Effect of repeated MDMA exposure on rat brain and behaviour

by

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Table of contents

Absract	5
List of abbreviations	7
CHAPTER 1: GENERAL INTRODUCTION	10
Introduction.....	10
Behavioural fundamentals of addiction	12
Studying addiction	13
Self-administration.....	14
Behavioural sensitisation.	18
Neurocircuitry of addiction.....	20
Ventral tegmental area and nucleus accumbens	20
Dopamine and reinforcement.....	20
Dopamine and incentive motivation.	22
Amygdala.....	26
Dorsal striatum.....	27
Prefrontal cortex.....	29
MDMA	31
MDMA/ecstasy use disorders	31
MDMA self-administration	32
Pharmacology of MDMA	33
Role of serotonin in self-administration	35
Effect of repeated MDMA exposure on serotonin and dopamine	37
Serotonin, dopamine, and MDMA self-administration	39
The current thesis	40
Δ FosB and immunohistochemistry	40
Microdialysis and LC-MS.....	45
Aims and objectives.....	51
CHAPTER 2: ΔFOSB AND IMMUNOHISTOCHEMISTRY	52
Introduction.....	52
Methods.....	53
Subjects	53
Surgical procedures.....	53
Apparatus	53

Self-administration.....	54
FosB-like immunohistochemistry	54
Statistical analysis	57
Results	57
Discussion.....	66
CHAPTER 3: LOCAL MDMA INFUSION	71
Introduction.....	71
Methods.....	72
Subjects	72
Surgical procedures.....	72
Apparatus	73
Drugs.....	73
Pretreatment and intra-striatal microinjections	73
Statistical analysis	75
Results	76
Discussion.....	80
CHAPTER 4: MICRODIALYSIS AND LC-MS	82
Introduction.....	82
Methods 1.....	84
Subjects	84
Drugs and reagents.....	84
Surgical procedures.....	85
Microdialysis.....	85
LC-MS	85
Data analysis	86
Results 1	86
Method development 1	89
Chromatography gradient	89
BzCl concentration.....	89
Probe implantation	93
Method 2	94
Results 2	94

Method development 2	95
BzCl concentration.....	95
LC-MS injection volume	100
Microdialysis.....	100
Quantification of MDMA	103
Methods 3	104
MDMA pretreatment and locomotor sensitisation	104
Statistical analysis.....	104
Results 3	105
Discussion	109
CHAPTER 5: UNTARGETED METABOLOMICS	112
Introduction	112
Methods	116
Sample collection, preparation, and analysis	116
Data processing	116
Data analysis	123
Metabolite annotation and MS/MS	124
Results	125
Metabolite annotation	134
Discussion	136
CHAPTER 6: GENERAL DISCUSSION	140
Summary of findings	140
Relevance and value of findings	142
Self-administration and Δ FosB	142
Behavioural sensitisation and the acquisition of self-administration	144
Metabolomics.....	146
Limitations	148
Future directions	151
Conclusions	153
References	155
Acknowledgements	202

Abstract

Rationale. $\pm$ 3,4-Methylenedioxymethamphetamine (MDMA; ‘ecstasy’) is a popular recreational drug of abuse. Like other drugs of abuse, a proportion of users develop symptoms that are characteristic of a Substance Use Disorder (SUD). The behavioural and neurobiological consequences of repeated misuse of MDMA are not well understood, however.

Objectives. The purpose of the present thesis was to investigate behaviourally relevant neuroadaptations that develop with repeated MDMA exposure in laboratory rats.

