# The effect of MDMA self-administration on MDMA-produced hyperactivity and c-fos expression

by

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**Abstract**

*Background:* MDMA preferentially releases serotonin (5HT) but following repeated exposure there is a decrease in this MDMA-produced effect. At the same time, some studies suggest an increase in MDMA-produced dopamine (DA) release following repeated exposure. The sensitised DA response is often accompanied by sensitisation of MDMA-produced locomotor activity. Because DAergic mechanisms have been implicated in the positively reinforcing properties of MDMA, these neuroadaptations might be relevant to MDMA self-administration.

*Objectives:* The main objective of this study was to determine whether MDMA self-administration and non-contingent MDMA exposure differentially affected the development of sensitisation to MDMA-produced hyperactivity. Additionally, the relationship between MDMA-produced hyperactivity and changes in c-fos expression in DA terminal regions was determined.

*Methods:* Triads of rats were designated 'master', 'yoked MDMA', or 'yoked saline'. Lever press responding by the master rat resulted in an intravenous infusion of MDMA for both the master rat and the yoked MDMA rat, as well as an equal infusion of vehicle for the yoked control rat. Daily tests continued until a total of 350 mg/kg MDMA had been self-administered. Three days following the last self-administration session, forward and vertical locomotion produced by MDMA (5.0 mg/kg, i.p) were measured during a 2 hr test. Rats were sacrificed immediately following the behavioural test, and c-fos immunohistochemistry was measured.

*Results:* Repeated MDMA exposure resulted in sensitised forward and vertical locomotor activity. Sensitisation of the increase in forward locomotion was produced only in rats that self-administered MDMA; non-contingent MDMA administration failed to sensitise this behavioural response. In contrast, sensitisation to MDMA-produced vertical activity was produced following both contingent and non-contingent MDMA exposure. C-fos expression was reduced in ventrolateral, and ventromedial areas of the dorsal striatum, as well as the infralimbic cortex, after MDMA exposure, regardless of whether the exposure was via self-administration or yoked administration. A selective decrease in c-fos expression in the nucleus accumbens (NAc) core and the cingulate cortex was produced by MDMA self-administration. There was a negative correlation between MDMA-produced forward locomotor activity and MDMA-produced c-fos expression in the NAc

core, cingulate cortex and infralimbic cortex. A negative correlation between rearing activity and MDMA-produced c-fos expression in the NAc core, NAc shell, cingulate cortex, and infralimbic cortex was also found.

*Conclusions:* These data provide evidence of behavioural sensitisation as a result of repeated MDMA exposure. Furthermore, MDMA-produced behavioural sensitisation was associated with a decrease in c-fos expression that was evident in the NAc and prefrontal cortex. Finally, region-specific changes in c-fos expression suggest an important role of neuroadaptations in the NAc core and the infralimbic cortex as a consequence of MDMA self-administration.

## Introduction

3,4-methylenedioxymethamphetamine (MDMA or 'ecstasy') is a ring substituted amphetamine derivative with structural similarities to both stimulants and hallucinogens (Green, Mechan, Elliott, O'Shea, & Colado, 2003). MDMA was first synthesised and patented by Merck pharmaceuticals in 1914, in an attempt to discover a novel clotting agent (Freudenmann, Öxler, & Bernschneider-Reif, 2006). It was not until the 1970s that the effects of MDMA were thoroughly investigated, when it was reported that MDMA produced psychoactive properties in humans (Shulgin & Nichols, 1978). In the 1980s, MDMA started to be used as an adjunct to psychotherapy. Because MDMA was reported to induce a feeling of being 'touched within' it was suggested that the drug would enhance the therapeutic experience (Shulgin, 1986). However it was not just in a clinical setting that MDMA became influential, its recreational use escalated in the early 1980s in the United States and abroad (Cohen, 1995). By 1985 the US drug enforcement agency classified MDMA a schedule I drug as a result of its high abuse potential, and lack of acceptance for use for clinical application (Green et al., 2003; Parrott, 2001).

In New Zealand, MDMA is scheduled as a class B drug under the Misuse of Drugs Act, 1975. According to the World Drug Report compiled by the United Nations Office on Drugs and Crime, global prevalence of ecstasy use has been decreasing in recent years (United Nations Office on Drugs and Crime, 2014). However ecstasy use in Oceania continues to be among the highest, with a prevalence rate of 2.9 percent. In New Zealand there has been an increase in ecstasy use since the early 2000s (Wilkins & Sweetsur, 2008), and it is the second most widely used illegal drug in the country (Global Drug Survey, 2014). High rates of MDMA use in New Zealand are concerning because although MDMA is commonly considered a 'safe' drug (Kalant, 2001; Solowij, Hall, & Lee, 1992), numerous surveys indicate that the frequency of use increases over time for some users (Degenhardt, Bruno, & Topp, 2010; Soar, Turner, & Parrott, 2006) and some meet the criteria for a substance use disorder (Cottler, Womack, Compton, & Ben-Abdallah, 2001).

### Pharmacology of MDMA

The primary neurochemical effect of MDMA is to increase the release of the neurotransmitter, serotonin (5HT) (Green et al., 2003; Nichols, Lloyd, Hoffman, Nichols, & Yim, 1982; Schmidt, 1987). The release of 5HT occurs via MDMA binding to, and reversing, the 5HT transporter, and by entering the neuron and reversing the action of the

vesicular monoamine transporter (Berger, Gu, & Azmitia, 1992; Rudnick & Wall, 1992). A vast amount of animal research has shown that MDMA increases levels of synaptic 5HT as measured by microdialysis (Gough, Ali, Slikker, & Holson, 1991; Gudelsky & Yamamoto, 2008; Mechan et al., 2002; Sabol & Seiden, 1998), and consequently produces a marked reduction in 5HT tissue levels during the first few hours following administration (Gough et al., 1991; Schmidt, 1987).

Like other drugs of abuse, MDMA also increases extracellular dopamine (DA) levels. This has been shown by *in vivo* microdialysis (Gough et al., 1991; Nash & Brodkin, 1991; Sabol & Seiden, 1998; Yamamoto & Spanos, 1988), and by *in vitro* experimental designs, using tissue slices (Johnson, Hoffman, & Nichols, 1986; Schmidt, 1987). However, the extracellular increase in 5HT produced by MDMA is substantially greater than that of extracellular DA (Schenk, 2011). Baumann and colleagues (2008) provided one of many studies to support this claim by showing that an inter-peritoneal administration of MDMA (1.5 mg/kg) increased 5HT in the nucleus accumbens by 500%, but failed to alter synaptic DA. This difference was even greater following administration of a higher dose of MDMA (7.5mg/kg) where 5HT increased by 3000%, and DA increased by 500% (Baumann, Clark, Franken, et al., 2008).

**Effects of MDMA on humans**

MDMA has been reported to produce relaxation, euphoria, increased empathy, and feelings of closeness to others (Cohen, 1995; Green, Cross, & Goodwin, 1995; Solowij et al., 1992; Verheyden, Henry, & Curran, 2003; Vollenweider, Gamma, Liechti, & Huber, 1998). Adverse psychological effects also occur, with recreational users reporting acute feelings of anxiety, overstimulation, panic, and loss of personal control (Cohen, 1995; Davison & Parrott, 1997; Verheyden et al., 2003; Vollenweider et al., 1998). In addition to these psychological effects, recreational doses of MDMA are associated with a number of physiological consequences, with the most reported adverse physiological response being hyperthermia (Cohen, 1995; Liechti, Baumann, Gamma, & Vollenweider, 2000). Experimental human studies confirmed a role of 5HT in these effects. For example, administration of a selective serotonin re-uptake inhibitor attenuated most of the subjective effects of MDMA, and a $5HT_2$ antagonist reduced MDMA-produced perceptual changes and emotional excitation (Liechti, Baumann, et al., 2000; Liechti, Saur, Gamma, Hell, & Vollenweider, 2000; Liechti & Vollenweider, 2001).

MDMA use can also produce long term psychological and physiological deficits. These include depersonalisation, insomnia, depression, and flashbacks (Cohen, 1995). Functional deficits in memory (M. J. Morgan, 1999; Parrott, Lees, Garnham, Jones, & Wesnes, 1998; von Geusau, Stalenhoef, Huizinga, Snel, & Ridderinkhof, 2004), and executive functioning and reasoning abilities (Verdejo-Garcıa, López-Torrecillas, de Arcos, & Pérez-Garcıa, 2005; Wareing, Fisk, & Murphy, 2000), have also been reported. Neuroimaging studies have shown a reduction in the density of 5HT transporter sites (McCann, Szabo, Scheffel, Dannals, & Ricaurte, 1998; Ricaurte, McCann, Szabo, & Scheffel, 2000; Semple, Ebmeier, Glabus, O'Carroll, & Johnstone, 1999) and reduced levels of the 5HT metabolite, 5-HIAA, in MDMA users (Ricaurte, Finnegan, Irwin, & Langston, 1990). The decrease in 5HT transporter binding was positively correlated with the extent of the MDMA use, suggesting that repeated use might increase the risk of 5HT deficits (McCann et al., 1998).

Studies that investigate the effects of MDMA in humans provide invaluable information, but there are also considerable limitations. Firstly, polydrug use is common among ecstasy users, and so the attribution of long term effects of drug use specifically to MDMA cannot easily be determined (Green et al., 2003). Although researchers attempt to control for this by including the histories of subjects as a co-variate, this information is obtained by self-report and retrospective surveys. The reliability of these reports is likely to be compromised due to MDMA's illegal status, functional deficits following MDMA use, or poor recall (Parrott, 2005; Topp, Hando, Dillon, Roche, & Solowij, 1999). Furthermore, it is difficult to attribute neuroadaptations to drug exposure rather than to a pre-existing condition (Green et al., 2003). Finally, there is limited information about purity or dose of MDMA consumed making it difficult to attribute long-term effects of drug use specifically to MDMA (Green et al., 2003).

Animal studies are not plagued by the same confounds that often limit interpretation of studies with humans. A major challenge that animal studies can face, however, is the ability to generalise to the human condition.

**The effects of repeated MDMA**

As was observed in humans who consumed ecstasy, repeated exposure to MDMA produced dose-dependent 5HTergic deficits in non-human primates and rats (Battaglia et al., 1987; O'Hearn, Battaglia, De Souza, Kuhar, & Molliver, 1988; Scanzello,

Hatzidimitriou, Martello, Katz, & Ricaurte, 1993; Thomasius et al., 2003). It is worth noting that both dose and frequency of MDMA administration impacted the magnitude of 5HT deficits. One study measured 5HT deficits following the administration of 4.0 mg/kg MDMA either daily for 4 days, twice-weekly for 8 weeks, or twice daily for 4 days, and found that only the latter dosing regimen decreased 5HT (O'shea, Granados, Esteban, Colado, & Green, 1998). Other studies have only found decreases in the density of 5HT reuptake sites after repeated exposure to high doses (20 mg/kg) of MDMA (Battaglia, Brooks, Kulsakdinun, & De Souza, 1988; Battaglia et al., 1987). Problematically, these regimens of regular, high doses have been criticised because human users rarely, if ever, experience this pattern of administration on initial exposure (Baumann & Rothman, 2009; Cole & Sumnall, 2003; De La Garza, Fabrizio, & Gupta, 2007; Meyer, Piper, & Vancollie, 2008). Most people consume 1-2 tablets during initial drug taking (De La Garza et al., 2007), raising questions concerning the validity of the findings from animal studies utilising repeated, high doses of MDMA.

Under some experimental conditions, procedures that allow animals to self-inject drugs provide a more valid means of determining consequences of drug-taking in humans. Most drugs that are abused by humans are self-administered by animals, and drugs that are not abused by humans are not self-administered by animals (Griffiths, Bigelow, & Henningfield, 1980). Animal models of self-administration can, therefore, be used to more reliably investigate the consequences of repeated, chronic MDMA exposure, and the neurobiological mechanisms underlying its abuse.

**Drug self-administration**

A number of routes of self-administration have been reported to successfully sustain drug-taking, including oral ingestion (Wikler, Martin, Pescor, & Eades, 1963), inhalation (Jarvik, 1967) intraperitoneal (Headlee, Coppock, & Nichols, 1955), and intracerebral (Olds & Olds, 1958). Intravenous (IV) self-administration is also a commonly used method (Schuster & Thompson, 1969). Weeks (1962) established the chronic IV catheter implant, which set the stage for studying the effects of IV drug self-administration. Animals are surgically implanted with a chronic IV catheter and, following recovery, they are trained to perform an operant (e.g. lever press) in order to receive an IV infusion of the drug of interest (O'Connor, Chapman, Butler, & Mead, 2011).

