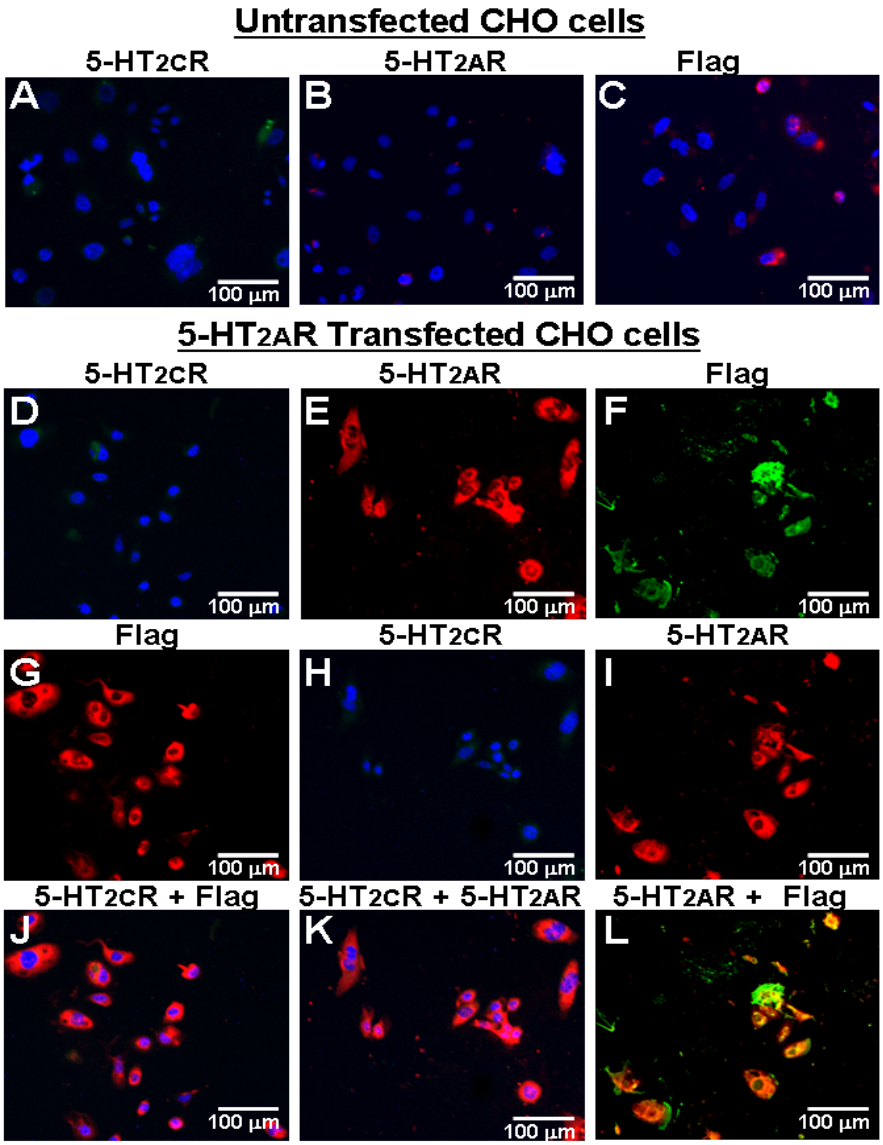

Figure 13. 5-HT$_{2C}$R, 5-HT$_{2A}$R and Flag Immunoreactivity in CHO cells. [A-C] Representative photomicrographs of untransfected CHO cells incubated with antibodies for the 5-HT$_{2C}$R [green; A], 5-HT$_{2A}$R [red, B], and Flag [red, C]. DAPI-stained nuclei (blue) indicate the location of CHO cells. [D-L] Representative photomicrographs of CHO cells transfected with the 5-HT$_{2A}$R containing the Flag tag sequence incubated with the 5-HT$_{2C}$R [green; D,H], 5-HT$_{2A}$R, [red; E,I], and Flag [green, F; red, G] antibodies. DAPI-stained nuclei (blue) are shown in images of 5-HT$_{2C}$R-IR in CHO/5-HT$_{2A}$R cells [D,H] to indicate the location of the CHO cells in the photomicrographs. [J] Overlay of images in D and G. [K] Overlay of images in E and H. [L] Overlay of images in F and I demonstrates double-labeling for 5-HT$_{2A}$R and Flag (yellow). Scale bars = 100 µm.

On the other hand, while IR for the SC 5-HT_{2C}R antibody was also undetectable in the CHO cells transfected with the 5-HT_{2A}R containing the Flag sequence **(Fig. 13D,H),** intense IR for the 5-HT_{2A}R (**Fig. 13E,I**) and Flag (**Fig. 13F,G**) were observed in CHO/5-HT_{2A}R transfected cells. To ensure efficiency of the transfection, double-label studies were conducted for 5-HT_{2C}R + Flag **(Fig. 13J)**, 5-HT_{2C}R + 5-HT_{2A}R **(Fig. 13K)**, and 5-HT_{2A}R + Flag **(Fig. 13L)**. The simultaneous application of two antibodies in each of the double-label experiments did not alter the pattern of staining of any individual antibody (data not shown). As shown in **Fig. 13L**, all cells containing 5-HT_{2A}R IR were co-labeled for Flag, suggesting that the 5-HT_{2A}R staining in these cells was due to the transfection of the 5-HT_{2A}R containing the Flag sequence. In addition, 5-HT_{2C}R-IR was not detected in cells immunoreactive for either Flag (**Fig. 13J**) or the 5-HT_{2A}R (**Fig. 13K**) suggesting that the SC 5-HT_{2C}R antibody does not bind to 5-HT_{2A}R.

2. 5-HT_{2C}R distribution in the VTA

5-HT_{2C}R immunoreactivity in the VTA

Intense 5-HT_{2C}R-IR was observed throughout the rostral (**Figs. 14-15**), middle (**Fig. 16**), and caudal levels (**Fig. 17**) of all subnuclei in the VTA. The 5-HT_{2C}R-IR in each subnucleus was most prominently expressed in cells bodies, although labeling of neuronal processes was also detected (see arrowheads **Figs. 14A, 15A**), albeit less frequently, particularly within the PBP, RLi, and CLi. Furthermore, tiny clusters of 5-HT_{2C}R-IR much smaller in diameter than the cell bodies were also observed (see open arrows, **Figs. 14C, 15C**). These small clusters, which may be indicative of terminal boutons, were most commonly present within the IF, RLi and CLi. Although all three patterns of staining were present throughout the VTA, analysis was focused upon the detection of 5-HT_{2C}R-IR in cell bodies, as this type of staining could be most easily and consistently detected and quantified.

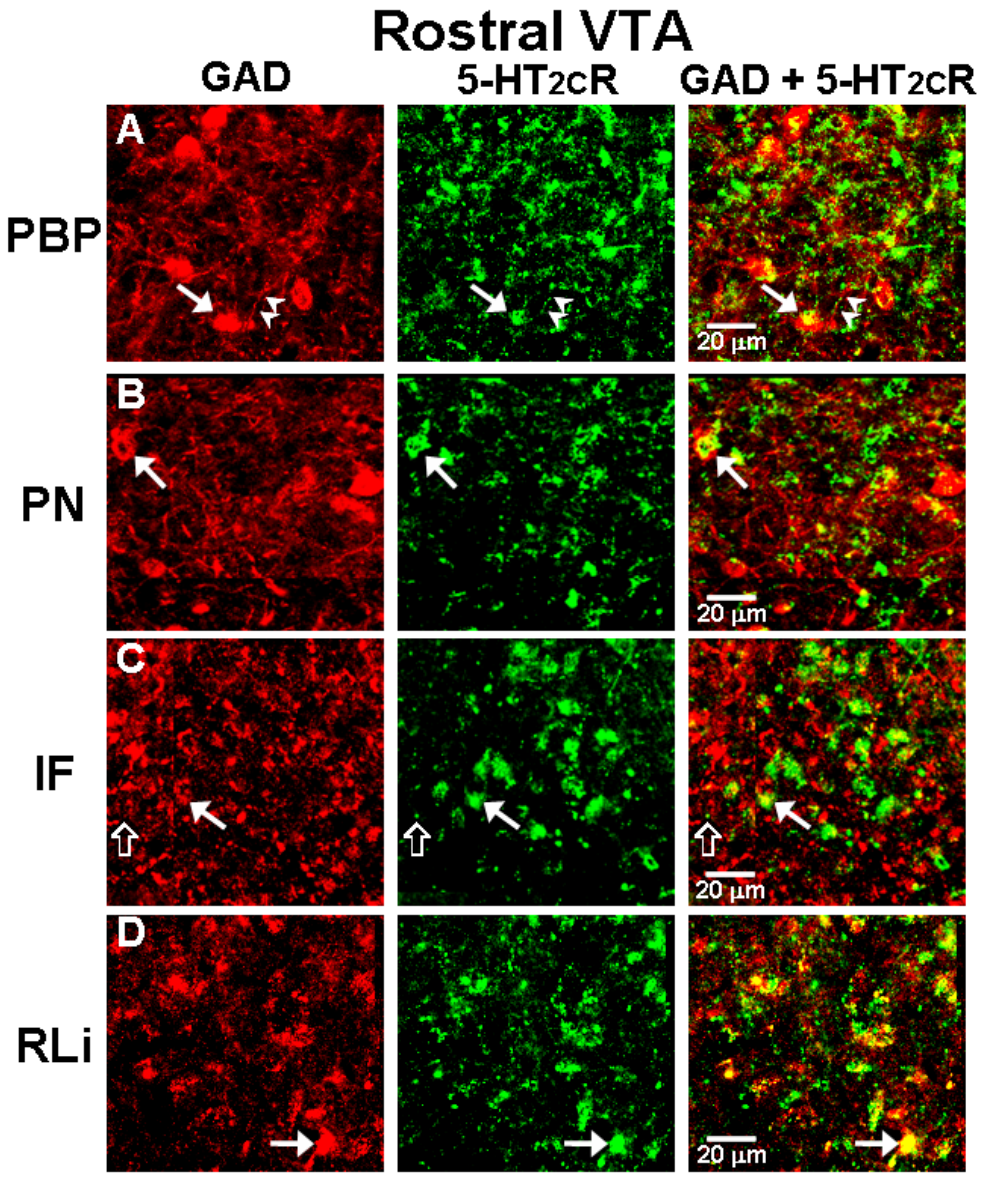

Figure 14. Co-localization of 5-HT_{2C}R and GAD immunoreactivity in the Rostral VTA. Representative photomicrographs displaying GAD-IR (red; left column) and 5-HT_{2C}R-IR (green, middle column) in the rostral levels of the PBP [A], PN [B], IF [C], and RLi [D]. Overlay of GAD (left) and 5-HT_{2C}R (middle) images indicate cells which contain immunoreactivity for both GAD and 5-HT_{2C}R (yellow, right column). ➞ GAD + 5-HT_{2C}R double-labeled cells; ⇨ potential terminal boutons; ➢ neuronal processes; Scale bar = 20 µm.

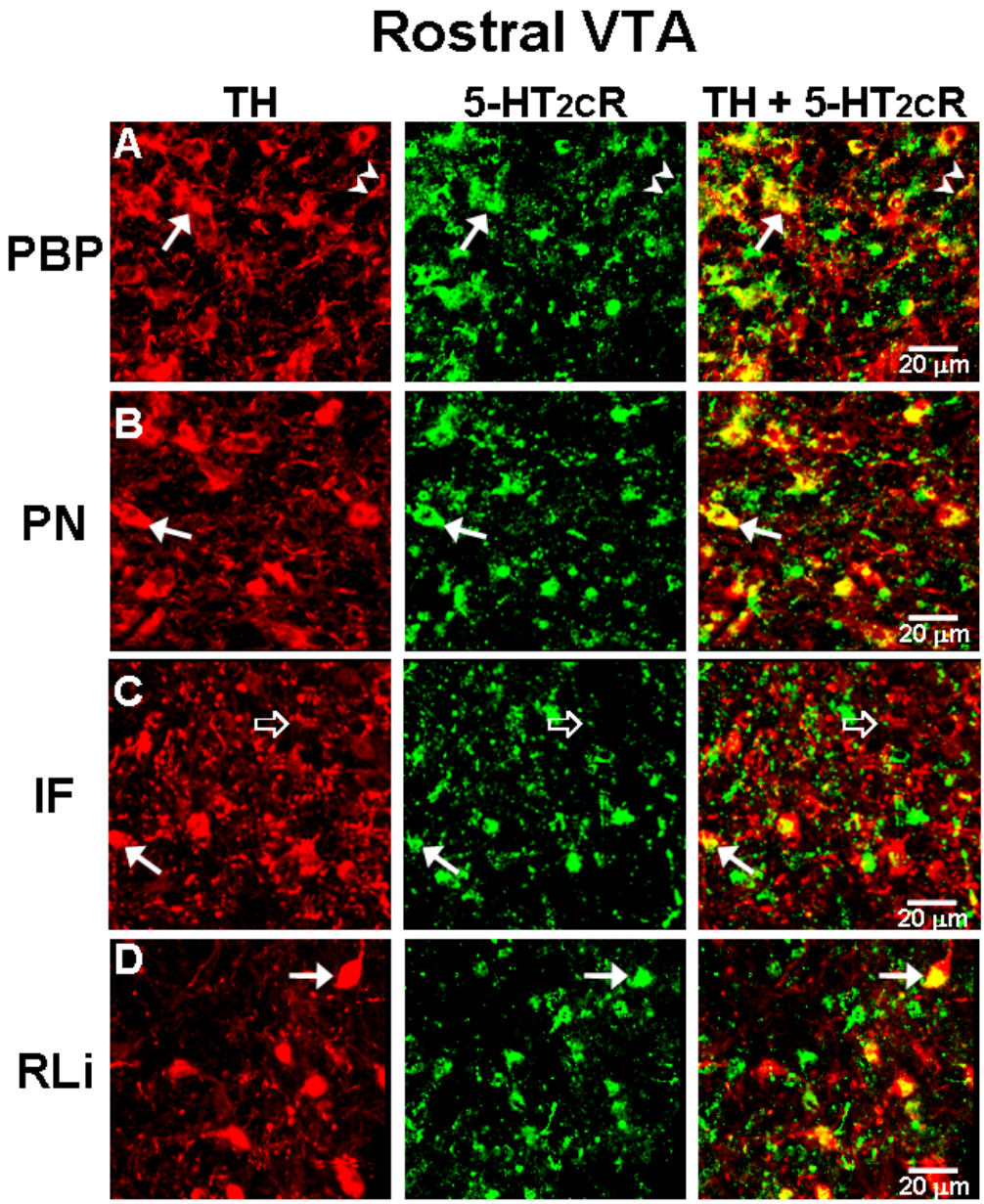

Figure 15. Co-localization of 5-HT_{2C}R and TH immunoreactivity in the Rostral VTA. Representative photomicrographs displaying TH-IR (red; left column) and 5-HT_{2C}R-IR (green, middle column) in the rostral levels of the PBP [A], PN [B], IF [C], and RLi [D]. Overlay of TH (left) and 5-HT_{2C}R (middle) images indicate cells which contain immunoreactivity for both TH and 5-HT_{2C}R (yellow, right column). ➞ TH + 5-HT_{2C}R double-labeled cells; ⇨ potential terminal boutons; ➢ neuronal processes; Scale bar = 20 µm.

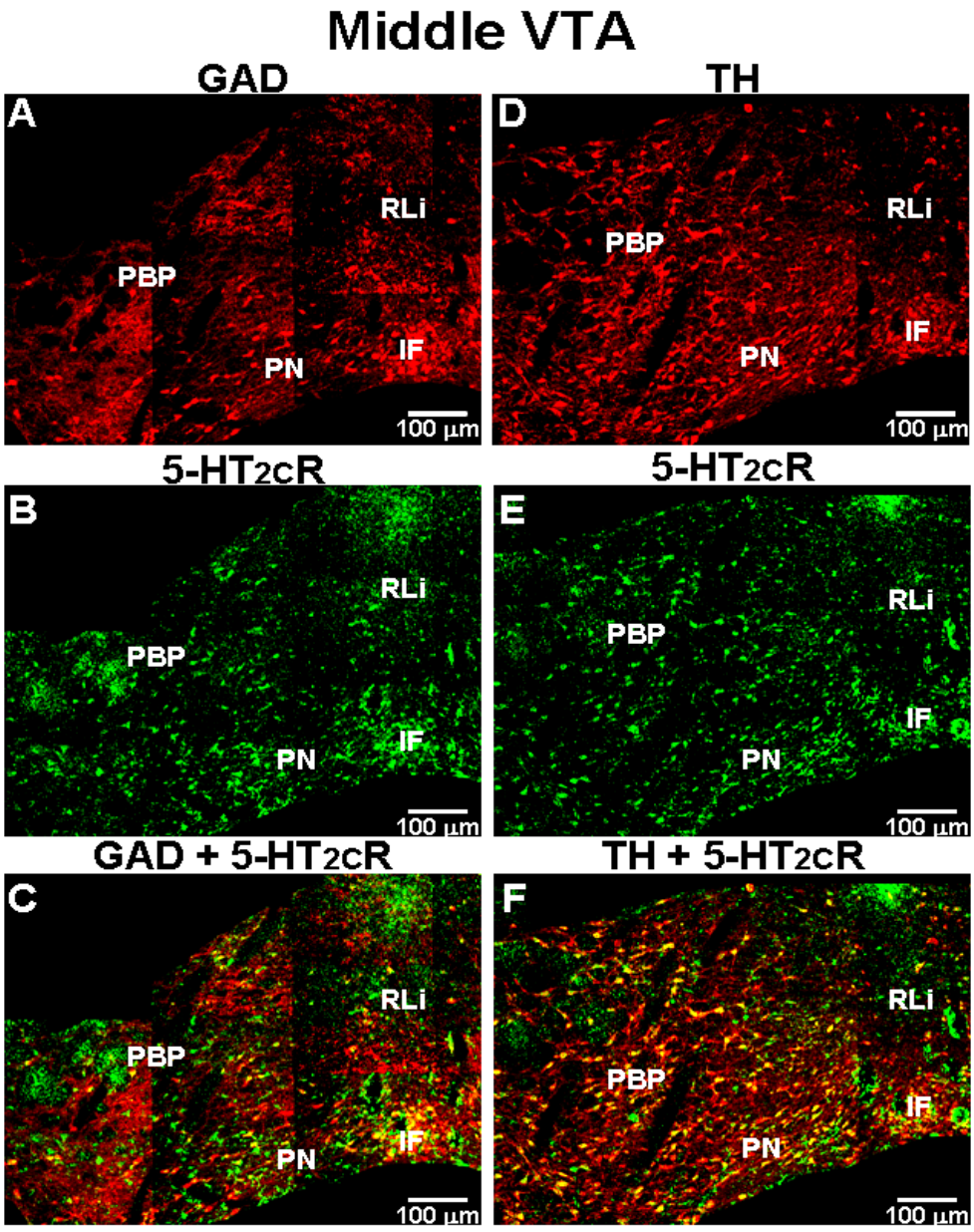

Figure 16. Co-localization of 5-HT$_{2C}$R/GAD and 5-HT$_{2C}$R/TH Immunoreactivity in the Middle VTA. Representative composite photomicrographs of the middle level of the VTA displaying GAD-IR [red; A], 5-HT$_{2C}$R-IR [green; B] and the overlay of B and C showing co-localization of GAD- and 5-HT$_{2C}$R-IR [yellow; C]. Photomicrographs of TH-IR [red; D], 5-HT$_{2C}$R-IR [green; E] and the overlay of D and E showing co-localization of TH- and 5-HT$_{2C}$R-IR [yellow; F] throughout the subnuclei (labeled) of the middle-VTA are also displayed. Scale bar = 100 µm.

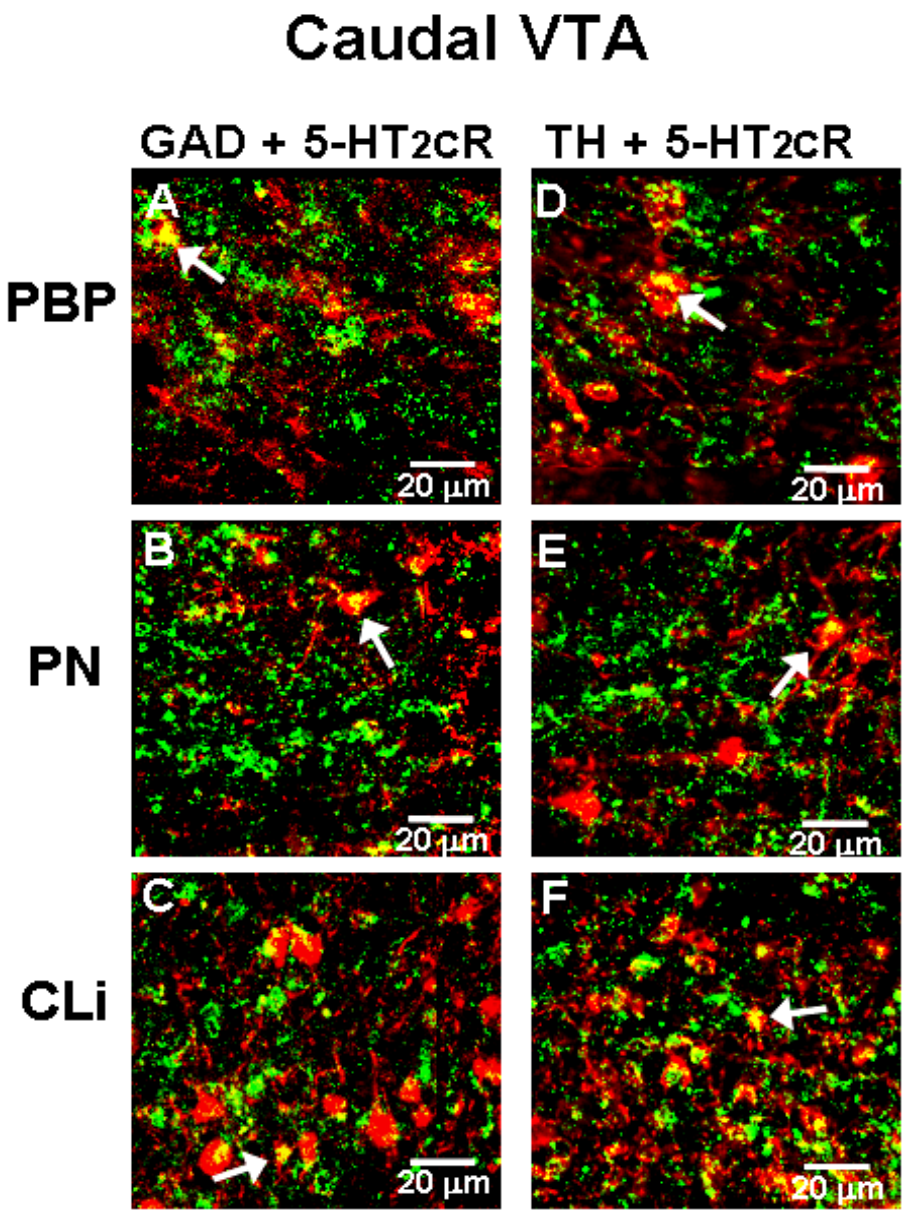

Figure 17. Co-localization of 5-HT_{2C}R/GAD and 5-HT_{2C}R/TH immunoreactivity in the Caudal VTA. Representative photomicrographs displaying the overlay images of [A-C] GAD (red) and 5-HT_{2C}R (green) immunoreactivity and [D-F] TH (red) and 5-HT_{2C}R (green) immunoreactivity in the caudal PBP [A,D], PN [B,E], an CLi [C,F]. Arrows indicate cells containing co-localization of 5-HT_{2C}R/GAD or 5-HT_{2C}R/TH IR (yellow). Scale bar = 20 µm.

As described in **Table 4**, the number of 5-HT_{2C}R-IR cells in the rostral-caudal levels of each subnucleus were as follows: PBP and PN: middle > rostral > caudal; IF: middle > caudal; RLi: rostral = middle = caudal CLi.

GAD and TH immunoreactivity in the VTA

Both GAD- (**Figs. 14, 16-17**) and TH-IR cells (**Figs. 15-17**.) and processes were clearly visible in each of the subnuclei throughout the rostral-caudal extent of the VTA, and the subnuclei were readily distinguished with either type of staining (see composite images, **Fig. 16**). The patterns of TH IR and morphology of TH-immunoreactive cells was similar to that described previously (Phillipson, 1979b; Swanson, 1982; Nocjar et al., 2002). Briefly, TH-IR cells in the IF were relatively small and tightly packed (**Figs. 15C, 16D**), while the cells in the RLi, just dorsal to the IF, were less dense, but similar in size or slightly larger and displayed a dorsal-ventral orientation (**Figs. 15D, 16D**). Cells in the caudal CLi were similar in size and orientation to the RLi TH-IR cells (**Fig. 17F**). The TH-IR cells in the PN and PBP were larger than those in other subnuclei. Cells in the PN subnucleus were typically oriented horizontally and followed along the interpeduncular nucleus (IP) found ventral to the VTA in middle and caudal sections (**Figs. 15B, 16D, 17E**). TH-IR cells in the PBP, on the other hand, were spread diffusely with no particular orientation (**Figs. 15A, 16D, 17A**). The number of TH-IR cells for each subnucleus were as follows: PBP: rostral = middle > caudal; PN: middle > rostral > caudal; IF: middle > rostral; RLi: rostral = middle < caudal CLi (**Table 4**).

GAD-IR cells exhibited similar morphology and followed similar patterns as that described above for TH-immunoreactive cells (**Figs. 14, 16-17**.). GAD-IR cells were more equally distributed throughout the different levels of each subnucleus than TH-IR cells. Similar numbers of cells were found in all levels of each subnucleus, except for the IF, where more cells were detected in the middle vs. rostral level, and in the PN where fewer cells were detected in the caudal vs. rostral or middle levels (**Table 4**).

When comparing the density of TH- vs. GAD-IR cells in each subnucleus **(Table 4)**, TH-IR cells were more prominent than GAD-IR cells in all levels of the IF, PBP, and

Table 4: Total number of TH-, GAD- and 5-HT2CR-immunoreactive cells detected in the various VTA subnuclei				
Subnucleus	**Bregma Location[a]**	**TH-immunoreactive cells[b]**	**GAD-immunoreactive cells[c]**	**5-HT2CR-immunoreactive cells[d]**
PBP				
Rostral	**-5.00 to -5.40**	**132.73 ± 9.09**	**88.70 ± 11.45**	**107.05 ± 6.51**
Mid	**-5.50 to -5.80**	**141.08 ± 12.88**	**99.22 ± 6.49**	**124.62 ± 7.55**
Caudal	**-5.90 to -6.30**	**109.29 ± 13.71**	**91.88 ± 8.54**	**73.73 ± 7.07**
PN				
Rostral	**-5.00 to -5.40**	**107.45 ± 8.87**	**100.50 ± 11.65**	**110.71 ± 9.12**
Mid	**-5.50 to -5.80**	**157.00 ± 16.87**	**112.91 ± 10.18**	**133.91 ± 9.01**
Caudal	**-5.90 to -6.30**	**80.25 ± 12.85**	**83.50 ± 8.06**	**87.31 ± 8.44**
IF				
Rostral	**-5.00 to -5.40**	**79.18 ± 3.52**	**64.36 ± 7.36**	**67.23 ± 4.26**
Mid	**-5.50 to -5.80**	**107.82 ± 11.12**	**84.56 ± 8.20**	**96.05 ± 6.55**
RIi				
Rostral	**-5.00 to -5.40**	**60.55 ± 5.56**	**71.82 ± 6.49**	**78.73 ± 4.77**
Mid	**-5.50 to -5.80**	**69.33 ± 4.91**	**63.36 ± 5.26**	**80.95 ± 6.38**
CIi				
Caudal	**-5.90 to -6.30**	**116.88 ± 10.30**	**73.14 ± 6.73**	**76.33 ± 7.70**

[a] Bregma locations according to the brain atlas of Paxinos and Watson (1998)

[b] TH: average of 10-12 rostral and mid sections, 8-9 caudal sections

[c] GAD: average of 9-11 rostral and mid sections, 7-8 caudal sections

[d] 5-HT2CR: average of 21-22 rostral and mid sections, 15-16 caudal sections

CLi, and the middle PN, while approximately equal numbers of cells were observed in both levels of the Rli and in the rostral and caudal PN.

Co-labeling for the 5-HT$_{2C}$R and GAD

5-HT$_{2C}$R-IR was co-localized in a subset of GAD-IR cells (**Figs. 14, 16-18**). The extent of co-localization varied slightly among the subnuclei, ranging from ~21 - 37% of total GAD-IR cells (**Fig. 18A**). There was not a significant main effect of subnucleus location on percentage of GAD/5-HT$_{2C}$R-co-labeled cells within in the rostral ($F_{3,38}$ = 1.84; p = 0.16), middle ($F_{3,36}$ = 1.84; p = 0.77), or caudal levels ($F_{2,20}$ = 0.26; p = 0.77; **Fig. 18A**). However, there was a significant main effect of rostro-caudal level on the percentage of co-labeled cells in the PBP ($F_{2,24}$ = 3.55; $p < 0.05$) and PN ($F_{2,26}$ = 1.84; p = 0.16) reflecting higher proportions of GAD/5-HT$_{2C}$R-co-labeled cells in the middle PBP compared to rostral PBP (**Fig 18A**).

Co-labeling for 5-HT$_{2C}$R and TH

5-HT$_{2C}$R-IR was also found to be co-localized in a subset of TH-IR cells (**Figs. 15-18**). The presence of 5-HT$_{2C}$R-IR within TH-IR cells was confirmed through examination of the images captured on a confocal microscope (**Fig. 19**), which demonstrated the presence 5-HT$_{2C}$R-IR throughout the cell membrane and cytoplasm if TH-IR and non-TH-IR cells. The extent of co-localization for 5-HT$_{2C}$R and TH was more variable than 5-HT$_{2C}$R/GAD co-labeling, ranging from ~17 – 48% of total TH-immunoreactive cells (**Fig. 18B**). A significant main effect of subnucleus location on the percentage of TH/5-HT$_{2C}$R-co-labeled cells observed in the middle ($F_{3,39}$ = 5.52; $p <$ 0.01) and caudal levels ($F_{2,20}$ = 4.34; $p < 0.01$), but not the rostral level of the VTA ($F_{3,40}$ = 2.06; p = 0.12; **Fig. 18B**). In the middle VTA, a significantly smaller proportion of total TH-IR cells co-labeled for 5-HT$_{2C}$R-IR was observed in RLi compared to the PBP, PN

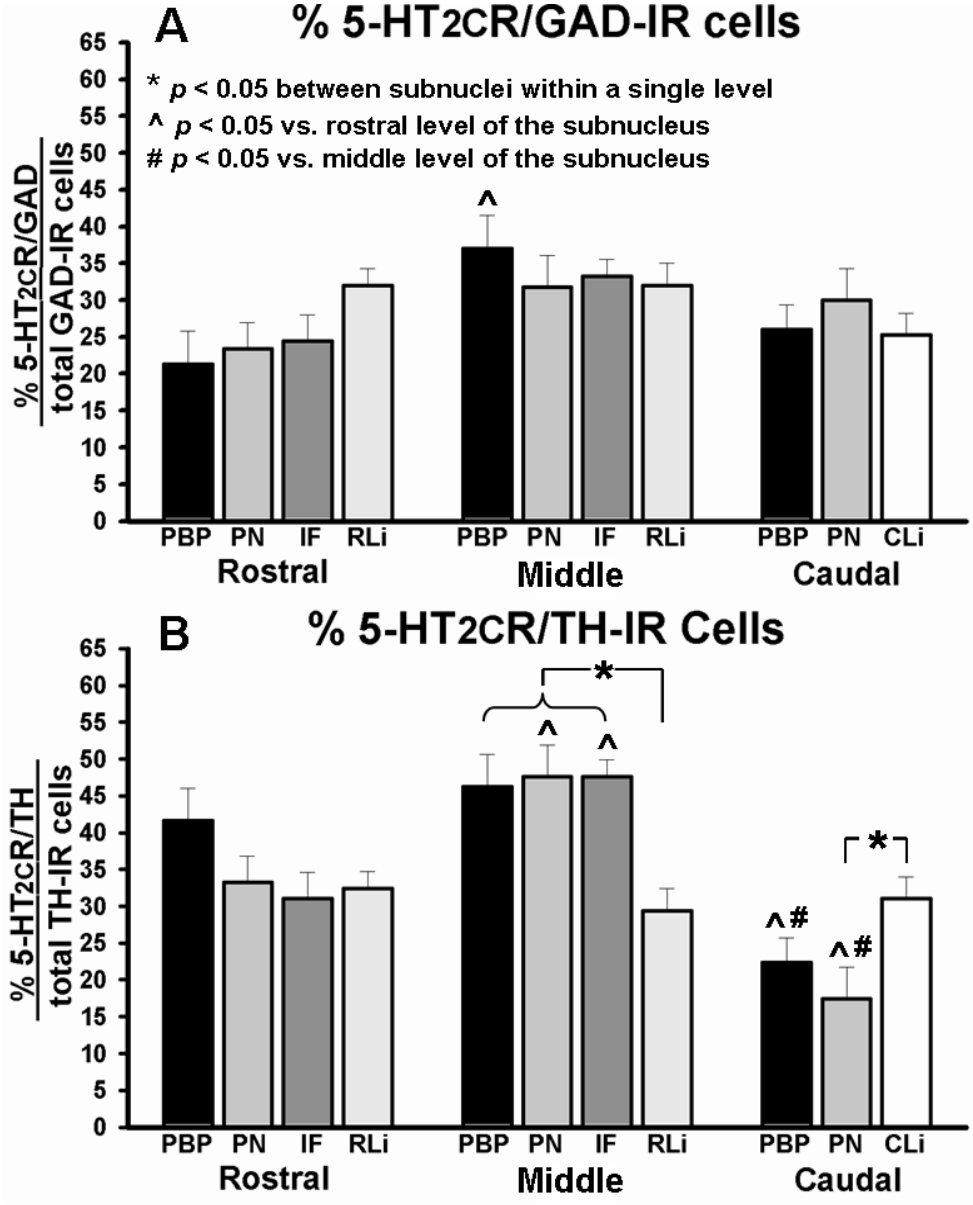

Figure 18. Percentage of 5-HT_{2C}R/GAD and 5-HT_{2C}R/TH co-localized cells in the various VTA subnuclei. Data represent the mean (± SEM; n = 7-12/group) percentage of [A] 5-HT_{2C}R/GAD co-localized cells and [B] 5-HT_{2C}R/TH co-localized cells in the rostral, middle and caudal levels of the VTA subnuclei. Data were calculated by dividing the % of co-labeled cells by the total number of GAD or TH labeled cells, respectively, in each subnucleus for each VTA tissue section. Resultant values were averaged for the middle, rostral and caudal levels of each subnucleus. * $p < 0.05$ between subnuclei; ^ $p < 0.05$ vs. rostral level of the subnucleus; ^ $p < 0.05$ vs. middle level of the subnucleus.

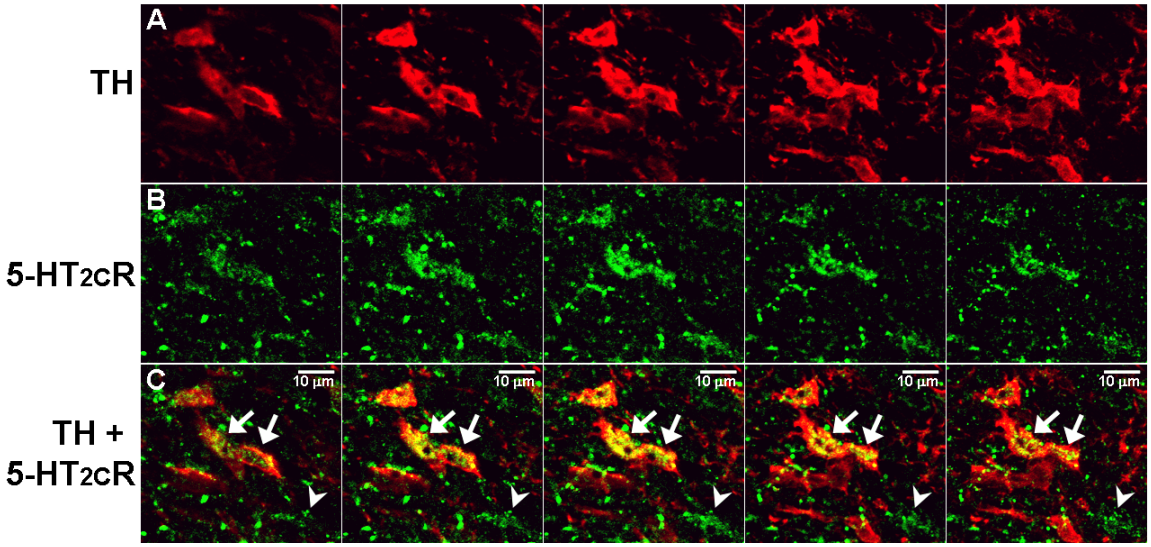

Figure 19. Co-localization of TH and 5-HT$_{2C}$R Immunoreactivity. Series of photomicrographs (from left to right) captured using a confocal microscope displaying TH [A; red] and 5-HT$_{2C}$R [B; green] immunoreactivity in the VTA. [C] Overlay of images in A and B shows co-localization of TH + 5-HT$_{2C}$R immunoreactivity (yellow) as indicated by arrows. Arrowheads point to a 5-HT$_{2C}$R-IR cell that is devoid of TH-IR. Scale bar = 10 µm.

and IF subnuclei. The significant effect in the caudal VTA, reflected a significantly higher percentage of TH/5-HT_{2C}R-co-labeled cells in the CLi compared to the PN.