Methods. First, the effect of chronic, long-access (6 hour) self-administration of MDMA on the accumulation of the transcription factor, Δ FosB, in the nucleus accumbens (core, shell), dorsal striatum (dorsomedial, dorsolateral, ventromedial, ventrolateral), prefrontal cortex (anterior cingulate, prelimbic, infralimbic, orbitofrontal), amygdala (central, basolateral), ventral tegmental area (anterior, posterior), and raphe (dorsal, median) was measured using immunohistochemistry. Second, the behavioural relevance of these findings was determined by examining the effect of bi-lateral intra-striatal (nucleus accumbens, dorsomedial striatum, dorsolateral striatum) microinjections of MDMA (200 μ g/1 μ L/side) on the expression of behavioural sensitisation following two days of withdrawal from a regimen of repeated, systemic MDMA exposure (10 mg/kg/day, i.p., for 5 days). Third, a procedure was developed to examine neurochemical correlates of sensitised MDMA-produced behaviour (0, 5, 10 mg/kg, i.p.) following the same regimen of repeated MDMA exposure. Samples were collected from the medial striatum using *in vivo* microdialysis and the extracellular concentrations of serotonin, dopamine, MDMA, and their metabolites were quantified using liquid chromatography coupled with quadrupole time-of-flight (Q-TOF) mass spectrometry. Lastly, a unique untargeted metabolomics procedure was developed to further analyse these microdialysis samples and to identify novel or unexpected metabolites that were relevant to the sensitised behavioural response produced by MDMA.

Results. MDMA self-administration produced region-dependant increases in Δ FosB. Significant increases in Δ FosB were observed in the nucleus accumbens core, the medial areas of the dorsal striatum, as well as all areas of the prefrontal cortex and amygdala. Small, but significant increases were also observed in the dorsal raphe. Increases were observed in the nucleus accumbens shell and the posterior tail of the ventral tegmental area, but these increases were not significant following statistical correction for multiple comparisons. Acute exposure to MDMA increased locomotor activity only when the drug was infused into the nucleus accumbens. Following repeated systemic exposure, behavioural sensitisation was expressed

when MDMA was infused into both the nucleus accumbens or the dorsomedial striatum, but not the dorsolateral striatum. Analysis of microdialysates from the medial striatum indicated that behavioural sensitisation was accompanied by small increases in baseline levels of extracellular serotonin and decreased MDMA-produced increases in serotonin, but these changes were not statistically significant. Behavioural sensitisation was also accompanied by increased extracellular concentrations of dopamine at baseline and following acute MDMA exposure, but these data were not statistically analysed due to small sample sizes. MDMA-produced extracellular concentrations of MDMA did not change with repeated exposure. Untargeted metabolomics revealed potential changes in MDMA and dopamine metabolism that might be relevant to the sensitised behavioural response.

Conclusions. The findings of the current research suggest that repeated MDMA exposure results in many of the same neuroadaptations that result from repeated exposure to other drugs of abuse. These included increased Δ FosB expression in many brain regions that are relevant to addiction, such as the nucleus accumbens, dorsal striatum, and prefrontal cortex. Dopaminergic mechanisms also appeared to be influenced and were associated with sensitised MDMA-produced behaviour. Surprisingly, serotonergic mechanisms were not significantly impacted by repeated MDMA exposure under the current conditions. Some of the procedures developed in this thesis are unique and may be of value for future research investigating the neurochemical underpinnings of addictive behaviour or other disease states.

List of abbreviations

3-MT:	3-Methoxytyramine
5,7-DHT:	5,7-Dihydroxytryptamine
5-HIAA:	5-Hydroxyindoleacetic acid
5-HT:	Serotonin
6-OHDA:	6-Hydroxydopamine
aCSF:	Artificial cerebrospinal fluid
ADAP:	Automated data analysis pipeline
AMPA:	α -Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANOVA:	Analysis of variance
AP:	Anteroposterior
AP-1:	Activator protein-1
ARC:	Activity-regulated cytoskeleton-associated protein
BLA:	Basolateral amygdala
BzCl:	Benzoyl chloride
CeA:	Central nucleus of the amygdala
CDK5:	Cyclin-dependent kinase 5
COMT:	Catechol- <i>O</i> -methyltransferase
C-P:	Caudate-putamen (dorsal striatum)
CPP:	Conditioned place preference
CRF:	Corticotrophin-releasing factor
DA:	Dopamine
DLS:	Dorsolateral striatum
DMS:	Dorsomedial striatum
DNA:	Deoxyribonucleic acid
DOPAC:	3,4-Dihydroxyphenylacetic acid
DSM:	Diagnostic and Statistical Manual of Mental Disorders
DV:	Dorsoventral
EIC:	Extracted ion chromatogram
ESI:	Electrospray ionisation
FR:	Fixed ratio
GABA:	Gamma-aminobutyric acid
GC-MS:	Gas chromatography-mass spectrometry