Self-administration studies uncovered an important role of DA in the reinforcing efficacy of drugs. DA antagonists attenuated the reinforcing effects of drugs of abuse as indicated by a dose-dependent compensatory increase in responding and a rightward shift in the dose-effect curve (Yokel & Wise, 1976). Furthermore, DA lesions blocked self-administration, while lesions of other brain systems did not (Roberts & Koob, 1982). As a result of many of these seminal studies, it is now commonly accepted that voluntary self-administration of drugs of abuse results from an increase in DA release in the mesolimbic DA system (Wise & Rompré, 1989).

There is also ample evidence that 5HT is inhibitory to drug self-administration. Serotonin uptake inhibitors are not abused (Howell & Byrd, 1995; Roberts et al., 1999), and the reinforcing efficacy of amphetamine analogues was negatively correlated with 5HT transporter affinity (Ritz & Kuhar, 1989). Based on these findings and because MDMA preferentially increases 5HT, MDMA would not be expected to be self-administered. However, a number of studies have reported MDMA self-administration in non-human primates (Fantegrossi, Ullrich, Rice, Woods, & Winger, 2002; Lamb & Griffiths, 1987) and rats (Ratzenboeck, Saria, Kriechbaum, & Zernig, 2001; Schenk, Gittings, Johnstone, & Daniela, 2003).

There are, however, a number of aspects of MDMA self-administration that differentiate it from the self-administration of other drugs of abuse. For example, the acquisition of MDMA self-administration was not dose-dependent; latency to meet a criterion for self-administration of a low dose was similar to latency to meet the criterion for self-administration of a higher dose (Schenk et al., 2007). This contrasts with the well-documented dose-response functions for latency to acquisition of self-administration of other drugs of abuse. For example, the latency to acquire cocaine or amphetamine self-administration was inversely related to the dose of drug (Carroll & Lac, 1997; Schenk et al., 1993). Additionally, the latency to acquisition of MDMA self-administration is considerably longer than is the latency to acquisition of self-administration of other drugs of abuse (Schenk et al., 2003). The most striking difference, however, is that only about 50% of animals acquire MDMA self-administration (Schenk, Colussi-Mas, Do, & Bird, 2012), which is considerably lower than what is generally seen in animals trained to self-administer cocaine (Lile, Ross, & Nader, 2005) or amphetamine (Carroll & Lac, 1997). These data suggest that, at least initially, MDMA is a less efficacious reinforcer than other psychostimulant drugs.

In contrast to the acquisition profile of MDMA self-administration, once acquired, responding maintained by MDMA infusions became dose-dependent (Schenk et al., 2003; Schenk et al., 2007). This behavioural change was accompanied by neuroadaptations in both 5HTergic (Reveron, Maier, & Duvauchelle, 2010), and DAergic systems (Colussi-Mas, Wise, Howard, & Schenk, 2010). Following repeated administration, the MDMA-produced increase in synaptic 5HT was reduced (Reveron et al., 2010), and the increase in synaptic DA was increased (Colussi-Mas et al., 2010). Thus, with repeated exposure, the pharmacology of MDMA becomes similar to amphetamine or cocaine.

Animals that self-administer large quantities of MDMA demonstrate behaviours consistent with some aspects of a substance use disorder. Substance use disorders are chronic relapsing disorders, and reinstatement of drug-seeking in animals has been used to explore the neuroadaptations that underlie the transition from drug use to drug abuse (De Wit & Stewart, 1981). Drug seeking can be produced by a priming injection of MDMA following the extinction of MDMA self-administration (Colussi-Mas et al., 2010; Schenk, 2008), and by exposure to stimuli that have been associated with self-administered MDMA (Ball, Walsh, & Rebec, 2007; Schenk, 2008). DAergic mechanisms are also implicated in MDMA-seeking following extensive self-administration. Extinguished MDMA self-administration was reinstated by direct and indirect DA agonists (Schenk, Gittings, & Colussi-Mas, 2011), but not by direct or indirect 5HT agonists (Schenk et al., 2011). Further, drug seeking was attenuated by DA antagonists (Schenk et al., 2011). These data further suggest that following MDMA self-administration, MDMA begins to preferentially activate DAergic substrates to potentiate the drug seeking response.

In summary, MDMA preferentially stimulates release of 5HT (Green et al., 2003). Following repeated exposure there is a decrease in MDMA-produced 5HT, and an increase in MDMA-produced DA (Colussi-Mas et al., 2010; Mayerhofer, Kovar, & Schmidt, 2001). It has been suggested that this increase in DA release underlies the development of high levels of MDMA self-administration (Schenk et al., 2011). Furthermore, following extensive self-administration of MDMA there is evidence of MDMA produced drug seeking, and sensitisation of DA release (Colussi-Mas et al., 2010), suggesting that sensitisation of DA underlies the development of high levels of MDMA self-administration.

**Behavioural Correlates of Sensitisation**

The sensitisation of DA release following repeated drug exposure is often mirrored by indices of behavioural sensitisation. A measure of behavioural sensitisation that is frequently employed when studying the effects of psychostimulants is locomotor activity. There are a number of studies that report a sensitised locomotor response following repeated exposure to various stimulants including cocaine (Kalivas & Stewart, 1991; Post & Contel, 1983), and amphetamine (Kuczenski & Segal, 1988; Robinson & Becker, 1986). Repeated administration of MDMA also produced sensitisation of MDMA-produced forward locomotor activity (Ball, Wellman, Fortenberry, & Rebec, 2009; Bradbury, Gittings, & Schenk, 2012; Colussi-Mas & Schenk, 2008; Kalivas, Duffy, & White, 1998; Spanos & Yamamoto, 1989) and vertical locomotor activity (Lettfuss, Seeger-Armbruster, & von Ameln-Mayerhofer, 2013; Schenk & Bradbury, 2015).

The acute hyperactive response to MDMA has been attributed, in part, to DAergic mechanisms since it was attenuated by neurotoxic DA lesions (Gold, Hubner, & Koob, 1989), or DA antagonists (Ball, Budreau, & Rebec, 2003; Daniela, Brennan, Gittings, Hely, & Schenk, 2004). Repeated exposure to MDMA sensitised MDMA-produced hyperactivity and enhanced DA release as measured by *in vivo* microdialysis (Baumann, Clark, & Rothman, 2008). Enhanced synaptic DA also underlies increased rearing activity (Thiel, Müller, Huston, & Schwarting, 1999), although relatively fewer investigations of this behaviour have been conducted. Thus, as has been suggested for sensitisation to the behavioural effects of other drugs, sensitisation to the effects of MDMA have been attributed to sensitised DAergic mechanisms.

Following repeated exposure animals also became sensitised to the reinforcing effects of stimulants, as indicated by decreased latency to acquire self-administration (Piazza, Deminiere, le Moal, & Simon, 1990; Suto et al., 2002), and an increase in motivation to self-administer drugs (Lorrain, Arnold, & Vezina, 2000; Mendrek, Blaha, & Phillips, 1998). Therefore, the neuroadaptations that underlie sensitisation of motor-activating effects of MDMA might provide information concerning the neuroadaptations underlying the development of MDMA self-administration.

**Importance of contingency of drug exposure**

Most studies that examine behavioural sensitisation test the effect of the drug of interest following repeated experimenter-administration. Several studies, however, have suggested that the magnitude of neurochemical effects of repeated exposure might depend

on whether drug is experimenter-administered or self-administered (Dworkin, Mirkis, & Smith, 1995; Hemby, Koves, Smith, & Dworkin, 1997; Hemby, Martin, Dworkin, & Smith, 1995; Miguéns et al., 2008; Stefanski, Ladenheim, Lee, Cadet, & Goldberg, 1999). For example, higher extracellular levels of DA were produced following self-administration of cocaine (Hemby et al., 1997), or amphetamine (Di Ciano, Blaha, & Phillips, 1996), when compared to the same amount administered non-contingently. Self-administration of methamphetamine produced a significant reduction in $D_1$- and $D_2$-like receptors, but no significant difference was produced following non-contingent, yoked methamphetamine administration (Stefanski et al., 1999). Furthermore, DA transporter binding was significantly enhanced in animals that received contingent administration of cocaine, compared to animals that received the drug non-contingently (Miguéns et al., 2008). Together, these studies suggest that the neuroadaptations produced by repeated exposure to drugs of abuse might depend on whether drug exposure is contingent or non-contingent.

One way to differentiate neuroadaptations that are produced by the pharmacological effects of the drug alone from those that are involved in the self-administration procedure is to utilise a yoked self-administration paradigm in which animals are grouped into triads. Responding by a master rat results in an intravenous infusion of drug for the master rat and a yoked drug rat, as well as an equal infusion of vehicle for a yoked control rat. Thus, by using the yoked self-administration paradigm one can assess the pharmacological effects of the drug and distinguish any differences between the neurobiological consequences of drug exposure that is contingent, or non-contingent. Figure 1 illustrates the basic mechanics of yoked self-administration.

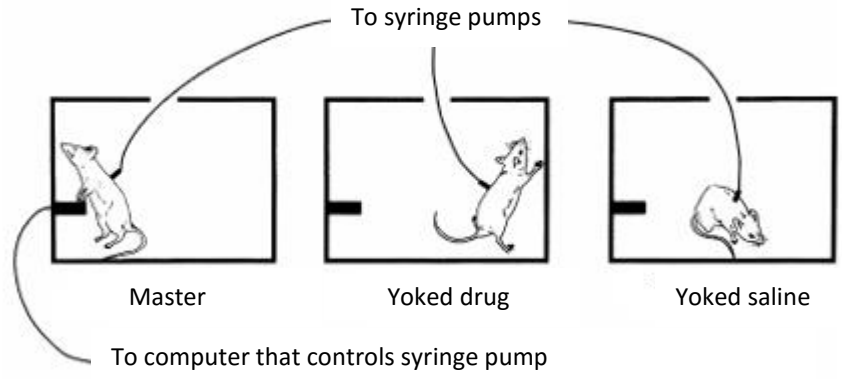



*Figure 1.* Yoked self-administration paradigm. The master rat (left) receives contingent drug delivery according to operant responding. The yoked drug rat (centre) non-

contingently receives the same number and pattern of IV drug infusions as the master rat. The yoked saline rat (right) non-contingently receives IV vehicle injections that are identical in number and pattern to the drug injections. A computer-controlled, three-chambered syringe pump enables the simultaneous delivery of these injections to all three rats. Image adapted from Haracz, Mash, and Sircar (1999).

**Neuroadaptations resulting from self-administration**

The transition from drug use to drug misuse has often been attributed to the sensitisation of central DA mechanisms that occurs as a result of repeated exposure (Gardner, 1997). Repeated MDMA self-administration produced a sensitised DA response to MDMA (Colussi-Mas et al., 2010). The mechanism for this sensitised DA response is currently unknown, but neuroadaptive responses of certain brain regions are likely candidates.

Previous research has suggested that neuroadaptations in the nucleus accumbens (NAc) play an important role in the development of drug taking (Wise, 2004) , whereas neuroadaptations in the dorsal striatum are invoked in the transition from drug self-administration to compulsive drug self-administration (Everitt & Robbins, 2013). Furthermore, neuroadaptations in the prefrontal cortex (PFC) have been suggested to play an important role in drug-seeking (Kalivas, 2009). Therefore, it is likely different phases of self-administration may reflect neuroadaptations in different DA circuits.

*NAc*: It is well established that the ventral striatum, including the NAc, plays a key role in mediating the reinforcing effects of stimulant drugs (Wise, 2004). This may not be surprising given that the NAc is a main projection site of DA-containing neurons from the ventral tegmental area. Previous research has highlighted the importance of the NAc since cocaine (McKinzie, Rodd-Henricks, Dagon, Murphy, & McBride, 1999) and amphetamine (Phillips, Robbins, & Everitt, 1994) are self-administered directly into the rat NAc. Furthermore, a $D_1$-like antagonist administered directly into the NAc attenuated the reinforcing effects of self-administered cocaine. In terms of effects of MDMA, various microdialysis studies have reported augmented release of DA in the NAc after repeated exposure to MDMA (Baumann, Clark, Franken, et al., 2008; Kalivas et al., 1998). These data suggest that, much like other drugs of abuse, repeated exposure to MDMA produced a sensitised DA response in the NAc.

