

**A BEHAVIOURAL ANALYSIS OF SEROTONERGIC
FUNCTIONAL STATUS FOLLOWING MDMA EXPOSURE**

by

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Abstract

Rationale +/- 3,4-Methylenedioxymethamphetamine (MDMA) produces effects on a number of neurochemical systems. Many studies have shown that repeated MDMA administration produces deficits in central serotonergic neurotransmission, which have been suggested to underlie some of the behavioural changes associated with use.

Objectives The present studies sought to evaluate the functional statuses of the serotonin transporter (SERT) and the serotonin_{2c} (5-HT_{2c}) and serotonin_{2a} (5-HT_{2a}) receptors following treatment with MDMA to determine whether behavioural deficits could be attributed to alterations in these proteins.

Methods Rats received a pretreatment regimen of MDMA (4 x 10mg/kg MDMA injections administered at 2h intervals) or the saline vehicle and, 2 weeks later, [³H] paroxetine binding was undertaken to assess densities of SERT. In other groups, dose-effect curves for MDMA-produced hyperactivity were determined. Additional groups were tested following a 12-week withdrawal period from MDMA in order to assess whether there was recovery of function. The functional status of the SERT was further examined by determining the effect of MDMA pretreatment on the reduction in MDMA-produced hyperactivity (0.0 – 10.0mg/kg) produced by the selective serotonin reuptake inhibitor, clomipramine (0.0 – 5.0mg/kg). The ability for the 5-HT_{2c} receptor agonist, m-CPP (0.0 – 2.5mg/kg) to produce hypolocomotion or increased emergence latency or for the 5-HT_{2a} receptor agonist, DOI (0.0 – 2.0mg/kg) to produce wetdog shakes (WDS) were examined in MDMA pretreated rats. The ability for the 5-HT_{2c} receptors to modulate MDMA-produced hyperactivity was assessed by examining the effect of MDMA pretreatment on the potentiation of MDMA-produced hyperactivity produced by the selective antagonist, RS102221 (0.0 – 1.0mg/kg). Conversely, the modulatory abilities of the 5-HT_{2a} receptors were assessed by examining the effect of MDMA pretreatment on the attenuation of MDMA-produced hyperactivity produced by the antagonist, ritanserin (0.0 – 10.0mg/kg).

Results MDMA pretreatment produced widespread reductions in SERT binding densities 2 weeks following administration. Prior exposure to MDMA rendered rats tolerant to MDMA-produced hyperactivity when tested 2, but not 12, weeks following MDMA administration. Two weeks following MDMA pretreatment rats were also less responsive to the clomipramine-produced attenuation of MDMA-produced hyperactivity. MDMA pretreatment failed to alter M-CPP -produced hypolocomotion or increased emergence latency, but decreased the ability for DOI to induce WDS. Further, MDMA pretreated rats exhibited tolerance to RS102221 as shown by a rightward shift in the dose effect curve and complete tolerance to ritanserin.

Conclusions Following MDMA pretreatment, the decreased SERT binding densities and inability of clomipramine to attenuate MDMA-produced effects might explain tolerance to the locomotor activating effects produced by MDMA. Functional recovery also occurred with extended abstinence from the drug, suggesting that MDMA produced transient serotonergic alterations. The results support the idea that the 5-HT_{2a} and 5-HT_{2c} receptors that modulate MDMA-produced hyperactivity are functionally distinct from the receptors that mediate m-CPP- and DOI-induced behavioural responses, as m-CPP-produced behaviours were resilient, yet RS102221-induced effects were reduced, by MDMA pretreatment. RS102221 is highly selective in comparison to ritanserin, yet there was only one dose that produced significant potentiation of MDMA-produced hyperactivity, whereas there were several effective ritanserin doses. This suggests that the 5-HT_{2a} receptors had a greater role in modulating MDMA-produced hyperactivity. Additionally, 5-HT_{2a} receptors might be more susceptible to MDMA-induced desensitisation than 5-HT_{2c} receptors, as MDMA pretreated rats exhibited some tolerance to the potentiating effects of RS102221 but were unresponsive to any ritanserin dose. In conclusion, MDMA-induced locomotor tolerance was attributable to decreased SERT densities and function as well as desensitisation of 5-HT_{2a} receptors that facilitate hyperactivity.

Introduction

Introduction to MDMA

Origins

3,4-Methylenedioxymethamphetamine (MDMA or 'ecstasy') is a ring-substituted derivative of phenylisopropylamine and is structurally similar to both methamphetamine and mescaline (McKenna and Peroutka, 1990; White et al., 1996). MDMA is a drug that has been purported to have unique subjective effects, with hallucinogenic- and psychostimulant-like properties. As a result, a novel drug class termed 'entactogens' (literal meaning 'producing a touching within') was proposed to include MDMA with structurally related compounds that also produce hallucinogenic and stimulant effects (Nichols, 1986).

MDMA was first synthesised by Merck pharmaceuticals and patented in 1914. In 1977, the United Kingdom Home Office scheduled MDMA as a class A drug on the premise that it had no known medicinal uses. Despite these legalities, MDMA was still used as an adjunct to psychotherapy in the late 1970's and early 1980's. It was also legally available in some regions of the United States such as Texas until 1985, when the United States Food and Drug Administration followed suit and added MDMA to schedule 1 of the Controlled Substances Act (Cole and Sumnall, 2003).

Use Patterns & Purity

MDMA became a popular recreational drug in the United Kingdom in the 1980's (Cole and Sumnall, 2003) where it became the most commonly used illicit drug at all night dance parties or 'raves' (Brown et al., 1995; Lenton et al., 1997; Weir, 2000; Wilkins, 2003; Engels and ter Bogt, 2004; Parks and Kennedy, 2004) and the increasing popularity of the 'rave subculture' during the 1990's led to more widespread use.

There are numerous reports suggesting that MDMA use has been steadily increasing. The World Drug Report compiled by the United Nations Office on Drugs and Crime (UNODC, 2004) presented long-term worldwide trends in production, trafficking and abuse of drugs and was based on a compilation of data obtained from the Annual Reports Questionnaire sent by governments to UNODC in 2003. The reports revealed that the consumption of certain illicit drugs such as heroin and cocaine were decreasing. However, cannabis was still the most commonly used illicit drug and use was accelerating. During the last decade, amphetamine-type stimulants (mainly MDMA) were the second most commonly used illicit drug. The main producers of amphetamine and MDMA were in Europe, including the Netherlands (the largest producer), Belgium, Poland, the Baltic States, the UK and Germany. The production of amphetamine-type stimulants increased globally during the last decade and was dominated by methamphetamine, followed by MDMA and amphetamine. The number of clandestine MDMA laboratories primarily in Europe rose almost 3-fold between 1992 and 2002. Global MDMA consumption has increased consistently over the last decade and continued to rise in 2002, although at a slower pace than in 2001. There appears to be a trend towards increasing use in developing countries, whilst some of the largest MDMA markets of Europe and North America stabilised, possibly due to the massive increases in popularity and use during the 1990's.

Reports and surveys suggest that MDMA use is prevalent and on the increase in New Zealand and Australia. National Drug Surveys in New Zealand indicated that MDMA use had increased by those aged between 15-45 years from 1.5% in 1998 to 3.4% in 2001 (Wilkins, 2003). MDMA-produced seizures have also increased within this time frame. The 2005 National Drug Strategy Household Surveys in Australia revealed that 22% of the populace aged between 20-29 years reported having used MDMA. Statistics showing recent drug use of the total population 14 years revealed that the frequency of MDMA use had increased from 2.9% in 2001 to 3.4% in 2004. In comparison, cocaine, cannabis and hallucinogen use had decreased during this period.

Regular MDMA use is usually associated with polydrug use. In a World Wide Web study, drug usage patterns were reported by novice, moderate, heavy and non-MDMA users (Scholey et al., 2004). It was found that MDMA users who did not take any other illicit substances were very rare and MDMA users reported significantly greater psychoactive drug usage than non-MDMA users. Experienced MDMA users took significantly more tablets on each occasion, and reported higher maximum weekly intake than moderate MDMA users.

Many regular MDMA users also described tolerance to the effects of MDMA, complaining that their acute drug reaction was weakened with repeated usage. Structured interviews were completed by 430 regular MDMA users recruited from London (68%) and Manchester (32%) in the UK (Verheyden et al., 2003). When asked how often participants combined other drugs with MDMA, 59% reported always mixing MDMA with another drug. Thus, MDMA appears to be increasing in use, availability and popularity. Regular users tend to be rave attendees (Brown et al., 1995; Lenton et al., 1997; Weir, 2000; Wilkins, 2003; Engels and ter Bogt, 2004; Parks and Kennedy, 2004) and also typically ingest a range of other drugs in addition to MDMA (Verheyden et al., 2003; Wilkins, 2003; Scholey et al., 2004). Reports suggest that prolonged use of MDMA leads to tolerance to the effects; the implications of this being that users take the drug more frequently and/or at higher doses to obtain the effects that were initially experienced; indeed, these predictions have been confirmed (Verheyden et al., 2003; Scholey et al., 2004; Parrott, 2005).

MDMA is usually administered orally and sold in tablet form; however, these tablets seldom contain 'pure' MDMA. The amount of MDMA within a tablet varies widely, and many contain other drugs such as amphetamine, 3,4-methylenedioxyethamphetamine (MDEA), 3, 4-methylenedioxyamphetamine (MDA), N-methyl-1-(1,3-benzodioxol-5-yl)-2-butanamine (MBDB), 4-bromo-2,5-dimethoxyphenethylamine (2C-B, 'Nexus'), ketamine, caffeine or ephedrine. The actual

MDMA content of ecstasy tablets has been reported to range from 34% to 75% (Sherlock et al., 1999; Bell et al., 2000; Cole et al., 2002; Cole and Sumnall, 2003).

Mechanisms and Pharmacology

MDMA induces massive increases in extracellular levels of serotonin (5-HT), dopamine (DA) and norepinephrine (NE) (Gough et al., 1991; White et al., 1996; Lyles and Cadet, 2003; Colado et al., 2004; Green et al., 2004). The greatly increased synaptic levels of these monoamines leads to the activation of numerous receptors (White et al., 1996). Therefore, the unique subjective effects experienced following ingestion of MDMA are likely a consequence of multiple 5-HT, NE and DA receptor activation. In addition, MDMA induces release of other neurotransmitters such as acetylcholine (Fischer et al., 2000), and has also been reported to inhibit gamma amino butyric acid (GABA) activity (Yamamoto et al., 1995). MDMA directly interacts with numerous brain recognition sites with highest affinity for the serotonin transporter (SERT) (Battaglia et al., 1988). MDMA also binds the NE transporter (NET), DA transporter (DAT), 5-HT₁ and 5-HT₂ receptors, D₁- and D₂-like receptors, α ₁- α ₂- and β ₂-adrenergic receptors, muscarinic and histamine receptors.

MDMA induces substantial extracellular 5-HT and DA release via exocytosis (Yamamoto and Spanos, 1988; Gough et al., 1991; Rudnick and Wall, 1992; Yamamoto et al., 1995; Shankaran and Gudelsky, 1999) and elevations in synaptic levels of these neurotransmitters have been associated with many behavioural and neurochemical effects observed following MDMA administration. Numerous reports also suggest that MDMA induces increased monoamine levels by interacting with carrier proteins on the plasma membrane. MDMA-produced increases in extracellular 5-HT might occur via the SERT (Hekmatpanah and Peroutka, 1990; Berger et al., 1992; Rudnick and Wall, 1992; Gudelsky and Nash, 1996; Iravani et al., 2000), since selective 5-HT reuptake inhibitors (SSRIs) such as imipramine or fluoxetine administered in conjunction with MDMA antagonised the

MDMA-induced synaptic 5-HT increase (Hekmatpanah and Peroutka, 1990; Berger et al., 1992; Rudnick and Wall, 1992; Gudelsky and Nash, 1996). Further supportive evidence showed that tetrodotoxin (TTX) did not entirely block MDMA-induced increases in synaptic 5-HT (Gudelsky and Nash, 1996), suggesting that release can occur in the absence of cell firing and 5-HT increases still occurred after Ca²⁺ depletion, which is normally an essential agent required for vesicular release (Schmidt et al., 1987b).

It is clear that MDMA blocks reuptake, however, there is still speculation as to whether MDMA also induces 5-HT release via SERT-mediated reverse transport (Hekmatpanah and Peroutka, 1990). MDMA was purported to produce elevations in intracellular 5-HT via interaction with vacuolar amine transporters located on storage vesicle membranes (Rudnick and Wall, 1992). MDMA disrupted the sequestration process, preventing the repackaging of cytosolic 5-HT into vesicles (Rudnick and Wall, 1992). In addition to increasing synaptic 5-HT, MDMA also inhibited tryptophan hydroxylase (TPH) (Stone et al., 1987; Schmidt and Taylor, 1987a; Stone et al., 1989a; Stone et al., 1989b; Garcia-Osta et al., 2004) and monoamine oxidase activity (Gu and Azmitia, 1993; Leonardi and Azmitia, 1994). MDMA-induced increases in synaptic levels of NE have been partly attributed to effects at the NET, as these were reduced by co-administration with the NE-uptake blocker, desmethylinipramine (Fitzgerald and Reid, 1990).

DAT blockade also contributed to MDMA-produced DA elevations, as reuptake inhibitors such as mazindol or GBR12909 co-administered with MDMA reduced DA outflow (Nash and Brodtkin, 1991; Shankaran et al., 1999). Increases in DA levels might involve more complex mechanisms than those involved with 5-HT and NE. It was found that TTX and ritanserin (5-HT₂ receptor antagonist) attenuated MDMA-induced DA release in the striatum and substantia nigra (Yamamoto et al., 1995). Conversely, it has also been discovered that direct infusion of 5-HT₂ receptor agonists into the nucleus accumbens or striatum increased DA levels in these areas (Benloucif and Galloway, 1991; Parsons and Justice, 1993). It was concluded that 5-HT₂ receptor activation modulates DA

release in addition to effects of MDMA at the DAT. The fact that TTX attenuated DA release was also indicative of a partly impulse-mediated mechanism (Yamamoto et al., 1995).

Time-course measurements of DA levels provide additional information regarding the mechanisms involved in MDMA-produced effects. Following administration of MDMA (10mg/kg), in vivo microdialysis showed an immediate increase in caudate DA, which was followed 70 minutes later by an increase in the nucleus accumbens (Yamamoto and Spanos, 1988). In another study direct infusion of MDMA via a dialysis probe into the nucleus accumbens caused an increase in DA that was delayed in relation to the 5-HT increase (White et al., 1994). It was suggested that MDMA may produce an initial but relatively small increase in DA, possibly via DAT-mediated mechanisms, whereas the later increase might be 5-HT₂ receptor-mediated (White et al., 1994). Local 5-HT application to the nucleus accumbens induced DA release (Jacocks and Cox, 1992), which supports the idea that MDMA-induced increases in extracellular 5-HT might be a trigger for DA release.

The stereoisomers of racemic (-/+) MDMA have different potencies, pharmacokinetics and effects on neurotransmitter release. Measurements of the half-life or clearance of each isomer type revealed that the (+) isomer has faster clearance; therefore drug-induced effects are shorter in duration (Fitzgerald et al., 1990). All derivatives have been reported to release comparable levels of 5-HT; however, the (+) MDMA isomer increases synaptic DA to a greater extent than (-) MDMA or the racemic compound (Johnson et al., 1986; Hiramatsu and Cho, 1990). These subtle differences in neurochemistry induced by these derivatives are reflected behaviourally in that (+) MDMA has more stimulant-like and (-) MDMA more hallucinogenic-like effects (Paulus and Geyer, 1992; Baker et al., 1995; Fantegrossi et al., 2003).

Physiological Effects

MDMA plasma concentrations are highest 2 hours following oral ingestion. The half-life of MDMA in humans is approximately 8 hours and most is excreted in the urine. Pharmacokinetic studies revealed that increasing the MDMA dose by a factor of 3 increased peak plasma concentrations by a factor of 6, thus there is a non-linear pharmacokinetic relationship between drug and plasma levels so that small increases in dose lead to relatively large changes in plasma levels (Mas et al., 1999; de la Torre et al., 2000a; de la Torre et al., 2000b).

The first physiological effects induced by MDMA begin to manifest approximately 15 minutes following ingestion. The stimulant-like characteristics of MDMA are represented by increases in heart rate, blood pressure and cardiac output (Vollenweider et al., 1998; Mas et al., 1999; Lester et al., 2000; de la Torre et al., 2000a; de la Torre et al., 2000b). Increases in muscle tension, as well as bruxism (grinding of teeth) are also commonly reported side effects (Greer and Tolbert, 1986; Siegel, 1986; Peroutka et al., 1988; Liester et al., 1992; Solowij et al., 1992; Cohen, 1995), as well as occasional nausea and vomiting (Downing, 1986).

MDMA administration produced dose-dependent increases in body temperature in laboratory animals (Gordon et al., 1991; Clemens et al., 2004). Controlled laboratory experiments involving human participants have not, however, found significant changes in body temperature as a result of acute MDMA administration (Vollenweider et al., 1998). This discrepancy may be due to the fact that hyperthermia was only found at higher ambient temperatures, whilst conversely, hypothermia occurred when the environment was colder (Dafters, 1994), suggesting that MDMA leads to a loss of thermoregulation. If so, this might explain the disparity between the Vollenweider (1998) results where no significant change in body temperatures occurred after MDMA, whereas MDMA-induced

hyperthermia has been reported to occur at dance parties (Coore, 1996; Smith et al., 2002; Parrott, 2004). As MDMA is usually consumed at crowded, hot venues where dance parties are held and people are actively dancing for many hours, ambient temperatures are high and dehydration is likely to occur. In some cases, MDMA-induced hyperthermia has been fatal, and cardiac arrhythmias, acute renal failure, rhabdomyolysis, hepatic toxicity and disseminated intravascular coagulation have been reported (Chadwick et al., 1991; Fahal et al., 1992; Screatton et al., 1992; Oranje et al., 1994; Dykhuizen et al., 1995; Khakoo et al., 1995; Malpass et al., 1999). However, fatalities as a result of MDMA ingestion are relatively rare, and as a result, it is perceived as a 'safe' drug (Greer and Tolbert, 1986; Grinspoon and Bakalar, 1986; Siegel, 1986). Many other MDMA-related adverse effects that do not entail fatality may go unrecorded (Whitaker-Azmitia and Aronson, 1989).

Subjective Effects

MDMA has been reported to have hallucinogenic-like effects or perceptual altering properties. Users have reported increased 'feelings of closeness' to others, enhanced insight, increased sensory awareness, euphoria, increased extraversion and elevated self-confidence; hence the street name 'ecstasy' (Downing, 1986; Greer and Tolbert, 1986; Solowij et al., 1992; Cohen, 1995, 1996; Cohen and Cocores, 1997; Liechti and Vollenweider, 2001a; Lyles and Cadet, 2003; Verheyden et al., 2003). When participants were pretreated with an SSRI, the positive MDMA-produced subjective effects were attenuated (Liechti et al., 2000b; Liechti et al., 2001; Liechti and Vollenweider, 2001). These findings suggest that binding to the SERT might produce many of these subjective effects following MDMA administration. In addition to this, the 5-HT_{2a/2c} receptor antagonist, ketanserin, prevented MDMA-produced perceptual changes (Liechti et al., 2000a; Liechti et al., 2001; Liechti and Vollenweider, 2001). These observations concur with other studies reporting that 5-HT_{2a} receptor activation produced hallucinogenic

effects (Jakab and Goldman-Rakic, 1998; Aghajanian and Marek, 1999; Nelson et al., 1999).

Negative side effects after MDMA ingestion such as paranoia, restlessness, depersonalisation, suppressed appetite, lack of energy, concentration difficulties, delirium and anxiety have been extensively documented (Peroutka et al., 1988; Liester et al., 1992; Vollenweider et al., 1998; Verheyden et al., 2003), and may explain why many discontinue use of this drug, or use it less frequently. Cognitive abilities are detrimentally affected during MDMA intoxication (Parrott and Lasky, 1998). Pre-drug baselines were compared to measures taken whilst the participants were intoxicated with MDMA at a Saturday night dance and the acute dose markedly reduced memory scores and impaired visual search tasks. There are numerous studies that have assessed cognitive abilities and psychiatric statuses in MDMA users, but an extensive review of this literature is beyond the scope of the present thesis. There was general consensus, however, that the long-term consequences of MDMA use were associated with memory and mood impairments (Peroutka et al., 1988; Liester et al., 1992; Vollenweider et al., 1998; Verheyden et al., 2003).

Behavioural Effects of MDMA on Laboratory Animals

Overview

MDMA administration produces ubiquitous neurochemical effects, and amongst these, substantial elevations in synaptic 5-HT and DA levels. As a consequence of this massive release of neurotransmitters, postsynaptic receptors are activated and several behaviours are induced. The MDMA-produced behaviours that have been characterised in the literature will be reviewed, and the contribution of 5-HT and DA, where relevant, will be outlined.

Stereotypy

Role of Dopamine

Stereotypy in the rat includes sniffing, repetitive head and limb movements, gnawing, biting and licking, which are typically drug-induced behaviours (Costall et al., 1975). Stereotypy has been reported following the administration of indirect or direct DA agonists, such as amphetamine or apomorphine. Dose response relationships showed that there was a significant relationship between amphetamine-induced increases in stereotypy and magnitude of dialysate DA release (Kuczenski and Segal, 1989; Kuczenski and Segal, 1990). In addition, the DA receptor antagonist, haloperidol, prevented amphetamine-produced stereotypy from occurring and reduced existing stereotypy when administered after amphetamine (Conti et al., 1997).

The majority of evidence indicates that D1- and D2-like DA receptor activation produces stereotypy. The D1- and D2-like DA receptor agonist, apomorphine, induces stereotypy when administered systemically (Gordon and Beck, 1984; Sandoval and Palermo-Neto, 1995). When two agonists selective for these receptor subtypes were administered directly into striata of rats, stereotypy was markedly enhanced by both and blocked by concomitant infusion of the selective D1-like receptor antagonist, SCH 23390, or the D2-like receptor antagonist, sulpiride (Bordi and Meller, 1989). Additionally, mice that were bred with differential expression of D1- and D2-like receptors showed differential stereotypy responses to apomorphine (Seale et al., 1984).

Differential activation of the D1- and D2-like receptor subtypes might produce different components of stereotypy behaviours. Amphetamine-produced stereotypy was differentiated into two components: sniffing and repetitive head and limb movements (low intensity component) and gnawing, biting and licking (high intensity component) (Costall et al., 1975). Low intensity components occurred at low doses of apomorphine and high intensity components at larger doses. Although tempting, it would be too simplistic to attribute low or high intensity behaviour with either receptor subtype, as the two behaviours never occurred independently. These observations suggest interactive relationships between the D1- and the D2-like receptors.

The striatum, namely the caudate putamen, is the site where these behaviours originate. Increased striatal DA dialysate levels were positively correlated with increasing stereotypy (Sharp et al., 1987). The intensity of stereotyped head and forepaw movements was closely correlated with the amount of DA released in striatum but not in the nucleus accumbens. In contrast, increased locomotor activity was correlated with the time course and amount of dialysate DA in the nucleus accumbens.

In addition to dopaminergic manipulations, central GABA systems have also been implicated in apomorphine-produced stereotypy. GABA manipulations facilitated apomorphine-produced stereotypy, and it was postulated that this was a consequence of disinhibition of dopaminergic circuits (Sandoval and Palermo-Neto, 1995). Indeed, stereotypy was induced by local impairment of GABA circuits in the caudate nuclei, which was attenuated by haloperidol. These findings suggest that GABA circuits exert inhibitory control over dopaminergic systems in the caudate region, and that disinhibition leads to DA-produced stereotypy behaviours (Kryzhanovsky and Aliev, 1981).

Role of Serotonin

Amphetamine also elevates striatal dialysate 5-HT at higher doses (Kuczenski and Segal, 1989), therefore some studies have examined a possible role for 5-HT in the expression of stereotypy. The majority of evidence suggests a minor, but inhibitory role for 5-HT in this drug-induced behaviour. For example, stereotypy induced by apomorphine or methamphetamine was enhanced when the synthesis of 5-HT was inhibited (Dickinson and Curzon, 1983) or 5-HT antagonists administered (Balsara et al., 1979). Similarly, amphetamine produced stereotypy when 5-HT was depleted (Segal, 1976; Dickinson and Curzon, 1983) and methamphetamine-induced stereotypy decreased following L-tryptophan administration (Balsara et al., 1979).

A study examined the contribution of both DA and 5-HT elevations to slow head shaking (SHS) and stereotyped gnawing (SG) induced by methamphetamine in rats

(Honma and Fukushima, 1979). SHS was mediated predominantly by serotonergic mechanisms, as 5-HT antagonists attenuated SHS, whilst the administration of the 5-HT precursor L-5-hydroxytryptophan and peripheral decarboxylase inhibitor enhanced these behaviours. Conversely, SG behaviours were predominantly mediated by DA, as tyrosine hydroxylase inhibitors blocked, whilst dopamine-beta-hydroxylase inhibitors and combined administration of L-3, 4-dihydroxyphenylalanine and Ro-4-4602 enhanced SG but did not affect SHS behaviours. SG was comparable to DA-mediated stereotyping behaviour, whereas SHS might be a component of the 5-HT syndrome (see next paragraph). These findings do highlight the importance of specifying which behaviours are defined as 'stereotypy', as some are clearly mediated by differential neurochemical mechanisms.

MDMA-Produced Stereotypy

As MDMA affects both 5-HT and DA systems, stereotypy has been observed following acute administration (O'Loinsigh et al., 2001). Consistent with its ability to increase DA to a greater extent than the -MDMA isomer, +MDMA was more effective at inducing stereotyped behaviour such as sniffing, head-weaving and turning (Hiramatsu et al., 1989). Another study reported that MDMA did not produce stereotypy behaviours following administration of 10 and 30mg/kg, whilst 5-HT syndrome behaviours and elevated locomotor activity were present and attributed to the predominant effects of MDMA on serotonergic systems (Matthews et al., 1989). These differential findings are consistent with the idea that elevated synaptic 5-HT levels inhibit the expression of stereotypy behaviours. Since MDMA elicits a relatively large efflux of 5-HT in the brain compared to other psychostimulants (Crespi et al., 1997), DA-mediated stereotypy would be an unexpected behavioural outcome. It is possible that difficulty in differentiating between stereotypy- and 5-HT syndrome-like behaviours might have led to these mixed findings.

Role of Serotonin

Serotonin syndrome (SS) behaviours resemble some components of DA-mediated stereotypy, but have been differentiated in that they can be induced by 5-HT agonists such as 5-methoxy-N, N-dimethyltryptamine (5-MeDMT) or d-lysergic acid diethylamide (LSD) (Lucki and Frazer, 1982). SS behaviours include flattened body posture, salivation, resting tremor, hunch back, Straub tail, hypothermia, lower lip retraction, piloerection and body tremors (Goodwin et al., 1987; Mokler et al., 1992; Kleven et al., 1997; Van Oekelen et al., 2003b; Morley et al., 2005). These behaviours were attenuated by 5-HT depletion (Goodwin et al., 1987), serotonergic lesions produced by 5,7-DHT (Goodwin et al., 1987) or 5-HT receptor antagonists (Kleven et al., 1997). The 5-HT_{1a} receptors have a major role in the expression of SS behaviours, as 5-HT_{1a} receptor agonists such as buspirone or 8-hydroxy-2- (di-n-propylamino) tetralin (8-OH-DPAT) produced dose-related increases in one or more elements of the SS (Blanchard et al., 1993; Kleven et al., 1997). The selective 5-HT_{1A} antagonist, N- [2-[4-(2-methoxyphenyl)-1-piperazinyl] ethyl]-N- (2-pyridinyl) cyclone xanecarboxamide (WAY 100635), attenuated SS produced by both agonists (Goodwin et al., 1987; Kleven et al., 1997).

MDMA-Produced Serotonin Syndrome

Acute MDMA produced dose-dependent increases in duration and intensity of SS behaviour, including low body posture, forepaw treading, head weaving, salivation and piloerection (Spanos and Yamamoto, 1989; Morley et al., 2005; Piper et al., 2005). The most consistently observed SS behaviour was head weaving in the rat, which was defined by Spanos and Yamamoto (1989) as a lateral side-to-side movement of the head with no net locomotion. These MDMA-produced behaviours were attenuated by pretreatment with the selective 5-HT_{1a} receptor antagonist, WAY 100635 (Morley et al., 2005). Head

weaving and forepaw treading have also been described as DA-mediated stereotypy behaviour (Honma and Fukushima, 1979), but because blockade of 5-HT receptors attenuated these behaviours, they were likely mediated by serotonergic mechanisms.

Anxiety

Overview

Behavioural paradigms such as the elevated plus maze (EPM), the emergence and open field tests have been used to measure anxiety as an avoidance response to open spaces or novel environments. Ultrasonic vocalisations in response to stressful stimuli have also been used as an index of anxiety and provides a measure of distress unrelated to the movement of the animal and thus avoids a confound that might distort other measures (Siemiakowski et al., 2001). Additionally, the social interaction test suggests anxiety when there is a decreased interaction time.

Role of Dopamine

There was indication that elevation of synaptic DA levels produced anxiogenesis, as amphetamine or DA reuptake inhibitors at doses subthreshold for locomotor activation produced anxiogenesis (File and Hyde, 1979; Simon et al., 1993; Silva et al., 2002).

Studies regarding the role of specific D1- or D2-like DA receptors have yielded a multitude of conflicting findings. Some studies showed that D1-like receptor agonists produced little change in anxiety tests (Rodgers et al., 1994; Bartoszyk, 1998). D2-like receptor agonists and antagonists have both produced anxiolysis in many instances. Quinpirole, a D2-like receptor agonist, reduced ultrasonic vocalisations (Bartoszyk, 1998) and apomorphine, a D1/D2-like receptor agonist, decreased avoidance behaviours (Talalaenko et al., 1994) suggesting anxiolytic effects. A substantial number of the D2-like receptors are autoreceptors, thereby limiting DA release upon activation (Cragg and Greenfield, 1997; Usiello et al., 2000). These anxiolytic effects were postulated to be due

to autoreceptor activation, thereby limiting DA synthesis and release. However, the fact that D2-like receptor antagonists have also produced anxiolysis (Costall et al., 1987; Bruhwyler et al., 1990; Rodgers et al., 1994; Talalaenko et al., 1994; Siemiatkowski et al., 2001) indicates that DA and associated receptors have an indeterminate role in anxiety as measured by these paradigms.

Role of Serotonin

A number of studies have suggested a role of 5-HT in anxiety. Depletion of central 5-HT induced by the selective 5-HT neurotoxin, 5,7-dihydroxytryptamine (5,7-DHT) or a tryptophan hydroxylase inhibitor, parachlorophenylalanine (pCPA) were found to increase time spent in the light chamber and in social interaction (File and Hyde, 1977; Clarke and File, 1982; Koprowska et al., 1999). Buspirone, a 5-HT_{1A} receptor agonist, produced anxiolysis in the open-field and elevated T maze tests (Moser, 1989; Angrini et al., 1998; Graeff et al., 1998; Belzung et al., 2001; Merali et al., 2003). In addition, another 5-HT_{1A} agonist, ipsapirone, decreased ultrasonic vocalisations and induced time and dose-dependent decreases in firing rates of serotonergic dorsal raphe neurons (Sommermeier et al., 1993). The vast majority of 5-HT_{1A} receptors are autoreceptors; therefore receptor activation limits 5-HT release, reducing synaptic 5-HT levels (Routledge et al., 1993; Sommermeier et al., 1993; Gartside et al., 1999; Hjorth et al., 2000).

These findings suggest that anxiety is inversely related to 5-HT level. Indeed, increasing synaptic 5-HT levels by administration of SSRIs such as fluoxetine and sertraline induced anxiogenesis in the EPM and social interaction tests (Martin et al., 1998; To et al., 1999; To and Bagdy, 1999; Kurt et al., 2000; Silva and Brandao, 2000; Bagdy et al., 2001) and SERT knockout mice exhibited significantly greater anxiety than their wildtype counterparts in an emergence test (Holmes et al., 2003). The absence of this transporter protein allows for prolongation of 5-HT in the synaptic cleft, thereby replicating the situation produced by SSRIs. In addition, the non-selective 5-HT agonist,

m-CPP, also induced anxiety in the emergence, EPM and social interaction tests (Kennett et al., 1989; Gibson et al., 1994; Kennett et al., 1994a; Meert et al., 1997; Bilkei-Gorzo et al., 1998; Graeff et al., 1998; Bagdy et al., 2001).

In contrast to the acute effects, chronic administration of SSRIs were anxiolytic (Burghardt et al., 2004), and this might reflect alterations in receptor responsiveness as a consequence of chronic activation (Kennett et al., 1994b; Kantor et al., 2001; Burghardt et al., 2004). Chronic fluvoxamine, paroxetine, clomipramine and fluoxetine reduced behavioural responses to m-CPP (Kennett et al., 1994b; Yamauchi et al., 2004). Numerous studies have also reported altered functional properties of 5-HT_{1a} receptors after chronic SSRI or 5-HT_{1a} agonist treatments (Schechter et al., 1990; Lund et al., 1992; Sim-Selley et al., 2000; Subhash et al., 2000; Cremers et al., 2004) and as this receptor has an established role in anxiety (Moser, 1989; Sommermeyer et al., 1993; Angrini et al., 1998; Beneytez et al., 1998; Graeff et al., 1998; Olivier et al., 1998; Belzung et al., 2001; Merali et al., 2003), it is likely that these alterations also underlie anxiolysis.

MDMA-Produced Anxiety

MDMA increased anxiety in the EPM (Lin et al., 1999; Morley and McGregor, 2000; Navarro and Maldonado, 2002; Ho et al., 2004; Sumnall et al., 2004; Morley et al., 2005) and emergence tests (Maldonado and Navarro, 2000; Morley and McGregor, 2000; Morley et al., 2005) but increased social interaction in rats (Morley and McGregor, 2000; Morley et al., 2005). Recent findings suggest that these discrepancies between paradigms were attributable to different neurochemical mechanisms. MDMA-produced anxiogenesis in the emergence test was unaffected by pretreatment with the selective 5-HT_{1a} antagonist, WAY 100635, whereas increased social interaction was attenuated by this antagonist (Morley et al., 2005). These mixed findings suggest that MDMA-produced ‘anxiety’ comprises several behavioural components, which might be mediated by differential mechanisms.

Decreased anxiety in the EPM after low dose and increased anxiety after high dose MDMA have been found (Lin et al., 1999; Ho et al., 2004). These differential effects might reflect activation of 5-HT receptors with varying affinities for 5-HT. The 5-HT₁ receptors have a higher affinity for 5-HT than the 5-HT₂ receptors (Zifa and Fillion, 1992), therefore following administration of low doses of MDMA, receptors such as presynaptic 5-HT_{1a} receptors might be activated and produce anxiolysis (Moser, 1989; Sommermeyer et al., 1993; Angrini et al., 1998; Beneytez et al., 1998; Graeff et al., 1998; Olivier et al., 1998; Belzung et al., 2001; Merali et al., 2003) whilst following administration of higher doses, receptors such as the 5-HT_{2c} receptor might become preferentially activated and produce anxiogenesis (Kennett et al., 1994a; Kennett et al., 1995; Mora et al., 1997; Bagdy et al., 2001; Campbell and Merchant, 2003). These findings highlight the complexity of serotonergic systems involved in anxiety.

Locomotion

Overview

MDMA exerts predominant effects on 5-HT systems, and it is these effects as well as significant elevations in extracellular DA levels that produce hyperactivity in laboratory animals. It has been demonstrated that the ability of MDMA to bind the SERT as well as the activation of numerous 5-HT and DA receptor subtypes all contribute to MDMA-produced hyperactivity.

Role of Dopamine

A role for DA in both basal and drug-induced locomotion has been demonstrated in numerous studies. In general, enhanced dopaminergic transmission increases locomotor activity. Administration of the D₂-like receptor antagonist, haloperidol, dose-dependently reduced baseline levels of locomotor activity in rats (Emerich et al., 1991). Conversely, administration of DA agonists, such as apomorphine (Irifune et al., 1995), DA reuptake

inhibitors such as GBR 12909 (Heikkila and Manzino, 1984; Ichihara et al., 1993; Schindler and Carmona, 2002; McGeehan et al., 2004) and the D1-like receptor agonist, SKF 82958 (Schindler and Carmona, 2002) significantly increased locomotor activity levels. These increases were clearly related to DA receptor activation, as the D1-like receptor antagonist SCH23390 (Ichihara et al., 1993) and haloperidol (Heikkila and Manzino, 1984) blocked GBR 12909-induced hyperactivity.

Stimulant-produced hyperactivity has also been largely attributed to increased synaptic DA levels. Stimulant drugs that produce hyperactivity activate the dopaminergic system. For example, amphetamine and cocaine bind to the DAT with high affinity (Calligaro and Eldefrawi, 1987; Amara and Sonders, 1998), and thus significantly increase synaptic levels of DA (Morgan et al., 1997; Lamensdorf et al., 1999; Chefer et al., 2003). Hypermotility coincided with increased DA outflow in the nucleus accumbens and caudate as revealed by microdialysis following psychostimulant administration (Di Chiara and Imperato, 1988). Indeed, hyperactivity produced by cocaine, amphetamine and other psychostimulants was attenuated by co-administration of a range of DA antagonists (Le et al., 1997; O'Neill and Shaw, 1999; Schindler and Carmona, 2002; Bubar et al., 2004; Daniela et al., 2004) or following neurotoxic 6-hydroxydopamine lesions (Roberts et al., 1975; Koob et al., 1981; Itoh et al., 1984; Louis and Clarke, 1998).