HHA:	3,4-Dihydroxyamphetamine
HHMA:	3,4-Dihydroxymethamphetamine
HMA:	4-Hydroxy-3-methoxyamphetamine
HMMA:	4-Hydroxy-3-methoxymethamphetamine
HPLC:	High-performance liquid chromatography
IHC:	Immunohistochemistry
i.p.:	Intraperitoneal
i.v.:	Intravenous
LC-MS:	Liquid chromatography-mass spectrometry
LOD:	Limit of detection
MDA:	3,4-Methylenedioxyamphetamine
MDMA:	3,4-Methylenedioxymethamphetamine
ML:	Mediolateral
mRNA:	Messenger ribonucleic acid
MS:	Mass spectrometry
MS/MS:	Tandem mass spectrometry
MSN:	Medium spiny neurons
m/z:	Mass-to-charge ratio
NAcc:	Nucleus accumbens
NE:	Norepinephrine (noradrenaline)
NF-kB:	Nuclear factor-kappaB
NMR:	Nuclear magnetic resonance
OFC:	Orbitofrontal cortex
OPLS:	Orthogonal partial least squares
OPLS-DA:	Orthogonal partial least squares discriminant analysis
PB:	Phosphate buffer
PBS:	Phosphate-buffered saline
PBST:	Phosphate-buffered saline containing 0.3% Triton X-100
PCA:	Principal components analysis
pCPA:	p-Chlorophenylalanine
PFA:	Paraformaldehyde
PFC:	Prefrontal cortex
PR:	Progressive ratio

PLS:	Partial least squares (projection to latent structures)
PLS-DA:	Partial least squares discriminant analysis
Q-TOF:	Quadrupole time-of-flight
QQQ:	Triple quadrupole mass spectrometry
RNA:	Ribonucleic acid
RSD:	Relative standard deviation
s.c.:	Subcutaneous
SD:	Standard deviation
SEM:	Standard error of the mean
SIMCA:	Soft Independent Modelling of Class Analogies
S/N:	Signal to noise ratio
SPSS:	Statistical Package for Social Sciences
SSRI:	Selective serotonin reuptake inhibitor
SUD:	Substance use disorder
SUS:	Shared and unique structures
TAAR1:	Trace amine-associated receptor 1
TIC:	Total ion chromatogram
VMAT:	Vesicular monoamine transporter
VTA:	Ventral tegmental area
RT:	Retention time
VIP:	Variable importance in projection
VMA:	Vanillylmandelic acid

CHAPTER 1: GENERAL INTRODUCTION

Parts of this chapter have been adapted with licensed permission from work published in Addiction Biology (van de Wetering & Schenk, 2019).

Introduction

Harmful use of tobacco, alcohol, and illicit drugs are some of the leading causes of preventable health loss in New Zealand, collectively accounting for over 130 000 years of healthy life lost annually due to premature death, illness, or disability (Ministry of Health, 2013, 2016). For comparison, that is over four times greater than that caused by breast and prostate cancer combined (Ministry of Health, 2013, 2016). The health issues associated with harmful substance use include overdose, increased risk of physical injury or suicide, various cancers, chronic diseases, and several neuropsychiatric disorders (Ministry of Health, 2013, 2016). The impact of harmful substance use also extends beyond the health of the individual. Relationship and family issues, domestic violence, child neglect, fetal drug exposure, crime, second-hand smoke, difficulty obtaining and maintaining employment, and traffic accidents are all examples of substance-related harm that affects others (Inter-Agency Committee on Drugs, 2015).