In addition, a significant main effect of rostro-caudal level on the percentage of co-labeled cells in each subnucleus were also observed for the PBP ($F_{2,27} = 7.49$; $p < 0.01$) and PN ($F_{2,27} = 14.47$; $p = 0.001$), and IF ($p < 0.001$; **Fig 18B**). A significantly lower proportion of TH/5-HT_{2C}R-co-labled cells were observed in the caudal PBP and PN subnuclei compared to the rostral or middle levels. Additionally, a significantly higher percentage of TH/5-HT_{2C}R was observed in the middle compared to rostral PN and IF subnuclei.

DISCUSSION

The present study is the first to report that 5-HT_{2C}R immunoreactivity is co-localized within subsets of both GAD- *and* TH-IR cells throughout the VTA subnuclei, suggesting that 5-HT_{2C}R are located on subpopulations of *both* GABA and DA cells in the VTA. While the percentage of 5-HT_{2C}R co-localization with GAD-IR was relatively similar across most subnuclei, the proportion of TH-IR cells co-labeled with 5-HT_{2C}R-IR differed significantly across a number of subnuclei, with the greatest incidence of co-localization occurring in the middle-levels of the VTA. These findings indicate that the ability of 5-HT_{2C}R to regulate the function of VTA neurons is much more complex than previously thought, potentially involving both direct and indirect modulation of DA mesolimbic circuits by 5-HT_{2C}R.

5-HT_{2C}R antibody specificity

In the present study, we performed a number of experiments to confirm the specificity of the 5-HT_{2C}R antibody used in the co-localization experiments. The first step was to compare the pattern of IR of two commercially available anti-5-HT_{2C}R antibodies, a rabbit polyclonal anti-5-HT_{2C}R antibody (SC 5-HT_{2C}R; Santa Cruz Biotechnology) and a mouse monoclonal anti-5-HT_{2C}R antibody (PH 5-HT_{2C}R; BD PharMingen). Similar to reports of immunohistochemical analyses conducted with anti-5-HT_{2C}R antibodies which are not commercially available (Clemett et al., 2000), IR for both antibodies appeared to label cell bodies indicated by the association of the clusters of 5-HT_{2C}R-IR with DAPI-stained nuclei. However, the IR for the SC 5-HT_{2C}R antibody was much more concentrated in these areas compared to PH 5-HT_{2C}R-IR, thereby appearing more analogous to previous reports of 5-HT_{2C}R staining (Clemett et al., 2000). This difference in staining intensity of the two antibodies may be due to the characteristics of their target epitopes. While the SC 5-HT_{2C}R antibody was raised against a peptide that mapped near the extracellular N-terminus of the human 5-HT_{2C}R, the PH 5-HT_{2C}R was raised against a GST fusion protein targeting the intracellular C-terminus of the human 5-HT_{2C}R. Although these two antibodies are highly reactive with the rat 5-HT_{2C}R, in order for the PH 5-HT_{2C}R antibody to bind to the receptor, it must first penetrate the cell membrane, while the SC 5-HT_{2C}R antibody does not have this restriction and thus may have greater accessibility to 5-HT_{2C}R binding sites (Goldenthal et al., 1985). Unfortunately, use of a detergent such as Triton X to permeableize cellular membranes and promote availability of intracellular binding sites (Polack and Van Noorden, 1997) actually seemed to reduce, rather than enhance, PH 5-HT_{2C}R antibody staining (data not shown), as has been demonstrated for some other membrane proteins (Goldenthal et al., 1985). Thus, since the staining with the SC 5-HT_{2C}R was more concentrated and more analogous to that observed in previous 5-HT_{2C}R immunohistochemical studies (Clemett et al., 2000), the subsequent specificity and distribution studies were performed using the SC 5-HT_{2C}R antibody.

In addition to expressing patterns of immunoreactivity similar to that reported for other 5-HT_{2C}R antibodies, SC 5-HT_{2C}R immunoreactivity was virtually eliminated in the VTA and other brain areas of 5-HT_{2C}R KO mice, compared to the intense immunoreactivity observed in the brains of WT littermates. These results thereby indicate that the SC 5-HT_{2C}R antibody does bind to the 5-HT_{2C}R as reduced 5-HT_{2C}R density was also observed in the 5-HT_{2C}R KO mice via autoradiography (Lopez-Gimenez et al., 2002). The 5-HT_{2C}R KO mice were developed by generating a mutation that truncates the receptor within the fifth transmembrane domain (Tecott et al., 1995). Thus, it is plausible that non-functional, truncated 5-HT_{2C}R proteins could still be produced in the brains of KO mice. This truncated receptor might be detected by the SC 5-HT_{2C}R antibody, which targets the N-terminus of the receptor, thereby resulting in the minor residual staining that was observed in the 5-HT_{2C}R KO mice. Alternatively, the residual staining present in the KO mice could indicate some non-specific binding of the SC 5-HT_{2C}R antibody, however, if this is the case, it is not likely that the antibody was binding to 5-HT_{2A}R, since immunoreactivity for the 5-HT_{2A}R antibody was unaltered in the 5-HT_{2C}R KO mice compared to the WT littermates and much more intense than the low levels of SC 5-HT_{2C}R immunoreactivity that remained in the 5-HT_{2C}R KO mice.

To further demonstrate that the SC 5-HT_{2C}R did not cross-react with the 5-HT_{2A}R, we also examined SC 5-HT_{2C}R immunoreactivity in CHO cells transfected with the 5-HT_{2A}R containing the Flag peptide sequence at the C-terminus. Although immunoreactivity for 5-HT_{2A}R and Flag antibodies was present in the 5-HT_{2A}R-transfected CHO cells, no SC 5-HT_{2C}R immunoreactivity was detected in the CHO/5-HT_{2A}R cells. As such, these studies demonstrated that the SC 5-HT_{2C}R antibody bound to 5-HT_{2C}R and did not cross-react with 5-HT_{2A}R, suggesting that this antibody was suitable for use in the 5-HT_{2C}R distribution studies.

5-HT_{2C}R distribution in the VTA

The present study revealed that the 5-HT_{2C}R is widely distributed in all subnuclei throughout the rostral-caudal extent of the VTA. Studies examining the distribution of 5-HT_{2C}R mRNA and protein throughout the brain have detected low to moderate levels of 5-HT_{2C}R mRNA in the in the VTA of rats (Hoffman and Mezey, 1989; Wright et al., 1995; Eberle-Wang et al., 1997) and monkeys (Lopez-Gimenez et al., 2001), and high levels of 5-HT_{2C}R protein in the ventral mesencephalon of rats (Clemett et al., 2000). Most other studies either did not specifically mention the VTA (Mengod et al., 1990; Abramowski et al., 1995; Sharma et al., 1997) or did not examine the VTA (Pasqualetti et al., 1999), while a single study reported undetectable levels of 5-HT_{2C}R protein in the VTA (Li et al., 2004). This latter study utilized the PH5-HT_{2C}R that, in our hands, proved to be unreliable, producing inconsistent results upon replication. Conversely, the SC5-HT_{2C}R antibody to utilized in the present studies has produced consistent, replicable staining in the VTA and other brain areas (PFC) similar to that described by Clemett and colleagues (2000).

Based upon analysis of mRNA expression, electrophysiology, and microdialysis studies, researchers have hypothesized that 5-HT_{2C}R are localized to GABA neurons within the VTA. For example, systemic administration of a 5-HT_{2C}R agonist, which would be expected to cause depolarization of neurons (Sheldon and Aghajanian, 1991), resulted in increased firing of non-dopaminergic neurons (presumably GABA neurons; (Di Giovanni et al., 2001) and decreased firing of DA neurons in the VTA (Di Giovanni et al., 2000). Although this hypothesis has been held for a number of years, we report the first evidence to show the co-localization of 5-HT_{2C}R protein in GABA neurons. Curiously, we also identify a co-localization of 5-HT_{2C}R in a subpopulation of DA neurons in the VTA.

The present study examined the distribution of 5-HT_{2C}R protein on neurons within the VTA via double-label immunofluorescence studies combining the SC 5-HT_{2C}R antibody with an anti-GAD 67 antibody to label GABA neurons, or an anti-TH

antibody to label DA neurons. Both TH- and GAD-immunoreactive cells were found to display a variety of sizes and shapes dependent upon the VTA subnucleus. However, since the size and shape of both types of cells were similar within each subnucleus, it does not appear that the neuronal type (i.e. GABA vs. DA) can be determined by these gross morphological features alone, as has been previously suggested (Domesick et al., 1983). The five subnuclei of the VTA could be readily distinguished for both TH- and GAD-immunoreactive cells, with the IF containing small densely packed cells, the PN containing larger horizontally-oriented cells, the PBP containing large diffuse cells with no particular orientation, and the RLi or CLi containing small to medium dorsal-ventral oriented cells (Phillipson, 1979b; Nocjar et al., 2002). Although reports on the relative concentration of different neuronal subtypes in the VTA vary widely (Yim and Mogenson, 1980; Swanson, 1982; Johnson and North, 1992), of the total number of TH and GAD cells detected in the present study, ~55% were TH-immunoreactive, while ~44% were GAD-immunoreactive. We detected a greater number of TH- than GAD-immunoreactive cells in all levels of the PBP and IF, and the middle PN, while a slightly larger number of GAD-positive vs. TH-positive neurons was observed in the rostral RLi.

Our results are the first to confirm that the 5-HT_{2C}R protein is localized to a subpopulation of GABA neurons within the VTA. In addition, we unexpectedly revealed that the 5-HT_{2C}R is also co-localized to a subpopulation of DA neurons in the VTA. The 5-HT_{2C}R appears to be prominently distributed in the membrane and cytoplasm of both TH-IR and GAD-IR perikarya, along with some labeling of neuronal processes. This type of cellular distribution is similar to that described for 5-HT_{2C}R in the substantia nigra (Clemett et al., 2000) as well as for 5-HT_{2A}R in the VTA (Cornea-Hebert et al., 1999; Doherty and Pickel, 2000; Nocjar et al., 2002). Some of the 5-HT_{2C}R-IR detected may, upon quick glance, appear to be localized within the vicinity of the nucleus. While the possibility of nuclear localization of some 5-HT_{2C}R cannot be ruled out at present as no studies have examined the ultrastructural localization of 5-HT_{2C}R in the VTA, this is more likely an artifact of the light microscopy techniques utilized, representing, instead,

IR of 5-HT_{2C}R in the peri-nuclear cytoplasm of these cells. The morphology DA and non-DA cells in the VTA are similar and vary in shape and size (Domesick et al., 1983). A proportion of these cells have large nuclei that appear to encompass the majority of the perikaryal space, permitting only a thin layer of cytoplasm surrounding the nucleus (Domesick et al., 1983). Indeed, close examination of TH-IR in the confocal images (see **Fig. 19**) demonstrates low-levels of TH-IR in the area representative of the nucleus that is indicative of the presence of cytoplasm potentially located behind the nucleus of that cell. This thin layer of cytoplasm is likely the origin of the 5-HT_{2C}R-IR detected in that area.

These preliminary results revealing co-localization of 5-HT_{2C}R in a subpopulation of DA neurons in the VTA are in contrast to a previous study by Eberle-Wang and colleagues (1997) who reported that 5-HT_{2C}R mRNA was not observed to co-localize with TH-mRNA in the VTA. This discrepancy may be due to the fact that only low levels of 5-HT_{2C}R mRNA were detected in the study. Furthermore, the details of the degree to which the co-localization studies for 5-HT_{2C}R and TH mRNA were conducted in the VTA were not provided. Thus, given that incidence of 5-HT_{2C}R/TH co-localization in the present study ranged from ~17 – 48% of all TH-IR neurons, among the different VTA subnuclei, the co-localization of 5-HT_{2C}R mRNA with TH mRNA might have gone unnoticed if only a small number of cells were examined.

The co-localization of 5-HT_{2C}R and GAD immunoreactivity varied mildly from ~21 - 37% of total GAD-immunoreactive cells, depending on subnucleus and rostral-caudal level. The greatest co-localization tended to occur in the middle levels of the VTA subnuclei, as well as in the rostral RLi, however the only significantly higher proportions of co-localized cells were detected in the middle compared to rostral PBP. As opposed to the minimal variation in proportions of total GAD-IR cells co-localized with 5-HT_{2C}R, the proportion of TH-IR cells containing 5-HT_{2C}R-IR significantly differed both across subnuclei and rostral-caudal levels. In general, the greatest incidence of co-localization for TH- and 5-HT_{2C}R-IR was detected in the middle-level PBP, PN and IF, while the lowest percentages of co-localization occurred in the caudal levels. Incidently, the PN,

PBP, and IF are also reported to contain the greatest populations of neurons projecting to the NAc (Swanson, 1982). In addition, both DA (Swanson, 1982) and GABA neurons (Van Bockstaele and Pickel, 1995) are reported to project from the subnuclei to the NAc. Thus, these results indicate that 5-HT_{2C}R have the potential to exert direct effects upon both DA and GABA neurons that project to the NAc, thereby providing multiple sites of action for 5-HT_{2C}R modulation of DA mesoaccumbens function.

Functional implications

The localization of 5-HT_{2C}R on both GABA and DA neurons in the VTA suggest that, in contrast to current dogma, 5-HT_{2C}R have the potential to exert direct influence upon the function of VTA DA neurons in addition to any indirect influence that may be mediated through 5-HT_{2C}R located on GABA neurons. The differences in co-localization, particularly of TH-IR and 5-HT_{2C}R-IR, across subnuclei and rostral-caudal level suggest that the 5-HT_{2C}R control of VTA DA neuron firing may be tightly regulated by discrete populations of 5-HT_{2C}R within different subnuclei.

The discovery of 5-HT_{2C}R localization on DA neurons is surprising considering that 5-HT_{2C}R are generally thought to exert an indirect inhibitory influence over DA mesocorticoaccumbens activation (Di Matteo et al., 2001). For example, systemic administration of the 5-HT_{2C}R agonists MK 212 (Di Giovanni et al., 2000) or RO 60,0175 has been shown to decrease basal VTA DA neuron firing (Di Matteo et al., 1999; Gobert et al., 2000) and DA release in the NAc (Di Matteo et al., 1999; Gobert et al., 2000; De Deurwaerdere et al., 2004) and PFC (RO 60,0175 only; Gobert et al., 2000), while systemic administration of the purported 5-$HT_{2C/2B}$R antagonist SB 206553 (Gobert et al., 2000) and the 5-HT_{2C}R antagonist SB 242084 (Di Matteo et al., 1999) enhance the basal firing rate of VTA DA neurons and DA release in the NAc (Di Matteo et al., 1999; Gobert et al., 2000) and PFC (Gobert et al., 2000). Conversely others have reported no effect of SB 242084 on basal DA neuron firing (Di Giovanni et al., 2000) or basal DA release in the NAc (Di Giovanni et al., 2000; Gobert et al., 2000) and no effect of RO

60,0175 on basal DA release in the NAc (Navailles et al., 2004)or PFC (Pozzi et al., 2002). Futhermore, the 5-HT_{2C}R antagonist SB 243213 was shown to reduce firing of spontaneously active VTA DA neurons (Blackburn et al., 2002). Thus, the discrepancies reported among these studies may be due to opposing influences of 5-HT_{2C}R located on GABA vs. DA neurons in the VTA competing against one another to modulate firing of VTA DA neurons and likewise DA release in terminal regions.

In further support of the present findings, studies examining the neurochemical and locomotor activating effects of intra-VTA infusion of 5-HT_{2C}R ligands suggest that the presence of the two subpopulations of 5-HT_{2C}R within the VTA, i.e. 5-HT_{2C}R located on DA and GABA neurons, likely results in negation of each others actions. For example, microinfusion RO 60,0175 into the VTA did not alter basal levels of DA measured in the PFC nor did intra-VTA infusion of the 5-HT_{2C}R antagonist SB 206553 affect basal DA levels in the NAc. Likewise, local administration of these compounds into the VTA also did not alter basal locomotor activation (McMahon et al., 2001; Fletcher et al., 2004). Conversely, local infusion of RO 60,0175 into the VTA was shown to reduce stress-evoked DA release in the PFC (Pozzi et al., 2002) and cocaine-evoked hyperactivity (Fletcher et al., 2004), while SB 206553 enhanced (+)-MDMA-induced DA release in the NAc (Bankson and Yamamoto, 2004). Thus these data suggest that the ability of 5-HT_{2C}R within the VTA to exert an inhibitory influence upon DA mesocorticoaccumbens activation may be dependent upon input from other brain regions, potentially via feedback loops to the VTA or interactions within the terminal regions.

Studies have demonstrated that various isoforms of 5-HT_{2C}R exist as a result of pre-mRNA editing which exhibit varying levels of constitutive activity (Herrick-Davis et al., 1999). Thus it is plausible that 5-HT_{2C}R isoforms present in the VTA have low levels of constitutive activity, and thus do not exert tonic inhibition over VTA DA neurons. Variations in the types of 5-HT_{2C}R isoforms expressed within the different subpopulations of 5-HT_{2C}R in the VTA (i.e. those located on GABA vs. DA neurons) also potentially add another level of complexity to the interplay of these 5-HT_{2C}R

subpopulations in control of DA mesocorticoaccumbens pathway activity. As such, future studies employing microinfusion of selective 5-HT_{2C}R ligands into the VTA are necessary to further delineate the contribution of particular subpopulations of 5-HT_{2C}R in the VTA on DA mesocorticoaccumbens pathway activation. In particular, systematic studies examining the effects of local 5-HT_{2C}R ligand infusion of the VTA on DA and GABA neuron activity and DA release are needed to examine how the differences in the topographical organization of 5-HT_{2C}R throughout the various rostral-caudal levels and subnuclei of the VTA differentially mediate these measures.

In summary, the present study is the first to demonstrate localization of 5-HT_{2C}R on a subpopulation of GABA neurons in the VTA, and, furthermore, that 5-HT_{2C}R are also localized to a subpopulation of DA neurons within the VTA. Although the distribution of the 5-HT_{2C}R on these two neuronal subtypes appears to vary slightly among the rostral-caudal levels of the various subnuclei, the incidence of co-localization of 5-HT_{2C}R with DA neurons appears to predominate in several subnuclei, particularly in the middle-VTA. While the functional implications of these differences in co-localization of 5-HT_{2C}R across subnuclei and rostral-caudal level are not known at present, they suggest that the 5-HT_{2C}R control of VTA DA neuron firing may be tightly regulated by discrete subpopulations of 5-HT_{2C}R within different subnuclei. However, further examination into the impact of these different 5-HT_{2C}R subpopulations through systematic microinfusion studies is necessary to fully understand how 5-HT_{2C}R in the VTA regulate activation of the DA mesocorticoaccumbens pathways.

CHAPTER 5:

LOCALIZATION OF 5-HT$_{2C}$ RECEPTORS ON DOPAMINE AND GABA NEURONS IN THE VENTRAL TEGMENTAL AREA THAT PROJECT TO THE NUCLEUS ACCUMBENS

INTRODUCTION

The DA mesoaccumbens pathway, consisting of DA neurons that originate in the VTA and terminate in the NAc, is known to play an integral role in mediating attention, motivation, cognition, and reward (Kalivas and Nemeroff, 1988). This pathway is highly modulated by the 5-HT system, which innervates both the cell body and terminal fields of the DA mesoaccumbens pathway (Azmitia and Segal, 1978; Herve et al., 1987). However, 5-HT has been shown to exert both excitatory and inhibitory influence upon this pathway (Bankson and Cunningham, 2001), an effect that likely is the result of 5-HT acting at the multitude of 5-HT receptor subtypes, many of which are present within the nuclei associated with the pathway (Hoyer et al., 2002).

A probable candidate for mediating, at least in part, the inhibitory influence of 5-HT on the DA mesoaccumbens pathway is the 5-HT$_{2C}$R, which inhibits tonic and phasic VTA DA neuron firing and DA release in the NAc (for review, see Di Matteo et al., 2002). The ability of 5-HT$_{2C}$R stimulation to result in inhibition of DA firing and release is thought to be mediated indirectly via depolarization of inhibitory GABA neurons that synapse onto DA cell bodies in the VTA (Di Matteo et al., 2002). However, the discovery that 5-HT$_{2C}$R are located on both GABA and DA neurons in the VTA (Chapter 4), indicates that the 5-HT$_{2C}$R may also exert a direct influence upon VTA DA neurons, and suggests that the ability of the 5-HT$_{2C}$R to exert its inhibitory actions may be multifaceted.

Efferents from the VTA project to a variety of areas throughout the brain including, but not limited to, the NAc, PFC, hypothalamus, and amygdala. A large

proportion of VTA neurons were shown to project to the NAc (Swanson, 1982), and the majority of these neurons seem to originate in the PN and PBP subnuclei of the VTA (Swanson, 1982). These are the same subnuclei in which the greatest proportion of 5-HT_{2C}R co-localization on TH and GAD-immunoreactive neurons were observed, as described in the Chapter 4. Taken together, these studies suggest that the 5-HT_{2C}R has a high potential to exert direct effects upon VTA DA neurons that project to the NAc. Interestingly, although 85% of the neurons projecting to the NAc were reported to be DA neurons (Swanson, 1982), a separate study reported that 39% of VTA projection neurons to the NAc contained GABA immunoreactivity (Van Bockstaele and Pickel, 1995). Although little is known of the actions that these GABA projection neurons exert in the NAc (see Discussion, Van Bockstaele and Pickel, 1995), the 5-HT_{2C}R may also be located on, and likewise exert influence upon, these VTA GABA projection neurons to the NAc.

The present study was conducted to examine the distribution of 5-HT_{2C}R on DA and GABA neurons that project to the NAc. To accomplish this, we combined retrograde tracing with the compound FluoroGold (FG), which can be detected by light microscopy using ultraviolet excitation/emission filters, and double-label immunofluroescence techniques for simultaneous detection of 5-HT_{2C}R and TH or GAD 67. The retrograde tracer FG, which has previously been shown to be effective in labeling mesoaccumbens neurons (Van Bockstaele et al., 1994), was unilaterally injected into the NAc shell of male Sprague-Dawley rats. Subsequently, double-label immunofluroescence for TH and the 5-HT_{2C}R or GAD and the 5-HT_{2C}R was performed on VTA sections of the FG-injected brains. Thus, cells that were labeled for FG, TH and 5-HT_{2C}R would indicate the localization of 5-HT_{2C}R on DA cells that project to the NAc, while co-localization of FG, GAD and 5-HT_{2C}R would reflect the presence of 5-HT_{2C}R on GABA neurons projecting to the NAc.

METHODS

Retrograde Tracing

Naïve male Sprague-Dawley rats (N = 6; virus antibody-free; Harlan, Houston,TX) weighing 250-300g were used in these studies. All rats were maintained in the colony room for a minimum of 7 days after arrival, where food and water was available *ad libitum*. Rats were deeply anesthetized using an intramuscular injection of 43 mg/kg of ketamine, 8.6 mg/kg of xylazine and 1.5 mg/kg of acepromazine in 0.9% saline. With the upper incisor bar of a stereotaxic instrument positioned at -3.8 mm below the interaural line and using the intersection of bregma and longitudinal sutures as the origin, FluoroGold (FG; Fluorochrome, Englewood, CO) was unilaterally injected into the NAc shell at 1.4 mm anterior to bregma, 0.75 mm lateral to the midline, and 8.0 mm ventral to the skull surface. A 1-2% FG solution (dissolved in 0.9% saline) was injected through a Hamilton syringe at a rate of 10 nL/min over 20 min for a total volume of 100 nL. Following infusion, rats received a single injection of sodium ampicillin after surgery and recovered for 1 week, during which the rats were handled and weighed daily. All experiments conformed to the NIH Guide for the Care and Use of Laboratory Animals and were approved by the UTMB Animal Care and Use Committee.

Seven days following FG infusion, rats were deeply anesthetized with pentobarbital (100 mg/kg, IP) then perfused transcardially with phosphate buffered saline (PBS) followed by ~500 ml of 3% paraformaldehyde in PBS. Brains were then removed, post-fixed for 2 h, then cryoprotected in 30% sucrose for 2 days at 4^{o} C. Using crushed dry ice, the brains were rapidly frozen and stored at -80^{o}C until sectioning. Coronal sections containing the NAc (30 µm; Bregma +0.70 mm through +2.0 mm) and VTA (20 µm; Bregma mm -4.8 through -6.5 mm) were taken from all brains using a cryostat (Leica) according to the atlas of Paxinos and Watson (Paxinos and Watson, 1998). Free floating sections were processed as described below.

Immunohistochemistry

The following antibodies were used in the present studies (for further details, see Chapter 4, **Table 2**): a goat polyclonal anti-5-HT_{2C}R antibody (5-HT_{2C}R; 1:100; Santa Cruz Biotechnology) a mouse monoclonal anti-TH antibody (1:3000; Immunostar); and a rabbit polyclonal anti-GAD 67 antibody (GAD; 1:150; Santa Cruz). Fluorescent-conjugated secondary antibodies (1:2000) obtained from Molecular Probes were utilized to visualize primary antibody staining: Alexa Fluor 488 donkey anti-goat, Alexa Fluor 555 donkey anti-rabbit, Alexa Fluor 555 donkey anti-mouse, The Alexa Fluor 488 antibody has an excitation/emission maxima of 491/515 and appears green, while the Alexa Fluor 555 antibodies have an excitation/emission maxima of 573/596 and appear red. Double-label immunohistochemistry experiments for 5-HT_{2C}R and TH and 5-HT_{2C}R and GAD were performed, as well as immunohistochemistry in the presence or absence of each antibody alone, on the FG-injected rat brains to determine the distribution of 5-HT_{2C}R on DA and GABA cells that project to the NAc. In addition, to examine the possibility of co-labeling of TH and GAD in the same cells, we also conducted double-label immunohistochemistry on a few of the FG-labeled VTA brain sections for TH and GAD using Alexa Fluor 488 goat anti-mouse, and Alexa Fluor 555 goat anti-rabbit secondary antibodies, respectively.

Free floating rat brain sections were washed using an orbital shaker in PBS (2x10 min), then incubated in 20 mM sodium acetate (1 x 15 min, RT), and washed again (3 x 10 min) with PBS. The sections were then incubated in a blocking serum (1.5% normal donkey or goat serum in PBS) for one hour (RT). The blocking serum was aspirated, and the sections were then incubated with primary antibodies diluted in 1.5% normal donkey or goat serum for 44 h on an orbital shaker at 4^{o}C. The sections were then washed with PBS (6 x 6 min) on an orbital shaker and incubated with the secondary antibodies diluted in 1.5% normal donkey or goat serum for 1 h at room temperature (protected from light). The sections were washed with PBS (3 x 10 min) and mounted using a 0.1% Drefts solution onto slides previously coated with gelatin chrom alum. The

slides were then coverslipped using Vectashield fluorescent mounting medium (Vector Laboratories), and stored protected from light at 4^{o}C until viewing.

Image Analysis

Digital images were captured from brain sections using an Olympus BX51 fluorescent microscope equipped with a Hamamatsu digital camera (Hamamatsu, Bridgewater, NJ) interfaced to a personal computer and were analyzed using Simple PCI software (version 5.1, Compix Inc., Imaging Systems, Cranberry Township, PA). A 20x or 40x objective was used to capture all photomicrographs for final magnification of 400x or 800x, respectively. Green fluorescence emitted by the Alexa Fluor 488 antibodies was visualized using a yellow GFP filter set (# 41017; Chroma Technology Corporation, Rockingham, VT), while the red fluorescence emitted by the Alexa Fluor 555 antibodies was visualized using a narrow band green excitation filter set (U-MNG2, Olympus). In addition, FG staining was visualized using a wideband ultraviolet excitation filter set (U-MWU2, Olympus). For each section three images of the same viewing area were captured, one for each filter set, and then resultant images were overlaid.

Rostro-caudal patterns of FG labeling and 5-HT$_{2C}$R-, TH-, and GAD-immunoreactivity in cells were analyzed at rostral (Bregma -5.00 to -5.40 mm), middle (Bregma -5.50 to -5.80 mm), and caudal (Bregma -5.90 to – 6.30 mm) levels of the VTA (Paxinos and Watson, 1998). Three to four FG/5-HT$_{2C}$R/TH-labeled sections and adjacent (when possible) FG/5-HT$_{2C}$R/GAD-labeled sections per each rostro-caudal level were examined from each rat (n = 3). For each section, a composite photomicrograph comprised of 20-30 individual images captured using the 20x objective was assembled to visualize the entire VTA. Each individual FG labeled cell was identified by hand as being labeled for FG alone, FG + 5-HT$_{2C}$R, FG + TH, FG + 5-HT$_{2C}$R + TH, FG + GAD, and FG + 5-HT$_{2C}$R + GAD and the number of each combination of labels was counted for each section using the "Region of Interest" function of the Simple PCI software.

For each section, the total number FG-labeled cells that contained FG alone, 5-HT_{2C}R (sum of FG/5-HT_{2C}R and FG/TH/5-HT_{2C}R or FG/GAD/5-HT_{2C}R) and TH (sum of FG/TH and FG/TH/5-HT_{2C}R) or FG/GAD (sum of FG/GAD and FG/GAD/5-HT_{2C}R) were calculated, and then averaged (± SEM) for rostral, mid and caudal levels. The percentage of FG/TH or FG/GAD co-labeled cells was determined by dividing the number of total FG/TH or FG/GAD co-labeled cells, respectively, by the total number of FG-labeled cells in that section. The resultant values were averaged (mean ± SEM) for rostral, middle, and caudal levels of each subnucleus. In addition, the percentage of FG/TH or FG/GAD cells that also contained 5-HT_{2C}R was determined for each section by dividing the number of FG/TH/5-HT_{2C}R or FG/GAD/5-HT_{2C}R co-labeled cells by the total FG/TH or FG/GAD co-labeled cells, respectively. Individual one way analysis of variance (ANOVA) was used to determine significant differences in co-labeling across the rostro-caudal levels of the VTA. The total number of FG-, FG/TH-, FG/GAD-, and FG/5-HT_{2C}R-co-labeled cells; the percentage of total FG cells labeled with TH-IR or GAD-IR; and the percentage of FG/TH or FG/GAD cells that contained 5-HT_{2C}R-IR were each examined for differences in the number of cells detected in the rostral, middle and caudal levels of the VTA. Significant effects were followed with post hoc analyses using the Student Newman Keuls procedure (Keppel, 1973).

RESULTS

Retrograde Labeling with FG

Of the six animals that received FG injections into the NAc, three animals had unilateral injections that were correctly placed in the NAc shell (NAcSh; **Fig. 20**). Thus, only these three brains were sectioned through the VTA and processed for immunohistochemistry. While the FG was primarily confined to the NAcSh, diffusion appeared to allow some distribution into the NAc core and surrounding regions including the ventral pallidum and the islands of calleja (**Fig. 20**).

VTA sections from the NAc FG-injected brains displayed large numbers of intensely labeled FG cells and processes. FG-labeled cells were visible throughout the rostro-caudal extent of the VTA (**Fig. 21**). The vast majority of FG-labeled cells were confined to the side of the brain ipsilateral to the injection site. However, a small number of FG-labeled cells were visible on the contralateral side of the VTA, as well as in the ipsilateral substantia nigra (data not shown).

The total number of FG-labeled cells counted in the VTA of each brain (11 sections/brain; n = 3 brains) ranged from 1521-2429. A significant main effect of rostro-caudal level on the distribution of FG cells was observed ($F_{2,63} = 45.82$ $p < 0.001$; **Table 5**). Significant differences in the number of FG-labeled cells detected was seen across the rostro-caudal levels, with the highest number of FG-labeled cells detected in the middle sections, followed by the rostral level, and the lowest number of FG-labeled cells present in the caudal VTA. Although not quantified by subnuclei, in general, the majority of FG-labeled cells were confined to the PBP, PN, and IF subnuclei, except in the caudal VTA, where most FG-labeled cells were found in the CLi (see **Figs. 23, 25**).

Distribution of TH-IR in FG-labeled cells

Immunofluorescence studies for TH in VTA sections following unilateral injection of FG into the NAcSh revealed that a subset of FG-labeled cells throughout the VTA contained immunoreactivity (IR) for TH (**Figs. 22, 23**). A main effect of rostro-caudal level was observed for the distribution of FG/TH cells in the VTA ($F_{2,30} = 28.06$; $p < 0.001$; **Table 5**). As observed for total FG-labeled cells, the number of FG/TH-co-labeled cells detected in each level was significantly different than the other levels, with the greatest overall abundance of FG/TH-co-labeled cells in the middle levels of the VTA. A main effect rostro-caudal level was also observed for the percentage of total FG cells containing TH-IR ($F_{2,30} = 11.46$ $p < 0.001$; **Table 5**). This effect was engendered by significantly lower percentages of total FG cells containing TH-IR detected in the rostral compared to middle or caudal levels of the VTA.

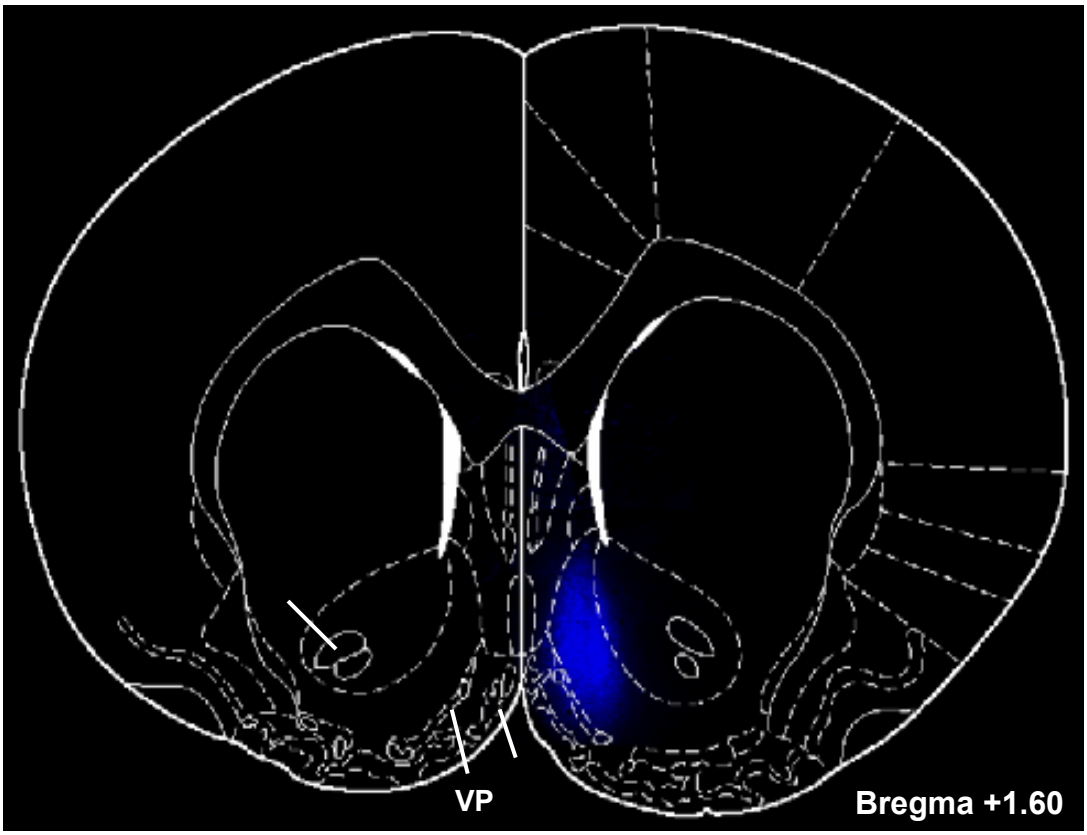

Figure 20. FG staining at injection site in the NAc. Schematic diagram depicting the NAc shell (NAcSh) and surrounding brain areas at Bregma +1.60 mm (Paxinos and Watson,1998) overlaid on top of a representative photomicrograph depicting a the FG injection (100 nL; 1-2% FG) correctly placed in the NAcSh. Other areas labeled include the anterior commisure (AC), caudate putamen (CPu), islands of calleja (ICj), NAc core (NAcC), and ventral pallidum (VP).