Role of Serotonin

Depletion of brain 5-HT in rats increased spontaneous daytime activity (Kostowski, 1968; Fibiger HC, 1971; Borbely, 1973; Jacobs, 1975; Marsden and Curzon, 1976; Steigrad et al., 1978; Dringenberg, 1995). Electrolytic lesions to mid-brain raphe nuclei reduced forebrain 5-HT levels and consequently increased spontaneous locomotor activity (Kostowski, 1968). Synthesis inhibition via pCPA also increased daytime activity, which was reversed when the precursor, L-tryptophan, was administered (Marsden and Curzon, 1976). The findings suggest that the increased daytime activity levels were related to 5-HT

levels reductions. One surprising anomaly was that despite the continuously falling 5-HT levels, 72 hours after pCPA when the depletions were the most severe, activity returned to control levels. This disparity between the neurochemical and behavioural data was explained in terms of compensatory alteration in 5-HT receptor responsiveness. It was postulated that 5-HT receptors had upregulated, which has been reported following 5-HT depletion (Lucki et al., 1989; Berendsen et al., 1991; Aguirre et al., 1995; Aguirre, 1998; Compan, 1998)

An inhibitory role of 5-HT in basal locomotion is supported by studies that have examined the effects of increased 5-HT neurotransmission. For example, pretreatment with agonists such as fluvoxamine (McMahon and Cunningham, 2001a), RO 60-0175 (Martin et al., 1998) and m-CPP (Sills et al., 1985; Aulakh et al., 1989; Lucki et al., 1989; Gleason and Shannon, 1998; Gleason et al., 2001; Takahashi et al., 2001) decreased locomotor activity. M-CPP-produced hypolocomotion was linked to activation of the 5-HT_{2c} receptor as it was attenuated by pretreatment with selective 5-HT_{2c} receptor antagonists (Kennett and Curzon, 1988; Klodzinska et al., 1989; Kennett et al., 1994, 1994a; Kennett et al., 1997; Takahashi et al., 2001). Agonists such as RO 60-0175 that were relatively selective for 5-HT_{2c} receptors also produced hypolocomotion (Martin et al., 1998), further supporting a role for this receptor in mediating the hypolocomotor effects produced by agents that increase serotonergic transmission.

Increased activity as a result of pCPA pretreatment has been reported to occur when animals were in familiar environments (Kostowski, 1968; Fibiger HC, 1971; Borbely, 1973; Jacobs, 1975; Marsden and Curzon, 1976; Dringenberg, 1995). Conversely, decreased locomotor activity was observed when animals that had been administered pCPA were placed in novel environments (Dringenberg, 1995). The decrease in activity in response to novel environments was thought to reflect anxiogenesis as opposed to locomotor effects. This suggests that in certain circumstances, anxiety might override locomotor effects. As described above, serotonergic neurotransmission has been implicated

in some forms of anxiety (Martin et al., 1998; Kurt et al., 2000; Silva and Brandao, 2000; Bagdy et al., 2001); therefore it has been problematic in some instances to clearly differentiate between anxiety-related behaviours and effects on locomotor activity. Some frequently used behavioural assays such as the EPM, emergence or social interaction tests used to measure anxiety might be affected by drug-produced hypolocomotion, which could then be incorrectly interpreted as an elevation in anxiety. Anxiety-related effects can, however, be dissociated from effects on locomotor activity by examining dose-effect relationships (Kennett et al., 1989; de Angelis, 1996). For example, m-CPP increased anxiety at doses that were below the threshold for hypolocomotion (Kennett et al., 1989).

Stimulant & MDMA-Produced Locomotion

Amphetamine-produced hyperactivity was potentiated by sertraline, fluoxetine or fenfluramine (Sills et al., 1999a, 1999b; Bankson, 2002). It was noted that there was an initial reduction in activity levels following drug administration, followed by potentiation. This potentiation was attributed to pharmacokinetics, as fluoxetine inhibits cytochrome P450 enzymes in the liver involved in amphetamine degradation. This was confirmed in that SSRI pretreated rats had significantly higher brain levels of amphetamine at a single point in time and the potentiation was thus not the result of elevated 5-HT levels or competitive binding.

Cocaine-produced hyperactivity was also potentiated by fluoxetine and fluvoxamine pretreatment (Herges and Taylor, 1998). Microdialysis was conducted whereby DA levels in the nucleus accumbens were measured following cocaine, SSRI and then the treatment with both drugs (Bubar, 2003). Cocaine significantly elevated accumbal DA levels, whereas either SSRI alone had no effect. When cocaine was combined with each SSRI, the DA levels were higher than when cocaine was administered alone; leading to the conclusion that elevated DA had yielded potentiation of cocaine-produced hyperactivity. 5-HT increases have been purported to elevate DA outflow via 5-HT₂

receptors (Benloucif and Galloway, 1991; Parsons and Justice, 1993). Alternatively, competitive binding might account for these findings, as cocaine binds with higher affinity to the SERT than the DAT. If SERT sites were occupied following SSRI pretreatment, a greater number of cocaine molecules would be available to bind DAT sites, thereby elevating DA levels and potentiating hyperactivity. The evidence suggests that despite the involvement of SERT binding and 5-HT increases in hyperactivity produced after amphetamine or cocaine, these drugs might produce SSRI-potentiated hyperactivity via different mechanisms.

Specific 5-HT receptor mechanisms have been implicated in psychostimulant-produced hyperactivity. Evidence suggests that the 5-HT_{1b} receptor plays a facilitatory role, as blockade attenuated, whilst agonists potentiated both amphetamine-(Papla et al., 2002) and cocaine-(Przegalinski et al., 2002) produced hyperactivity. 5-HT_{2a} receptor blockade produced similar effects, as the 5-HT₂ receptor antagonist, ritanserin, attenuated both cocaine and amphetamine-produced hyperactivity (O'Neill et al., 1999). These effects were further explored in cocaine-studies, where attenuation of hyperactivity by ritanserin was attributed to the 5-HT_{2a} receptor, because a selective 5-HT_{2a} receptor antagonist was equipotent at reducing cocaine-produced hyperactivity (O'Neill et al., 1999; McMahon and Cunningham, 2001b; Fletcher PJ, 2002a; Filip et al., 2004) and an agonist potentiated these effects (Filla et al., 2004). The 5-HT_{2c} receptor had an inhibitory influence on these behavioural effects, as blockade potentiated (McCreary and Cunningham, 1999; Fletcher PJ, 2002a; Filip et al., 2004), whilst 5-HT_{2c} receptor agonists attenuated (Grottick et al., 2000) hyperlocomotive effects produced by cocaine. A role for the 5-HT_{2b} receptors was discounted, as 5-HT_{2b} antagonists/agonists were without effect (Fletcher PJ, 2002a; Filla et al., 2004). These findings indicate that the activation of numerous 5-HT receptor subtypes modulates the locomotive effects produced by both cocaine and amphetamine administration in a similar manner.

MDMA produces dose-dependent hyperactivity (Spanos and Yamamoto, 1989; Callaway et al., 1990; Kehne et al., 1996a) and this behavioural effect has been attributed to increases in both synaptic DA and 5-HT. MDMA-produced hyperactivity was mirrored by increased DA outflow (Yamamoto and Spanos, 1988; Bubar, 2003) and was attenuated by DA receptor antagonists (Kehne et al., 1996a; Bubar et al., 2004; Daniela et al., 2004) or 6-hydroxydopamine lesions (Gold et al., 1989b). Increased DA outflow might be related to direct effects on the DAT, or increased cell firing. A study monitored single striatal dopaminergic neurons of freely moving rats to determine levels of excitation in this area following MDMA administration and also to observe whether this was correlated with the pattern of activity (Ball et al., 2003). The results showed that 5mg/kg MDMA produced significant neuronal excitation in the striatum that was positively correlated with increased locomotor activity. When the D1-like receptor antagonist, SCH 23390, was administered in conjunction with MDMA, a late onset of hyperactivity was observed and this was associated with a delay in neuronal excitation. Administration of the D2-like receptor antagonist, eticlopride with MDMA, completely abolished hyperactivity, and subsequently no neuronal excitation was detected. The behavioural data reflected the electrophysiological recordings in that increased DA outflow, excited striatal neurons and MDMA-induced hyperactivity were all interrelated (Ball et al., 2003).

The different behavioural effects of the MDMA stereoisomers also demonstrate the important role that DA plays in MDMA-produced hyperactivity. The (+) MDMA isomer increased DA in striatal areas to a greater extent than its (-) congener (Johnson et al., 1986; Hiramatsu and Cho, 1990) and induced greater levels of hyperactivity than comparable doses of (+/-) MDMA or the (-) isomer (Paulus and Geyer, 1992; Fantegrossi et al., 2003).

Evidence has shown that MDMA-produced 5-HT outflow and binding to the SERT are also necessary for full expression of MDMA-produced hyperactivity. Rats that had sustained 5-HT depletion following 5,7-DHT lesions or pCPA administration showed attenuation of MDMA-produced hyperactivity (Callaway et al., 1990; Kehne et al., 1996a).

The administration of SSRIs such as fluoxetine, sertraline and zimelidine completely or partially attenuated MDMA-produced hyperactivity (Callaway et al., 1990; Callaway and Geyer, 1992b) and SERT knockout mice did not exhibit hyperactivity following MDMA administration (Bengel et al., 1998), suggesting that MDMA binding to the SERT is critical for the full expression of MDMA-produced hyperactivity. It is possible that this attenuation was due to reduced synaptic 5-HT levels, as SERT selective compounds dose-dependently prevented MDMA-produced increases in synaptic levels of 5-HT (Hekmatpanah and Peroutka, 1990; Berger et al., 1992; Rudnick and Wall, 1992; Gudelsky and Nash, 1996). Following MDMA administration, SERT-produced 5-HT elevations might be necessary to activate facilitatory 5-HT receptors involved in hyperactivity, as antagonism of the 5-HT_{1b} or the 5-HT_{2a} receptors attenuated MDMA-produced hyperactivity (Kehne et al., 1996a; McCreary et al., 1999; Bankson, 2002; Herin et al., 2005).

The majority of reports indicate that both DA and 5-HT play a role in MDMA-produced hyperactivity. MDMA is a drug that produces substantial increases in synaptic levels of these neurotransmitters and therefore provides an ideal tool for studying possible interactive effects. Given the inhibitory role for 5-HT upon basal locomotor activity, it might seem counter-intuitive that MDMA produces dose-dependent hyperactivity (Spanos and Yamamoto, 1989; Callaway et al., 1990; Kehne et al., 1996a; Fletcher PJ, 2002b; Herin et al., 2005) when it has higher affinity for the SERT than the DAT (Battaglia et al., 1988) and increases synaptic 5-HT levels substantially (Gough et al., 1991; White et al., 1996; Lyles and Cadet, 2003; Colado et al., 2004; Green et al., 2004). It is likely that interactions between DA and 5-HT systems can explain these seemingly inconsistent results. The combination of 5-HT and DA release might be synergistic when considering MDMA-produced hyperactivity. This was demonstrated in an eloquent study that examined the effects of selective SERT and DAT reuptake inhibitors on locomotor activity (McMahon and Cunningham, 2001a). When low dose of a DA reuptake inhibitor that did

not alter locomotor activity was administered with varying fluvoxamine doses, dose-dependent hyperactivity was produced by the combination. A 5-HT_{2a} receptor antagonist attenuated this hyperactivity (Gold, 1988b; Kehne et al., 1996a; Herin, 2001; Bankson, 2002; Fletcher PJ, 2002b; Herin et al., 2005), suggesting that hyperactivity produced by the reuptake inhibitor combination might have similar mechanisms to that produced by MDMA. Increased 5-HT and DA input might be synergistic because the 5-HT_{1b} and 5-HT_{2a} receptors contribute to MDMA-produced hyperactivity (Kehne et al., 1996a; McCreary et al., 1999; Fletcher PJ, 2002a; Herin et al., 2005) by facilitating DA outflow (Benloucif and Galloway, 1991; Galloway et al., 1993; Schmidt et al., 1994; Iyer, 1996; Yan, 2000; Kuroki et al., 2003).

A Behavioural Analysis of Serotonergic Integrity After MDMA Pre-Exposure: The Present Study

Tolerance to MDMA Following Pre-Exposure

MDMA Users

There are mixed reports concerning the consequences of long-term MDMA use. Anxiety (Cohen, 1996; Schifano et al., 1998; Parrott, 2002a; Parrott et al., 2002b), memory impairments (Bolla et al., 1998; Verkes et al., 2001; Curran and Verheyden, 2003; Hanson and Luciana, 2004), cognitive difficulties (McCann et al., 1999), mood disturbances (Cohen, 1996; Parrott and Lasky, 1998; Schifano et al., 1998; MacInnes et al., 2001; Parrott, 2002a; Verheyden et al., 2003; de Win et al., 2004) and suicidal tendencies (Cohen, 1996; Shannon, 2000; Kalant, 2001) have all been reported. Contrary to these findings, some studies have reported minimal impact of prior MDMA exposure (Verheyden et al., 2003). A consistently reported consequence is tolerance to the subjective effects of the drug following regular use (Verheyden et al., 2003; Scholey et al., 2004;

Parrott, 2005). Because tolerance to MDMA has been so frequently reported, neurochemical changes associated with use have been investigated.

Since most of the subjective effects produced by MDMA have been attributed to 5-HT mechanisms (Liechti et al., 2000b; Liechti et al., 2001; Liechti and Vollenweider, 2001), alterations in serotonergic neurotransmission have been a focal point. Cerebrospinal fluid (CSF) 5-HIAA levels provide a means of assessing brain 5-HT turnover in humans, as lowered 5-HIAA concentration coincided with decreased tissue 5-HT levels in non-human primates after MDMA (Ricaurte et al., 1988b; Insel et al., 1989; Taffe et al., 2001; Taffe et al., 2003). MDMA users exhibited reduced 5-HIAA levels, suggesting central 5-HT depletions (McCann et al., 1994; Bolla et al., 1998; McCann et al., 1999). The ability of MDMA to elevate synaptic 5-HT levels could be compromised when there are central 5-HT depletions, producing tolerance.

It is also possible that MDMA exposure alters 5-HT metabolism. For example, following a tryptophan augmented drink, MDMA users had significantly higher plasma tryptophan increases than controls, possibly as a consequence of disrupted serotonergic metabolism (Curran and Verheyden, 2003). It is possible that these results were due to TPH inhibition, as this was a consequence of MDMA exposure in animals (Stone et al., 1987; Schmidt and Taylor, 1987a; Stone et al., 1989a; Stone et al., 1989b; Garcia-Osta et al., 2004). Thus, elevated plasma tryptophan levels in MDMA users might have reflected an impaired ability to metabolise tryptophan.

There are other physiological measures of the 5-HT systems that have been used to assess serotonergic function in MDMA users. Single Photon Emission Computed Tomography (SPECT) or Photon Emission Tomography (PET) scans following radioligand injection revealed evidence of altered serotonergic neurotransmission. SERT (McCann et al., 1998; Semple et al., 1999; Buchert et al., 2003; Thomasius et al., 2003) and 5-HT_{2a} receptor binding densities (Reneman et al., 2002) were significantly decreased in participants who were currently using MDMA. Following abstinence, there was a

significant increase in 5-HT_{2a} receptor binding densities in the occipital cortices of this group (Reneman et al., 2002). Because receptors are sensitive to changes in synaptic levels of 5-HT and up- or down-regulate in response to these levels (Teeple, 1990; Lohse, 1993; Cooper, 2002), these results might reflect altered 5-HT turnover and/or levels. Length of abstinence from MDMA use was also positively correlated with recovery of SERT binding (Semple et al., 1999; Buchert et al., 2003; Thomasius et al., 2003).

5-HT receptor desensitisation might contribute to the diminished effects of MDMA, as users were tolerant to the subjective and physiological effects of a range of 5-HT agents. For example, the ability of the 5-HT agonists, m-CPP or fenfluramine, to increase plasma levels of prolactin and cortisol was decreased (McCann et al., 1999; Verkes et al., 2001; Hatzidimitriou et al., 2002) and additionally, users did not experience m-CPP-produced anxiety or negative effect (McCann et al., 1999). These reduced m-CPP-related subjective effects and blunted neuroendocrine responses are consistent with reduced 5-HT₂ receptor densities resulting from MDMA exposure (Reneman et al., 2002).

Similar mechanisms might underlie tolerance to the subjective effects of MDMA, as both a 5-HT_{2a/2c} receptor antagonist (Liechti et al., 2000a; Liechti et al., 2001; Liechti and Vollenweider, 2001) and a SERT blocker attenuated MDMA-produced subjective effects (Liechti et al., 2000b; Liechti et al., 2001; Liechti and Vollenweider, 2001), therefore decreased 5-HT_{2a} receptor and SERT binding densities in MDMA users might contribute to tolerance to MDMA. 5-HT_{2a} receptor activation has been associated with hallucinogenic drug effects (Jakab and Goldman-Rakic, 1998; Aghajanian and Marek, 1999; Nelson et al., 1999), therefore, it is possible that decreased receptor densities might contribute to tolerance to some of the perception-altering properties of MDMA.

MDMA Pre-Treatment in Animal Studies

There were also mixed findings with regards to the effects of MDMA pre-exposure on baseline behaviour in laboratory animals. Anxiogenesis (Morley et al., 2001; Gurtman

et al., 2002; Bull, 2003; McGregor et al., 2003; Bull, 2004; Clemens et al., 2004; Thompson et al., 2004; Morley et al., 2005), anxiolysis (Mechan et al., 2002b; Piper and Meyer, 2004) or no change in anxiety (Bull, 2003; Bull, 2004; Ho et al., 2004; Sumnall et al., 2004) or locomotor (Callaway and Geyer, 1992a; McNamara et al., 1995; Piper and Meyer, 2004) measures were reported following MDMA pre-treatment.

Decreased 5-HT tissue levels have been reported following MDMA exposure (Battaglia et al., 1987; Commins et al., 1987; Ricaurte et al., 1988b; Ricaurte et al., 1988c; Insel et al., 1989; Molliver, 1990; Scanzello et al., 1993; Aguirre et al., 1995; Colado and Green, 1995; Fischer et al., 1995; McNamara et al., 1995; Sabol et al., 1996; Sexton et al., 1999; Mayerhofer et al., 2001; Boot, 2002; McGregor et al., 2003; Clemens et al., 2004; Sumnall et al., 2004; Wang et al., 2004; Nair and Gudelsky, 2006) and both decreases or no changes to basal 5-HT dialysate levels were measured in MDMA pretreated rats (Matuszewich et al., 2002). Despite inconsistencies in basal dialysate results, 5-HT release was consistently impaired in response to pharmacological, physiological or stressful stimuli in MDMA pretreated animals (Gartside et al., 1996; Shankaran and Gudelsky, 1999; Matuszewich et al., 2002). These findings might explain why laboratory animals tolerant to the behavioural effects produced by an acute MDMA challenge showed little change in baseline behaviour (Callaway and Geyer, 1992a; Shankaran and Gudelsky, 1999) and suggest that pharmacological challenge might be required for functional impairments to be evident.

Animal studies provide evidence to support the idea that tolerance to MDMA is due to serotonergic deficits produced following chronic exposure. MDMA exerts predominant effects on the 5-HT system, as it has highest affinity for the SERT (Battaglia et al., 1988; Rudnick and Wall, 1992), inhibits vesicular 5-HT uptake (Brodin, 1993), inhibits TPH (Stone et al., 1987; Schmidt and Taylor, 1987a; Stone et al., 1989a; Stone et al., 1989b; Garcia-Osta et al., 2004) and induces widespread 5-HT release (Gough et al., 1991; White et al., 1996; Lyles and Cadet, 2003; Colado et al., 2004; Green et al., 2004). These

serotonergic changes likely instigate many of the other neurological changes reported after MDMA treatment, such as altered 5-HT receptor status (Scheffel et al., 1992; Aguirre et al., 1995; Aguirre, 1998; Reneman et al., 2002; McGregor et al., 2003) and SERT binding densities (Battaglia et al., 1987; Scanzello et al., 1993; Yau et al., 1994; Aguirre et al., 1995; Colado and Green, 1995; Lew et al., 1996; O'Shea et al., 1998; Scheffel et al., 1998; Colado et al., 1999; Sexton et al., 1999; Brown, 2000; Boot, 2002; McGregor et al., 2003; Sumnall et al., 2004). The most robust long-term effect of MDMA administration is 5-HT depletion (Battaglia et al., 1987; Commins et al., 1987; Ricaurte et al., 1988b; Ricaurte et al., 1988c; Insel et al., 1989; Molliver, 1990; Scanzello et al., 1993; Aguirre et al., 1995; Colado and Green, 1995; Fischer et al., 1995; McNamara et al., 1995; Sabol et al., 1996; Sexton et al., 1999; Mayerhofer et al., 2001; Boot, 2002; McGregor et al., 2003; Clemens et al., 2004; Sumnall et al., 2004; Wang et al., 2004; Nair and Gudelsky, 2006) and this might lead to relevant downstream changes.

Consistent with these findings, a relationship between altered serotonergic neurotransmission and tolerance was demonstrated. MDMA pretreatment (10mg/kg X4, total = 40mg/kg) and subsequent microdialysis revealed an impaired ability of acute MDMA to increase striatal 5-HT (Shankaran and Gudelsky, 1999). MDMA-produced behaviours were also suppressed, as 5-HT syndrome and hyperthermic responses were attenuated in pretreated rats. MDMA-produced hyperactivity (Kehne et al., 1996a; Bankson, 2002; Fletcher PJ, 2002b; Herin et al., 2005), 5-HT syndrome and hyperthermia have been associated with 5-HT receptor activation (Goodwin et al., 1986a; Goodwin et al., 1987b; Murthy and Pranzatelli, 1992; Aguirre et al., 1995; Kleven et al., 1997; Aguirre, 1998), therefore impaired ability to elevate synaptic 5-HT levels and reduced postsynaptic receptor activation might lead to behavioural tolerance. Indeed, MDMA-produced hyperactivity is largely dependent upon activation of receptor subtypes such as the 5-HT_{1b} and 2a receptors (Kehne et al., 1996a; McCreary et al., 1999; Fletcher PJ, 2002a; Herin et al., 2005). Decreased binding densities of these receptor subtypes were reported post

MDMA exposure (Scheffell et al., 1992; Reneman et al., 2002; McGregor et al., 2003), therefore tolerance might reflect reduced availability of 5-HT to activate receptors that facilitate MDMA-produced hyperactivity.

There was evidence to suggest that tolerance to MDMA following preexposure was due to 5-HT depletions. MDMA pretreated rats were tolerant to MDMA, but exhibited an augmented response to amphetamine challenge (Gately et al., 1986). These observations were purported to relate to MDMA-induced 5-HT depletions, as 5-HT depletion has potentiated locomotor response to amphetamines (Segal, 1976). Further, the response to amphetamine confirms that tolerance to MDMA-produced hyperactivity was not likely generalised impairment of motor function or insurmountable fatigue (Callaway and Geyer, 1992a).

MDMA pretreated rats were tolerant not only to the activating effects of MDMA 36 hours post initial exposure, but also to hyperactivity produced by the 5-HT₁ receptor agonist, RU24969 (Callaway and Geyer, 1992a; Rempel et al., 1993). MDMA pretreatment also produced tolerance to the effects of other serotonergic compounds, suggesting that tolerance is indeed reflective of changes to 5-HT systems targeted by these drugs. For example, MDMA pretreated rats were tolerant to the effects of the 5-HT₂ receptor agonist, DOI, following an 8-week withdrawal period (Bull, 2004) and to the ability of 8-OHDPAT to produce SS-like behaviours (Piper et al., 2006). These findings suggest that 5-HT₁ and 5-HT₂ receptor desensitisation might account for reduced behavioural response to the 5-HT agonists.

MDMA-produced serotonergic deficits have recovered over time. Following a withdrawal period, TPH levels increased as did TPH mRNA expression in cortical regions (Garcia-Osta et al., 2004). 5-HT levels and SERT binding returned to control values 16-32 weeks post MDMA exposure (Scanzello et al., 1993; Fischer et al., 1995; Lew et al., 1996; Sabol et al., 1996). SERT recovery showed regional distribution, with densities in some areas such as the hippocampus (Scanzello et al., 1993; Fischer et al., 1995; Sabol et al.,

1996; Sumnall et al., 2004) and occipital cortex (Lew et al., 1996) slower to recover than others. Downregulated 5-HT₂ receptor binding densities (Scheffel et al., 1992; Reneman et al., 2002) also recovered over time, probably in response to increases in synaptic 5-HT levels. These neurochemical findings are consistent with the notion that MDMA produces neuroadaptations that recover over time (Wang et al., 2004; Wang et al., 2005).

MDMA pretreated rats (10mg/kg twice daily for 4 days) that were tolerant to the activating effects of MDMA 3 days afterwards exhibited recovery following a 3 weeks withdrawal (Callaway and Geyer, 1992a). Substantial 5-HT depletions were evident in hippocampus and striatum immediately after MDMA pretreatment and these depletions persisted beyond 3 weeks. Tolerance to MDMA might be due to functional desensitisation of the postsynaptic receptors that mediate the behavioural effects of MDMA rather than depleted 5-HT levels (Callaway and Geyer, 1992a) since 5-HT_{1b} and 5-HT_{2a/2c} receptors underwent region and MDMA-dose specific alteration (McGregor et al., 2003).

Since MDMA alters serotonergic systems, DA neurotransmission might be affected because the systems are interrelated. No changes, however, to DA levels or metabolism were found after monkeys had received MDMA when analyses of brain tissue and CSF were conducted (Ricaurte et al., 1988a; Ricaurte et al., 1988b; Ricaurte et al., 1988c). In rats, no changes to DAT binding densities were reported following MDMA exposure (Battaglia et al., 1987; Lew et al., 1996; McGregor et al., 2003). No change (Callaway and Geyer, 1992a), elevated (McNamara et al., 1995; Mayerhofer et al., 2001) or lowered (Commins et al., 1987) DA levels in the striatum, nucleus accumbens and hippocampus have been reported following administration of high dose MDMA. When DA neurotransmission was affected by MDMA, it was possible that this was a secondary occurrence to changes in the serotonergic system. Numerous 5-HT receptors have the ability to modulate the dopaminergic system; suggesting that changes in function/number of these receptors might impact DA neurotransmission in affected areas (Daniela et al., 2004). In addition to this, GABA has been found to influence MDMA-produced DA

release in the nucleus accumbens (Bankson and Yamamoto, 2004) and decreased GABA-1 transporters has been reported following MDMA exposure (Peng and Simantov, 2003).

The present study is investigating tolerance to the behavioural response to MDMA following preexposure, but MDMA pretreatment has also produced sensitisation to the activating effects of MDMA (Spanos and Yamamoto, 1989; Dafters, 1995; Kalivas et al., 1998; McCreary et al., 1999; Ramos et al., 2004, 2005). Sensitisation was consistently produced by repeated and intermittent MDMA dosing regimens (Spanos and Yamamoto, 1989; Kalivas et al., 1998; Ramos et al., 2004, 2005). When tolerance (Callaway and Geyer, 1992a) or no changes (McNamara et al., 1995) in MDMA-produced hyperactivity were reported, MDMA was administered at higher doses over shorter periods and behavioural testing was conducted shortly thereafter. A withdrawal period was important for the development of sensitisation to psychostimulants (Kolta et al., 1985; Kalivas and Duffy, 1993; Heidbreder et al., 1996; Kalivas et al., 1998). This was specifically demonstrated for MDMA, as immediately following an 8-day MDMA pretreatment period, rats that were administered lower MDMA doses showed a sensitised response to MDMA; but those that had received the higher dose pretreatment did not display sensitisation (Kalivas et al., 1998). Interestingly, after an 11-day withdrawal period, both low and high MDMA groups exhibited sensitisation.

The MDMA dosing regimen might also determine which neurotransmitter systems are affected. Whilst tolerance to MDMA was associated predominantly with serotonergic changes, altered dopaminergic neurotransmission was reported in animals that were sensitised to MDMA. For example, following a chronic and intermittent MDMA pretreatment regimen, microdialysis measuring DA levels produced after acute MDMA administration showed potentiated DA increases in these animals (Kalivas et al., 1998). These differential neurochemical changes likely explain the different behavioural profiles following an MDMA challenge.

Present Study

Outline

The present study utilised behavioural measures to determine whether functional changes to two different 5-HT receptor subtypes or the SERT might account for the suppressed behavioural response to MDMA following pre-exposure in rats. Since there have been no consistent reports regarding changes to baseline behaviour following MDMA exposure, most of the experiments assessed behaviour following acute MDMA administration. It has been widely reported that even low doses of MDMA produce serotonergic deficits, but these deficits only become evident following pharmacological challenge. There have been attempts to assess 5-HT receptor status following MDMA preexposure, but the range of varying methodologies has made it difficult to draw unambiguous conclusions. The present study provides several behavioural assays to assess the SERT and 5-HT_{2a/2c} receptors in rats that received the same MDMA pretreatment regimen.

MDMA produces several behavioural effects, but MDMA-produced hyperactivity was the primary behavioural assay used in this thesis. This behaviour was selected over other MDMA-produced behaviours for several reasons. Firstly, stereotypy predominantly involves dopaminergic mechanisms (Gordon and Beck, 1984; Sandoval and Palermo-Neto, 1995) and has not been consistently reported as an MDMA-produced behavioural effect (Matthews et al., 1989). Secondly, although MDMA-produced 5-HT syndrome (Kleven et al., 1997), anxiety (Martin et al., 1998; To et al., 1999; To and Bagdy, 1999; Kurt et al., 2000; Silva and Brandao, 2000; Bagdy et al., 2001) and hyperactivity (Callaway et al., 1990; Kehne et al., 1996a) have been attributed to serotonergic systems, the advantage that the locomotor activity paradigm has over these more qualitative assessments is that clear dose-dependent effects are visible. It is therefore possible to gauge the sensitivities of target binding sites, as time course data and dose effect curves allow specific versus non-

specific effects of pharmacological manipulation to be determined. Finally, both the 5-HT_{2a} (Kehne et al., 1996a; Bankson, 2002; Herin et al., 2005) and 5-HT_{2c} (Gold, 1988b; Hutson et al., 2000; Herin, 2001; Bankson, 2002; Fletcher PJ, 2002b; Fletcher et al., 2006) receptor subtypes and the SERT (Callaway et al., 1990; Callaway and Geyer, 1992b) have identifiable roles in MDMA-produced hyperactivity, thus use of this single paradigm enabled a comparative assessment.

Although MDMA-produced hyperactivity was the primary assay, 5-HT₂ receptor agonists were also administered to MDMA pretreated rats in order to determine whether any other functional changes had occurred that were not visible in locomotor activity. 5-HT_{2c} agonists produce hypolocomotion (Klodzinska et al., 1989; Kennett et al., 1994a; Martin et al., 1998; McCreary et al., 1999; Gleason et al., 2001), whereas 5-HT_{2a} agonists tend to increase locomotor activity (Darmani et al., 1996; Hillegaart et al., 1996; Kaur and Ahlenius, 1997; Bull, 2004; Ross et al., 2005). In addition to these locomotor effects, 5-HT_{2c} receptor activation increases emergence latency (Bilkei-Gorzo et al., 1998) and 5-HT_{2a} receptor activation produces wetdogshakes (Kleven et al., 1997; Willins and Meltzer, 1997; Bartoszyk et al., 2003). These 5-HT₂ agonist-induced behaviours were measured in MDMA and saline pretreated rats in order to determine whether the receptor subtypes were differentially affected by MDMA exposure.

MDMA Pretreatment and Hyperactivity

An MDMA pretreatment regimen (10mg/kg X4, total 40mg/kg MDMA) and 2-week withdrawal time were selected for the present study, as serotonergic deficits (Scanzello et al., 1993; Fischer et al., 1995; Sumnall et al., 2004; Nair and Gudelsky, 2006) and behavioural tolerance (Shankaran and Gudelsky, 1999) following this protocol are well documented. The 2-week recovery period was also selected because evidence suggests that MDMA-produced serotonergic deficits are most pronounced after a short withdrawal period. For example, 5-HT levels were depleted by approximately 20-30% 6 hours

following MDMA exposure, whilst 30 days later, 5-HT levels had further reduced by 80% (Reneman et al., 2002). Similarly with SERT binding, decreases in SERT sites were visible 24 hours after the last dose of MDMA, but regions such as dorsal striatum exhibited greatest reductions 2 weeks later (Battaglia et al., 1991). Since suppressed behavioural responsiveness to MDMA is probably related to these deficits, it was expected that at the 2-week time point, rats might exhibit maximal tolerance to MDMA. In order to verify that the regimen used in the present study produced comparable serotonergic deficits to previous reports, SERT binding was measured across multiple brain regions 2 weeks following preexposure.

Many of the deficits produced by MDMA have been reported to recover over a longer period of time (Scanzello et al., 1993; Fischer et al., 1995), which concurs with recent reports suggesting that MDMA does not induce irreversible neurological changes (Wang et al., 2004; Wang et al., 2005). TPH activity (Garcia-Osta et al., 2004), 5-HT levels (Scanzello et al., 1993; Fischer et al., 1995), SERT binding (Scanzello et al., 1993; Fischer et al., 1995) and 5-HT₂ receptor densities (Scheffel et al., 1992; Reneman et al., 2002) returned to control levels following an adequate post MDMA withdrawal period. Behavioural measures of the integrity of the 5-HT system would also be expected to show similar time-dependent recovery. As the neurological recovery following the selected pretreatment regimen has been well documented, the present study sought to determine whether there was a functional correlate. MDMA-produced hyperactivity was also assessed after 12-week withdrawal period, as previous studies showed that serotonergic deficits were apparent at the earlier test period but had largely dissipated approximately 12 weeks following exposure (Scanzello et al., 1993; Fischer et al., 1995).

The Serotonin Transporter

Overview

The SERT controls the synaptic actions of 5-HT by rapidly clearing released neurotransmitter. The transporters are sites of action for SSRIs and high affinity targets for drugs such as cocaine, amphetamine and MDMA. Displacement of MDMA binding to SERT ablates 5-HT release (Hekmatpanah and Peroutka, 1990; Berger et al., 1992; Rudnick and Wall, 1992; Gudelsky and Nash, 1996) and also diminishes behavioural effects. For example, when SSRIs were administered in combination with MDMA, hyperactivity was attenuated (Callaway et al., 1990). One of the most consistently reported consequences of MDMA exposure was reduced SERT binding densities (Battaglia et al., 1987; Scanzello et al., 1993; Yau et al., 1994; Aguirre et al., 1995; Colado and Green, 1995; Lew et al., 1996; O'Shea et al., 1998; Scheffel et al., 1998; Colado et al., 1999; Sexton et al., 1999; Brown, 2000; Boot, 2002; McGregor et al., 2003; Sumnall et al., 2004), thus it was hypothesised that loss of SERT sites might contribute to behavioural suppression.

Distribution

In situ hybridisation studies have revealed that SERT is primarily expressed by serotonergic neurons (Blakely et al., 1994). The highest SERT binding densities were observed in the midbrain and thalamus, with the lowest in the cerebellum and occipital cortex (Brust et al., 2003). Specifically, SERT-rich brain areas were the globus pallidus, thalamus, hypothalamus, substantia nigra, interpeduncular nucleus, amygdala, and raphe nucleus, where the dorsal raphe nucleus had the highest 5-HT uptake (Lin et al., 2004).

Neurochemical Roles

Neural activity, hormones, aging, environmental factors and pharmacological agents have the ability to regulate SERT-mediated 5-HT uptake, radioligand binding and mRNA expression (Jayanthy and Ramamoorthy, 2005). Much of the detail regarding SERT

regulatory mechanisms are beyond the scope of this thesis, but second messenger mechanisms are affected by amphetamines, and have therefore been considered.

Second messengers regulate SERT via phosphorylation of the transporter protein, which alters turnover rate, plasma membrane insertion or sequestration from the plasma membrane (Jayanthi and Ramamoorthy, 2005). Phosphorylation state and transporter regulation is a balance between the actions and localisation of protein kinases and phosphatases, as application of protein kinase C (PKC) activators reduced the transport capacity for 5-HT. Also, agents that maintained a phosphorylation state, such as phosphatase inhibitors, rapidly reduced transport capacity (Ramamoorthy et al., 1998).

SERT-selective substrates can influence transporter trafficking and plasma membrane residency (Ramamoorthy and Blakely, 1999). Since transporters are regulated by kinase-dependent mechanisms, it is possible that substrates influence transporter surface expression by regulating kinase-mediated transporter phosphorylation. For example, SERT substrates such as 5-HT, amphetamines, SSRIs and cocaine control PKC-mediated SERT phosphorylation and surface redistribution (Ramamoorthy and Blakely, 1999).

Chronic administration of SSRIs, paroxetine or sertraline, decreased SERT binding densities in the hippocampus, but had no effect on 5-HT levels or SERT mRNA expression (Benmansour et al., 1999). Since multiple kinase pathways regulate SERT, whole brain kinase mRNA expression was studied following chronic fluoxetine or citalopram treatment (Rausch et al., 2002). Chronic treatment with either SSRI produced downregulation in PKC-delta, PKC-gamma, stress-activated protein kinase, cAMP-dependent protein kinase beta isoform, Janus phosphokinase and phosphofructokinase M, whereas acute treatment produced no changes. These findings suggest that decreased SERT binding following chronic SSRI exposure might involve changes in SERT-regulatory mechanisms rather than SERT mRNA expression.

Behaviours Produced by Selective Reuptake Inhibitors

SSRIs primarily target the SERT and are clinically effective as antidepressants. The forced swim test (FST) is the most widely used model for assessing pharmacological antidepressant activity (Cryan et al., 2005). The test is based on the observation that rodents eventually develop immobility when they are placed in a cylinder of water after they stop active escape behaviours, such as climbing or swimming. Generally, it has been observed that drugs that increase serotonergic neurotransmission increase swimming, whilst those that affect catecholaminergic mechanisms affect climbing behaviours (Cryan et al., 2005). The FST is used as a screen in antidepressant discovery research due to its sensitivity to a broad range of antidepressant drugs, including not only SSRIs, but also tricyclics, monoamine oxidase inhibitors and atypical antidepressants. The exceptions are psychomotor stimulants, which also reduce immobility in the FST but are not clinically effective as antidepressants. In order to distinguish between stimulants and SERT-selective compounds, locomotor activity tests are typically conducted alongside FST tests.

Most clinically established SSRIs, such as fluvoxamine and fluoxetine, decrease locomotor activity (Rodriguez Echandia et al., 1983; To et al., 1999; McMahon and Cunningham, 2001a). Also consistent with elevated synaptic serotonergic levels, SSRI administration decreased exploration of open arms in the EPM (Matto et al., 1996; Silva et al., 1999; Kurt et al., 2000; Pollier et al., 2000) and social interaction (To et al., 1999; To and Bagdy, 1999). However, the role for SSRIs in anxiety tests are not clear, as acute administration has also induced anxiolysis (Molewijk et al., 1995; Bagdy et al., 2001). These findings likely reflect the range of pharmacological properties, in addition to 5-HT reuptake inhibition, exhibited by different SSRI compounds. For example, anticholinergic activity as well as some affinity for the D2-like, H1 histamine and adrenergic receptors have been reported (McTavish and Benfield, 1990; Fujishiro et al., 2002).