Harmful substance use also carries a significant economic burden. A 2016 study estimated that the annual cost of illicit drug use in New Zealand was \$1.8 billion (McFadden Consultancy, 2016). Older estimates on the annual cost of illicit drug use and alcohol use combined were upwards of \$6 billion (Slack, Nana, Webster, Stokes, & Wu, 2009), while the annual cost associated with tobacco use was estimated to be as high as \$1.6 billion (O'Dea & Thompson, 2007). These estimates include the cost of healthcare-associated with substance use related accidents, injuries, and illnesses, the cost of intervention and rehabilitation programs, the costs to the criminal justice system, and the cost of welfare payments for people who have become incapacitated through substance use (Inter-Agency Committee on Drugs, 2015; McFadden Consultancy, 2016; O'Dea & Thompson, 2007; Slack et al., 2009).

Although not every instance of drug use is harmful, the health, financial, and societal consequences caused by individuals who develop a harmful pattern of substance use characteristic of addiction are severe. Drug addiction is a chronic, relapsing disorder characterised by compulsive drug-seeking and drug-taking behaviour despite adverse consequences. In the fifth edition of the Diagnostic and Statistical Manual of Mental Disorders

(DSM-V), drug addiction is clinically defined as a Substance Use Disorder (SUD), which is characterised by 11 diagnostic criteria (Table 1.1) and is measured along a continuum from mild (2 – 3 criteria) to severe (6+ criteria; American Psychiatric Association [APA], 2013). In order to develop new strategies for the prevention and treatment of SUDs, a better understanding of the various factors underlying harmful drug use and the development of SUDs are required.

Table 1.1.

DSM-V Substance Use Disorder criteria

-
1. The substance is often taken in larger amounts or over a longer period than was intended.
 2. Inability to cut down or control substance use despite a desire to do so.
 3. A great deal of time is spent in activities necessary to obtain, use, or recover from the substance.
 4. Craving, or a strong desire or urge to use the substance.
 5. Repeated usage results in a failure to fulfil major role obligations at work, school, or home.
 6. Continued usage despite having persistent or recurrent social or interpersonal problems caused or exacerbated by the effects of the substance.
 7. Important social, occupational, or recreational activities are given up or reduced because of substance use.
 8. Recurrent usage in situations in which it is physically hazardous.
 9. Continued usage despite knowledge of having a persistent or recurrent physical or psychological problem that is likely to have been caused or exacerbated by the substance.
 10. Tolerance, as evidenced by a need for markedly increased amounts of the substance to achieve the desired effect and/or a markedly diminished effect with continued use of the same amount of the substance.
 11. Withdrawal, as evidenced by the emergence of the characteristic group of withdrawal symptoms as the amount of substance in the body decreases and/or the substance is taken to relieve or avoid such withdrawal symptoms.
-

Source: (APA, 2013).

Behavioural fundamentals of addiction

Changes in the reinforcement processes (Skinner, 1938) underlying drug-taking behaviour are an important factor driving the progression of a SUD. Most drugs that are misused produce rewarding subjective effects (e.g. euphoria). This can function as a positive reinforcer, increasing the likelihood of subsequent drug-taking behaviour. However, it is well known that with repeated drug exposure, tolerance to the rewarding effects of many drugs of abuse can quickly develop (Fischman & Schuster, 1981; Fischman, Schuster, Javaid, Hatano, & Davis, 1985; Foltin & Fischman, 1991). Thus, while positive reinforcement may underlie initial, recreational drug-taking behaviour, it does not explain continued drug-use when the rewarding effects of the drug have dramatically decreased due to tolerance.

In this situation, continued drug-taking behaviour may be better explained by negative reinforcement. Repeated drug use can lead to the development of withdrawal symptoms; aversive physical, mental, or emotional states that occur following the cessation of drug intake. These withdrawal symptoms can be avoided or alleviated by further drug-use (Ahmed & Koob, 2005; Koob, 2013). Thus, even when the drug no longer produces any pleasurable rewarding effects due to tolerance, the removal or avoidance of these aversive withdrawal symptoms can act as a negative reinforcer for continued drug-taking behaviour.