*Dorsal striatum*: The sensitised DA response in the NAc is thought to be important during the development of drug-taking, but the shift to compulsive drug-seeking has been attributed to other neural circuits (Everitt & Robbins, 2005). It is important to acknowledge that while drug taking is often goal directed and develops because of the pleasurable subjective effects produced by the drug, following repeated exposure these pleasurable effects are reduced (Robinson & Berridge, 2000). Nonetheless, drug-taking often persists and drug-seeking develops. This transition from goal-directed behaviour to habitual, stimulus-response behaviour has been suggested to depend upon a switch of drug-produced effects from the ventral striatum (in particular the NAc) to the dorsal striatum (Everitt & Robbins, 2005, 2013; Vanderschuren, Di Ciano, & Everitt, 2005). The importance of DA in the dorsal striatum during drug-seeking is highlighted in a study that showed reduced cocaine-seeking behaviour following the administration of a DA antagonist directly into the dorsal striatum (Vanderschuren et al., 2005). These data are further supported by the observation that cocaine-seeking under a second-order schedule is associated with an increase in extracellular DA in the dorsal striatum (Ito, Dalley, Robbins, & Everitt, 2002).

Similar to cocaine, drug seeking in rats trained to self-administer MDMA was positively correlated with MDMA-produced DA release in the striatum (Colussi-Mas et al., 2010). In addition, repeated exposure to MDMA produced a decrease in 5HT levels in the striatum (Do & Schenk, 2013; O'shea et al., 1998; Scanzello et al., 1993; Schenk et al., 2007), as well as reduced 5HT release in the striatum (Baumann, Clark, Franken, et al., 2008).

*PFC:* The PFC has also been suggested to play a key role in the development of drug-seeking (Kalivas, 2009). Along with the NAc, the PFC is one of the main projection areas of DA-containing cells of the ventral tegmental area (Sesack & Pickel, 1992). This pathway has been extensively studied and has been shown to play a crucial role in behavioural sensitisation to psychostimulants (Pierce & Kalivas, 1997; Vanderschuren & Kalivas, 2000). However, the PFC not only receives DAergic innervation from the VTA, it also sends major glutamatergic projections from the PFC to the NAc (Sesack & Pickel, 1992). It has been suggested that these glutamatergic projections from the PFC play an important role in controlling drug-taking behaviour (Kalivas, 2009), and that drug use disrupts the excitatory glutamatergic outputs from the PFC resulting in compulsive drug-seeking behaviour (Kalivas, 2009). The importance of the neural circuit is revealed by the

fact that the reinstatement of drug-seeking, and the associated increase in glutamate in the NAc, are both abolished by inactivation of PFC glutamatergic neurons that project to the NAc (McFarland, Lapish, & Kalivas, 2003).

Of interest, ibotinic acidic lesion of the PFC prevented the development and expression of behavioural sensitisation following repeated MDMA exposure (Ramos, Goñi-Allo, & Aguirre, 2005). The inability of MDMA to produce behavioural sensitisation following impaired functioning of the PFC highlights the importance of this neural circuit in the development of MDMA-taking. 5HT receptors are also densely localised in the PFC and have been shown to modulate DA release (Alex & Pehek, 2007). Previous research suggests that there is a reduction in 5HT in the PFC following the self-administration of MDMA (Do & Schenk, 2013; Schenk et al., 2007), and this finding aligns with what was found in former heavy ecstasy users (McCann et al., 1998). Furthermore, it has been suggested that the prelimbic cortex, a sub-region of the PFC, plays an important role in MDMA-seeking (Ball & Slane, 2012). Together these data suggest that, much like other drugs of abuse, neuroadaptations in the PFC play an important role in the development of MDMA-seeking.

Overall it is clear that ascending projections of midbrain dopamine neurons to the nucleus accumbens, dorsal striatum, and prefrontal cortex play an important role in modulating the reinforcing efficacy of drugs of abuse, including MDMA. It is likely that neuroadaptations within these structures are induced by repeated MDMA exposure. The shift from drug-taking to drug-seeking most likely represents a shift at the neural level. Although MDMA initially induces a large 5HTergic response, following repeated administration the 5HT response is reduced and the DA response is increased (Colussi-Mas et al., 2010; Kalivas et al., 1998), with the result that MDMA becomes comparable to other drugs of abuse. If so, similar neuroadaptations may underlie the development of compulsive MDMA use (Schenk et al., 2011).

**Immediate Early Gene c-fos**

The immediate early genes are a group of transcription factors that play an important role in the regulation of brain development and function (Hughes & Dragunow, 1995). In this large family of over 100 members, the proto-oncogene, c-fos, has been extensively mapped following a variety of manipulations as a measure of neural activation (Curran & Morgan, 1995). C-fos is expressed in neurons following the entry of

calcium through voltage-gated channels into the cell (J. I. Morgan & Curran, 1986), and so expression of c-fos in specific neurons often occurs following recent activation. Fos, the protein of c-fos, can be detected within neurons using immunohistochemical techniques (Menetrey, Gannon, Levine, & Basbaum, 1989; J. I. Morgan, Cohen, Hempstead, & Curran, 1987; Mugnaini, Berrebi, Morgan, & Curran, 1989), thereby allowing an *in vivo* map of cellular responses to a given stimulus.

A number of studies have mapped the neural substrates mediating the long term effects of exposure to drugs of abuse by measuring changes in c-fos expression (Harlan & Garcia, 1998). Acute administration of cocaine (Ennulat, Babb, & Cohen, 1994; Graybiel, Moratalla, & Robertson, 1990; Hope, Kosofsky, Hyman, & Nestler, 1992; Moratalla, Elibol, Vallejo, & Graybiel, 1996; Steiner & Gerfen, 1993) and amphetamine (Graybiel et al., 1990; Jaber et al., 1995; Konradi, Leveque, & Hyman, 1996; Persico, Schindler, O'Hara, Brannock, & Uhl, 1993; Wang, Smith, & McGinty, 1995) stimulated a rapid increase in c-fos expression in a number of brain regions, including the dorsal striatum, NAc and PFC. In comparison, more complex changes in c-fos expressions were seen after repeated drug administration. C-fos expression was reduced in the dorsal striatum, NAc and PFC, following repeated exposure to cocaine (Ennulat et al., 1994; Hope et al., 1992; Moratalla et al., 1996; Steiner & Gerfen, 1993; Todtenkopf & Stellar, 2000) or amphetamine (Jaber et al., 1995; Konradi et al., 1996; Persico et al., 1993). In the majority of these studies rats were tested within 24 hours of the last drug treatment. Following a longer withdrawal period the results are equivocal. For example, some studies report no change in c-fos expression in the striatum (Ostrander et al., 2003), while others report a sensitised c-fos induction (Norman, Lu, Klug, & Norgren, 1993). Thus, results of studies are difficult to compare due to widely varied drug treatment regimens and lengths of withdrawal.

There was an increase in c-fos expression in the PFC and striatum, following cocaine (Daunais, Roberts, & McGinty, 1993; Gao, Limpens, Spijker, Vanderschuren, & Voorn, 2015; Larson et al., 2010; Neisewander et al., 2000; Zahm et al., 2010), and methamphetamine (Cornish, Hunt, Robins, & McGregor, 2012; Krasnova et al., 2013), self-administration, however the magnitude of the increase in c-fos expression was variable. For example, methamphetamine produced similar increases in c-fos expression in subjects with extensive methamphetamine self-administration experience, and yoked saline controls (Cornish et al., 2012), while c-fos expression in response to cocaine was

lower in cocaine self-administration subjects, compared to yoked saline controls (Larson et al., 2010; Zahm et al., 2010). Furthermore, Larson and colleagues (2010) suggest that yoked cocaine animals only showed reduced c-fos expression in the dorsal striatum.

There are a limited number of studies that have investigated the changes in c-fos expression as a result of MDMA exposure, and all of the available data focus on non-contingent administration of MDMA. Nevertheless, these studies indicate that acute administration of MDMA increased c-fos expression in a number of brain regions, including the PFC, NAc, and dorsal striatum (Colussi-Mas & Schenk, 2008; Dragunow, Logan, & Laverty, 1991; Hargreaves, Hunt, Cornish, & McGregor, 2007; Hashimoto, Tomitaka, Narita, Minabe, & Iyo, 1997; Stephenson, Hunt, Topple, & McGregor, 1999; Won et al., 2003). Furthermore, c-fos expression was attenuated in various brain regions following repeated exposure to MDMA (Colussi-Mas & Schenk, 2008). More research needs to be done in order to understand the neuroadaptations that occur following repeated exposure to MDMA, particularly following contingent exposure. Furthermore, by correlating changes in c-fos expression with behavioural data it is possible to reveal changes in brain regions that are functionally relevant.

**The present study**

The present research was undertaken to determine whether the contingency of chronic exposure to MDMA would produce differential changes in the activation of neural populations. Firstly, in an attempt to replicate previous research, forward and vertical locomotor activity were examined following MDMA self-administration and non-contingent MDMA administration. Then the distribution of c-fos expression was examined in order to determine whether c-fos expression would differ between rats that self-administered MDMA, and rats that received the same amount of MDMA non-contingently. Lastly, forward and vertical locomotor activity were correlated with c-fos expression in order to investigate whether the behavioural data could be explained by changes in c-fos expression in specific brain regions.

## Methods

### Subjects

Sprague-Dawley rats (n=81) bred in the vivarium at the Victoria University of Wellington, New Zealand were used. All experiments were approved by the Animal Ethics Committee of Victoria University of Wellington. Rats were housed in groups of four until reaching weights of 290-330g, thereafter they were housed individually in hanging polycarbonate boxes. The housing facility was temperature- (19-21°C) and humidity- (55%) controlled and maintained on a 12h light/dark cycle (lights on at 0700). All testing was completed during the light cycle and rats had free access to food and water except during testing.

### Surgery

Rats were deeply anesthetised using a combination of ketamine (90mg/kg, IP) and xylazine (9mg/kg, IP). The scalp and right side of the chest were shaved and swabbed with ethanol (75%) and vetadine. Eye lubricant was also used to prevent drying. An incision was made on the skull and the tissue was removed before an incision was made above the right external jugular vein. The vein was isolated and a length of silastic tubing was inserted into the vein and subcutaneously passed to an exposed section of the skull. The distal length of the catheter, comprising a 2cm piece of 22 gauge stainless steel tubing was attached to the skull using embedded jeweller's screws and dental cement. An electrolyte replacement (Hartmann's solution, 12ml, s.c) as well as an analgesic (carprofen, 5.0mg/kg, s.c) was administered following surgery. Post-operative care on the two days following surgery consisted of carprofen (5.0mg/kg, s.c) to reduce pain and inflammation, and catheters were infused with 0.15-0.2 ml of solution containing heparin (30IU/ml) to prevent coagulation and penicillin (250 000 IU/ml). Self-administration testing began at least 5-7 days after surgery, once pre-surgery weight was reached. A weekly test of the catheter patency was conducted by administration of pentobarbital (20mg/kg, i.v). Immediate lack of the automatic righting reflex confirmed patency.

### Apparatus

*Self-administration* testing was conducted in a temperature- (19-21°C) and humidity- (55%) controlled environment using operant chambers (Medical Associates, ENV-001) equipped with two levers and a stimulus light. Rats were randomly assigned to groups of three and assigned as 'master', 'yoked MDMA' or 'yoked saline'. Depression

of the right lever (i.e. the "active" lever) by the master rat resulted in a 12 second infusion of MDMA (0.1ml) for the master and yoked MDMA rats or an infusion of vehicle (3IU/ml heparinised saline) for the yoked control rat. Infusions were paired with the illumination of a light stimulus located above the lever. Depression of inactive lever had no programmed consequence for any rat. Both active and inactive lever responses were recorded for all rats.

Each day before testing, catheters were flushed with 0.2mls of the heparin-penicillin solution and the exposed metal tubing was connected to a 20ml drug syringe through a length of micro bore tubing. The tubing was connected through a steel spring for protection and fed through a swivel mechanism (Harvard Apparatus) to the syringe which was loaded into a pump (Razel, Model A. Motor: 1rpm). All drug infusions and data collection were controlled by Med Associates software.

*Locomotor activity* was conducted in clear Plexiglas chambers (Med Associates Inc., USA; model ENV-515) measuring 42 x 42 x 30cm, located within sound attenuating boxes. Forward locomotion was measured using two sets of sixteen infra-red beams and sensors spaced evenly along the side of each box produced squares measuring 25 x 25mm. The interruption of three adjacent beams was recorded as one activity count. In addition, vertical locomotion (rearing) was measured using a second set of identical infra-red sensors located 14cm above the floor of the chamber. A white noise generator was used during the experiments to mask any outside noise. At the end of each session, chambers were wiped with Virkon 'S' disinfectant (Southern Veterinary Supplies, NZ) to control for any olfactory confounds. All experiments were run in the dark except for a red light that was used to illuminate the room during drug administration.