Anxiolysis as measured in the EPM (Griebel et al., 1999; Silva et al., 1999) and social interaction tests (To et al., 1999; To and Bagdy, 1999) were reported following chronic SSRI treatment. In the FST paradigm, SSRIs are effective following both acute

and chronic administrations. Low doses of fluoxetine (2-5mg/kg) that were inactive following acute administration, became active after chronic administration in the FST (Detke et al., 1997). It has also recently been observed that an acute fluoxetine dose (5mg/kg) that did not produce any observable behavioural effects, significantly reduced immobility in the FST following a 14-day treatment regimen (Vazquez-Palacios et al., 2004). These findings probably reflect adaptive changes in postsynaptic receptors that mediate these effects as a consequence of enhanced synaptic 5-HT levels.

Effects of MDMA on the Serotonin Transporter

Acute Effects of MDMA Administration

MDMA binding to the SERT was necessary for full expression of MDMA-produced hyperactivity, as coadministration of SSRIs such as fluoxetine, sertraline and zimelidine completely or partially attenuated MDMA-produced hyperactivity (Callaway et al., 1990; Callaway and Geyer, 1992b) and SERT knockout mice did not exhibit MDMA-produced hyperactivity (Bengel et al., 1998). SSRIs produce hypomotility (Rodriguez Echandia et al., 1983; To et al., 1999; McMahon and Cunningham, 2001a), but attenuation of hyperactivity was not likely due to non-selective behavioural suppression, as MDMA-induced reductions in rears or holepokes were not attenuated (Callaway et al., 1990; Callaway and Geyer, 1992b). Also, amphetamine-produced hyperactivity was augmented by SSRI pretreatment, indicating a selective effect on MDMA-produced hyperactivity (Sills et al., 1999a, 1999b). These differential effects might be explained in that amphetamine has highest affinity for the DAT (Rothman and Baumann, 2003), thus hyperactivity is probably driven mostly by dopaminergic mechanisms in comparison to MDMA.

Neurochemical Effects of MDMA Exposure

One of the most frequently reported consequences of MDMA exposure was reduced SERT binding densities and 5-HT depletions. These observations were initially thought to reflect 5-HT neurotoxicity but measuring SERT densities appears to have limited use as an index of MDMA-produced neurotoxicity. The SERT is not a structural element of the nerve terminals and is therefore susceptible to pharmacological regulation, maturation, aging and food restriction (Zhou et al., 1996; Sumnall et al., 2004). Deficits in serotonergic systems could alternatively reflect persistent adaptive changes in gene expression or protein levels rather than neurotoxicity (Baumann et al., 2006). In studies that have assessed MDMA as a potential neurotoxin, silver staining (Switzer, 2000) and FluoroJade Blue have been utilised. Fluoro Jade Blue is an anionic fluorescein derivative that selectively stains degenerating terminals, axons and cell bodies (Schmued and Hopkins, 2000). Since neuronal damage is accompanied by hypertrophy of astrocytes or 'reactive gliosis', an increase in the expression of glial fibrillary acidic protein (GFAP) has also been used as an indicator of neurotoxicity (O'Callaghan and Sriram, 2005).

Rats have been pretreated with excessively high doses of MDMA (80-600mg/kg) and silver-markers for neurotoxicity examined (Commins et al., 1987; Jensen et al., 1993). Extensive damage and degenerating nerve terminals were observed in striatum and parietal cortex, indicating that these doses of MDMA could cause neuronal damage. However, it was suggested that these doses induced non-specific neuronal damage due to excessive sympathetic activation and hyperthermia (Baumann et al., 2006). Indeed, silver staining was not confined to 5-HT neurons and the staining pattern did not correspond to 5-HT innervation or pattern of 5-HT depletions. When Fluoro Jade Blue was used to assess the effects of 10-40mg/kg MDMA, doses of 20mg/kg or greater produced neurotoxicity, but again, staining did not overlap with the pattern of 5-HT deficits and toxicity occurred in the rats with the highest body temperatures (Schmued, 2003).

GFAP expression studies indicated that moderate doses MDMA were not neurotoxic, yet extremely high doses produced non-selective damage. MDMA

pretreatment (10-30mg/kg daily for 7 days) markedly reduced 5-HT levels in the cortex, hippocampus and striatum, but GFAP expression was unaffected (O'Callaghan and Miller, 1993). However, when a separate group was treated at higher doses (75-150mg/kg twice daily for 2 days), GFAP expression was elevated in several brain regions but expression changes did not correlate to the degree of 5-HT depletions.

The 5-HT and DA neurotoxins 5,7-DHT and methamphetamine respectively, increased GFAP expression. MDMA was administered at doses that produced comparable decreases in SERT binding and 5-HT levels to these treatments, but MDMA had no effect on GFAP expression (O'Callaghan and Miller, 1993; Pubill et al., 2003). Indeed, two other substituted amphetamines, fenfluramine and p-chloroamphetamine (PCA) decreased 5-HT levels by approximately 50-60%, but neither affected SERT or GFAP expression (Rothman et al., 2003; Wang et al., 2004; Wang et al., 2005). These findings suggest that drug-induced decreases in 5-HT levels and reduced SERT binding densities can occur in the absence of neurotoxicity.

Since decreased SERT binding was not due to degenerating 5-HT nerve terminals, it was hypothesised that central 5-HT depletions might have altered SERT binding properties. MDMA, 5,7-DHT or a tryptophan hydroxylase inhibitor, pCPA all depleted 5-HT levels, yet only 5,7-DHT (Gobbi et al., 1994; Neumaier et al., 1996) and MDMA (Battaglia et al., 1987; Scanzello et al., 1993; Yau et al., 1994; Aguirre et al., 1995; Colado and Green, 1995; Lew et al., 1996; O'Shea et al., 1998; Scheffel et al., 1998; Colado et al., 1999; Sexton et al., 1999; Brown, 2000; Boot, 2002; McGregor et al., 2003; Sumnall et al., 2004) reduced SERT binding. Effects on SERT binding might, therefore, be dependent on the properties of the compound administered and not on the ability to reduce 5-HT levels. 5,7-DHT reduced SERT binding, 5-HT levels, altered SERT mRNA expression, increased GFAP expression and reduced plasma membrane SERT levels (Wang et al., 2004; Wang et al., 2005). MDMA did not, however, affect SERT mRNA, GFAP expression or SERT protein levels in plasma membrane, suggesting that the SERT might undergo

conformational/functional change after MDMA exposure and that decreased 5-HT levels were not a necessary prerequisite.

MDMA pretreatment might affect SERT regulatory mechanisms, as MDMA and other substituted amphetamines induced PKC translocation from the cytosol to the plasma membrane (Kramer et al., 1998). MDMA pretreatment (20mg/kg X2) increased membrane-bound PKC by 48% (Kramer et al., 1995). PKC translocation was triggered by 5-HT_{2a} receptor and SERT activation, as selective blockade of either site attenuated MDMA-induced PKC translocation (Kramer et al., 1997). The administration of fluoxetine or 5-HT_{2a} antagonists prior to MDMA pretreatment were protective against MDMA-produced serotonergic deficits (Malberg et al., 1996; Sprague et al., 1998), thus it is possible that 5-HT_{2a} receptor or SERT activation leads to MDMA-produced PKC translocation. Since PKC regulates the SERT (Jayanthi and Ramamoorthy, 2005), MDMA-induced PKC alteration could affect transport capacity and possibly the ability of selective ligands to bind this site.

MDMA-induced reductions in SERT binding recovered over time following drug abstinence, suggesting that changes to SERT regulatory mechanisms/conformation were transient (Scanzello et al., 1993; Fischer et al., 1995; Lew et al., 1996; Sabol et al., 1996). Recovering SERT binding densities did not correlate well with the restoration of 5-HT levels, so 5-HT levels might not instigate initial binding decreases or have a role in the recovery process. However, if decreased SERT binding were related to the effects of MDMA on PKC, it is possible that changes in 5-HT_{2a} receptor densities might initiate recovery. Since MDMA exposure induces a downregulation/desensitisation of 5-HT_{2a} receptors (Reneman et al., 2002; McGregor et al., 2003; Bull, 2004) and decreased SERT binding, reduced 5-HT_{2a} and SERT-mediated input might reverse the effects of MDMA on PKC. Future studies might more closely examine the role of PKC in MDMA-induced reductions in SERT binding and whether it also plays a role in the recovery process.

MDMA pretreatment produced substantial SERT binding reductions in most brain regions examined (Battaglia et al., 1987; Scanzello et al., 1993; Yau et al., 1994; Aguirre et al., 1995; Colado and Green, 1995; Lew et al., 1996; O'Shea et al., 1998; Scheffel et al., 1998; Colado et al., 1999; Sexton et al., 1999; Brown, 2000; Boot, 2002; McGregor et al., 2003; Sumnall et al., 2004), but the direct effects on behaviour have yet to be elucidated. Studies that have examined the consequences of MDMA exposure on baseline and drug-induced behaviours have reported SERT binding status as verification that MDMA had affected serotonergic transmission. There have been no attempts to directly associate altered SERT status with behaviour. Since the SERT regulates synaptic 5-HT levels and thus neurotransmission, any behaviour that is related to 5-HT neurotransmission might be disrupted by changes to the transporters. Therefore, studies that assessed the behavioural consequences of MDMA exposure and SERT binding densities will be reviewed to determine whether any behavioural changes appear to correlate with SERT binding status.

Low (1 X 5mg/kg over 4h) or high (4 X 5mg/kg over 4hrs) dose MDMA was administered with a withdrawal period of 10 weeks (McGregor et al., 2003). Only the high dose MDMA group exhibited significant 5-HT level decreases but both groups showed SERT binding reductions. Low dose MDMA produced significantly lower densities in the medial hypothalamus, whereas high dose lowered densities in almost every brain region examined, thus the higher dose produced more widespread serotonergic disruptions than the low dose group. The high dose group also exhibited significantly greater behavioural deficits, as evidenced by decreased social interaction and increased emergence latency. Since no measures of dopaminergic transmission had changed, it was concluded that altered 5-HT neurotransmission had affected baseline behaviours in this case.

A follow-on study attempted to reverse the deficits produced by the high dose MDMA regimen by administering oral doses of fluoxetine in drinking water (Thompson et al., 2004). Fluoxetine alone reduced 5-HIAA levels in all regions examined, reflecting

decreased turnover of 5-HT. Neurochemical analyses were not conducted on the MDMA/fluoxetine pretreated group due to the long half life of fluoxetine metabolites, but behavioural tests showed that most deficits were ameliorated by chronic fluoxetine treatment, however, decreased social interaction remained unaffected. Although these findings do not point to specific mechanisms, the effects of chronic fluoxetine treatment support previous hypotheses that altered serotonergic neurotransmission produced most of the behavioural deficits. It would have been interesting to assess SERT binding sites in the MDMA/fluoxetine group, as this could have provided a clue as to whether the behavioural recovery was associated with SERT binding status.

Young Listar Hooded rats were pretreated with low or high dose MDMA (7.5 or 15.0mg/kg twice daily for 3 days) that produced modest 5-HT decreases with no changes in cortical paroxetine binding (Fone et al., 2002; Bull, 2003). Rats exhibited decreased social interaction without any effect on locomotor activity, suggesting in this instance that reduced SERT binding densities might not be a necessary prerequisite for decreased social interaction. It was interesting that these regimens did not reduce SERT binding when lower doses in numerous other studies have consistently produced this result. It cannot be assumed from these data that there were no changes to SERT densities when only a single region was assessed, as alternatively, MDMA might not have produced serotonergic deficits in these rats. The rats were only around 165g in weight at the time of MDMA pretreatment, whereas most other studies have utilised animals that were in the 250-350g-weight range (McGregor et al., 2003; Sumnall et al., 2004). It was suggested by the authors that young rats might be less susceptible to the 5-HT altering properties of MDMA. For example, the MDMA-induced increases in free radical formation, which have been attributed to the production of serotonergic deficits, did not appear to occur in the neonatal rat brain (Colado et al., 1997). It was also suggested that in earlier developmental periods, MDMA might be metabolised differently.

MDMA pretreated rats administered a pharmacological challenge prior to behavioural testing have produced some inconsistent findings (Callaway and Geyer, 1992a; Bull, 2003; Bull, 2004; Sumnall et al., 2004). The response to an acute dose of MDMA that produced anxiogenesis in the EPM was assessed in MDMA (10mg/kg X 4 over 6 hrs) pretreated rats following a 2-week withdrawal (Sumnall et al., 2004). In both MDMA pretreated and control rats, 10mg/kg MDMA produced an anxiogenic profile in the maze, suggesting that there were no pretreatment differences. Despite these behavioural similarities, MDMA pretreated rats exhibited significant decreases in paroxetine binding and lowered hippocampal 5-HT and 5-HIAA levels compared to controls. The results were somewhat surprising, as MDMA pretreatment would be expected to produce tolerance to MDMA, as has been reported for MDMA-produced locomotor activity (Callaway and Geyer, 1992a), milk drinking (Zacny et al., 1990), 5-HT syndrome and hyperthermia (Shankaran et al., 1999). Recent data collected in our laboratory from rats that received an identical pretreatment regimen have indicated that a dose of 3.3mg/kg MDMA increased emergence latency, and that MDMA pretreated rats were tolerant to these effects (unpublished data). Sumnall's (2004) findings might be explained in that the single dose of MDMA was too high and produced non-selective effects, as there were decreased closed and open arm entries, suggesting that this dose was affecting locomotor activity. Sumnall suggested that the EPM might not be a behavioural measure sensitive enough to gauge MDMA-induced functional deficits.

MDMA pretreated rats (total of 40 or 80mg/kg MDMA) were tested following either a 3-day (Callaway and Geyer, 1992a) or 8-week (Bull, 2004) withdrawal period. Both regimens produced 5-HT depletions, but cortical SERT binding was unaffected in the low dose group. SERT binding was not assessed after the high dose regimen, but considering studies that used comparable methodologies have shown decreased binding, it was likely that decreases were present. Also, since only one brain area was assessed following low dose MDMA, it is possible that decreased SERT binding had occurred in

other regions. Following the high dose regimen, rats were tolerant to the activating effects of 3.0mg/kg MDMA (Callaway and Geyer, 1992a) and similarly, the low dose produced tolerance to a 5-HT₂ agonist (Bull, 2004). These findings indicate that MDMA pretreatment had affected serotonergic systems and that behavioural tolerance was evident, but the extent that SERT binding was affected could not be determined.

The inability to determine SERT binding status in past studies highlights the importance of conducting multiregional SERT binding assays. Additionally, it has been reported that SERT binding is differentially affected by MDMA pretreatment regimen and the age and strain of the rats (Colado et al., 1997; McGregor et al., 2003). As a consequence, comparisons between studies are equivocal. In order to make valid comparisons and avoid the aforementioned confounds, use of the same pretreatment methodologies are vital. For example, Bull and colleagues utilised different withdrawal times and pretreatment regimens in two studies comparing the effects of MDMA preexposure on the behavioural responsiveness to an m-CPP (2003) or DOI (2004) challenge. It was concluded that MDMA preexposure did not alter response to m-CPP, yet the response to DOI was ablated. However, because the experimental procedures were different, differences might be attributable to procedure.

Experiment Details

To assess the role of the SERT in MDMA-produced hyperactivity, a SERT-selective compound, clomipramine, was administered in combination with MDMA. Clomipramine was selected for these experiments because it is highly selective for the SERT, in fact, more selective than the commonly used fluoxetine (Wu et al., 2005). According to ligand binding analysis, the potency of clomipramine for the SERT was at least 100-fold higher than for the NET or DAT.

It has been reported that SSRIs prevented MDMA-produced hyperactivity (Callaway et al., 1990; Callaway and Geyer, 1992b), so it was expected that clomipramine

would similarly attenuate hyperactivity. SSRIs also produced hypomotility by enhancing synaptic 5-HT levels (Rodriguez Echandia et al., 1983; To et al., 1999; McMahon and Cunningham, 2001a), so the ability of clomipramine to affect baseline locomotor activity was investigated for use as a second behavioural assay. SSRI-induced hypolocomotion was consistently reported and most likely to yield dose-dependent effects, whereas there were mixed findings in anxiety and FST measures. In addition, as MDMA pretreated rats exhibit tolerance to MDMA, examining the inhibitory abilities of clomipramine might be confounded, as low activity levels might not be further reduced. For these reasons, it was deemed necessary to provide a SERT-mediated assay wherein MDMA was excluded.

The effects of clomipramine on MDMA-produced hyperactivity were assessed in MDMA pretreated and control rats. The MDMA pretreatment regimen produced significant SERT binding reductions in multiple brain regions, therefore, it was expected that pharmacological SERT manipulation would yield little behavioural response in MDMA pretreated rats. These findings would be consistent with the idea that reduced SERT binding densities might be a candidate for tolerance to MDMA-induced hyperactivity.

The 5-HT₂ Receptors

Overview

The 5-HT₂ receptor family presently includes three subtypes; the 5-HT_{2a}, 5-HT_{2b} and 5-HT_{2c} receptors. The subtypes are similar in terms of their molecular structure, pharmacology and signal transduction pathways (Barnes and Sharp, 1999). The 5-HT₂ receptors are coupled positively to phospholipase C and lead to increased accumulation of inositol phosphates and intracellular calcium. The availability of selective antagonists has allowed receptor distributions to be mapped and the pharmacological and behavioural roles

to be studied. There are currently no selective agonists available, which limits further progress to some degree.

Selective antagonists for the 5-HT_{2a} and 5-HT_{2c}, but not the 5-HT_{2b}, receptor subtypes modulate MDMA-produced hyperactivity by affecting dopaminergic neurotransmission. Changes to these receptor subtypes might contribute to locomotor tolerance to MDMA, as dopaminergic mechanisms affect locomotor activity and it was reported that 5-HT₂ receptors were desensitised or downregulated following MDMA exposure. The 5-HT₂ receptors were selected over other 5-HT receptor subtypes that also modulate MDMA-produced locomotor activity because there were more consistent effects of MDMA exposure on these receptors and the selective ligands were available. Also, in addition to MDMA-produced locomotor activity, the 5-HT_{2c} and 5-HT_{2a} receptors have been implicated in a number of other behavioural functions and are widely distributed throughout the central nervous system. Since tolerance to MDMA has been observed in other behaviours in addition to locomotor activity, it is possible that 5-HT₂ receptor desensitisation might also explain general behavioural suppression.

The 5-HT_{2c} Receptor Subtype

Distribution

Extensive work has been conducted to map the distribution of the 5-HT_{2c} receptor within the mammalian central nervous system (CNS). 5-HT_{2c} receptor m-RNA labelling was the most abundant and widespread of the 5-HT receptor subtypes investigated (Wright et al., 1995) and greater 5-HT_{2c} receptor distribution was found to that of the 5-HT_{2a} receptor (Pompeiano et al., 1994; Wright et al., 1995). The distribution of this receptor subtype is consistent across rat, human and monkey (Abramowski et al., 1995). Dense 5-HT_{2c} receptor distributions were found in the hippocampus, amygdala, thalamus, hypothalamus, cerebral cortex, nucleus accumbens, caudate putamen, olfactory system, midbrain and brain stem (Molineaux et al., 1989; Abramowski et al., 1995; Eberle-Wang et

al., 1997; Clemett et al., 2000; Lopez-Gimenez et al., 2001). 5-HT_{2c} mRNA was co-expressed with glutamic acid carboxylase but not tyrosine hydroxylase mRNA in substantia nigra, suggesting expression in GABA but not dopaminergic or NE neurons (Eberle-Wang et al., 1997). Patterns of mRNA receptor expression indicated that this receptor subtype was located mainly in postsynaptic regions (Lopez-Gimenez et al., 2001) but some expression was also found in dorsal raphe nuclei neurons, suggesting a presynaptic function in this region (Clemett et al., 2000). Higher mRNA densities were found in the dorsal raphe nucleus as opposed to the median raphe nucleus and since the two regions have projections to different brain regions, there could be functional implications of this distribution disparity.

Neurochemical Mechanisms

Many investigations have shown that 5-HT_{2c} receptors modulate DA outflow, thus providing a means whereby the serotonergic system can alter DA neurotransmission. Microdialysis studies have shown that 5-HT_{2c} receptor blockade increased, whilst 5-HT_{2c} agonist decreased DA levels in striatum, nucleus accumbens and frontal cortex (Millan et al., 1998; De Deurwaerdere, 1999; Di Giovanni, 1999; Gobert et al., 2000). When haloperidol was administered at doses that blocked the D₂-like autoreceptor, thereby significantly elevating striatal DA, DA outflow was potentiated by the 5-HT_{2b/2c} antagonist SB 206553 (Lucas et al., 2000). Potentiated cocaine-produced DA increases in the striatum and nucleus accumbens were also found after 5-HT_{2c} antagonist pretreatment (Navailles et al., 2004). Additional support for an inhibitory role on DA outflow for the 5-HT_{2c} receptors was demonstrated in that 5-HT_{2c} receptor knockout mice had higher DA levels in the nucleus accumbens than wildtypes following cocaine administration (Rocha et al., 2002). These findings suggest that 5-HT_{2c} receptor activation has an inhibitory influence on DA mechanisms.

This inhibitory influence of the 5-HT_{2c} receptor on dopaminergic neurotransmission was demonstrated further in electrophysiological studies. The basal firing rate of DA neurons in the ventral tegmental area and substantia nigra increased following 5-HT_{2c} antagonist administration (Di Giovanni, 1999; Gobert et al., 2000). Conversely, the 5-HT_{2c} agonist, Ro 60-0175 caused a dose-dependent reduction in the firing of the ventral tegmental area dopaminergic neurons and a decrease in DA levels in the nucleus accumbens (Di Matteo, 2000; Gobert et al., 2000).

The inhibitory role of the 5-HT_{2c} receptor on DA outflow might relate to GABA, as 5-HT_{2c} receptors have been localised on GABA neurons (Stanford and Lacey, 1996; Eberle-Wang et al., 1997; Liu et al., 2000). An investigation used microdialysis to simultaneously monitor GABA and DA levels in the ventral tegmental area and nucleus accumbens respectively, following MDMA administration (Bankson and Yamamoto, 2004). GABA levels were significantly increased after MDMA, as were DA levels. When SB 206553 was perfused into the ventral tegmental area, GABA efflux was markedly reduced and accumbal DA levels potentiated. It was postulated that elevated 5-HT levels following MDMA had activated 5-HT_{2c} receptors on GABA neurons in the ventral tegmental area, with a resulting GABA efflux. This efflux inhibited accumbal DA outflow and SB 206553 disinhibited DA.

A splice variant of the 5-HT_{2c} receptor has been isolated and localised in brain tissue of the rat, mouse and human (Canton et al., 1996). Recently it has been reported that 5-HT_{2c} receptor mRNA undergoes post-transcriptional editing to yield multiple 5-HT_{2c} receptor isoforms with different distributions in the brain. The 5-HT_{2c} receptor undergoes editing of the second intracellular loop (IL2) at 5 sites. The non-edited receptor contains isoleucine, asparagine and isoleucine (INI) at positions 156, 158, 160, whilst the two principal edited isoforms have valine, serine, valine (VSV) or valine, glycine and valine (VGV) at these positions. It is known that the IL2 plays a role in G-protein coupling and it is therefore not surprising that following editing, reduced G-protein coupling efficiency,

agonist affinity and basal activity levels of isoforms have been observed (Herrick-Davis et al., 1999; Niswender et al., 1999; Wang et al., 2000; Berg et al., 2001; Visiers et al., 2001).

Indeed, reports support propositions that mRNA editing can produce 5-HT_{2c} receptor subpopulations with reduced functional capacities. The 5-HT_{2c} receptor stimulates PLC resulting in production of inositol phosphate (IP) and diacylglycerol and these compounds can be measured as an index of receptor activity (Niswender et al., 1999). A relatively large proportion of 5-HT_{2c}-ini receptors exist in a G-protein coupled state compared to the 5-HT_{2c}-vsv and 5-HT_{2c}-vgv receptors (Niswender et al., 1999). This was cited as the first evidence to show that different isoforms have different G-protein coupling efficiencies. In addition, some receptors have the ability to induce effective G-protein coupling in the absence of an agonist, and this is termed constitutive activity. The non-edited 5-HT_{2c}-ini receptor has the capacity to spontaneously change conformation to produce an active state whereby it can interact with G-proteins, thus exhibiting constitutive activity as measured by IP production. In contrast, the 5-HT_{2c}-vgv isoform yielded only a modest amount of IP formation. The edited isoform also had lower affinity for 5-HT. Brain regions that contain the non-edited receptor might be more sensitive to 5-HT and it was postulated that editing might occur to modulate receptor responses to endogenous 5-HT at serotonergic synapses.

Agonists do not have the same effect on the edited 5-HT_{2c} receptor isoforms, in that they do not exhibit agonist-directed trafficking in the same manner as the non-edited receptor does (Berg et al., 2001). Agonist directed trafficking theory states that agonists promote the formation of ligand-specific receptor conformations which have different capacities to couple to/activate signalling molecules (G-proteins). The relative efficacy of agonists at the 5-HT_{2c}-ini receptor isoform differed depending on whether arachidonic acid (AA) or IP were measured (Berg et al., 1998). Relative to 5-HT, DOI/bufotenin/LSD preferentially activated the AA pathway, whereas quipazine/TFMPP favoured IP accumulation, demonstrating agonist directed trafficking. The edited isoforms did not show

these different second messenger responses to these agonists, suggesting that only the non-edited receptor appears to have the ability to ‘direct traffic’. It is possible that DA-modulatory abilities are related to the activation of specific pathways. 5-HT_{2c} receptor editing might therefore reduce the ability of agonists to activate the pathways that enable the receptor to affect DA outflow.

Paradoxically, a facilitatory role for the 5-HT_{2c} receptor on DA outflow has also been documented. When a non-selective 5-HT₂ agonist, DOI, was perfused into either the nucleus accumbens (Yan, 2000) or the striatum (Lucas and Spampinato, 2000), dose-dependent increases in extracellular DA levels occurred. SB 206553 and TTX attenuated increased DA levels in these areas (Lucas and Spampinato, 2000; Yan, 2000). A role for the 5-HT_{2b} receptor was discounted, as no changes to DA outflow occurred when a 5-HT_{2b} agonist or antagonist was administered (Di Matteo, 2000; Gobert et al., 2000). It was concluded that DOI had increased DA via 5-HT_{2c} receptors, as a receptor-mediated process is also TTX-sensitive. As a result, it was postulated that different subpopulations of 5-HT_{2c} receptors might exist with differential functions.

Finally, some evidence suggests the existence of presynaptic 5-HT_{2c} receptors and consequent ability to limit 5-HT release to some degree. 5-HT_{2c} receptor manipulations do not affect basal 5-HT levels, as numerous studies report no effect on dialysate 5-HT levels in any brain region studied following 5-HT_{2c} antagonist or agonist administration (Millan et al., 1998; Gobert et al., 2000; Cremers et al., 2004). However, a recent study examined the effects of 5-HT_{2c} antagonists on elevated 5-HT levels produced after the administration of SSRIs (Cremers et al., 2004). Significantly elevated 5-HT levels were recorded in rats following SSRI administration, then SB 242084 and RS102221 significantly potentiated 5-HT levels, whilst 5-HT_{2a} antagonists had no effect.

Behavioural Roles

The 5-HT_{2c} receptors limit basal locomotor activity, as activation produced dose-dependent hypolocomotion following the 5-HT₂ agonist, m-CPP (Sills et al., 1985; Aulakh et al., 1987; Kennett and Curzon, 1988; Aulakh et al., 1989; Bagdy et al., 1989; Klodzinska et al., 1989; Lucki et al., 1989; Freo et al., 1990; Kennett et al., 1994a; Bonhaus, 1997; Kennett et al., 1997a; Gleason and Shannon, 1998; Gleason et al., 2001) or other more selective 5-HT_{2c} agonist administration (Martin et al., 1998). Hypolocomotion following 5-HT_{2c} receptor activation was attenuated by 5-HT_{2c} antagonists (Kludzinska et al., 1989; Kennett et al., 1994a; Martin et al., 1998; McCreary et al., 1999; Gleason et al., 2001) but not 5-HT_{2a} or 5-HT_{2b} antagonists (Gleason et al., 2001). In addition, there were no changes to basal locomotion in 5-HT_{2c} receptor knockout mice after m-CPP administration (Heisler and Tecott, 2000; Dalton et al., 2004). It is unknown whether the mechanisms for m-CPP-induced 5-HT_{2c} receptor activation and subsequent hypolocomotion were attributable to downstream effects on dopaminergic or other neurotransmitter systems.

Activation of the 5-HT_{2c} receptors with m-CPP also increased the time spent in peripheral areas in the open field test (Meert et al., 1997), decreased time spent in open arms of the EPM and increased emergence latency (Bilkei-Gorzo et al., 1998), which are all properties that are consistent with an anxiogenic compound. These m-CPP-produced behavioural effects were attenuated by selective 5-HT_{2c} antagonists (Kennett et al., 1994a; Kennett et al., 1995; Mora et al., 1997; Bagdy et al., 2001; Campbell and Merchant, 2003) and some of the 5-HT_{2c} antagonists produced opposite effects to those produced by m-CPP when administered alone (Kennett et al., 1994a; Kennett et al., 1994c; Griebel et al., 1997; Kennett et al., 1997a; Martin et al., 2002).

Bilateral infusions of m-CPP into the basolateral amygdala (BLA) altered open field behaviours, ultrasonic vocalisation and latency to approach a novel object. Rats pretreated with SB242084 or SB 200646A showed significant attenuation of these m-CPP-produced behaviours (Kennett et al., 1994a; Campbell and Merchant, 2003). 5-HT_{2c}

receptor agonists were also applied to dorsal or ventral hippocampi and ensuing behaviour in the EPM assessed (Alves et al., 2004). Ventral hippocampal administration reduced open arm entries, whereas these agonists were without effect in the dorsal hippocampus. These studies indicate that 5-HT_{2c} receptors in the BLA and ventral hippocampus might play an important role in 5-HT_{2c} receptor-mediated behavioural effects.

Few studies have examined the role of the 5-HT_{2c} receptors in MDMA-produced behaviours. As MDMA and m-CPP produce widespread 5-HT₂ receptor activation, 5-HT_{2c} receptor activation probably contributes to the acute behavioural effects produced by both compounds. Recently, a role for the 5-HT_{2c} receptor in MDMA-induced increased emergence latency was discounted due to the inability of the 5-HT_{2c/2b} antagonist, SB 206553 (2mg/kg), to antagonise these effects (Morley et al., 2005). However, the long antagonist pretreatment time and use of only one dose of SB 206553 might not have allowed for the optimal effects of this antagonist to be behaviourally apparent. The non-selectivity of SB 206553 for the 5-HT_{2c} receptor might also confound these results, as the 5-HT_{2b} (Kennett et al., 1996; Duxon et al., 1997; Kennett et al., 1998; Nic Dhonnchadha et al., 2003) and 5-HT_{2c} receptors (Kennett et al., 1994a; Kennett et al., 1995; Mora et al., 1997; Bagdy et al., 2001; Campbell and Merchant, 2003) have opposite roles in related paradigms.

Role in Stimulant-Produced Effects

5-HT_{2c} receptor antagonists potentiated cocaine (McCreary et al., 1999; Fletcher PJ, 2002a; Filip et al., 2004), amphetamine, methylphenidate, nicotine, morphine, phencyclidine and MDMA-produced hyperactivity (Gold, 1988b; Hutson et al., 2000; Herin, 2001; Bankson, 2002; Fletcher PJ, 2002b; Fletcher et al., 2006), whilst 5-HT_{2c} agonists attenuated morphine, nicotine and cocaine-produced hyperactivity (Willins and Meltzer, 1998; Grottick et al., 2000; Grottick et al., 2001; Di Matteo et al., 2004). These behavioural effects have been attributed to the ability of the 5-HT_{2c} receptor to modulate

mesolimbic DA (Millan et al., 1998; Gobert et al., 2000; Lucas et al., 2000; Lucas and Spampinato, 2000; Yan, 2000). These behavioural studies have been paired with evidence for 5-HT_{2c} receptor-mediated altered dopaminergic neurotransmission. 5-HT_{2c} receptor knockout mice display enhanced hyperactivity paired with significantly higher accumbal DA dialysate levels when compared to wildtypes following cocaine administration (Rocha et al., 2002). Secondly, pretreatment with SB242084 potentiated the increase in extracellular levels of DA in the nucleus accumbens and striatum elicited by 15mg/kg of cocaine (Navailles et al., 2004).

The ability of 0.5mg/kg SB242084 to potentiate hyperactivity produced by drugs that affect mainly DA or 5-HT systems were compared (Fletcher et al., 2006). Fenfluramine and citalopram both selectively increased synaptic 5-HT levels at doses that did not significantly affect locomotor activity. Interestingly, the SB242084 and fenfluramine (5.0mg/kg) combination potentiated locomotor activity, but the lower dose of fenfluramine was ineffective and there were no interactions between SB242084 and citalopram. It was postulated that activation of facilitatory 5-HT_{2a} and 5-HT_{1b} receptor subtypes in addition to 5-HT_{2c} receptor blockade had produced fenfluramine/SB242084-induced hyperactivity. This suggests that the inability of SSRIs to affect baseline locomotor activity might be related to simultaneous activation of facilitatory and inhibitory receptors. It was suggested that failure of the citalopram/SB242084 combination to potentiate activity might have been due to an inability of citalopram to elevate synaptic 5-HT levels high enough to activate facilitatory receptors. Alternatively, these observations might relate to the fact that fenfluramine is a substituted amphetamine with some DA-releasing abilities. Fenfluramine, PCA, amphetamine and MDMA released DA via the DAT (Crespi et al., 1997), thus was likely to affect these mechanisms to a greater extent than citalopram. The fenfluramine dose selected did not significantly affect extracellular DA levels, but it is possible that significant 5-HT_{2c}-receptor-induced potentiation occurs only when there is some activation of dopaminergic neurotransmission. Indeed, supporting

this idea, it was reported that the hyperactivity induced by a 5-HT_{1b} agonist, RU24969, was not potentiated by SB242084 (Fletcher et al., 2006).

SB242084 potentiated both methylphenidate and amphetamine-produced hyperactivity at both doses tested, and it was confirmed that these doses had little effect on 5-HT levels (Fletcher et al., 2006). The antagonist-produced potentiation indicated that 5-HT_{2c} receptor blockade enhances the stimulant effects of drugs that selectively increase dopaminergic neurotransmission. In opposition to these observations, SB206553, a mixed 5-HT_{2b/2c} receptor antagonist, did not alter the DA-elevating abilities of 2.0mg/kg amphetamine. The inability of the antagonist to affect amphetamine-induced DA outflow in this instance might be dose-dependent. Indeed, Fletcher et al. (2006) reported that the behavioural response to 1mg/kg amphetamine was unaffected by SB242084 pretreatment, whereas effects of the 0.25 and 0.5mg/kg doses were significantly potentiated. It was suggested that the modulatory effects of 5-HT_{2c} receptor blockade on amphetamine-produced hyperactivity might be 'overwhelmed' by relatively high synaptic DA levels.

In addition to the differential behavioural effects produced by the combination of SB242084 and the DA/5-HT releasing drugs, differential doses and pharmacological properties of 5-HT_{2c} antagonists have produced inconsistencies in behavioural and neurochemical results. For example, a high dose of SB 206558 potentiated cocaine-produced hyperlocomotion, whilst lower doses produced attenuation (McCreary et al., 1999). These findings raise the possibility of the existence of two distinct 5-HT_{2c} receptor populations: pre- and postsynaptic receptors. The presynaptic receptors might be located in the hippocampus since bilateral m-CPP infusions increased locomotor activity that was blocked by co-infusion of SB 242084 (Takahashi et al., 2001). Additionally, 5-HT_{2c} receptor antagonists have different DA-modulatory abilities. SB 206553 significantly increased DA levels in the striatum and nucleus accumbens, whereas this did not occur following SB242084 administration (Gobert et al., 2000; De Deurwaerdere et al., 2004), SB 206553 was an agonist for differential second messenger responses (De Deurwaerdere

et al., 2004), whereas even though SB 242084 was comparable, it displayed low efficacy agonism towards the phospholipase C (PLC) response. It was proposed that disparate DA-modulatory abilities might reflect activation of differential second messenger pathways.

5-HT_{2c} receptor-mediated behavioural effects might also produce inconsistent results in some instances, as it has been suggested that two distinct 5-HT_{2c} receptor subpopulations exist. Prefrontal cortical infusions of 5-HT_{2c} agonists significantly attenuated cocaine-produced hyperactivity (Filip and Cunningham, 2003) but a significant potentiation of cocaine-produced hyperactivity was produced when the agonists were applied to the nucleus accumbens shell (Filip, 2002). 5-HT_{2c} antagonists enhanced cocaine-produced locomotor activity when applied to the prefrontal cortex (Filip and Cunningham, 2003), but hyperactivity was attenuated when they were infused into the nucleus accumbens shell (McMahon et al., 2001b; Filip, 2002). These results suggest that a population of 5-HT_{2c} receptors in the prefrontal cortex are inhibitory whereas a population in the nucleus accumbens shell is facilitatory. The effects of local agonist/antagonist application to the prefrontal cortex are comparable to those produced following systemic administration, suggesting a predominant effect in the prefrontal cortex (McCreary et al., 1999; Grottick et al., 2000; Fletcher PJ, 2002a)

Effects of MDMA Exposure

5-HT receptor changes after MDMA exposure might be attributable to altered serotonergic neurotransmission. Indeed, altered receptor densities in response to changing levels of 5-HT have been reported. For example, 5-HT_{1b} receptors significantly increased (Manrique et al., 1994; Compan, 1998), whilst 5-HT_{1a} receptors decreased (Gobbi et al., 1994) in number following 5,7-DHT or pCPA treatment. 5-HT_{2a/2c} receptor binding was, however, consistently reduced following either 5-HT depletion (Skrebuhhova et al., 1999), agonist (Koshikawa et al., 1985; Fone, 1998) or antagonist treatments (Scheffel et al., 1992), suggesting atypical regulation properties (Van Oekelen et al., 2003a). MDMA

exposure produced dose-dependent decreases in 5-HT_{2a/2c} receptor densities in the cortex septum and caudate putamen (McGregor et al., 2003), likely a consequence of altered serotonergic transmission. Further ligand binding studies have revealed decreases in 5-HT_{2a} (Reneman et al., 2002) and 5-HT₂ (Scheffel et al., 1992) receptor densities in the frontal cortex 6 hours post MDMA exposure. Since 5-HT₂ receptors in the choroid plexus remained unchanged following MDMA (Scheffel et al., 1992), the effects might be regionally selective.