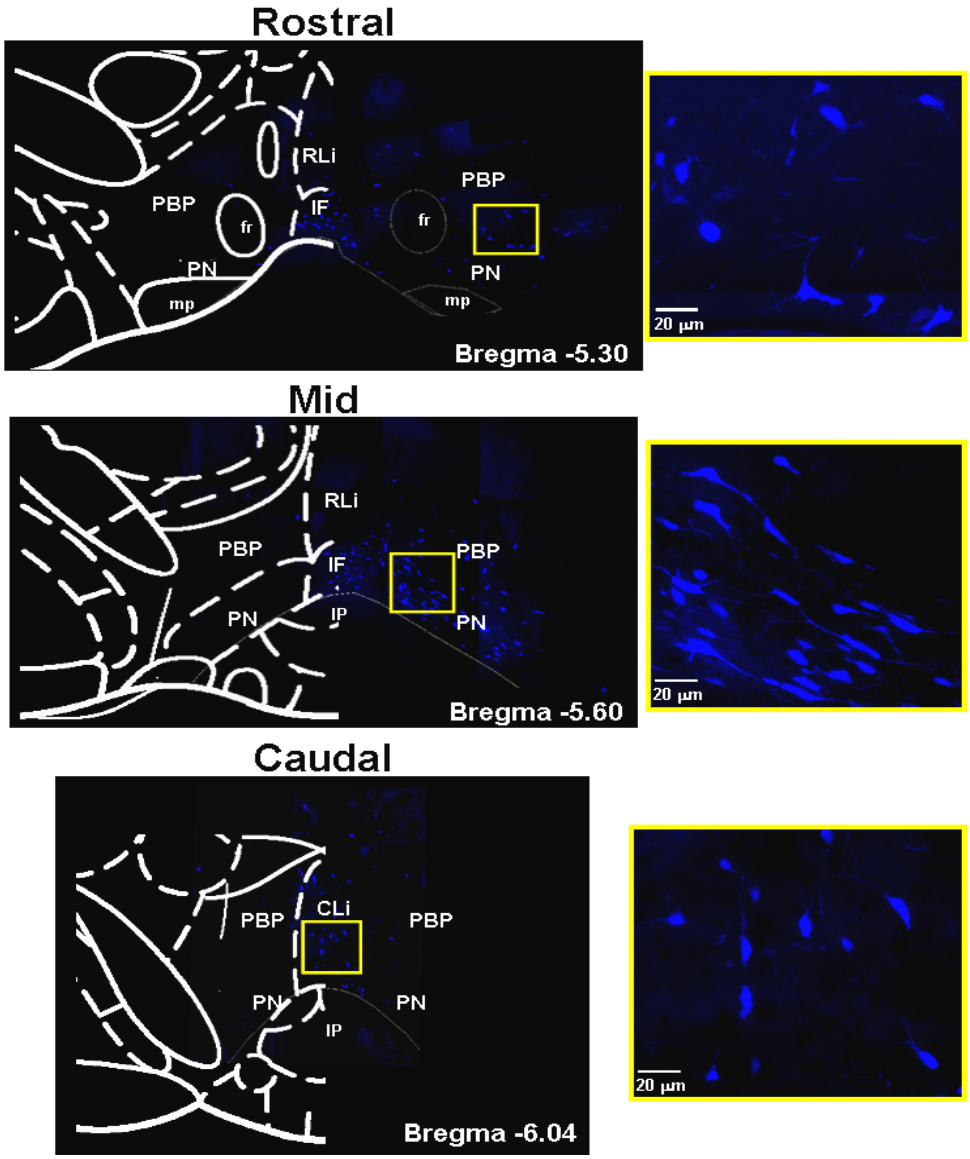

Figure 21. FG-labeled cells in the VTA following NAc FG infusion. Schematic diagrams depicting the five subnuclei of the VTA [parabrachial pigmented nucleus (PBP), paranigral nucleus (PN), intrafascicular nucleus (IF), rostral linear raphe nucleus (RLi) and caudal linear raphe nucleus (CLi; Paxinos and Watson, 1998) overlaid on top of representative photomicrographs displaying FG (blue) labeling in the rostral [Bregma -5.30 mm; A], middle [Bregma -5.60 mm; B], and caudal [Bregma -6.04 mm; C] levels of the VTA one week following infusion of FG into the NAc shell. Other brain regions labeled for orientation purposes include: fasiculus retroflexus (fr), mammillary peduncle (mp), and interpeduncular nucleus (IP). [D, E, F] Higher power magnification of yellow boxed regions in A, B, and C, respectively. Scale bars = 20 µm.

Table 5: Total FG-labeled cells containing immunoreactivity for TH, GAD, and 5-HT_{2C}R (2C)

Level Bregma Location[a]	Rostral -5.00 to -5.40	Middle -5.50 to -5.80	Caudal -5.90 to -6.30	ANOVA
Totals:				
FG [b]	78.71 ± 6.34	124.13 ± 6.84*	37.39 ± 5.23*^	$F_{2,63} = 45.82$; $p < 0.001$
FG/TH [b]	55.00 ± 4.67	108.92 ± 10.65*	28.44 ± 10.06*^	$F_{2,30} = 28.06$; $p < 0.001$
FG/GAD [b]	61.83 ± 8.48	100.33 ± 7.84*	33.78 ± 6.13*^	$F_{2,30} = 19.05$; $p < 0.001$
FG/2C [c]	61.08 ± 4.54	101.21 ± 5.28*	27.50 ± 4.06*^	$F_{2,63} = 59.01$; $p < 0.001$
Percentages:				
%FG/TH [d]	72.59 ± 3.13	88.80 ± 1.20*	82.95 ± 3.42*	$F_{2,30} = 11.46$; $p < 0.001$
%FG/TH/2C [e]	83.09 ± 2.24	86.76 ± 2.28	77.52 ± 5.01	$F_{2,30} = 2.33$; $p < 0.12$
%FG/GAD [f]	78.31 ± 2.06	79.01 ± 1.72	85.36 ± 3.27	$F_{2,30} = 2.86$; $p = 0.07$
%FG/GAD/2C [g]	82.68 ± 1.98	87.76 ± 2.47	76.83 ± 5.04	$F_{2,30} = 2.19$; $p < 0.13$

[a] Bregma locations according to the brain atlas of Paxinos and Watson (1998)

[b] average (± SEM) per section of 12 rostral and mid sections, 9 caudal sections

[c] average (± SEM) number of total FG/5-HT_{2C}R-co-labeled cells per section in 24 rostral and mid, 18 caudal sections

[d] average (± SEM) of FG/TH-co-labeled cells/total FG cells in 12 rostral and mid, 9 caudal sections

[e] average (± SEM) of FG/GAD-co-labeled cells/total FG cells in 12 rostral and mid, 9 caudal sections

[f] average (± SEM) of FG/TH/5-HT2CR-co-labeled cells/total FG/TH-co-labeled cells in 12 rostral and mid, 9 caudal sections

[g] average (± SEM) of FG/GAD/5-HT2CR-co-labeled cells/total FG/GAD-co-labeled cells in 12 rostral and mid, 9 caudal sections

* $p < 0.05$ vs. rostral level

^ $p < 0.05$ vs. middle level

Examination of the distribution of FG/TH-co-labeled cells within the different subnuclei across the rostro-caudal levels of the VTA revealed that FG/TH-co-labeled cells appeared to be most abundant within the PN subnucleus, compared to the other subnuclei in the rostral (**Fig. 23, top**) and middle levels (**Fig. 23, middle**), with few co-labeled cells present in the RLi. Conversely, the majority of FG/TH-co-labeled cells in the caudal VTA were present in the CLi (**Fig. 23, bottom**). These results are displayed in **Fig. 23**, which illustrates the distribution of the TH/FG-co-labeled cells detected in a one rostral, middle, and caudal section of the VTA of a single rat injected with FG into the NAcSh.

Distribution of 5-HT_{2C}R-IR in FG-labeled cells

5-HT_{2C}R immunoreactivity was found to be co-localized on a subset of FG-labeled cells throughout the VTA (**Figs. 22-25**). A main effect of rostro-caudal level was observed for the distribution of FG/5-HT_{2C}R-co-labled cells in the VTA ($F_{2,63} = 59.01$; $p < 0.001$; **Table 5**). This effect reflected significant differences in the percentage of total FG-labeled cells that contained 5-HT_{2C}R-IR between all three levels of the VTA. The greatest numbers of FG/5-HT_{2C}R co-localized cells were detected in the middle VTA (101.21 ± 5.28/section), followed by the rostral VTA (61.08 ± 8.48/section), with the fewest number of FG/5-HT_{2C}R co-localized cells found in the caudal VTA (27.50 ± 4.06).

Distribution of 5-HT_{2C}R-IR in FG/TH-co-labeled cells

Immunoreactivity for the 5-HT_{2C}R was observed to be present in the majority of the FG/TH-co-labeled cells (**Figs. 22, 23; Table 5)**, ranging from ~78 – 88% of total FG/TH-co-labeled cells throughout the VTA. However, there was no main effect of rostro-caudal level on the percentage of FG/TH-co-labeled cells containing 5-HT_{2C}R-IR ($F_{2,30} = 2.33$; $p = 0.12$; **Table 5**). Examination of the distribution of FG/TH/5-HT_{2C}R-co-

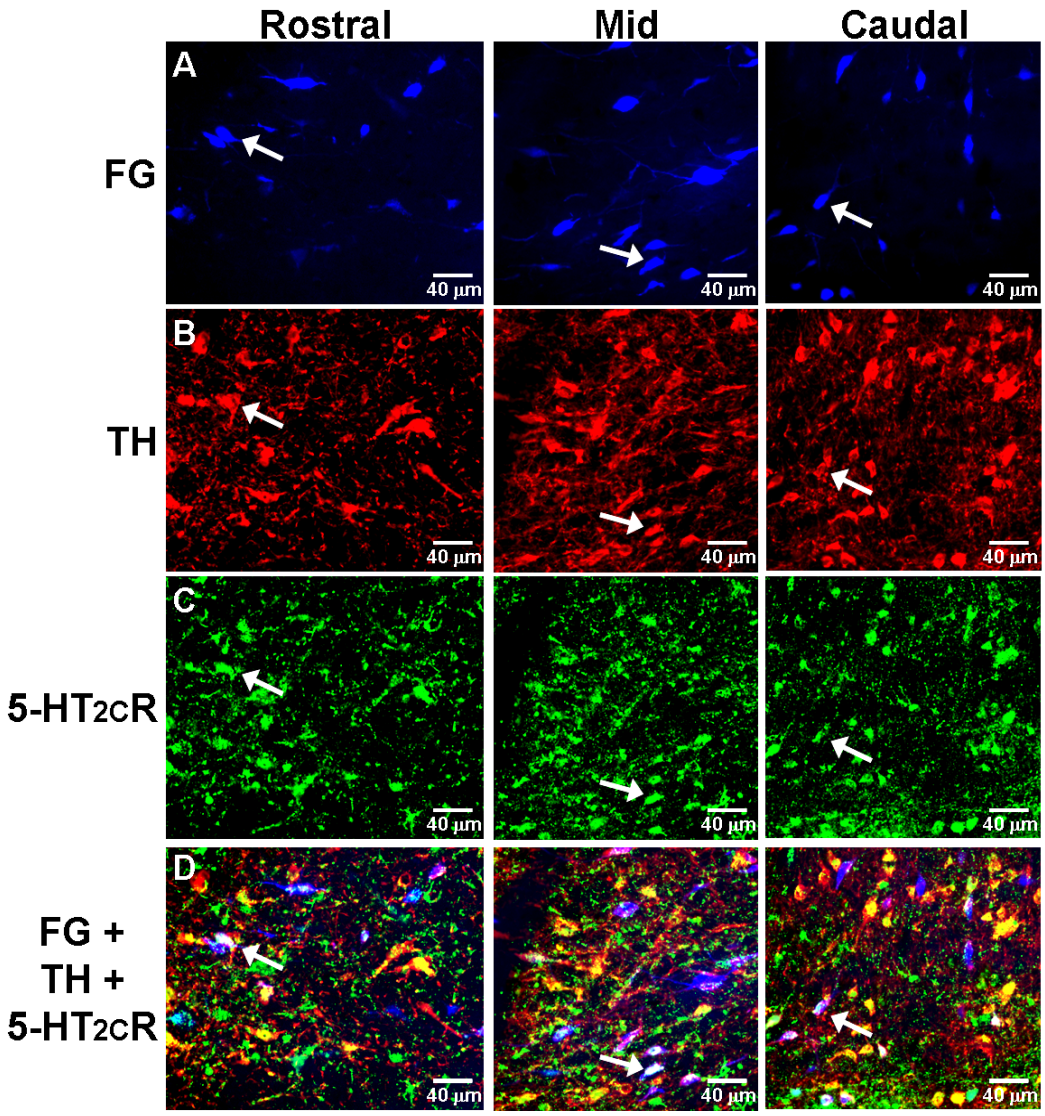

Figure 22. Co-localization of TH and 5-HT$_{2C}$R immunoreactivity with FG-labeled cells in the VTA. Representative photomicrographs depicting FG labeling [blue; A], TH-IR [red, B], and 5-HT$_{2C}$R-IR [green, C] in the rostral [left column], middle [middle column], and caudal [right column] levels of the VTA. [D] overlay of images in A, B, and C. Arrows represent cells triple-labeled for FG, TH, and 5-HT$_{2C}$R (which appear white). Scale bars = 40 µm.

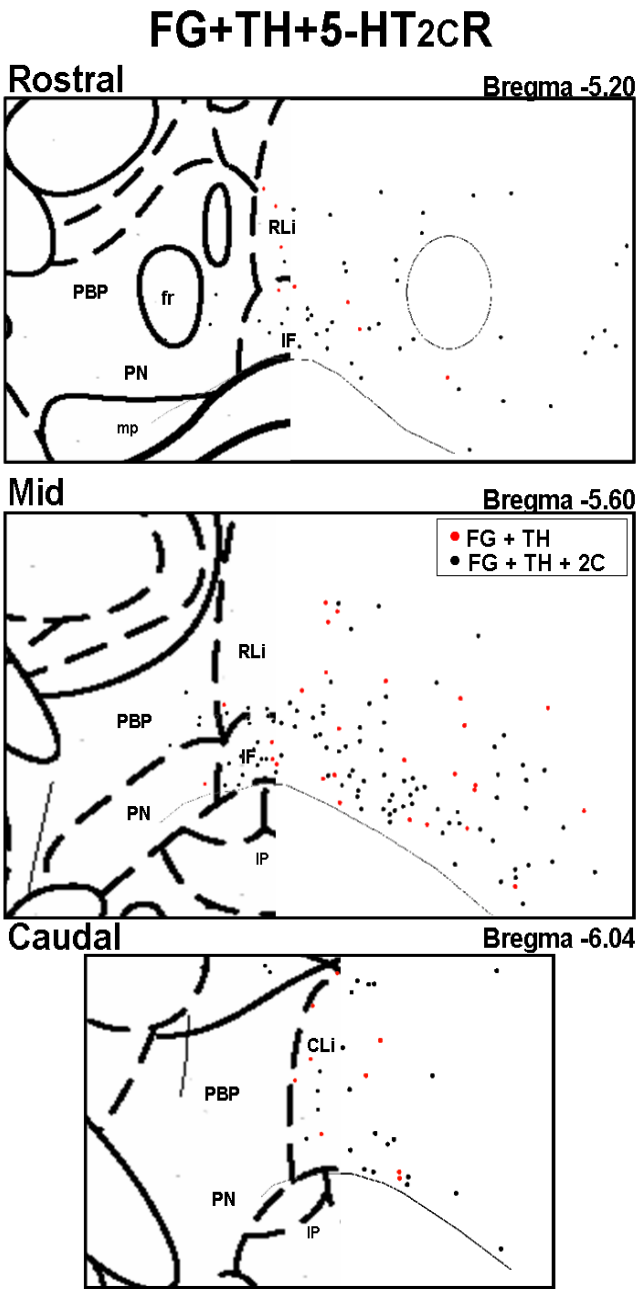

Figure 23. FG/TH- and FG/TH/5-HT$_{2C}$R-co-labeled cells in the VTA. Schematic representation of the distribution of FG/TH- (red) and FG/TH/5-HT$_{2C}$R-co-labled cells (black) in the five subnuclei across the rostral [Bregma -5.20 mm, top], mid [Bregma -5.60 mm; middle], and caudal [Bregma -6.04 mm; bottom] levels of the VTA (Paxinos and Watson, 1998}. Data represent the number and distribution of cells counted in one of three brains injected with FG in the NAcSh. See **Fig. 21** for explanation of abbreviations.

labeled cells across the subnuclei of the VTA (**Fig. 23**) revealed that the co-labeled cells were present in all subnuclei across all levels of the VTA. The distribution patterns of FG/TH/5-HT_{2C}R cells followed that of the total FG/TH cells, with the greatest abundance in the middle level PN subnucleus (**Fig. 23, middle**).

Distribution of FG/GAD-co-labeled cells

A subset of FG cells throughout the VTA contained GAD-IR (**Figs. 24, 25**). A main effect of rostro-caudal level was observed for the total number of FG/GAD-co-labeled cells ($F_{2,30}$ = 19.05; $p < 0.001$; **Table 5**), which also reflected significant differences between all three levels of the VTA, with the greatest number of FG/GAD-co-labeled cells detected in the middle level. However, there was no main effect of rostro-caudal level on the percentage of FG-labeled cells containing GAD-IR ($F_{2,30}$ = 2.86; $p = 0.07$; **Table 5**).

The distribution of the FG/GAD-co-labeled cells in the VTA subnuclei in one rostral, middle, and caudal section from a single rat injected with FG into the NAcSh is illustrated in **Fig. 25**. The sections displayed in this figure are adjacent to those utilized to illustrate FG/TH-co-labeled cells in **Fig. 23**. FG/GAD-co-labeled cells were present in all subnuclei of the VTA, with the fewest cells observed in the RLi. The FG/GAD-co-labeled cells in the rostral VTA appeared to be most abundant within the PBP subnucleus versus other subnuclei (**Fig. 25, top**), while numbers of FG/GAD-co-labeled cells in the middle VTA were equally distributed between PBP and PN (**Fig. 25, middle**), and those in the caudal VTA were similar between the PBP and CLi (**Fig. 25, bottom**).

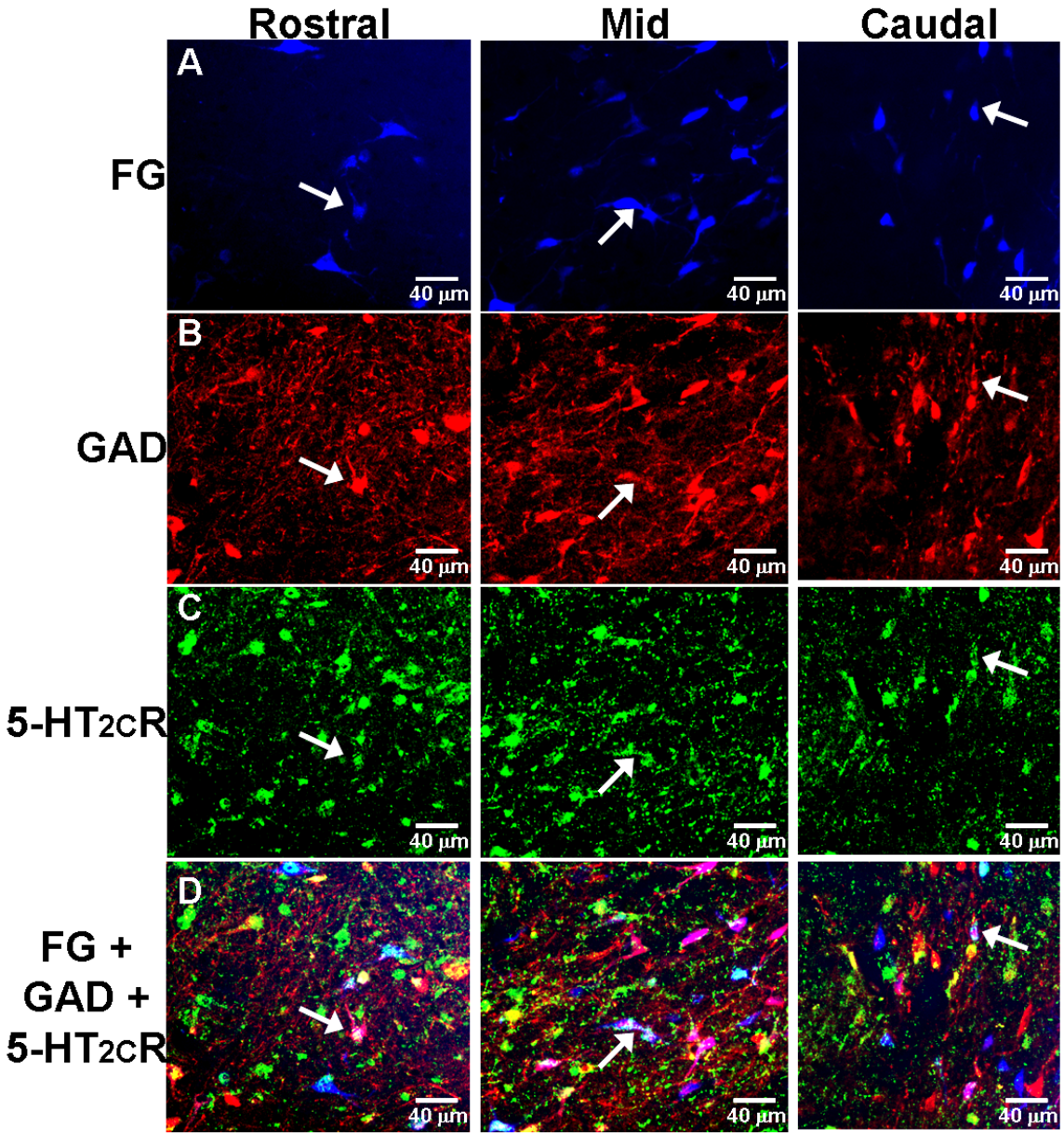

Figure 24. Co-localization of GAD and 5-HT$_{2C}$R immunoreactivity with FG-labeled cells in the VTA. Representative photomicrographs depicting FG labeling [blue; A], GAD-IR [red, B], and 5-HT$_{2C}$R-IR [green, C] in the rostral [left column], middle [middle column], and caudal [right column] levels of the VTA. [D] Overlay of images in A, B, and C. Arrows represent cells triple-labeled for FG, TH, and 5-HT$_{2C}$R. Scale bars = 40 µm.

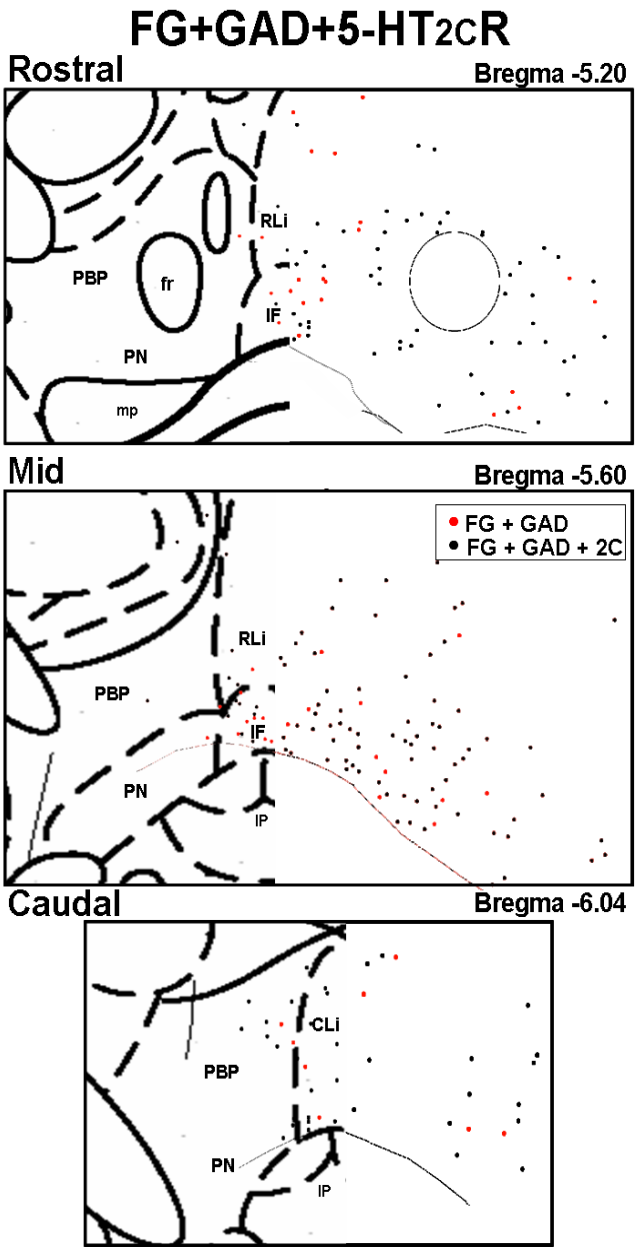

Figure 25. FG/GAD- and FG/GAD/5-HT$_{2C}$R-co-labeled cells in the VTA. Schematic representation of the distribution of FG/GAD- (red) and FG/GAD/5-HT$_{2C}$R-co-labled cells (black) in the five subnuclei across the rostral [Bregma -5.20 mm, top], mid [Bregma -5.60 mm; middle], and caudal [Bregma -6.04 mm; bottom] levels of the VTA (Paxinos and Watson, 1998). Data represent the number and distribution of cells counted in one of three brains (sections adjacent to those in **Fig. 23**) injected with FG in the NAcSh. See **Fig. 21** for explanation of abbreviations.

Distribution of 5-HT_{2C}R-IR in FG/GAD-labeled cells

Immunoreactivity for the 5-HT_{2C}R was observed to be present in the majority of the FG/GAD-co-labeled cells (**Figs. 24,25; Table 5)**, ranging from ~77 – 88% of total FG/GAD-co-labeled cells throughout the VTA. However, there was no main effect of rostro-caudal level on the percentage of FG/GAD-co-labeled cells containing 5-HT_{2C}R-IR ($F_{2,30}$ = 2.19; p = 0.13; **Table 5**). Examination of the distribution of FG/GAD/5-HT_{2C}R-co-labeled cells across the subnuclei of the VTA (**Fig. 25**) revealed that the co-labeled cells were present in all subnuclei across all levels of the VTA. The distribution patterns of FG/GAD/5-HT_{2C}R cells followed that of the total FG/GAD cells described above, there was a noticeably smaller number of FG/GAD/5-HT_{2C}R-labeled cells in the IF subnucleus, particularly in the rostral level of the VTA (**Fig. 25, top**).

TH and GAD-co-localization in the VTA

The observation that sum of the percentages of total FG cells labeled with TH and GAD was greater than 100% for all levels of the VTA (**Table 5**) brought us to examine whether some of the FG-labeled cells contained immunoreactivity for both TH and GAD, when labeled in the same section. Although a thorough analysis of the distribution was not conducted, we examined one section/level of VTA from one of the NAc FG-injected brains for TH and GAD co-localization. From these few sections, it appeared that >60% of the total FG-labeled cells contained immunoreactivity for *both* TH and GAD (**Figs. 26, 27**), as did a large number of non-FG labeled cells (**Fig. 26**). TH/GAD co-localization was present throughout the various subnuclei of the VTA, but were particularly prominent within the mid PN and IF, and the caudal CLi (**Fig. 27**).

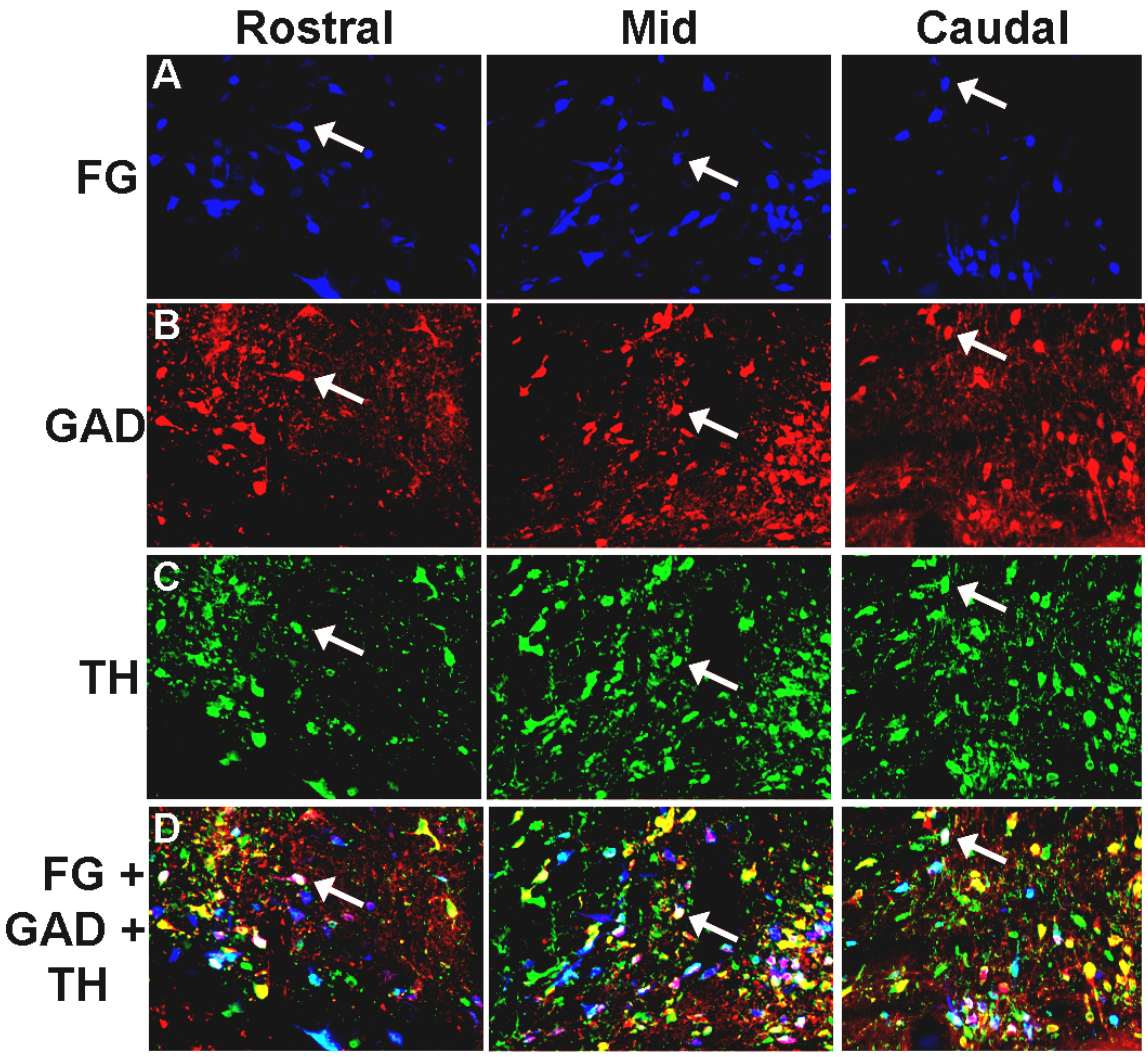

Figure 26. Co-localization of TH and GAD immuonoreactivity with FG-labeled cells in the VTA. Representative photomicrographs depicting FG labeling [blue; A], GAD-IR [red, B], and TH-IR [green, C] in the rostral [left column], mid [middle column], and caudal [right column] levels of the VTA. [D] overlay of images in A, B, and C show cells triple-labeled for FG, TH, and 5-HT_{2C}R (usually white) as indicated by the arrows, and cells double-labeled for TH- and GAD-IR in yellow) Scale bars = 40 μm.

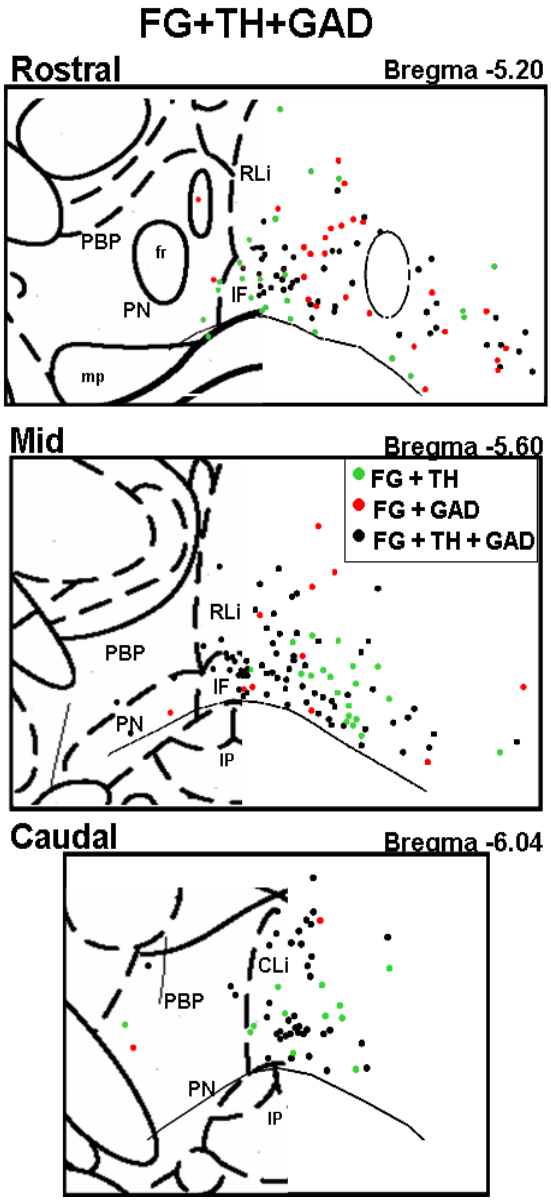

Figure 27. FG/TH-, FG/GAD-, and FG/TH/GAD-co-labeled cells in the VTA. Schematic representation of the distribution of FG/TH- (green), FG/GAD- (red) and FG/TH/GAD-co-labled cells (black) in the five subnuclei across the rostral [Bregma -5.20 mm, top], mid [Bregma -5.60 mm; middle], and caudal [Bregma -6.04 mm; bottom] levels of the VTA. Data represent the distribution of cells observed in one brain (same brain as in **Figs. 23,25**) injected with FG in the NAcSh. See **Fig. 21** for explanation of abbreviations.

DISCUSSION

Using combined retrograde tracing and double-label immunofluorescence techniques, the present study demonstrates that a large number of neurons projecting from the VTA to the NAc can be detected via retrograde labeling with FG. These FG-labeled neurons were most frequently detected in the middle level of the VTA, and similar proportions of the FG-labeled neurons detected contained immunoreactivity for TH or GAD. In addition, across the rostro-caudal extent of the VTA, >70% of both FG/TH and FG/GAD co-labeled cells also contained immunoreactivity for the 5-HT_{2C}R, suggesting that 5-HT_{2C}R are located on the majority of DA and GABA neurons in the VTA identified as projecting to the NAc. Furthermore, although all the subpopulations of NAc-projecting neurons were most prominent in the middle level of the VTA, modest differences in the distribution of these different neuronal subpopulations were observed among the VTA subnuclei, with a larger number of FG-labeled neurons in the rostral PBP containing GAD immunoreactivity. These studies suggest that 5-HT_{2C}R have the potential to exert direct influence upon both DA and GABA VTA neurons that project to the NAcSh. Furthermore, the observation that a large proportion of FG-labeled (and non-FG-labeled) cells contain immunoreactivity for *both* TH and GAD, adds additional complexity to the framework of the VTA.

The present study demonstrates that unilateral injection of FG into the NAc shell resulted in intense labeling of a large number of cells in the ipsilateral VTA. Although FG-labeling was detected in all rostro-caudal levels of the VTA, significantly greater numbers of FG-labeled cells were detected in the middle level of the VTA, compared to rostral or caudal levels, although this may be related to the site of FG injection in the NAc. In addition, FG-labeled neurons were generally confined to the PN, PBP, and IF subnuclei in rostral and mid levels, but were primarily located in the CLi in the caudal VTA. These results coincide with previous reports describing the distribution of VTA neurons that project to the NAc (Swanson, 1982; Van Bockstaele et al., 1994).

Immunofluorescent staining for TH, to identify DA neurons, and GAD 67, to identify GABA neurons, revealed that these VTA projection neurons to the NAc are comprised of both DA and GABA neurons. Previous studies examining DA (Swanson, 1982) or GABA (Van Bockstaele and Pickel, 1995) projections from the VTA to the NAc reported that ~85% of these neurons were DA neurons (Swanson, 1982), while a separate study reported 36% of the projections neurons contained GABA. Conversely, in the present study, when the co-localization of FG with immunoreactivity for DA and GAD was examined in adjacent sections, we found that >70% of the FG-labeled neurons contained immunoreactivity for TH *and* >70% of FG-labeled neurons contained GAD. Thus, the sum of these percentages was greater than 100% of the FG cells. This numeric discrepancy may be due to the fact that the sections were examined and images were captured using light microscopy. Using this method, it is possible that some of the immunoreactivity that was designated to be part of certain cell was actually present in terminals adjacent to that cell, thus resulting in a high number of false positive cells and an over-estimation of the number of cells that contained immunoreactivity for TH and/or GAD. Alternatively, the high percentages of TH- and GAD-immunoreactivity being detected in FG-labeled cells could indicate that some of the cells contained immunoreactivity for both TH and GAD. Indeed, examination of a small number of VTA sections that were double-labeled for TH and GAD, revealed that >60% of all FG-labeled cells as well as a large number of non-FG labeled cells contained immunoreactivity for both TH and GAD in the same cell.