**Procedure**

*Self-administration:* Rats were weighed and catheters were flushed with the penicillin/heparin solution. The rats were then placed in the operant chamber boxes and connected to the tubing/spring apparatus for self-administration training. Rats assigned to the 'master' group were trained to self-administer MDMA, whereas rats assigned to the 'yoked MDMA' and 'yoked saline' groups were both manually connected to the light and pump mechanism of the master rat.

*Training*: Self-administration sessions were conducted during 2hr sessions, 6 days per week. Each self-administration session began with an experimenter-delivered infusion of drug to clear the line of penicillin solution. Thereafter, depression of the active lever

resulted in an infusion of MDMA (1.0 mg/kg) to the master and yoked MDMA rats, and saline to the yoked saline rats, according to a Fixed Ratio 1 (FR1) schedule. Tests continued until a total of 90 infusions (90 mg/kg) had been self-administered by the master rat or 25 days, whichever came first. A total of 15 master rats, and the associated triads, failed to meet this criterion and were not included for further testing (Aronsen, Bukholt, & Schenk, 2016; Bradbury et al., 2014; Schenk et al., 2012). For the remaining 36 rats (12 triads) the dose was reduced to 0.5mg/kg until an additional 150 infusions were administered. When responding varied by less than 20% over three consecutive days the reinforcement schedule was then changed to FR2. Then once responding on the FR2 schedule had stabilised (varied by less than 20% over three consecutive days) the reinforcement schedule was changed to FR5 and rats remained on this schedule until a total of 350 mg/kg MDMA had been self-administered.

A total of 12 triads met the initial criterion of 90 infusions of MDMA (1.0 mg/kg/infusion). Some triads did not proceed for various reasons: three triads due to loss of catheter patency, three triads due to MDMA toxicity, and two triads due to an inner ear infection. This is an abnormally high attrition rate, but only complete triads were included in the current study. The remaining four triads completed testing and self-administered 350 mg/kg MDMA. The training stage varied but an average of 14.5 (*SD* = 3.52) daily sessions was required to meet the initial criterion of 90 mg/kg and an additional 15.75 (*SD* = 0.50) days was required to meet the subsequent 350 mg/kg criterion.

*Locomotor activity:* Horizontal and vertical activity were measured during three daily test sessions that commenced the day following the last session. On each day of testing the rats were habituated to the chamber for 30 minutes before receiving an injection of either saline vehicle (i.p) on the first two days, or MDMA (5 mg/kg, i.p) on the test day. After the injection, animals were immediately returned to the activity chambers and activity was then measured for 2 hours post-injection. This time frame was used because c-fos expression peaks between 1 to 3 hours after a stimulation (Kovács, 1998). After both habituation sessions the rats were returned to the home cage, but on the test days animals were euthanized immediately after the activity session. Testing was completed between 0700 and 1000.

Rats were deeply anesthetised with sodium pentobarbital (50mg/kg, i.p) and perfused transcardially with 60ml of 0.1% heparinised saline followed by 200ml of 4% paraformaldehyde solution in 0.1M phosphate buffer (PB) (pH 7.2). Perfusions were

performed using a perfusion pump (EYLA micro tube pump MP-3, Tokyo Rikakikai Co., Ltd, Tokyo, Japan) at a speed of 1450ml/hr (or 24.16 ml/min). Brains were then rapidly removed, placed in 4% paraformaldehyde fixative overnight, cryoprotected in 20% glycerol containing 0.1M PB and 0.05% sodium azide at 4°C, before being frozen for 4min in isopentane at -40°C. Brains were stored at -80°C until further processing.

*Immunohistochemistry:* Coronal sections (35μm thick) were cut on a sliding microtome (Microm HM 450) connected to a freezing unit (Microm KS 34, Microm International GmbH part of Thermo Fisher Scientific, Auckland, New Zealand) chilled to -40°C. Tissue slices were stored in 0.1M phosphate buffered saline (PBS) with 0.05% sodium azide at 4°C until immunological staining. Areas of interest included the dorsal striatum, the nucleus accumbens, and the prefrontal cortex. These areas were selected using images from Paxinos & Watson's Rat Brain Atlas (Paxinos & Watson, 2005).

*c-fos:* The revelation of c-fos positive nuclei was conducted as per the protocol reported in Colussi-Mas and Schenk (2008). Free-floating sections were washed in PBS containing 0.3% Triton X-100 (PBST) (3x10min) and incubated overnight with the primary rabbit anti-Fos antibody (Ab-5; Calbiochem, EMD Biosciences, Darmstadt, Germany) diluted to 1:20000 in PBST and 1% bovine serum albumin. The anti-Fos antibody was raised against a synthetic peptide (SGFNADYEASSSRC) which corresponds to amino acids 4-17 of the human Fos protein. It recognises the ~55 kDa c-Fos and the ~62 kDa v-Fos proteins, and does not cross-react with the ~39 kDa Jun protein (manufacturer's data sheet). During washing and incubation, sections were gently agitated on a tilting rocker at room temperature.

The following day, sections were washed with PBST (3 x 10mins) before being incubated for 90 min with the secondary biotinylated goat anti-rabbit antibody (Vector Laboratories, Burlingame, CA, USA) diluted 1:1000 in PBST. This was followed by another three washes in PBST (3x10mins) and a 60 min incubation with a preformed avidin-biotinylated horseradish peroxidase complex diluted 1:1000 in PBST. Sections were washed in PBST (3x10mins) and c-fos was revealed by a reaction with 50mM Tris HCl (pH 7.4) buffer, containing 0.02% 3,3'-diaminobenzidine, 0.8% nickel chloride and 0.003% $H_2O_2$, resulting in blue-black staining precipitate. This reaction was stopped with a further three washes in PBST. Finally, the sections were mounted on gelatin-coated slides and left to dry overnight. The slides were then stained with neutral red, rinsed in distilled $H_2O$, dehydrated in EtOH (70%, 95% x 2, 100% x 2) and rinsed twice in Histo-

Clear. The slides were then coverslipped with DePeX mounting medium. A control without the primary antibody was stained to ensure nonspecific staining was not present.

*Cell counts and analysis of immunohistochemical data:* Sections were magnified using an Olympus BX-51 microscope equipped with a MBF Biosciences camera (CX 9000) (x 20 magnification) and computerised image analysis system Neurolucida (MBF Biosciences, v. 8). Cells with dark blue or black nuclei were manually counted.

The distribution of positively stained nuclei for the c-fos antibody was determined. For each structure the number of Fos-immunoreactive (IR) cells was bilaterally counted in sections taken at 210-µm intervals, according to the rostrocaudal extension of the structure. For each structure a template was determined according to its shape and size (Fig. 2). The density of Fos-IR cells was calculated as the number of FOS-IR cells/mm$^2$ for each section. An average was then calculated for all sections within a structure for each rat.

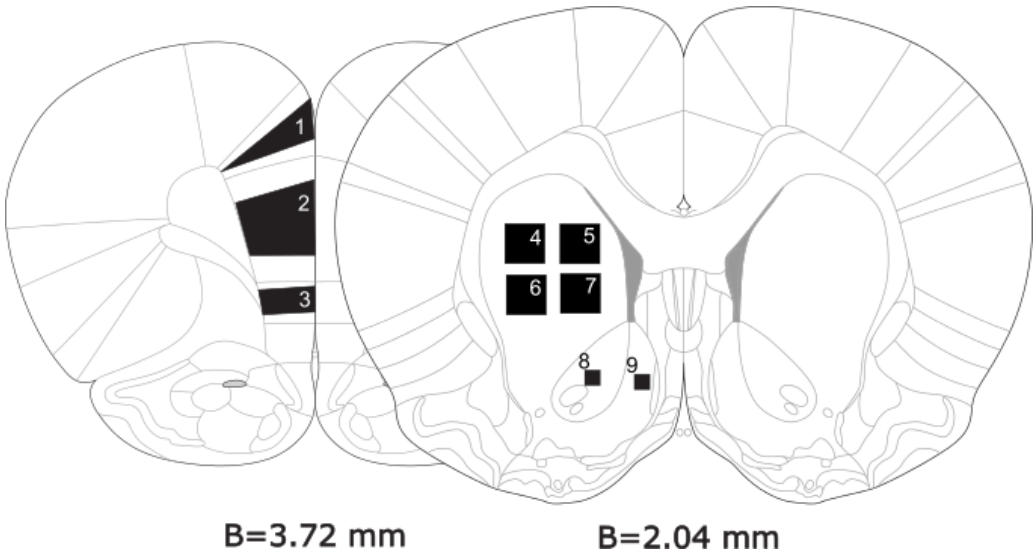



*Figure 2.* A schematic representation of the template used to quantify Fos-immunoreactive cells. Adapted from Paxinos and Watson (2005). 1. Cingulate cortex; 2. Prelimbic cortex; 3. Infralimbic cortex; 4. Dorsal striatum, dorsolateral part; 5. Dorsal striatum, dorsomedial part; 6. Dorsal striatum, ventrolateral part; 7. Doral striatum, ventromedial part; 8. Nucleus accumbens core; 9. Nucleus accumbens shell.

**Drugs**

MDMA-HCl (ESR, Porirua, New Zealand) for self-administration was dissolved in a sterile solution of heparinised saline (3IU heparin, 0.9% NaCl). MDMA for non-

contingent injections was dissolved in sterilised saline. Intravenous infusions were administered at a volume of 0.1 ml/kg and intraperitoneal injections were 1.0 ml/kg. All drug doses were calculated based on salt weights.

**Data Analysis**

Data were analysed using SPSS statistics package (SPSS Inc.; version 19.0 for Windows 7). An alpha level $\alpha=.05$ was adopted for all analyses. Where appropriate, post hoc tests were conducted using the Tukey HSD method. To examine the effect of condition on lever responding, a 3(group) x 2 (lever) repeated measures analysis of variance (ANOVA) was used. Condition (master, yoked, control) was the between subjects factor, and lever (active, inactive) was the within subjects factor.

To determine whether MDMA increased locomotor activity relative to saline, forward and vertical locomotor activity were assessed using separate analyses for master, yoked MDMA and yoked saline rats by a 2 (0, 5 mg/kg) x 30 (time) repeated measures ANOVA. The effect of MDMA exposure on forward and vertical locomotor activity was assessed by a 3 (master, yoked, control) x 30 (time) mixed model ANOVA, with time as the within subjects factor. The effect of MDMA exposure on c-fos density was assesed using a one-way ANOVA for each sub-region.

Correlations between the density of Fos-IR cells and total forward locomotor activity, as well as correlations between the density of Fos-IR cells and total vertical locomotor activity, were obtained using Pearson's correlation. For each rat the density of Fos-IR was plotted against the corresponding value for total forward locomotor activity counts, or total vertical locomotor activity counts.

## Results

### *Part I:* Behavioural effects of MDMA

*Self-administration*

The effect of self-administration condition (master, yoked, control) lever responding (active, inactive) over the last 5 days self-administration is shown in figure 3. ANOVA revealed a significant interaction between lever and self-administration condition ($F(2,9) = 121.46$, $p<.05$), as well as a main effect of lever ($F(1,9) = 126.76$, $p<.05$), and self-administration condition ($F(2,9) = 67.51$, $p<.05$). Post hoc analyses showed a significant preference for the active lever in the master condition, which was not seen in the yoked MDMA or yoked saline conditions.