In addition to downregulation, the 5-HT_{2c} receptor also undergoes functional change in response to changing levels of synaptic 5-HT. PCPA resulted in 80% reduction in 5-HT/5-HIAA and initiated 5-HT_{2c} receptor mRNA editing (Gurevich, 2002). Isoforms with higher sensitivity to 5-HT were expressed. Prolonged stimulation of 5-HT_{2c} receptors achieved by frequent injections of DOI produced isoforms that were less responsive to 5-HT. Similar editing of the 5-HT_{2c} receptor might also occur as a neuroadaptive response to altered synaptic 5-HT levels after MDMA exposure.

Functional receptor changes might affect associated behaviour, however, the response to a 5-HT_{2c} receptor agonist was not altered by prior MDMA exposure (Bull, 2003). Bull et al. (2003) pretreated young Listar Hooded rats (15mg/kg twice daily for 3 days) and examined baseline and m-CPP-produced behavioural responses in locomotor, social interaction, open field and EPM tests. Three weeks later, MDMA pretreated rats exhibited decreased social interaction in comparison to controls. Two days later, a 2.5mg/kg dose of m-CPP was administered to both pretreatment groups to examine locomotor activity and open field behaviour. This m-CPP dose did not significantly affect baseline locomotor activity or open field behaviour in either pretreatment group. Four days after these tests, a 1.0mg/kg dose of m-CPP produced equipotent behavioural effects in both pretreatment groups, by reducing time spent in the open maze arms. Decreased 5-HT levels were observed in the hippocampus and frontal cortex of MDMA pretreated rats, whereas the cortex and striatum showed no changes. It was concluded that MDMA

pretreatment had not affected 5-HT_{2c} receptor function, despite the fact that some serotonergic depletion and decreased social interaction was evident. Since social interaction was the only measure affected by MDMA exposure, the type of anxiety detected by the EPM likely involves distinct brain mechanisms from that produced by the social interaction test (File, 1992).

It is possible that the protocols used did not reveal underlying 5-HT_{2c} receptor changes. The 2.5mg/kg dose did not significantly produce hypolocomotion in the control rats, thus was a 'subthreshold' dose for the intended effect. Since MDMA pretreated rats were suspected to be less sensitive to m-CPP, it was not surprising that no differences were observed when administering this dose that did not induce significant hypolocomotion in controls. This study would have benefited from examining dose effect curves, as left or rightward shifts would have revealed changes in receptor sensitivities. Although no changes were observed in the EPM following the 1.0mg/kg m-CPP dose, it has been demonstrated that different paradigms involve different mechanisms. Thus, if a dose that produced significant hypolocomotion were used in the locomotor tests, it might have been possible to see pretreatment differences even if there were none evident in the EPM.

The regimen used in this study might not have produced 5-HT_{2c} receptor changes. A laboratory group have utilised various MDMA dosing regimens (7.5 or 15mg/kg twice daily for 3 days, and 5mg/kg hourly for 4 hours on 2 days), rat strains (Lister Hooded or Wistar rats) and withdrawal times (3 or 8 weeks), and consistently shown modest 5-HT depletions with no effect

on cortical SERT binding (Fone et al., 2002; Bull, 2003; Bull, 2004). Since most other studies have reported SERT binding reductions, these findings seem unusual. There was, however, a common difference these studies and others; young, small rats were used. As previously mentioned, younger rats have been purported to be less susceptible to MDMA-induced serotonergic alteration (Colado et al., 1997). Had these experiments been conducted using older rats; greater effects on serotonergic markers might have been

evident. Since MDMA-induced alterations to serotonergic neurotransmission probably initiate changes to postsynaptic receptors, functional changes to 5-HT_{2c} receptors might only be evident in older rats.

5-HT_{2c} Receptor Experiments in the Present Study

It has been consistently reported that 5-HT_{2c} receptor antagonists potentiated MDMA-produced hyperactivity (Gold, 1988b; Herin, 2001; Bankson, 2002; Fletcher PJ, 2002b; Fletcher et al., 2006). The present study aimed to replicate these findings using a selective 5-HT_{2c} receptor antagonist, RS102221. This compound was chosen because it was 100-fold more selective for the 5-HT_{2c} receptor compared to the 5-HT_{2a} and 5-HT_{2b} receptor subtypes (Bonhaus, 1997). The ability of RS102221 to potentiate MDMA-produced locomotor activity was then assessed in MDMA and vehicle pretreated rats.

There have been few investigations into the effects of MDMA on the binding or functional properties of the 5-HT_{2c} receptors. It was reported that MDMA pretreatment decreased 5-HT₂ receptor binding, but the 5-HT_{2a} and 5-HT_{2c} subtypes were not differentiated due to the use of non-selective ligands (Scheffel et al., 1992; McGregor et al., 2003). It is possible that MDMA pretreatment could have produced 5-HT_{2a} receptor downregulation, whilst the 5-HT_{2c} receptor remained unchanged or vice versa. Despite these considerations, there is evidence to suggest that mRNA editing of the 5-HT_{2c} receptor occurred in response to changing synaptic 5-HT levels (Gurevich, 2002), therefore, altered receptor function might occur following MDMA exposure. Generally, tolerance to serotonergic compounds has been documented after MDMA exposure, so the massive 5-HT efflux produced by the MDMA pretreatment regimen might lead to 5-HT_{2c} receptor desensitisation.

5-HT_{2c} receptor desensitisation might be expected to produce a potentiated locomotor response to MDMA. This suggests that MDMA pretreatment might result in little or no change to 5-HT_{2c} receptors, since tolerance to MDMA-induced hyperactivity

was evident in pretreated animals. Although 5-HT_{2c} receptor desensitisation/downregulation might not explain tolerance to MDMA, it might contribute to other aspects of behavioural suppression. The existence of distinct 5-HT_{2c} receptor populations with differential behavioural roles has been documented. It is possible that some populations and associated behavioural functions are affected by MDMA, whilst others are not. In order to characterise the status of several 5-HT_{2c} receptor populations post MDMA exposure, m-CPP-produced behaviours were also examined.

Activation of 5-HT_{2c} receptors produced dose-dependent hypolocomotion and increases in emergence latency following m-CPP administration (Sills et al., 1985; Aulakh et al., 1987; Kennett and Curzon, 1988; Aulakh et al., 1989; Bagdy et al., 1989; Klodzinska et al., 1989; Lucki et al., 1989; Freo et al., 1990; Kennett et al., 1994a; Bonhaus, 1997; Kennett et al., 1997a; Gleason and Shannon, 1998; Gleason et al., 2001). The social interaction, open field and the EPM were not as easily quantifiable and less likely to exhibit dose dependent effects compared to hypolocomotion and emergence latency. Although emergence latency has been used as an anxiety measure, in the present study, it was used only to pharmacologically determine shifts in receptor responsiveness.

This study extended work by Bull and colleagues (2003), where no functional change to 5-HT_{2c} receptor-mediated behaviour following MDMA pretreatment was reported, although corresponding neurochemical data revealed that there was minimal effect of MDMA on serotonergic parameters. The present study utilised different subjects, MDMA pretreatment regimens and testing protocols. The primary objective was to investigate behavioural tolerance to MDMA challenge, therefore, all behavioural tests were performed upon MDMA pretreated rats that exhibited tolerance to MDMA-produced hyperactivity. Several doses of m-CPP that produced significant behavioural effect were examined in the hypolocomotion and emergence latency paradigms and compared between pretreatment groups.

In order to determine what role the 5-HT_{2c} receptors might have in behavioural suppression to MDMA, the first novel assay utilised MDMA and a selective 5-HT_{2c} receptor ligand. It was hypothesised that the 5-HT_{2c} receptor population being assessed by this paradigm had a minor role in locomotor tolerance, so the purpose was mainly to dissociate 5-HT_{2c} from 5-HT_{2a} receptor effects. It has been purported that different 5-HT_{2c} receptor populations have diverse behavioural roles, thus the effect of MDMA pretreatment on other 5-HT_{2c} receptor-produced behaviours were also examined, even though these might not directly relate to locomotor tolerance.

The 5-HT_{2a} Receptor Subtype

Distribution

High 5-HT_{2a} receptor densities were observed predominantly in forebrain areas such as the neocortex, also in caudate nucleus, nucleus accumbens, olfactory tubercle, piriform cortex, red nucleus, claustrum, mamillary bodies, pontine nuclei and cranial motor nuclei (Pazos et al., 1985b; Lopez-Gimenez et al., 1997; Barnes and Sharp, 1999). Immunocytochemical localization of 5-HT_{2a} receptor distributions in the parabrachial and paranigral regions of the ventral tegmental area revealed that approximately 40% of the 5-HT_{2a}-labeled dendrites contained tyrosine hydroxylase, suggesting that they were localised on dopaminergic neurons and might exist as heteroreceptors in this region (Doherty and Pickel, 2000). 5-HT_{2a} and 5-HT_{2c} receptor mRNA expression was distinct but overlapped in several brain regions. There were high levels of 5-HT_{2a} and 5-HT_{2c} receptor expression in the anterior olfactory nucleus, piriform cortex, endopiriform nucleus, claustrum, pyramidal cell layer of the ventral part of CA3, taenia tecta, substantia nigra and several brainstem nuclei. In the caudate-putamen, expression of both receptors was present but with different distributions (Pompeiano et al., 1994).

Neurochemical Mechanisms

As shown by receptor distributions, some 5-HT_{2a} receptors have been localised to DA neurons and can therefore modulate dopaminergic neurotransmission. In vivo microdialysis demonstrated that 5-HT_{2a} receptors facilitate DA release, but there were indications that this receptor only has a modulatory role following increased dopaminergic neurotransmission. This was demonstrated when microdialysis was used to determine what effect amphetamine, the 5-HT_{2a} agonist, DOI, and the 5-HT_{2a} antagonist, M100907 had on DA release. When MDL100907 was perfused via reverse microdialysis into the prefrontal cortex (PFC) or nucleus accumbens (NAc), no change in basal DA dialysate levels occurred (Kuroki et al., 2003). Perfusion of the 5-HT₂ agonist, DOI, into the striatum or nucleus accumbens did not alter dialysate DA levels at the doses used (Ichikawa and Meltzer, 1995). These findings indicate that the 5-HT_{2a} receptor has minimal role in basal DA-release modulation, which reaffirms findings from other studies (De Deurwaerdere, 1999; Di Giovanni, 1999).

Systemically administered amphetamine increased dopaminergic neurotransmission, as evidenced by a marked increase in extracellular DA levels in the mPFC, striatum and NAc (Ichikawa and Meltzer, 1995; Kuroki et al., 2003). When DOI was administered in conjunction with amphetamine, DA increases were significantly potentiated in both areas compared to amphetamine alone. MDL100907 pretreatment had no effect on the elevated amphetamine-produced DA levels, however, MDL100907 reversed the DOI-produced potentiation of DA (Kuroki et al., 2003). It was concluded that DA potentiation following DOI administration was a result of 5-HT_{2a} receptor activation.

The 5-HT_{2a} receptors also have an active role in DA modulation following MDMA administration. An electrophysiology study revealed that MDMA-induced 5-HT_{2a} receptor activation increased neuronal firing in the striatum (Ball and Rebec, 2005). SR46349B, a 5-HT_{2a} receptor antagonist, blocked nearly all MDMA (5.0mg/kg)-induced striatal excitations. It was not ascertained whether the neurons affected were serotonergic or dopaminergic, but microdialysis studies suggest that DA neurons were activated. MDMA

significantly elevated striatal extracellular DA levels, which were dose-dependently attenuated by MDL100907 (Schmidt et al., 1994). Intrastriatal infusion of tetrodotoxin (TTX) reduced both basal and MDMA-stimulated DA efflux and eliminated the effect of MDL100907, suggesting that DA elevations were impulse mediated via 5-HT_{2a} receptor activation.

MDL100907 did not attenuate amphetamine-induced DA elevations in the PFC or NAc, whereas this antagonist blocked amphetamine-(Auclair et al., 2004) and MDMA-(Schmidt et al., 1994) induced DA elevations in the striatum. Different brain regions might be differentially susceptible to 5-HT_{2a} receptor manipulations, as some areas might have higher receptor densities than others. It is also likely that 5-HT_{2a} receptors with differential functional capacities exist.

MDMA-produced striatal DA increases involved a PKC-mediated process (Nair and Gudelsky, 2004). Since 5-HT_{2a} receptors use PKC for intracellular signalling (Kramer et al., 1997), it is possible that PKC-mediated DA increases occurred following 5-HT_{2a} receptor activation. PKC inhibitors were administered via dialysis probes to MDMA pretreated rats, which attenuated MDMA-produced striatal dialysate DA increases, whereas PKC activators increased DA release. Conversely, it was found that intracortical infusion of PKC inhibitors increased MDMA-produced DA levels. These findings suggest that PKC-mediated signalling occurred differentially in mesocorticolimbic and nigrostriatal regions, which might provide indirect evidence for the existence of 5-HT_{2a} receptor populations with different functional roles.

Role of the 5-HT_{2a} Receptor in Baseline and Stimulant-Induced Behaviour

Due to the scarcity of selective 5-HT_{2a} agonists, the behavioural effects of 5-HT_{2a} receptor activation have been difficult to ascertain. Selective 5-HT_{2a} receptor antagonists used in conjunction with 5-HT₂ agonists have allowed certain behaviours to be attributed, with some degree of confidence, to 5-HT_{2a} receptor activation. Wetdog shakes (WDS) in

the rat and head twitches in the mouse are behavioural markers commonly associated with 5-HT_{2a} receptor activation. These behaviours have been observed following administration of the 5-HT₂ agonist, DOI (Kennett et al., 1994a; Darmani et al., 1996; Kugaya et al., 1996; Willins and Meltzer, 1997; Bartoszyk et al., 2003; Bull, 2004) and were attributed to 5-HT_{2a} receptor activation because they were blocked by 5-HT_{2a} antagonists (Kleven et al., 1997; Willins and Meltzer, 1997; Bartoszyk et al., 2003), whilst 5-HT_{2c} antagonists had no effect (Kennett et al., 1994a; Schreiber et al., 1995; Willins and Meltzer, 1997). The 5-HT₂ antagonists, ketanserin and ritanserin, produced dose-related decreases in DOI-induced head twitches (Kleven et al., 1997). Also, in rats trained to discriminate DOI from saline in a drug discrimination paradigm, ketanserin and ritanserin blocked the discriminative stimulus effects of the training dose by more than 50%. In contrast, 5-HT₁ antagonists failed to affect the discriminative stimulus effects of DOI. It was concluded that DOI-induced head twitches and discriminative stimulus effects were mediated via the 5-HT_{2a} receptors.

DOI administration also produced hyperthermia (Pranzatelli and Pluchino, 1991; Mazzola-Pomietto et al., 1995) and further studies have attributed hyperthermia to the 5-HT_{2a} receptor (Mazzola-Pomietto et al., 1995; Nisijima et al., 2001; Fantegrossi et al., 2003; Herin et al., 2005). A monoamine oxidase inhibitor and 5-hydroxy-L-tryptophan, a precursor of 5-HT, were administered to rats to induce the 5-HT syndrome (Nisijima et al., 2001). Body temperatures increased to more than 40 degrees C, and all of the animals died 90 minutes later. Pretreatment with ritanserin and pipamperone, both 5-HT_{2a} receptor antagonists, prevented these eventualities. The selective 5-HT_{2a} receptor antagonist, MDL100907, attenuated hyperthermia produced by 8 and 12mg/kg MDMA in Sprague Dawley rats (Herin et al., 2005). Additionally, pretreatment with ketanserin, MDL100907 or fluoxetine significantly attenuated MDMA-induced hyperthermia in mice (Fantegrossi et al., 2003). There was however, a conflicting finding where MDL100907 failed to affect 12.5mg/kg MDMA-induced hyperthermia in Dark Agouti rats (Mechan et al., 2002a).

These differences might be related to the use of different rat strains, as the ability to attenuate MDMA-produced hyperthermia by several MDL100907 doses has been demonstrated (Herin et al., 2005).

Evidence suggests that 5-HT_{2a} receptor activation tends to produce anxiolysis, although there were inconsistencies between reports. DOI produced anxiolysis in mice in two different measures of anxiety: the four plates test (FPT) and the EPM (Nic Dhonnchadha et al., 2003). The 5-HT_{2c} antagonist, RS102221 and 5-HT_{2c/2b} antagonist, SB206553 had no effect on the DOI-produced anxiolysis in either paradigm. The 5-HT_{2a} receptor was implicated when the specific antagonist, SR46349, significantly inhibited the number of open arm entries induced by DOI in the EPM and inhibited the anti-punishment action of DOI in the FPT. In concurrence with these findings, decreased ultrasonic vocalisations in rats produced by DOI administration was attributed to 5-HT_{2a} receptor activation, as these effects were attenuated by MDL100907 (Schreiber et al., 1998).

A report revealed anxiogenesis in the EPM (Setem et al., 1999) and increased ultrasonic vocalisations following 5-HT_{2a} antagonist administration (Olivier et al., 1998), however, 5-HT_{2a} receptor antagonists have also been found to have no effect on anxiety (Kehne et al., 1996b). One study showed that DOI produced anxiogenesis in the EPM in rats (Bull, 2004), making it difficult to conclude what the role of the 5-HT_{2a} receptor is in anxiety. As DOI is a mixed 5-HT₂ receptor agonist with affinity for the 5-HT_{2c} and 5-HT_{2b} receptor subtypes, it is possible that drug doses determine what receptors are preferentially bound and the resulting behavioural outcome. It is possible that the 5-HT_{2a} receptor has no role in anxiety, and that behaviour following DOI administration is determined by the ratio of 5-HT_{2c} receptors that are activated compared to the 5-HT_{2b} receptors. Both subtypes have opposite roles in anxiety; for example, activation of 5-HT_{2c} induced anxiogenesis (Kennett et al., 1994a; Kennett et al., 1995; Mora et al., 1997; Bagdy et al., 2001; Campbell and Merchant, 2003), whilst 5-HT_{2b} receptor activation produced

anxiolysis (Kennett et al., 1996; Duxon et al., 1997; Kennett et al., 1998; Nic Dhonnchadha et al., 2003).

It does not appear that the 5-HT_{2a} receptor has a major role in locomotor activity under basal conditions, as antagonists such as MDL100907 and ketanserin do not affect baseline activity levels (Moser et al., 1996; McMahon and Cunningham, 2001b; Herin et al., 2005). There are reports documenting a modest increase in forward locomotion following administration of the agonist, DOI (Darmani et al., 1996; Hillegaart et al., 1996; Kaur and Ahlenius, 1997; Bull, 2004; Ross et al., 2005). These findings suggest, that although m-CPP and DOI are both relatively non-selective 5-HT₂ receptor agonists, DOI preferentially binds the 5-HT_{2a}, whilst m-CPP binds the 5-HT_{2c} receptor, evidenced by its ability to induce hypolocomotion. The 5-HT_{2a} receptor also modulates hyperactivity induced by psychostimulants. MDL100907, ritanserin and ketanserin blocked hyperactivity induced by amphetamine, cocaine and MDMA (Sorensen et al., 1993; Moser et al., 1996; Kehne et al., 1996a; O'Neill et al., 1999; McMahon and Cunningham, 2001b; Fletcher PJ, 2002a; Fletcher PJ, 2002b; Broderick et al., 2004; Herin et al., 2005).

Co-administration of fluoxetine and cocaine potentiated hyperactivity above that produced by cocaine alone (Herges and Taylor, 1998). This potentiation was blocked when ketanserin was administered in addition to the other two drug treatments. These data suggest that the SSRI-induced potentiation was partly attributable to enhanced synaptic 5-HT that in turn activated the 5-HT_{2a} receptors. It has been hypothesised that the ability of 5-HT_{2a} antagonists to attenuate stimulant-produced hyperactivity might be due to influences on DA release (Schmidt et al., 1994; Yan, 2000; Kuroki et al., 2003). Indeed, 5-HT_{2a}-mediated effects on dopaminergic neurotransmission have been linked to increased locomotor activity. Electrophysiology revealed that MDMA increased neuronal firing in the striatum and that 5-HT_{2a} receptor blockade attenuated both striatal excitations and MDMA-produced hyperactivity (Ball and Rebec, 2005). Local perfusion with a 5-HT_{2a}

antagonist into the ventral tegmental area blocked both the D-amphetamine-induced locomotor activity and DA release in the nucleus accumbens (Auclair et al., 2004).

The ability of a selective 5-HT_{2a} antagonist, MDL100907, to attenuate MDMA-produced forward locomotion was demonstrated and compared to the less selective 5-HT₂ antagonist, ritanserin (Kehne et al., 1996a). The antagonists were administered in conjunction with 20.0mg/kg MDMA, which was the dose selected for these studies because it produced the highest levels of hyperactivity, thus making attenuation clearly visible. Both antagonists significantly attenuated MDMA-produced forward locomotion, whilst rearing behaviours were unaffected. These results indicate that the attenuating ability of ritanserin was a 5-HT_{2a} receptor-mediated effect and also, because 5-HT_{2a} receptor manipulations selectively affected forward locomotion, differential mechanisms probably underlie these MDMA-produced behaviours.

A more recent study examined the ability of several MDL100907 doses to attenuate MDMA-produced hyperactivity and hyperthermia (Herin et al., 2005). The results revealed that the 5-HT_{2a} antagonist attenuated hyperthermia and hyperactivity produced by the highest doses of MDMA, whilst lower doses were not affected. These findings concur with previous findings indicating that 5-HT_{2a} receptors facilitate MDMA-produced hyperactivity only at the highest doses (Bankson, 2002). Hyperactivity produced by low dose MDMA was attenuated by 5-HT_{1b} receptor blockade but unaffected by 5-HT_{2a} antagonists. These results were explained in that 5-HT has higher affinity for the 5-HT₁ than the 5-HT₂ receptors, thus the 5-HT₁ receptors have a greater role in hyperactivity produced by low dose MDMA. At higher MDMA doses, higher synaptic 5-HT levels allow for activation of 5-HT₂ receptors. Additionally, it was suggested that the MDMA molecule might bind to the 5-HT_{2a} receptors at the higher doses, because MDMA has affinity for this site (Battaglia et al., 1988).

Evidence suggests that the 5-HT_{2a} receptor might be involved in MDMA-produced hyperactivity and hyperthermia, but the time course for the attenuation of these effects were not consistent with a common mechanism (Herin et al., 2005).

Effects of MDMA Exposure

An elegant study using autoradiography examined the effects of MDMA exposure on 5-HT_{2a} receptors in multiple brain regions, and compared MDMA users to rats (Reneman et al., 2002). The aim was to determine what the immediate effects of MDMA exposure were and then map a time course of receptor binding densities in both humans and rats. All subjects were injected with radiolabelled R91150, a selective 5-HT_{2a} receptor ligand, whereupon humans underwent SPECT scans, whilst rat brains were removed and regions assayed with a gamma counter. In the rat studies, these data were correlated with 5-HT levels in each brain area.

There were three groups of human participants: MDMA-naïve controls, recent MDMA users and ex-MDMA users. In order to be classified as an MDMA user, it was required that a lifetime previous use of 50 tablets had occurred. The recent users had been abstinent for 1 week, whilst the ex-users had been abstinent for at least 2 months. Male Wistar rats were treated with MDMA (10mg/kg X2 per day for 4 consecutive days, 80mg/kg total) and separate groups were analysed at the 6h, 3 and 30 days post exposure time points.

Recent MDMA users exhibited significant decreases in 5-HT_{2a} receptor binding densities in frontal, parietal and occipital cortices. These decreases were not apparent in ex-MDMA users, and there were significantly increased densities in the occipital cortex of this group. The rats showed a similar pattern, as there were significant decreases in 5-HT_{2a} receptor densities in all 3 regions 6 hours post MDMA. At the 3-day time point, only the occipital cortex still showed significant decreases, whilst the other areas had returned to control values. However, at 30 days post MDMA, there was a significant upregulation in

the frontal cortex, whilst other areas were comparable to control levels. The 5-HT levels were significantly decreased at 6hrs in the frontal and occipital cortices, and over time, 5-HT levels decreased progressively until all regions exhibited approximately 80% decreases at the 30-day time point. Bull (2004) suggested that the magnitude and duration of synaptic 5-HT depletion might be the primary determinant of any compensatory change in expression of the 5-HT_{2a} receptor, but all areas exhibited similar 5-HT depletions.

It was hypothesised that the receptors were following synaptic 5-HT gradients. The massive release of 5-HT initiated by MDMA upon acute exposure was thought to lead to the initial downregulation 6 hours following MDMA in rats. Although 5-HT levels have been purported to recover over time, this study has shown that levels continue dropping up to 30 days after MDMA. This is in agreement with Battaglia and co-workers that showed the most extensive depletion in 5-HT uptake sites in some regions 2 weeks after MDMA treatment. A more recent study, however, suggests that depleted 5-HT levels are not necessary for 5-HT_{2a} receptor changes and that the observed ‘upregulation’ in Reneman’s study (2002) might have been misinterpretation of the data (McGregor et al., 2003).

McGregor and colleagues assessed the effects of a high (5mg/kg every hour for 4 hours on 2 consecutive days, 40mg/kg total) or low dose MDMA (5mg/kg every day for 2 consecutive days, 10mg/kg total) regimen on 5-HT_{2a/2c} receptor densities after 10 weeks. [¹²⁵I] DOI was used for the binding assays, and although low dose MDMA reduced DOI binding in most regions, only the decrease in the piriform cortex was significant in comparison to controls. The high dose group showed significantly decreased binding in nearly every region examined. 5-HT levels were depleted in most regions after the high, but not after the low dose of MDMA. These results led McGregor to suggest that Reneman (2002) might have observed ‘upregulation’ due to the excessively high doses of MDMA used for pretreatment. MDMA exposure induced 80% decreases in 5-HT levels, thus McGregor suggested that the ‘increased binding to 5-HT_{2a} receptors’ might reflect

increased availability to bind due to the lack of competition with depleted endogenous 5-HT.

Another study that used the same pretreatment regimen (5mg/kg every hour for 4 hours on 2 consecutive days, 40.0mg/kg total) and rat strain (Wistars) and a similar withdrawal period (8 weeks) to McGregor has revealed evidence of desensitised 5-HT_{2a} receptor-mediated behavioural effects (Bull, 2004). The ability of 1mg/kg DOI to induce WDS was compared between MDMA and saline pretreated groups. One day later, response to the same DOI dose was assessed in the EPM. DOI significantly induced WDS in saline pretreated rats, however, significantly MDMA pretreated rats exhibited fewer WDS. In these studies, DOI induced an increase in locomotor activity, which was equivalent between pretreatment groups. In the EPM, DOI significantly reduced the percentage of open to total arm entries and the percentage of time spent in the open arms of the EPM in saline- but not MDMA-pretreated rats. It would be interesting to have seen whether the low dose MDMA group from McGregor's study would have also exhibited tolerance to DOI, even though there were no significant 5-HT depletions.

5-HT_{2a} Receptor Experiments in the Present Study

In order to assess the role of the 5-HT_{2a} receptor in tolerance to MDMA-produced hyperactivity, ritanserin, a 5-HT₂ antagonist, was administered in combination with MDMA. Ritanserin was a readily available ligand, and although relatively non-selective for the 5-HT_{2a} receptor, has demonstrated comparable ability to a highly selective 5-HT_{2a} antagonist to attenuate MDMA-produced hyperactivity (Kehne et al., 1996a) and DOI-produced head twitches (Kleven et al., 1997). These effects of ritanserin were not attributable to 5-HT_{2c} or 5-HT_{2b} receptor blockade, as 5-HT_{2c} receptor antagonists potentiate hyperactivity, whilst 5-HT_{2b} receptors have little role in this behaviour. In the present study, the ability of ritanserin (0.0, 2.5 and 5.0mg/kg) to attenuate MDMA-produced hyperactivity was assessed in MDMA and saline pretreated rats in order to

determine the effect of prior MDMA exposure on the ability of 5-HT_{2a} receptors to modulate hyperactivity.

The 5-HT₂ receptor agonist, DOI, produces modest increases in locomotor activity and WDS in rats that have been attributed to the 5-HT_{2a} receptor. The present study also aimed to assess the ability of DOI to produce these effects in MDMA pretreated rats as additional receptor assays. A comparable methodology to that used by Bull (2004) and colleagues was used in that the ability of a single 1mg/kg dose of DOI to produce hypermotility and WDS was assessed. A single dose was used because preliminary studies revealed that these behavioural effects were not dose-dependent. This is probably due to the fact that DOI is not selective for the 5-HT_{2a} receptor, and additional receptors are activated at higher doses, negating specific 5-HT_{2a} receptor-mediated effects. The development of more selective 5-HT_{2a} agonists might allow for dose-dependent studies to be conducted in the future, but the use of a single dose in this study will be strengthened by the use of multiple assays.

Chronic DOI treatment produced tolerance to the discriminative stimulus properties of DOI (Smith et al., 1999). In addition, significant decreases in 5-HT_{2a} binding were observed, whereas no changes to 5-HT_{2c} receptors occurred. These findings indicate that the 5-HT_{2a} receptors might be more susceptible to up- or downregulation following continuous activation. The degree to which MDMA pretreatment might mimic DOI in this instance is uncertain, as the drugs were administered using different pretreatment regimens and had different pharmacological properties. It is also possible however, that the 5-HT_{2a} receptors might be more susceptible to up- or downregulation following MDMA-produced alterations in serotonergic neurotransmission. 5-HT_{2a} receptor, with little change to 5-HT_{2c} receptors, might explain tolerance to the activating effects of MDMA.

Summary

The present study investigated the effects of MDMA exposure on the functional statuses of the SERT and 5-HT_{2c} and 5-HT_{2a} receptor subtypes. The existing literature is difficult to interpret due to the use of varying methodologies that have produced conflicting findings. The present study aimed to resolve these confounds by administering the same MDMA pretreatment regimen prior to behavioural tests to allow direct comparisons. A well-characterised MDMA pretreatment regimen was used to these ends, as previous work has demonstrated that this regimen selectively affected 5-HT systems in rats, without inducing non-selective neurotoxicity (Scanzello et al., 1993; Fischer et al., 1995).

The MDMA pretreatment regimen produced tolerance to several behavioural effects of MDMA, including locomotor activity, which were attributable to serotonergic deficits (Shankaran and Gudelsky, 1999). Extensive neurochemical studies have documented the time course for recovery of these deficits, therefore, the present study sought to determine whether functional recovery was evident 12 weeks later. Since it was presumed that functional recovery would reflect serotonergic recovery, the effect on behaviour of repeatedly activating 5-HT systems was investigated. Weekly injections with a relatively high dose of MDMA were administered to observe the effect on behavioural recovery.

Paroxetine binding was conducted as verification that the pretreatment regimen used in the present study produced comparable deficits to those previously reported (Scanzello et al., 1993; Fischer et al., 1995). The present study also included the brain stem region, which has not previously been assessed. This site has the highest SERT densities of any brain region (Lin et al., 2004), thus SERT alteration would be expected to produce widespread changes in serotonergic neurotransmission.

Although SERT binding has frequently been measured in studies examining the effects of MDMA exposure, it has usually been used as an index of ‘neurotoxicity’ or as verification for the presence of serotonergic deficits. The present study aimed to extend these reports and determine the functional implication of MDMA-induced decreases in SERT binding densities. SERT integrity is vital for the full expression of MDMA-produced hyperactivity (Callaway et al., 1990), as 5-HT released via the SERT activates 5-HT receptors that have a major contribution towards the expression of MDMA-produced hyperactivity (Bankson, 2002). It was, therefore, hypothesised that decreased SERT densities might contribute to behavioural tolerance to MDMA following preexposure. The SERT selective ligand, clomipramine, blocks MDMA-produced hyperactivity in control rats by preventing MDMA-induced 5-HT release (Piffl et al., 2005). The ability of clomipramine to attenuate MDMA-produced hyperactivity in MDMA pretreated rats was assessed. If decreased SERT binding was responsible for tolerance to MDMA-produced hyperactivity, it would be expected that MDMA-produced hyperactivity would be unaffected by clomipramine pretreatment.

Several studies have demonstrated a role for the 5-HT_{2a} and 5-HT_{2c} receptors in MDMA-produced hyperactivity (Herin et al., 2005; Fletcher et al., 2006). It has also been shown that these receptors are vulnerable to MDMA-induced downregulation/desensitisation (Reneman et al., 2002; McGregor et al., 2003; Bull, 2004). There have been behavioural studies that suggest that 5-HT_{2c} receptor-mediated m-CPP-produced behaviours remained unchanged (Bull, 2003), whilst 5-HT_{2a} receptor-mediated DOI-produced behaviours were diminished following MDMA exposure (Bull, 2004). The present study aimed to replicate these findings using improved methodology and a pretreatment regimen that produced tolerance to MDMA.

5-HT_{2a} receptor desensitisation, as shown by decreased DOI responsiveness, might explain locomotor tolerance to MDMA, as 5-HT_{2a} receptor activation facilitates MDMA-produced hyperactivity (Schmidt et al., 1994; Kehne et al., 1996a; Herin et al., 2005).

However, evidence suggests that the receptors that modulate MDMA-produced hyperactivity influence DA release (Schmidt et al., 1994; Di Matteo, 2000; Gobert et al., 2000) and that these functions might be distinct from behaviour produced by agonists such as m-CPP and DOI.

Two novel assays were developed that combined MDMA with 5-HT_{2c} or 5-HT_{2a} receptor antagonists in order to assess DA-modulatory function following MDMA exposure. These assays were intended to reveal relative contributions of each receptor subtype to the tolerance to MDMA-produced hyperactivity. 5-HT_{2c} receptor antagonists potentiate, whilst 5-HT_{2a} antagonists attenuate, MDMA-produced hyperactivity. The ability for respective antagonists to modulate MDMA-produced hyperactivity was examined in MDMA pretreated animals. It was hypothesised that MDMA exposure would decrease the ability for the 5-HT_{2a} receptor antagonist to attenuate hyperactivity to a greater extent than the ability for the 5-HT_{2c} receptor antagonist to potentiate hyperactivity, as this might explain tolerance to MDMA-produced hyperactivity.

Methods

Subjects

The subjects were male Sprague-Dawley rats, weighing between 250-350g. The animals were bred at Victoria University in Wellington, New Zealand and were housed singly in a temperature- (21°C) and humidity- (79%) controlled room. There was a controlled 12-hr light/dark cycle with lights on at 0700. Food and water were available ad libitum except during testing periods. All experimental protocols were consistent with OLAW regulations and approved by the Animal Ethics Committee of Victoria University.

Apparatus

Initial experimental work was conducted in measuring apparatus developed in-house at Victoria University. The chamber used for rat locomotor activity measurement was a wooden box lined with Perspex (50 x 50 x 20cm). Activity of an animal was recorded as the number of times it crossed infrared light beams. These beams formed an 8 x 8 lattice 2.2 cm above the bottom and were 5cm apart. Whenever a light beam was broken, a computer recorded it with software designed by university technicians.

Following the failure of this equipment, Med-PC Activity Monitor apparatus was acquired and subsequent experiments were carried out using the new equipment. As a consequence, the older apparatus was utilized for much of the preliminary work in Part 1, whereas Part 2 was conducted mostly using Med PC equipment. The measurements from the old (activity counts) versus the new Med-PC (ambulatory counts) equipment yielded different numbers, but drug profiles and effective doses, were, in most cases, equivalent between apparatus.

The Activity Monitor Version 5 program (Med Associates Inc) measured forward locomotion and emergence latency. The system consisted of a subject containment chamber, infrared red sources and sensors, a system power supply, an environment data

source controller, connecting cables, PC/environment interface card and data analysis software. The subject location was tracked using 16 evenly spaced infrared sources and sensors positioned around the periphery of the 4-sided chamber (40 X 40cm). If the infrared beam failed to reach the sensor, the system registered this event as a broken beam and assumed the presence of the subject. The program specifically recorded forward locomotion by dividing the chamber into zones, or 'boxes '. It was possible to set the 'box size' and for these experiments, the size was set to be the approximate dimensions of a rat. When the rat travelled distance and crossed the box perimeters into an adjacent box, this was registered as ambulatory counts.

Emergence latency was measured using a version of the emergence test developed by McGregor's laboratory group (McGregor et al., 2003, Morley et al., 2005). For these tests, wooden hide boxes (16.5 X 25cm) with lift-up lids and slide doors were placed in the top left corner of the activity chambers. The hide box lid was lifted, the animal placed inside and the lid closed for habituation before the slide door was opened 15 minutes later. Full body emergence from the hide box was determined when distance travelled was equivalent to the length of a rat, as measured by the Med Associates software. There were infrared cameras situated above each locomotor activity chamber to verify emergence and also to observe qualitative behaviours, such as wetdog shakes.

Before and after each animal had been in the test chambers, the interiors and hide boxes were wiped with Virkon 'S' disinfectant (Southern Veterinary Supplies, Palmerston North). All behavioural tests were conducted during the light phase of the light cycle and the test room was illuminated with red light. A white noise generator ran throughout experiments to reduce auditory disturbance and experimenters remained outside the test room during experiments.

Procedures

Part 1

Overview

Assays to assess the role of the SERT, 5-HT_{2c} or 5-HT_{2a} receptors in MDMA-induced hyperactivity, hypolocomotion, emergence latency and wetdog shakes were developed. These experiments examined the dose effects of several ligands for the aforementioned target sites. The objectives were to develop paradigms where significant pharmacologically induced behavioural effects were evident, and to determine which doses and drug pretreatment times produced these effects.

When designing experimental procedures for locomotor activity tests, it was necessary to consider drug type. For example, MDMA is a stimulant that induces hyperactivity, thus to observe the most pronounced MDMA-induced change in locomotion, it was necessary to administer MDMA when baseline activity levels were low. When rats were initially placed in activity boxes, there were relatively high levels of activity for the first 15 minutes, and following this time, activity slowed. Therefore, prior to MDMA administration, the animals were habituated for 30 minutes to the chambers to lower baseline activity levels.