Co-localization of GABA and catecholamines in the same cell has been reported throughout the brain (Kosaka et al., 1987; Hedou et al., 2000), although co-localization of GABA and TH was originally not thought to occur in the VTA (Kosaka et al., 1987; Bayer and Pickel, 1991; Van Bockstaele and Pickel, 1995). However, more recent studies have demonstrated evidence for co-localization of TH and GAD in neurons of the VTA and the closely related substantia nigra (SN). For example, studies have shown the co-localization of TH and GAD mRNA in the SN and lateral VTA (Gonzalez-Hernandez et

al., 2001) as well as the co-localization of TH and GAD protein in the SN neurons via immunofluorescence (Hedou et al., 2000). In addition, the presence of TH and GAD mRNA in the same cell has been demonstrated in the VTA and/or SN via single-cell RT-PCR (Klink, 2001; Korotkova 2003). However, we are the first to demonstrate the presence of TH and GAD protein in the same cells in the VTA. Although we can only speculate that the presence of both TH and GAD equates to the production of both DA and GABA in these cells, the large proportion of the FG labeled neurons containing immunoreactivity for both enzymes suggests the possibility of simultaneous release of the two neurotransmitters within the NAc. Unfortunately, the functional implications of TH and GAD co-localization are not known at the present time. Thus, further studies are necessary to understand the full implications of TH and GAD co-localization, and the potential that these neurotransmitters are co-released from the same neurons.

This study is the first to report the presence of 5-HT_{2C}R-IR in the subpopulations of TH- and GAD-IR neurons that project from the VTA to the NAc. The present findings coincide with the results of the studies described in Chapter 4 demonstrating the co-localization of 5-HT_{2C}R-IR in both TH-IR as well as GAD-IR cells in the VTA. Thus, it appears that the 5-HT_{2C}R has the ability to exert direct control over both DA and GABA neurons located in the VTA that project to the NAcSh. Considering that the 5-HT_{2C}R is known to increase intracellular Ca^{++} (Hoyer et al., 2002) and induce neuronal depolarization (Sheldon and Aghajanian, 1991), stimulation of 5-HT_{2C}R located directly on DA neurons would be expected to enhance VTA DA output. Indeed, systemic administration of the 5-HT_{2C}R antagonist SB 243214 was shown to inhibit firing of spontaneously active VTA DA neurons (Blackburn et al., 2002). However, this study contrasts all other studies which demonstrate that 5-HT_{2C}R antagonists increase, or have no effect on basal VTA DA neuron firing rates (Di Matteo et al., 1999; Di Giovanni et al., 2000; Gobert et al., 2000) as well as DA release in the NAc (Di Matteo et al., 1999; Di Giovanni et al., 2000; Gobert et al., 2000; De Deurwaerdere et al., 2004). Although this discrepancy cannot be fully explained at the present time, the disparity is likely due

to the competing influence of 5-HT_{2C}R localized to GABA neurons in the VTA, which act to indirectly inhibit VTA DA neuron firing and DA release in the NAc through induction of GABA release. For example, local infusion of the 5-HT_{2C}R agonist mCPP into the VTA induced firing of non-DA (presumably GABA) neurons in the VTA (Di Giovanni et al., 2001), while infusion of the 5-HT_{2C}R antagonist SB 206553 into the VTA blocked local GABA release while simultaneously enhancing DA release evoked by systemic administration of (±)-MDMA (Bankson and Yamamoto, 2004). This 5-HT_{2C}R-induced inhibition of VTA DA neuron firing and DA release in the NAc is thought to be mediated through induction of GABA release from axonal collaterals that synapse upon DA neurons within the VTA (Steffensen et al., 1998). However, the discovery of 5-HT_{2C}R on GABA neurons projecting to the NAc (present study) in combination with evidence for an interaction of DA and GABA terminals within the NAc (Pickel et al., 1988) provide the potential for the 5-HT_{2C}R within the VTA to also modulate accumbal DA release through the action of GABA released in the NAc.

Little is known about the functional role of the GABA mesoaccumbens circuit. Researchers have suggested that VTA GABA neurons may also be involved mediating the reinforcing and rewarding properties of drugs via non-DA related mechanisms (Steffensen et al., 1998), potentially playing a critical role in cortical activation and attention to rewarding stimuli (Steffensen et al., 2001). GABA terminals within the NAc have been shown to form synaptic junctions with both GABAergic and non-GABAergic cells and dendrites as well as TH-IR terminals (Pickel et al., 1988), although the majority of GABA terminals seem to principally interact with non-GABAeric dendrites (Pickel et al., 1988), which are likely processes of aspiny acetycholine (ACh) interneurons (Rada et al., 1993; See **Fig**. **28**) However, it is not known which of these GABA terminal synapses are derived from GABA neurons that originate in the VTA. The functional interactions of GABA, ACh, and DA within the NAc are extremely complex and unclear at present (**Fig. 28**); however they appear to be involved in both direct and indirect feedback inhibition loops to the VTA (Domesick, 1988). Thus further investigation into

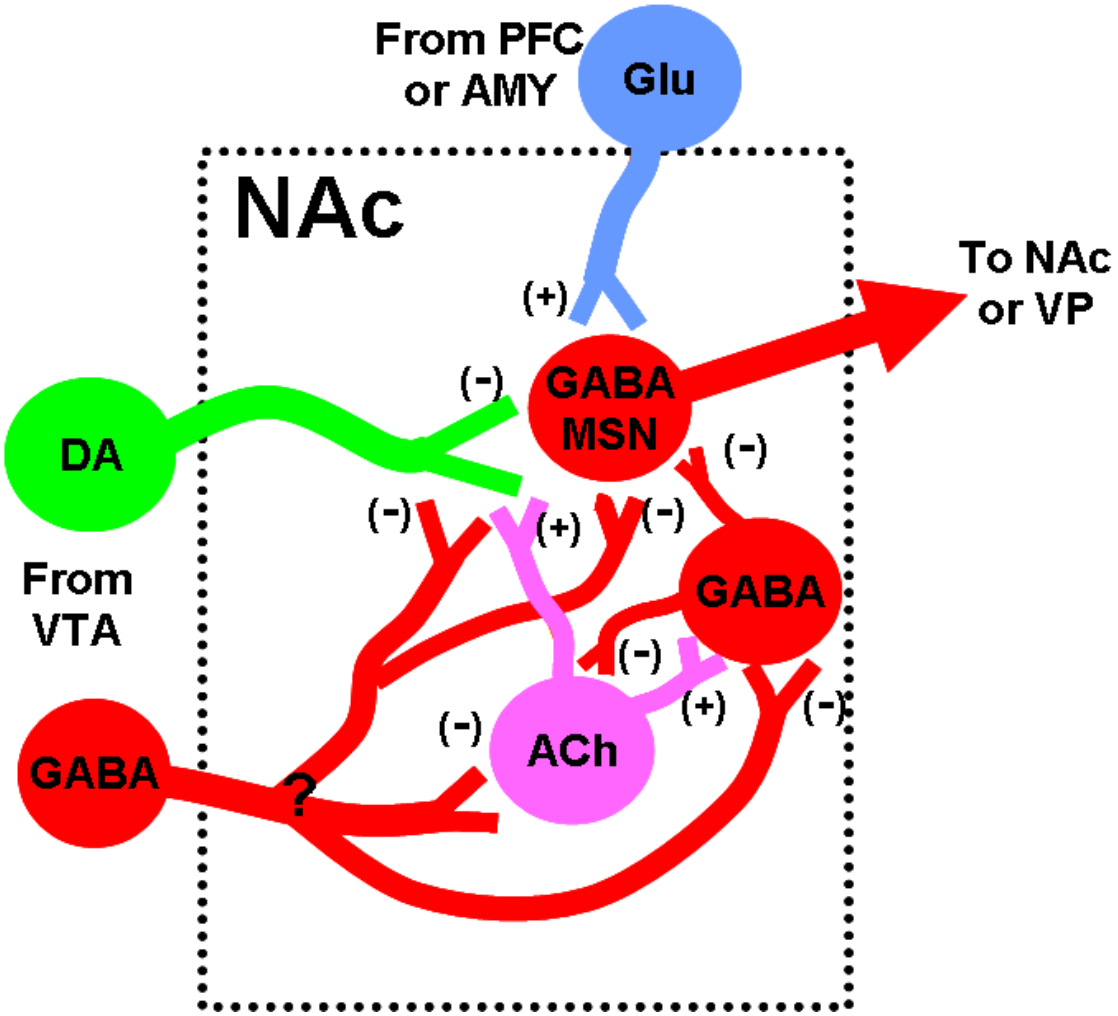

Figure 28. Potential interactions of VTA GABA neurons projecting to the NAc. Illlustration depicts the interactions of GABA terminals with DA, acetylcholine (ACh), glutamate (glu), GABA interneurons, and GABA medium spiny neurons (MSN) in the NAc based upon electron microscopic analysis of GABA terminals in the NAc (Pickel et al., 1988). Thus, GABA neurons projecting from the VTA to the NAc have the potential to synapse on ACh interneurons, GABA interneurons, DA terminals, or GABA medium spiny projection neurons that feedback to the VTA either directly or via the ventral pallidum (VP).

the complex interactions of these neuronal systems within the NAc are necessary before we can begin to understand the consequences of 5-HT_{2C}R-induced GABA release in the NAc.

Analysis of the distribution of TH- and GAD–IR on FG-labeled cells across the rostro-caudal extent of the VTA revealed that while similar proportions of FG/GAD-IR cells were observed across all three levels of the VTA, a significantly lower number of FG-labeled cells contained TH-IR in the rostral VTA compared to the middle and caudal levels. Thus, even though similar proportions of total FG/TH- and FG/GAD-co-labeled cells contained 5-HT_{2C}R-IR throughout the rostro-caudal levels of the VTA, discrete differences in the distribution of 5-HT_{2C}R on these neuronal subpopulations may be apparent. Further examination into the distribution of the different subtypes of FG-co-labeled cells within the subnuclei at each rostro-caudal level of the VTA revealed what appears to be a greater abundance of FG/GAD/5-HT_{2C}R- than FG/TH/5-HT_{2C}R-co-labeled cells in the rostral PBP. The opposite trend was observed in the middle PN, where a greater number of FG/TH/5-HT_{2C}R-co-labeled cells than FG/GAD/5-HT_{2C}R-co-labeled cells were observed. Thus, discrete topographical differences appear to exist in the relative proportion of 5-HT_{2C}R on these DA vs. GABA mesoaccumbens neurons across the different levels/subnuclei of the VTA.

The lack of functional studies examining the differences in the particular subnuclei of the VTA make it difficult to speculate on the impact of the topographical distribution of 5-HT_{2C}R on DA vs. GABA mesoaccumbens function. One might expect that differences in serotonergic innervation to the different VTA subnuclei (Herve et al., 1987) or even to the different neuronal subpopulations may greatly impact the potential of different subpopulations of 5-HT_{2C}R to exert their respective actions. In addition, this regional distribution may also affect electrophysiological recordings as well as studies involving microinfusion of ligands into the VTA. These topographical differences may help explain certain anomalies that have been discovered following systemic and local administration of 5-HT_{2C}R ligands. For example, in electrophysiological studies,

Blackburn et al (1996) noted that systemic administration of a 5-HT_{2C}R antagonist decreased spontaneous firing of VTA DA neurons, while all other studies noted enhancement (Di Matteo et al., 1999; Gobert et al., 2000) or no effect (Di Giovanni et al., 2000) of systemic 5-HT_{2C}R antagonists on VTA DA neuron firing. Additionally, microinfusion of a 5-HT_{2C}R agonist into the VTA was shown to reduce cocaine evoked hyperactivity (Fletcher et al., 2004), while intra-VTA infusion of a 5-HT_{2C}R antagonist had no effect on cocaine-evoked hyperactivity (McMahon et al., 2001). Thus, these discrepancies may be accounted for by differential activation of 5-HT_{2C}R expressed on DA vs. GABA neurons based upon slight variations in anatomical placements of electrodes or cannula.

Although we were not able to directly examine whether 5-HT_{2C}R are localized to the mesoaccumbens neurons displaying co-localization for TH and GAD, given that >70% of FG-labeled neurons contained 5-HT_{2C}R immunoreactivity and >60% of FG-labeled neurons contained co-localization of TH- and GAD-immunoreactivity, it may be inferred that a minimum of 30% of these neurons likely overlap. Unfortunately, since the characteristics of these TH/GAD-IR mesoaccumbens neurons are unknown at the present time, it is difficult to speculate how stimulation of 5-HT_{2C}R with neurons containing both DA and GABA would contribute to the overall interactions of 5-HT_{2C}R, DA, and GABA within the VTA.

In summary, the present study reveals that 5-HT_{2C}R immunoreactivity is co-localized with subsets of TH- and GAD-immunoreactive neurons in the VTA that project to the NAc, with modest differences in distribution of these neuronal subtypes across the rostral-caudal levels of the VTA subnuclei. In addition, TH and GAD immunoreactivity appeared to be co-localized in a large population of the mesoaccumbens-projecting neurons. These novel findings provide a complex picture of 5-HT_{2C}R/TH/GAD interaction within the VTA and suggest that regulation of NAc DA release by 5-HT_{2C}R may be multifaceted. However, further investigation is required to fully understand the

functional effects of both the 5-HT_{2C}R localization on DA and GABA mesoaccumbens neurons as well as the co-localization of TH and GAD within these neurons.

CHAPTER 6:

CONCLUSIONS

The studies described here were conducted to gain further insight into the functional adaptations of 5-HT_{2C}R in response to repeated (+)-MDMA administration and whether these adaptations may contribute to behavioral sensitization, as well as to examine the distribution of 5-HT_{2C}R on neurons within the VTA and the potential sites of action for these receptors to influence activity of DA mesoaccumbens neurons. Indeed, these studies demonstrate that diminished function of the 5-HT_{2C}R is likely to play an integral role in the induction and early expression of (+)-MDMA sensitization, while enhanced expression of 5-HT_{2C}R in the VTA may contribute to the persistence of enhanced (+)-MDMA-evoked hyperactivity. This up-regulation of 5-HT_{2C}R may occur on DA and/or GABA neurons in the VTA, since 5-HT_{2C}R were shown to be expressed in both types of neurons, including subpopulations of each that directly project to the NAc.

In general, evidence in the literature indicates that the 5-HT_{2C}R serves to exert an overall inhibition of behavioral effects elicited by a variety of compounds (for reviews, see Higgins and Fletcher, 2003; Giorgetti and Tecott, 2004), and that this receptor is a prominent mediator of the broad serotonergic influence in the effects of psychostimulants, antidepressants, and antipsychotics (see Higgins and Fletcher, 2003; Giorgetti and Tecott, 2004). The inhibitory influence of 5-HT_{2C}R on behavior, which is likely due to inhibition of DA mesocorticoaccumbens pathway activation (Di Giovanni et al., 2000; Di Matteo et al., 2002), has been demonstrated through pharmacological manipulation of 5-HT_{2C}R as well in 5-HT_{2C}R KO mice. The 5-HT_{2C}R KO mice, for example, have been shown to display enhanced hyperactivity, reinforcing effects, and NAc DA release in response to cocaine administration compared to wildtype littermates (Rocha et al., 2002). In keeping with this notion, the studies described in Chapters 2 and 3 reveal that a decrease in the functional responsiveness of 5-HT_{2C}R was associated with an enhancement of (+)-MDMA evoked hyperactivity, or behavioral sensitization, during

early withdrawal from repeated pretreatment with either (+)-MDMA or the 5-HT_{2C}R agonist MK 212. Thus, since repeated (+)-MDMA pretreatment can induce diminution of 5-HT_{2C}R function, and, in turn, the loss of 5-HT_{2C}R function can induce enhancement of (+)-MDMA-evoked hyperactivity, these results suggest that the diminished function of the 5-HT_{2C}R is an integral component of the induction and early expression of (+)-MDMA sensitization.

The experiments described in Chapters 4 and 5 reveal that in contrast to current dogma, 5-HT_{2C}R are localized to subpopulations of both GABA and DA neurons (and those that potentially release both neurotransmitters) in the VTA. Examination of the few studies in the literature employing intra-VTA 5-HT_{2C}R ligand infusion (McMahon et al., 2001; Pozzi et al., 2002; Fletcher et al., 2004; Bankson and Yamamoto, 2004) suggests that under basal conditions these two subpopulations of 5-HT_{2C}R within the VTA (i.e., 5-HT_{2C}R located on DA vs. GABA neurons) counteract one another, neutralizing any influence that either population alone would exert upon DA mesocorticoaccumbens activation (**Fig. 29**). Conversely, under circumstances in which extrinsic influences upon the VTA are engaged, such as in response to systemic 5-HT_{2C}R ligand (Di Matteo et al., 1999; Di Giovanni et al., 1999; Gobert et al., 2000) or psychostimulant (Fletcher et al., 2004; Bankson and Yamamoto, 2004) administration, the indirect inhibitory influence upon DA mesoaccumbens output mediated by populations of 5-HT_{2C}R located on VTA GABA neurons seems to be enhanced, thereby superseding the effect of the direct depolarizing action of 5-HT_{2C}R located directly on VTA DA neurons that project to the NAc (and potentially the PFC).

The enhanced inhibition of DA mesocorticoaccumbens output upon systemic 5-HT_{2C}R ligand or psychostimulant administration may be a result of feedback to the VTA from extrinsic sources that converge upon GABA and/or DA neurons to influence their firing rates (**Fig. 30**). For example, glutamate neurons from the PFC have been shown to

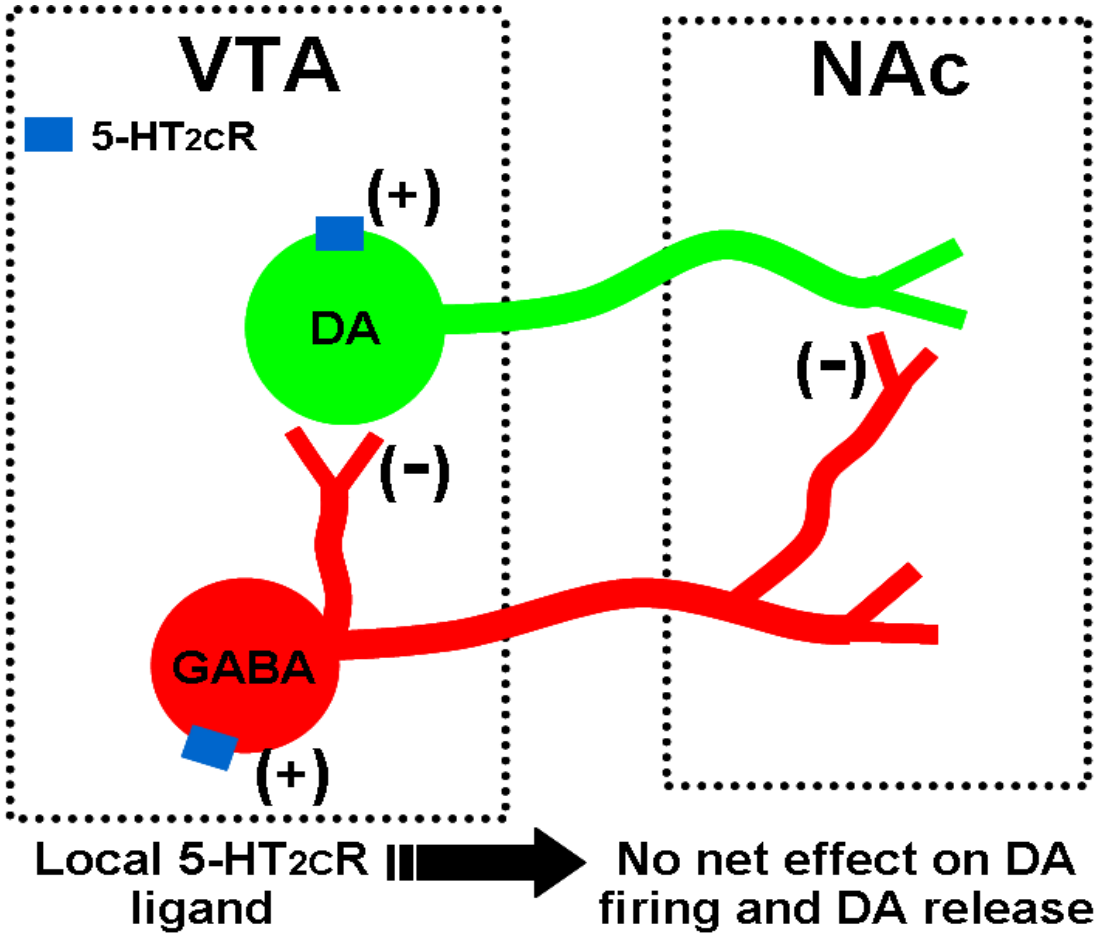

Figure 29. Sites of 5-HT$_{2C}$R interaction with DA and GABA neurons in the VTA. Illustration depicts the localization of 5-HT$_{2C}$R (blue squares) on both DA (green) and GABA (red) neurons in the VTA. Stimulation 5-HT$_{2C}$R localized to VTA DA neurons would be expected to induce depolarization of DA neurons and increase release of DA in the NAc. Stimulation of 5-HT$_{2C}$R on GABA neurons would likewise induce GABA neuron depolarization resulting in release of GABA both locally in the VTA and in the NAc, resulting in inhibition of DA neuron firing and decreased DA release. The competing influence of these two VTA 5-HT$_{2C}$R subpopulations would be expected to result in no net effect of local 5-HT$_{2C}$R ligand administration into the VTA on DA neuron firing and DA release in the NAc.

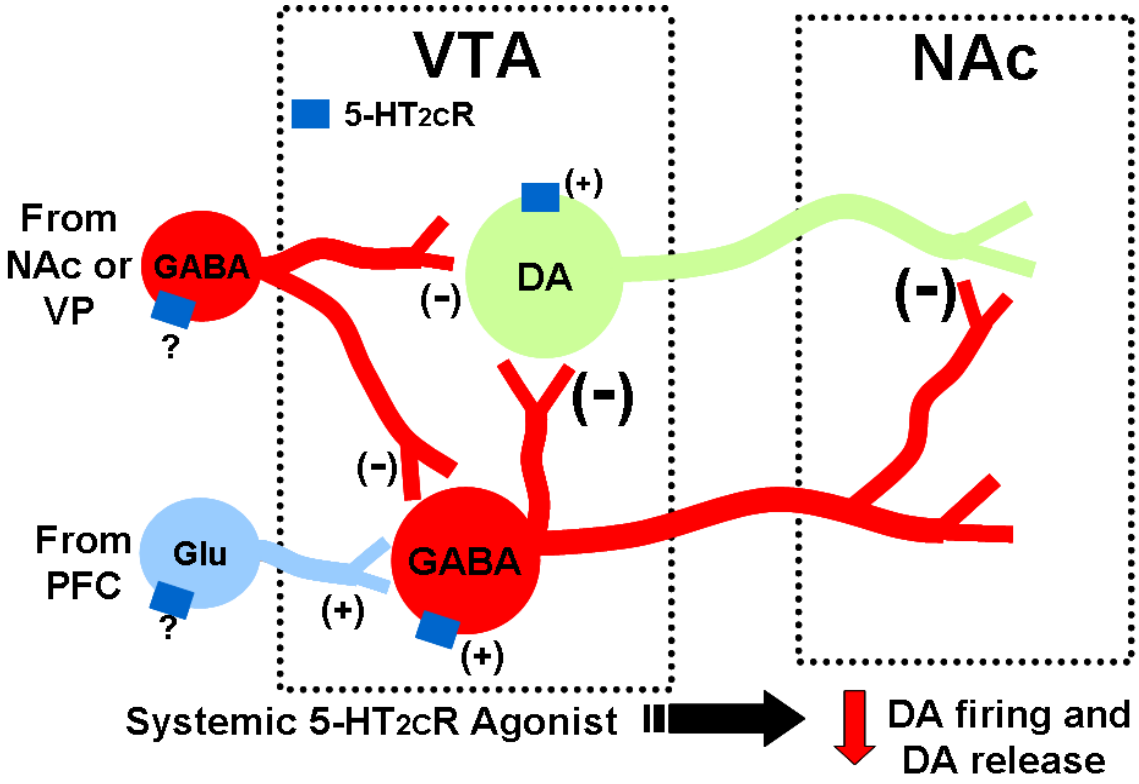

Figure 30. Extrinsic influence upon 5-HT$_{2C}$R interaction with DA and GABA neurons in the VTA. Illustration depicts the innervation of VTA DA and GABA neurons by GABA neurons (red) from the NAc or ventral pallidum (VP; Kalivas et al., 1993) and the selective innervation of GABA mesoaccumbens neurons by glutamate neurons (Glu; light blue) from the PFC (Carr and Sesack, 2000b). Convergence of these inputs onto VTA GABA neurons enhances the inhibitory influence of GABA upon DA neuron firing and DA release, thereby overriding the stimulatory effect of 5-HT$_{2C}$R (blue squares) located directly on VTA DA neurons. The potential for localization of the 5-HT$_{2C}$R on GABA neurons in the NAc and/or VP and Glu neurons in the PFC is also illustrated. The dominance of GABA inhibition upon engagement of extrinsic influences into the VTA following systemic 5-HT$_{2C}$R agonist administration would result in a decrease in DA neuron firing and DA release in the NAc (or vice versa following 5-HT$_{2C}$R antagonist administration).

exclusively innervate VTA GABA, but not DA, neurons that project to the NAc, thus providing a source for selective stimulation of GABA neurons that may in turn inhibit DA neurons projecting to the NAc (Carr and Sesack, 2000b). In addition, feedback from GABA neurons that originate in the NAc and/or ventral pallidum may work in concert to inhibit VTA DA and GABA neurons (Kalivas et al., 1993a; Steffensen et al., 1998; Carr and Sesack, 2000b; Korotkova et al., 2004). Collectively these influences would work together with the populations of 5-HT_{2C}R on VTA GABA neurons to promote inhibition of VTA DA neuron firing and DA release in terminal regions. Furthermore, since the 5-HT_{2C}R has been localized to the NAc, PFC, and VP (Pompeiano et al., 1994; Eberle-Wang et al., 1997), stimulation of 5-HT_{2C}R in these regions may also be involved in modulation of these feedback circuits to the VTA (**Fig. 30**).

The influence of the different subpopulations of 5-HT_{2C}R in the VTA (or other regions) is likely altered as a consequence of the recurring indirect or direct stimulation of 5-HT_{2C}R during (and subsequent to) repeated (+)-MDMA or MK 212 pretreatment, respectively. No studies to date have examined the potential alterations in the influence of different populations of 5-HT_{2C}R on DA neuron firing or DA release in terminal regions following repeated (+)-MDMA or other psychostimulant activation. However, the suggestion of diminished 5-HT_{2C}R function in early withdrawal from repeated (+)-MDMA or MK 212 pretreatment in the present studies may indicate a reduction in the indirect inhibitory influence of VTA 5-HT_{2C}R via GABA neurons on DA neuron firing and control of DA release in terminal regions. These early alterations in 5-HT_{2C}R function are seemingly not due to changes in the levels of 5-HT_{2C}R expression, but rather are likely due to forms of receptor regulation such as differential mRNA editing. The unique regulation of 5-HT_{2C}R function through mRNA editing is enhanced in response to both increased levels of 5-HT and repeated 5-HT agonist treatment (Gurevich et al., 2002a). Enhancement of 5-HT_{2C}R mRNA editing leads to expression of 5-HT_{2C}R isoforms with reduced agonist affinity and functional efficacy (Herrick-Davis et al., 1999), and thus could account for the reduced functional efficacy of the 5-HT_{2C}R agonist

in the MK 212 and (+)-MDMA pretreated rats. Furthermore, increased expression of edited isoforms were also detected in the PFC human suicide victims with a history of major depressive disorder (Gurevich et al., 2002b), suggesting a link between 5-HT_{2C}R mRNA editing and depression. Thus, changes in 5-HT_{2C}R mRNA editing may be an important factor in the depressive component of the sequelae observed in early withdrawal from an MDMA binge, as well as during withdrawal from other drugs of abuse, in particular those that have been shown to cross-sensitize with MDMA (Callaway and Geyer, 1992b; Morgan et al., 1997; Kalivas et al., 1998).

Interestingly, the persistent enhancement of (+)-MDMA-evoked hyperactivity observed at the 2 wk withdrawal time-point in repeated MK 212, but not (+)-MDMA pretreated rats, was associated with an unexpected up-regulation of 5-HT_{2C}R protein expression in the VTA (see Chapter 4). One may speculate based on the anatomical data that this paradoxical observation is due to increased expression of the 5-HT_{2C}R within VTA DA neurons, thereby causing the direct depolarizing effect on VTA DA neurons to dominate over the inhibitory influence that other populations of 5-HT_{2C}R may exert through their actions at GABA neurons. As noted in the Discussion in Chapter 4, this loss of inhibitory influence of 5-HT_{2C}R may also function to unmask actions of other 5-HT_{2C}R, namely the 5-HT_{1B}R and the 5-HT_{2A}R, that serve to facilitate expression of behavioral sensitization, resulting in cooperative actions of these receptors upon DA mesococorticoaccumbens pathway activation.

In conclusion, the studies conducted herein demonstrate that both the initiation and maintenance of enhanced (+)-MDMA-evoked hyperactivity is associated with alterations in function and/or expression of 5-HT_{2C}R, particularly within subpopulations of 5-HT_{2C}R that function to modulate DA release within the mesocorticoaccumbens "reward" circuit. The 5-HT_{2C}R exerts a complex modulatory influence upon VTA mesocorticoaccumbens pathway activation that is mediated by a variety of subpopulations of 5-HT_{2C}R located on neurons both within and outside of the VTA. The localization of the 5-HT_{2C}R on subpopulations of both GABA and DA neurons in the

VTA indicates that although an indirect inhibitory action of 5-HT_{2C}R tends to dictate the firing patterns of DA mesocorticoaccumbens neurons, the potential for a dominant stimulatory effect upon DA mesocorticoaccumbens pathway activation also exists, which may be influential in the maintenance of sensitization.

The intricacy of 5-HT_{2C}R modulation of DA mesocorticoaccumbens reward pathway activation in combination with the role for the 5-HT_{2C}R in the induction and maintenance of sensitization suggest that 5-HT_{2C}R may be a potential target for the development of therapies to modulate the efficacy of reward pathway stimulation. The 5-HT_{2C}R agonist RO-60,0175 has been shown to reduce the self-administration of both food (Grottick et al., 2000; Fletcher et al., 2004) and several drugs of abuse including cocaine (Grottick et al., 2000; Fletcher et al., 2004), nicotine (Grottick et al., 2001) and ethanol (Tomkins et al., 2002), suggesting that 5-HT_{2C}R agonists may be effective in reducing the reinforcing value of compounds that engage the DA mesocorticoaccumbens reward pathways. Indeed, 5-HT_{2C}R agonists are being developed for Phase I clinical trials for treatment of obesity (Vernalis Announcement, 2002), and if proven effective may pave the way for the examination of these compounds as treatments for drug dependence to reduce the reinforcing value of the drugs. Furthermore, 5-HT_{2C}R agonists have also been shown to reduce the ability of cocaine to reinstate responding for cocaine self-administration (Grottick et al., 2000) suggesting that administration of 5-HT_{2C}R agonists may also prove to be effective in preventing relapse to drug use. Alternatively, the 5-HT_{2C}R antagonist agomelatine (S 20098) was shown to be useful in the treatment of major depressive disorder and alleviating anxiety associated with depression (Loo et al., 2002), suggesting that 5-HT_{2C}R antagonists may be useful in alleviating the depressive and anxiolytic components of psychostimulant withdrawal. Taken together these studies indicate a potential for use of 5-HT_{2C}R ligands in the treatment of drug dependence, prevention of relapse, and/or the alleviation of withdrawal symptoms. However, follow-up studies to those described herein are necessary to better understand the implications of

5-HT_{2C}R locale and adaptations in response to repeated ligand administration prior to initiation of 5-HT_{2C}R ligands in treatment for drug dependence or psychiatric disorders.

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APPENDIX A[1]

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Selective serotonin reuptake inhibitors enhance cocaine-induced locomotor activity and dopamine release in the nucleus accumbens☆

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Abstract

The role for serotonin (5-HT) in mediating the behavioral effects of cocaine may be related in part to the ability of 5-HT to modulate the function of the dopamine (DA) mesoaccumbens pathways. In the present study, the ability of the selective serotonin reuptake inhibitors (SSRIs) fluoxetine (10 mg/kg, IP) and fluvoxamine (10 and 20 mg/kg, IP) to alter cocaine (10 mg/kg, IP)-induced hyperactivity and DA release in the nucleus accumbens (NAc) was analyzed in male Sprague–Dawley rats. Systemic administration of either fluoxetine or fluvoxamine enhanced cocaine-induced locomotor activity in a dose-dependent manner; fluoxetine (10 mg/kg, IP) also enhanced cocaine (10 mg/kg, IP)-induced DA efflux in the NAc. To test the hypothesis that the NAc serves as the locus of action underlying these effects following systemic cocaine administration, fluoxetine (1 and 3 μg/0.2 μl/side) or fluvoxamine (1 and 3 μg/0.2 μl/side) was microinfused into the NAc shell prior to systemic administration of cocaine (10 mg/kg, IP). Intra-NAc shell infusion of 3 μg of fluoxetine or fluvoxamine enhanced cocaine-induced hyperactivity, while infusion of fluoxetine (1 μM) through the microdialysis probe implanted into the NAc shell enhanced cocaine (10 mg/kg, IP)-induced DA efflux in the NAc. Thus, the ability of systemic injection of SSRIs to enhance cocaine-evoked hyperactivity and DA efflux in the NAc is mediated in part by local actions of the SSRIs in the NAc.

Keywords: Cocaine; Fluoxetine; Fluvoxamine; Locomotor activity; Dopamine microdialysis; Nucleus accumbens

1. Introduction

As cocaine abuse and dependence continue to afflict our society, emphasis has been placed upon understanding the neurobiology of cocaine to ultimately enable more effective management of cocaine dependence. The hyperlocomotive, discriminative stimulus, and reinforcing properties of cocaine are thought to be principally controlled via elevation of dopamine (DA) transmission, in particular that of the DA mesoaccumbens pathway (for review see Erinoff and Brown, 1994). However, in addition to enhancing efflux of DA via blockade of the DA reuptake transporter (DAT), cocaine also blocks the ability of the serotonin (5-HT) and norepinephrine (NE) reuptake transporters to transfer their respective neurotransmitter out of the synapse, thereby increasing the extracellular concentrations of 5-HT and NE as well as DA (Koe, 1976). Serotonin neurons innervate both the ventral tegmental area (VTA) and nucleus accumbens (NAc), the origin and terminal of the DA mesoaccumbens pathway, respectively (Broderick and Phelix, 1997), and 5-HT is capable of modulating DA efflux in the NAc

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(Parsons and Justice, 1993). Thus it is not surprising that manipulation of the 5-HT system has also been shown to alter the behavioral effects of cocaine (Walsh and Cunningham, 1997).

Selective serotonin reuptake inhibitors (SSRIs) are frequently used as tools to pharmacologically elevate synaptic 5-HT levels (Jordan et al., 1994), and are reported to augment the in vivo effects of indirect catecholamine agonists. For example, acute treatment with the SSRI fluoxetine enhanced amphetamine-induced hyperactivity as well as amphetamine-induced release of DA in the NAc (Sills et al., 1999a), and pretreatment with the SSRI fluvoxamine enhanced hyperactivity induced by the DA and NE reuptake blocker mazindol (McMahon and Cunningham, 2001b). While the SSRIs fluoxetine and fluvoxamine are also reported to potentiate the behavioral effects of cocaine (Cunningham and Callahan, 1991; Herges and Taylor, 1998; Reith et al., 1991), conflicting results have been observed (Reith et al., 1991). However, it is important to note that several variables, including sex, species, dose, and route of administration, differed across these studies. Thus in an effort to clarify the interaction between SSRIs and cocaine, we have chosen to study the effects of the SSRIs fluoxetine and fluvoxamine on cocaine-induced hyperactivity and NAc DA efflux in male Sprague–Dawley rats.

In addition to testing the hypothesis that systemic administration of these SSRIs will dose-dependently enhance cocaine-induced hyperactivity and DA efflux in the NAc, we have also tested the hypothesis that the NAc is a principle site of action for SSRIs to enhance the in vivo effects of cocaine. Studies have shown that cocaine administration preferentially increases DA in the shell of the NAc relative to the core (Pontieri et al., 1995) and that the extracellular concentration of DA in the NAc shell is positively correlated with increases in locomotor activity (Hedou et al., 1999). Thus, the SSRIs were microinfused into the NAc shell prior to a systemic cocaine injection, and locomotor activity and NAc DA efflux were assessed.

2. Materials and methods

2.1. Animals

Male Sprague–Dawley rats ($n = 42$, Harlan, Houston, TX; $n = 52$, IFFA CREDO, Lyon, France) weighing 300–350 g at the beginning of the experiment were used. The rats were housed 2 or 3 per cage in standard plastic rodent cages in a colony room maintained at 21±2°C and at 40–50% humidity under a 12 h light-dark cycle (lights on at 0700 h). Rats surgically fitted with indwelling bilateral or unilateral guide cannulae (for microdialysis) were housed individually. Each rat was provided with continuous access to tap water and rodent chow except during experimental sessions. All experiments were conducted during the light phase of the light–dark cycle (between 0900–1400 h), and were carried out in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and approval from the Institutional Animal Care and Use Committee, and with the International European Ethical Standards (86/609-EEC) and the French National Committee (*décret* 87/848) for the care and use of laboratory animals. All efforts were made to minimize animal suffering and to reduce the number of animals used.