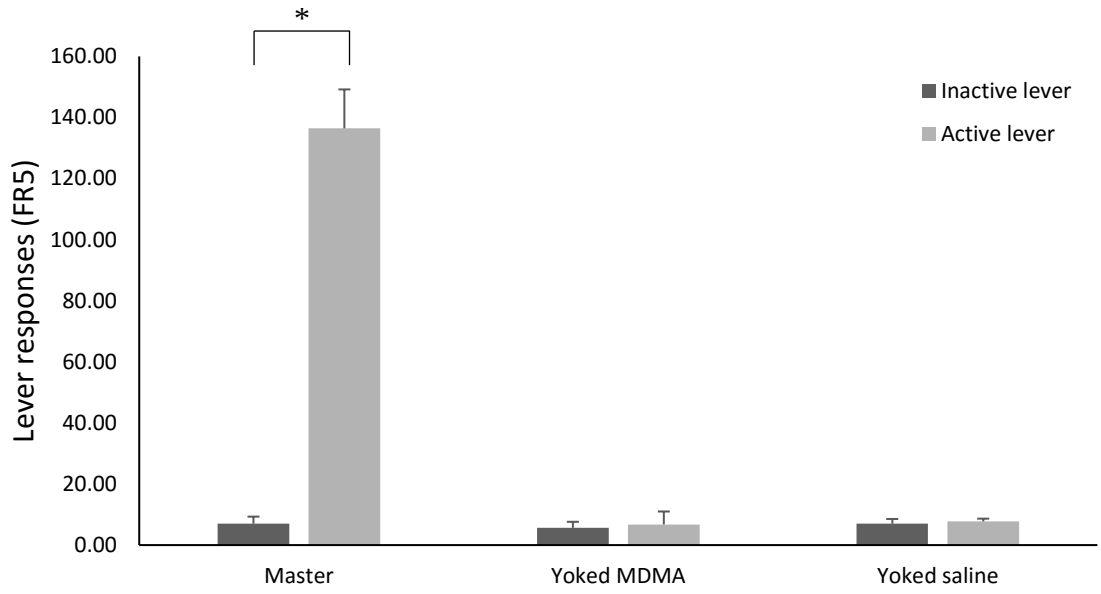



*Figure 3.* The effect of self-administration condition on lever responding. Bars represent average responding over the last 5 days of self-administration (+SEM). *=p<0.05

*Forward locomotor activity*

Figure 4 shows the effect of an MDMA challenge injection (0.0, 5.0 mg/kg, i.p) on forward locomotor activity, with activity for 0.0 mg/kg MDMA taken from the second day of locomotor activity testing. ANOVA revealed significant interactions between MDMA dose and time in the master ($F(29,87) = 4.24$, $p<.05$), yoked MDMA ($F(29,87) = 4.29$, $p<.05$), and yoked saline conditions ($F(29,87) = 4.29$, $p<.05$). A significant effect of MDMA dose was found in the master condition ($F(1,3) = 10.49$, $p<.05$), but not in the

yoked MDMA ($F(1,3) = 9.03$, *ns*), or yoked saline conditions ($F(1,3) = 6.49$, *ns*). There was a significant effect of time in the master ($F(29,87) = 4.34$, $p<.05$), yoked MDMA ($F(29,87) = 3.53$, $p<.05$), and yoked saline conditions ($F(29,87) = 2.29$, $p<.05$). Post hoc analyses showed a significant difference between saline and MDMA at multiple time points for the master (t20-t70), yoked MDMA (t15-t25, and t35-t60), and yoked saline conditions (t20, t30, t35, t45, t50, t65).

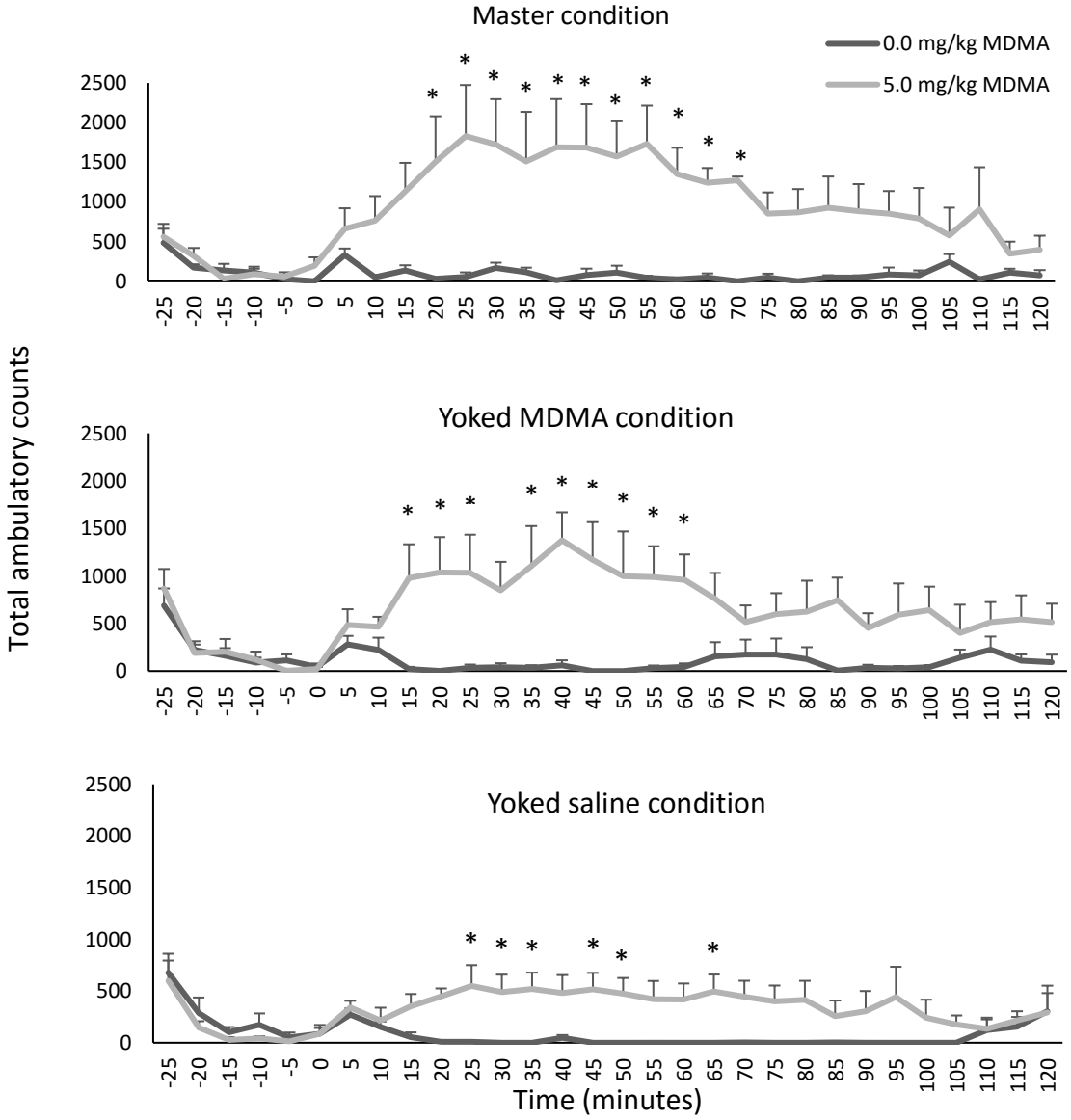



*Figure 4.* Effect of challenge injection (0.0, 5.0 mg/kg, i.p) on forward locomotor activity in the master, yoked MDMA, and yoked saline conditions. Challenge injections were administered at time 0. Error bars represent SEM. * $p < .05$ compared to 0.0 mg/kg MDMA.

Time course data for forward locomotor activity following an MDMA challenge injection (5 mg/kg) are shown in figure 5. ANOVA revealed an interaction between time and self-administration condition ($F(58,261) = 1.53$, $p<.05$), and a significant main effect of time ($F(29,261) = 9.97$, $p<.05$), but no main effect of condition ($F(2,9) = 2.12$, *ns*). Post hoc Tukey tests revealed a significant increase in forward locomotor activity in the master condition, compared to yoked saline condition, at multiple time points (t25, t30, t40, t45, t55).

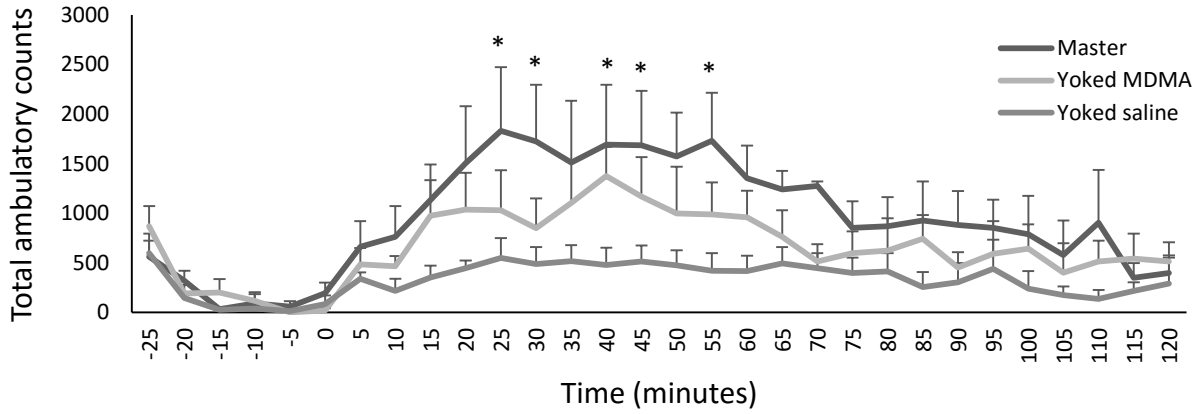



*Figure 5.* Time course of data from figure 4 showing effect of an MDMA challenge injection (5 mg/kg, i.p) on forward locomotor activity in each self-administration condition. The MDMA challenge was administered at time 0. Error bars represent SEM.* $p < 0.05$, master compared to yoked saline.

*Vertical locomotor activity*

Figure 6 shows the effect of MDMA dose (0.0, 5.0 mg/kg MDMA) on vertical locomotor (rearing) activity, with activity for 0.0 mg/kg MDMA taken from the second day of locomotor activity testing. ANOVA revealed a significant interaction between MDMA dose and time for the master ($F(29,87) = 3.09$, $p<.05$), yoked MDMA ($F(29,87) = 3.47$, $p<.05$), and yoked saline conditions ($F(29,87) = 1.90$, $p<.05$). No significant effect of MDMA dose was found in the master ($F(1,3) = 8.71$, *ns*), yoked MDMA ($F(1,3) = 7.96$, *ns*), or yoked saline group ($F(1,3) = 0.96$, *ns*), however a significant effect of time was found for the master ($F(29,87) = 2.75$, $p<.05$), yoked MDMA ($F(29,87) = 3.82$, $p<.05$), and yoked saline conditions ($F(29,87) = 5.44$, $p<.05$). Post hoc analyses showed a significant difference between 0.0 and 5.0 mg/kg MDMA doses at multiple time points

for the master (t25-t40, and t50) and yoked MDMA (t30-t45) conditions, but no significant differences were found in the yoked saline condition.

Time course data following MDMA administration are shown in figure 7. ANOVA revealed an interaction between time and self-administration condition ($F(58,261) = 1.83$, $p<.05$) and a significant main effect of time ($F(29,261) = 4.81$, $p<.05$), but no main effect of self-administration condition ($F(2,9) = 3.51$, *ns*). Post hoc Tukey tests revealed a significant increase in in vertical activity in the master condition, compared to the yoked saline condition, at two time points (t25, t30), as well as a significant increase in vertical activity in the yoked MDMA condition, compared to the yoked saline condition, at multiple time points (t30, t35, t40, t45).

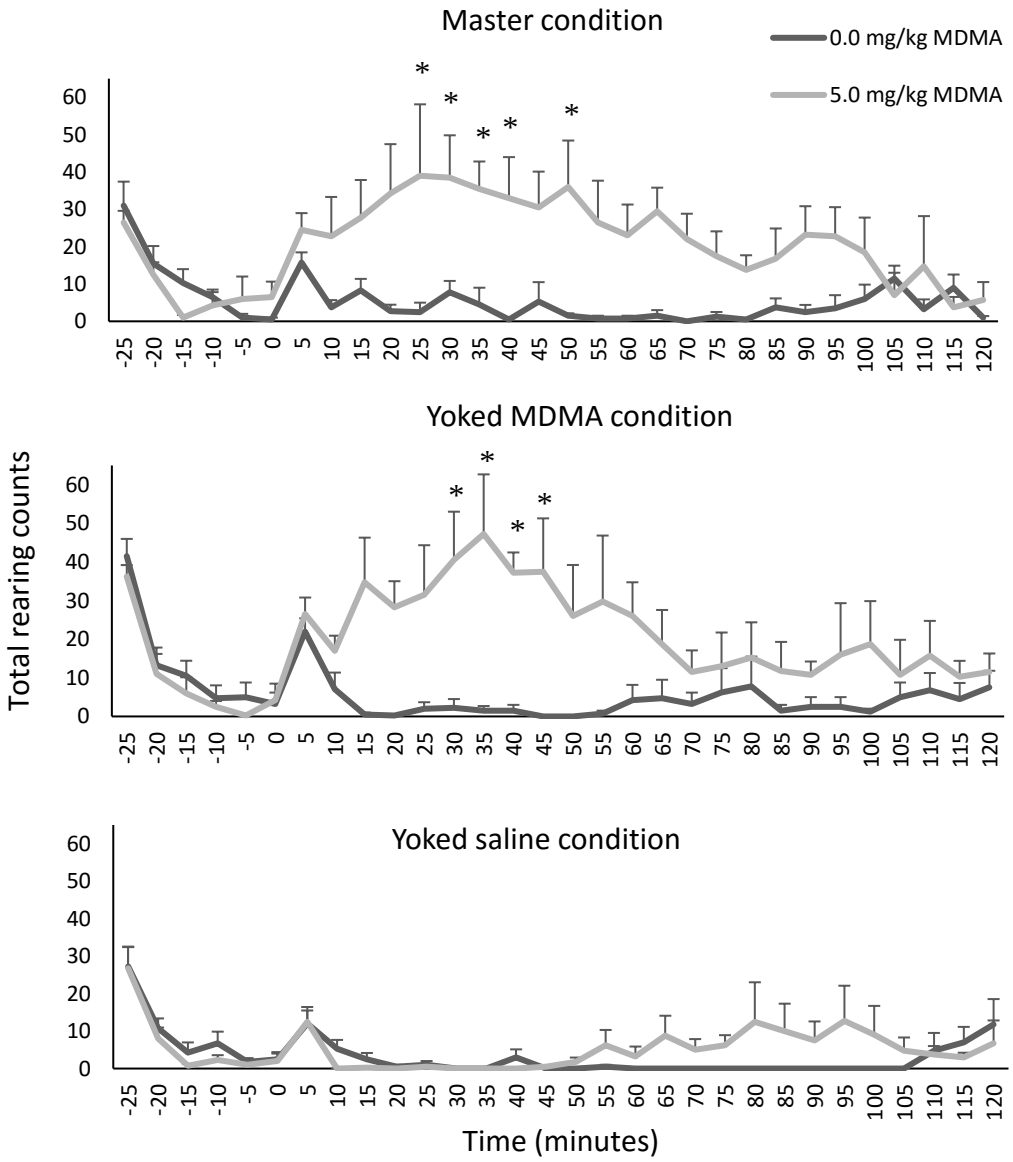



*Figure 6.* Effect of MDMA challenge injection (0.0, 5.0 mg/kg, i.p) on vertical locomotor