Conversely, serotonergic compounds such as clomipramine and m-CPP tend to produce hypomotility. As previously mentioned, rats are most active immediately after being placed in the activity chamber. In order to observe suppressive effects on locomotion, the drugs were administered to allow peak behavioural effects to coincide with the first 15 minutes of highest baseline activity.

Unless otherwise stated, all experiments were of a between subjects design where no repeated testing occurred. In an attempt to minimise subject numbers, several trial experiments were conducted where rats were repeatedly tested. It was observed that prior

exposure to the test chambers and MDMA altered subsequent behavioural responses. Additionally, in some instances, the numbers of rats per group differs in the results compared to those outlined in the methods, as data from rats that received misplaced injections was discarded.

MDMA-Produced Hyperactivity

Dose Effects of MDMA

Groups of drug-naïve rats (n=8 per group) were placed in the activity chambers and allowed a 30-minute habituation period. Thereafter, rats were administered MDMA (old boxes, 0.0, 5.0, 10.0, 15.0 or 20.0mg/kg, IP; Med-PC boxes, 0.0, 2.5, 5.0, 10.0 or 20.0mg/kg, IP) and locomotor activity recorded for a further 60 minutes.

The Serotonin Transporter

Dose Effects of Clomipramine on Baseline Locomotor Activity

Separate groups of drug-naïve rats (n=9 per group) were administered clomipramine (0.0, 1.25 or 5.0mg/kg, IP) in the homecage. Thirty minutes later, the rats were placed in the Med-PC activity chambers and locomotor activity was recorded for a 30-minute period thereafter.

Dose Effects of Clomipramine on MDMA-Produced Hyperactivity

Groups of drug-naïve rats (n=8 per group) were administered clomipramine (0.0, 0.3, 1.25, 5.0, 10.0 or 20.0mg/kg, IP) and placed in Med-PC activity chambers. Thirty minutes later, MDMA (5.0mg/kg, IP) was administered and locomotor activity recorded for the final 60 minutes.

Using identical methodology, drug-naïve rats (n=10 per group) were administered the effective clomipramine doses (0.0, 1.25 or 5.0mg/kg, IP) with a higher MDMA

(10mg/kg, IP) dose. The highest clomipramine doses of 10.0 and 20.0mg/kg were excluded due to lack of selectivity.

The 5-HT_{2c} Receptor

Dose Effects of m-CPP on Baseline Locomotor Activity

m-CPP (0.0, 0.3, 0.6, 1.25, 2.5 and 5.0mg/kg, IP) was administered to drug-naïve rats (n=8 per group), which were then placed in the old activity chambers and activity recorded for 60 minutes. m-CPP has a rapid onset of action, thus the suppressive effects were expected within the first 15 minutes.

Dose Effects of m-CPP on Emergence Latency

Drug-naïve rats (n=6 per group) were administered m-CPP (0.0, 0.6 or 1.25mg/kg) before being placed in hide boxes situated in Med-PC chambers. These doses did not affect baseline locomotor activity. Rats were placed in the hide box for 15 minutes following m-CPP injection and emergence latency into the test arena was measured for 40 minutes after the sliding door was opened.

Dose Effects of MDMA Following RS102221 Pretreatment

Groups of drug-naïve rats (n=8 per group) were used to test the effect of RS102221 (0.0 or 0.5 mg/kg) on MDMA- (0.0, 5.0, 10.0 or 20.0mg/kg, IP) produced hyperactivity in the old apparatus. Rats were administered RS102221 and placed in the old activity boxes for 30 minutes. Thereafter, rats received an injection of MDMA (10.0mg/kg) and locomotor activity was measured for a further 60 minutes. MDMA dose was selected as a moderate dose that did not produce maximal effects (based on MDMA dose effect data in old equipment), thus allowing for potentiation.

Dose Effects of RS102221 on MDMA-Produced Hyperactivity

The potentiating effects of several RS102221 doses (0.0, 0.06, 0.50, 1.25, 1.0 and 2.0mg/kg, IP) were assessed against 10mg/kg MDMA (see previous study), which was the dose where significant RS102221-induced potentiation of hyperactivity was evident. Groups of drug-naïve rats (n=6 per group) were administered RS102221 and MDMA and placed in the old activity chambers using an identical methodology as specified above.

The 5-HT_{2a} Receptor

Dose Effects of DOI on Wetdog Shakes and Baseline Locomotor Activity

Groups of drug-naïve rats (n=6 per group) were administered DOI (0.0, 1.0 or 2.0mg/kg, IP) and placed in the Med-PC chambers, using DOI doses and pretreatment times based on Bull's experiments (2004). Locomotor activity and qualitative behaviour on video cameras were simultaneously recorded for a 30-minute test period. Analyses of videos by an observer blind to experimental condition were conducted 15-minute post DOI injection time point for the duration of 10 minutes. The number of wetdog shakes (WDS) were counted in 2-minute blocks for the recording period, where a WDS was defined as a 'paroxysmic shudder of the head and trunk' (Darmani and Ahmad, 1999).

Dose Effects of Ritanserin on MDMA-Produced Hyperactivity

The effects of several ritanserin doses (0.0, 2.5, 5.0 or 10.0mg/kg, IP) on MDMA (10mg/kg, IP)-produced hyperactivity were assessed. Groups of drug-naïve rats (n=10 per group) were placed in the Med-PC chambers and administered ritanserin, then 30 minutes later, received an MDMA injection and recorded for 60 minutes. A dose of MDMA that produced maximal levels of hyperactivity was selected (see MDMA dose effect data for Med-PC equipment) in order for ritanserin-induced attenuation to be clearly discernable.

Part 2

Overview

The objective of Part 2 was to use behavioural assays developed in Part 1 to assess the effects of prior MDMA exposure. With the exception of the first experiment that examined the effects of repeated MDMA exposure, no repeated testing occurred.

MDMA Pretreatment

Pretreatment Procedures

Rats were placed in individual cages for the day of treatment, whereupon they received an injection regimen comprising 4 injections of either MDMA (10mg/kg per injection, IP) or the saline vehicle administered at 2 hr intervals (total MDMA exposure = 40.0 mg/kg) in the home cage. This regimen was selected because it has been demonstrated in other studies to produce significant effects on 5-HT neurotransmission 2 weeks following treatment (Scanzello et al., 1993, Fischer et al., 1995, Shankaran and Gudelsky, 1999, Sumnall et al., 2004) and was relatively well tolerated with a 10 percent mortality rate. The rats were housed in pairs during a recovery period of either 2 or 12 weeks before commencement of behavioural tests.

Paroxetine Binding

A group of MDMA-pretreated (n=5) and control (n=5) rats were sacrificed and the brains were removed and rapidly frozen in 2-methyl butane at -40°C and stored at -80°C. The levels of SERT were measured in membrane homogenates of frontal cortex, striatum, hippocampus, and brain stem using [³H] paroxetine as previously described (Backstrom et al., 1989) with minor modifications. Briefly, brain tissues were suspended 1:20 in ice-cold buffer (10 mM sodium phosphate, pH 7.4) and homogenized with a Kinematica GmbH Polytron (Brinkmann, New Haven, CT) for 15 seconds at setting 4 and left for 30 minutes

on ice. The brain tissue homogenate was centrifuged for 10 minutes, 4°C at 30,000-x g; the resulting pellet was resuspended and re-centrifuged. Binding assays were conducted with 5 mg of membrane tissue in a total volume of 1 ml containing either 0.06 nM or 0.2 nM [³H] paroxetine for 2 hours at 25°C. Non-specific binding was determined in the presence of 1 µM clomipramine. Radioactivity was measured by liquid scintillation spectrometry and counted at 35% efficiency. Data were analysed using PRISM™ (v3.0, GraphPad, San Diego, CA) and the density values were corrected for occupancy at two concentrations of radioligand.

MDMA-Produced Hyperactivity

Repeated Testing Regimen

Two weeks after pretreatment, groups of MDMA pretreated animals (n=14) and controls (n=15) were first habituated to the old activity chambers for 30 minutes, then administered an MDMA (0.0 or 10.0mg/kg, IP) injection and activity recorded for 60 minutes. This behavioural test was conducted weekly for 1 month, where a within subjects design was utilised to determine the effects of repeated MDMA exposure.

Comparison Between 2- and 12-Week Recovery Periods

Two or 12 weeks following pretreatment, separate groups of MDMA pretreated animals (2 weeks: n=27; 12 weeks: n=32) and controls (2 weeks: n=32; 12 weeks: n=36) were first habituated to the Med-PC activity chambers for 30 minutes. Following this habituation period, they were injected with MDMA (0.0, 2.5, 5.0 or 10.0mg/kg, IP) and activity recorded at 5-minute intervals for an additional 60 minutes.

The Serotonin Transporter

Clomipramine-Induced Attenuation of MDMA-Produced Hyperactivity

MDMA (n=38) and saline (n=37) pretreated rats were used to test the effect of several doses of clomipramine that did not significantly affect baseline locomotor activity (0.0, 0.3, 1.25 or 5.0mg/kg, IP) on MDMA- (5.0 mg/kg, IP) produced hyperactivity. This dose of MDMA was chosen because clomipramine produced dose-dependent attenuation of hyperactivity at this dose, whereas the higher MDMA dose was not significantly attenuated by clomipramine (see clomipramine dose effect study). Rats were administered the clomipramine injection and placed in the activity boxes for 30 minutes. Thereafter, they received an injection of MDMA and activity was recorded for a further 60 minutes.

The 5-HT_{2c} Receptor

m-CPP-Induced Hypolocomotion

MDMA (n=29) and saline (n=28) pretreated rats were administered a dose of m-CPP (0.0, 0.6, 1.25 or 2.5mg/kg, IP) and locomotor activity recorded for 30 minutes thereafter.

m-CPP-Induced Increased Emergence Latency

MDMA (n=20) and saline (n=34) pretreated rats were administered m-CPP (0.0, 0.6 or 1.25mg/kg, IP) before being placed in the hide box. Rats were placed in the hide box for 15 minutes following an m-CPP injection and emergence latency into the test arena was measured for 40 minutes after the sliding door was opened.

RS102221 Induced Potentiation of MDMA-Produced Hyperactivity

Groups of MDMA (n=24) and saline (n=33) pretreated rats were used to test the effect of RS102221 (0.0, 0.5 and 1.0mg/kg, IP) on MDMA- (5.0 mg/kg, IP) produced hyperactivity. Rats were administered RS102221 and placed in the activity boxes for 30 minutes. Thereafter, they received an injection of MDMA and locomotor activity was measured for a further 60 minutes.

DOI-Induced Wetdog Shakes

Groups of MDMA (n=12) and saline (n=12) pretreated rats were administered DOI (0.0 or 1.0mg/kg, IP), placed in the Med-PC chambers and video taped for 25 minutes. The 1.0 and 2.0mg/kg DOI doses did not produce significantly different behavioural effects (see Part 1), therefore only a single test dose was utilised. Analyses of videos by an observer blind to experimental condition were conducted 15 minutes post DOI injection for 10 minutes. The numbers of wetdog shakes (WDS) were counted in 2-minute blocks for the recording period. Locomotion was not recorded, as neither DOI dose tested in Part 1 altered baseline activity.

Ritanserin-Induced Attenuation of MDMA-Produced Hyperactivity

MDMA (n=32) and saline (n=50) pretreated rats were used to assess the ability of several ritanserin doses (0.0, 2.5, 5.0 or 10.0mg/kg, IP) to attenuate MDMA (10mg/kg, IP)-produced hyperactivity. Rats were placed in the Med-PC chambers and administered ritanserin, then 30 minutes later, received an MDMA injection and recorded for 60 minutes.

Drug Preparation

8-[5-(2,4-Dimethoxy-5-(4-trifluoromethylphenylsulphonamido)phenyl-5-oxopentyl]-1,3,8-triazaspiro[4.5]decane-2,4-dione hydrochloride (RS102221, Tocris) and ritanserin (Tocris) were suspended in a solution of 1% polysorbate 80 (Tween® 80). 1-(3-Chlorophenyl) piperazine hydrochloride (m-CPP, Tocris), 3-chloro-10,11-dihydro-N,N-dimethyl-5H-dibenz[b,f]azepine-5-propanamine hydrochloride (clomipramine, Tocris), +/- 2,5-dimethoxy-4-iodo-phenylisopropylamine (DOI, Sigma Aldrich) and +/- 3,4-methylenedioxymethamphetamine hydrochloride (MDMA, ESR) were dissolved in 0.9% saline. Injections were intraperitoneal and administered in a volume of 1ml/kg. All drugs

were prepared immediately prior to behavioural tests and drug weights refer to the salt.

Statistical Analyses

The binding data were analysed by 1-way ANOVAs followed by Tukey's Post Hoc tests (PRISM™ v3.0, Graphpad, San Diego, CA). Differences between treatment groups were considered statistically significant at $p < 0.05$. Time course data for locomotor activity tests were analysed using 3-Way ANOVAs (time X pretreatment X drug dose) with time as the repeated measure. Univariate analyses followed by Tukey post hoc tests determined whether there were significant differences as a result of pretreatment in the event of main effects or interactions.

Results

Part 1 – Preliminary Work

MDMA-Produced Hyperactivity

Dose Effects of MDMA on Locomotor Activity in Old Equipment

Figure 1 shows the time course and dose-dependency for the hyperactivity produced by MDMA (0.0, 5.0, 10.0, 15.0 and 20.0mg/kg, IP). A 2-Way interaction (time X MDMA dose) with time as a repeated measure revealed a significant interaction between these factors ($F(13,110)=13.19$, $p<.001$) and a main effect of MDMA dose ($F(4,35)=18.80$, $p<.001$). Post hoc analyses showed that the 10.0, 15.0 and 20.0mg/kg doses of MDMA significantly potentiated locomotor activity above baseline.

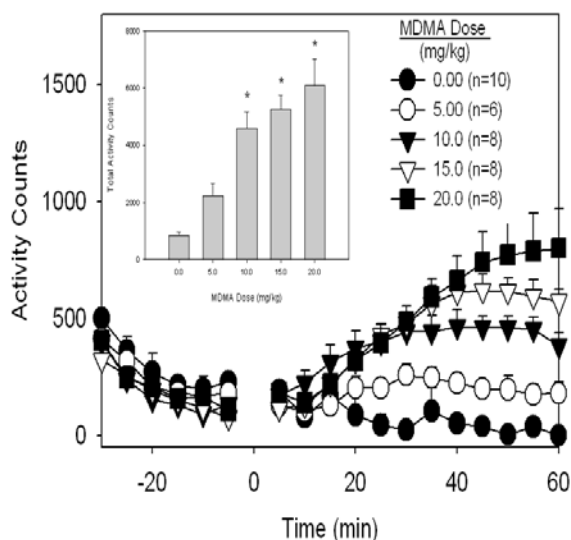


Figure 1 Activity counts produced after MDMA (0.0, 5.0, 10.0, 15.0 or 20.0mg/kg, IP at time=0) administration in drug-naïve rats. The time course of the mean activity counts produced following the MDMA injection is shown (+SEM). The inset shows total activity counts at each MDMA dose for the 60-minute period post MDMA injection (+SEM). Significant difference relative to vehicle group * $p<.001$.

Dose Effects of MDMA on Locomotor Activity in Med-PC Equipment

Figure 2 also shows the time course and dose-dependency for the hyperactivity produced by MDMA (0.0, 2.5, 5.0, 10.0 and 20.0mg/kg, IP), but in the Med-PC apparatus. A 2-Way interaction (time X MDMA dose) with time as a repeated measure revealed a significant interaction between these factors ($F(21,192)=12.08$, $p<.001$) and a main effect of MDMA dose ($F(4,37)=37.44$, $p<.001$). Post hoc analyses showed that the 5.0, 10.0 and 20.0mg/kg doses of MDMA significantly potentiated locomotor activity above baseline.

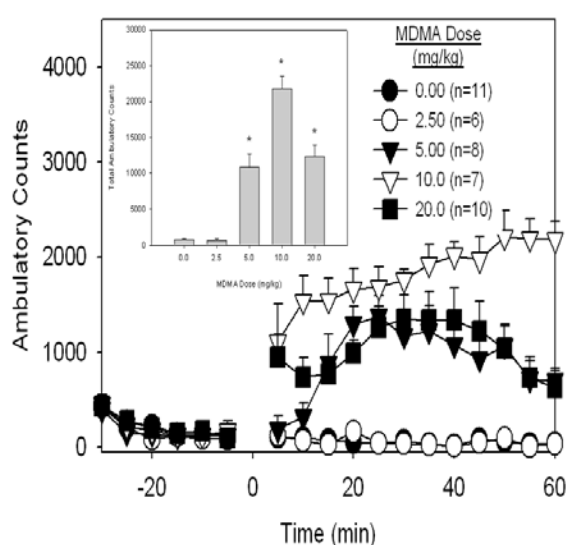


Figure 2 Activity counts produced after MDMA (0.0, 2.5, 5.0, 10.0 or 20.0mg/kg, IP at time=0) administration in drug-naïve rats. The time course of the mean ambulatory counts produced following the MDMA injection is shown (+SEM). The inset shows total ambulatory counts at each MDMA dose for the 60-minute period post MDMA injection (+SEM). Significant difference relative to vehicle group * $p<.001$.

The Serotonin Transporter

Dose Effects of Clomipramine on Baseline Locomotor Activity

Figure 3 shows the time course for the hypolocomotor effects of clomipramine (0.0, 1.25 and 5.0mg/kg, IP). Clomipramine did not affect baseline locomotor activity as a 2-

Way interaction (time X clomipramine dose) with time as a repeated measure failed to reach significance ($F(7,90)=.374$, $p>.05$).

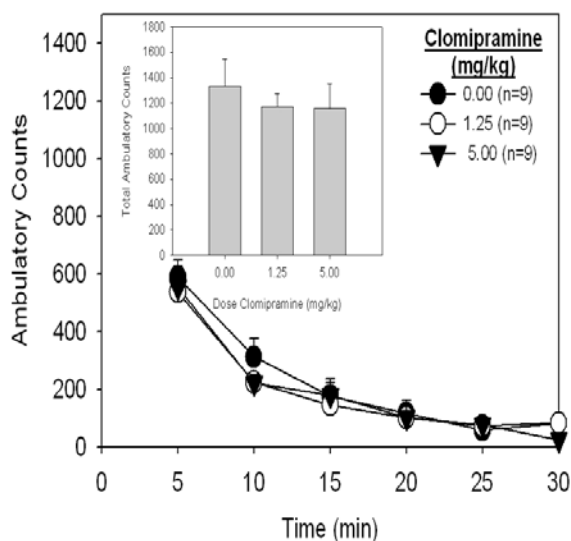


Figure 3 Ambulatory counts produced following clomipramine (0.0, 1.25 or 5.0mg/kg, IP) pretreatment. The time course for mean ambulatory counts are shown, where clomipramine was administered 30 min prior to behavioural tests (+SEM). The inset shows total locomotor activity during the 30 min recording period (+SEM).

Dose Effects of Clomipramine on MDMA-Produced Hyperactivity

Figure 4 shows the ability of clomipramine (0.0, 0.3, 1.25, 5.0, 10.0 or 20.0mg/kg, IP) to dose-dependently attenuate MDMA-produced hyperactivity (5.0mg/kg, IP). A 2-Way ANOVA (time X clomipramine dose) with time as the repeated measure showed a significant interaction between these factors ($F(18,142)=2.62$, $p<.01$) and there was also a main effect of clomipramine dose ($F(5,39)=5.09$, $p<.01$). Post hoc tests showed that the 5.0, 10.0 and 20.0mg/kg doses significantly attenuated hyperactivity compared to baseline, whereas the 1.25mg/kg dose narrowly missed significance.

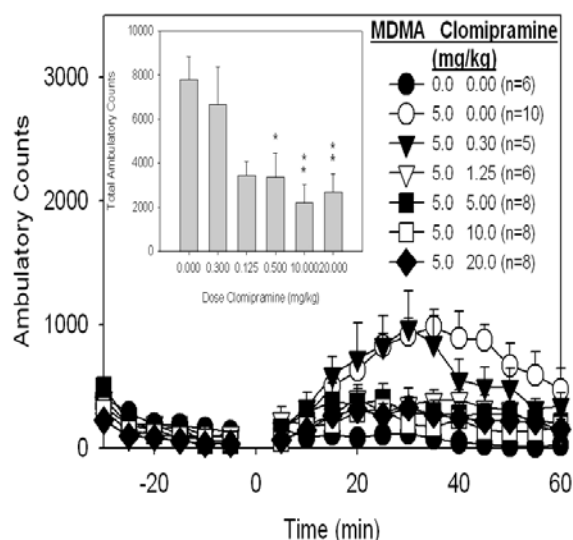


Figure 4 Ambulatory counts produced following clomipramine (0.0, 0.3, 1.25, 5.0, 10.0 or 20.0mg/kg, IP) and MDMA (0.0 or 5.0mg/kg, IP) administration. The time course for mean ambulatory counts are shown, where clomipramine was administered 30 min prior to MDMA (time= 0 min) (+SEM). The inset shows total MDMA-produced hyperactivity during the 60-minute post-MDMA injection period at each clomipramine dose (+SEM). Significant difference relative to vehicle group * $p < .05$, ** $p < .01$.

Figure 5 shows the ability of clomipramine (0.0, 1.25 or 5.0mg/kg, IP) to dose-dependently attenuate hyperactivity produced by a higher dose of MDMA (10.0mg/kg, IP). A 2-Way ANOVA (time X clomipramine dose) with time as the repeated measure failed to show significance ($F(9,115)=.814$, $p > .05$) and there was no main effect of clomipramine on hyperactivity produced by this MDMA dose ($F(2,27)=1.14$, $p > .05$).

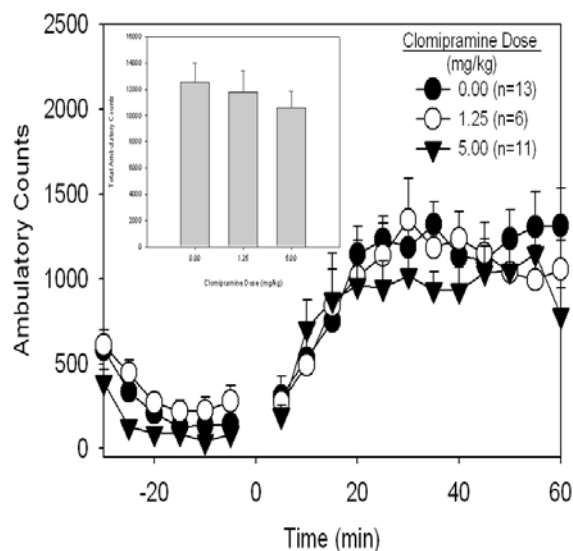


Figure 5 Ambulatory counts produced following clomipramine (0.0, 1.25 or 5.0mg/kg, IP) and MDMA (10.0mg/kg, IP) administration. The time course for mean ambulatory counts are shown, where clomipramine was administered 30 min prior to MDMA (+SEM). The inset shows total MDMA-produced hyperactivity during the 60 min post-MDMA injection period at each clomipramine dose (+SEM).

The 5-HT_{2c} Receptor

Dose Effects of m-CPP on Baseline Locomotor Activity

Figure 6 shows the hypolocomotive effects of m-CPP on baseline locomotor activity. A 2-Way ANOVA (time X m-CPP dose) where time was the repeated measure showed a significant interaction between these factors ($F(38,381)=2.46, p<.001$) and a main effect of m-CPP dose ($F(5,50)=3.54, p<.001$). Post hoc analyses revealed that the 5.0mg/kg m-CPP dose significantly attenuated baseline locomotor activity.

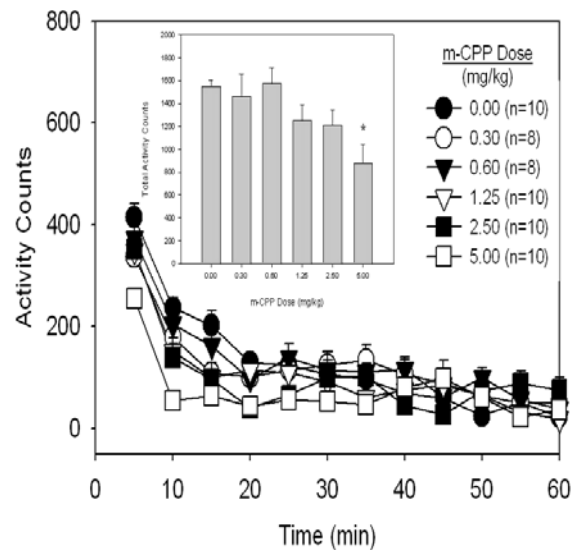


Figure 6 Activity counts produced following m-CPP (0.0, 0.60, 0.60, 1.25, 2.5, 5.0mg/kg, IP) pretreatment. The time course for mean ambulatory counts are shown, where m-CPP was administered at time=0 and activity recorded for 60 minutes (+SEM). The inset shows total locomotor activity during the 60 min recording period (+SEM). Significant difference relative to vehicle group * $p<.05$.

Dose Effects of m-CPP on Emergence Latency

Figure 7 shows increased emergence latency time induced by m-CPP. There was a main effect of m-CPP dose on emergence latency time (2,34)=3.81, $p<.05$). Post hoc tests showed that the 1.25mg/kg dose significantly increased emergence latency.

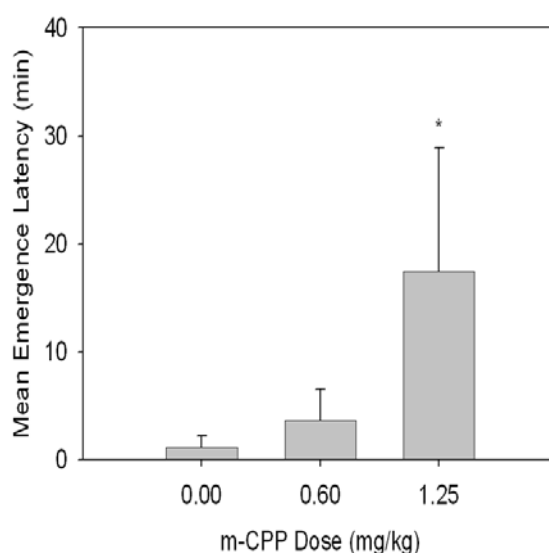


Figure 7 Emergence latency times following m-CPP (0.0, 0.60 or 1.25mg/kg, IP) pretreatment for the 40-minute recording period (+SEM). Significant difference relative to vehicle group * $p < .05$.

Dose Effects of MDMA Following RS102221 Pretreatment

Figures 8A, 8B, 8C and 8D show the time course and dose-dependency of MDMA-produced hyperactivity in rats pretreated with RS102221 (0.0 or 0.5mg/kg). A 3-Way ANOVA (time X RS102221 dose X MDMA dose) with time as a repeated measure showed no significant interaction between these factors ($F(10,170)=1.80$, $p > .05$). There was a main effect of MDMA ($F(3,50)=44.5$, $p < .001$) but not RS102221 dose ($F(1,50)=2.08$, $p > .05$). ANOVAs for each dosage group revealed no significant differences as a result of RS102221 pretreatment for rats that received the 0.0 (Figure 8A) ($F(1,13)=.024$, $p > .05$), 5.0 (Figure 8B) ($F(1,11)=.004$, $p > .05$) or 20.0mg/kg (Figure 8D) ($F(1,13)=.001$, $p > .05$) doses, however, 10mg/kg MDMA-produced hyperactivity was potentiated by RS102221 (Figure 8C) ($F(1,13)=9.21$, $p < .05$).

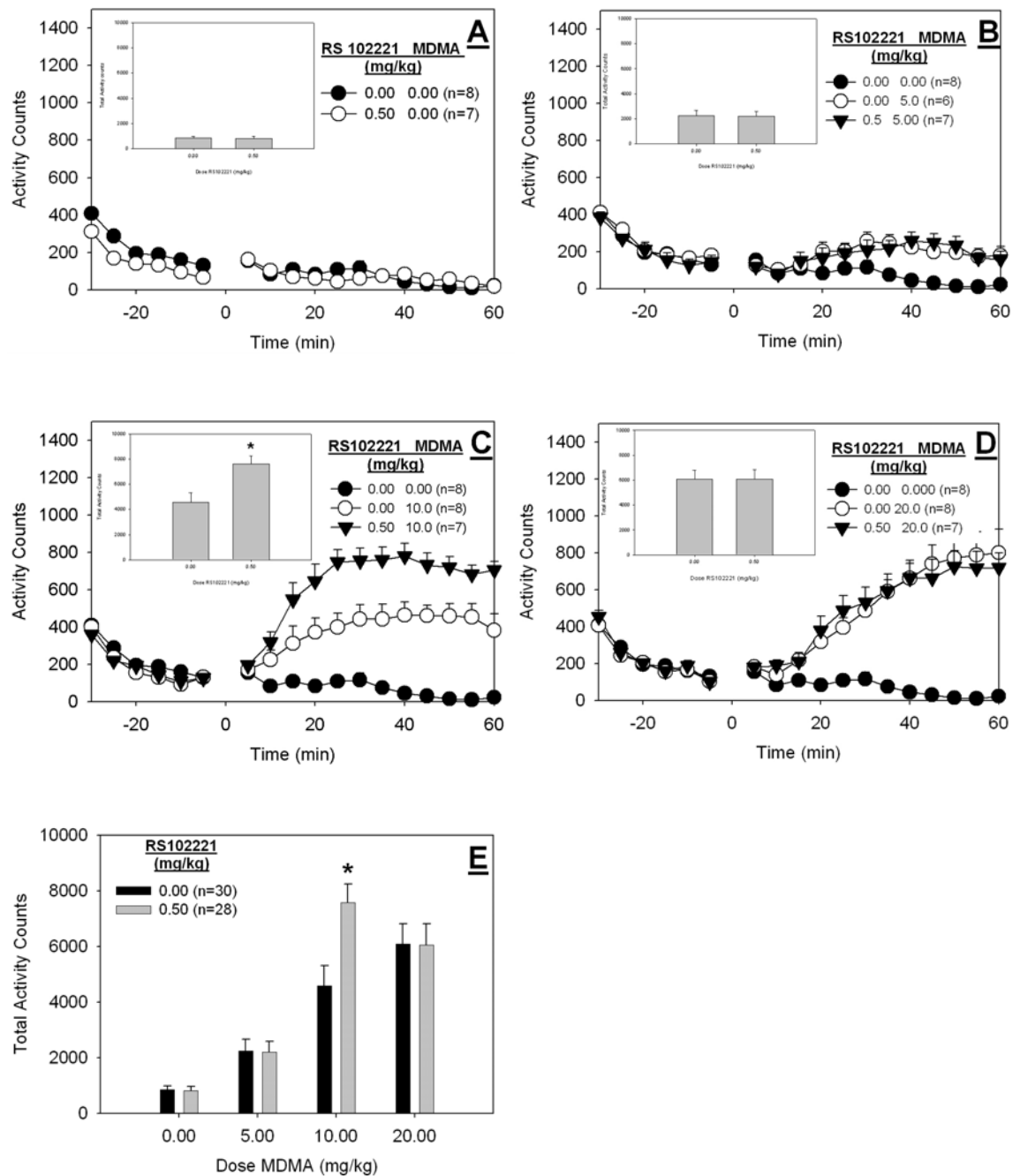


Figure 8 The time course of the mean activity counts produced after 0.0 (Figure 8A), 5.0 (Figure 8B), 10.0 (Figure 8C) or 20.0mg/kg (Figure 8D) MDMA administration is shown (+SEM). Insets show total activity counts during the 60-minute period post MDMA injection for the 0.0 or 0.5mg/kg RS102221 groups (+SEM). Figure 8E shows total activity produced at all MDMA doses for the 60-minute period post MDMA injection for the 0.0 or 0.5mg/kg RS102221 groups (+SEM). Significant difference relative to the 0.0mg/kg RS 102221 group * $p < .05$.

The ability of RS102221 to potentiate MDMA-produced hyperactivity was observed at the 10.0mg/kg MDMA dose, as demonstrated in 8C. This MDMA dose was selected to assess the dose effects of RS102221. Figure 9 shows the time course and dose-dependency for the ability of RS102221 (0.0, 0.06, 0.50, 1.25, 1.0 and 2.0mg/kg, IP) to potentiate MDMA (10.0mg/kg, IP)-produced hyperactivity. A 2-Way interaction (time X RS102221 dose) with time as a repeated measure failed to reach significance ($F(19,131)=1.25$, $p>.05$), but there was a significant main effect of RS102221 dose on MDMA-produced hyperactivity ($F(5,34)=3.15$, $p<.05$).

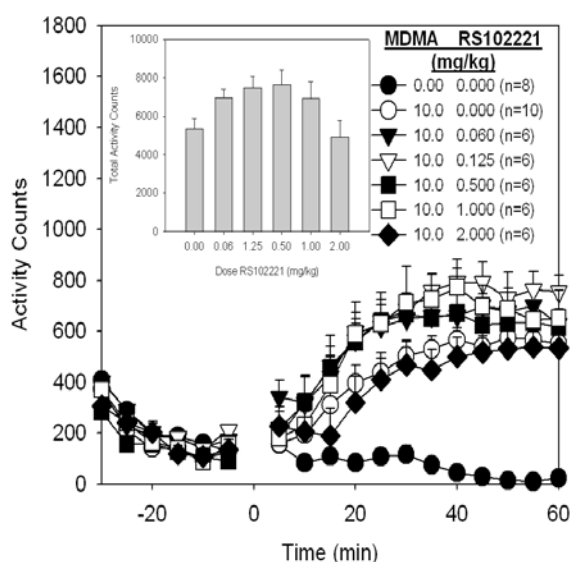


Figure 9 Activity counts produced after RS102221 (0.0, 0.06, 0.5, 1.25, 1.0 or 2.0mg/kg, IP at time=-30) and MDMA (0.0 or 10.0mg/kg, IP at time =0)) administration in drug-naïve rats. The time course of the mean activity counts produced after RS102221 and MDMA pretreatment are shown (+SEM). The inset shows total ambulatory counts at each RS102221 dose for the 60-minute period post MDMA injection (+SEM).

Dose Effects of DOI on Wetdog Shakes

Table 1 shows the number of wetdog shakes (WDS) produced in a 10-minute recording period 15-minutes following a DOI injection (0.0, 1.0 or 2.0mg/kg, IP). Univariate analyses revealed that there was a significant main effect of DOI treatment ($F(2,14)=3.94$, $p<.05$). Post hoc tests showed that both the 1.0 and 2.0mg/kg DOI doses significantly increased WDS behaviour, but there were no differences between doses. The animals that received DOI exhibited different behavioural characteristics to control rats. For example, DOI-treated rats did not curl up and sleep, were constantly moving with a wobbling gait around the chamber periphery and there was also evidence of 5-HT syndrome with a flattened posture. Additionally, some rats, but not all that received DOI, exhibited back muscle contractions, which was purported to be a 5-HT_{2a} receptor mediated effect (Bull, 2004). Back muscle contractions were not recorded, as they originate from 5-HT_{2a} receptors located near the brain stem and were separate from 5-HT_{2a} receptor-mediated WDS. Also, WDS were consistently produced by DOI administration, whereas back muscle contractions were not always observed.

DOI Dose (mg/kg)	Number WDS
0.0 (n=4)	1 ± 0.4
1.0 (n=7)	5.3 ± 0.8 *
2.0 (n=6)	5.3 ± 1.5 *

Table 1 Mean total number of DOI-induced WDS during a 10-minute recording period + SEM. Significant difference relative to vehicle group * $p<.05$

Dose Effects of DOI on Baseline Locomotor Activity

Figure 10 shows the effects of DOI pretreatment on baseline locomotor activity (0.0, 1.0 and 2.0mg/kg, IP). The time course for the mean ambulatory counts over the 30-minute recording period is shown (+SEM). DOI pretreatment did not affect locomotor activity measures as revealed by a 2-Way ANOVA (time X DOI dose) with time as a repeated measure ($F(5,35)=.822$, $p>.05$).

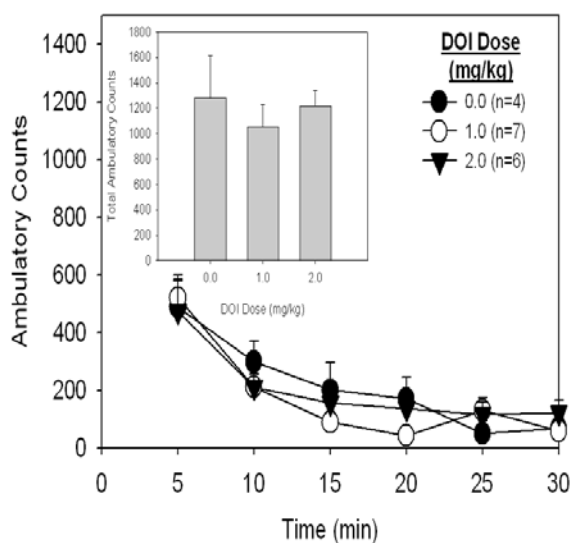


Figure 10 Ambulatory counts produced following DOI (0.0, 1.0 or 2.0mg/kg, IP) pretreatment. The time course for mean ambulatory counts are shown, where DOI was administered at time=0 (+SEM). The inset shows total ambulatory counts during the 30-minute recording period (+SEM).

Dose Effects of Ritanserin on MDMA-Produced Hyperactivity

Figure 11 shows the time course and dose-dependency for the ability of ritanserin (0.0, 2.5, 5.0 and 10.0mg/kg, IP) to attenuate MDMA (10.0mg/kg, IP)-produced hyperactivity. A 2-Way ANOVA (time X ritanserin dose) with time as a repeated measure showed a significant interaction between these factors ($F(12,194)=2.00$, $p<.05$). There was a main effect of ritanserin dose on MDMA-produced hyperactivity ($F(3,49)=4.38$, $p<.01$).

and post hoc analyses revealed that the 5.0mg/kg dose significantly attenuated ambulatory counts compared to baseline ($P<0.05$).