2.2. Drugs

The following drugs were used: cocaine hydrochloride (National Institute of Drug Abuse, Research Triangle Park, NC, USA; and Calaire Chimie, Calais, France); fluvoxamine maleate (Solvay, Marietta, GA), and fluoxetine hydrochloride, kindly provided by Ely Lilly (Indianapolis, IN, USA). All other chemicals and reagents were the purest commercially available (Merck Eurolab, Sigma).

All the drugs used were dissolved in sterile saline (0.9% NaCl), and injected either intraperitoneally (IP) in a volume of 1 ml/kg, or intracranially (fluvoxamine and fluoxetine only) in a volume of 0.2 µl/side. All solutions injected centrally were adjusted to pH 7.0. All drug doses were calculated as the salt. In reverse microdialysis experiments, fluoxetine was directly dissolved in the artificial cerebro-spinal fluid (aCSF, see Surgery and microdialysis, section 2.4.1).

2.3. Behavioral experiments

2.3.1. Surgery and cannulae implantation

Rats underwent surgical implantation of 22-gauge stainless steel bilateral guide cannulae (Small Parts Inc., Miami Lakes, FL, USA). Each rat was anesthetized using an intramuscular (IM) injection of 43 mg/kg of ketamine, 8.6 mg/kg of xylazine and 1.5 mg/kg of acepromazine in physiological saline (0.9% NaCl). With the upper incisor bar of a stereotaxic instrument (Kopf Instruments, Tujunga, CA, USA) positioned at −3.8 mm below the interaural line and using the intersection of the bregma and longitudinal sutures as the origin, the ventral surfaces of the bilateral guide cannulae were positioned 2 mm above the NAc shell (AP = + 1.7 mm, ML = ± 0.75 mm, and DV = −6 mm; Paxinos and Watson, 1998). The guide cannulae were fastened to the skull with stainless steel screws (Small Parts Inc.) and cranioplastic cement (Plastics One, Inc., Roanoke, VA, USA) and were fitted with 28-gauge stainless steel bilateral obturators (Plastics One, Inc.). Rats received a single injection of 300,000 units (IM) of sodium ampicillin after surgery and were allowed a 1-week recovery period

during which rats were handled and weighed daily. Following the initial 1-week recovery period, each rat was habituated to the brief confinement associated with intracranial microinjections by removing the 28-gauge internal obturator, gently restraining the rat for approximately 3 min, and replacing the obturator. For each intra-NAc shell microinjection, the obturators were removed and two internal cannulae (Plastics One) were positioned so as to extend 2 mm below the tips of the bilateral guide cannulae. The bilateral internal cannulae were attached to two 5-µl syringes (Hamilton Co., Reno, NV, USA) via PE-50 tubing (Clay-Adams, Parsippany, NJ, USA). A microsyringe drive (Baby Bee, Bioanalytical Systems, West Lafayette, IN, USA) driven by a programmable controller (Bee Hive Controller, Bioanalytical Systems) delivered a volume of 0.2 µl/side at a rate of 0.1 µl/min. The injection cannulae remained in place for an additional 1 min to allow for diffusion away from the cannulae tips.

2.3.2. Measurement of locomotor activity

2.3.2.1. Apparatus Locomotor activity was monitored and quantified using a modified open field activity system under low light conditions (San Diego Instruments, San Diego, CA). Each clear plexiglass chamber (40 cm × 40 cm × 40 cm) was housed within a sound-attenuating enclosure and was surrounded with a 4 × 4 photobeam matrix located 4 cm from the floor surface. Interruptions of the photobeams resulted in counts of activity in the peripheral and central fields of the chamber. Activity recorded in the inner 16 × 16 cm of the open field was counted as central activity while the field bounded by the outer 12 cm band registered peripheral activity. Another horizontal row of 16 photobeams, located 16 cm from the floor surface, provided each chamber with a measurement of vertical activity (rearing). Separate counts of peripheral, central, and vertical activity were made by the control software (Photobeam Activity Software, San Diego Instruments) and stored for subsequent statistical evaluation. Video cameras positioned above the chambers permitted continuous observation of behavior without disruption.

2.3.2.2. Behavioral protocols: systemic injections All rats were habituated to the test environment for 3 h/day on each of the 2 days before the start of the experiment, and on each test day for 1 h before the administration of drugs. Using a repeated measures design and four or six test sessions, one group of rats (n = 15) received either saline (1 ml/kg) or fluoxetine (10 mg/kg, IP), and a second group of rats (n = 8) received an injection of either saline (1 ml/kg, IP) or fluvoxamine (10 or 20 mg/kg, IP); each injection was followed 60 min later by an injection of either saline (1 ml/kg, IP) or cocaine (10 mg/kg, IP). Measurements of locomotor activity began immediately after the cocaine injection and were taken for a total of 60 min. Test sessions were conducted every 2 days, and the order of fluoxetine or fluvoxamine tests were counterbalanced for each rat. Systemic cocaine injections were given every other test.

2.3.2.3. Behavioral protocols: intra-NAc shell microinfusions Surgically-implanted rats were habituated to the test environment for 3 h/day on each of the 2 days before the start of the experiment, and on each test day for 1 h before the administration of drugs. Using a repeated measures design and six test sessions, one group of rats (n = 8) received an intra-NAc shell microinjection of either saline (0.2 µl/side) or fluoxetine (1 or 3 µg/0.2 µl/side), and a second group of animals (n = 11) received an intra-NAc shell microinjection of either saline (0.2 µl/side) or fluvoxamine (1 or 3 µg/0.2 µl/side); each microinjection was immediately followed by an IP injection of either saline (1 ml/kg) or cocaine (10 mg/kg). Measurements of locomotor activity began immediately after the systemic injection and were taken for a total of 60 min. The interval between test sessions was 3–7 days, and the order of microinjections was counterbalanced for each rat. Systemic cocaine injections were given every other test and only once per week.

2.3.3. Histology

At the completion of the study, rats that had received guide cannulae were overdosed with chloral hydrate (800 mg/kg, i.p.), the brains were removed and stored in a 20% sucrose/10% formalin solution for at least 3 days before sectioning. Brain sections (50 µm) were mounted onto gelatin-coated glass slides. The brain sections were defatted, stained with cresyl violet, cleared with xylene and cover-slipped. The cannulae placements were verified using a light microscope. Only those animals whose cannulae were within the shell of the NAc were included for statistical analysis (Paxinos and Watson, 1998). No significant tissue damage was evident upon histological examination of sections.

2.4. Microdialysis experiments

2.4.1. Surgery and microdialysis

Microdialysis experiments were performed in freely-moving animals according to procedures previously described (Lucas et al., 2001). Briefly, a siliconized stainless steel guide-cannula (Carnegie Medicin, Phymep, Paris, France) was stereotaxically implanted under chloral hydrate (400 mg/kg, IP) anesthesia just above the right shell of the nucleus accumbens (NAc) (coordinates in mm, relative to the interaural point: AP = 10.6, L = 0.9, V = 4; Paxinos and Watson, 1998), and permanently fixed to the skull with stainless steel screws and methylacrylic cement. Five to seven days after surgery, a microdialysis probe (CMA 11, cuprophan, 240 µm outer

diameter, 2 mm length, Carnegie Medicin, Phymep) was filled with aCSF, containing (in mM): 154.1 Cl^-, 147 Na^+, 2.7 K^+, 1 Mg^{2+}, and 1.2 Ca^{2+}, adjusted to pH 7.4 with 2 mM sodium phosphate buffer, and lowered through the guide-cannula so that the tip of the probe reached a depth value of 2 mm above the interaural point. After an 18 h stabilization period, the probe was connected to a two channel liquid swivel (Carnegie Medicin, Phymep) and perfused at a constant flow rate of 2 μl/min with the aCSF by means of a microperfusion pump (CMA100, Carnegie Medicin, Phymep). Dialysate fractions (30 μl) were collected every 15 min. The in vitro recovery of the probes was approximately 10% for DA. At the end of each experiment, the animal was sacrificed with an overdose of chloral hydrate (800 mg/kg, IP), and its brain was removed and fixed with 10% paraformaldehyde in NaCl (0.9%). The location of the probe was determined histologically on serial coronal sections (60 μm) stained with cresyl violet. Only those animals (40 of a total cohort of 52 animals) whose cannulae were within the shell of the NAc were included for statistical analysis.

2.4.2. Chromatographic analysis

Dialysate samples were immediately analyzed by reverse-phase HPLC coupled with electrochemical detection, as previously described (Bonhomme et al., 1995). The mobile phase [containing (in mM) 70 NaH_2PO_4, 0.1 Na_2EDTA, 0.7 triethylamine and 0.1 octylsulfonic acid plus 10% methanol, adjusted to pH 4.8 with ortophosphoric acid] was delivered at 1 ml/min flow rate (system LC-10AD-*VP*, Shimadzu, Touzard & Matignon, Paris, France) through a Hypersyl column (C18; 4.6 × 150 mm; particle size, 5 μm; Shimadzu, Touzard & Matignon). Detection of DA was carried out with a coulometric detector (Coulochem II, ESA, Paris, France) coupled to a dual electrode analytic cell (model 5011). The potential of the electrodes was set at −175 and +175 mV. Output signals were recorded on a computer (system class VP-4, Shimadzu, Touzard & Matignon). Under these conditions, the sensitivity for DA was 0.5 pg/30 μl with a signal/noise ratio of 3:1.

2.4.3. Pharmacological treatments

Pharmacological treatments were performed after the stabilization of DA levels in the perfusate. A stable baseline, defined as three consecutive samples in which DA contents varied by less than 10%, was generally obtained 120 min after the beginning of the perfusion (stabilization period).

Fluoxetine (10 mg/kg, IP) was administered 60 min prior to cocaine injection (10 mg/kg, IP). When locally applied into the NAc, fluoxetine (1 μM) was administered through the microdialysis probe by means of a 3-way liquid switch system (CMA111, Carnegie Medicin, Phymep), and taking into account the total dead volume of the perfusion system.

Fluoxetine perfusion started 30 min before 10 mg/kg, IP cocaine administration, and was maintained over 120 min. In each experimental group, animals received either drugs or their appropriate vehicle. In the case of local perfusion, control rats underwent the same manipulation as animals treated with fluoxetine, but syringes contained aCSF only. Rats received only one pharmacological treatment.

2.5. Statistical analyses

2.5.1. Behavioral experiments

Locomotor activity data are presented as mean total activity counts (±S.E.M.) for the 60 min observation period and the dependent measures were total horizontal (peripheral + central activity counts) or vertical activity observed during the test session. Because group comparisons were specifically defined prior to the start of the experiment, planned comparisons were conducted in lieu of an overall F test in a multifactorial ANOVA; this statistical analysis has been supported in a number of statistical tests (e.g. Keppel, 1973). Thus, each experiment was subjected to a one-way ANOVA for repeated measures with levels of the treatment factor corresponding to the drug combinations administered to that group. Planned, pairwise comparisons of the treatment means were made with least significant difference test (Keppel, 1973; SAS for Windows, Version 8.1). Horizontal and vertical activity were also divided into separate 15-min time bins and analyzed with a two-way ANOVA with time as a repeated measures factor to examine treatment x time interactions. Significant interactions were followed by planned pairwise comparisons using Fisher's Least Significant Difference test. A repeated measures ANOVA across the days of testing was utilized to establish if there was a main effect of treatment order. All comparisons were conducted with an experiment wise error rate of $\alpha = 0.05$.

2.5.2. Microdialysis experiments

DA content in each sample was expressed as the percentage of the average baseline level calculated from the 3 fractions collected before the first drug administration. Data correspond to the mean ±S.E.M. of the percentage obtained in each experimental group.

The effect of cocaine on DA extracellular levels was assessed by using a one-way ANOVA with time as a repeated measures factor, for the 8 samples that followed its administration. The ability of fluoxetine (pretreatment) to modify the action of cocaine (treatment) was evaluated by a two-way ANOVA (pretreatment × treatment) with time as a repeated measures factor for the 8 samples that followed cocaine administration. When significant, the two-way ANOVA

was followed by a post-hoc Tukey's test to allow adequate multiple comparisons among groups. A separate one-way ANOVA with time as repeated measures factor was performed to assess whether systemic or local fluoxetine administration modified DA dialysate content by itself (12 or 10 samples in the case of systemic or local fluoxetine administration, respectively). In all cases, $p < 0.05$ was chosen as a criterion for significance.

3. Results

3.1. Behavioral experiments

3.1.1. Effects of systemic injections of fluoxetine on basal and cocaine-stimulated locomotor activity

Horizontal (Fig. 1) and vertical activity counts (data not shown) were assessed following IP injection of saline or fluoxetine (10 mg/kg) 60 min prior to an IP injection of saline or cocaine (10 mg/kg). A significant main effect of drug was observed for total horizontal ($F_{3,56} = 13.54$, $p < 0.001$) and total vertical activity counts ($F_{3,56} = 5.58$, $p < 0.01$) summed across the 60 min session. Cocaine significantly increased total horizontal activity (Fig. 1A) in rats pretreated with saline or fluoxetine (10 mg/kg) as compared to saline-saline controls ($p < 0.05$). Cocaine significantly increased total vertical activity in rats pretreated with fluoxetine ($p < 0.05$), but not in rats pretreated with saline as compared to saline-saline controls (data not shown). Pretreatment with 10 mg/kg of fluoxetine significantly enhanced cocaine-induced horizontal activity ($p < 0.05$; Fig. 1A). Administration of fluoxetine alone did not significantly alter basal horizontal (Fig. 1A) or vertical activity (data not shown).

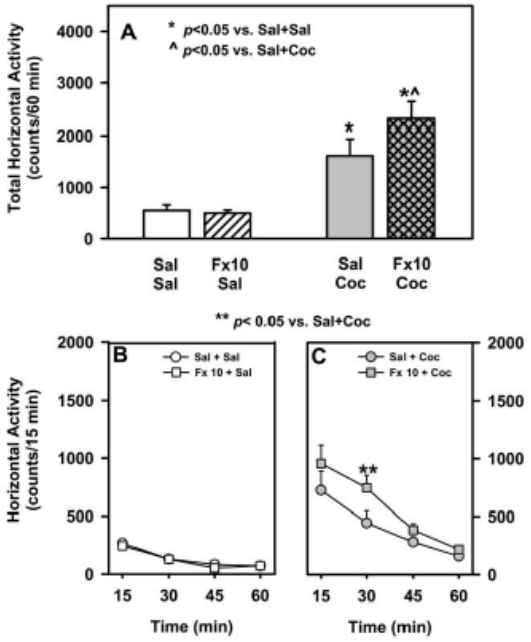

Fig. 1. Horizontal activity following systemic pretreatment with fluoxetine. (A). Mean total horizontal activity (counts/60 min) (±SEM; $n = 15$) following systemic pretreatment with saline (Sal; 1 ml/kg, IP) or fluoxetine (Fx; 10 mg/kg, IP) followed 1 h later by treatment with saline (1 ml/kg) or cocaine (Coc; 10 mg/kg, IP). * activity levels that were significantly different ($p < 0.05$) from Sal-Sal controls. ^activity levels that were significantly different ($p < 0.05$) from Sal-Coc controls based upon Fisher's Least Significant Difference test. Time course of horizontal activity plotted in 15-min bins across the 60-min test session following IP injection of saline of fluoxetine (10 mg/kg) followed by treatment injection of saline (B) or cocaine (10 mg/kg, IP) (C). ** activity levels that were significantly different from Sal-Coc controls at the corresponding time point based upon Fisher's Least Significant Difference test.

A significant treatment x time interaction was observed for horizontal activity ($F_{9,126} = 6.60$; $p < 0.001$) and vertical activity ($F_{9,126} = 2.72$; $p < 0.01$) divided into 4 separate 15-min time bins. Cocaine-induced increases in horizontal activity were significantly enhanced by pretreatment with 10 mg/kg of fluoxetine during the second interval ($p < 0.05$; Fig. 1C). Cocaine-induced increases in vertical activity were not significantly enhanced by pretreatment with fluoxetine (data not shown). Fluoxetine did not significantly alter spontaneous horizontal (Fig. 1A and B) or vertical activity (data not shown) at any time point after saline injection.

3.1.2. Effects of systemic injections of fluvoxamine on basal and cocaine-stimulated locomotor activity

Horizontal (Fig. 2) and vertical activity counts (data not shown) were assessed following IP injection of saline or fluvoxamine (10 or 20 mg/kg) 60 min prior to an IP injection of saline or cocaine (10 mg/kg). A significant main effect of drug was observed for total horizontal ($F_{5,36} = 8.21$, $p < 0.001$) and total vertical activity ($F_{5,36} = 14.85$, $p < 0.001$) summed across the 60 min session. Cocaine significantly increased both total horizontal activity (Fig. 2A) and total vertical activity (data not shown) in rats pretreated with saline or fluvoxamine (10 and 20 mg/kg) as compared to saline–saline controls ($p < 0.05$). Pretreatment with 20 mg/kg of fluvoxamine significantly enhanced cocaine-induced horizontal ($p < 0.05$; Fig. 2A) and vertical activity ($p < 0.05$; data not shown). Administration of doses of fluvoxamine alone did not significantly alter basal horizontal (Fig. 2A) or vertical activity (data not shown).

A significant treatment × time interaction was observed for horizontal activity ($F_{15,90} = 7.48$; $p < 0.001$) and vertical activity ($F_{15,90} = 4.73$; $p < 0.001$) divided into 4 separate 15-min time bins. Cocaine-induced increases in horizontal activity were significantly enhanced by pretreatment with 20 mg/kg of fluvoxamine during the first, third, and fourth intervals

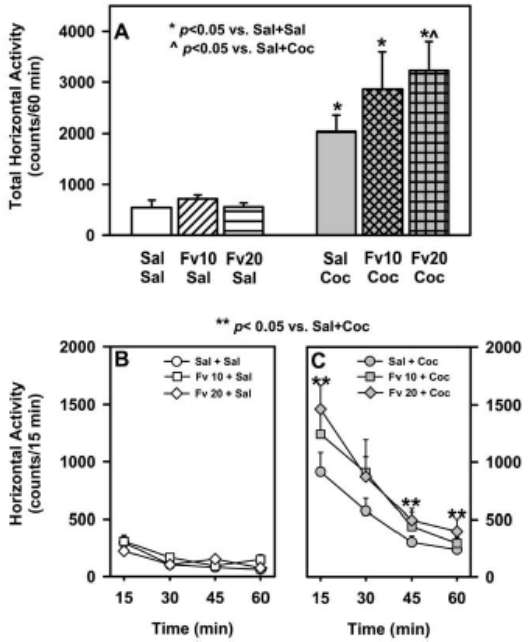

Fig. 2. Horizontal activity following systemic pretreatment with fluvoxamine. (A). Mean total horizontal activity (counts/60 min) (±SEM; $n = 8$) following systemic pretreatment with saline (Sal; 1 ml/kg, IP) or fluvoxamine (Fv; 10 or 20 mg/kg, IP) followed 1 h later by treatment with saline (1 ml/kg) or cocaine (Coc; 10 mg/kg, IP). * activity levels that were significantly different ($p < 0.05$) from Sal-Sal controls. ^activity levels that were significantly different ($p < 0.05$) from Sal-Coc controls based upon Fisher's Least Significant Difference test. Time course of horizontal activity plotted in 15-min bins across the 60-min test session following IP injection of saline of fluvoxamine (10 or 20 mg/kg) followed by treatment injection of saline (B) or cocaine (10 mg/kg, IP) (C). ** activity levels that were significantly different from Sal-Coc controls at the corresponding time point based upon Fisher's Least Significant Difference test.

($p < 0.05$; Fig. 2C). Cocaine-induced increases in vertical activity were also significantly enhanced by pretreatment with 20 mg/kg fluvoxamine during the first, second, and fourth intervals ($p < 0.05$; data not shown). Pretreatment with 10 mg/kg of fluvoxamine did not significantly alter cocaine-induced horizontal or vertical activity at any of the four intervals (Fig. 2C). Fluvoxamine did not significantly alter spontaneous horizontal (Fig. 2A and B) or vertical activity (data not shown) at any time point after saline injection.

3.1.3. *Effects of intra-NAc shell microinjections of fluoxetine on basal and cocaine-stimulated locomotor activity*

Of the 8 rats originally cannulated and tested, 7 rats exhibited cannula placements that were bilaterally positioned in the center of the NAc shell; only data from these 7 rats were included in the analyses. Horizontal (Fig. 3) and vertical activity counts (data not shown) were assessed following pretreatment with intra-NAc shell saline or fluoxetine (1 or 3 μg/0.2 μl/side) and systemic treatment with saline or cocaine (10 mg/kg). A significant main effect of drug was observed for total horizontal ($F_{5,36} = 10.25$; $p < 0.001$), but not vertical, activity counts ($F_{5,36} = 2.07$; $p > 0.05$) summed across the 60 min session. Systemic cocaine injection significantly increased horizontal activity following intra-NAc shell saline and 1 or 3 μg of fluoxetine as compared to saline-saline controls ($p < 0.05$; Fig. 3A). Intra-NAc shell pretreatment with 3 μg of fluoxetine significantly enhanced cocaine-induced horizontal activity (Fig. 3A; $p < 0.05$). Systemic cocaine also significantly increased total vertical activity following pretreatment with 1 μg intra-NAc fluoxetine ($p < 0.05$; data not shown), however cocaine alone or in combination with 3 μg intra-NAc fluoxetine did not alter vertical activity as com-

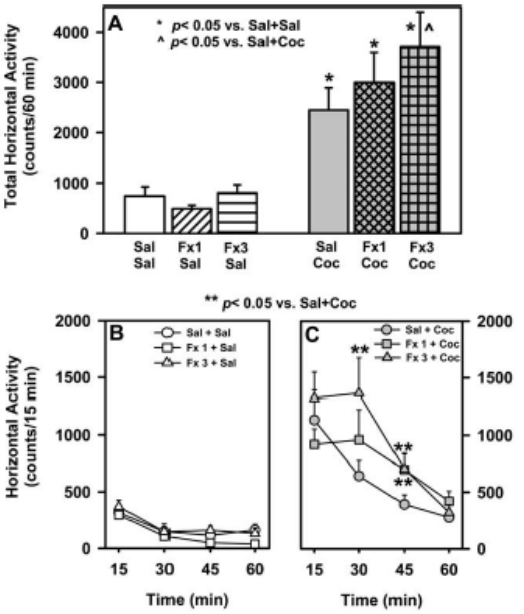

Fig. 3. Horizontal activity following intra-NAc shell pretreatment with fluoxetine. (A) Mean total horizontal activity (counts/60 min) (±SEM; $n = 7$) following intra-NAc shell pretreatment with saline (Sal; 0.2 μl/side) or fluoxetine (Fx; 1 or 3 μg/0.2 μl/side) followed by treatment with saline (1 ml/kg, IP) or cocaine (Coc; 10 mg/kg, IP). * activity levels significantly different ($p < 0.05$) from Sal-Sal controls. ^activity levels significantly different ($p < 0.05$) from Sal-Coc controls based upon Fisher's Least Significant Difference test. Time course of horizontal activity plotted in 15-min bins across the 60-min test session following pretreatment injection of saline (0.2 μl/side) or fluoxetine (1 or 3 μg/0.2 μl/side) followed by systemic treatment injection of saline (B) or cocaine (10 mg/kg, IP) (C). ** activity levels that were significantly different from Sal-Coc controls at the corresponding time point based upon Fisher's Least Significant Difference test.

pared to saline-saline controls ($p > 0.05$; data not shown). Intra-NAc shell pretreatment with fluoxetine alone did not significantly alter basal horizontal (Fig. 3A) or vertical activity (data not shown). A one-way ANOVA for repeated measures showed that the dose-dependent enhancement of cocaine-evoked hyperactivity by intra-NAc fluoxetine was not dependent on the order of treatment ($F_{7,48} = 0.78$; $p > 0.05$).

A significant treatment × time interaction was observed for horizontal activity ($F_{15,90} = 5.86$; $p < 0.001$) and vertical activity ($F_{15,90} = 2.57$; $p < 0.01$) divided into 4 separate 15-min time bins. Cocaine-induced increases in horizontal activity were significantly enhanced by pretreatment with 3 μg of fluoxetine during the second and third intervals, and by pretreatment with 1 μg of fluoxetine during the third interval ($p < 0.05$; Fig. 3C). In addition, cocaine-induced increases in vertical activity were significantly attenuated by pretreatment with 3 μg of fluoxetine at the first interval and enhanced by pretreatment with 1 μg of fluoxetine at the third and fourth intervals (data not shown). Intra-NAc shell pretreatment with fluoxetine did not significantly alter spontaneous horizontal (Fig. 3A and B) or vertical activity (data not shown) at any time point after saline injection.

3.1.4. Effects of intra-NAc shell microinjections of fluvoxamine on basal and cocaine-stimulated locomotor activity

Of the 11 rats originally cannulated and tested, 7 rats exhibited cannulae placements bilaterally positioned in the center of the NAc shell; only data from these 7 rats were included in the analyses. Horizontal (Fig. 4) and vertical activity counts (data not shown) were determined following pretreatment with intra-NAc shell saline or fluvoxamine (1 or 3 μg/0.2 μl/side) and systemic treatment with saline or cocaine (10 mg/kg). A significant main effect of drug was observed for total horizontal activity ($F_{5,36} = 6.35$, $p < 0.001$), but not total vertical activity ($F_{5,36} = 1.77$, $p > 0.05$) summed across the 60 min session. Systemic cocaine significantly increased total horizontal activity following intra-NAc shell saline or fluvoxamine (1 or 3 μg; $p < 0.05$; Fig. 4A) as compared to saline–saline controls. Systemic cocaine also significantly increased total vertical activity following intra-NAc shell pretreatment with fluvoxamine (1 μg; $p < 0.05$; data not shown), however cocaine did not significantly increase vertical activity following intra-NAc pretreatment with saline or 3 μg fluvoxamine, as compared to saline-saline controls ($p < 0.05$; data not shown). Intra-NAc shell pretreatment with 3 μg of fluvoxamine significantly enhanced cocaine-evoked horizontal activity (Fig. 4A; $p < 0.05$) and 1 μg of fluvoxamine significantly enhanced cocaine-evoked vertical activity ($p < 0.05$; data not shown). Intra-NAc pretreatment with fluvoxamine (1 or 3 μg) alone did not alter

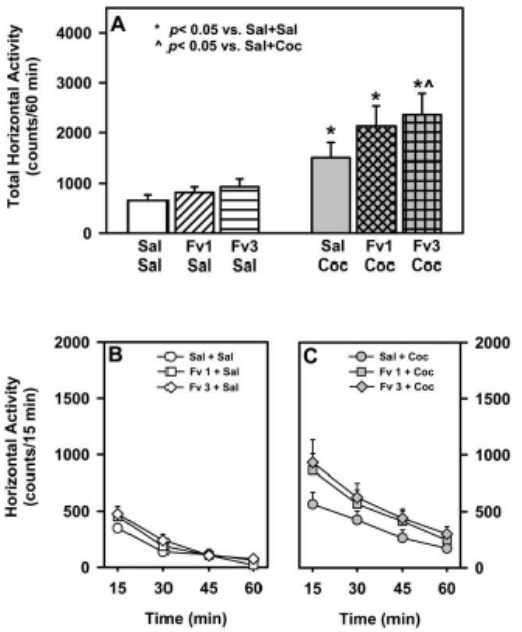

Fig. 4. Horizontal activity following intra-NAc shell pretreatment with fluvoxamine. (A) Mean total horizontal activity (counts/60 min) (±SEM; $n = 7$) following intra-NAc shell pretreatment with saline (Sal; 0.2 μl/side) or fluvoxamine (Fv; 1 or 3 μg/0.2 μl/side) followed by treatment with saline (1 ml/kg, IP) or cocaine (Coc; 10 mg/kg, IP). * activity levels significantly different ($p < 0.05$) from Sal-Sal controls. ^activity levels significantly different ($p < 0.05$) from Sal-Coc controls based upon Fisher's Least Significant Difference test. Time course of horizontal activity plotted in 15-min bins across the 60-min test session following pretreatment injection of saline (0.2 μl/side) or fluvoxamine (1 or 3 μg/0.2 μl/side) followed by systemic treatment injection of saline (B) or cocaine (10 mg/kg, IP) (C).

basal horizontal (Fig. 4A) or vertical activity (data not shown). A one-way ANOVA for repeated measures showed that the dose-dependent enhancement of cocaine-evoked hyperactivity by intra-NAc fluvoxamine was not dependent on the order of treatment ($F_{7,48} = 0.63$; $p > 0.05$).

There was not a significant treatment × time interaction observed for horizontal activity ($F_{10,60} = 1.45$; $p > 0.05$) or vertical activity ($F_{10,60} = 1.92$; $p > 0.05$) divided into 4 separate 15-min time bins.

3.2. Microdialysis experiments

3.2.1. Basal extracellular DA concentration in dialysates from the NAc shell

All measurements were carried out 120 min after the beginning of perfusion, by which time a steady state was achieved. Absolute basal levels of DA in dialysate collected from the NAc shell were (without adjusting for probe recovery) 2.9 ± 0.5 pg/30 μl.

3.2.2. Effects of the systemic administration of fluoxetine on cocaine-induced DA outflow

Administration of cocaine (10 mg/kg, IP) enhanced DA outflow in the NAc [one-way ANOVA, $F_{1,9} = 105.8$, $p < 0.001$; Fig. 5]. The maximal increase was observed 30 min after drug injection (368 ± 14% over baseline values). The IP administration of fluoxetine (10 mg/kg) significantly enhanced the increase in DA outflow induced by cocaine [two-way ANOVA, $F_{1,15} = 24.9$, $p < 0.001$; Fig. 5A]. NAc DA outflow in the fluoxetine + cocaine group was increased up to 614 ± 27.2% of baseline 30 min after cocaine administration. Thereafter, DA outflow remained significantly higher than values found in the saline + cocaine group throughout the entire experimental period (Tukey's test, $p < 0.001$ vs the saline + cocaine group). Systemic administration of fluoxetine alone had no significant effect on basal DA outflow [one-way ANOVA $F_{1,8} = 2.8$, $p > 0.05$].

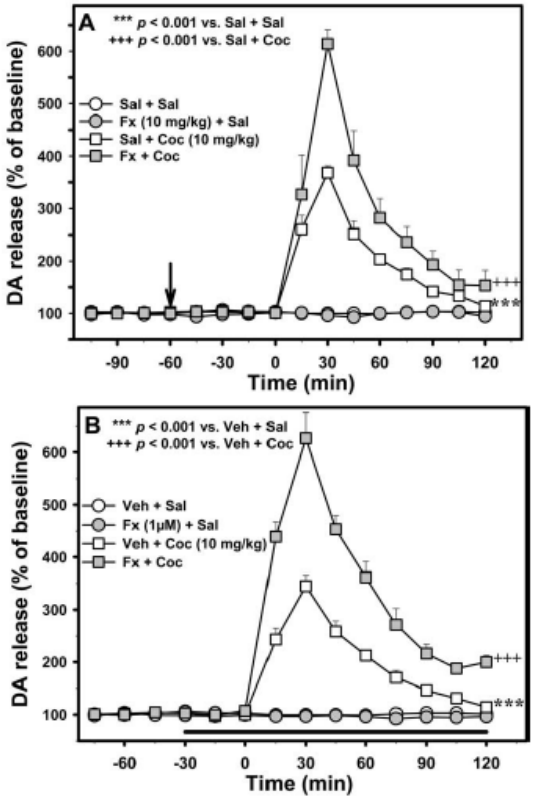

Fig. 5. Cocaine-induced DA outflow in the NAc shell following systemic or intra-NAc fluoxetine. Time course of the effect of the systemic (10 mg/kg, IP) (A) or intra-NAc (1 μM) (B) administration of fluoxetine on cocaine-stimulated DA outflow in the NAc shell of freely-moving rats. Data, obtained from 4–6 animals per group, are presented as the mean ±S.E.M. percentages of the baseline calculated from the three samples preceding the first drug administration. Cocaine (Coc; 10 mg/kg, IP) was administered at time zero. When systemically administered, fluoxetine (Fx; 10 mg/kg, IP) was injected (vertical arrow) 60 min before cocaine administration (A) whereas its local infusion (1 μM) (horizontal bar) started 30 min before cocaine administration (B). Systemic and local fluoxetine administration potentiated the increase in DA outflow induced by cocaine. *** $p < 0.001$ vs. the vehicle (Veh)+saline (Sal) group; +++ $p < 0.001$ vs. the Veh+Coc group (Tukey's test).

3.2.3. Effects of the intra-NAc infusion of fluoxetine on cocaine-induced DA outflow

Local infusion of fluoxetine (1 μM) potentiated the facilitatory effect of cocaine on DA outflow in the NAc [two-way ANOVA, $F_{1,17} = 27.7$, $p < 0.001$; Tukey's test, $p < 0.001$ vs the vehicle + cocaine group; Fig. 5B]. The maximal effect was observed 30 min after cocaine administration and reached 627 ± 49% of basal values in the fluoxetine + cocaine group compared to 345 ± 20% in the vehicle + cocaine group. Dialysate DA content remained significantly higher in the fluoxetine + cocaine group throughout the entire experimental period (Tukey's test, $p < 0.001$ vs the saline + cocaine group). Intra-NAc perfusion of fluoxetine alone had no significant effect on basal DA outflow [one-way ANOVA $F_{1,8} = 5.3$, $p > 0.05$].

4. Discussion

In the present study, systemic and intra-NAc administration of the SSRIs fluoxetine and fluvoxamine were shown to enhance cocaine-evoked locomotor activity in male rats. In addition, delivery of fluoxetine systemically or through the dialysis probe into the NAc shell enhanced cocaine-evoked DA efflux in the NAc. Thus the present results suggest that SSRIs enhance the behavioral effects of cocaine via actions localized, in part, to the NAc.

In support of previous findings (Herges and Taylor, 1998; Reith et al., 1991), systemic pretreatment with the SSRIs fluoxetine (10 mg/kg) or fluvoxamine (10 or 20 mg/kg) 1 h prior to systemic cocaine (10 mg/kg) administration enhanced cocaine-evoked locomotor activity. Since similar doses of these SSRIs have been shown to enhance extracellular 5-HT (Jordan et al., 1994), these data suggest that elevated levels of 5-HT augment the locomotor stimulant effects of cocaine. Studies show that elevated accumbal 5-HT modulates DA efflux in the NAc (Parsons and Justice, Jr., 1993), which is considered to be an important process in the generation of stimulant-induced hyperactivity. Thus, we hypothesize that the ability of SSRIs to enhance cocaine-induced locomotor activity is due to the enhancement of 5-HT levels and consequent 5-HT-mediated augmentation of DA efflux in the NAc. In support of this hypothesis, the present results confirm that systemic administration of

fluoxetine (10 mg/kg) enhanced cocaine-induced DA efflux in the NAc, as measured by microdialysis in the NAc shell.

To more closely explore the potential for 5-HT transporters (SERT) in the NAc to serve as a principle site of action for SSRIs to augment cocaine-evoked hyperactivity and DA efflux, SSRIs were infused directly into the NAc shell prior to systemic cocaine injection. Hyperactivity evoked by a systemic injection of cocaine (10 mg/kg) was enhanced by fluoxetine or fluvoxamine infused into the NAc shell, and in addition, intra-NAc shell infusion of fluoxetine enhanced cocaine-evoked DA efflux in the NAc. Thus, administration of SSRIs directly into the NAc produces a similar enhancement of systemic cocaine-induced hyperactivity and DA efflux in the NAc as observed with systemic administration of SSRIs, indicating that the NAc is a key site for the action of SSRIs to enhance cocaine-evoked locomotor activity and DA efflux in the NAc.

The extent of DA efflux in the NAc following psychostimulant administration has been correlated not only with hyperactivity, but also with the discriminative stimulus and reinforcing properties of the drugs (for review see Erinoff and Brown, 1994). Thus, enhancement of cocaine-induced NAc DA efflux by SSRIs might be expected to enhance both the discriminative stimulus and reinforcing effects as well as hyperactivity at a given dose of cocaine. Indeed the SSRIs fluvoxamine and fluoxetine have been shown to enhance the discriminative stimulus effects of cocaine (Callahan and Cunningham, 1995; Cunningham and Callahan, 1991), a result that is consistent with current observations. In addition, fluoxetine has been shown to attenuate the rate of cocaine self-administration on a fixed ratio (FR) schedule of reinforcement (Carroll et al., 1990). This observed attenuation may be due to the ability of fluoxetine to enhance cocaine-induced DA efflux in the NAc resulting in less cocaine intake required to achieve the desired levels of NAc DA efflux. However, a limitation of the FR self-administration paradigm is the difficulty in interpreting attenuations in the rate of self-administration (Richardson and Roberts, 1996). On a progressive ratio (PR) schedule of cocaine self-administration, fluoxetine has been shown to reduce the breaking point (Richardson and Roberts, 1991) suggesting that the reinforcing value of the drug is decreased (Richardson and Roberts, 1991), an observation inconsistent with present and cited studies. Contrary to these results, additional studies found SSRIs had no effect on cocaine self-administration (Porrino et al., 1989; Tella, 1995). Full dose response curves for the effects of SSRIs on cocaine self-administration under both FR and PR schedules would provide more insight as to the nature and specificity of the interaction.