activity in the master, yoked MDMA, and yoked saline conditions. Challenge injections were administered at time 0. Error bars represent SEM. * $p < .05$ compared to 0.0 mg/kg MDMA.

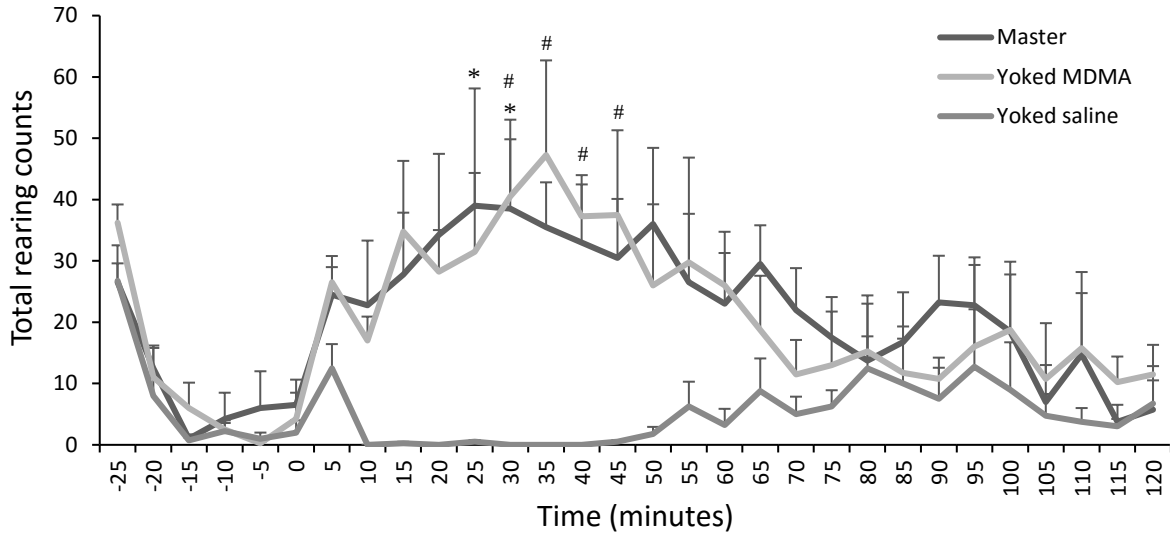
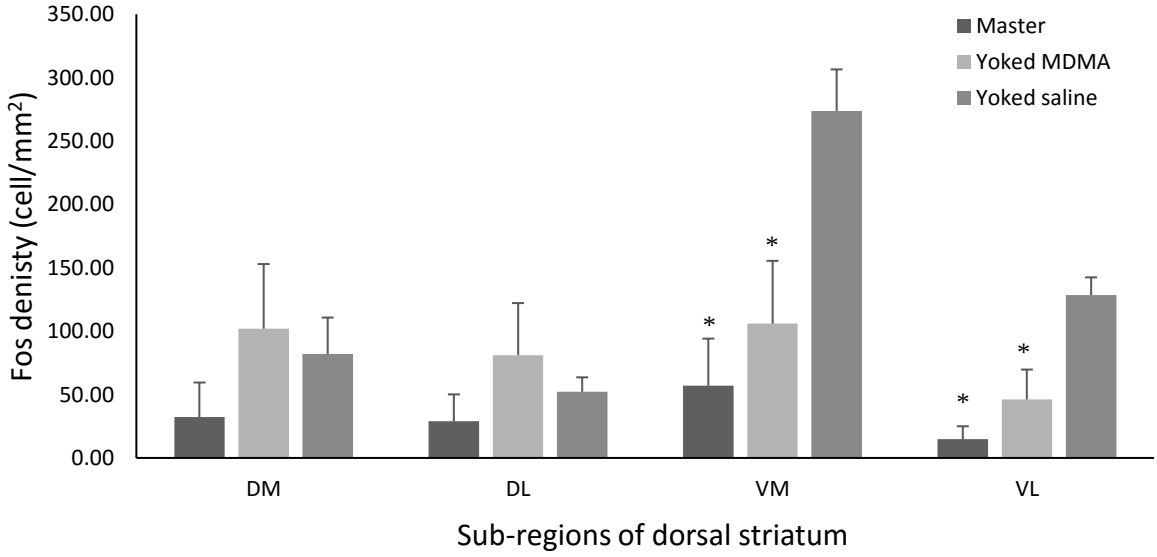



*Figure 7.* Time course of data from figure 6 showing effect of an MDMA challenge injection (5 mg/kg, i.p) on vertical locomotor activity in each self-administration condition. The challenge MDMA injection was administered at time 0. Error bars represent SEM.* $p < 0.05$, master compared to yoked saline. # $p < 0.05$, yoked MDMA compared to yoked saline.

***Part II:*** **c-fos expression**



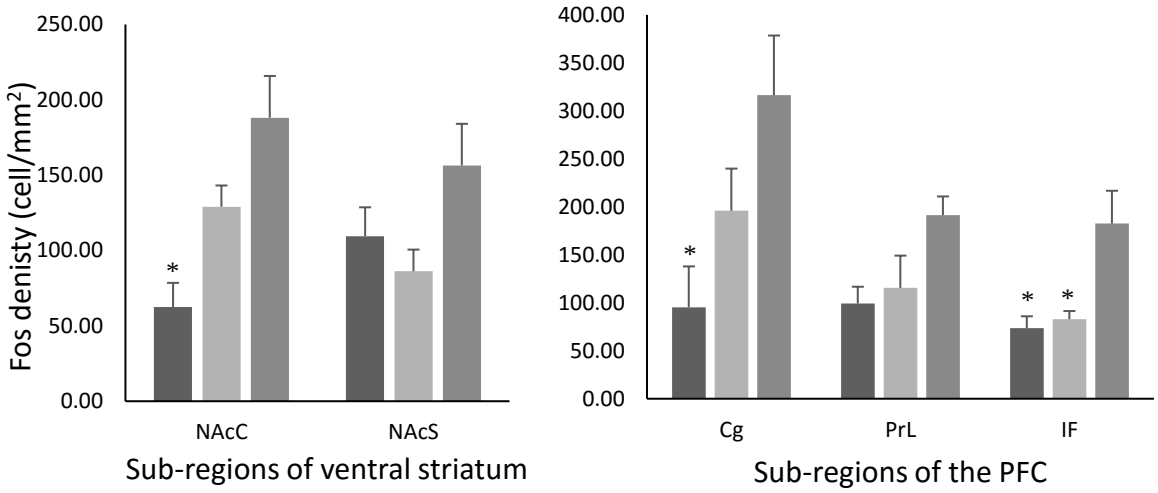


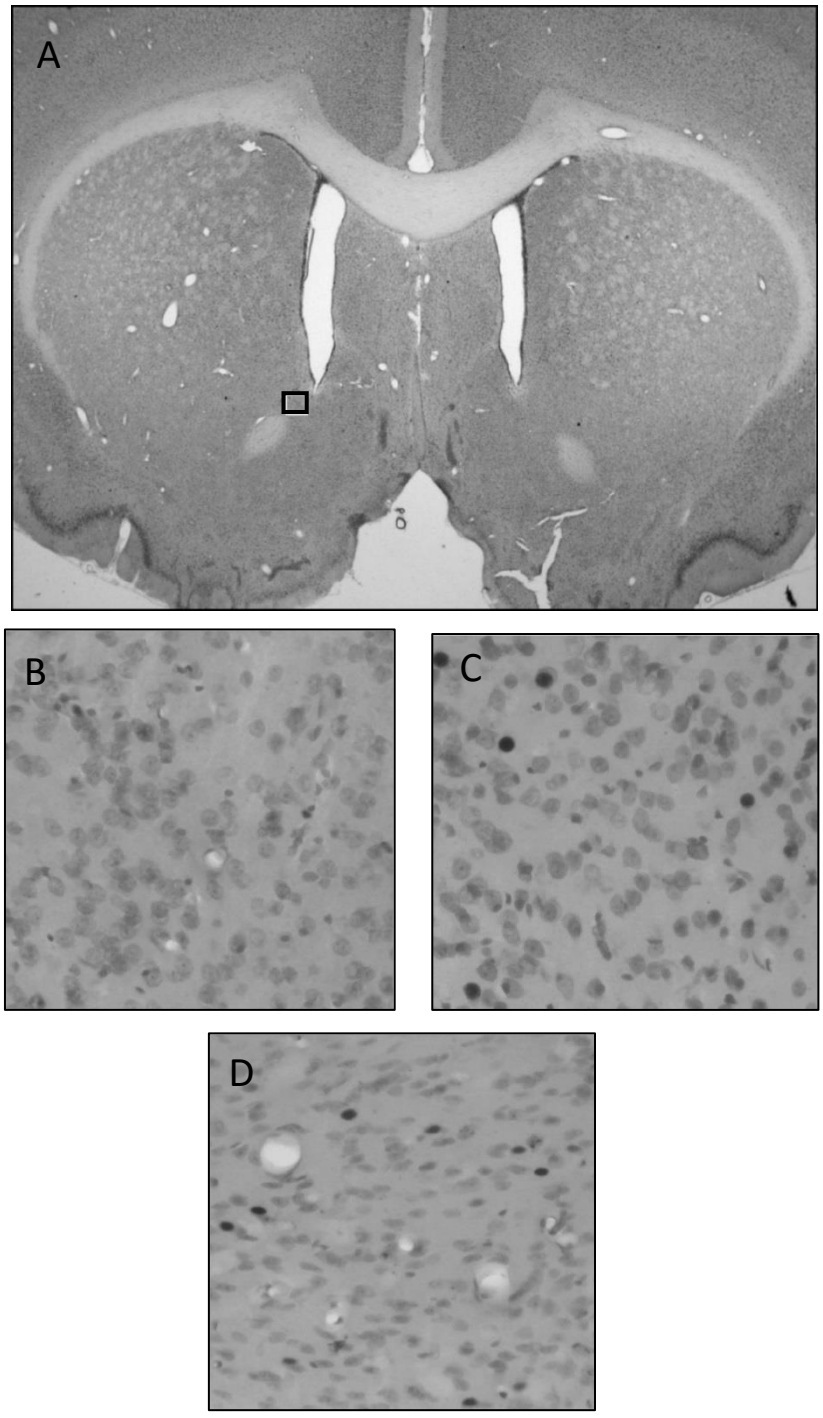

*Figure 8.* Quantification of c-fos density in brain structures after an MDMA challenge injection (5 mg/kg). Data are expressed as the mean number of Fos-IR cells/mm$^2$ in each structure (+SEM). *p < 0.05, compared to yoked saline.

In the two sub regions of the ventral striatum ANOVA revealed a significant main effect of self-administration condition in the nucleus accumbens core ($F(2,9) = 9.68$, $p<.05$), and no significant effect of self-administration condition in the nucleus accumbens shell ($F(2,9) = 2.87$, *ns*). Post hoc tests showed that c-fos density was significantly lower in the master condition compared to the yoked MDMA and yoked saline conditions, as shown in figure 9.