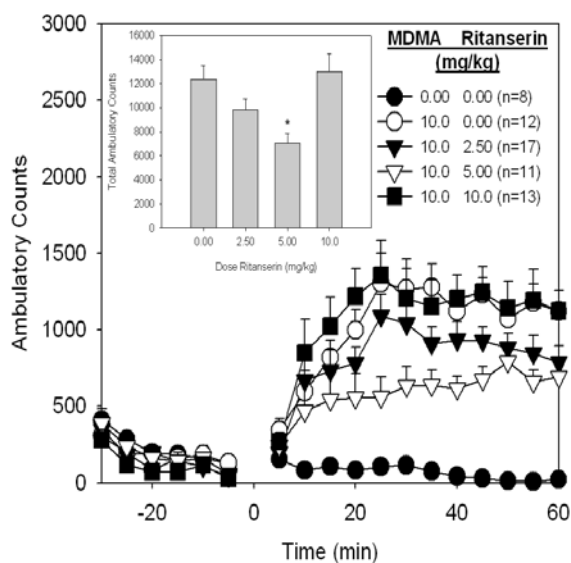


Figure 11 Ambulatory counts produced after ritanserin (0.0, 2.5, 5.0 or 10.0mg/kg, IP at time=-30) and MDMA (0.0 or 10.0mg/kg, IP at time =0)) administration in drug-naïve rats. The time course of the mean ambulatory counts produced after ritanserin and MDMA pretreatment are shown (+SEM). The inset shows total ambulatory counts at each ritanserin dose for the 60-minute period post MDMA injection (+SEM). Significant difference relative to vehicle and 10mg/kg MDMA group * $p<.05$.

Part 2 – Effects of MDMA Pretreatment

MDMA-Produced Hyperactivity

MDMA-Produced Hyperactivity 2-5 Weeks Post Exposure

Figure 12 shows MDMA-produced hyperactivity (0.0 or 10.0mg/kg) 2-5 weeks following MDMA pretreatment. Tolerance to the locomotor-activating effect of MDMA was observed two weeks later, and with repeated weekly administrations, the effect of the 10.0 mg/kg dose decreased in control rats such that the response was comparable to rats that had initially received 40.0mg/kg MDMA pretreatment by 5 weeks. A 3-Way ANOVA (week X pretreatment X MDMA dose) where week was the repeated measure, did not reveal a significant interaction between these factors ($F(2,56)=.74$, $p>.05$) but there were main effects of pretreatment ($F(1,25)=10.15$, $p<.01$) and MDMA dose ($F(1,25)=55.92$, $p<.001$). Within subjects contrasts revealed that there were significant differences between weeks 2 and 5 on the effects of pretreatment ($F(1,25)=.04$, $p<.05$) and MDMA dose ($F(1,25)=14.45$, $p<.01$).

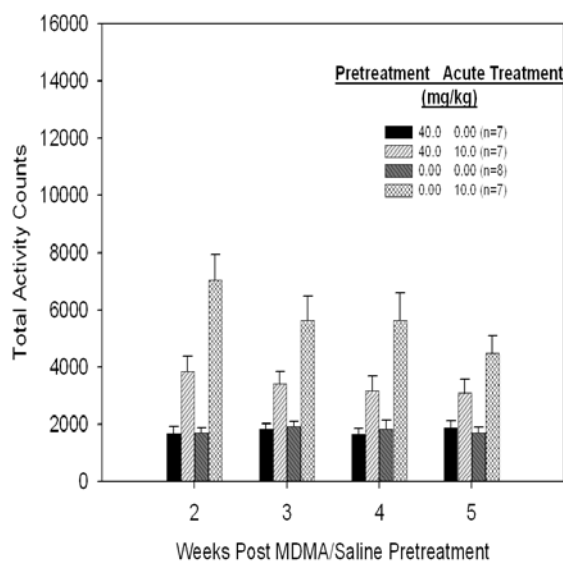


Figure 12 Total activity counts produced for the 60-minute period following an acute MDMA (0.0 or 10.0mg/kg, IP) injection in MDMA (40mg/kg) or vehicle pretreated groups at the 2, 3, 4 and 5 week time points post pretreatment (+SEM).

Dose Effects of MDMA-Produced Hyperactivity 2- and 12-Weeks Post Exposure

Figures 13A, 13B, 13C and 13D show the time course and dose-dependency of MDMA-produced hyperactivity for saline and MDMA-pretreated rats following a 2-week withdrawal period. A 3-Way ANOVA (time X pretreatment X MDMA dose) with time as a repeated measure showed a significant interaction between these factors ($F(13, 227)=1.96$, $p<.05$) and main effects of MDMA dose ($F(3,51)=84.33$, $p<.001$) and pretreatment ($F(1,51)=21.64$, $p<.001$). ANOVAs on total ambulatory counts for each dosage group revealed no significant differences as a result of pretreatment for rats that received either the 0.0 (Figure 13A) or 2.5 ($F(1,15)=.380$, $p>.05$), 2.5 (Figure 13B) ($F(1,10)=.007$, $p>.05$) mg/kg doses. MDMA-produced hyperactivity was greater for the saline pretreated rats that received 5.0 (Figure 13C) ($F(1,14)=8.07$, $p<.05$) and 10.0 mg/kg (Figure 13D) ($F(1,12)=16.42$, $p<.01$) doses.

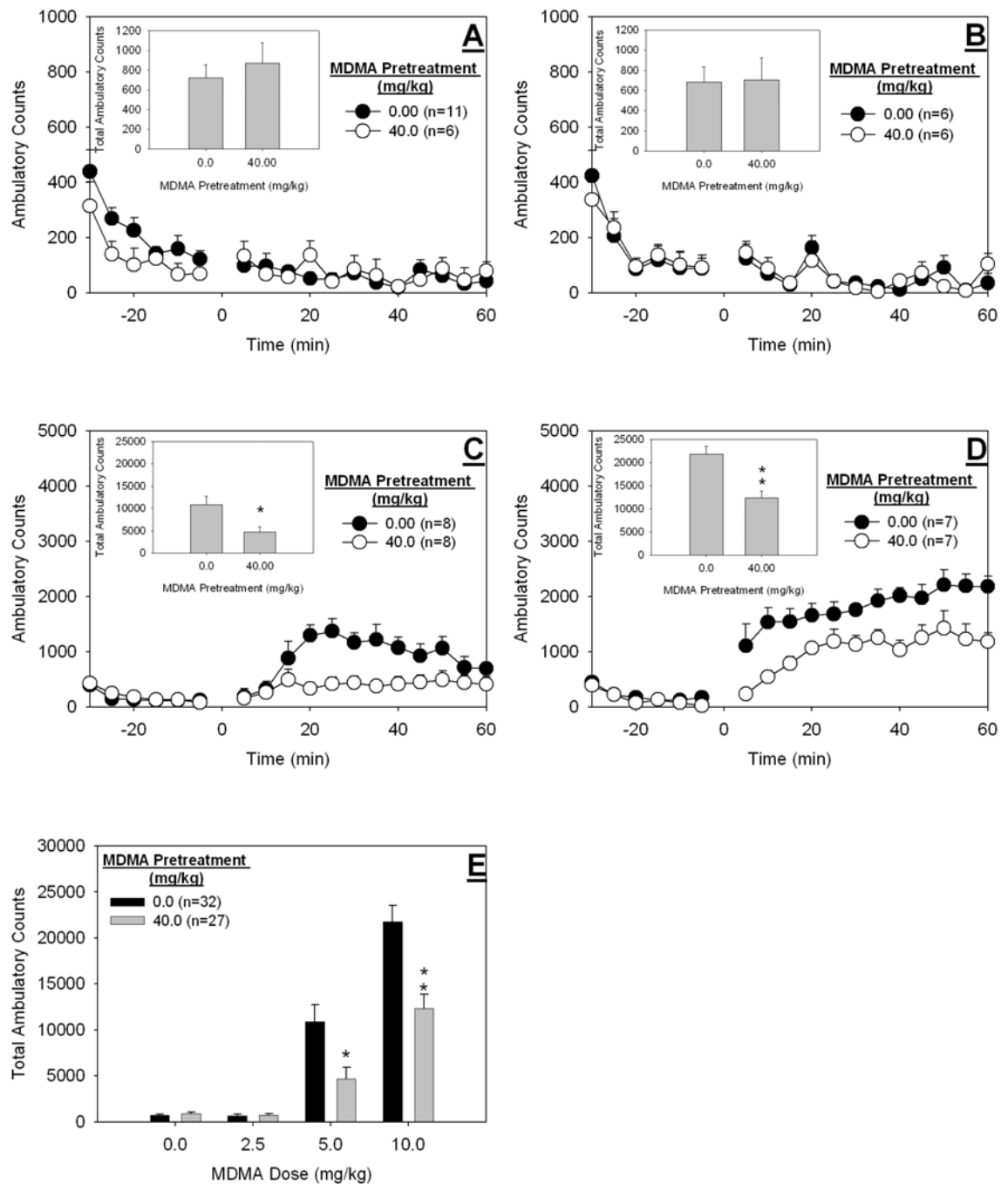


Figure 13 Ambulatory counts produced after acute MDMA (0.0, 2.5, 5.0 or 10mg/kg) administration 2 weeks following saline or MDMA pretreatment. The time course of the mean ambulatory counts produced after 0.0 (Figure 13A), 2.5 (Figure 13B), 5.0 (Figure 13C), or 10.0mg/kg (Figure 13D) MDMA are shown (+SEM). Insets show total ambulatory counts during the 60-minute period post MDMA injection for the two pretreatment groups (+SEM). Figure 13E shows total ambulatory counts produced by all MDMA doses for the 60-minute period post MDMA injection for the two pretreatment groups (+SEM). Significant difference relative to saline pretreated group * $p < .05$ ** $p < .01$.

Figures 14A, 14B, 14C and 14D show the time course and dose-dependency of MDMA-produced hyperactivity for saline and MDMA-pretreated rats after a 12-week withdrawal period. A 3-Way ANOVA (time X pretreatment X MDMA dose) with time as a repeated measure failed to reveal a significant interaction between these factors ($F(11,214)=1.04$, $p>.05$) and ANOVAs did not reveal differences as a result of pretreatment for any of the doses tested (0.0 mg/kg; ($F(1,14)=3.11$, $p>.05$); 2.5 mg/kg; ($F(1,14)=.071$, $p>.05$); 5.0 mg/kg; ($F(1,18)=2.00$, $p>.05$) and 10.0 mg/kg ($F(1,14)=.162$, $p>.05$). There was still a main effect of MDMA on locomotor activity levels ($F(3,60)=47.89$, $p<.001$), but pretreatment did not affect overall responses ($F(1,60)=1.16$, $p>.05$).

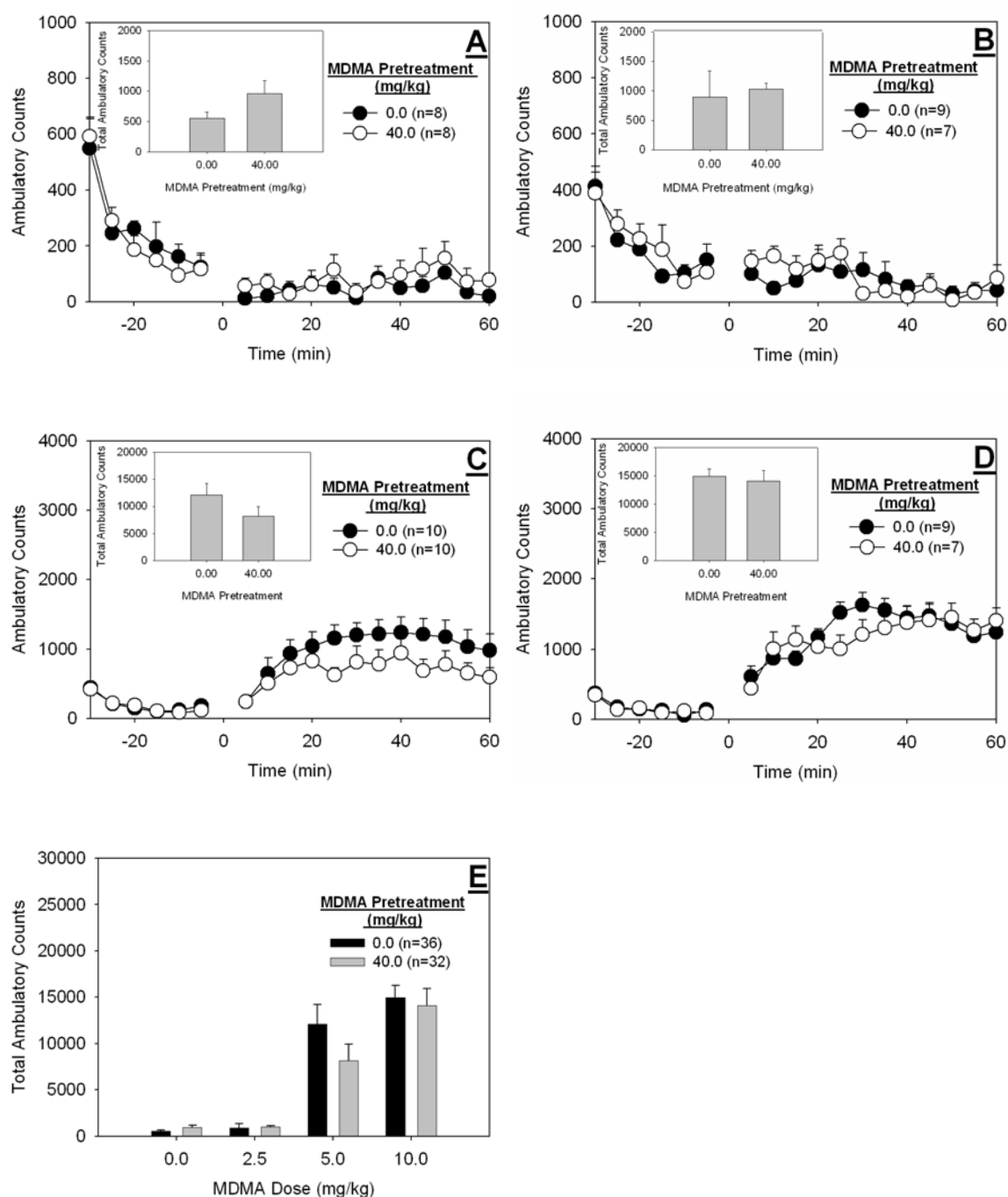


Figure 14 Ambulatory counts produced after acute MDMA (0.0, 2.5, 5.0 or 10.0mg/kg) administration in rats 12 weeks following saline or MDMA pretreatment. The time course of the mean ambulatory counts produced after 0.0 (Figure 14A), 2.5 (Figure 14B), 5.0 (Figure 14C) or 10.0mg/kg (Figure 14D) MDMA (+SEM) are shown. Insets show total ambulatory counts at each MDMA dose for the 60-minute period post MDMA injection for the two pretreatment groups (+SEM). Figure 14E shows total ambulatory counts produced during the 60-minute period post MDMA injection for the two pretreatment groups (+SEM).

Paroxetine Binding Densities

Specific [^3H] paroxetine binding was measured in regional assays of brain tissue homogenates from frontal cortex, hippocampus and striatum. B_{max} values were determined by assaying at two concentrations of radioligand (0.06 and 0.2 nM) and correcting for occupancy ($K_D=0.25$ nM, (Sanchez et al., 2001)). MDMA pretreated rats had significantly lower levels of SERT binding compared to the control animals in all regions assayed ($p<0.05$).

	CONTROL	MDMA
Frontal Cortex	29.25 ± 3.29	15.44 ± 2.17 * (53% of control)
Caudate Putamen	47.35 ± 2.15	23.85 ± 5.92 *** (50% of control)
Hippocampus	29.96 ± 3.22	15.32 ± 2.65 *(51% of control)
Brain Stem	47.52 ± 4.28	17.64 ± 4.22 *** (37% of control)

Table 2 Regional measurements of SERT densities following MDMA pretreatment. Bmax values are expressed as the mean + SEM. * $p<0.05$, ** $p<0.01$ and * $p<0.001$**

Clomipramine-Induced Attenuation of MDMA-Produced Hyperactivity

Figures 15A and 15B show the time course for the dose effects of clomipramine (0.0, 0.3, 1.25 or 5.0mg/kg) on MDMA (5.0mg/kg, IP)-produced locomotor activity in saline and MDMA-pretreated rats. A 3-Way ANOVA with time as the repeated measure (time X clomipramine dose X pretreatment) failed to reveal a significant interaction between these factors ($F(11,247)=1.41$, $p>0.05$). There were main effects of pretreatment ($F(1,67)=5.10$, $p<0.05$) and clomipramine dose ($F(3,67)=8.74$, $p<0.001$). Post hoc tests

showed that clomipramine significantly attenuated MDMA-produced hyperactivity in the saline pretreated rats at the 1.25 and 5.0mg/kg doses, whereas clomipramine did not affect MDMA pretreated rats.

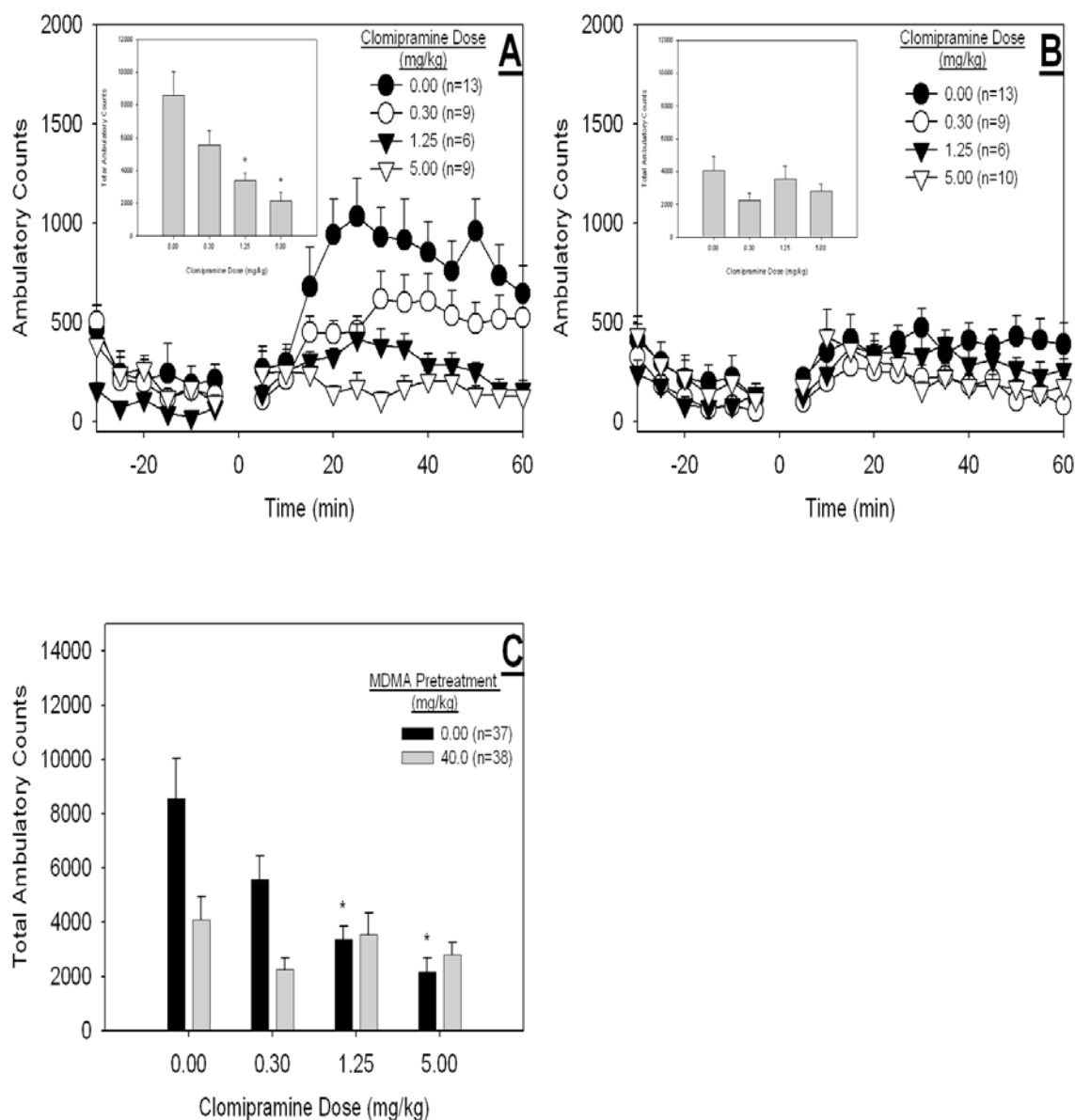


Figure 15 Ambulatory counts produced after acute MDMA (5.0mg/kg) and clomipramine (0.0, 0.3, 1.25 or 5.0mg/kg) administration in rats that had received saline or MDMA pretreatment. The top panels show the time course of hyperactivity in saline (Figure 3A) and MDMA (Figure 3B) pretreated rats (+SEM). Insets show total ambulatory counts following each clomipramine dose for the 60-minute period post MDMA injection for each of the pretreatment groups (+SEM). Figure 3C shows total ambulatory counts produced at all clomipramine doses for the 60-minute period

post MDMA injection for the two pretreatment groups (+SEM). Significant difference relative to vehicle group within pretreatment condition * $p < .01$.

The 5-HT_{2c} Receptor

m-CPP-Induced Hypocomotion

Figures 16A and 16B show the time course for the dose effects of m-CPP (0.0, 0.60, 1.25 and 2.5mg/kg) on baseline locomotion in saline and MDMA-pretreated rats. m-CPP did not produce differential effects on the two pretreatment groups as revealed by a 3-Way ANOVA with time as the repeated measure (time X m-CPP dose X pretreatment) that did not show a significant interaction ($F(12,199)=1.12$, $p > .05$). m-CPP did significantly alter behaviour, as there was a main effect of m-CPP dose ($F(3,49)=14.45$ $p < .001$) but not pretreatment ($F(1,49)=3.96$, $p > 0.05$). Post hoc tests showed that the 2.5mg/kg dose significantly lowered ambulatory counts in saline-, whilst both the 1.25 and 2.5mg/kg doses were effective in MDMA-pretreated rats.

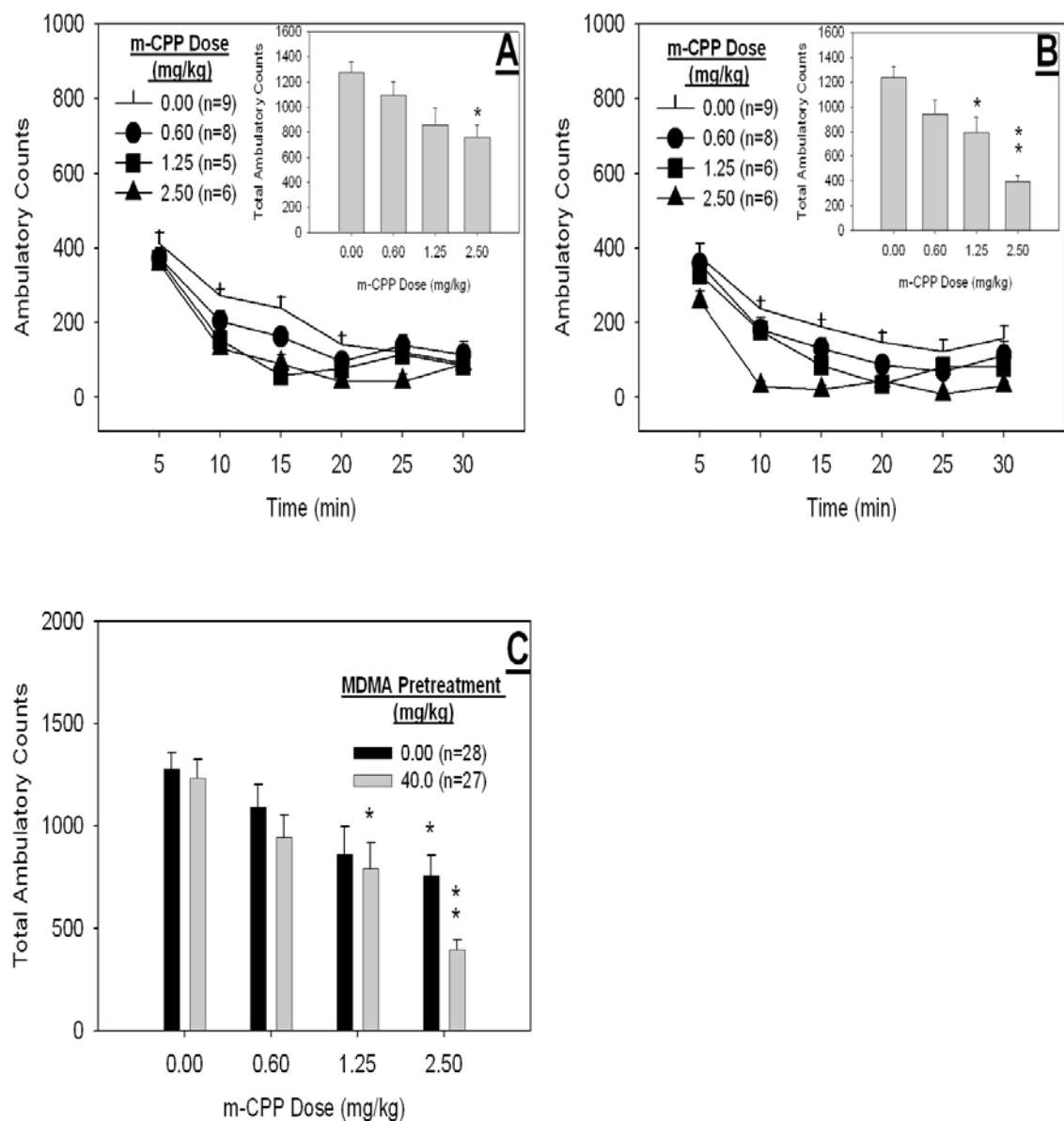


Figure 16 Ambulatory counts produced after acute m-CPP administration (0.0, 0.6, 1.25 or 2.5mg/kg). Top panels show the time course of the mean ambulatory counts in saline (Figure 16A) and MDMA (Figure 16B) pretreated rats (+SEM). Insets show total ambulatory counts following each m-CPP dose for the 30-minute recording period for each of the pretreatment groups (+SEM). Figure 16C shows total ambulatory counts produced at all m-CPP doses for the 30-minute recording period for the two pretreatment groups (+SEM). Significant difference relative to vehicle within pretreatment group * $p < .05$ ** $p < .001$.

m-CPP-Induced Increased Emergence Latency

Figure 17 shows mean emergence latency times (minutes) following m-CPP (0.0, 0.6 or 1.25mg/kg) administration in saline and MDMA-pretreated rats. There were no differences as a result of pretreatment in the ability of m-CPP to increase emergence latencies as shown by a 2-Way ANOVA (m-CPP dose X pretreatment) that failed to reveal a significant interaction between these factors ($F(2,47)=.03$, $p>.05$). There was a main effect of m-CPP dose ($F(2,47)=27.32$, $p<.001$) but not of pretreatment ($F(1,47)=.023$, $p>.05$). Post hoc analyses showed that the 1.25mg/kg dose produced significantly higher emergence latencies than baseline in both pretreatment conditions.

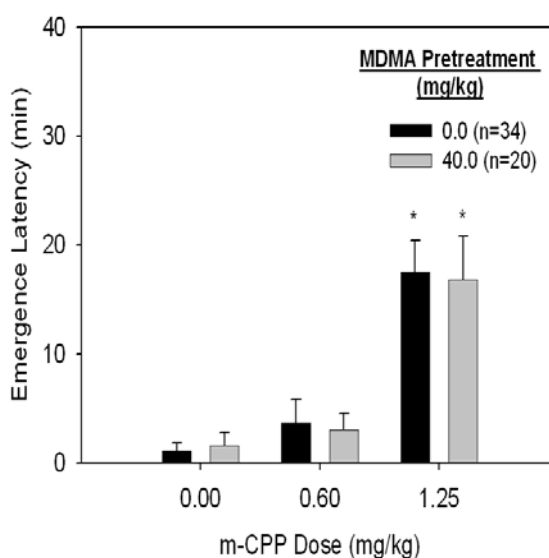


Figure 17 Mean emergence latencies for MDMA and saline pretreated rats following administration of m-CPP (0.0, 0.6 or 1.25mg/kg) (+SEM). Significant difference relative to vehicle group within pretreatment condition* $p<.001$.

RS102221-Induced Potentiation of MDMA-Produced Hyperactivity

Figures 18A and 18B show the time course for the dose effects of RS102221 (0.0, 0.5 and 1.0mg/kg) on MDMA (5.0mg/kg, IP)-produced locomotor activity in saline and MDMA-pretreated rats. A 3-Way ANOVA (time X pretreatment X RS102221 dose) revealed a significant interaction ($F(11,281)=2.45$, $p<.01$) where there was a main effect of

pretreatment ($F(1,52)=9.17$, $p<.01$) but not RS102221 dose ($F(2,52)=.911$, $p>.05$). Post hoc analyses showed that only the 0.5mg/kg RS102221 dose significantly increased activity counts above the vehicle group in saline pretreated rats. The response of the MDMA pretreated group to 1.0mg/kg RS102221 was significantly greater than the response to the 0.5mg/kg dose, indicating a rightward dose-effect shift ($p<.05$).

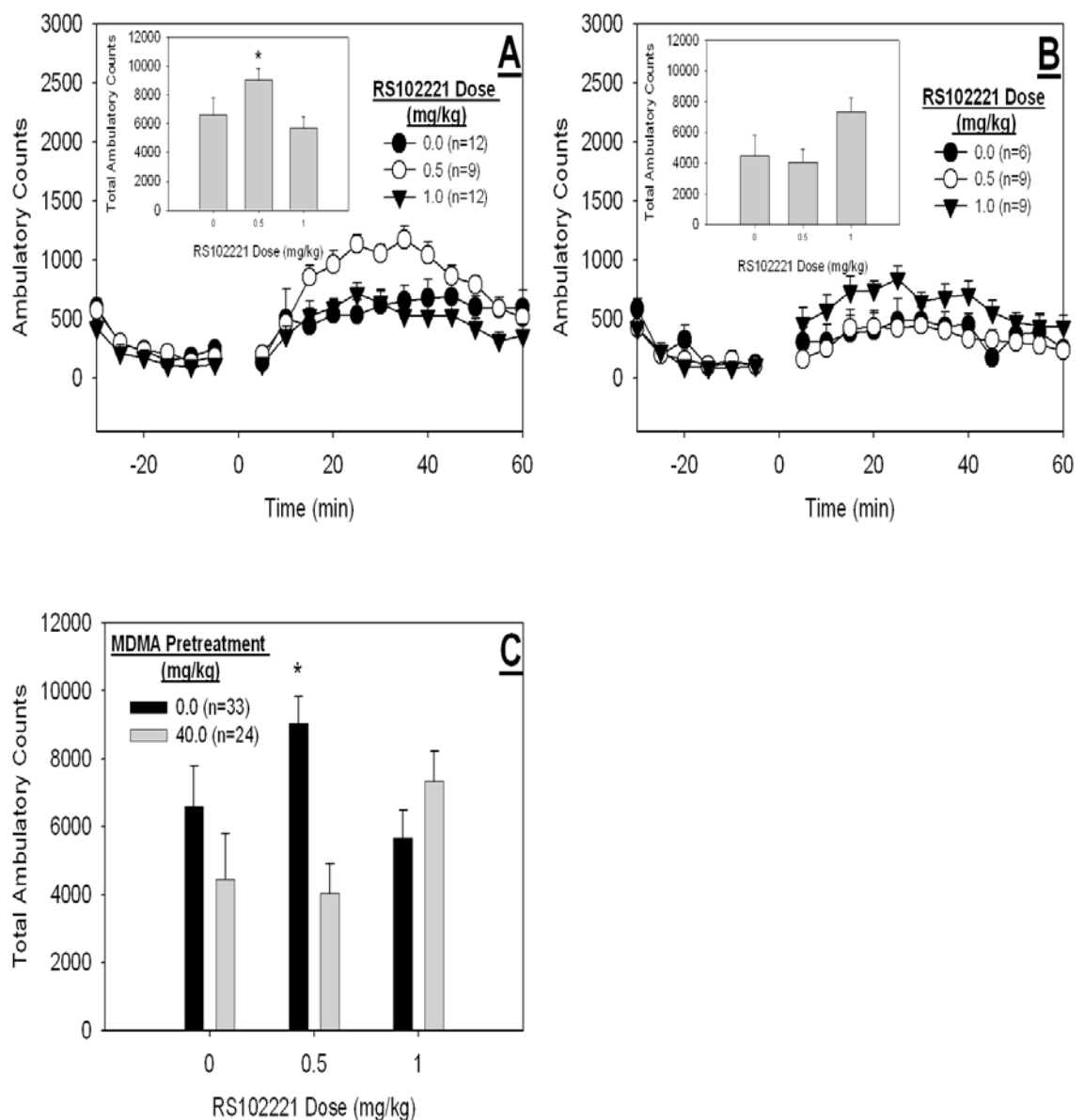


Figure 18 Ambulatory counts produced following MDMA (5.0mg/kg) and RS 102221 (0.0, 0.5 or 1.0mg/kg) administration, where top panels show the time course of the mean ambulatory counts in saline (Figure 18A) and MDMA (Figure 18B) pretreated rats (+SEM). Insets show total ambulatory counts at each RS 102221 dose for the 60-minute period post MDMA injection for each of the pretreatment groups (+SEM). Figure 18C shows total ambulatory counts produced following all RS 102221 doses for the 60-minute period post MDMA injection for the two pretreatment groups (+SEM). Significant difference relative to vehicle group within pretreatment condition* $p < .05$.

DOI-Induced Wetdog Shakes

Table 3 shows that DOI produced significantly more WDS in saline than MDMA pretreated rats. Univariate analyses (pretreatment X DOI dose) revealed a significant interaction between these factors ($F(1,18)=16.38$, $p<.01$) as well as main effects of DOI dose ($F(1,18)=18.50$, $p<.001$) and pretreatment ($F(1,18)=14.40$, $p<.01$).

DOI (mg/kg)	SALINE	MDMA
0.0 (n=6 per group)	1.0 ± 0.4	1.2 ± 0.3
1.0 (n=6 per group)	6.5 ± 1.1 *	1.3 ± 0.2

Table 3 Mean total number of DOI-induced WDS during a 10-minute recording period in MDMA and saline pretreatment groups + SEM. Significant difference relative to vehicle group * $p<.001$

Ritanserin-Induced Attenuation of MDMA-Produced Hyperactivity

Figures 19A and 19B show the time course for the dose effects of ritanserin (0.0, 2.5, 5.0 and 10.0mg/kg) on MDMA (10.0mg/kg, IP)-produced locomotor activity in saline and MDMA-pretreated rats. A 3-Way ANOVA with time as the repeated measure (time X ritanserin dose X pretreatment) revealed a significant interaction between these factors ($F(16,387)=1.92$, $p>.05$) and a main effect of ritanserin dose ($F(3,74)=10.16$, $p<.001$). Post hoc analyses showed that all doses of ritanserin dose-dependently and significantly attenuated MDMA-produced hyperactivity in the saline pretreated rats, where the 10mg/kg dose appeared to lose some selectivity. The MDMA pretreated group was unresponsive to ritanserin, although there was a tendency towards attenuation.

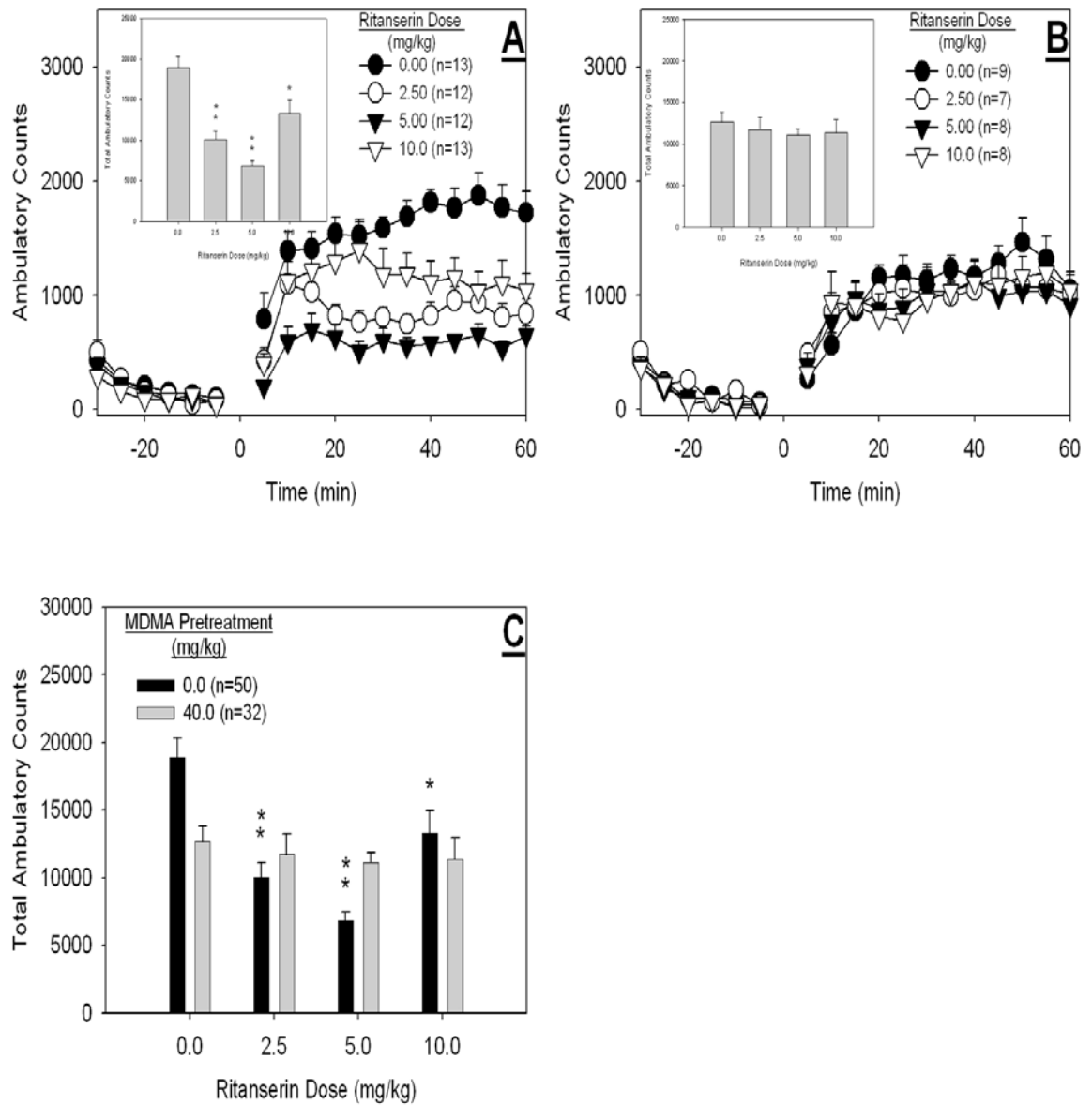


Figure 19 Ambulatory counts produced following MDMA (10.0mg/kg) and ritanserin (0.0, 2.5, 5.0 or 10.0mg/kg) administration, where top panels show the time course of the mean ambulatory counts in saline (Figure 19A) and MDMA (Figure 19B) pretreated rats (+SEM). Insets show total ambulatory counts at each ritanserin dose for the 60-minute period post MDMA injection for each of the pretreatment groups (+SEM). Figure 19C shows total ambulatory counts produced following all ritanserin doses for the 60-minute period post MDMA injection for the two pretreatment groups (+SEM). Significant difference relative to vehicle group within pretreatment condition* $p<.01$, ** $p<.001$.