A logical mechanism by which SSRIs enhance cocaine-evoked hyperactivity and DA release is via indirect activation of 5-HT receptors. Antagonism of 5-HT_{1B} (Castanon et al., 2000), 5-HT_{2A} (McMahon and Cunningham, 2001a), 5-HT_{2C} (McCreary and Cunningham, 1999), or 5-HT_3 (Reith, 1990) receptors has been shown to attenuate the locomotor response to cocaine, suggesting that stimulation of each of these receptors by 5-HT released by SSRIs could act to augment cocaine-evoked locomotor activity. Unlike the 5-HT_{1B} and 5-HT_{2C} receptors which appear to exert control over basal mesoaccumbens DA activity (Di Giovanni et al., 1999; Yan and Yan, 2001), evidence suggests that 5-HT_{2A} and 5-HT_3 receptors do not exhibit tonic control over NAc DA release (De Deurwaerdère et al., 1998; De Deurwaerdère and Spampinato, 1999). Rather, activating these receptors appears to predominantly enhance NAc DA release under conditions in which the DA system is activated (i.e. following cocaine administration or electrical stimulation of the dorsal raphe nucleus; De Deurwaerdère et al., 1998; De Deurwaerdère and Spampinato, 1999). The effects of 5-HT_{2A} and/or 5-HT_3 receptor stimulation on DA release are consistent with the present results which demonstrate that both systemic and intra-NAc fluoxetine have the ability to enhance systemic cocaine-induced DA efflux to a similar extent, without altering basal extracellular DA concentration in the NAc. Our findings support previous studies which indicate that 5-HT efflux generated by SSRIs does not affect basal DA efflux (Guan and McBride, 1988; Lucas et al., 2000), but facilitates DA efflux under conditions in which DA transmission is activated (Lucas et al., 2000). In addition, these results are consistent with the present observation that the chosen doses of fluvoxamine and fluoxetine were able to enhance cocaine-evoked hyperactivity, but did not alter basal locomotor activity. Thus, it is plausible that stimulation of 5-HT_{2A} and/or 5-HT_3 receptors may contribute to the enhancement of cocaine-evoked hyperactivity and DA release achieved by SSRI administration observed in the present study.

Alternative theories to explain why SSRIs enhance DA-mediated behaviors and DA efflux in the presence of cocaine but not when administered alone propose mechanisms that do not involve modulation of DA transmission by 5-HT. Pozzi and colleagues (1999) hypothesized that fluoxetine-induced increases in DA concentration in the prefrontal cortex were not dependent upon 5-HT, but rather occurred via blockade of NE uptake. While a possible explanation for the results observed with fluoxetine which has moderate affinity for the NE transporter (NET; K_i = 240 nM; Tatsumi et al., 1997), this postulate is unlikely to hold true for fluvoxamine, which has relatively low affinity for NET (K_i = 1300 nM; Tatsumi et al., 1997), but is efficacious in enhancing cocaine-induced activity and enhancing cocaine-induced increases in NAc DA levels.

A second mechanism to explain the state-dependent effect of fluoxetine on DA efflux in the NAc is an alter-

ation in the distribution of cocaine binding to monoamine transporters. Cocaine has higher affinity for SERT than DAT (Kozikowski et al., 1998), thus theoretically, more cocaine molecules could bind to SERT than to DAT. However, in the presence of fluoxetine, the ratio of cocaine binding to SERT and DAT may shift, as fluoxetine would compete with cocaine for the binding site on SERT. Fluoxetine has higher affinity for SERT than does cocaine (Damaj et al., 1999; Owens et al., 1997), therefore it is plausible that fewer cocaine molecules are able to bind to SERT in the presence of fluoxetine than when cocaine is administered alone, thereby rendering more cocaine available to bind DAT. Thus, the present behavioral and neurochemical effects could be explained by increased blockade of DAT by cocaine in the presence of SSRIs, which also would likely result in enhanced DA efflux in the NAc.

Tella and Goldberg (1993) reported that when cocaine was administered intravenously (IV), pretreatment with fluoxetine or other monoamine reuptake inhibitors caused a rapid, but brief (<5 min), enhancement of plasma cocaine levels in a time frame consistent with the distribution phase of IV cocaine, suggesting altered distribution characteristics of IV cocaine in the presence of monoamine reuptake inhibitors. However, to our knowledge, brain drug levels have not been measured following administration of fluoxetine plus cocaine. Therefore, whether changes in plasma cocaine concentrations correspond to changes in brain cocaine concentration and altered cocaine binding distribution specifically in the brain is unknown. In addition, the effect of fluoxetine on plasma cocaine levels was not dose-dependent (Tella and Goldberg, 1993), while the present results show a clear dose-dependent effect. Furthermore, fluoxetine has been shown to potentiate increases in DA efflux in the NAc induced by the selective DA/NE uptake inhibitor bupropion (Li et al., 2002), and the SSRI fluvoxamine is able to enhance the locomotor activating effects of the DA/NE uptake inhibitor mazindol (McMahon and Cunningham, 2001b). In these instances, since the drug molecules bind to different transporters, competition between the SSRIs and the respective DA/NE uptake inhibitor for binding sites should be nominal. Thus the enhancement of extracellular DA levels and DA-mediated behaviors is not likely to be due to altered distribution characteristics, but rather due to the influence of 5-HT on DA release. These studies demonstrate that 5-HT has the ability to enhance NAc DA efflux and DA mediated behaviors and thus provide supporting evidence for our proposed mechanism that the enhancement of cocaine-induced hyperactivity and DA efflux in the NAc by SSRIs is due to the influence of 5-HT on DA efflux. Nonetheless, to rule out the potential mechanistic involvement of an altered cocaine distribution, binding studies could be performed to measure the amount of cocaine binding to SERT and/or DAT in the presence or absence of fluoxetine.

Potential pharmacological interactions between the SSRIs fluoxetine or fluvoxamine and cocaine must also be considered. Sills et al. (1999b) proposed a pharmacokinetic mechanism to explain the facilitation of amphetamine-induced hyperactivity and DA efflux in the NAc by systemic fluoxetine. In this study animals pretreated with fluoxetine had higher levels of amphetamine in the NAc than animals given amphetamine alone and the authors postulated that fluoxetine interfered with the metabolism of amphetamine. A similar mechanism is plausible for an interaction between cocaine and fluvoxamine since fluvoxamine is known to inhibit the P450 3A4 (Fleishaker and Hulst, 1994), which contributes to the metabolism of cocaine to norcocaine (Ladona et al., 2000). Thus, if P450 enzymes are inhibited by fluvoxamine, the amount of cocaine available in the brain could increase. It is difficult to attribute our findings solely to a pharmacokinetic interaction between the SSRIs and cocaine, since we saw a similar behavioral response with the SSRI fluoxetine as we did with fluvoxamine, but fluoxetine has little effect on P450 3A4 (von Moltke et al., 1995). In addition, our results show that intra-NAc infusion of either fluvoxamine or fluoxetine caused an immediate enhancement of cocaine-induced locomotor activity. This time course would be unexpected if the observed effects were dependent upon P450 3A4 mediated metabolism of cocaine in the NAc. Nonetheless, measurements of the concentration of cocaine following SSRI treatment by microdialysis would be necessary to confirm or deny this hypothesis.

In conclusion, the present study provides additional evidence for a facilitory role of 5-HT in the effects of cocaine mediated by the DA mesoaccumbens pathway. Both systemic and intra-NAc administration of SSRIs enhanced cocaine-evoked locomotor activity, as well as the elevation of NAc DA efflux following cocaine administration, suggesting that SERT in the NAc is a potential site of action for SSRIs to augment the in vivo effects of cocaine. The lack of effect of the SSRIs on both basal locomotor activity as well as basal DA release in the NAc suggests that DA activation is required for positive modulation of NAc DA concentrations by SSRI generated 5-HT efflux. The effects of SSRIs may be mediated by a number of 5-HT receptors, however the relative contributions of each receptor to the wide spectrum of effects of systemically administered SSRIs on cocaine-induced changes in DA neurotransmission and the subsequent behavioral effects must be further investigated.

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APPENDIX B[2]

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ORIGINAL INVESTIGATION

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Effects of dopamine D_1- or D_2-like receptor antagonists on the hypermotive and discriminative stimulus effects of (+)-MDMA

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Abstract *Rationale:* Both dopamine (DA) and serotonin (5-HT) release are evoked by (+)-MDMA; however, little is known of the contribution of DA D_1- and D_2-like receptors (D_1R and D_2R, respectively) in the behavioral effects of (+)-MDMA. *Objectives:* To test the hypothesis that a D_1R or D_2R antagonist would attenuate the hypermotive or discriminative stimulus effects of (+)-MDMA. *Methods:* Male Sprague-Dawley rats (n=164) were pretreated with the D_1R antagonist SCH 23390 (3.125–50 μg/kg, SC) or the D_2R antagonist eticlopride (12.5–50 μg/kg, SC) prior to treatment with (+)-MDMA (3 mg/kg, SC) and locomotor activity was recorded using photobeam monitors. Twelve additional rats trained to discriminate (+)-MDMA (1 mg/kg, IP) from saline in a two-lever water-reinforced FR20 task were administered SCH 23390 (6.25 μg/kg, IP) or eticlopride (12.5 μg/kg, IP) prior to (+)-MDMA (0.375–1.0 mg/kg, IP). Rats were then placed in the drug discrimination chambers and the percent (+)-MDMA appropriate responding and response rate were measured. *Results:* Both SCH 23390 and eticlopride blocked (+)-MDMA-evoked hyperactivity in a dose-related manner; the highest doses of the antagonists also effectively suppressed basal locomotor activity. In rats trained to discriminate (+)-MDMA from saline, SCH 23390 (6.25 μg/kg), but not eticlopride (12.5 μg/kg), blocked the stimulus effects of (+)-MDMA without altering response rate. *Conclusion:* These data indicate that DA released indirectly by (+)-MDMA administration results in stimulation of D_1R and D_2R to enhance locomotor activity. Furthermore, the D_1R appears to play a more prominent role than the D_2R in the discriminative stimulus properties of (+)-MDMA.

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Introduction

Over the past decade, there has been a sharp rise in the use of the substituted amphetamine 3,4-methylenedioxymethamphetamine (MDMA), commonly known as "ecstasy." Use of MDMA is especially popular in young adults, among whom the awareness of the possible dangers associated with MDMA has recently begun to rise (Johnston et al. 2003). Likewise, there has been a push to better understand the mechanisms underlying the actions of MDMA in order to better delineate the potential dangers associated with MDMA use.

Two common behavioral measures employed to study the mechanisms of psychostimulants in rodents are measurement of locomotor activity and drug discrimination. In general, the ability of a drug to elicit locomotor hyperactivity correlates with its ability to enhance dopamine (DA) release in the nucleus accumbens (NAc), the terminal region of the DA mesoaccumbens pathway or "reward" pathway (for review, see Erinoff and Brown 1994). Thus, measurement of locomotor activity can be used as an indirect measure of activation of the "reward" circuit (Wise and Bozarth 1987). The NAc has also been implicated in the recognition of the "interoceptive cue" or stimulus effects elicited by psychoactive drugs as demonstrated using the drug discrimination assay, which is thought to model the subjective effects of drugs in humans (for review, see Callahan et al. 1997).

Indeed, (+)- and (±)-MDMA do elicit hyperactivity (Gold et al. 1988; Callaway et al. 1990) and can be readily discriminated from saline in the drug discrimination task (Schechter 1989; present study). Additionally, (±)-MDMA has been shown to enhance extracellular DA release in the NAc (White et al. 1994) at least partially due to reversal of the DA transporter (DAT; Rudnick and Wall 1992). However, unlike its parent compound amphetamine,

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which is primarily a DA releaser, (±)-MDMA is actually more potent at releasing serotonin (5-HT; IC_{50}=56.6 ±2.1 nM) via reversal of the 5-HT transporter (SERT; Rudnick and Wall 1992) than DA (IC_{50}=376±16 nM; Rothman et al. 2001). Likewise, many of the behavioral effects of (+)- and (±)-MDMA, including the hypermotive and discriminative stimulus effects, are thought to be mediated by 5-HT neurotransmission (Schechter 1989; Callaway et al. 1990; Baker et al. 1997; Bankson and Cunningham 2001; Frankel and Cunningham 2003). As such, the majority of research has focused on a 5-HTergic contribution to the effects of (+)- and (±)-MDMA. However emerging evidence of an important role for 5-HT-DA interactions in the mechanisms of action of (+)- and (±)-MDMA (for review, see Bankson and Cunningham 2001) has shifted attention to discerning the contribution of the DA system in addition to 5-HT to the effects of MDMA.

Although (±)-MDMA has higher affinity for SERT and is more potent at reversing SERT than DAT, (±)-MDMA evokes a proportionately larger amount of DA release compared to 5-HT release in the NAc (White et al. 1994). Because (+)-MDMA-evoked DA release can be accounted for by both direct actions of (+)-MDMA on DAT as well as via 5-HT stimulated DA release (Koch and Galloway 1997), it is difficult to separately allocate the contribution of 5-HT and DA to these effects. (+)- or (±)-MDMA-induced DA release can be attenuated not only by the DAT inhibitor GBR 12909, but also with the selective 5-HT reuptake inhibitor fluoxetine (Gudelsky and Nash 1996; Koch and Galloway 1997). Additionally, antagonists for the 5-HT_{1B}, 5-HT_{2A}, and 5-HT_{2C} receptors that modulate (+)- and (±)-MDMA-induced locomotor hyperactivity (see Bankson and Cunningham 2001) are also known to modulate basal (Benloucif et al. 1993; Ng et al. 1999; Lucas and Spampinato 2000) and/or stimulated DA release (Parsons et al. 1999; Porras et al. 2002). Thus, since each of these receptors has the capacity to alter DA activity, the observed effects of serotonergic compounds on (+)- and (±)-MDMA-induced locomotor activity may ultimately be related to the ability of these compounds to modulate DA mesoaccumbens pathway activation. Thus it is important to determine the role of DA and the DA receptors in mediating the behavioral effects of (+)- and (±)-MDMA.

Two families of DA receptors, the D_1-like and the D_2-like receptors (D_1R and D_2R, respectively), have been shown to be important in the expression of the hypermotive and discriminative stimulus effects of DA indirect agonists such as cocaine and amphetamine (Callahan et al. 1991; Callahan and Cunningham 1993; Schechter 1997; O'Neill and Shaw 1999). The D_1R family contains the D_1R and D_5R that are primarily post-synaptic and positively coupled to cAMP (for review, see Lachowicz and Sibley 1997). The D_2R family, which includes the D_2R, D_3R, and D_4R, are located both pre- and post-synaptically and are negatively coupled to cAMP, but stimulate PLA2 (Lachowicz and Sibley 1997). Both D_1R and D_2R mRNA and protein are found in high concentrations within the basal forebrain, and are prominently represented in the ventral tegmental area (VTA) and NAc (Dearry et al. 1990; Bouthenet et al. 1991; Landwehrmeyer et al. 1993); D_3R, D_4R, and D_5R mRNA are also present within the brain, but other than high concentrations of D_3R in the NAc shell, these receptors appear to be much less concentrated than the D_1R and D_2R (Bouthenet et al. 1991; Meador-Woodruff et al. 1992; O'Malley et al. 1992; Landwehrmeyer et al. 1993). Blockade of D_1R or D_2R with respective antagonists suppresses basal or stimulated locomotor activation (Agmo and Soria 1999; Chausmer and Katz 2001). Likewise, both D_1R and D_2R have been implicated in control of cocaine- and amphetamine- stimulated locomotor hyperactivity (O'Neill and Shaw 1999) and thus are postulated to be important in (+)- and (±)-MDMA-induced locomotor hyperactivity as well. Indeed, Kehne and colleagues (1996) demonstrated that the D_1R antagonist SCH 23390 and the D_2R antagonist haloperidol blocked (±)-MDMA-induced locomotor hyperactivity. This study used a single dose of the antagonists and a relatively high dose (20 mg/kg) of (±)-MDMA.

The first goal of the present study was to further extend the results of Kehne and colleagues (1996) to test the hypothesis that an antagonist of D_1R or D_2R will attenuate (+)-MDMA-induced locomotor activation in a dose-related manner. In the present experiment, we chose a relatively low dose (3 mg/kg) of (+)-MDMA, which is described to produce robust locomotor hyperactivity (McCreary et al. 1999; Bankson and Cunningham 2002). As the more active isomer, (+)-MDMA is more potent than either (±)-MDMA or (−)-MDMA at eliciting hyperactivity (Callaway et al. 1990). As mentioned above, both D_1R and D_2R antagonists have been shown to effectively suppress basal locomotor activity. Thus, we also aimed to uncover doses of both antagonists that might attenuate (+)-MDMA-induced hyperactivity without altering basal locomotor activation. A second goal of the present study was to determine if administration of a D_1R or D_2R antagonist would alter the discriminative stimulus properties of (+)-MDMA when administered at doses shown to block (+)-MDMA-induced hyperactivity.

Materials and methods

Animals

Adult male Sprague-Dawley rats (n=176; Harlan Sprague-Dawley, Inc., Indianapolis, Ind., USA) weighing 225–350 g at the beginning of the experimental procedures were used. The animals were housed two or four to a cage for drug discrimination or locomotor activity assays, respectively, in a temperature (21–23ºC) and humidity (40–50%) controlled environment and lighting was maintained under a 12-h light-dark cycle (lights on at 7:00 a.m. to 7:00 p.m.). For locomotor activity assays, food and water were available ad libitum (except during habituation and testing procedures). In drug discrimination assays, food was available ad libitum, except during the experimental sessions; however, the amount of water each animal received was restricted to that given during operant training sessions, after test sessions (10–15 min) and on weekends (36 h). All

experimental protocols were carried out in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council 1986) and with the approval by the Institutional Animal Care and Use Committee.

Drugs

The followings drugs were used: (+)-MDMA was obtained from the National Institutes on Drug Abuse (Research Triangle, N.C., USA), R-(+)-7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine hydrochloride [R-(+)-SCH 23390] and S-(−)-3-chloro-5-ethyl-N-[(1-ethyl-2-pyrrolidinyl)methyl]-6-hydroxy-2-methoxybenzamidehydro-chloride [S-(−)-eticlopride] were obtained from Sigma Aldrich Co. (St Louis, Mo., USA). All drugs were dissolved in sterile saline (0.9% NaCl); doses refer to the weight of the salt. Drug injections were administered subcutaneously (SC) and intraperitoneally (IP) in locomotor activity and drug discrimination experiments, respectively.

Locomotor activity experiments

Apparatus

Locomotor activity was monitored and quantified under low light conditions using a modified open field activity system (San Diego Instruments, San Diego, Calif., USA) housed within sound attenuated chambers. Each of the eight chambers consisted of a clear Plexiglas open field (40×40×40 cm). Two photobeam matrices are used for recording activity: at 4 cm from the floor, a 4×4 matrix counts horizontal activity and at 16 cm from the floor horizontal photobeams count vertical (rearing) activity. The control software (Photobeam Activity Software; San Diego Instruments) was used to count horizontal and vertical activity and data were stored for subsequent statistical evaluation. Video cameras located above the chambers were used to monitor activity continuously without disruption of behavior.

Behavioral procedures

The locomotor activity experiments were conducted in three separate groups of animals according to pretreatment: the first group (n=36) was administered lower doses of D_1R antagonist SCH 23390 (3.125 or 6.25 μg/kg, SC), the second group (n=64) was administered higher doses of SCH 23390 (12.5, 25, or 50 μg/kg, SC), and the third group (n=64) received the D_2R antagonist eticlopride (12.5, 25, or 50 μg/kg, SC). Rats were habituated to the activity monitors for 3 h/day on the 2 days prior to the start of the experiment. On the test day, rats were habituated to the locomotor activity monitors for 30 min prior to receiving pretreatment with either the assigned dose of SCH 23390, eticlopride, or saline, followed 30 min later by treatment with (+)-MDMA (3 mg/kg, SC) or saline (1 ml/kg, SC). Recording of activity in 5-min time epochs for 90 min began immediately following the treatment injection.

Statistical analysis

Total activity counts were summed for each individual animal throughout the 90-min session. Data are presented as mean total activity counts (±SEM) with the dependent measure of total horizontal or vertical activity recorded during the testing session. Since group comparisons were specifically defined prior to the start of the experiment, planned comparisons were conducted in lieu of an overall F-test in a multifactorial anaysis of variance (ANOVA); this statistical analysis has been supported in a number of statistical tests (e.g. Keppel 1973). Significant interactions were followed up with a priori comparisons using Fisher's least significant difference procedure (Keppel 1973). Time course data were broken down into six separate 15-min time bins and a three-way ANOVA was used to detect pretreatment×treatment×time interactions. In the case that a significant interaction was present, differences between treatment groups were determined at each 15-min time point using a one-way ANOVA. All planned comparisons were assessed with Fisher's least significant difference test with the error rate (α) set at 0.05.

Drug discrimination experiments

Apparatus

The procedures were conducted in 12 two-lever operant chambers (Lafayette Instruments Model 80001, Lafayette, Ind., USA or Med Associates Model ENV-001, St Albans, Vt., USA) housed in sound attenuating chambers (Lafayette Instruments Model 80015, or Med Associates Model ENV-015). The operant chambers contained two levers with a water dispenser centered between the levers. Illumination was provided by a 28-V house light; ventilation and masking noise were supplied by a blower. An interface (Med Associates) connected the chambers to a PC computer running Med-PC for Windows software (Med Associates) that controlled and recorded all experimental events.

Design and procedures of drug discrimination analyses

Rats (n=12) were trained to discriminate an injection of (+)-MDMA (1.0 mg/kg, IP) from saline (1 ml/kg, IP) administered 20 min before daily (Monday to Friday) 30-min sessions. In order to maintain a pseudorandomized drug administration schedule, (+)-MDMA and saline were administered irregularly with the restriction that neither training condition prevailed for more than three consecutive sessions. Initially, training began under a schedule of continuous water reinforcement on a fixed ratio (FR) 1 with only the stimulus-appropriate (drug or saline) lever present ("errorless training"); the schedule of reinforcement was incremented until all rats were responding reliably (>300 bar presses on treatment appropriate lever) under an FR 20 schedule for each experimental condition. For half of the rats, responses on the right lever were reinforced following drug administration; for the remaining rats, responses on the left lever were reinforced following drug administration. To control for the possible development of position cues based upon olfactory stimuli, a pseudo-random relationship was maintained between the lever programmed to deliver reinforcement for each consecutive rat run in the same experimental chamber (Extance and Goudie 1981). After responding stabilized on an FR 20 schedule of reinforcement, both levers were presented simultaneously and rats were required to respond on the stimulus-appropriate (correct) lever in order to obtain reinforcement (water). At the start of discrimination training, the session time was shortened from 30 min to 20 min; there were no programmed consequences for responding on the incorrect lever. After responding stabilized, training sessions were shortened from 20 min to 15 min. This phase of training continued until the performance of all rats reached criterion (individual mean accuracies of at least 80% correct prior to the first reinforcer for ten consecutive sessions).

Drug discrimination test procedures

Test sessions were then initiated and were conducted once or twice a week with training (maintenance) sessions intervening on other days. Test sessions included all rats that met the 80% accuracy criterion during the preceding (+)-MDMA and saline maintenance sessions. During test sessions, rats were placed in the chamber as during training sessions and upon completion of 20 responses on either lever or after the session time (15 min) had elapsed, a single

(water) reinforcer was delivered, the house light was turned off and the rat was removed from the chamber. After being returned to the home cages, all rats were allowed 10–15 min of free access to water.

Two pharmacological test manipulations were performed during test sessions in all rats (n=12). In substitution tests, rats were administered (+)-MDMA (0.375, 0.5, 0.75 or 1.0 mg/kg, IP), SCH 23390 (6.25 μg/kg, IP), eticlopride (12.5 μg/kg, IP), or saline (1 ml/kg, IP). In combination tests, rats were tested for lever selection following administration (IP) of SCH 23390 (6.25 μg/kg) or eticlopride (12.5 μg/kg) prior to a dose of (+)-MDMA (0.375, 0.5, 0.75 or 1.0 mg/kg). SCH 23390 or eticlopride were administered 30 min and (+)-MDMA 20 min prior to placement in the operant chambers. The doses of SCH 23390 and eticlopride were chosen based upon the results of the current locomotor experiments, as these doses of the antagonists attenuated (+)-MDMA-induced hyperactivity without significantly altering basal locomotor activity.

Statistical analysis

During training sessions, accuracy was defined as the percentage of correct total responses before the delivery of the first reinforcer; during test sessions, performance was expressed as the percentage of (+)-MDMA-appropriate responses to the total responses prior to the first reinforcer. Response rates (responses per min) were also evaluated during training and test sessions as a measure of behavioral disruption. Response rates were calculated as the total number of responses emitted on either lever before the completion of the first FR 20 on the stimulus appropriate lever (training sessions) or prior to completion of 20 responses on either lever (test sessions), divided by the number of minutes taken to complete the first ratio. During test sessions, data from rats that did not complete the FR 20 within the allotted 15 min period were excluded from analyses. For (+)-MDMA substitution tests, Student's t-test for repeated measures were used to compare the percentage of (+)-MDMA-appropriate lever responding and response rates during test sessions with the corresponding values for the previous (+)-MDMA or saline sessions. Because two comparisons to each test data point were conducted, the experimentwise error rate (α) was adjusted to P<0.025 (0.05÷2 comparisons). For combination tests, a two-way ANOVA for repeated measures was used to assess whether the percentage of (+)-MDMA-appropriate lever responding and response rates observed across four doses of (+)-MDMA (0.375. 0.5, 0.75 or 1.0 mg/kg) differed in the presence versus the absence of a fixed dose of a DA antagonist; a priori comparisons at each dose of (+)-MDMA in the presence and absence of the test drug were conducted using Student's t-test. All comparisons were made with an experimentwise type I error rate (α) set at 0.05 (Keppel 1973), except where noted above where α was set at 0.025.

Results

Locomotor activity experiments

Effects of D_1R antagonist SCH-23390 on spontaneous and (+)-MDMA-evoked activity

Locomotor activity was assessed following pretreatment with SCH 23390 (3.125, 6.25, 12.5, 25, or 50 μg/kg) or saline (1 ml/kg) and treatment with (+)-MDMA (3 mg/kg) or saline (1 ml/kg; Figs. 1 and 2). As stated in Materials and methods, this experiment was conducted in two groups of animals, one of which received lower doses of SCH 23390 (3.125, 6.25 μg/kg) and the other which received higher doses of SCH 23390 (12.5, 25, 50 μg/kg). Statistical analyses comparing the total horizontal or vertical activity counts of the lower dose and higher dose groups determined that there were no significant differences in the levels of horizontal [F(1,13)=0.65, P=0.44] or vertical activity [F(1,13)=0.01, P=0.92] for the Sal/Sal rats in the two groups, nor was there a significant difference in the levels of horizontal [F(1,13)=0.38, P=0.55] or vertical activity [F(1,13)=0.06, P=0.81] for the Sal/(+)-MDMA rats between the two groups (P>0.05). Therefore, data from the two groups were pooled and the mean total horizontal or vertical activity counts for all Sal/Sal (n=14) or Sal/MDMA (n=14) treated rats served as the comparison for tests of all doses of SCH 23390.

There was a main effect of drug treatment observed for both total horizontal [F(11,97)=24.22, P<0.01] and vertical activity [F(11,97)=12.82, P<0.01]. (+)-MDMA significantly increased both mean total horizontal (Fig. 1) and vertical activity (Fig. 2) compared with saline (P<0.05).

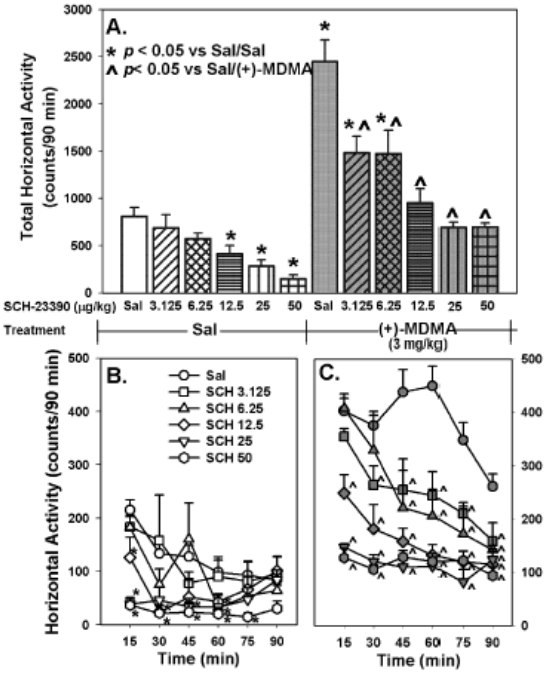

Fig. 1 Effects of the D_1R antagonist SCH 23390 on horizontal hyperactivity induced by (+)-MDMA. **A** Datapoints are represented as mean total horizontal activity (counts/90 min; SEM) in rats pretreated with saline (*Sal*; 1 ml/kg, SC; n=14) or SCH 23390 (*SCH*; 3.125, 6.25, 12.5, 25 or 50 μg/kg, SC; n=6–8) followed by treatment with saline (1 ml/kg, SC) or (+)-MDMA (3 mg/kg, SC). **B**,**C** Datapoints represent the timecourse for horizontal activity in 15-min time bins (SEM) across the 90-min session; pretreatment with saline (1 ml/kg, SC, *circles*) or SCH 23390 [3.125 (*square*), 6.25 (*triangle*), 12.5 (*diamond*), 25 (*inverted triangle*) or 50 μg/kg (*hexagon*), SC] followed by treatment with **B** saline (1 ml/kg, SC; *open symbols*) or **C** (+)-MDMA (3 mg/kg, SC; *closed symbols*). The *asterisk* (*) indicates activity levels significantly different (P<0.05) from Sal/Sal controls. The *caret* (^) indicates activity levels significantly different (P<0.05) from Sal/(+)-MDMA controls

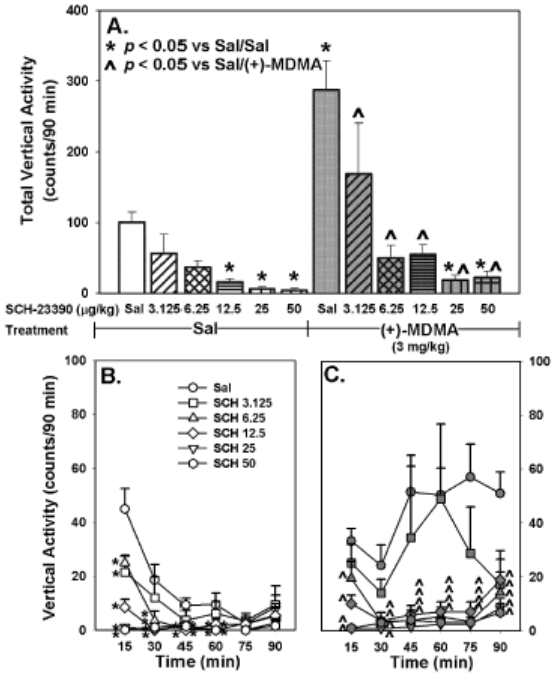

Fig. 2 Effects of the D_1R antagonist SCH 23390 on vertical hyperactivity induced by (+)-MDMA. **A** Datapoints are represented as mean total vertical activity (counts/90 min; SEM) in rats pretreated with saline (*Sal*; 1 ml/kg, SC; n=14) or SCH 23390 (*SCH*; 3.125, 6.25, 12.5, 25 or 50 µg/kg, SC; n=6–8) followed by treatment with saline (1 ml/kg, SC) or (+)-MDMA (3 mg/kg, SC). **B**, **C** The datapoints represent the timecourse for vertical activity in 15-min time bins (SEM) across the 90-min session; pretreatment with saline (1 ml/kg, SC; *circles*) or SCH 23390 [3.125 (*square*), 6.25 (triangle), 12.5 (*diamond*), 25 (*inverted triangle*) or 50 µg/kg (*hexagon*), SC] followed by treatment with **B** saline (1 ml/kg, SC; *open symbols*) or **C** (+)-MDMA (3 mg/kg, SC; *closed symbols*). The *asterisk* (*) indicates activity levels significantly different (P<0.05) from Sal/Sal controls. The *caret* (^) indicates activity levels significantly different (P<0.05) from Sal/(+)-MDMA controls

Pretreatment with all doses of SCH 23390 significantly decreased (+)-MDMA-induced horizontal (Fig. 1) and vertical hyperactivity (Fig. 2; P<0.05). While the two lower doses of SCH 23390 (3.125 and 6.25 µg/kg) did not alter basal activity, the three higher doses (12.5, 25, 50 µg/kg) significantly suppressed spontaneous horizontal (Fig. 1) and vertical locomotor activity (Fig. 2) in addition to blocking (+)-MDMA-induced hyperactivity (P<0.05). The doses of 25 and 50 µg/kg of SCH 23390 prevented the ability of (+)-MDMA to induce vertical activity; in fact, levels of vertical activity seen were lower than basal levels of vertical activity (Fig. 2; P<0.05).

A significant pretreatment×treatment×time interaction was shown for horizontal [Fig. 1; F(25,599)=1.90, P<0.05] and vertical activity [Fig. 2; F(25,599)=2.47, P<0.05] separated into six different 15-min time bins when comparing all doses of SCH 23390. Examination of the timecourse revealed that the higher doses of SCH 23390 (12.5, 25 and 50 µg/kg) significantly attenuated (+)-MDMA-evoked horizontal hyperactivity at all time points (P<0.05; Fig. 1C), while the lower doses of SCH 23390 (3.125, 6.25 µg/kg) suppressed (+)-MDMA-induced horizontal hyperactivity from 30–90 min and 45–90 min, respectively. Pretreatment with the lower doses of SCH 23390 (3.125 and 6.25 µg/kg) had no effect upon spontaneous horizontal activity, except for a significant suppression of activity with 6.25 µg/kg at 60 min; higher doses of SCH 23390 (12.5, 25 µg/kg) significantly decreased spontaneous horizontal activity during the first 60 min of the session except at the 45 and 30 min time points, respectively, while the 50 µg/kg dose of SCH 23390 suppressed spontaneous horizontal activity throughout the first 75 min of the session (Fig. 1B). All doses of SCH 23390 significantly attenuated (+)-MDMA-evoked vertical activity at all time points (P<0.05; Fig. 2C), except the lowest dose (3.125 µg/kg) at which a significant suppression was observed only at the 90-min time point. Spontaneous vertical activity was suppressed by all doses of SCH 23390 for the first 60 min of the session, except for the lowest dose (3.125 µg/kg), which only suppressed basal vertical activity at the 15 min time point (Fig. 2B).

Effects of D_2R antagonist eticlopride on spontaneous and (+)-MDMA-evoked activity

Locomotor activity was assessed following pretreatment with eticlopride (12.5, 25, 50 µg/kg) or saline (1 ml/kg) and treatment with (+)-MDMA (3 mg/kg) or saline (1 ml/kg; Figs. 3 and 4). There was a significant main effect of treatment observed for both horizontal [F(7,63)=23.90, P<0.01] and vertical activity [F(7,63)=9.80, P<0.01]. (+)-MDMA significantly increased horizontal (Fig. 3) and vertical activity (Fig. 4) when compared to control animals (P<0.05). Pretreatment with all doses of eticlopride significantly decreased (+)-MDMA-induced horizontal (Fig. 3) and vertical hyperactivity (Fig. 4; P<0.05). Eticlopride significantly suppressed spontaneous horizontal activity at the two higher doses (25 and 50 µg/kg), but not at the lowest dose (12.5 µg/kg; Fig. 3); however, all of the doses of eticlopride significantly suppressed spontaneous vertical activity (Fig. 4; P<0.05). As seen with the higher doses of SCH 23390, levels of vertical activity following 25 and 50 µg/kg eticlopride in combination with (+)-MDMA were lower than basal levels of vertical activity (Fig. 4; P<0.05).