The transition from positive reinforcement to negative reinforcement is well described by the opponent-process theory of addiction/motivation (Koob, Caine, Parsons, Markou, & Weiss, 1997; Koob & Le Moal, 2008b; Solomon & Corbit, 1974). According to this theory, drug addiction progresses due to the automatic modulation of drug-produced positive and negative emotional states by the central nervous system. Initially, there are high levels of positive drug effects (e.g. euphoria) and low levels of negative drug effects (e.g. withdrawal symptoms). With repeated drug use, however, the positive effects of the drug decrease and the negative effects of the drug increase, which provides the motivation for continued drug taking via negative reinforcement. Although both positive and negative reinforcement certainly play crucial roles in the development of a SUD, they do not explain all aspects of the disorder (T. E. Robinson & Berridge, 1993).

It is now well established that drug craving and the risk of relapse can persist even after decades of abstinence, when most withdrawal symptoms have long since subsided (Hyman & Malenka, 2001; O'Brien, Childress, Ehrman, & Robbins, 1998; Wise, 2000; Wise & Bozarth, 1987). Craving and relapse can be precipitated by exposure to various environmental stimuli such as drug paraphernalia as well as the people and places that have been repeatedly associated

with the drug (Childress, Ehrman, McLellan, & O'Brien, 1988; Childress et al., 1993, 1999; Ehrman, Robbins, Childress, & O'Brien, 1992). These stimuli can develop conditioned reinforcing properties, capable of reinforcing behaviour in the absence of the primary drug reinforcer, despite not being intrinsically rewarding (Daniela, Gittings, & Schenk, 2006; Schenk & Partridge, 2001; Schuster & Woods, 1968; S. G. Smith & Davis, 1973; Stewart, de Wit, & Eikelboom, 1984). Unlike the primary reinforcing effects of the drug, which undergo tolerance following repeated exposure, conditioned reinforcers can maintain their strength as a reinforcer over extended periods of time, and may even become more effective reinforcers of behaviour as a function of abstinence (Grimm, Hope, Wise, & Shaham, 2001; P. Li et al., 2015; X. Li, Caprioli, & Marchant, 2015).

Conditioned reinforcers can also acquire incentive motivational properties, becoming highly salient and capable of motivating approach or consummatory behaviour (Bindra, 1974, 1978; Bolles, 1972). Thus, exposure to drug-associated conditioned reinforcers can produce intense feelings of desire and craving for the drug, which can then motivate behaviour towards the acquisition and consumption of the drug they were associated with (Childress et al., 1988, 1993, 1999; Ehrman et al., 1992; Franken, Hendriks, Stam, & Van den Brink, 2004; Garavan et al., 2000). As will be discussed in greater detail below, it has been suggested that hypersensitivity to the incentive motivational properties of drugs and drug-associated stimuli plays a major role in the development of persistent drug-craving and drug-seeking behaviour that is characteristic of SUDs (Berridge & Robinson, 2016; T. E. Robinson & Berridge, 1993, 2008).

Studying addiction

In order to provide more effective behavioural and pharmaceutical treatments for SUDs, it is vital to further our understanding of the mechanisms that mediate the shift from positive to negative reinforcement, the sensitisation of incentive salience attributed to drugs and drug-associated stimuli, and the inability to exert self-control over drug-taking and drug-seeking behaviour. In recent decades, much progress has been made in this regard, with substantial evidence supporting the idea that a SUD is a disorder of the brain brought on by persistent drug-induced changes in various brain mechanisms that are involved in these processes (Berridge & Robinson, 2016; Everitt & Robbins, 2005; Gardner, 2011; Kalivas & Volkow, 2005; Volkow, Koob, & McLellan, 2016; Weiss, 2005).

Neuroimaging studies in individuals that suffer from a SUD have provided valuable insight into some of the brain mechanisms underlying a SUD (Goldstein & Volkow, 2011;

Koob & Volkow, 2010). Studies using human participants have several limitations, however. Firstly, researchers will often have incomplete or inaccurate information regarding drug-use history; consumption patterns, drug doses, overall intake, polydrug use, and drug purity can all vary considerably and are unlikely to be recalled accurately in the self-report questionnaires that are typically used. Secondly, varied personal histories and traits can limit the conclusions that can be drawn. For example, anxiety, depression, and high impulsivity are not only predisposing factors for drug abuse but also possible consequences of drug abuse (Parrott, 2006, 2013; Rogers et al., 2009). Finally, in an experimental setting, the administration of addictive and potentially harmful drugs to humans has obvious ethical limitations, with only low doses of some drugs being approved due to safety reasons, which limits the experimental investigation of causal relationships.