Overview

The present study investigated the contribution of decreased SERT densities and/or reduced responsiveness of 5-HT_{2c} and 5-HT_{2a} receptors to MDMA-induced tolerance. The MDMA pretreatment regimen used in the present study previously produced tolerance to several behavioural effects of MDMA which were attributable to serotonergic deficits (Shankaran and Gudelsky, 1999). Paroxetine binding verified that the pretreatment regimen produced comparable decreases in SERT binding densities to those previously reported (Scanzello et al., 1993; Fischer et al., 1995). Neurochemical studies have documented the time course for recovery of these deficits, so the present study investigated whether recovery of function was also evident. Since it was presumed that functional and neurochemical recoveries were related, the effect on tolerant locomotor behaviour of repeatedly challenging 5-HT systems with MDMA was investigated. Further, the functional implications of decreased SERT densities were assessed by examining the ability of the SERT selective ligand, clomipramine, to block MDMA-produced hyperactivity in tolerant animals.

Previous investigations revealed that 5-HT_{2c} receptor-mediated m-CPP-produced behaviours remained unchanged (Bull, 2003), whilst 5-HT_{2a} receptor-mediated DOI-produced behaviours were diminished following MDMA exposure (Bull, 2004). The present study aimed to determine whether these findings were replicable, using improved methodologies. However, the functions whereby 5-HT_{2a} and 5-HT_{2c} receptors modulate MDMA-produced hyperactivity might be distinct from behaviour produced by agonists such as m-CPP and DOI. To these ends, two novel assays were developed that combined MDMA with 5-HT_{2c} or 5-HT_{2a} receptor antagonists to directly assess receptor ability to modulate MDMA-produced hyperactivity following MDMA exposure.

MDMA-Produced Hyperactivity

Acute MDMA Response

MDMA produced dose-dependent increases in locomotor activity in both measuring apparatus (Figure 1 and 2), which concurs with similar studies (Kehne et al., 1996; Herin et al., 2005). Dose-dependent MDMA-induced hyperactivity has also been reliably paired with dose-dependent elevations in 5-HT and DA levels in the nucleus accumbens (Baumann et al., 2005). Release of DA contributes to hyperactivity, as increased DA outflow and concomitant hyperactivity after MDMA administration (Yamamoto and Spanos, 1988; Bubar, 2003) was attenuated by DA receptor antagonists (Kehne et al., 1996; Bubar et al., 2004; Daniela et al., 2004) or 6-hydroxydopamine lesions (Gold et al., 1989b). However, the effects of MDMA on 5-HT release are predominant compared to other psychostimulants (Crespi et al., 1997; Baumann et al., 2005), which has been attributed to the high affinity for the SERT (Battaglia et al., 1988).

MDMA-induced 5-HT levels were elevated more rapidly than DA immediately following administration. MDMA has some affinity for the DAT, but it is likely that increased 5-HT levels indirectly release DA via facilitatory 5-HT receptors, such as the 5-HT_{1b} and 5-HT_{2a} subtypes (Benloucif and Galloway, 1991; Parsons and Justice, 1993; Yamamoto et al., 1995). The differential time course for the elevation of 5-HT and DA levels after MDMA support this idea. For example, following MDMA administration, microdialysis revealed an increase in DA that was delayed in relation to 5-HT increases (White et al., 1994). It was suggested that MDMA might produce an initial but relatively small increase in DA, probably via DAT-mediated mechanisms, whereas the later increase might be 5-HT₂ receptor-mediated (White et al., 1994). This idea was further supported in that local 5-HT application to the nucleus accumbens induced DA release (Jacocks and Cox, 1992) and TTX largely attenuated DA elevations, suggesting impulse-mediated mechanisms (Yamamoto et al., 1995).

Evidence suggests that 5-HT is the main contributor to MDMA-produced hyperactivity, as 5-HT depletion following 5,7-DHT lesions or pCPA administration attenuated the expression of MDMA-produced hyperactivity (Callaway et al., 1990; Kehne et al., 1996a). The MDMA 5-HT-releasing mechanism was purported to be SERT-mediated (Hekmatpanah and Peroutka, 1990; Berger et al., 1992; Rudnick and Wall, 1992; Gudelsky and Nash, 1996; Pifl et al., 2005), supported by the observation that pretreatment with SSRIs such as clomipramine, fluoxetine, sertraline and zimelidine completely or partially attenuated MDMA-produced hyperactivity (Callaway et al., 1990; Callaway and Geyer, 1992b) and SERT knockout mice did not exhibit hyperactivity following MDMA administration (Bengel et al., 1998). Additionally, pretreatment with 5-HT_{1b}, 5-HT_{2a} and 5-HT_{2c} receptor ligands modulate the expression of MDMA-produced hyperactivity (Bankson, 2002; Herin et al., 2005; Fletcher et al., 2006).

The different measuring apparatus used in the present study yielded different MDMA dose effect curves. The MDMA used for each experiment, however, was prepared in a similar manner and equivalent doses administered. These discrepancies can be explained in terms of the differential mechanisms by which these apparatus measured locomotor activity. The MDMA dose effect data produced by the older equipment shows an ascending curve, where the 20.0mg/kg MDMA dose appears to produce maximal activity levels (Figure 1). Any beam break was counted by the computer as an ‘activity count’, thus all forward locomotion, head weaving, tail movements would all be counted as ‘activity’. Behaviours such as head weaving are components of the 5-HT syndrome, which tend to be exhibited predominantly at the higher MDMA doses (Spanos and Yamamoto, 1989). This equipment, therefore, did not allow the differentiation of the aforementioned behaviours.

The newer Med-PC equipment was considerably more sensitive in that it could differentiate between forward locomotion (where distance was travelled in order for the movement to be registered as an ‘ambulatory count’) and head weaving (termed

‘stereotypy counts’, which are small movements made when the animal remains stationary). The MDMA dose effect curve produced by the newer equipment revealed that 10.0mg/kg MDMA produced maximal levels of forward locomotion, whilst 20.0mg/kg did not produce greater forward locomotion than the 5.0mg/kg dose (Figure 2). The combined data from the old and new equipment indicate that the 20.0mg/kg MDMA dose produced non-selective behavioural activation. There were high levels of beam-breaks after 20.0mg/kg MDMA as shown by the older apparatus (Figure 1), but the Med-PC data revealed that forward locomotion was reduced at this dose (Figure 2). In order to examine the effects of MDMA pretreatment on the ability of MDMA to increase forward locomotion as measured using the Med-PC equipment, the 2.5, 5.0 and 10.0mg/kg MDMA doses were selected because the 20.0mg/kg dose produced non-selective effects.

Effects of MDMA Pretreatment

Acute Response to MDMA

Despite large decreases in SERT binding densities (Table 2), baseline locomotor behaviour following vehicle challenge was unaffected by MDMA pretreatment (Figure 12). These findings contrast with the hyperactivity that has been observed following neurotoxic 5,7-DHT lesions (Kostowski, 1968; Fibiger HC, 1971; Borbely, 1973; Jacobs, 1975; Marsden and Curzon, 1976; Steigrad et al., 1978; Dringenberg, 1995). Thus, MDMA-pretreatment must produce deficits that are not as extensive or widespread as these other more radical and neurotoxic treatments. It is not likely that higher MDMA doses than those used in this study would have altered baseline activity, as pretreatment with a 160.0mg/kg MDMA did not alter baseline activity (McNamara et al., 1995). These findings are consistent with the idea that MDMA exposure produces serotonergic alterations rather than neurotoxicity (Baumann et al., 2006). The present results also indicate that MDMA-produced serotonergic deficits are not functionally evident under

baseline conditions. This concurs with other studies that have also reported no change in locomotor (McNamara et al., 1995; Bull, 2003) or EPM (Bull, 2004; Sumnall et al., 2004) behaviours following MDMA pretreatment.

Behavioural alteration following MDMA pretreatment might depend upon which behaviour is examined. For example, although locomotor (McNamara et al., 1995; Bull, 2003) and EPM (Bull, 2004; Sumnall et al., 2004) behaviours were consistently unaffected following MDMA pretreatment, altered anxiety states were detected in the social interaction and emergence tests (Gurtman et al., 2002; McGregor et al., 2003; Thompson et al., 2004). Although 5-HT has been shown to have a role in numerous baseline behaviours (locomotor activity, emergence behaviour, social interaction, EPM tests and the FST), there are also varying contributions from other neurotransmitter systems, brain regions and 5-HT receptor subtypes. Since MDMA-produced serotonergic deficits were dose and brain region-dependent (McGregor et al., 2003), it follows that some behaviours might be more susceptible to MDMA pretreatment than others. For instance, social interaction has been associated with postsynaptic 5-HT_{1A} receptor activation (Morley et al., 2005), and MDMA substantially alters functional properties, mRNA expression and binding densities of this receptor (Aguirre et al., 1995; Aguirre et al., 1997; Aguirre, 1998). This might explain why decreased social interaction has been frequently reported following MDMA exposure. Other behaviours that might be less reliant on single contributors and involve input from numerous 5-HT receptors and neurotransmitter systems, such as exploratory EPM behaviour (Rodgers et al., 1997), might be less affected.

Extracellular 5-HT dialysate levels in MDMA pretreated rats were comparable to controls (Matuszewich et al., 2002), which might also explain unchanged baseline locomotion. Indeed, it was observed that 5-HT release was consistently impaired in response to pharmacological, physiological or stressful stimuli in MDMA pretreated animals (Gartside et al., 1996; Shankaran and Gudelsky, 1999; Matuszewich et al., 2002). These findings might explain why rats tolerant to the behavioural effects produced by an

acute MDMA challenge showed little change in baseline behaviour (Callaway and Geyer, 1992a; Shankaran and Gudelsky, 1999) and suggest that pharmacological challenge might be required for functional impairments to be evident.

Indeed, MDMA pretreatment in the present study produced locomotor tolerance to the MDMA (5.0 and 10.0mg/kg) doses that produced significant hyperactivity (Figure 12 and 13E). The 2.5mg/kg MDMA dose did not significantly elevate baseline ambulatory counts (Figure 13B), and there were no differences as a function of pretreatment. This is consistent with the idea that the serotonergic system must be pharmacologically challenged to observe deficits, but challenged to an extent where significant behavioural effects are evident.

Tolerance to the activating effects of MDMA has been associated with serotonergic deficits. Sprague Dawley rats that received an identical pretreatment regimen to the present study exhibited diminished dialysate striatal 5-HT release and tolerance to the behavioural effects of acute MDMA (Shankaran and Gudelsky, 1999). It was unclear whether these observations were attributable to MDMA-induced attenuation of 5-HT releasing abilities or vesicular 5-HT depletions, however, MDMA-induced increases in dialysate DA levels were comparable between pretreatment groups, suggesting that the diminished serotonergic response was associated with the tolerant behavioural profile. Further, pharmacologically induced 5-HT depletion attenuated the ability for MDMA to produce hyperactivity (Callaway et al., 1990; Kehne et al., 1996a), which presumably mirrors the tolerance to MDMA observed by Shankaran et al. (1999) and the present results. Since the present study utilised a regimen that produced extensive 5-HT depletions and reduced SERT densities (Scanzello et al., 1993; Fischer et al., 1995), tolerance was likely due predominantly to serotonergic deficits.

Pharmacokinetics could affect behavioural response to acute MDMA challenge. MDMA is demethylenated by the CYP2D1 hepatic cytochrome P450 enzymes in the rat (Kumagai et al., 1994). This enzyme is polymorphically expressed, therefore different rat

strains have been purported to have differential metabolism of MDMA (Malpass et al., 1999). For example, the Dark Agouti rat exhibits enzymic deficiencies whereas the Sprague Dawley strain has more effective CYP2D1 enzyme capacity. Behavioural differences in response to MDMA were observed, which were purported to relate to differential drug metabolism. Dark Agouti rats did not exhibit increased locomotor activity following 2.0, 5.0 or 10.0mg/kg MDMA, whilst Sprague Dawley rats showed dose-dependent increases. The relationship between the acute locomotor response to MDMA and enzyme function was unclear, but these findings suggest that tolerance to MDMA could be related to impaired enzyme function. The effects of MDMA exposure on these hepatic enzymes has not been characterised, but it has been reported that methamphetamine pretreatment decreased cytochrome P450 enzyme activity (Yamamoto et al., 1988). It is possible that enzymic deficiencies and altered MDMA metabolism were contributors to behavioural tolerance.

Although kinetics might have a role, behavioural tolerance to MDMA was relatively selective. For example, MDMA pretreated rats that were tolerant to the effects of MDMA exhibited a sensitised response to amphetamine challenge (Callaway and Geyer, 1992a). Amphetamine and MDMA are metabolised via similar pathways (Yamamoto et al., 1984; Kumagai et al., 1994), therefore the pharmacokinetics explanation could not account for these differences. Since diminished 5-HT levels have potentiated the locomotor response to amphetamine (Segal, 1976), it is most likely that these differential responses were attributable to MDMA-induced 5-HT depletions. These differential responses also indicate that behavioural tolerance to MDMA is not a general suppression of locomotor behaviour, as in this instance, tolerance to both drugs would have been expected.

The specificity of tolerance for certain serotonergic compounds has been reported. For example, m-CPP-produced hypolocomotion and anxiogenesis in the EPM were not altered by MDMA pretreatment (Bull, 2003), whereas behavioural responses to DOI (Bull, 2004) or the 5-HT₁ agonist, RU24969 (Callaway and Geyer, 1992a) were diminished. These

findings suggest that the receptors and associated mechanisms that contribute to MDMA-produced hyperactivity undergo desensitisation or downregulation, producing tolerance to compounds that selectively target affected systems.

Repeated MDMA Exposure

In the present study, MDMA and vehicle pretreatment groups were challenged on a weekly basis with MDMA for 1 month. Tolerance to MDMA was persistent in the MDMA pretreated group, whereas the saline pretreated group appeared to become increasingly tolerant to the 10.0mg/kg MDMA challenge over the 4-week period (Figure 12). Within subject comparisons revealed that there were significant differences of MDMA and pretreatment between weeks 2 and 5. These effects were attributed to the decreasing locomotor response of the saline pretreated group to MDMA, since responses of other groups were relatively stable. Interestingly, these now tolerant rats had been administered a total of 40.0mg/kg MDMA after the last test day, which was equivalent to the total dose administered initially to the MDMA pretreated rats. The level of hyperactivity in this group was comparable to the MDMA pretreated group at week 2, suggesting that these procedures, although spread over a 4-week period, might have produced equivalent serotonergic deficits.

Contrary to the present findings, repeated administration of MDMA followed by a withdrawal period, has produced an augmented response to subsequent challenge (Spanos and Yamamoto, 1989; Dafters, 1995; Kalivas et al., 1998; McCreary et al., 1999; Ramos et al., 2004, 2005). The pretreatment regimen and consequent duration of drug exposure might determine whether tolerance or sensitisation to MDMA is expressed. Indeed, chronic cocaine administration via osmotic minipumps produced tolerance to the locomotor effects of cocaine (Reith et al., 1987; Izenwasser and French, 2002; Hope et al., 2005), whereas repeated daily injections produced sensitisation (Reith et al., 1987; Martin-Iverson and Burger, 1995; Izenwasser and French, 2002; Hope et al., 2005). Intermittent exposure to

methamphetamine also resulted in behavioural sensitisation (Hirabayashi et al., 1991; Kuribara, 1996; Davidson et al., 2005) whereas chronic exposure via minipump administration resulted in tolerance to the behavioural effects of the drug (Kuribara, 1996; Davidson et al., 2005). Similarly, either sensitised or tolerant responses have been observed following either intermittent or chronic exposure to methylphenidate (McNamara et al., 1993; Gaytan et al., 1997; McDougall et al., 1999) or morphine (Kuribara, 1996).

Regimens typically used to produce locomotor sensitisation to MDMA might involve a specific set of changes to the receptors that produce potentiated hyperactivity. Sensitisation-producing regimens usually involve consecutive daily MDMA injections in a test chamber followed by a withdrawal period before MDMA challenge (Spanos and Yamamoto, 1989; Dafters, 1995; Kalivas et al., 1998; McCreary et al., 1999; Ramos et al., 2004, 2005). The present study indicates that pretreatment protocols necessary to produce sensitisation might be more specific than those required to produce tolerance, as 40.0mg/kg MDMA administered either in 1 day, or over 1 month both produced locomotor tolerance.

Different MDMA pretreatment regimens might differentially affect receptor populations that produce hyperactivity. For example, decreased sensitivity of receptors that facilitate MDMA-produced hyperactivity, such as the 5-HT1b or 5-HT2a subtypes, might contribute to tolerance. Conversely, receptor supersensitisation might lead to a sensitised behavioural response. Recently, repeated treatment with a 5-HT2a receptor agonist produced a sensitised response to MDMA challenge (Ross et al., 2006), suggesting that MDMA-produced sensitisation might be partly mediated via the 5-HT2a receptor. Additionally activation of the inhibitory 5-HT2c receptors in the prefrontal cortex prevented the expression of locomotor sensitisation in MDMA-pretreated animals (Ramos et al., 2005). Conversely, chronic MDMA exposure produced tolerance to MDMA and 5-HT1 (Callaway and Geyer, 1992a) and 5-HT2 (Bull, 2004) receptor agonists, suggesting decreased receptor responsivities.

It is possible that environmental conditioning might affect the expression of sensitisation or tolerance. Only one previous investigation has reported locomotor tolerance to MDMA challenge following pretreatment (Callaway and Geyer, 1992a). The animals were pretreated with MDMA in the home cage and exhibited tolerance to MDMA-produced hyperactivity when placed in the test chamber and injected with MDMA 3 days later. It has been well established that environmental conditioning significantly enhances the sensitised locomotor response (Badiani et al., 1997; Bonate et al., 1997; Uslander et al., 2001). These principles also apply to MDMA, as MDMA pretreated rats exhibited potentiated locomotion in response to environmental cues previously paired with the drug (Gold and Koob, 1989a). It was suggested that because there were no drug-test chamber pairings in Callaway and Geyer's study (1992), a sensitised response was not observed (Kalivas et al., 1998). The present study refutes this claim, as the saline pretreated group that received weekly MDMA injections in the test chamber were exposed to 4 drug-test chamber pairings, but did not exhibit sensitisation. Additionally, activity of the rats that received saline did not change with each week, reducing the possibility that progressive tolerance might have been due to increasing familiarity with the test chamber. These observations indicate that tolerance was predominantly attributable to MDMA-produced pharmacological alterations rather than conditioning effects.

The MDMA dosing regimen used in the present study was not selected to replicate human use patterns, as species differences, such as pharmacokinetic and neurophysiological considerations (de la Torre and Farre, 2004) render the task of determining equivalent doses difficult. However, the tolerance to the behavioural effects of MDMA reported in this study might be highly relevant to the effects reported by humans who consume MDMA. Indeed, the most frequently reported consequence of MDMA use in humans was tolerance to the subjective effects of the drug (Verheyden et al., 2003; Scholey et al., 2004; Parrott, 2005). It is of interest to note that recreational MDMA users typically

ingest the drug on weekends (de Almeida and Silva, 2003), which might be comparable to the rat model in the present study that also produces tolerance (Figure 12).

Comparison Between 2- and 12-Week Recovery Periods

The data from both the present (Table 2) and past studies indicate that MDMA exposure altered serotonergic neurotransmission. Despite these extensive changes, SERT binding reductions and 5-HT depletions produced by the pretreatment regimen used in this thesis exhibited significant recovery to control levels over time (Scanzello et al., 1993; Fischer et al., 1995). Other studies have also shown recovery after a similar time period following MDMA pretreatment for other markers of serotonergic integrity. For example, TPH levels increased as did TPH mRNA expression in cortical regions (Garcia-Osta et al., 2004) and 5-HT_{2a} receptor binding densities (Reneman et al., 2002) recovered to control levels. These neurochemical findings are consistent with the notion that MDMA produces neuroadaptations that recover over time.

In order to elucidate whether tolerance to MDMA-produced hyperactivity (Figure 13) also exhibited recovery over time, a 12-week period after MDMA exposure before behavioural testing was imposed for additional groups of rats. Following this more extensive period, differences in responsiveness to MDMA between pretreatment groups were no longer apparent (Figure 14). These findings concur with Callaway and Geyer's (1992a) study, where a higher MDMA dose treatment regimen was used (10mg/kg X 8, total 80mg/kg MDMA) and tolerance to MDMA occurred after 3 days and recovery 3 weeks later.

Comparisons between the 2 and 12-week experiments reveal that the dose effects of MDMA differ between the saline pretreated groups. For instance, the response to 5.0mg/kg MDMA was equivalent between groups, whereas the 12-week rats showed a much lower response to the 10.0mg/kg MDMA dose (Figure 13E and 14E). Although the differences between pretreatment groups were not significant in the 12-week rats, there appears to be a

tendency for lower responsiveness to MDMA in the MDMA pretreated group. It was a possibility that there were no pretreatment differences because control values were too low. However, this was a between subjects design, where the only difference between the 2 and 12 week groups was recovery time and rat age at the time of the behavioural tests. Aging has been associated with decreased 5-HT-containing fibres, 5-HT levels, 5-HT receptor densities (Keck and Lakoski, 1997), SERT binding sites (Kakiuchi et al., 2001) and DA receptor densities (Hyttel, 1987) in the rat brain. Consistent with these neurochemical changes, locomotor response to amphetamine was reduced in older rats (Huang et al., 1995). Reduced response and a different MDMA dose-effect profile exhibited by the 12-week group were consistent with the idea that the aging process involves substantial changes the serotonergic system. Further, the comparable response to MDMA between pretreatment groups demonstrates considerable neuroadaptation following MDMA exposure, as aging was also continually diminishing serotonergic function.

Data from the present thesis (Figure 12) suggests that repeated exposure to MDMA might prevent or slow the recovery of function in the MDMA pretreated group. Tolerance diminished 3 weeks after MDMA preexposure in rats that received much higher doses of MDMA than those used in the present study (Callaway and Geyer, 1992a). Thus it was expected that the MDMA pretreated rats in the present study would have started showing behavioural recovery by 3 weeks. However, tolerance persisted over the 7-week period when MDMA was administered weekly. It is possible that persistent tolerance might be related to the inability for MDMA-produced serotonergic deficits, such as reduced SERT binding densities or 5-HT depletions, to recover when MDMA is constantly being administered. In support for this suggestion, our laboratory conducted paroxetine binding on rats that had received the MDMA pretreatment regimen used in the present study and compared this group to MDMA self-administering rats that were receiving MDMA daily.

The results showed that both groups exhibited comparable decreases in SERT binding densities (unpublished results).

The Serotonin Transporter

Preliminary Clomipramine Experiments

Clomipramine pretreatment did not significantly affect baseline locomotor activity, although there was a tendency towards hypolocomotion (Figure 3). Doses beyond 5.0mg/kg were not assessed in this experiment, because clomipramine non-selectively binds 5-HT receptors and has cholinergic effects at these doses (McTavish and Benfield, 1990) and the aim was to develop a SERT-selective behavioural assay. These results were not surprising, as the ability for SSRIs to produce hypolocomotion was reported as a modest effect (Rodriguez Echandia et al., 1983; To et al., 1999; McMahon and Cunningham, 2001a). These findings concur with existing literature showing that acute SSRI administration produce few consistent and easily measurable behavioural effects.

The behavioural effects of SSRIs, however, are consistently evident when they are combined with MDMA. In the present study, clomipramine dose-dependently attenuated 5.0mg/kg MDMA-produced hyperactivity (Figure 4). These findings concur with a similar study, where SSRI pretreatment inhibited MDMA-produced hyperactivity (Callaway et al., 1990). MDMA-produced increases in extracellular 5-HT occur via the SERT (Hekmatpanah and Peroutka, 1990; Berger et al., 1992; Rudnick and Wall, 1992; Gudelsky and Nash, 1996; Iravani et al., 2000; Pifl et al., 2005), as shown when SSRIs administered in conjunction with MDMA antagonised the MDMA-induced synaptic 5-HT increase (Hekmatpanah and Peroutka, 1990; Berger et al., 1992; Rudnick and Wall, 1992; Gudelsky and Nash, 1996). In vitro experiments showed that clomipramine alone did not affect SERT-mediated 5-HT release, but completely abolished MDMA-induced release (Pifl et al., 2005). The clomipramine-induced attenuation of MDMA-produced hyperactivity is

attributable to decreased 5-HT release, as this leads to reduced activation of postsynaptic 5-HT receptors that facilitate MDMA-produced hyperactivity (Bankson, 2002).

It is possible that the effects of SSRIs on locomotion were due to general behavioural suppression, but the evidence suggests that these effects were SERT-selective. Firstly, although SSRIs have been purported to produce hypolocomotion, doses that did not significantly affect baseline activity levels (Figure 3) significantly attenuated MDMA-produced hyperactivity (Figure 4). Additionally, Callaway et al. (1990) reported that only MDMA-produced forward locomotion, but not increases in holepokes and rearings, were attenuated by SSRI pretreatment.

When clomipramine was combined with 10.0mg/kg MDMA, there was a tendency towards attenuation, but these effects were not significant (Figure 5). The inability of clomipramine to affect behaviour produced by high-dose MDMA might reflect saturated SERT-binding and concomitant binding to lower affinity sites. For example, at the 10.0mg/kg MDMA dose, all SERT sites might be occupied, thus sites such as the NET and DAT with lower affinity for MDMA could be bound (Battaglia et al., 1988). Hyperactivity at the highest dose, therefore, might have a larger NE and DA component than that produced by the lower MDMA dose. This might explain why SERT blockade did not greatly impact hyperactivity produced by the 10.0mg/kg MDMA dose.

Effects of MDMA Pretreatment

Paroxetine Binding

The paroxetine binding data showed significant and region-dependent reduction of SERT sites (Table 2), which concurs with other studies that used this pretreatment regimen (Scanzello et al., 1993; Fischer et al., 1995). The present results extended these findings to include the brain stem region, which showed 37% reductions of control levels. It is surprising that no studies that have assessed the impact of MDMA exposure on behaviour

have assessed brain stem SERT densities, as 5-HT neurons originating in the brain stem raphe nuclei project to numerous other structures (Molliver, 1987). Altered neurotransmission in this region would have the potential to affect a large number of brain functions. Since it was reported that the raphe nucleus contained the highest densities of SERT (Lin et al., 2004), decreases in this population following MDMA pretreatment would likely affect ascending structures. For example, MDMA might not significantly elevate brain stem 5-HT levels in MDMA pretreated rats, as this is a predominantly SERT-mediated effect (Hekmatpanah and Peroutka, 1990; Berger et al., 1992; Rudnick and Wall, 1992; Gudelsky and Nash, 1996; Iravani et al., 2000; Pifl et al., 2005). Decreased 5-HT release in this region might reduce the activation of serotonergic neurons that project to other structures in the brain that are involved in the behavioural responses to MDMA. Indeed, the brainstem projects to the hippocampus, frontal cortex and caudate putamen (Molliver, 1987) and these regions exhibited MDMA-induced SERT binding decreases (Table 2), which might be independent from those observed in the brain stem.

The frontal cortex and hippocampus have both been associated with 5-HT-mediated hyperactivity. Local infusions of 5-HT receptor agonists/antagonists into the frontal cortex modulated stimulant-produced hyperactivity (Filip and Cunningham, 2003) and it was also demonstrated that 5-HT_{2c} receptors in this region mediated locomotor sensitisation to MDMA following a chronic exposure regimen (Ramos et al., 2005). The hippocampus contributes to hyperactivity, as systemic administration of a monoamine oxidase inhibitor that elevated hippocampal 5-HT levels produced hyperactivity (Takahashi et al., 2000). A role for the hippocampus in these 5-HT-mediated effects was verified when hyperactivity was induced by direct 5-HT or monoamine oxidase infusion. It is possible that hippocampal 5-HT_{2c} receptor activation contributed to this hyperactivity, as hyperactivity was elevated by the direct infusion of m-CPP into this area (Takahashi et al., 2001). 5-HT_{2c} receptor activation in most regions produces hypomotility (Klodzinska et al., 1989; Kennett et al., 1994a; Martin et al., 1998; McCreary et al., 1999; Gleason et al., 2001), but

evidence suggests that a population exists in the hippocampus with a facilitatory role, as a selective 5-HT_{2c} antagonist attenuated m-CPP-produced hyperactivity.

Behaviour is the product of complex interactions between different brain structures and neurotransmitter systems. Indeed, it appears that the frontal cortex and hippocampus interact and contribute to 5-HT-mediated hyperactivity, but the striatum might have less of a role. Systemic administration of a monoamine oxidase inhibitor increased 5-HT levels in all three regions, which was paired with hyperactivity (Takahashi et al., 2000). Subsequent experiments showed that hyperactivity was only exhibited following local infusions into the hippocampus or prefrontal cortex and not the striatum. Further, these regions were interconnected, as local infusion of tetrodotoxin (TTX) into the hippocampus prevented hyperactivity produced by 5-HT infusion into either area. However, TTX infusion into the prefrontal cortex failed to affect hippocampal-mediated hyperactivity, suggesting that the hippocampus was directly involved in locomotor activity and that the prefrontal cortex might facilitate hippocampal control of locomotion.

MDMA pretreatment reduced SERT binding densities to the greatest extent in the brain stem, thereby producing widespread alterations in serotonergic neurotransmission. The brain stem projects to the hippocampus, striatum and frontal cortex, which would receive altered signals. Additionally, these regions also exhibited decreased SERT binding densities, although to a lesser degree. It was not clear whether these decreases were independent from those observed in the brain stem, but since the hippocampus and frontal cortex are involved in 5-HT mediated hyperactivity, these local SERT changes would also be expected to affect MDMA-produced locomotor behaviour.

Clomipramine-Induced Attenuation of MDMA-Produced Hyperactivity

The behavioural effects of MDMA pretreatment were assessed using 5.0mg/kg MDMA, as this was the only dose where significant clomipramine-induced attenuation of MDMA-produced hyperactivity was evident (Figure 4). The results showed that

clomipramine significantly attenuated MDMA-produced hyperactivity in the saline pretreated rats (Figure 15A), which closely resembled the results obtained in the preliminary studies (Figure 4). The 1.25mg/kg clomipramine dose significantly attenuated hyperactivity in the saline pretreated group, whereas it just missed significance in the preliminary work (Figure 4). The response to 5.0mg/kg MDMA was equivalent between control groups from both experiments, indicating that the 1.25mg/kg clomipramine was the 'threshold dose' for attenuation.

It is of interest to note that clomipramine failed to completely block MDMA-produced hyperactivity (Figures 4 and 15), even when doses as high as 20.0mg/kg were administered (Figure 4). These findings suggest that effects at the SERT cannot fully account for hyperactivity produced by MDMA and indicate that other mechanisms might also play a role. Indeed, hyperactivity following administration of psychostimulant drugs has frequently been attributed to effects on central dopaminergic systems (Roberts et al., 1975; Koob et al., 1981; Di Chiara and Imperato, 1988; Gold et al., 1989b; Kehne et al., 1996; Le et al., 1997; Louis and Clarke, 1998; O'Neill and Shaw, 1999; Schindler and Carmona, 2002; Bubar et al., 2004; Daniela et al., 2004). MDMA, like other drugs, increases synaptic DA in addition to its effects on the 5-HT system (Yamamoto and Spanos, 1988; Gough et al., 1991; Rudnick and Wall, 1992; Yamamoto et al., 1995; Shankaran and Gudelsky, 1999) and DA release contributes to MDMA-produced hyperactivity (Gold et al., 1989b; Kehne et al., 1996; Bubar et al., 2004; Daniela et al., 2004). These findings suggest that residual MDMA-induced hyperactivity exhibited by clomipramine-pretreated animals was possibly attributable to elevated DA or NE levels.

Ascending doses of clomipramine failed to affect MDMA-produced hyperactivity in the MDMA pretreated rats (Figure 15B). The preliminary data suggest that residual hyperactivity evident at the highest clomipramine doses, as previously discussed, was due mostly to DA and NE release. Decreased SERT densities in MDMA pretreated rats might, therefore, produce a comparable situation to high dose clomipramine pretreatment in drug-

naïve rats. In this instance, administering clomipramine would not be expected to produce any notable behavioural effect. The present data suggest that the MDMA-produced hyperactivity in the pretreated rats was not mediated by remaining SERT sites, as even the highest clomipramine dose was ineffectual.

Alternatively, the hyperactivity exhibited by the MDMA pretreated rats was too low to be attenuated further. It might have resolved this question to examine the effects of clomipramine pretreatment in conjunction with 10.0mg/kg MDMA, as this would have presumably raised the activity levels higher in the MDMA pretreated rats and perhaps allowed for clomipramine-induced attenuation to become evident. The results from the preliminary studies showed, however, that this dose of MDMA appeared to produce hyperactivity that was highly resistant to SERT-manipulation (Figure 5).

There have been no previous attempts to assess the functional status of the SERT following MDMA pretreatment, thus these novel results are the first demonstration that MDMA-reduced SERT binding densities have functional consequences. These experiments were fraught with difficulties, as SERT manipulations are not usually apparent in behavioural models, particularly the acute effects. Although the results must be interpreted cautiously, it can be tentatively concluded when combined with the paroxetine binding data that MDMA pretreated rats were unresponsive to clomipramine due to extensive SERT binding reductions. Additionally, MDMA-produced hyperactivity in MDMA pretreated rats might be mediated predominantly by non-serotonergic mechanisms.

The 5-HT_{2c} Receptor

Preliminary m-CPP Experiments

m-CPP pretreatment produced relatively dose-dependent hypolocomotion in preliminary studies (Figure 6). These findings concur with previous work showing that m-

CPP (Sills et al., 1985; Aulakh et al., 1987; Kennett and Curzon, 1988; Aulakh et al., 1989; Bagdy et al., 1989; Klodzinska et al., 1989; Lucki et al., 1989; Freo et al., 1990; Kennett et al., 1994a; Bonhaus, 1997; Kennett et al., 1997a; Gleason and Shannon, 1998; Gleason et al., 2001) or other more selective 5-HT_{2c} agonists (Martin et al., 1998) produced hypolocomotive effects. Hypolocomotion following 5-HT_{2c} receptor activation has been reliably attenuated by 5-HT_{2c} antagonists (Klodzinska et al., 1989; Kennett et al., 1994a; Martin et al., 1998; McCreary et al., 1999; Gleason et al., 2001) but not 5-HT_{2a} or 5-HT_{2b} antagonists (Gleason et al., 2001). In addition, there were no changes to basal locomotion in 5-HT_{2c} receptor knockout mice after m-CPP administration (Heisler and Tecott, 2000; Dalton et al., 2004). These findings indicate that the suppressive effects of m-CPP were 5-HT_{2c} receptor-mediated and not due to general sedation, although the mechanisms for m-CPP-induced hypolocomotive effects are yet to be determined.

The results indicate that only the 5.0mg/kg m-CPP dose significantly attenuated baseline locomotor activity (Figure 6). Generally, when rats were placed in the test chambers there were initially high activity levels during the habituation phase, which became almost non-existent over time. For these reasons, it was difficult to significantly decrease these activity levels any lower and explains why differences were relatively small between vehicle- and m-CPP-pretreated rats.

The doses selected for emergence were based on the experiment examining the hypolocomotive effects of m-CPP on saline and MDMA pretreated rats (Figure 16). Doses were selected that did not significantly affect baseline locomotion. These doses were not based on the preliminary study examining the hypolocomotive effects of m-CPP (Figure 6), because this was conducted in the old recording equipment. The 5.0mg/kg m-CPP dose significantly decreased baseline locomotion in the preliminary studies, but it was evident that the 2.5mg/kg dose significantly attenuated activity in the saline pretreated rats measured by the new equipment (Figure 16A). These differential effects suggest a lack of consistency between experiments, however, the old apparatus was much less sensitive.

Since the differences in activity between groups were relatively small due to the nature of this assay, it is not surprising that the old recording program failed to detect a significant difference between vehicle and the 2.5mg/kg m-CPP dose. The preliminary studies did, however, indicate which doses to use in the MDMA pretreated rats and provided verification that the results obtained by other researchers regarding the hypolocomotive effects of m-CPP (Sills et al., 1985; Aulakh et al., 1987; Kennett and Curzon, 1988; Aulakh et al., 1989; Bagdy et al., 1989; Klodzinska et al., 1989; Lucki et al., 1989; Freo et al., 1990; Kennett et al., 1994a; Bonhaus, 1997; Kennett et al., 1997a; Gleason and Shannon, 1998; Gleason et al., 2001) were replicable.

In the present study, m-CPP decreased emergence latency (Figure 7), which was consistent with previous findings (Bilkei-Gorzo et al., 1998). Although the effects appeared to be dose-dependent, extensive variability between subjects meant that only the 1.25mg/kg m-CPP dose produced significant increases in emergence latency. This variability might be due to the 'all or nothing' nature of this test, as each rat only received one time score for full body emergence. Conversely, activity counts provide numerous scores within 5-minute blocks, so gradual changes in behaviour and dose-dependent effects are easily observable. Despite this apparent weakness of the m-CPP emergence assay, the lack of confounding locomotor effects were indicative that the test was measuring an alternate function of the 5-HT_{2c} receptor. Indeed, a role for the 5-HT_{2c} receptor was established in that m-CPP-increased emergence latency was attenuated by 5-HT_{2a/2c} antagonists (Bilkei-Gorzo et al., 1998). Additionally, it has been demonstrated that the selective 5-HT_{2c} receptor antagonist, RS102221, dose-dependently attenuated the effects of m-CPP on emergence latencies (Jones 2006, VUW PhD thesis).