A significant pretreatment×treatment×time interaction was shown for horizontal [Fig. 3; F(15,383)=2.36, P<0.01] and vertical activity [Fig. 4; F(15,383)=4.03, P<0.01] separated into six different 15-min time bins. All doses of eticlopride were shown to significantly attenuate (+)-MDMA-evoked horizontal (Fig. 3C) or vertical (Fig. 4C) hyperactivity at all time points except for the lowest dose (12.5 µg/kg), which did not alter (+)-MDMA-evoked vertical activity during the initial 15 min of the session. While the lowest dose (12.5 µg/kg) of eticlopride only suppressed spontaneous horizontal activity at the 60-

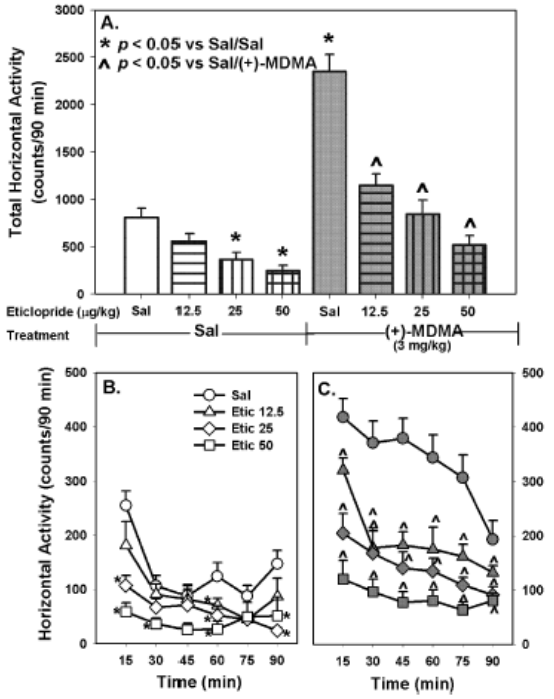

Fig. 3 Effects of the D_2R antagonist eticlopride on horizontal hyperactivity induced by (+)-MDMA. **A** The datapoints represent mean total horizontal activity (counts/90 min; SEM; n= 8) in rats pretreated with saline (*Sal*; 1 ml/kg, SC) or eticlopride (*Etic*; 12.5, 25 or 50 μg/kg, SC) followed by treatment with saline (1 ml/kg, SC) or (+)-MDMA (3 mg/kg, SC). **B**,**C** The datapoints represent timecourse for horizontal activity in 15-min time bins (SEM) across the 90-min session; pretreatment with saline (1 ml/kg, SC, *circles*) or eticlopride [12.5 (*triangle*), 25 (*diamond*) or 50 μg/kg (*square*), SC] followed by treatment with **B** saline (1 ml/kg, SC; *open symbols*) or **C** (+)-MDMA (3 mg/kg, SC; *closed symbols*). The *asterisk* (*) indicates activity levels significantly different (P<0.05) from Sal/Sal controls. The *caret* (^) indicates activity levels significantly different (P<0.05) from Sal/(+)-MDMA controls

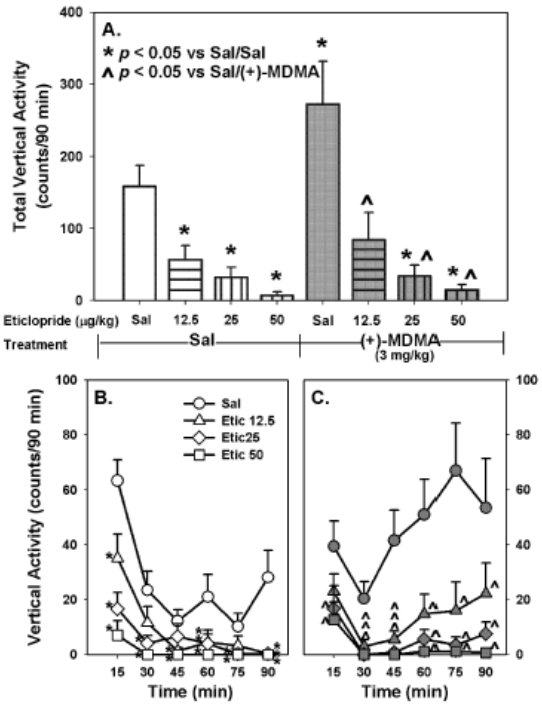

Fig. 4 Effects of the D_2R antagonist eticlopride on vertical hyperactivity induced by (+)-MDMA. **A** The datapoints represent mean total vertical activity (counts/90 min; SEM; n=6–8) in rats pretreated with saline (*Sal*; 1 ml/kg, SC) or eticlopride (*Etic*; 12.5, 25 or 50 μg/kg, SC) followed by treatment with saline (1 ml/kg, SC) or (+)-MDMA (3 mg/kg, SC). **B**,**C** The datapoints represent timecourse for vertical activity in 15-min time bins (SEM) across the 90-min session. Pretreatment with saline (1 ml/kg, SC, *circles*) or eticlopride [12.5 (*triangle*), 25 (*diamond*) or 50 μg/kg (*square*), SC] or followed by treatment with **B** saline (1 ml/kg, SC; *open symbols*) or **C** (+)-MDMA (3 mg/kg, SC; *closed symbols*). The *asterisk* (*) indicates activity levels significantly different (p<0.05) from Sal/Sal controls. The *caret* (^) indicates activity levels significantly different (P<0.05) from Sal/(+)-MDMA controls

min time point, pretreatment with 25 μg/kg and 50 μg/kg eticlopride significantly decreased spontaneous levels of horizontal activity at 15, 60 and 90 min and at 15, 30, 60 and 90 min, respectively (Fig. 3B). Spontaneous vertical activity was significantly suppressed by 50 μg/kg eticlopride at all time points and 25 μg/kg at all but the 45 min time point, while the lowest dose (12.5 μg/kg) of eticlopride suppressed vertical activity at all time points except 30 and 75 min (P<0.05; Fig. 4B).

Drug discrimination experiments

(+)-MDMA dose-response tests

The discrimination between (+)-MDMA (1.0 mg/kg) and saline was acquired in an average of 32 sessions (range: 18–61). Throughout acquisition, response rates (±SEM) during (+)-MDMA sessions (31.12±1.05 responses/min) were not significantly different from saline sessions (35.85 ±1.18 responses/min). (+)-MDMA (0.375–1.0 mg/kg) elicited a dose-related increase in (+)-MDMA-appropriate responding (Fig. 5A, B), whereas saline administration resulted in <10% (+)-MDMA-appropriate responding (Fig. 5A, B). Response rates were stable across all test doses of (+)-MDMA and did not differ from the response rates observed on the previous maintenance days (P<0.025; Fig. 5C, D).

Effects of the D_1R antagonist SCH 23390 and the D_2R antagonist eticlopride on the stimulus effects of (+)-MDMA

Neither SCH 23390 (6.25 μg/kg) nor eticlopride (12.5 μg/kg) mimicked (+)-MDMA, rather each drug produced

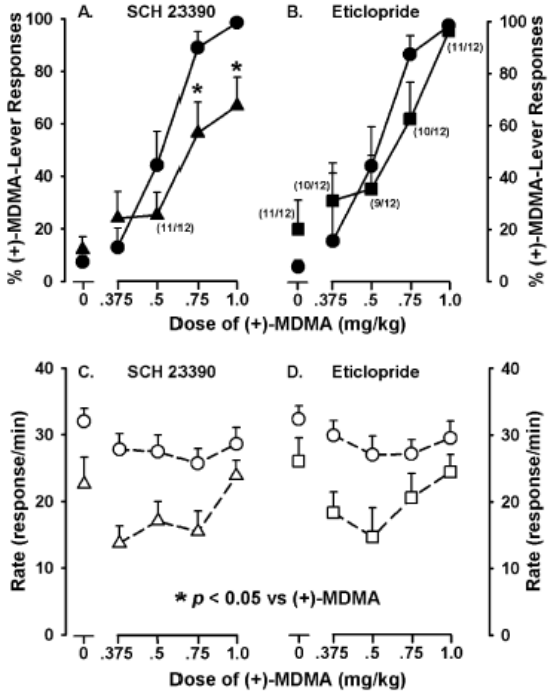

Fig. 5 Effects of the D_1R antagonist SCH 23390 and D_2R antagonist eticlopride in rats trained to discriminate (+)-MDMA. Datapoints represent mean (SEM) **A**,**B** percentage of (+)-MDMA-lever responses (*closed symbols*) and **C**,**D** response rate (*open symbols*) for substitution and combination tests with **A**,**C** SCH 23390 (SCH; 6.25 μg/kg, IP; *triangles*) and **B**,**D** eticlopride (*Etic*; 12.5 μg/kg, IP; *squares*) in rats trained to discriminate (+)-MDMA (1 mg/kg, IP; *circles*) from saline. Data points for substitution tests with saline (*circles*), SCH 23390 (*triangle*) or eticlopride (*square*) are represented by 0 mg/kg dose of (+)-MDMA on the *x*-axis. The number (*n*) of rats completing a FR 20 compared with the number tested (*N*) is 12/12, unless otherwise noted adjacent to individual points. The *asterisk* (*) illustrates datapoints significantly different from performance following the respective dose of (+)-MDMA administration alone ($P<0.05$)

primarily saline-like responding (Fig. 5A, B). Response rates following administration of either SCH 23390 (6.25 μg/kg) or eticlopride (12.5 μg/kg) were not statistically different than the previous (+)-MDMA session (Fig. 5C, D).

A two-way ANOVA revealed a significant pretreatment×treatment interaction for the combination of D_1R antagonist SCH 23390 (6.25 μg/kg) with (+)-MDMA (0.375–1.0 mg/kg) on the percentage of (+)-MDMA-appropriate lever responding [$F(3,32)=3.58$, $P<0.05$; Fig. 5A], but not on response rate [$F(3,32)=1.77$, $P=0.173$; Fig. 5C]. Pretreatment with SCH 23390 was shown to significantly attenuate (+)-MDMA-appropriate lever responding when administered prior to 0.75 and 1.0 mg/kg (+)-MDMA ($P<0.05$). Conversely, there was no significant pretreatment×treatment interaction for the combination of the D_2R antagonist eticlopride (12.5 μg/kg) with (+)-MDMA (0.375–1.0 mg/kg) on the percentage of (+)-MDMA-appropriate responding [$F(3,26)=1.56$, $P=0.223$; Fig. 5B) or the response rate [$F(3,26)=1.44$, $P=0.255$; Fig. 5D].

Although eticlopride (12.5 μg/kg) did not alter the percentage of (+)-MDMA appropriate responding or response rate in animals that completed the FR20, several animals (n=1–3/test session) were excluded from the analyses because they did not complete the FR20 in the allotted 15-min period (Fig. 5B), suggesting that the combination of eticlopride with (+)-MDMA was moderately disruptive to responding. SCH 23390 (6.25 μg/kg), on the other hand, was disruptive to only one subject when combined with (+)-MDMA (0.5 mg/kg; Fig. 5A). Experiments employing higher doses of SCH 23390 (12.5 μg/kg) or eticlopride (25 μg/kg) in combination with the training dose of (+)-MDMA (1 mg/kg) were discontinued upon observation that these doses of the antagonists disrupted responding in greater than 50% of subjects tested (data not shown).

Discussion

The results of the present study support a role for DA receptors in both the hypermotive and discriminative stimulus effects of (+)-MDMA. In support of our hypothesis, administration of the D_1R antagonist SCH 23390 or the D_2R antagonist eticlopride attenuated (+)-MDMA-induced hyperactivity in a dose-related manner. While most doses used in the present study also suppressed spontaneous locomotor activity, lower doses of both SCH 23390 (3.125 and 6.25 μg/kg) and eticlopride (12.5 μg/kg) attenuated (+)-MDMA-induced hyperactivity without significantly altering basal locomotor activity. Thus, the ability of D_1R and D_2R antagonists to attenuate (+)-MDMA-induced hyperactivity as demonstrated in the present study extend the findings of Kehne and colleagues (1996), suggesting that D_1R and D_2R stimulation are integral in the ability of (+)-MDMA to elicit locomotor hyperactivity. Based upon these results, a low dose of SCH 23390 (6.25 μg/kg) and eticlopride (12.5 μg/kg) were chosen to determine the ability of these antagonists to block or shift the dose-response curve for the stimulus properties of (+)-MDMA. SCH 23390 significantly attenuated (+)-MDMA-appropriate responding, resulting in a rightward shift in the (+)-MDMA dose-response curve. Conversely, eticlopride had no effect upon (+)-MDMA-appropriate responding; however, eticlopride moderately disrupted overall lever-responding when combined with any dose of (+)-MDMA tested. The lack of an effect of a D_2R antagonist on the discriminative stimulus effects of (±)-MDMA has previously been reported (Schechter 1989; Goodwin et al. 2003), although, to our knowledge, this is the first report of a significant attenuation of (+)-MDMA-appropriate responding by a D_1R antagonist, suggesting a role for D_1R in the discriminative stimulus properties of (+)-MDMA.

While much of the research investigating the mechanisms of action underlying the effects of MDMA have

focused on the role of 5-HT and the 5-HT receptors (Schechter 1989; Callaway et al. 1990; Baker et al. 1997; Bankson and Cunningham 2001; Frankel and Cunningham 2003), the importance of DA in the behavioral effects of MDMA has become increasingly evident. Early studies demonstrated that 6-hydroxydopamine lesions of DA terminals in the NAc were found to attenuate (±)-MDMA-induced hyperactivity (Gold et al. 1989). As validated here, both D_1R and D_2R antagonists will also attenuate (+)- (present study) or (±)-MDMA-induced hyperactivity (Kehne et al. 1996). These results suggest a fundamental role for the DA system, and in particular DA in the NAc, in the locomotor activating effects of MDMA. Studies discerning the role of DA in the discriminative stimulus effects of MDMA, however, are limited and the results vary depending upon the paradigm used, the isomer chosen, and the time point of administration (Baker et al. 1997; for review, see Cole and Sumnall 2003). A DA component is suggested by the observation that (±)-MDMA mimics the stimulus effects of the DA releaser amphetamine (Glennon et al. 1988) and vice versa (Oberlender and Nichols 1988; Schechter 1989; but see Baker et al. 1997). Additionally, the present results demonstrating that the D_1R antagonist SCH 23390 interferes with the discriminative stimulus effects of (+)-MDMA supplies further evidence for a role for DA in this behavioral effect of (+)-MDMA.

On the other hand, the inability of the D_2R antagonist eticlopride to alter the stimulus effects of (+)-MDMA is in keeping with previous reports (Schechter 1989; Goodwin et al. 2003), all of which cumulatively suggest that the D_2R stimulation may not be an integral component of the discriminative stimulus properties of (+)-MDMA. Interestingly, the role of DA in the stimulus effects of MDMA may be dependent upon the interval of time at which the discrimination is trained relative to the injection of MDMA. Schechter (1989) reported that in rats trained to discriminate (±)-MDMA at a much longer time point (105 min) after injection relative to that typically used (20 min; present results; Schechter 1989; Goodwin et al. 2003), haloperidol did attenuate (±)-MDMA-appropriate responding. Because there is some evidence to suggest that the later time point coincides with the maximal (±)-MDMA-evoked DA release (60–120 min; Yamamoto and Spanos 1988), it is possible that D_1R and D_2R may play differential roles time-locked to variant neurochemical profiles seen after MDMA administration (Yamamoto and Spanos 1988; Schechter 1989). Nonetheless, although further investigation is required to elucidate the potential contribution of D_2R in the discriminative stimulus effects of (+)-MDMA, the present results suggest that D_1R play a prominent role in the discriminative properties of (+)-MDMA.

While the present and previous studies (Gold et al. 1988; Kehne et al. 1996) suggest that DA receptors are involved in the behavioral effects of MDMA, the mechanistic contribution of different receptor subtypes in the control of the hypermotive and discriminative stimulus effects of MDMA has been difficult to decipher. Some of the difficulty may be due to a lack of selectivity of the ligands employed for the D_1R versus D_2R classes and/or for the subtypes of receptors within the two classes. The antagonists employed in the present study, while enabling differentiation of the contribution of D_1R versus D_2R, do not have the ability to distinguish between the individual subtypes of receptors within the respective classes. For example, the D_2R antagonist eticlopride shows moderate selectivity for D_2 (K_i=0.07 nM; Lawler et al. 1999) over D_1 (K_i=113 nM; Hall et al. 1986) receptors but only displays a slight preference for D_2 versus D_3 (K_i=0.36 nM) or D_4 receptors (K_i=64 nM; Lawler et al. 1999). SCH 23390 on the other hand has preferential selectivity for D_1 (K_i=0.37 nM; Lawler et al. 1999) over D_2 (K_i=2367 nM; Levant et al. 1992), but virtually no selectivity over D_5 receptors (K_i=0.47 nM; Lawler et al. 1999). Thus, interpretation of the results of the present study is limited to differentiation between the D_1R and D_2R classes, but not within each class. As such, the availability of more selective compounds for subtypes within the D_1R and D_2R classes is necessary to fully understand the contribution of each of the receptor subtypes to the behavioral effects of (+)-MDMA.

In addition to a lack of selective ligands, another obstacle in the quest for deciphering the individual actions of DA receptor subtypes to control (+)-MDMA-induced behaviors is the apparent synergism between D_1R and D_2R in elicitation of DA-related behaviors (for reviews, see Waddington and O'Boyle 1989; Jackson and Westlind-Danielsson 1994). With regard to locomotor activation, D_1R tone appears to be necessary in order to "enable" D_2R-mediated locomotor stimulation, whereas changes in D_2R tone can alter expression of D_1R agonist-induced hypermotility (see Waddington and O'Boyle 1989). For example, systemic or intra-NAc administration of inactive doses of the D_1R agonist SKF 38393 in combination with suppressive doses of the D_2R agonists RU 24213 or quinpirole, respectively, induced hyperactivity (Starr and Starr 1987; Canales and Iversen 2000). Furthermore, concurrent stimulation of D_1R and D_2R is required to evoke locomotor stimulation in DA-depleted mice (Jackson and Hashizume 1986; but see Arnt 1985), suggesting that simultaneous activation of D_1R and D_2R is important in eliciting locomotor activation. In the present study, (+)-MDMA-induced hyperactivity was blocked by administration of either D_1R or D_2R antagonists suggesting that activation of both receptors are necessary to elicit hyperactivity. Furthermore, (+)-MDMA administered following the higher doses of SCH 23390 or eticlopride, never evoked activity levels greater than baseline levels. Thus, the inability of (+)-MDMA to elicit hyperactivity following the higher doses of SCH 23390 or eticlopride may be due to the lack of synergistic interactions of D_1R and D_2R when one or the other of the receptors is blocked (see Waddington and O'Boyle 1989). These data further speak to the importance of D_1R and D_2R in (+)-MDMA-induced hyperactivity and support the theory that concomitant D_1R and D_2R stimulation may be necessary for elicitation of hyperactivity.

A synergism between D_1R and D_2R can also be inferred from studies of the stimulus effects cocaine, *d*-amphetamine, and (+)- or (±)-MDMA. Coinciding with the inhibitory actions of the D_1R antagonist SCH 23390 on the recognition of the stimulus cue of (+)-MDMA observed in the present study, SCH 23390 completely blocked the discriminative cues for both cocaine and *d*-amphetamine (Nielsen et al. 1989; Smith et al. 1989; Callahan et al. 1991). Conversely, D_1R agonists produce either partial or no substitution for cocaine or *d*-amphetamine (Nielsen et al. 1989; Smith et al. 1989; Callahan et al. 1991). An opposite effect is observed for D_2R ligands, as D_2R antagonists do not consistently block cocaine or (+)- or (±)-MDMA drug discrimination, (present study; Schechter 1989; Callahan et al. 1994; Goodwin et al. 2003), while D_2R agonists reliably substitute for both cocaine and *d*-amphetamine (Smith et al. 1989; Callahan et al. 1991). Thus, these results suggest that D_1R may be necessary, but not sufficient, to mimic the interoceptive cues of cocaine and *d*-amphetamine (Callahan et al. 1994). Furthermore, the inability of D_2R antagonists to fully antagonize the discriminative stimulus cues of psychostimulants may be a result of underlying D_1R stimulation that is retained in these conditions.

Both SCH 23390 and eticlopride suppressed spontaneous locomotor activity in a dose-related manner thereby generating the potential of nonspecific behavioral competition between the suppressive effects of the antagonists vs. the stimulatory effects of (+)-MDMA on locomotor activity. However, examination of the results following administration of the lower doses of the antagonists in the locomotor activity experiments reveals that while 3.125 and 6.25 μg/kg SCH 23390 and 12.5 μg/kg eticlopride produced a modest, non-significant suppression of spontaneous locomotor activity (15%, 30%, and 31%, respectively; see Figs 1A and 5A), these antagonists significantly decreased (+)-MDMA-induced hyperactivity by 39%, 40% and 51% for SCH 23390 at 3.125, 6.25 μg/kg and eticlopride at 12.5 μg/kg, respectively (see Figs 1A and 5A). The greater degree of attenuation produced when the antagonists were administered in combination with (+)-MDMA suggests that the blockade of (+)-MDMA-induced hyperactivity at the lower doses of antagonists was probably not a result of non-specific behavioral suppression, but rather that both D_1R and D_2R play a vital role in the ability of (+)-MDMA to induce hyperactivity. Results from the drug discrimination experiments conducted with the D_1R antagonist SCH 23390 provide further evidence of specific actions of DA receptors in the behavioral effects of (+)-MDMA. The 6.25 μg/kg dose of SCH 23390 was demonstrated to significantly reduce (+)-MDMA-appropriate responding, without altering rates of responding and causing only slight disruption of responding. These results suggest that the effects of SCH 23390 on (+)-MDMA appropriate responding are due to the ability of D_1R antagonism to alter the discriminative stimulus properties of (+)-MDMA, and not interference with responding due to the suppressive effects of the drug.

An unusual finding in the present study was that (+)-MDMA consistently and robustly enhanced vertical (rearing) activity, since (+)-MDMA is most commonly reported to suppress or have no effect on vertical activity (Callaway et al. 1990; McCreary et al. 1999; Bankson and Cunningham 2002). This consistent and robust enhancement of vertical activity by (+)-MDMA was, however, blocked by administration of either the D_1R or D_2R antagonists. Interestingly, the suppressant effects of the higher doses (25 and 50 μg) of SCH 23390 or eticlopride on vertical activity were so intense that (+)-MDMA-evoked vertical activity was significantly lower than that seen in saline treated controls. These data suggest that both D_1R and D_2R activation are necessary to maintain normal rearing activity as well as to induce vertical hyperactivity upon (+)-MDMA administration.

While enhancement of vertical activity by (+)-MDMA is not commonly observed, several studies have reported that suppression of vertical activity occurred only within the first 30 min following (+)-MDMA administration (Callaway et al. 1990; Gold et al. 1988), as was the case in the present study (Figs 4D and 5C). Furthermore, in addition to the enhanced vertical activity observed in the present study, increases in vertical activity have previously been reported following injection of (+)-MDMA directly into the NAc (Callaway and Geyer 1992) and following systemic (+)-MDMA administration in combination with a $5\text{-HT}_{2C}R$ antagonist (Bankson and Cunningham 2002). Unfortunately, the reasons underlying the inconsistencies in the vertical activity response to (+)-MDMA administration are not fully understood.

This phenomenon may be due to behavioral competition between the stimulation of vertical activity and the induction of the 5-HT syndrome by (+)-MDMA. The 5-HT syndrome is characterized, among other effects, by flat body posture and splayed hind limbs (Spanos and Yamamoto 1989). The presence of these components of the 5-HT syndrome may interfere with the ability of the rat to stand on its hind-legs (rear), thereby revealing an inverse relationship between expression of flat body posture and rearing. Indeed, individual differences in response to administration of (+)-MDMA are observed with rearing seen less frequently in animals exhibiting flat body posture (unpublished observations; data not shown). While flat body posture is consistently observed following administration of the current dose (3 mg/kg, SC) of (+)-MDMA, the intensity and duration of the effect appears to vary among individual rats, with some animals displaying flat body posture throughout the duration of the test session and others only for approximately the first 30 min following administration (unpublished observations). Thus, the ability of (+)-MDMA to induce vertical activity may vary depending on the extent of the induction of 5-HT syndrome, and in particular flat body posture. In the present study, seven out of 22 Sal-MDMA treated rats displayed flat body posture; however, only one rat continued to express the behavior after the initial 30-min period (data not shown). Thus the low incidence of flat body posture observed in the present study may have

contributed to the ability of (+)-MDMA to enhance vertical activity.

In conclusion, the present study suggests that DA D_1R and D_2R play critical roles in the expression of basal and (+)-MDMA-induced horizontal and vertical hyperactivity and furthermore that D_1R also contribute to the discriminative stimulus properties of (+)-MDMA. These findings combined with previous reports of the importance of 5-HT in the behavioral effects of (+)-MDMA lends favor to a role for 5-HT-DA interactions in the mechanisms of MDMA action (for review, see Bankson and Cunningham 2001). However, the nature of 5-HT-DA interactions, which has only begun to be characterized, appears to be extremely complex, and may reveal that these two neurotransmitter systems are highly interdependent (see Gudelsky and Nash 1996; Bankson and Cunningham 2001). Future studies investigating the nature of this interaction as well as studies determining the contribution of specific DA receptor subtypes within the D_1R- and D_2R-like classes will further elucidate the role of these systems in the behavioral effects of (+)-MDMA.

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APPENDIX C[3]

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Contribution of Serotonin (5-Hydroxytryptamine; 5-HT) 5-HT$_2$ Receptor Subtypes to the Hyperlocomotor Effects of Cocaine: Acute and Chronic Pharmacological Analyses

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ABSTRACT

The role of serotonin (5-hydroxytryptamine; 5-HT) 5-HT$_2$ receptor subtypes (5-HT$_{2A}$R, 5-HT$_{2B}$R, and 5-HT$_{2C}$R) in acute cocaine-evoked hyperactivity was compared with their contribution to the development and expression of locomotor sensitization upon repeated, intermittent treatment with cocaine (10 mg/kg/day for 5 days) in male Wistar rats. Cocaine-evoked hyperactivity was significantly enhanced by pretreatment with the preferential 5-HT$_{2A}$R agonist 1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane (DOI) and the 5-HT$_{2C}$R antagonist SDZ SER-082 [(+)-*cis*-4,5,7a,8,9,10,11,11a-octahydro-7*H*-10-methylindolo(1,7-BC)(2,6) naphthyridine fumarate]. The 5-HT$_{2A}$R antagonist SR 46349B [1(*Z*)-[2-(dimethylamino)ethoxyimino]-1(2-fluorophenyl)-3-(4-hydroxyphenyl)-2(*E*)-propene] and the preferential 5-HT$_{2C}$R agonist MK 212 [6-chloro-2-(1-piperazinyl)pyrazine HCl] (2 mg/kg) significantly attenuated acute cocaine-evoked hyperactivity; however, a lower dose of MK 212 (0.3 mg/kg) enhanced cocaine-evoked hyperactivity. The 5-HT$_{2B}$R agonist BW 723C86 (1-[5-(2-thienylmethoxy)-1*H*-3-indolyl]propan-2-amine HCl) and the 5-HT$_{2B}$R antagonist SB 204741 [*N*-(1-methyl-5-indolyl)-*N*′-(3-methyl-5-isothiazolyl) urea] had no effect on cocaine-evoked hyperactivity. Repeated treatment with cocaine alone resulted in a 2-fold increase in hyperactivity upon challenge with cocaine 5 days after termination of the cocaine regimen (sensitization). The 5-HT$_{2A}$R antagonist SR 46349B also blocked cocaine-evoked hyperactivity following repeated cocaine treatment, whereas the other 5-HT$_2$R ligands were ineffective. When any of the 5-HT$_2$R ligands was coadministered with cocaine during the treatment regimen (10 mg/kg/day for 5 days), the development of sensitization was unchanged as measured by the level of cocaine-evoked hyperactivity upon challenge 5 days after termination of the treatment. The present study implies that 5-HT$_{2A}$R and 5-HT$_{2C}$R exert oppositional influence upon hyperactivity evoked by acute administration of cocaine; this balance is altered following repeated cocaine administration.

Cocaine enhances dopamine (DA), serotonin (5-hydroxytryptamine; 5-HT), and norepinephrine neurotransmission through inhibition of their respective reuptake inhibitors (Koe, 1976). Enhancement of DA, particularly within the DA mesoaccumbens ("reward") pathway, is important in the locomotor-stimulant, reinforcing, and discriminative stimulus effects of cocaine (Pettit et al., 1984; Delfs et al., 1990; Callahan et al., 1997). However, the 5-HT system has also been shown to play a vital role in the modulation of DA mesoaccumbens pathways (Schmidt et al., 1992; De Deurwaerdere and Spampinato, 1999; Di Matteo et al., 1999; Gobert et al., 2000) and has been implicated in the mediation of cocaine-evoked behaviors, including cocaine-induced hyperactivity (McCreary and Cunningham, 1999; McMahon and Cunningham, 2001; McMahon et al., 2001; Filip and Cunningham, 2002, 2003; Fletcher et al., 2002, 2004; Bubar et al., 2003).

The 5-HT$_{2A}$ receptor (5-HT$_{2A}$R) and the 5-HT$_{2C}$R appear to have opposing influences on DA neurotransmission and psy-

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ABBREVIATIONS: DA, dopamine; 5-HT, 5-hydroxytryptamine, serotonin; 5-HT$_2$R, serotonin$_2$ receptor; SR 46349B, 1(*Z*)-[2-(dimethylamino)ethoxyimino]-1(2-fluorophenyl)-3-(4-hydroxyphenyl)-2(*E*)-propene; MK 212, 6-chloro-2-(1-piperazinyl)pyrazine HCl; DOI, 1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane; SDZ SER-082, (+)-*cis*-4,5,7a,8,9,10,11,11a-octahydro-7*H*-10-methylindolo(1,7-BC)(2,6) naphthyridine fumarate; BW 723C86, 1-[5-(2-thienylmethoxy)-1*H*-3-indolyl]propan-2-amine HCl; SB 204741, *N*-(1-methyl-5-indolyl)-*N*′-(3-methyl-5-isothiazolyl) urea; ANOVA, analysis of variance; M100907, *R*-(+)-(2,3-dimethoxyphenyl)-1-[2-(4-fluorophenylethyl)]-4-piperidine-methanol; SB 242084, 6-chloro-5-methyl-1-[[2-(2-methylpyridyl-3-oxy)-pyrid-5-yl]carbamoyl]indoline; SB 206553, *N*-3-pyridinyl-3,5-dihydro-5-methyl-benzo(1,2-*b*:4,5-*b*′)dipyrrole-1(2*H*)-carboxamide hydrochloride; GABA, γ-aminobutyric acid; NAc, nucleus accumbens; PFC, prefrontal cortex; VTA, ventral tegmental area.

chostimulant-evoked behaviors. Microdialysis assays suggest that the 5-HT$_{2A}$R can enhance DA neurotransmission under "stimulated" conditions such as after amphetamine administration (Schmidt et al., 1992) or dorsal raphe nucleus stimulation (De Deurwaerdere and Spampinato, 1999), whereas the 5-HT$_{2C}$R appears to exert inhibitory control over brain DA pathways (De Deurwaerdere and Spampinato, 1999; Di Matteo et al., 1999; Gobert et al., 2000). In keeping with a potentiative role for the 5-HT$_{2A}$R over DA mesoaccumbens circuits, 5-HT$_{2A}$R antagonists have been shown to block the hyperlocomotor (Filip et al., 2001; McMahon and Cunningham, 2001) and discriminative stimulus effects (McMahon and Cunningham, 2001; but see Callahan and Cunningham, 1995; Meert and Janssen, 1992), as well as relapse to self-administration evoked by cocaine (Fletcher et al., 2002). Conversely, systemic administration of brain-penetrant 5-HT$_{2C}$R antagonists has been shown to potentiate these same behavioral effects of cocaine (McCreary and Cunningham, 1999; Fletcher et al., 2002). These data suggest that the 5-HT$_2$R family may be functional and oppositional regulators of the neural substrates that control responsiveness to cocaine.

Modifications in serotonin function may be involved in the processes that underlie "behavioral sensitization" (Cunningham et al., 1992; Filip et al., 2001; Przegalinski et al., 2001). Behavioral sensitization is the enhancement of locomotor hyperactivity and stereotypies demonstrated upon challenge with cocaine during withdrawal from repeated, intermittent cocaine administration (for review, see Vanderschuren and Kalivas, 2000). This behavioral model has been used extensively to analyze the neural modifications associated with chronic cocaine exposure and withdrawal (White and Kalivas, 1998).

The present series of experiments was conducted to compare the ability of selective agonists and antagonists for specific 5-HT$_2$R subtypes to modulate locomotor activity evoked by acute cocaine administration versus their ability to modulate the acquisition or expression of locomotor sensitization to cocaine. A repeated cocaine treatment regimen of 10 mg/kg/day for 5 days has previously been shown to induce locomotor sensitization when expression is measured 5 days after the last treatment injection (Filip et al., 2001; Przegalinski et al., 2001). To examine the ability of the 5-HT$_2$R ligands to alter *acquisition* of cocaine sensitization, the ligands were administered before each daily injection of cocaine during the repeated treatment regimen, whereas the ability of the 5-HT$_2$R ligands to alter *expression* of sensitization was determined via administration of the ligands before challenge with cocaine 5 days after the termination of repeated cocaine treatment. We hypothesized that the selective 5-HT$_{2A}$R antagonist SR 46349B and the preferential 5-HT$_{2C}$R agonist MK 212 would limit acute cocaine (10 mg/kg)-evoked hyperactivity and the development and/or expression of sensitization to cocaine, whereas the preferential 5-HT$_{2A}$R agonist 1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane (DOI) and the selective 5-HT$_{2C}$R antagonist SDZ SER-082 were expected to enhance these cocaine-evoked behaviors. Although DOI has moderate affinity for all three 5-HT$_2$R subtypes (see Table 1), the effects of DOI on acute cocaine-evoked hyperactivity are thought to be primarily mediated by the 5-HT$_{2A}$R, since the behavioral effects of DOI (e.g., wet dog shakes) are preferentially blocked by 5-HT$_{2A}$R, but not 5-HT$_{2B/2C}$R, antagonists (Kennett, 1993; Schreiber et al., 1995). Since 5-HT$_{2B}$R expression in the brain is low (Duxon et al., 1997), the 5-HT$_{2B}$R agonist BW 723C86 and the 5-HT$_{2B}$R antagonist SB 204741 were predicted to have little or no effect on acute cocaine-evoked hyperactivity or cocaine sensitization.

Materials and Methods

Animals

Male Wistar rats (n = 832; Institute of Pharmacology Polish Academy of Sciences, Krakow, Poland) weighing 250 to 270 g at the beginning of the experiment were used. The rats were housed eight per cage in standard plastic rodent cages (57 cm × 35 cm × 20 cm) in a colony room maintained at 21 ± 2°C and at 40 to 50% humidity under a 12-h light/dark cycle (lights on at 7:00 AM) and had continuous access to tap water and rodent chow except during experimental sessions. All experiments were conducted during the light phase of the light/dark cycle (between 9:00 Am and 3:00 PM) and were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and with approval from the Bioethics Commission as compliant with the Polish Law (21 August 1997).

Drugs

The following drugs, their full chemical names (when relevant), the supplier, and the route of injection, respectively, were as follows: BW 723C86 [1-[5-(2-thienylmethoxy)-1*H*-3-indolyl]propan-2-amine HCl; Tocris Cookson, Bristol, UK; i.p.], cocaine HCl (Merck, Darmstadt, Germany; i.p.), DOI [1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane HCl; Sigma-Aldrich, St. Louis, MO; i.p.], MK 212 [6-chloro-2-(1-piperazinyl)pyrazine HCl; Tocris Cookson; i.p.], SB 204741 [*N*-(1-methyl-5-indolyl)-*N'*-(3-methyl-5-isothiazolyl) urea; Tocris Cookson; i.p.], SDZ SER-082 [(+)-*cis*-4,5,7a,8,9,10,11,11a-octahydro-7*H*-10-methylindolo(1,7-BC)(2,6) naphthyridine fumarate; Tocris Cookson; i.p.], and SR 46349B [1(*Z*)-[2-(dimethylamino)ethoxyimino]-1(2-fluorophenyl)-3-(4-hydroxyphenyl)-2(*E*)-propene; Sanofi-Sythelabo, Paris, France; s.c.]. To achieve dissolution, DOI, MK 212, and SDZ SER-082 were dissolved in saline (0.9% NaCl), BW 723C86

TABLE 1
Affinity (K_i, nM) of ligands for 5-HT$_2$R subtypes

Ligand	Receptor Subtype			Reference
	5-HT$_{2A}$R	5-HT$_{2B}$R	5-HT$_{2C}$R	
BW 723C86	>3891	12	125	Baxter et al. (1995)
DOI	50[a]	39[a]	15[a]	Kennett (1993)
MK 212	15,848	1258	630	Kennett (1993)
SB 204741	5011	15	>1000	Baxter et al. (1995)
SR 46349B	5.8[b]	>100	120[b]	Rinaldi-Carmona et al. (1992)
SDZ SER-082	630[c]	58	15[c]	Nozulak et al. (1995)

[a] EC_{50} (nM).
[b] IC_{50} (nM).
[c] K_D (nM).

and SB 204741 were suspended in aqueous 1% Tween solution, and SR 46349B was dissolved in two to three drops of ethanol and diluted as required in distilled water. All drugs were injected in a volume of 1 ml/kg. The doses of drugs were chosen based upon their functional selectivity at a particular 5-HT_2R (Cunningham et al., 1986; Rinaldi-Carmona et al., 1992; Schreiber et al., 1995; Kennett et al., 1997; Gobert et al., 2000); the affinity profiles for each of the 5-HT_2R ligands are presented in Table 1.

Apparatus

Locomotor activity was monitored and quantified in clear Plexiglas chambers (43 cm × 43 cm × 25 cm) housed inside Opto-Varimex activity monitors surrounded with a 15 × 15 array of photocell beams located 3 cm from the floor surface (Columbus Instruments, Columbus, OH). Interruptions of these photobeams resulted in horizontal activity defined as distance traveled (expressed in centimeters). Records of horizontal activity were made by the control software (Columbus Instruments) for subsequent statistical evaluation.

Procedures

Effects of 5-HT_2R Ligands on Acute Cocaine-Evoked Locomotor Activity. Rats were habituated to the test environment for 2 h/day on each of the 2 days before the start of the experiment, and on each test day for 1 h before the start of the test session. Animals were tested only one time, and separate groups of animals (n = 8/group) were pretreated with either the 5-HT_{2A}R agonist DOI (0.1–1 mg/kg), 5-HT_{2A}R antagonist SR 46349B (0.25–1 mg/kg), 5-HT_{2B}R agonist BW 723C86 (3–10 mg/kg), 5-HT_{2B}R antagonist SB 204741 (1–3 mg/kg), 5-HT_{2C}R agonist MK 212 (0.1–2 mg/kg), 5-HT_{2C}R antagonist SDZ SER-082 (0.25–1 mg/kg), or the appropriate vehicle 30 min (DOI, BW 723C86, MK 212, SDZ SER-082) or 40 min (SR 46349B, SB 204741) before an i.p. treatment injection of either saline (1 ml/kg) or cocaine (10 mg/kg). Measurements of locomotor activity began immediately after the second (saline or cocaine) injection and lasted 60 min.