It is for these reasons that much of our current understanding of the brain mechanisms underlying compulsive drug-taking and drug-seeking behaviour has come through the use of various animal models such as self-administration, conditioned place preference (CPP), behavioural sensitisation, or drug discrimination (Carter & Griffiths, 2009; Gardner, 2000; Koob & Volkow, 2010; O'Brien & Gardner, 2005; Overton, 1987; Panlilio & Goldberg, 2007; Shaham, Shalev, Lu, De Wit, & Stewart, 2003; Tzschentke, 2007). The use of non-human animal subjects allows for researchers to carefully control important experimental variables such as the age at which the animals are exposed to the drug, the drug dose and drug purity, as well as the duration and extent of drug exposure. Important genetic, environmental, and developmental factors can also be carefully controlled and investigated. Furthermore, a wider range of drug doses and drug types can be administered than what is ethically viable with human participants. Although no animal model can fully emulate a SUD, they do allow for the investigation of specific aspects of the disorder (Koob & Volkow, 2010). The self-administration paradigm is one such procedure that has proved to be particularly valuable in unravelling the behavioural and neurobiological mechanisms underlying drug-taking behaviour.

Self-administration. In the standard self-administration paradigm, laboratory animals are trained to perform a simple behavioural response (e.g. lever press, key peck, nose poke) in order to receive a drug reward. The drug is usually administered via an indwelling intravenous (i.v.) catheter (Weeks, 1962), but other methods (e.g. oral, inhalation, transdermal, intracranial) can also be used depending on the species of animal or drug being used (Mattox & Carroll, 1996; McBride, Murphy, & Ikemoto, 1999; Samson, Pfeffer, & Tolliver, 1988). The

frequency of responding maintained by drug infusions is typically compared to the responses made on a second control manipulandum (e.g. a second lever) that has no programmed consequence or against responding maintained by a vehicle/placebo infusion. A preference in responding on the manipulandum that results in the drug reward suggests that the drug is acting as a reinforcer (e.g. Corrigan & Coen, 1989; Schenk, Colussi-Mas, Do, & Bird, 2012; Williamson, 1997). Further evidence that the drug is acting as a reinforcer include the eventual cessation of responding (extinction) when the drug is removed or replaced with vehicle/placebo and the reinstatement of extinguished responding when the drug is subsequently reintroduced (e.g. Gerber & Stretch, 1975; Grove & Schuster, 1974; Pickens & Harris, 1968). Likewise, when the consequences associated with each manipulandum (i.e. drug vs no drug) are reversed, a corresponding reversal in the preference in responding can also be observed (e.g. Goeders & Smith, 1983; Pickens & Harris, 1968).

Self-administration of various drugs of abuse has been reliably demonstrated in non-human primates (Deneau, Yanagita, & Seevers, 1969; Gerber & Stretch, 1975; Goldberg, Woods, & Schuster, 1971; Lamb & Griffiths, 1990; Thompson & Schuster, 1964), dogs (Risner & Jones, 1975), cats (Balster, Kilbey, & Ellinwood, 1976), rats (Collins, Weeks, Cooper, Good, & Russell, 1983; Weeks & Collins, 1964), mice (Criswell, 1983), fish (Bossé & Peterson, 2017), and even invertebrates such as crayfish (Datta, van Staaden, & Huber, 2018) and ants (Entler, Cannon, & Seid, 2016). Rodents (rats and mice) are the most commonly used subjects, however. With few exceptions (i.e. hallucinogens), drugs that are abused by humans are readily self-administered by laboratory animals, whereas drugs that are not abused by humans do not maintain self-administration in laboratory animals (Deneau et al., 1969; O'Connor, Chapman, Butler, & Mead, 2011; Schuster & Thompson, 1969). Accordingly, the self-administration procedure has proved to be an excellent method for evaluating the abuse liability of drugs and is used as a pre-clinical screening method to assess the abuse liability of novel pharmaceutical compounds (Ator & Griffiths, 2003; Food and Drug Administration [FDA], 2010).