Preliminary RS102221 Experiments

MDMA-produced hyperactivity was potentiated by RS102221 pretreatment (Figures 8, 9 and 18A), which concurs with previous studies. 5-HT_{2c} receptor antagonists

have potentiated cocaine (McCreary et al., 1999; Fletcher PJ, 2002a; Filip et al., 2004), amphetamine, methylphenidate, nicotine, morphine, phencyclidine and MDMA-produced hyperactivity (Gold, 1988b; Hutson et al., 2000; Herin, 2001; Bankson, 2002; Fletcher PJ, 2002b; Fletcher et al., 2006). These behavioural effects were attributed to the ability of the 5-HT_{2c} receptor to modulate mesolimbic DA (Millan et al., 1998; Gobert et al., 2000; Lucas et al., 2000; Lucas and Spampinato, 2000; Yan, 2000), for example, pretreatment with SB242084 potentiated the increase in extracellular levels of DA in the nucleus accumbens and striatum elicited by 15mg/kg of cocaine (Navailles et al., 2004).

Since there were no reports where RS102221 had been combined with MDMA, the first experiment aimed to determine which MDMA doses (if any) were most potentiated by the 0.5mg/kg RS102221 (Figure 8). The results showed that RS102221 significantly potentiated 10.0mg/kg MDMA-produced hyperactivity (Figure 8C), which was in agreement with previous findings using selective 5-HT_{2c} antagonists (Fletcher et al., 2006). This MDMA dose was subsequently utilised to assess the effects of a range of RS102221 doses. The results showed that there was a significant main effect of RS102221 dose on MDMA-produced hyperactivity, but the differences between doses were not significant (Figure 9). The 0.5mg/kg dose produced maximal potentiation, whereas the 1.0 and 2.0mg/kg RS102221 doses produced non-selective effects. Similarly in saline pretreated rats, there was potentiation of MDMA-produced hyperactivity following the lower dose of 0.5mg/kg but not following 1.0mg/kg RS102221 (Figure 18A). Non-selective binding might account for these observations, as RS102221 might also block 5-HT_{2a} receptors at this higher dose. Activation of 5-HT_{2a} receptors facilitated MDMA-induced DA outflow (Schmidt et al., 1992b; Schmidt et al., 1994) and selective 5-HT_{2a} receptor antagonists attenuated MDMA-produced hyperactivity (Gold, 1988b; Kehne et al., 1996; Herin, 2001; Bankson, 2002; Fletcher PJ, 2002b; Herin et al., 2005). The additional effect on the 5-HT_{2a} receptors following higher dose administration might be expected to

counteract the effect of selective 5-HT_{2c} receptor antagonism produced by lower RS102221 dose administration.

Of interest, it has been suggested that there are 5-HT_{2c} receptor populations with differential effects on DA (Millan et al., 1998; De Deurwaerdere, 1999; Di Giovanni, 1999; Di Matteo, 2000; Gobert et al., 2000; Lucas et al., 2000; Lucas and Spampinato, 2000; Yan, 2000) and locomotor activity (Takahashi et al., 2001; Filip, 2002; Filip and Cunningham, 2003; Dalton et al., 2004). It is equally possible that the lower RS102221 dose produced potentiated locomotor activity via inhibitory receptors located in an area such as the prefrontal cortex, as locally applied 5-HT_{2c} receptor antagonists potentiated, whilst 5-HT_{2c} receptor agonists attenuated stimulant-produced hyperactivity in this area (Filip and Cunningham, 2003). Following administration of higher RS102221 doses and more widespread 5-HT_{2c} receptor blockade, facilitatory 5-HT_{2c} receptors in the nucleus accumbens or hippocampus might also be affected. 5-HT_{2c} agonists applied directly into this area potentiated stimulant-produced hyperactivity, whereas 5-HT_{2c} antagonists produced attenuation (Takahashi et al., 2001; Filip, 2002), thus, blockade of these receptors might be expected to counteract the potentiated behavioural effects produced by prefrontal 5-HT_{2c} receptor blockade.

The non-significant effects between doses were of little concern, because all of these preliminary studies were conducted in the old measuring apparatus. These results provided an indication as to which doses to use in later studies, and also verified that RS102221 potentiated MDMA-produced hyperactivity.

The ability of the 5-HT_{2c} receptor to modulate MDMA-produced behaviour is likely due to effects on DA systems. Microdialysis studies have shown that 5-HT_{2c} receptor blockade increased, whilst 5-HT_{2c} agonist decreased DA levels in striatum, nucleus accumbens and frontal cortex (Millan et al., 1998; De Deurwaerdere, 1999; Di Giovanni, 1999; Gobert et al., 2000). Potentiated cocaine-produced DA increases in the striatum and nucleus accumbens were also found after 5-HT_{2c} antagonist pretreatment (Navailles et al., 2004).

Additional support for an inhibitory role on DA outflow for the 5-HT_{2c} receptors was demonstrated in that 5-HT_{2c} receptor knockout mice had higher DA levels in the nucleus accumbens than wildtypes following cocaine administration (Rocha et al., 2002). As these findings suggest that 5-HT_{2c} receptor activation inhibits DA release, antagonists allow dopaminergic disinhibition, thereby potentiating MDMA-produced hyperactivity.

It is possible that NE might contribute to the potentiation of hyperactivity. In addition to releasing 5-HT and DA, MDMA, amphetamine, methylphenidate and cocaine increase NE neurotransmission (Florin et al., 1994; Kuczenski and Segal, 1997; Rothman et al., 2001). SB242084 also elevated NE levels (Millan et al., 1998), thus it was possible that this might have been contributing or producing the potentiated behavioural response. A role for NE is not clear, however, as selective NE reuptake inhibitors do not stimulate activity (Brocco et al., 2002; Davids et al., 2002) although amphetamine-produced locomotor activation was blocked by prazosin and clonidine, and enhanced by propranolol (Vanderschuren et al., 2003). The majority of the evidence from other compounds that have been assessed indicates that dopaminergic effects are predominant

The possibility that the potentiation of stimulant-produced hyperactivity was related to altered pharmacokinetics was investigated. The selective 5-HT_{2c} antagonist, SB242084, has reliably potentiated amphetamine, methylphenidate, morphine and MDMA-produced hyperactivity (Fletcher et al., 2006). It was established that SB242084 had negligible affinity for several major cytochrome enzymes responsible for metabolism of cocaine, amphetamine, nicotine, morphine and fenfluramine (Bromidge et al., 1997). It was also reported that SB242084 did not alter brain levels of MDMA, amphetamine or cocaine (Fletcher PJ, 2002). These findings suggest that the ability of SB242084 to potentiate MDMA-produced hyperactivity was not related to altered pharmacokinetics. Although RS102221 is a different pharmacological compound, these findings also suggest that the potentiated response were not attributable to pharmacokinetics.

MDMA pretreatment did not affect the ability of m-CPP to produce hypolocomotion (Figure 16). These findings replicate a similar study that reported that m-CPP-induced hypolocomotion and decreased EPM and open field exploration were unaltered by MDMA preexposure (Bull, 2003). The present findings extend this work, as the MDMA pretreatment regimen used has been extensively characterised and shown to produce serotonergic (Scanzello et al., 1993; Fischer et al., 1995; Shankaran and Gudelsky, 1999; Sumnall et al., 2004; Nair and Gudelsky, 2006) and behavioural (Shankaran and Gudelsky, 1999) deficits. The pretreatment regimen used by Bull and colleagues (2003) had not previously been characterised and only mild 5-HT depletions in hippocampus and frontal cortex were reported.

The young Listar Hooded rats received higher MDMA doses (15.0mg/kg twice daily for 3 days, total 90.0mg/kg) (Bull, 2003) than the rats in the present study. Although the present results suggest that tolerance can be produced despite variation in pretreatment regimen (Figure 12), there was concern that the Listar rats in Bull's study were somewhat more resilient to the effects of MDMA because they were young and small relative to those used in similar studies. These explanations likely account for more extensive MDMA-produced serotonergic deficits evident in older Sprague Dawley rats used in the present study when compared to the young Listar Hooded rats (Colado et al., 1997).

Given these different effects on serotonergic parameters, it was a possibility that that the lack of change in m-CPP-induced behavioural responses following MDMA pretreatment might have been attributable to more resilient rats rather than no change to 5-HT_{2c} receptors. Additionally, an m-CPP dose was used in the hypolocomotion tests that did not significantly attenuate baseline activity in the saline pretreated rats. This was problematic, as the MDMA pretreated rats were expected to exhibit tolerance as opposed to sensitisation to m-CPP, thus a dose that did not significantly affect baseline behaviour

would not be expected to show pretreatment differences. The results from the present study show that there were no pretreatment differences in response to an m-CPP dose that induced significant locomotor decreases. Additionally, the present study utilised rats that were tolerant to the activating effects of MDMA (Figures 12 and 13) and displayed extensive serotonergic deficits. These results suggest that the tolerance to MDMA was pharmacologically specific and indicates that the hypolocomotive function of the 5-HT_{2c} receptors was not altered by MDMA pretreatment.

MDMA pretreated rats exhibited greater responsiveness to m-CPP at the highest doses (Figure 16B), however, these differences were not substantial enough to produce a significant main effect of pretreatment. These findings do, however, reflect the complexity of behaviour and suggest the contribution of other neurotransmitters. In addition to predominant effects on the 5-HT_{2c} receptor (Fiorella et al., 1995), m-CPP also binds other 5-HT receptor subtypes (Harel-Dupas et al., 1991; Kahn and Wetzler, 1991), the SERT (Eriksson et al., 1999) and alters cortical acetylcholine release (Zhelyazkova-Savova et al., 1999). 5-HT and acetylcholine contribute to hypolocomotive effects, as a role for acetylcholine in these effects has been established (Salas et al., 2004; Howell et al., 2005). Since MDMA pretreated rats exhibited significant SERT binding reductions (Table 2), and likely 5-HT receptor density changes (Scheffel et al., 1992; Aguirre, 1998; Shankaran et al., 1999; Reneman et al., 2002; McGregor et al., 2003), m-CPP would likely be displaced from binding targets. Despite changes in 5-HT systems, the acetylcholine response to drug challenge was unaltered in MDMA pretreated rats that received an identical pretreatment to the one used in the present study (Nair and Gudelsky, 2006). The differential dose-response profile exhibited by different pretreatment groups in the present study might, therefore, reflect a greater cholinergic contribution to m-CPP-induced hypolocomotion.

MDMA pretreatment did not affect latency to emerge for the groups that received a saline injection (Figure 17). These findings correspond to other reports documenting no change in baseline open field (McNamara et al., 1995; Bull, 2003) or EPM (Bull, 2004;

Sumnall et al., 2004) exploratory behaviours following MDMA pretreatment and indicate that MDMA exposure does not detrimentally affect associated mechanisms. Conversely, decreased social interaction is a widely reported consequence of MDMA exposure (Morley et al., 2001; Fone et al., 2002; Bull, 2003; McGregor et al., 2003; Thompson et al., 2004), suggesting that the mechanisms involved are dissociable from the aforementioned tests and more susceptible to MDMA-induced change.

The present results were in direct contrast with the only other study that has conducted similar experiments (McGregor et al., 2003). In these experiments, MDMA pretreated rats exhibited delayed emergence in comparison to controls following a 12-week recovery period. However, in our laboratory, rats that received the same pretreatment regimen as the present study did not show any pretreatment differences in emergence following a 12-week recovery period either (data not shown). These differential findings are possibly related to rat strain, as McGregor and colleagues (2003) used the Wistar rat. Indeed, Wistar rats have displayed differential baseline and drug-induced behaviour in the emergence test when compared to the Sprague Dawley strain (Pare et al., 2001). These findings suggest that the emergence test is a relatively sensitive measure, and only comparisons between studies that utilised similar methodologies should be made.

In a similar manner to the hypocomotion experiment, MDMA pretreatment did not affect m-CPP-induced increased emergence latency (Figure 17). These findings also concur with Bull (2003), who assessed open field and EPM behaviour and did not find any pretreatment differences, despite the use of different m-CPP assays, rat strains and ages and MDMA pretreatment regimens. These findings suggest that the 5-HT_{2c} receptor mechanisms underlying these m-CPP-produced behaviours were unaffected by MDMA exposure.

Effects of MDMA Pretreatment on RS102221-Potentiated MDMA-Produced Hyperactivity

MDMA-pretreated rats were less responsive to RS102221-produced potentiation of MDMA-produced hyperactivity (Figure 5B), suggesting receptor desensitisation as a function of MDMA exposure. Preliminary investigations revealed that MDMA pretreated rats also exhibited tolerance to the effect of SB200646, a 5-HT_{2b/2c} antagonist (Australasian Winter Conference on Brain Research Abstracts, 2003). The present study utilised RS102221 due to higher 5-HT_{2c} receptor selectivity, but both compounds are 5-HT_{2c} receptor antagonists. These findings suggest that tolerance to the effects of these antagonists was related to 5-HT_{2c} receptor desensitisation. Dose-effect studies with RS102221 show that potentiation of MDMA-produced hyperactivity was shifted to the right in the MDMA pretreated group (Figure 5B), as the response to 1.0mg/kg RS102221 was significantly greater than the response to the 0.5mg/kg dose. These findings concur with other studies that have suggested reduced 5-HT₂ receptor binding densities (Scheffel et al., 1992; Reneman et al., 2002; McGregor et al., 2003) following MDMA exposure.

Reduced 5-HT_{2c} receptor functional response might be attributable to mRNA editing. mRNA editing of 5-HT_{2c} receptors has been reported to produce receptor isoforms with altered functional properties (Herrick-Davis et al., 1999; Niswender et al., 1999; Wang et al., 2000; Berg et al., 2001; Visiers et al., 2001) and editing has occurred in response to fluctuating 5-HT levels (Gurevich, 2002). For example, 5-HT_{2c} receptor agonist administration produced an isoform with decreased sensitivity to 5-HT, as evidenced by decreased ability to activate G-proteins (Gurevich, 2002). The implications are that less sensitive 5-HT_{2c} receptor isoforms might also have reduced ability to modulate DA outflow under these circumstances. Conversely, 5-HT depletion produced 5-HT_{2c} receptor isoforms with increased sensitivity to 5-HT (Gurevich, 2002). Since MDMA induces massive 5-HT release (Gough et al., 1991; White et al., 1996; Lyles and

Cadet, 2003; Colado et al., 2004; Green et al., 2004) followed by central serotonergic depletions (Battaglia et al., 1987; Commins et al., 1987; Ricaurte et al., 1988a; Ricaurte et al., 1988b; Insel et al., 1989; Molliver, 1990; Scanzello et al., 1993; Aguirre et al., 1995; Colado and Green, 1995; Fischer et al., 1995; McNamara et al., 1995; Sabol et al., 1996; Sexton et al., 1999; Mayerhofer et al., 2001; Boot, 2002; McGregor et al., 2003; Clemens et al., 2004; Sumnall et al., 2004; Wang et al., 2004), mRNA editing of the 5-HT_{2c} receptor might occur. The RS102221 data from the present study suggest that the initial MDMA-produced 5-HT efflux might have produced desensitised 5-HT_{2c} receptor isoforms that were only responsive to the highest RS102221 dose.

5-HT_{2c} Receptor Conclusions

Despite large decreases in SERT binding (Table 2) and a tendency towards a rightward shift in the dose-effect curve for RS102221-produced potentiation of MDMA-produced hyperactivity, the behavioural effects of m-CPP were not altered by prior exposure to MDMA (Figures 16C and 17). This dissociation might reflect the existence of separate 5-HT_{2c} receptor populations with differential vulnerability to MDMA exposure. Indeed, evidence suggests that there are 5-HT_{2c} receptor populations that not only have different functional roles (Filip and Cunningham, 2003), but also anatomical locations. For instance, the ability to modulate DA release and inhibit stimulant-induced hyperactivity has been attributed to receptors located in the prefrontal cortex, whereas facilitatory 5-HT_{2c} receptors have been identified in the nucleus accumbens (Filip, 2002).

In contrast, there is no evidence to suggest that the receptors that mediate m-CPP-produced behaviours alter DA release. Additionally, m-CPP-responsive 5-HT_{2c} receptors might have differential locations, as local m-CPP injection into the ventral hippocampus and basolateral amygdala reduced exploratory behaviour in the EPM (Alves et al., 2004) and altered open field behaviours, ultrasonic vocalisation and latency to approach a novel

object (Kennett et al., 1994a; Campbell and Merchant, 2003), however, administration to other regions such as the dorsal hippocampus did not alter behaviour.

It was purported that MDMA exposure produced region-dependent alterations in 5-HT_{2a/2c} receptor binding, where the most significant downregulations were evident in the caudate putamen, insular cortex, cingulate cortex, lateral septum, frontal cortex and entorhinal cortex (McGregor et al., 2003). The potentiating effects of systemically administered 5-HT_{2c} receptor antagonists have been attributed to 5-HT_{2c} receptors in the prefrontal cortex (Filip and Cunningham, 2003), thus the observation that 5-HT₂ binding was most severely affected in this area might explain desensitised responses to RS102221 manipulation in MDMA pretreated rats. Conversely, 5-HT₂ receptor binding in the amygdala and hippocampus were not significantly affected, which were the sites where 5-HT_{2c} receptors that mediated m-CPP-produced behaviours were localised (Kennett et al., 1994a; Campbell and Merchant, 2003; Alves et al., 2004). It could be tentatively concluded that the 5-HT_{2c} receptor sites involved in the m-CPP-induced responses were more resistant to MDMA exposure than those receptors that modulate hyperactivity.

Reduced 5-HT_{2c} receptor responsivity, as evident in the RS102221 experiments, cannot explain tolerance to the behavioural effects of MDMA (Figure 12 and 13E). The effect of 5-HT_{2c} receptor activation was to attenuate stimulant-produced hyperactivity (Grottick et al., 2000), therefore desensitised 5-HT_{2c} receptors would be expected to potentiate MDMA-produced hyperactivity. Even though there was a small decrease in the potency of RS102221 as a function of MDMA exposure, this effect is not likely relevant to the behavioural deficits observed. These findings indicate that the 5-HT_{2c} receptor does not contribute significantly to the expression of MDMA-produced hyperactivity and that MDMA-produced desensitisation is not sufficient to counteract the tolerance attributable to other serotonergic deficits.

Preliminary DOI Experiments

DOI administration significantly increased the frequency of wetdog shakes (WDS) over the 10-minute recording period (Table 1) in concurrence with previous reports (Schreiber et al., 1995; Granoff and Ashby, 1998; Bull, 2004). Indeed, both WDS and back muscle contractions (BMC) have been used as an index of 5-HT_{2a} receptor activation in the rat, as these behaviours were attenuated by ritanserin and the more selective 5-HT_{2a} receptor antagonist, MDL100907 (Fone et al., 1989). It was also established that these 5-HT_{2a} receptor-mediated behaviours had different anatomical origins, as BMC were localised to the spinal cord, whereas WDS were related to receptor activation in the cortex and brainstem (Fone et al., 1989; Fone et al., 1991).

It was observed that some rats exhibited BMC, but the occurrence was inconsistent (results not presented). The occurrence of both behaviours confirmed that DOI was activating 5-HT_{2a} receptors, but the unreliability of the BMC response provided reason for exclusion from the present results. The inconsistent BMC response might reflect the inability of systemically administered DOI to reach spinal cord areas, as studies reporting DOI-induced BMC injected DOI locally into the spinal regions (Fone et al., 1989; Fone et al., 1991).

DOI-induced WDS were not dose-dependent, as the 1.0 and 2.0mg/kg doses produced equivalent behaviour. There was, however, a previous report where DOI produced dose-dependent increases in the head twitch response (Granoff and Ashby, 1998). Head twitch behaviour was observed for 1 hour rather than the 10-minute assessment period used in the present study. Differences between doses might have been apparent in this study because the time-course for the behavioural effects was not assessed. It is possible that the highest dose might have had a longer duration of action, thus producing more behaviour. The WDS in the present study were counted in 2-minute blocks

in order to verify that there were no dose-induced differences in time course for these effects, and since none were evident, the data was presented as totals.

Measuring WDS rather than head twitches might also explain the lack of DOI dose effects. Different definitions of what constitutes a 'WDS' as opposed to a 'head twitch' might explain these observations. A WDS was defined as a 'paroxysmic shudder of the head and trunk' (Darmani and Ahmad, 1999) whereas a head twitch was a 'rapid rhythmic shaking of the head in a radial motion' (Granoff and Ashby, 1998). Rats in the present study had to exhibit a shudder that included the head and the trunk in order to be counted as a WDS. The counts would probably have been much higher if all head twitches had been included. However, the present methodology was based on a study where MDMA pretreated rats exhibited tolerance to the ability of DOI to produce WDS (Bull, 2004). Bull only used the 1.0mg/kg DOI dose, thus the significant effect of the 1.0mg/kg dose in the present study and the knowledge that an MDMA pretreatment difference had been observed previously at this dose provided justification to examine the effects of MDMA pretreatment using this model.

DOI did not affect baseline locomotor activity in the present study (Figure 10), however, an increase (Darmani et al., 1996; Hillegaart et al., 1996; Kaur and Ahlenius, 1997; Bull, 2004; Ross et al., 2005), no change (Darmani et al., 1994; Hawkins et al., 2002) or a decrease in locomotion following DOI administration (Granoff and Ashby, 1998) have all been reported. It is possible that these mixed results were due to the use of different measuring methods and apparatus. For example, line crosses were manually recorded as indices of locomotor activity (Hawkins et al., 2002), whereas other studies utilised total number of beam breaks as counted by a computer (Granoff and Ashby, 1998). There was one notable study where DOI-produced locomotor activity was assessed as separate components (Hillegaart et al., 1996). DOI (1.0mg/kg) administration decreased total locomotor counts and rearing, but increased forward locomotion and peripheral activity. The DOI-produced changes were however, mild in comparison to another more

effective 5-HT agonist. These differential effects of DOI on several components of locomotor behaviours suggest that ‘total beam breaks’ or ‘line crosses’ were not sensitive enough to observe DOI-produced behavioural effects.

Although the present study assessed forward locomotion, no DOI effects were visible, whereas Hillegardt and colleagues (1996) observed an increase. Again, these differences were attributable to measuring methodology. Forward locomotion was defined as ‘successive interruptions of photocell beams when the animal was moving in the same direction’ (Hillegardt et al., 1996), whereas the Activity Monitor program in the present study divided the chamber into zones, or ‘boxes’ and when the rat travelled distance and crossed the box perimeters into an adjacent box, this was registered as an ambulatory count. The Activity Monitor program simultaneously recorded forward locomotion as well as ‘stereotypy counts’, which were all beam breaks made whilst the rat was within a ‘box’. As the program can differentiate between behaviours, the DOI data were subsequently re-analysed to determine whether stereotypy counts were influenced by drug treatment (results not shown). It was evident that the doses of DOI administered in the present study did not significantly alter ambulatory or stereotypy counts. Interestingly, the same DOI dose and rat strain/age were utilised as subjects for both the present study and Hillegardt’s work (1996), but the present results suggest that DOI does not produce robust effects on these behavioural paradigms.

It has been suggested that the ability for DOI to increase locomotor activity might be due to effects on DA release (Herin et al., 2005). It is well established that 5-HT_{2a} receptor activation facilitates stimulant-induced hyperactivity due to the ability to increase dopaminergic neurotransmission, however, DOI administration did not alter DA release in the mPFC, striatum and NAc (Ichikawa and Meltzer, 1995; Kuroki et al., 2003). Amphetamine administration produced a marked increase in extracellular DA levels in these areas, and when DOI was administered in conjunction with amphetamine, DA increases were significantly potentiated in all areas. Additionally, pretreatment with the

selective antagonist, MDL100907, reversed the DOI-produced potentiation of DA, thereby implicating 5-HT_{2a} receptor activation (Kuroki et al., 2003). Although these studies did not report concomitant locomotor responses, the effects on DA release largely determine the expression of hyperactivity. Thus, when DOI-induced increases in activity were previously reported, it was unlikely that the underlying mechanisms were DA-related. The behavioural results from the present study are consistent with the idea that 5-HT_{2a} receptor manipulations might only exert consistent effects on locomotor activity under conditions of increased monoamine neurotransmission.

Preliminary Ritanserin Experiments

Ritanserin pretreatment dose-dependently attenuated 10.0mg/kg MDMA-produced hyperactivity (Figure 11). The 10.0mg/kg ritanserin dose produced some non-selective effects, as evidenced by the inability to significantly attenuate MDMA-produced hyperactivity. It would be expected that increasing ritanserin dose would eventually antagonise 5-HT_{2c} receptors, thus negating the inhibitory effects from 5-HT_{2a} receptor blockade. Despite the non-selective nature of ritanserin, evidence suggests that the ability to attenuate MDMA-produced hyperactivity is a 5-HT_{2a} receptor-mediated effect. The selective 5-HT_{2a} antagonist, MDL100907, attenuated MDMA-produced forward locomotion in a comparable manner to ritanserin (Kehne et al., 1996), whilst MDMA-induced rearing behaviours were unaffected. These results indicate that these 5-HT_{2a} receptor manipulations selectively altered forward locomotion.

The 5-HT_{2a} receptor-mediated attenuation of MDMA-produced hyperactivity was attributable to the ability for these receptors to facilitate DA release. An electrophysiology study revealed that MDMA-induced 5-HT_{2a} receptor activation increased neuronal firing in the striatum, as a 5-HT_{2a} receptor antagonist blocked nearly all MDMA-induced striatal excitations.(Ball and Rebec, 2005). Additionally, MDMA significantly elevated striatal

extracellular DA levels, which were dose-dependently attenuated by MDL100907 (Schmidt et al., 1994).

Effects of MDMA Pretreatment on DOI-Induced Behaviour

MDMA pretreated rats did not exhibit WDS following 1.0mg/kg DOI administration (Table 3). These results support previous findings where Wistar rats that had received MDMA pretreatment (5mg/kg every hour for 4 hours on 2 consecutive days, 40.0mg/kg total) and an 8 week withdrawal period subsequently showed no DOI-induced WDS in comparison to controls (Bull, 2004). The present findings suggest that MDMA-induced desensitisation of 5-HT_{2a} receptors involved in this response was a relatively robust effect, since rat strain and differential pretreatment/withdrawal times did not significantly change the outcome.

Bull (2004) used the same rat strain, pretreatment regimen and similar withdrawal time to a study that conducted extensive DOI-binding assays (McGregor et al., 2003). It was reported that the caudate putamen, lateral septum, insular cortex, cingulate cortex, piriform cortex, perirhinal cortical area, medial hypothalamic area and the medial and lateral thalamic nuclei exhibited significant [¹²⁵I] DOI binding decreases. WDS were localised generally to the cortex and brainstem (Fone et al., 1989; Fone et al., 1991), therefore it was possible that there were downregulations in 5-HT_{2a} receptor populations that mediate DOI-induced WDS following MDMA pretreatment.

There was another study that reported no change in the ability for DOI to produce head twitches in the rat following MDMA pretreatment (Granoff and Ashby, 1998). Male Sprague Dawley rats of the same age and size to the present study were used, but rats received 20.0mg/kg MDMA twice daily for 4 consecutive days, totalling 160.0mg/kg. Comparable (and lower) doses than 160.0mg/kg MDMA have produced non-specific neurotoxicity (Commins et al., 1987; Jensen et al., 1993; Schmued, 2003). These effects were purported to relate to drug-induced hyperthermia and not pharmacological effects of

MDMA (Baumann et al., 2006). Indeed, during the MDMA dose-effect experiments in the present investigation, it was occasionally observed that a single 20.0mg/kg MDMA administration resulted in lethality, presumably due to hyperthermia. The MDMA exposure regimen used by Granoff and Ashby (1998) did deplete brain 5-HT levels below 80% as intended, but this regimen would not likely have selectively affected serotonergic systems, making it difficult to compare these results to the present study.

Effects of MDMA Pretreatment on Ritanserin-Attenuated MDMA-Produced Hyperactivity

Ritanserin pretreatment had no effect on MDMA-produced hyperactivity in MDMA pretreated rats (Figure 19B). These results indicate that the 5-HT_{2a} receptors involved in this response were completely desensitised. Firstly, three ritanserin doses were tested that encompassed the range of effective doses, as the highest 10.0mg/kg dose started to produce non-selective effects. Tolerance to ritanserin would have been expected to shift the dose effect curve to the right in a similar manner to the RS102221 response (Figure 18B), but instead, there was no indication of attenuation at any dose. Secondly, although MDMA pretreated rats were tolerant to 10.0mg/kg MDMA, there were still sufficient levels of activity present for significant attenuation to occur. For example, the hyperactivity exhibited by the saline pretreated rats that received 2.5mg/kg ritanserin was comparable to the levels exhibited by the MDMA pretreated rats that received no ritanserin. MDMA-produced hyperactivity exhibited by the saline pretreated group was attenuated further following 5.0mg/kg ritanserin administration, whereas the MDMA rats remained completely unresponsive.

There was some variability evident between the preliminary study (Figure 11) and the response of the saline pretreated group (Figure 19A). This variability was due to higher response to 10.0mg/kg MDMA by the saline pretreated rats. The reasons for these differences were unclear, but the response to ritanserin was equivalent between studies. It appears that the saline pretreated rats were more responsive to ritanserin because the higher

baseline made the differences between doses more significant. The 10.0mg/kg ritanserin dose also significantly attenuated activity, whereas it had not in the preliminary study. However, it was evident that 10.0mg/kg ritanserin did not attenuate activity to the same extent as the 5.0mg/kg dose. Despite these differences, both experiments had internal control groups and the same pattern/profile of ritanserin effects were observed each time.

Failure of ritanserin pretreatment to affect MDMA-produced hyperactivity might reflect the extensive 5-HT_{2a} receptor binding decreases that have been reported following MDMA exposure (Reneman et al., 2002; McGregor et al., 2003). Approximately 40% of 5-HT_{2a} receptor-labelled dendrites contained tyrosine hydroxylase in the ventral tegmental area, suggesting that these receptors were localised on dopaminergic neurons (Doherty and Pickel, 2000). 5-HT_{2a} receptors were expressed on DA neurons in several A10 subnuclei in the ventral tegmental area that project to mesolimbic forebrain regions, which could potentially produce widespread changes to frontal dopaminergic neurotransmission (Nocjar et al., 2002). The behavioural results from the present study indicate that the 5-HT_{2a} receptors found on/around DA neurons were desensitised and/or downregulated following MDMA pretreatment.

5-HT_{2a} Receptor Conclusions

The inability of DOI or ritanserin to produce any behavioural response indicates that MDMA exposure produced widespread 5-HT_{2a} receptor desensitisation. Evidence strongly suggests that DOI-induced behaviours and the ability for ritanserin to modulate MDMA-produced hyperactivity might be related to different 5-HT_{2a} receptor functions and/or different populations. The present results, therefore suggest desensitisation of the receptors involved in both functions. These are novel findings, as there have been no previous attempts to distinguish between 5-HT_{2a} receptor-related responses.

The ritanserin results indicate that tolerance to the activating effects of MDMA could largely be attributed to complete desensitisation of the 5-HT_{2a} receptors involved in

modulating DA. Desensitised 5-HT_{2a} receptors that produced DOI-induced WDS do not, however, explain decreased locomotor response to MDMA, as the role for these receptors in locomotion (and DA release) was negligible. However, tolerance in other MDMA-produced behaviours might be attributable to these receptors. Indeed, DOI administration has produced hyperthermia (Pranzatelli and Pluchino, 1991; Mazzola-Pomietto et al., 1995) and further studies have attributed hyperthermia to the 5-HT_{2a} receptor (Mazzola-Pomietto et al., 1995; Nisijima et al., 2001; Fantegrossi et al., 2003; Herin et al., 2005). The selective 5-HT_{2a} receptor antagonist, MDL100907, attenuated hyperactivity and hyperthermia produced by 8 and 12mg/kg MDMA in Sprague Dawley rats (Herin et al., 2005). Although the 5-HT_{2a} receptor was implicated in both MDMA-produced behaviours, the time course for the attenuation of these effects were not consistent with a common mechanism (Herin et al., 2005). These studies suggest that 5-HT_{2a} receptor-mediated hyperthermia might be related to the receptors that mediate DOI-produced behaviours. Tolerance to DOI and MDMA-induced hyperthermia might be due to downregulation/desensitisation of similar 5-HT_{2a} receptor populations.

Conclusions

MDMA pretreatment reduced SERT binding densities in brain regions that have been implicated in 5-HT mediated hyperactivity (Table 2). MDMA pretreated rats were tolerant to MDMA-produced hyperactivity (Figures 12 and 13) and unresponsive to the attenuating effects of the SSRI, clomipramine (Figure 15B). Since 5-HT release via the SERT contributes to the majority of MDMA-produced hyperactivity (Callaway et al., 1990; Callaway and Geyer, 1992b), reduced SERT densities probably decreased 5-HT release in response to acute MDMA challenge, producing a tolerant behavioural response. Tolerance might also potentially reflect desensitised 5-HT₂ receptors that contribute to the expression of MDMA-produced hyperactivity.

Indeed, both the 5-HT_{2c} and 5-HT_{2a} receptor subtypes modulated MDMA-produced hyperactivity, but the present results indicated that 5-HT_{2a} receptors had a greater role. For example, the 5-HT_{2c} receptor antagonist, RS102221, is a highly selective ligand for its target (Bonhaus, 1997) in comparison to the 5-HT_{2a/2b/2c} antagonist, ritanserin, yet there was only one dose that produced significant potentiation of MDMA-produced hyperactivity in control rats (Figure 18A). In contrast, ritanserin produced significant attenuation of hyperactivity at several doses (Figure 19A). Additionally, if 5-HT_{2c} receptors were significant contributors to MDMA-produced hyperactivity, desensitisation would have produced a sensitised, and not a tolerant, locomotor response to MDMA challenge. Cell recordings following MDMA administration support the idea for a greater role for the 5-HT_{2a} receptor in MDMA-produced hyperactivity (Ball and Rebec, 2005). 5-HT_{2a} receptor antagonist pretreatment prevented MDMA-induced striatal excitations, which paralleled attenuation of MDMA-induced hyperactivity, whilst a 5-HT_{2c} receptor antagonist had comparatively little effect on neuron excitability or hyperactivity.

Interestingly, 5-HT_{2a} receptors that facilitate MDMA-produced hyperactivity might be more susceptible to MDMA-induced desensitisation than 5-HT_{2c} receptors. The present study showed that MDMA pretreated rats exhibited tolerance to the potentiating effects of RS102221 (Figure 18B), as revealed by a rightward shift in the dose effect curve. In contrast, MDMA pretreated rats showed no response to any ritanserin dose (Figure 19B). The results suggest that the 5-HT_{2a} receptors not only have a greater role in MDMA-produced hyperactivity, but also were also more susceptible to MDMA-induced desensitisation. Given these findings, tolerance to MDMA-produced hyperactivity was attributable to 5-HT_{2a} receptor desensitisation.

There is evidence to suggest that the 5-HT_{2a} and 5-HT_{2c} receptors that modulate MDMA-produced hyperactivity are functionally distinct from the receptors that mediate m-CPP- and DOI-induced behavioural responses. The dissociation in the present results

supports this idea, whereby m-CPP-produced behaviours were resilient (Figures 16B and 17), yet RS102221-induced effects were reduced (Figure 18B), by MDMA pretreatment. The 5-HT_{2a} receptors that mediate DOI-produced effects might also be separate to those involved in the modulation of MDMA-produced hyperactivity. In order for the 5-HT_{2a} receptors that modulate stimulant release to exert behavioural affect, monoamine neurotransmission must be increased, and antagonists/agonists then modulate DA release (Ichikawa and Meltzer, 1995; Kuroki et al., 2003) and subsequent locomotor behaviour (Schmidt et al., 1994). However, behavioural effects produced by DOI occur independently to effects on monoamine neurotransmission, as DOI did not alter DA neurotransmission on its own (Ichikawa and Meltzer, 1995; Kuroki et al., 2003). These represent either differential functions of the same 5-HT_{2a} receptors, or functions of separate 5-HT_{2a} receptors. MDMA pretreatment produced tolerance to DOI-induced WDS (Table 3), but it was unclear whether this would directly impact tolerance to MDMA-produced hyperactivity. It was evident that MDMA pretreatment did not affect 5-HT_{2c} receptors involved in m-CPP produced behaviours, but receptors associated with DOI-produced effects might have a role in tolerance to other 5-HT_{2a} receptor-mediated MDMA behaviours, such as hyperthermia.

Tolerance to the subjective effects of MDMA might be related to the presence of decreased SERT densities in users (McCann et al., 1998; Semple et al., 1999; Buchert et al., 2003; Thomasius et al., 2003), as SERT blockade with citalopram reduced the positive drug effects (Liechti et al., 2000b; Liechti et al., 2001; Liechti and Vollenweider, 2001). In addition to SERT binding, activation of 5-HT receptor subtypes might also contribute to MDMA-produced effects in humans. Activation of the 5-HT_{2a} receptor has been associated with hallucinogenic-related drug effects (Jakab and Goldman-Rakic, 1998; Aghajanian and Marek, 1999; Nelson et al., 1999) and pretreatment with ketanserin, a 5-HT_{2a/2c} antagonist, decreased MDMA-produced perceptual changes and emotional excitation (Liechti et al., 2000a; Liechti et al., 2001; Liechti and Vollenweider, 2001).

Since MDMA exposure in humans has also been reported to decrease 5-HT_{2A} binding densities (Reneman et al., 2002), changes to this particular receptor subtype might explain tolerance in MDMA-related perceptual changes.

Return to control values of MDMA-produced 5-HT depletions and SERT binding reductions was reported by 12 weeks (Scanzello et al., 1993; Fischer et al., 1995). These neurochemical findings following the pretreatment regimen used in the present study were in correspondence with the behavioural data, showing a recovery of function (Figure 14). These findings further implicate serotonergic deficits as the major contributors to behavioural tolerance to MDMA and also show that these effects are reversible. The present study showed that repeated weekly exposure to MDMA resulted in persistent tolerance in MDMA pretreated rats, suggesting that drug abstinence is necessary for recovery to occur.

Indeed, abstinence from MDMA led to recovery of SERT binding in ex-users (Semple et al., 1999; Buchert et al., 2003; Thomasius et al., 2003) but behavioural effects of MDMA following abstinence have not been measured in humans. Because effects in humans are comparable to the effects reported in laboratory animals in these experiments, the present data suggest that tolerance to the effects of MDMA might also diminish in humans who abstain from drug use for significant period of time. However, chronic use of MDMA without an abstinence period to allow for serotonergic recovery might result in persistent tolerance to MDMA.

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