Effects of 5-HT_2R Ligands on the Acquisition of Behavioral Sensitization to Cocaine. On each day for 5 consecutive days, rats (n = 8/group) were removed from their home cage, weighed, and injected with either DOI (0.1–1 mg/kg), SR 46349B (0.25–1 mg/kg), BW 723C86 (3–10 mg/kg), SB 204741 (1–3 mg/kg), MK 212 (0.1–2 mg/kg), or SDZ SER-082 (0.25–1 mg/kg) and returned to their home cage; 30 to 40 min later, rats received an injection of cocaine (10 mg/kg) and were immediately returned to their home cage. Control rats (n = 8/group) were injected with the appropriate vehicle (1 ml/kg; see *Drugs*, above) before an injection of saline (1 ml/kg) or cocaine (10 mg/kg) each day for 5 consecutive days in a similar manner. All injections occurred between 10:30 AM and 1:30 PM. On days 3 and 4 following the last repeated injection, rats were habituated to the test environment for 2 h/day. On the 5th day after the last repeated injection, rats were habituated to the test environment for 40 min. The rats were then removed from the test environment to receive a challenge of cocaine (10 mg/kg) and immediately returned to the test environment, and locomotor activity was recorded for 60 min. Each rat underwent only one test session.

Effects of 5-HT_2R Ligands on the Expression of Behavioral Sensitization to Cocaine. Rats (n = 8/group) were removed from their home cage, weighed, and injected with vehicle (1 ml/kg) or cocaine (10 mg/kg) and immediately returned to their home cage each day for 5 days. All injections occurred between 10:30 AM and 1:30 PM. On days 3 and 4 following the last repeated injection, rats were habituated to the test environment for 2 h/day. On the 5th day after the last repeated injection, rats were habituated to the test environment and briefly removed from the test environment after 1 h of habituation to receive a pretreatment injection of an appropriate vehicle (1 ml/kg), DOI (0.1–1 mg/kg), SR 46349B (0.25–1 mg/kg), BW 723C86 (3–10 mg/kg), SB 204741 (1–3 mg/kg), MK 212 (0.1–2 mg/kg), or SDZ SER-082 (0.25–1 mg/kg) and returned to the test environment. Thirty minutes (DOI, BW 723C86, MK 212, SDZ SER-082) or 40 min later (SR 46349B, SB 204741), the rats received an i.p. treatment injection of either saline (1 ml/kg) or cocaine (10 mg/kg) and were returned to the test environment, and their locomotor activity was recorded for 60 min. Each rat underwent only one test session.

Data Analysis

For analyses of the effects of acute administration of 5-HT_2R ligands on cocaine-induced hyperactivity, data are presented as mean horizontal distance traveled in centimeters (±S.E.M.) for the 60-min observation period. The data were analyzed using a two-way analysis of variance (ANOVA) for the factors of pretreatment [0 mg/kg (i.e., vehicle) and different doses of the 5-HT_2R ligand], treatment (0 or 10 mg/kg cocaine), and the pretreatment × treatment interaction. The Student-Newman-Keuls procedure was used to analyze preplanned, pairwise comparisons; all comparisons were conducted with an experimentwise error rate of $\alpha = 0.05$.

For the sensitization experiments, the dependent measure was horizontal activity (mean total distance traveled in cm ± S.E.M.) observed during the 60-min challenge test 5 days after the last repeated treatment. Because group comparisons on challenge days were specifically defined before the start of the experiment, these planned comparisons were conducted in lieu of an overall F test in a mulifactorial ANOVA; this analysis has been supported in a number of statistical texts (e.g., Keppel, 1973). Each experiment was subjected to a one-way ANOVA with levels of the treatment factor corresponding to the dose of 5-HT_2R ligand + cocaine administered either during the repeated treatment regimen (acquisition of sensitization) or upon challenge 5 days after repeated cocaine treatment (expression of sensitization). Subsequent a priori comparisons between means representing changes from baseline activity for horizontal activity were made using a Student's t test (SAS for Windows, Version 8.1; SAS Institute, Cary, NC), which were conducted with an experimentwise error rate of $\alpha = 0.05$.

Results

Hyperactivity Induced by Acute Cocaine Administration

Effects of the 5-HT_{2A}R Agonist DOI on Cocaine-Induced Hyperactivity. A main effect of pretreatment ($F_{3,56} = 4.74$, $p < 0.01$), treatment ($F_{1,7} = 42.12$, $p < 0.001$), and a pretreatment × treatment interaction ($F_{3,56} = 2.90$, $p < 0.05$) was observed for total horizontal activity summed across the 1-h session. DOI (0.1–1 mg/kg) administered before a systemic saline injection did not alter basal locomotor activity ($p > 0.05$). Pretreatment with DOI dose dependently increased the horizontal activity induced by cocaine (10 mg/kg), reaching significance at the highest dose (1 mg/kg) of DOI tested ($p < 0.05$; Fig. 1A).

Effects of the 5-HT_{2A}R Antagonist SR 46349B on Cocaine-Induced Hyperactivity. A main effect of pretreatment ($F_{3,56} = 6.99$, $p < 0.001$), treatment ($F_{1,7} = 29.49$, $p < 0.001$), and a pretreatment × treatment interaction ($F_{3,56} = 2.95$, $p < 0.05$) was observed for total horizontal activity summed across the 1-h session. Pretreatment with SR 46349B (0.25–1 mg/kg) dose dependently attenuated cocaine-induced horizontal activity ($p < 0.05$); activity levels observed following pretreatment with 1 mg/kg SR 46349B were significantly decreased ($p < 0.001$; Fig. 1B) to levels that were not significantly different from vehicle + saline controls ($p > 0.05$). SR 46349B (0.25–1 mg/kg) tested alone did not significantly alter basal locomotor activity ($p > 0.05$).

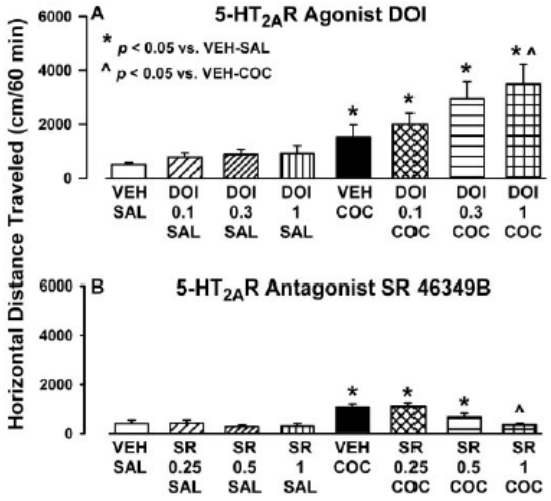

Fig. 1. Basal and cocaine-evoked hyperactivity following pretreatment with the 5-HT$_{2A}$R ligands. Data represent the mean horizontal distance traveled (±S.E.M.) summed over the 60-min recording period after injection of vehicle (VEH) or the 5-HT$_{2A}$R agonist DOI (0.1, 0.3, or 1 mg/kg) (A), or VEH or the 5-HT$_{2A}$R antagonist SR 46349B (SR; 0.25, 0.5 or 1 mg/kg) (B) followed by an injection of saline (SAL) or cocaine (COC; 10 mg/kg). Data points represent the mean of data from eight rats. ⋆, $p <$ 0.05 versus VEH-SAL; ∧, $p < 0.05$ versus VEH-COC.

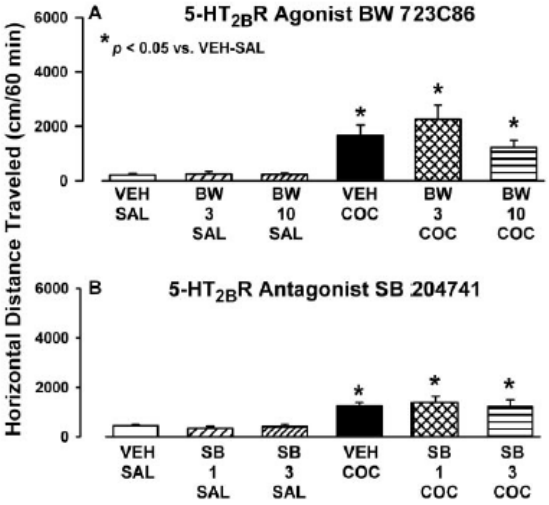

Fig. 2. Basal and cocaine-evoked hyperactivity following pretreatment with the 5-HT$_{2B}$R ligands. Data represent the mean horizontal distance traveled (±S.E.M.) summed over the 60-min recording period after injection of vehicle (VEH) or the 5-HT$_{2B}$R agonist BW 723C86 (BW; 3 or 10 mg/kg) (A), or VEH or the 5-HT$_{2B}$R antagonist SB 204741 (SB; 1 or 3 mg/kg) (B) followed by an injection of saline (SAL) or cocaine (COC; 10 mg/kg). Data points represent the mean of data from eight rats. ⋆, $p < 0.05$ versus VEH-SAL.

Effects of the 5-HT$_{2B}$R Agonist BW 723C86 on Cocaine-Induced Hyperactivity. A main effect of treatment ($F_{1,5} = 40.31$, $p < 0.001$), but not pretreatment ($F_{2,42} = 1.67$, $p > 0.05$) or a pretreatment × treatment interaction ($F_{2,42} = 1.58$, $p > 0.05$), was observed for total horizontal activity summed across the 1-h session. Neither of the doses of BW 723C86 (3 and 10 mg/kg) significantly altered either basal or cocaine-induced horizontal activity ($p > 0.05$; Fig. 2A).

Effects of the 5-HT$_{2B}$R Antagonist SB 204741 on Cocaine-Induced Hyperactivity. A main effect of treatment ($F_{1,5} = 42.00$, $p < 0.001$), but not pretreatment ($F_{2,42} = 0.04$, $p > 0.05$) or a pretreatment × treatment interaction ($F_{2,42} = 0.33$, $p > 0.05$), was observed for total horizontal activity summed across the 1-h session. Neither of the doses of SB 204741 (1 and 3 mg/kg) significantly altered basal or cocaine-induced horizontal activity ($p > 0.05$; Fig. 2B).

Effects of 5-HT$_{2C}$R Agonist MK 212 on Cocaine-Induced Hyperactivity. A main effect of pretreatment ($F_{4,70} = 5.08$, $p < 0.01$), treatment ($F_{1,9} = 44.39$, $p < 0.001$), and a pretreatment × treatment interaction ($F_{4,70} = 4.28$, $p < 0.01$) was observed for total horizontal activity summed across the 1-h session. Pretreatment with 0.3 mg/kg MK 212 enhanced cocaine-induced horizontal activity ($p < 0.05$), whereas 2 mg/kg MK 212 significantly reduced cocaine-induced increases in locomotor activity ($p < 0.05$) to levels that were not significantly different from vehicle + saline controls ($p > 0.05$; Fig. 3A). However, 2 mg/kg MK 212 significantly reduced basal locomotor activity ($p < 0.05$; Fig. 3A).

Effects of the 5-HT$_{2C}$R Antagonist SDZ SER-082 on Cocaine-Induced Hyperactivity. A main effect of pretreatment ($F_{3,56} = 15.97$, $p < 0.01$), treatment ($F_{1,7} = 64.25$, $p < 0.001$), and a pretreatment × treatment interaction ($F_{3,56} = 17.32$, $p < 0.001$) was observed for total horizontal activity summed across the 1-h session. Pretreatment with SDZ SER-082 increased the horizontal activity induced by cocaine (10 mg/kg) in a dose-dependent manner; a significant enhancement was observed after 1 mg/kg SDZ SER-082 ($p <$ 0.001; Fig. 3B). SDZ SER-082 (0.25–1 mg/kg) did not alter basal locomotor activity ($p > 0.05$).

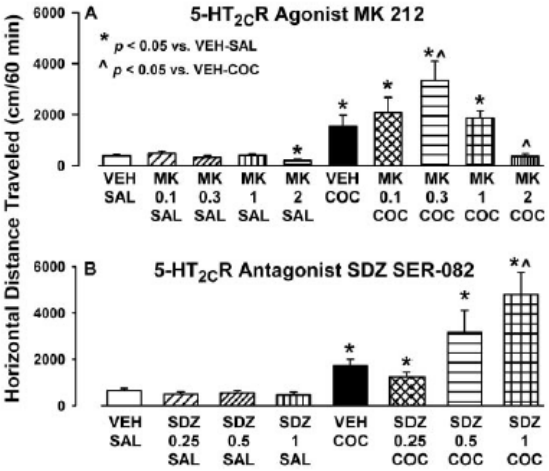

Fig. 3. Basal and cocaine-evoked hyperactivity following pretreatment with the 5-HT$_{2C}$R ligands. Data represent the mean horizontal distance traveled (±S.E.M.) summed over the 60-min recording period after injection of vehicle (VEH) or the 5-HT$_{2C}$R agonist MK 212 (MK; 0.1, 0.3, 1 or 2 mg/kg) (A), or VEH or the 5-HT$_{2C}$R antagonist SDZ SER-082 (SDZ; 0.25, 0.5 or 1 mg/kg) (B) followed by an injection of saline (SAL) or cocaine (COC; 10 mg/kg). Data points represent the mean of data from eight rats. ⋆, $p < 0.05$ versus VEH-SAL; ∧, $p < 0.05$ versus VEH-COC.

TABLE 2

Effects of the 5-HT_2R ligands on the development of cocaine sensitization

Rats were treated repeatedly with vehicle (VEH) + saline (SAL), vehicle + cocaine (COC; 10 mg/kg), or a 5-HT_2R ligand + cocaine (10 mg/kg) for 5 days. Five days after termination of the repeated regimen, rats were challenged with cocaine (10 mg/kg) and locomotor activity was measured. Data points represent the mean horizontal distance traveled (cm ± S.E.M.; 8 rats/group) in response to the cocaine challenge.

Repeated Treatment	Distance Traveled	Repeated Treatment	Distance Traveled
	cm ± S.E.M.		*cm ± S.E.M.*
VEH/SAL	971 ± 184	VEH/SAL	1317 ± 275
VEH/COC	4158 ± 527*	VEH/COC	3967 ± 499*
DOI 0.1/COC	4854 ± 454*	SR 46349B 0.25/COC	4653 ± 609*
DOI 0.3/COC	5034 ± 685*	SR 46349B 0.5/COC	2855 ± 971
DOI 1.0/COC	4412 ± 618*	SR 46349B 1.0/COC	3732 ± 995*
VEH/SAL	2335 ± 581	VEH/SAL	2335 ± 851
VEH/COC	5915 ± 844*	VEH/COC	5915 ± 844*
BW 723C86 3.0/COC	5200 ± 858*	SB 204741 1.0/COC	4992 ± 868*
BW 723C86 10.0/COC	6321 ± 1012*	SB 204741 3.0/COC	3934 ± 914
VEH/SAL	1266 ± 379	VEH/SAL	1784 ± 296
VEH/COC	4333 ± 1023*	VEH/COC	5997 ± 788*
MK 212 0.1/COC	3769 ± 616*	SDZ SER-082 0.25/COC	4862 ± 1002*
MK 212 0.3/COC	5102 ± 784*	SDZ SER-082 0.5/COC	4780 ± 966*
MK 212 1.0/COC	3947 ± 633*	SDZ SER-082 1.0/COC	5746 ± 1213*
MK 212 2.0/COC	3366 ± 729*		

* $p < 0.05$ vs. VEH/SAL.

Cocaine Sensitization

Rats repeatedly treated with cocaine (5 days) displayed an ~2-fold increase in locomotor activity when challenged with cocaine (10 mg/kg) 5 days after the last treatment injection compared with the effect of the acute injection of cocaine (10 mg/kg) in vehicle-treated (5 days) rats (Table 2; Figs. 4–6), indicating that behavioral sensitization was detected at 5 days after termination of the repeated cocaine treatment utilized in these experiments.

Effects of 5-HT_2R Ligands on the Development of Cocaine Sensitization. Rats received five daily pretreat-

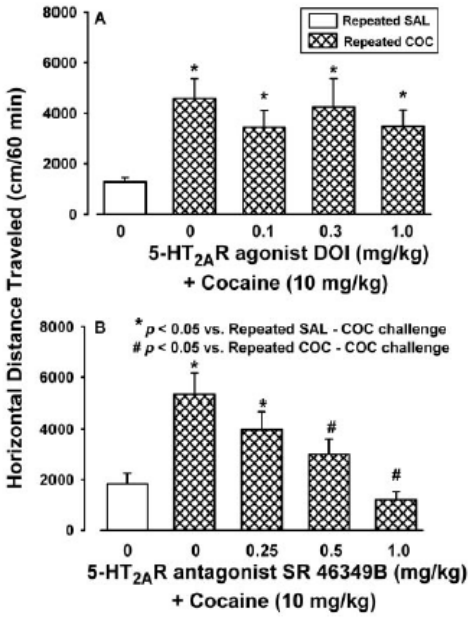

Fig. 4. Challenge with 5-HT_{2A}R ligands + cocaine following the repeated cocaine sensitization regimen. Rats were treated repeatedly with saline (SAL; 1 ml/kg; open bars) or cocaine (COC; 10 mg/kg; hatched bars) once a day for 5 days. Five days after the last repeated treatment, rats were challenged with the 5-HT_{2A}R agonist DOI (0, 0.1, 0.3, or 1.0 mg/kg) + cocaine (10 mg/kg) (A), or the 5-HT_{2A}R antagonist SR 46349B (0, 0.25, 0.5, or 1 mg/kg) + cocaine (10 mg/kg) (B). Data points represent the mean horizontal distance traveled (±S.E.M.) over the 1-h recording period from eight rats. ⋆, $p < 0.05$ versus repeated SAL-COC challenge group; #, $p < 0.05$ versus repeated COC-COC challenge group.

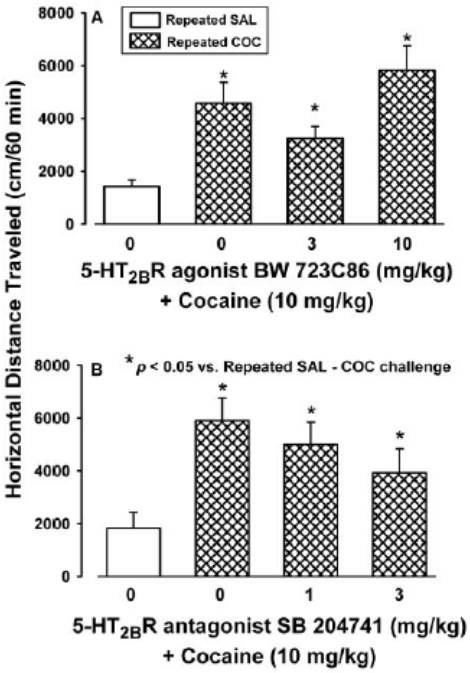

Fig. 5. Challenge with 5-HT_{2B}R ligands + cocaine following the repeated cocaine sensitization regimen. Rats were treated repeatedly with saline (SAL; 1 ml/kg; open bars) or cocaine (COC; 10 mg/kg; hatched bars) once a day for 5 days. Five days following the last repeated treatment, rats were challenged with [A] the 5-HT_{2B}R agonist BW 723C86 (0, 3 or 10 mg/kg) + cocaine (10 mg/kg), or [B] the 5-HT_{2B}R antagonist SB 204741 (0, 1, or 3 mg/kg) + cocaine (10 mg/kg). Data points represent the mean horizontal distance traveled (± S.E.M.) over the 1-h recording period from 8 rats. ⋆, $p < 0.05$ versus repeated SAL-COC challenge group.

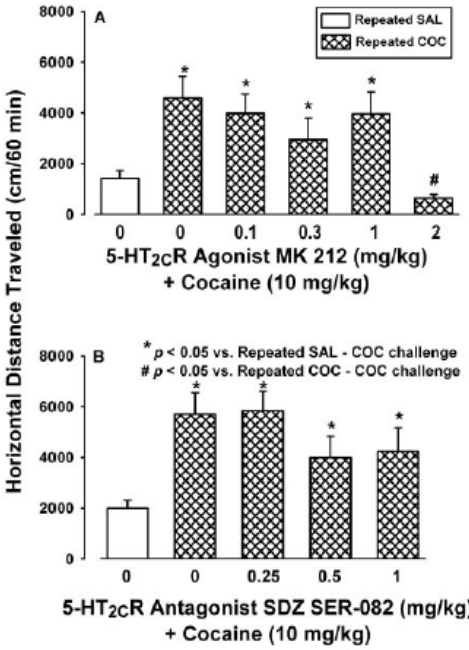

Fig. 6. Challenge with 5-HT$_{2C}$R ligands + cocaine following the repeated cocaine sensitization regimen. Rats were treated repeatedly with saline (SAL; 1 ml/kg; open bars) or cocaine (COC; 10 mg/kg; hatched bars) once a day for 5 days. Five days after the last repeated treatment, rats were challenged with the 5-HT$_{2C}$R agonist MK 212 (0, 0.1, 0.3, 1, or 2 mg/kg) + cocaine (10 mg/kg) (A), or the 5-HT$_{2C}$R antagonist SDZ SER-082 (0, 0.25, 0.5, or 1 mg/kg) + cocaine (10 mg/kg) (B). Data points represent the mean horizontal distance traveled (±S.E.M.) over the 1-h recording period from eight rats. ⋆, $p < 0.05$ versus repeated SAL-COC challenge group; #, $p < 0.05$ versus repeated COC-COC challenge group.

ments with vehicle or a 5-HT$_2$R ligand followed by an injection of saline or cocaine (10 mg/kg). Five days after termination of the repeated regimen, the animals were challenged with cocaine (10 mg/kg) and locomotor activity was measured. A main effect of treatment was observed for horizontal activity summed across the 60-min session in the experimental groups pretreated with DOI ($F_{4,34} = 10.767$, $p < 0.001$), SR 46349B ($F_{4,35} = 3.90$ $p < 0.05$), BW 723C86 ($F_{3,28} = 7.93$, $p < 0.001$), SB 204741 ($F_{3,28} = 3.68$, $p < 0.05$), MK 212 ($F_{5,42} = 6.03$, $p < 0.001$), and SDZ SER-082 ($F_{4,34} = 3.48$, $p < 0.05$). Rats repeatedly dosed with vehicle + cocaine or any 5-HT$_2$R ligand + cocaine combination exhibited significantly higher levels of activity upon challenge with cocaine compared with animals repeatedly injected with vehicle + saline (sensitization; Table 2). In all cases, the degree of hyperactivity seen upon challenge with cocaine on the test day was similar regardless of the pharmacological regimen imposed during the repeated treatment ($p > 0.05$; Table 2).

Effects of 5-HT$_2$R Ligands on Expression of Cocaine Sensitization. Rats were treated daily with vehicle or cocaine (10 mg/kg/day for 5 days). Five days after the last injection, the animals were challenged with vehicle or a 5-HT$_2$R ligand followed by cocaine (10 mg/kg) and locomotor activity was measured.

Effects of the 5-HT$_{2A}$R Agonist DOI on Expression of Cocaine Sensitization. A main effect of treatment was observed for total horizontal activity summed across the 60-min session ($F_{4,34} = 2.97$, $p < 0.05$). However, pretreatment with DOI (0.1–1 mg/kg) did not significantly alter hyperactivity expressed upon challenge with cocaine (10 mg/kg) 5 days after termination of the repeated cocaine regimen ($p > 0.05$; Fig. 4A).

Effects of the 5-HT$_{2A}$R Antagonist SR 46349B on Expression of Cocaine Sensitization. A main effect of treatment was observed for total horizontal activity summed across the 60-min session ($F_{4,35} = 17.21$, $p < 0.001$). Pretreatment with SR 46349B (0.25–1 mg/kg) dose dependently reduced the hyperactivity induced upon challenge with cocaine (10 mg/kg) 5 days after termination of the repeated cocaine regimen; pretreatment with 0.5 and 1.0 mg/kg SR 46349B significantly suppressed cocaine-evoked hyperactivity ($p < 0.05$; Fig. 4B).

Effects of the 5-HT$_{2B}$R Agonist BW 723C86 on Expression of Cocaine Sensitization. A main effect of treatment was observed for total horizontal activity summed across the 60-min session ($F_{3,27} = 12.44$, $p < 0.001$). BW 723C86 had no effect on the expression of sensitization since pretreatment with BW 723C86 (3 or 10 mg/kg) did not significantly alter hyperactivity induced by challenge with cocaine (10 mg/kg) 5 days after termination of the repeated cocaine regimen ($p > 0.05$; Fig. 5A).

Effects of the 5-HT$_{2B}$R Antagonist SB 204741 on Expression of Cocaine Sensitization. A main effect of treatment was observed for total horizontal activity summed across the 60-min session ($F_{3,28} = 4.87$, $p < 0.01$). None of the doses of SB 204741 significantly altered hyperactivity expressed upon challenge with cocaine (10 mg/kg) 5 days after termination of the repeated cocaine regimen ($p > 0.05$; Fig. 5B).

Effects of the 5-HT$_{2C}$R Agonist MK 212 on Expression of Cocaine Sensitization. A main effect of treatment was observed for total horizontal activity summed across the 60-min session ($F_{5,40} = 12.64$, $p < 0.001$). Pretreatment with MK 212 (0.1–1 mg/kg) did not significantly alter hyperactivity expressed upon challenge with cocaine (10 mg/kg) 5 days after termination of the repeated cocaine regimen. However, the highest dose (2 mg/kg) of MK 212 significantly reduced hyperactivity induced by challenge with cocaine ($p < 0.01$; Fig. 6A); this dose of MK 212 also suppressed basal locomotor activation (see Fig. 3A).

Effects of the 5-HT$_{2C}$R Antagonist SDZ SER-082 on Expression of Cocaine Sensitization. A main effect of treatment was observed for total horizontal activity summed across the 60-min session ($F_{4,34} = 7.36$, $p < 0.001$). Pretreatment with SDZ SER-082 (0.25–1 mg/kg) did not significantly alter hyperactivity expressed upon challenge with cocaine (10 mg/kg) 5 days after termination of the repeated cocaine regimen ($p > 0.05$; Fig. 6B).

Discussion

The present studies were conducted to compare the ability of 5-HT$_2$R agonists and antagonists to alter acute cocaine-evoked hyperactivity with the ability of these same ligands to alter the acquisition and/or expression of cocaine sensitization. The results suggest that the 5-HT$_{2A}$R plays a stimulatory role in cocaine hyperactivity induced by either acute cocaine administration or challenge with cocaine 5 days after

termination of the sensitization regimen, while having no influence upon the acquisition of locomotor sensitization. Conversely, the 5-HT_{2C}R appears to have an inhibitory role in cocaine hyperactivity induced by acute cocaine administration, while having little influence upon the acquisition of sensitization or cocaine-evoked hyperactivity following the sensitizing cocaine regimen. The 5-HT_{2B}R has no overt role in elicitation of cocaine-evoked hyperactivity or cocaine sensitization.

The present observations following administration of the 5-HT_{2A}R antagonist SR 46349B support and extend previous findings that the selective 5-HT_{2A}R antagonist M100907 (McMahon and Cunningham, 2001; Fletcher et al., 2002) and the nonselective 5-HT_2R antagonist ketanserin (Filip et al., 2001; McMahon and Cunningham, 2001) attenuated cocaine-evoked hyperactivity at doses of the antagonists that did not alter basal activity levels. SR 46349B (present study) and ketanserin (Filip et al., 2001) also attenuated hyperactivity induced by challenge with cocaine 5 days after termination of the sensitizing cocaine regimen. In keeping with these results, we are the first to report that the preferential 5-HT_{2A}R agonist DOI enhanced the locomotor-activating effects of cocaine administered acutely, at doses of DOI that did not alter basal locomotor activity. After a sensitizing regimen of cocaine, however, DOI is no longer capable of further enhancing hyperactivity seen upon cocaine challenge 5 days after termination of the repeated cocaine regimen. In addition, cotreatment with neither DOI nor SR 46349B during the repeated cocaine regimen effectively altered the course of sensitization, suggesting that the role for 5-HT_{2A}R in acquisition of cocaine sensitization is minimal. This may be attributed to the rapid desensitization and down-regulation of 5-HT_{2A}R that can occur following repeated administration of either 5-HT_{2A}R agonists or antagonists (for review, see Gray and Roth, 2001) and, potentially, cocaine (e.g., Darmani et al., 1997; but see Baumann and Rothman, 1998). Thus, although the 5-HT_{2A}R appears to be integral for the enhancement of hyperactivity induced by acute administration of cocaine, the functional role of the 5-HT_{2A}R appears to be altered with repeated cocaine administration.

Loss of the enhancement of cocaine-induced hyperactivity by the preferential 5-HT_{2A}R agonist DOI was observed in rats exposed to repeated cocaine administration. An alteration in the expression of the 5-HT_{2A}R or its downstream components after repeated cocaine administration may account for this observation. Repeated cocaine administration has been shown to result in short-term supersensitivity of 5-HT_{2A}R during withdrawal (Baumann and Rothman, 1998) that appears to be associated with an increase in membrane-associated $G_{q/11}$ protein expression, rather than a specific increase in 5-HT_{2A}R (B_{max}) expression (Carrasco et al., 2003). Since cocaine-induced 5-HT efflux is enhanced in cocaine-sensitized animals (Parsons and Justice, 1993), it is possible that the 5-HT_{2A}R is in a state of maximal stimulation following cocaine challenge in the sensitized animals. Under these circumstances, DOI may be unable to provide any additional stimulation of 5-HT_{2A}R over levels of activation induced by endogenous 5-HT, which accumulates in the synapse after cocaine administration alone (Parsons and Justice, 1993). Conversely, the high-affinity 5-HT_{2A}R antagonist SR 46349B may effectively compete with 5-HT for 5-HT_{2A}R binding sites (Rinaldi-Carmona et al., 1992), and thus, SR 46349B blockade of 5-HT_{2A}R would continue to exert its inhibitory effect upon hyperactivity evoked by cocaine challenge, as was observed in the present study. This hypothesis is further supported by the present observation that, in animals treated with the repeated cocaine regimen, the levels of hyperactivity induced by challenge with cocaine alone were greater than those elicited by acute administration of DOI + cocaine in animals that had not been previously exposed to cocaine. Thus, the present results suggest that adaptation of the 5-HT_{2A}R or its downstream signaling components may occur following repeated cocaine administration and that these modifications may contribute to the inability of the 5-HT_{2A}R agonist to modulate the sensitized response to cocaine.

Alternatively, it is important to note that DOI is not a selective 5-HT_{2A}R agonist (see Table 1), and thus may also act at other 5-HT_2R subtypes. Considering the opposing actions of the 5-HT_{2A}R and 5-HT_{2C}R on cocaine-evoked hyperactivity (present results; McMahon and Cunningham, 2001; Fletcher et al., 2002), simultaneous stimulation of 5-HT_{2A}R and 5-HT_{2C}R by DOI may have contributed to the lack of effect of DOI on cocaine-evoked hyperactivity following the cocaine sensitization regimen.

Administration of the selective 5-HT_{2C}R antagonists SDZ SER-082 (present study) or SB 242084 (Fletcher et al., 2002) as well as the 5-$HT_{2B/2C}$R antagonist SB 206553 (McCreary and Cunningham, 1999) enhanced cocaine-evoked hyperactivity, suggesting that the 5-HT_{2C}R exerts an inhibitory influence on acute cocaine-evoked hyperactivity. Interestingly, a biphasic effect of the preferential 5-HT_{2C}R agonist MK 212 on cocaine-evoked hyperactivity was observed; enhancement and suppression were elicited by a low (0.3 mg/kg) and high dose of MK 212 (2 mg/kg), respectively. The high dose of MK 212 (2 mg/kg) was also shown to significantly suppress basal locomotor activation. A reciprocal biphasic effect was previously demonstrated following administration of the 5-$HT_{2B/2C}$R antagonist SB 206553, with lower doses suppressing and higher doses enhancing cocaine-evoked hyperactivity (McCreary and Cunningham, 1999); however, a significant biphasic effect was not observed following administration of the selective 5-HT_{2C}R antagonist SDZ SER-082 in the present study. The biphasic nature of the response may be due to the lack of complete selectivity of either MK 212 or SB 206553 for the 5-HT_{2C}R and, in particular, the lack of selectivity over the 5-HT_{2B}R. However, the inability of the selective 5-HT_{2B}R agonists and antagonists to alter cocaine-evoked hyperactivity as observed in the present and other studies (Fletcher et al., 2002) suggests that this distinction is unlikely attributable to the 5-HT_{2B}R.

A potential explanation for the biphasic effects of 5-HT_{2C}R agonists and antagonists on cocaine-induced hyperactivity is the differential influence of various populations of 5-HT_{2C}R within the DA mesolimbic pathways. In the ventral tegmental area (VTA), the origin of the DA mesolimbic pathways, 5-HT_{2C}Rs appear to be located on both DA and γ-aminobutyric acid (GABA) neurons (Eberle-Wang et al., 1997; Bubar and Cunningham, 2003). Systemic administration of 5-HT_{2C}R antagonists has been shown to either decrease (Blackburn et al., 2002) or increase (Di Matteo et al., 1999) the firing rate of spontaneously active VTA DA neurons, suggesting that different populations of 5-HT_{2C}Rs within the

VTA may differentially activate VTA DA neurons (Blackburn et al., 2002). In addition, 5-HT$_{2C}$Rs are also located in the nucleus accumbens (NAc) and prefrontal cortex (PFC), the terminal regions of the DA mesolimbic pathways. Microinfusion of 5-HT$_{2C}$R agonists into the NAc (Filip and Cunningham, 2002) or PFC (Filip and Cunningham, 2003) enhanced or reduced, respectively, cocaine-evoked hyperactivity, with reciprocal effects observed following antagonist administration (Filip and Cunningham, 2002, 2003). These data suggest that 5-HT$_{2C}$Rs in the NAc and PFC exert opposing influence upon cocaine-evoked hyperactivity. Thus, the ability of systemically administered 5-HT$_{2C}$R agonists and antagonists to alter cocaine-evoked hyperactivity in a biphasic manner may be attributed to the oppositional influence of 5-HT$_{2C}$R populations within and/or between brain regions associated with the DA mesolimbic pathways.

In contrast to the profound effects of 5-HT$_{2C}$R ligands on cocaine-evoked hyperactivity following acute cocaine administration, neither MK 212 nor SDZ SER-082 effectively altered the acquisition of sensitization. The 5-HT$_{2C}$R antagonist SDZ SER-082 also had no effect on cocaine-evoked hyperactivity 5 days after the repeated cocaine sensitization regimen, whereas only the highest dose of the 5-HT$_{2C}$R agonist MK 212 (2 mg/kg), which suppressed basal activity alone, was effective in suppressing cocaine-evoked hyperactivity. The lack of effects of the 5-HT$_{2C}$R ligands on cocaine-evoked hyperactivity following the cocaine sensitization regimen suggests that repeated cocaine administration results in alterations in the contribution of 5-HT$_{2C}$R to cocaine-evoked hyperactivity.

The loss of effect of both the 5-HT$_{2C}$R agonist and antagonist following the cocaine sensitization regimen suggests that the function of these receptors or their downstream signaling components is altered significantly following repeated exposure to cocaine. Unfortunately, to our knowledge, alterations in the sensitivity or expression of the 5-HT$_{2C}$R or its key signaling components subsequent to repeated cocaine administration have not yet been examined. Recent evidence from our laboratory, however, suggests that 5-HT$_{2C}$R sensitivity is decreased during withdrawal from a sensitizing regimen of the 5-HT/DA releaser (+)-3,4-methylenedioxymethamphetamine (Bubar et al., 2002); thus a similar consequence may occur following repeated cocaine administration. Since the 5-HT$_{2C}$R appears to predominantly exert an inhibitory influence upon DA mesoaccumbens pathway activation (De Deurwaerdere and Spampinato, 1999; Di Matteo et al., 1999; Gobert et al., 2000) and cocaine-evoked hyperactivity (present study; Fletcher et al., 2002), down-regulation of the 5-HT$_{2C}$R following repeated cocaine administration would likely result in a decrease in 5-HT$_{2C}$R-mediated inhibition. This loss of 5-HT$_{2C}$R inhibition would be expected to enhance activation of the DA mesoaccumbens pathway and cocaine-evoked hyperactivity. This hypothesis is consistent with enhanced cocaine-evoked DA release (Vanderschuren and Kalivas, 2000) and sensitization of hyperactivity, as well as the loss of influence of the 5-HT$_{2C}$R ligands observed following repeated cocaine administration (present results).

In conclusion, the present results confirm previous evidence that the 5-HT$_{2A}$R and 5-HT$_{2C}$R exert opposing modulatory actions on hyperactivity evoked by acute cocaine administration, whereas 5-HT$_{2B}$Rs do not appear to be involved in elicitation of cocaine-evoked hyperactivity. In addition, we provide evidence that repeated cocaine administration may result in adaptations that contribute to the expression of sensitization and that alter the ability of 5-HT$_{2A}$R and 5-HT$_{2C}$R ligands to modulate cocaine-evoked hyperactivity.

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VITA

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