In the three sub-regions in the prefrontal cortex ANOVA revealed a significant main effect of self-administration condition in the cingulate cortex ($F(2,9) = 4.82$, $p<.05$) and in the infralimbic cortex ($F(2,9) = 7.85$, $p<.05$), but no significant main effect of self-administration condition in the prelimbic cortex ($F(2,9) = 3.99$, *ns*). As illustrated in figure 8, post hoc analyses showed, in the cingulate cortex, c-fos density was significantly lower in the master condition compared to the yoked MDMA and yoked saline

conditions, and in the infralimbic cortex c-fos density was significantly lower in the master and yoked MDMA conditions, compared to the yoked saline condition.

*Figure 9.* Effect of self-administration condition on c-fos expression in the nucleus




accumbens core following a challenge injection of 5.0 mg/kg MDMA. (A) Microscopy image of a coronal section of a rat brain after staining with neutral red. (B-D) higher-magnification images (20x objective) of the boxed area depicted in A. (B) master, (C) Yoked MDMA, (D) Yoked saline group.

***Part III:*** **The relationship between behaviour and c-fos expression**

Figure 10 shows correlations between the MDMA-produced forward locomotor response and c-fos density in the areas of interest. Significant negative correlations between c-fos density and total ambulatory counts were found in the cingulate cortex and the infralimbic cortex, and a negative correlation between c-fos density and total ambulatory counts in the nucleus accumbens core approached significance.

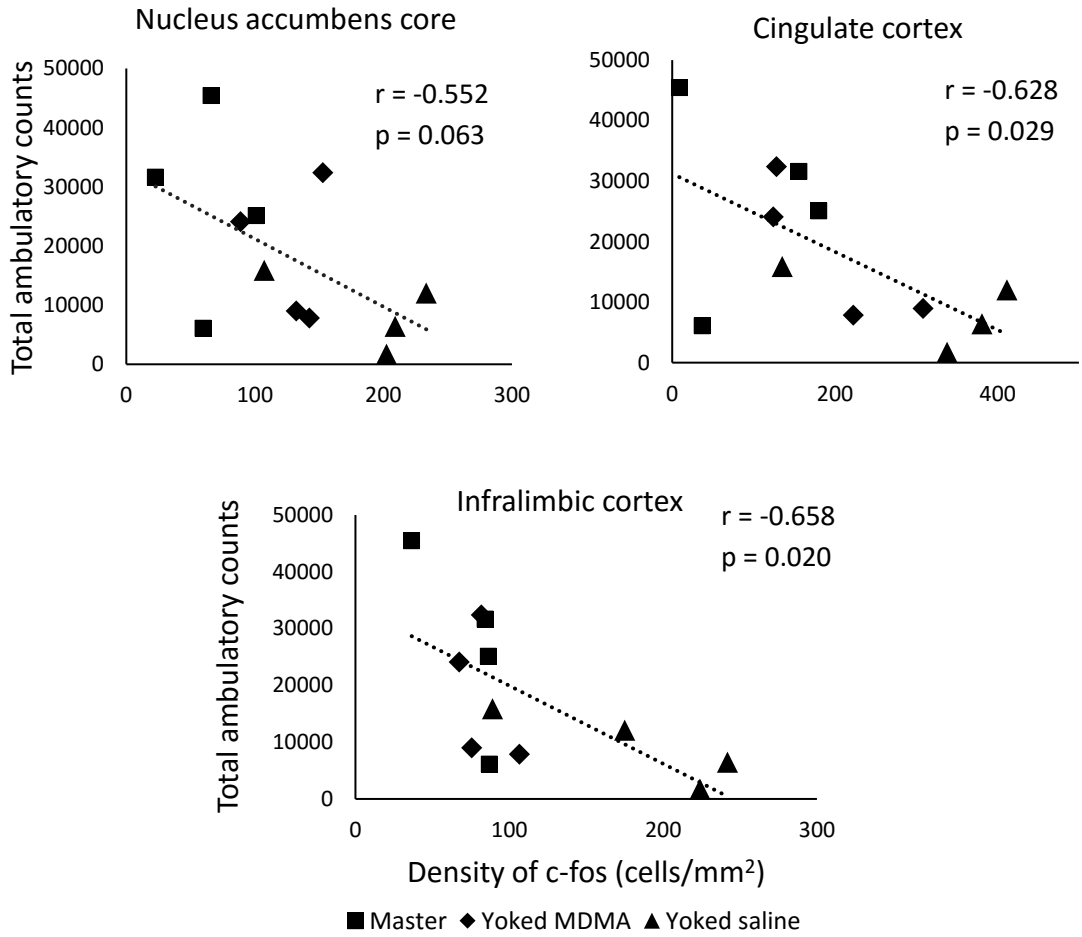



*Figure 10.* Correlations between total ambulatory counts and c-fos density in one subregion of ventral striatum (NAcC), and two sub-regions of the prefrontal cortex (Cg, IF).

Figure 11 shows correlations between MDMA-produced vertical locomotor activity and c-fos density. Significant negative correlations were found between c-fos density and total rearing counts in the cingulate cortex and the infralimbic cortex, while negative correlations between c-fos density and total rearing counts in the nucleus accumbens core, and nucleus accumbens shell, approached significance.

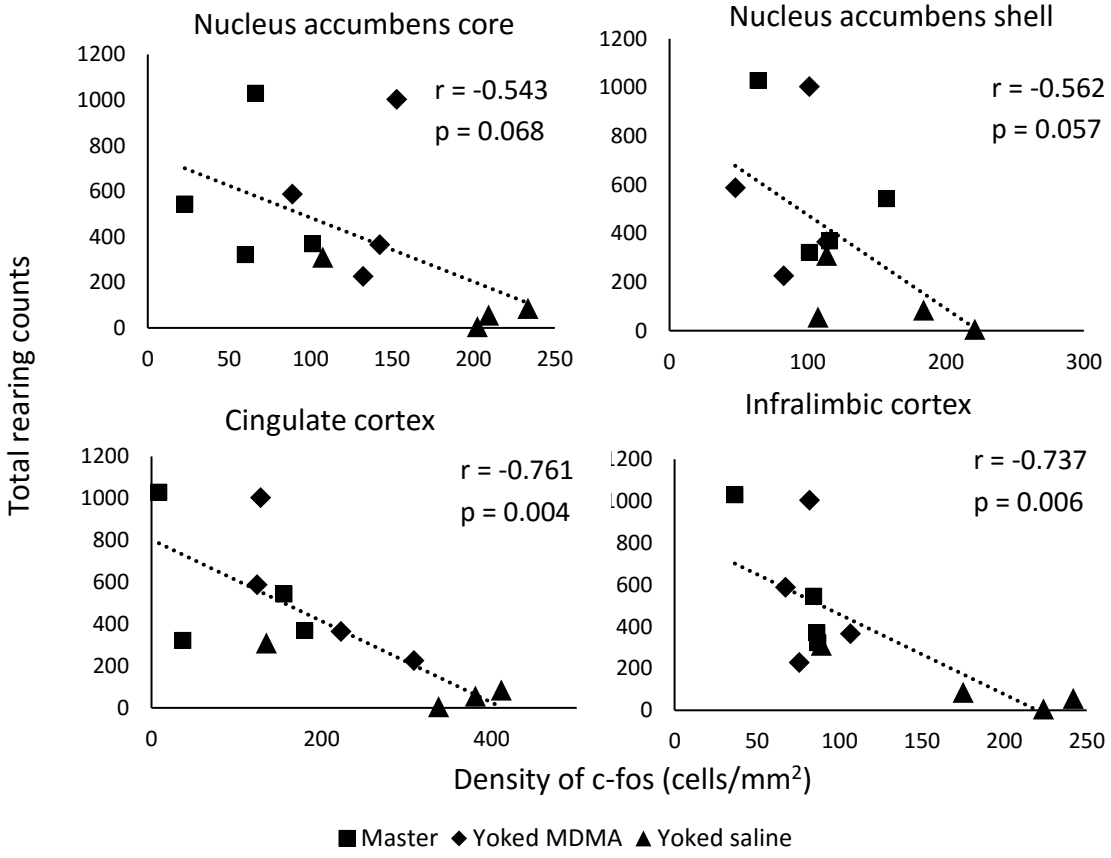



*Figure 11.* Correlations between total rearing counts and c-fos density in two subregions of ventral striatum (NAcC, NAcS), and two sub-regions of the prefrontal cortex (Cg, IF).

## Discussion

The main objective of this thesis was to determine whether MDMA-produced forward locomotor activity and vertical locomotor activity became sensitised following MDMA self-administration. Additionally, the relationship between MDMA-produced hyperactivity and c-fos expression in specific brain regions was determined.

The first experiment compared the effect of method of MDMA exposure on the subsequent locomotor activating effects of MDMA. There was an MDMA-produced increase in forward locomotor activity in rats from all three conditions (MDMA self-administration, yoked MDMA, and yoked saline) as has previously been reported (McCreary, Bankson, & Cunningham, 1999). Sensitisation was produced by MDMA exposure, as has also been previously reported (Ball, Budreau, & Rebec, 2006; Bradbury et al., 2012; Kalivas et al., 1998; Schenk & Bradbury, 2015; Spanos & Yamamoto, 1989). The magnitude of the sensitised response, however, depended on method of MDMA exposure. Specifically, after an MDMA challenge injection, forward locomotion was significantly increased only for the group that had received self-administered MDMA; the locomotor activating effect of MDMA in the group that received yoked infusions of MDMA was not significantly greater than the MDMA-produced increase in forward locomotion of the saline pre-exposed group. Given that previous research has reported sensitisation of MDMA-produced forward locomotion following repeated experimenter-administered MDMA it was surprising that the yoked MDMA animals failed to show a sensitised locomotor response. This differential effect of MDMA exposure method might indicate that different neuroadaptations are produced by contingent vs non-contingent exposure to MDMA.

There are data that indicate that, indeed, neurochemical effects of repeated drug exposure might depend on whether the drug is experimenter-administered or self-administered (Dworkin et al., 1995; Hemby et al., 1997; Hemby et al., 1995; Stefanski et al., 1999). For example, following 25 days of cocaine exposure, an intravenous cocaine infusion produced greater extracellular DA levels in a group of rats that had self-administered cocaine, compared to the rats that had received yoked cocaine delivery (Hemby et al., 1997). In the case of MDMA it has been reported that self-administration produced an augmented DA response to MDMA (Colussi-Mas et al., 2010). However, no studies have been conducted to compare MDMA produced DA efflux following self-administration and yoked administration. If the DAergic response to MDMA is greater

after MDMA self-administration compared to yoked administration, as was seen with cocaine (Hemby et al., 1997), this could explain behavioural differences observed in the present study.

In addition to forward locomotor activity, the effect of method of MDMA exposure on subsequent MDMA-produced vertical locomotor activity was also assessed. As has been previously reported (O'Loinsigh, Boland, Kelly, & O'Boyle, 2001), acute administration of MDMA did not produce vertical locomotor activity in animals that were not previously exposed to MDMA. An increase in vertical locomotor activity was, however, evident in both groups that received MDMA exposure. This finding is consistent with findings from previous studies that also found an increase in vertical locomotor activity following repeated experimenter-administered (Lettfuss et al., 2013), or self-administered (Schenk & Bradbury, 2015) MDMA. Because increased vertical locomotor activity has been attributed to increased DA release (Thiel et al., 1999), an increase in vertical locomotor activity following repeated MDMA exposure may reflect the increase in MDMA-produced DA release produced following pre-exposure (Colussi-Mas et al., 2010; Kalivas et al., 1998).

Alternatively, vertical locomotor activity might emerge as a result of 5HT deficits produced by repeated exposure to MDMA. Acute administration of MDMA produced elements of 5HT syndrome, including flat-body posture and forepaw treading (Spanos & Yamamoto, 1989), behaviours that are incompatible with vertical locomotor activity. Repeated MDMA exposure attenuated MDMA-produced increases in extracellular 5HT levels, and resulted in tolerance to these 5HT-mediated behaviours (Baumann, Clark, Franken, et al., 2008). Although 5HT syndrome in response to MDMA has not been assessed after MDMA self-administration, the dosing regimen used in the present study has been reported to produce 5HT depletions (Do & Schenk, 2013). Therefore, tolerance to MDMA-produced 5HT syndrome behaviours might explain why rearing emerges following repeated MDMA exposure.