The pattern of drug self-administration maintained by laboratory animals is similar to that exhibited by humans who misuse drugs. For example, opiate self-administration is more constant and moderate, with little voluntary abstinence periods, while psychostimulant and alcohol self-administration is more binge-like, alternating between bursts of responding and abstinence (Deneau et al., 1969; Gardner, 2000; Harrigan & Downs, 1978; Kramer, Fischman, & Littlefield, 1967; Pickens & Harris, 1968; Schenk, Gittings, Johnstone, & Daniela, 2003; Winger & Woods, 1973; Yokel & Pickens, 1973). Like humans, laboratory animals are also

capable of regulating their drug-intake. When a range of drug doses are available for self-administration, responding changes in a compensatory manner as a function of drug dose so that drug-intake remains relatively constant (Goldberg, Hoffmeister, Schlichting, & Wuttke, 1971; Pickens & Harris, 1968; Weeks & Collins, 1964). Likewise, when the work required to receive a drug injection is increased (i.e. an increase in the fixed ratio [FR] schedule), there is a proportional increase in the number of responses, within limits (Goldberg, Hoffmeister, et al., 1971; Pickens & Harris, 1968; Schenk et al., 2012). This suggests that laboratory animals titrate their drug-intake to maintain blood/brain levels that are reinforcing, as do humans (Gardner, 2000). In order for self-administration to be more than just a model of drug-taking, however, other features of a SUD must also be evident and able to be investigated.

In most self-administration procedures, the delivery of the drug reinforcer occurs concurrently with the delivery of a discrete, neutral stimulus such as a light or tone. These stimuli are presumed to be analogous of the various environmental stimuli that become associated with drug-taking in humans (e.g. the people, places, and drug paraphernalia associated with the drug). Given repeated pairings of these stimuli with the drug over several self-administration sessions, these stimuli can become conditioned reinforcers, able to maintain responding in the absence of the primary drug reinforcer (Daniela et al., 2006; Schenk & Partridge, 2001; Schuster & Woods, 1968; S. G. Smith & Davis, 1973; Stewart et al., 1984), and acquire incentive motivational properties (T. E. Robinson & Berridge, 1993). Failure to present such stimuli can delay or even prevent the acquisition of self-administration of some drugs (Caggiula et al., 2002; Schenk & Partridge, 2001; Schindler, Cogan, Thorndike, & Panlilio, 2011).

To study the important role of these conditioned stimuli in maintaining drug-seeking and drug-taking behaviour, researchers have employed more complex schedules of reinforcement that are designed to model the sequence of stimuli-exposure, drug-seeking, and drug-taking, that is typical in humans (Panlilio & Goldberg, 2007). For instance, under second-order or chained schedules of reinforcement, responding is primarily reinforced by the presentation of these conditioned-stimuli, and only after a certain number of conditioned reinforcers have been earned, does the drug reinforcer become available (Goldberg, Kelleher, & Morse, 1975; Schindler, Panlilio, & Goldberg, 2002). Removal of these stimuli dramatically decreases responding, suggesting that they are acting as conditioned reinforcers and are maintaining drug-seeking behaviour until the drug itself becomes available.