Taken together, these data suggest that repeated MDMA exposure resulted in sensitised forward and vertical locomotor activity in response to MDMA. It is possible that these sensitised responses could be explained by a reduction in the 5HT response and/or a sensitised DA response to MDMA. Such neuroadaptations would be consistent with previous findings that have shown an increase in MDMA-produced DA release

(Colussi-Mas et al., 2010), and a reduction in MDMA-produced 5HT release (Reveron et al., 2010) following MDMA self-administration.

The second part of this study mapped MDMA-produced neuronal activation of cells as a function of MDMA exposure. Acute exposure to MDMA (Dragunow et al., 1991; Hashimoto et al., 1997; Stephenson et al., 1999), cocaine (Ennulat et al., 1994; Graybiel et al., 1990), and amphetamine (Graybiel et al., 1990; Jaber et al., 1995) has been shown to increase c-fos expression in various brain regions including the dorsal striatum, NAc, and PFC. Repeated exposure to various psychostimulants resulted in an attenuation of this increase in c-fos expression (Ennulat et al., 1994; Hope et al., 1992; Jaber et al., 1995; Konradi et al., 1996), and similar effects were reported following repeated exposure to MDMA (Colussi-Mas & Schenk, 2008).

In the present study, repeated MDMA exposure produced a decrease in c-fos expression in the ventromedial and ventrolateral dorsal striatum, and in the infralimbic cortex, compared to yoked saline controls. Because vertical activity was sensitised in both MDMA exposure conditions (MDMA self-administration and yoked MDMA), the data suggest that the non-selective decrease in c-fos expression in these regions might be important for the expression of these sensitised behaviours. C-fos expression in the NAc core and the cingulate cortex was, however, selectively decreased by MDMA self-administration. This might be relevant to the selective sensitised response following MDMA self-administration. A decrease in c-fos expression in the NAc shell was produced following cocaine self-administration (Larson et al., 2010; Zahm et al., 2010), suggesting that there might be different consequences depending on the drug that is self-administered. In fact, methamphetamine-produced c-fos expression in the NAc was not altered at all following methamphetamine self-administration (Cornish et al., 2012). These findings are, however, difficult to interpret because Cornish et al. (2012) failed to invoke a withdrawal period. It is well documented that a withdrawal period is required for sensitisation of methamphetamine-produced locomotor activity (Hamamura et al., 1991; Nishikawa, Mataga, Takashima, & Toru, 1983). This might explain why Cornish et al. (2012) failed to show a sensitised behavioural response, and also failed to observe changes in c-fos expression.

The PFC is also implicated in self-administration. Specifically, drug-produced hypofrontality of the PFC has been suggested to underlie the loss of inhibitory cognitive

control over drug seeking (Kalivas, 2009). There was a decrease in c-fos expression in the prelimbic cortex and cingulate cortex as a function of repeated MDMA exposure. Similar results have been reported following repeated cocaine exposure (Todtenkopf & Stellar, 2000). While other self-administration studies have not focussed various sub-divisions of the PFC (Larson et al., 2010; Zahm et al., 2010), the present study measured c-fos in three sub-regions and highlights the importance of the cingulate cortex in the development of MDMA self-administration. This is a novel finding that suggests that the cingulate cortex might play an important role in sensitisation to the behavioural effects following MDMA self-administration.

The NAc receives glutamatergic projections from the PFC, and disruption of these projections plays an important role in the development of compulsive drug-seeking (Kalivas, 2009). Thus, it is possible that changes in c-fos expression after MDMA self-administration reflect neuroadaptations in the glutamate projections from the PFC to the NAc. Because c-fos is a general marker of neuronal activation it is not possible to identify the specific neurochemical systems that were activated by MDMA. Use of double labelling in future studies might provide more information. Alternatively, the impact of selective agonists on c-fos expression following MDMA self-administration would be informative.

The current study used c-fos immunohistochemistry to identify neuronal activation following repeated exposure to MDMA. While this method has been regarded as extremely useful for mapping neural activity (Kovács, 1998) and has been used for studying effects of drugs of abuse (Harlan & Garcia, 1998), there are some limitations that must be taken into account. Since c-fos expression is low under basal conditions, immunohistochemistry permits the detection of neurons following recent activation. However, this approach does not allow for the detection of neurons that are under net synaptic inhibition, so there is risk of missing a potential decrease in neuronal activation. Furthermore, neurons with high activity do not always express c-fos (Kovács, 1998), leaving the possibility of an underestimation of c-fos expression in regions involved in the drug response.

The combined investigation of c-fos expression and behavioural responses, however, provides important information concerning the relationship between brain and behaviour. The third part of the current study attempted to determine whether specific

changes in neural activation were correlated with forward and vertical locomotor activity. A negative relationship between forward and vertical locomotor activity and MDMA-produced c-fos expression in the cingulate cortex and infralimbic cortex was found. Furthermore, the negative relationship between forward locomotor activity and c-fos expression in the nucleus accumbens approached significance, as did the negative relationship between vertical locomotor activity and c-fos expression in the NAc core and NAc shell. It is possible that these correlations would become significant if the sample size had been larger, however given the length of time required to conduct the current experiment and the large attrition rate, it was not possible to increase the sample size.

The sensitisation of forward locomotor activity and vertical locomotor activity following repeated drug exposure have been attributed to enhanced DAergic neurotransmission (Brennan, Carati, Lea, Fitzmaurice, & Schenk, 2009; Thiel et al., 1999). Accordingly, one might have expected c-fos expression to increase rather than decrease. One possible explanation is that the decrease in c-fos expression reflects MDMA-produced neuroadaptations in intracellular mechanisms.

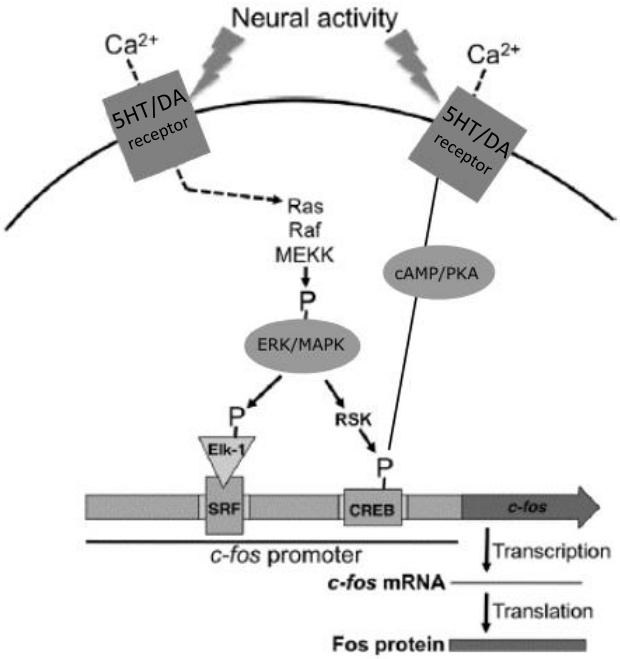



*Figure 12.* Schematic drawing to illustrate the molecular mechanism of c-fos expression. Image adapted from Cruz, Rubio, and Hope (2015).

Figure 12 shows the molecular mechanisms involved in c-fos expression. C-fos is a marker of post-synaptic activation, and is expressed following a cascade of molecular events. Strong neural activity at G-protein coupled receptors (GPCR), including 5HT and DA receptors, induces calcium influx (Kroeze, Sheffler, & Roth, 2003). Once the calcium influx is sufficient, ERK/MAPK are phosphorylated and activated via the Ras-Raf-MEKK cascade. The phosphorylation of ERK/MAPK leads to the phosphorylation of Elk-1 that is associated with SRF, and also phosphorylates CREB via RSK. The c-fos promoter is then activated and c-fos is transcribed. Transcribed c-fos mRNA, and the translated protein, Fos, can then be used as a marker of strongly activated neurons.

Phosphorylation of CREB can also occur via the cAMP/PKA pathway (Grady & Bunnett, 1997). The effect of activation of GPCR on CREB phosphorylation depends on the protein subunit. Stimulation of GPCR that are coupled to a Gs or Gq subunit activates the cAMP/PKA pathway, while stimulation of a GPCR coupled to a Gi subunit inhibits the cAMP/PKA pathway (Grady & Bunnett, 1997). Activation of the cAMP/PKA pathway increases CREB phosphorylation, and therefore enhances transcription of c-fos (Grady & Bunnett, 1997). Activation of $D_2$ receptor subtypes inhibits the cAMP/PKA pathway, while activation of some 5HT (e.g. $5HT_{2A}$ and $5HT_{2C}$) and DA (e.g. $D_1$) receptor subtypes stimulates the cAMP/PKA pathway (Hoyer, Hannon, & Martin, 2002; Surmeier, Ding, Day, Wang, & Shen, 2007).

Changes in MDMA-produced increases in extracellular DA or 5HT and the subsequent impact on activation of post-synaptic receptors would, therefore, be expected to alter c-fos expression. Repeated MDMA self-administration decreased MDMA stimulated 5HT release (Reveron et al., 2010), and increased DA release (Colussi-Mas et al., 2010), suggesting, in the absence of any relevant post-synaptic changes, a decrease or increase, respectively, of 5HT and DA receptor activation. This alone might explain the observed decrease in c-fos expression.

Repeated exposure to MDMA, however, could provide an explanation for changes in c-fos expression. Repeated exposure to MDMA decreased the density of some 5HT receptors in rats (McGregor et al., 2003; Scheffel, Lever, Stathis, & Ricaurte, 1992) and humans (Reneman et al., 2002). Specifically, repeated MDMA administration produced a decrease in $5HT_{2A/2C}$ receptor density in the striatum and PFC (McGregor et al., 2003). Because $5HT_{2A}$ and $5HT_{2C}$ receptors are coupled to a Gq G-protein subunit (Hoyer et al.,

2002), activation would be expected to increase CREB phosphorylation. Thus, a decrease in $5HT_{2A}$ and $5HT_{2C}$ receptors would be expected to decrease c-fos expression. Consistent with this idea, a selective $5HT_{2A}$ antagonist decreased cocaine-induced c-fos expression (Szucs, Frankel, McMahon, & Cunningham, 2005), but effects on MDMA-produced c-fos expression have not been tested.

Repeated exposure to MDMA also altered behavioural responses to $D_1$ and $D_2$ receptor agonists (Bradbury et al., 2012). Sensitisation to the effects of a $D_2$ agonist was pronounced following a sensitising regimen of MDMA exposure (Bradbury et al., 2012). Because $D_2$ receptors are coupled to a Gi G-protein subunit (Surmeier et al., 2007), activation would be expected to reduce CREB phosphorylation. Therefore, an upregulation of $D_2$ receptors would be expected to decrease c-fos expression.

Alterations in c-fos expression produced by MDMA self-administration in the present study might, therefore, reflect changes in CREB phosphorylation, as has been demonstrated following repeated methamphetamine administration (McCoy et al., 2011). The effects of repeated MDMA on CREB phosphorylation in the striatum, NAc, or PFC has not yet been reported. Further research is needed in order to identify the effect of repeated MDMA exposure on CREB phosphorylation.

In summary, the decrease in c-fos produced by repeated exposure to MDMA might be due to either changes in stimulated 5HT or DA release, the effects of such changes on receptor expression, or changes in intracellular mechanisms that influence the phosphorylation of CREB. It should be noted, however, that the regulation of c-fos expression is complex, and likely to be affected by various external variables. For example previous research suggested that c-fos expression is mediated by other transcription factors, such as ΔFosB (Renthal et al., 2008). ΔFosB bound to the c-fos promotor region, and reduced the transcriptional activity of c-fos in the striatum (Renthal et al., 2008). While it is well-documented that repeated exposure to psychostimulants increased ΔFosB in the striatum (Larson et al., 2010; Perrotti et al., 2005), the effect of MDMA exposure on ΔFosB has not yet been reported. If ΔFosB levels are increased as a result of repeated MDMA exposure this may explain the reduction in c-fos that was found in the present study.

Interestingly, after a two week withdrawal period, evidence of MDMA-produced 5HT syndrome behaviours started to reappear (Schenk & Bradbury, 2015). Measurement

of c-fos following this longer withdrawal period might help to tease apart the various possibilities for the mechanism behind this important recovery.

Because of the correlational design of the present study, we can only conclude that the behavioural responses to MDMA are associated with changes in c-fos expression. Nevertheless, the expression of c-fos in the dorsal striatum, NAc, and PFC likely lead to long-term changes in the expression of target genes involved in neural activity. It is likely that neuroadaptations in these regions are important for regulating the development of MDMA self-administration. Thus, further research of the different neuroadaptations produced by MDMA self-administration should investigate the role of 5HT and DA in these regions.

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