Another method developed to investigate the role of these stimuli in drug-seeking is the reinstatement paradigm (de Wit & Stewart, 1981, 1983; Shaham et al., 2003; Stewart & de Wit, 1987). In this procedure, following an extensive history of drug self-administration, drug reinforcement is discontinued until responding has extinguished. External stimuli are then used to provoke relapse of drug-seeking behaviour, as measured by the reinstatement of responding. Three types of stimuli have been found to reinstate responding; the presentation of conditioned stimuli/cues (Katner, Magalong, & Weiss, 1999; McFarland & Ettenberg, 1997; Meil & See, 1996), re-exposure to a single dose of drug (de Wit & Stewart, 1981, 1983; Stewart & de Wit, 1987), and stress (Erb, Shaham, & Stewart, 1996; Shaham, Rajabi, & Stewart, 1996; Shaham & Stewart, 1995). Notably, these are all stimuli that are known to be prominent triggers of craving and relapse in humans, which is one of the defining characteristics of a SUD (criteria 4 of SUD).

An uncontrolled increase in drug intake is another defining characteristic of a SUD (criteria 1 of SUD), yet this is not typically observed under the limited-access (e.g. 1 hour per day) self-administration conditions most commonly used with laboratory animals. As previously mentioned, laboratory animals will maintain a relatively constant drug-intake across sessions, even when different drug-doses or reinforcement schedules are used. When daily session lengths are increased (e.g. 6 hours per day), however, a significant escalation in drug intake has been observed over successive test sessions (Ahmed & Koob, 1998; Ahmed, Walker, & Koob, 2000; Highgate & Schenk, 2018; Kitamura, Wee, Specio, Koob, & Pulvirenti, 2006; O'Dell et al., 2007; A. J. Roberts, Heyser, Cole, Griffin, & Koob, 2000). Importantly, these extended-access self-administration conditions can also exacerbate, or are often necessary for, the development of several other features that are characteristic of SUDs (Edwards & Koob, 2013).

For example, following unlimited access to some drugs, preferential attention was directed towards the acquisition of drugs as opposed to other naturally reinforced behaviours (criteria 7 of SUD; Bozarth & Wise, 1985; Deneau et al., 1969). Extended self-administration access also resulted in drug-taking that was resistant to effects of punishment (e.g. foot shock; criteria 8 of SUD; Deroche-Gamonet, Belin, & Piazza, 2004; Pelloux, Everitt, & Dickinson, 2007; Vanderschuren & Everitt, 2004). Furthermore, there was resistance to extinction of responding when the drug was no longer available following a history of extended access self-administration (criteria 3 of SUD; Ahmed et al., 2000; Perry, Morgan, Anker, Dess, & Carroll, 2006). Subjects with a history of extended access self-administration also worked significantly

harder to obtain the drug under progressive ratio (PR) schedules, making hundreds or even thousands of responses to obtain a single dose of the drug (criteria 3 of SUD; Paterson & Markou, 2003; Walker & Koob, 2007; Wee, Mandyam, Lekic, & Koob, 2008; but see Liu, Roberts & Morgan, 2005). Moreover, both drug- and cue-induced reinstatement of extinguished drug-seeking was enhanced in animals following an escalation of intake under extended access self-administration conditions (criteria 4 of SUD; Deroche-Gamonet et al., 2004; Deroche-Gamonet, Le Moal, & Piazza, 1999; Kippin, Fuchs, & See, 2006; Mantsch, Yuferov, Mathieu-Kia, Ho, & Kreek, 2004; Vanderschuren & Everitt, 2004). It is for these reasons that long-access self-administration sessions and an escalation of drug intake have been suggested to be of particular importance for the comprehensive and valid modelling of a SUD (Ahmed, 2011; Edwards & Koob, 2013).

The self-administration procedure has been invaluable for studying the mechanisms underlying the maintenance and reinstatement of drug-taking/seeking behaviour (Carter & Griffiths, 2009; Gardner, 2000; Koob & Volkow, 2010; Panlilio & Goldberg, 2007; Shaham et al., 2003). Under the right conditions, several features of a SUD can be observed, and various manipulations can be employed to study them. Self-administration has also been useful for examining predisposing factors of drug abuse (e.g. D. Belin, Berson, Balado, Piazza, & Deroche-Gamonet, 2011; D. Belin, Mar, Dalley, Robbins, & Everitt, 2008; Bird & Schenk, 2013; Horger, Shelton, & Schenk, 1990; Lynch, 2006; Piazza, Deminière, le Moal, & Simon, 1990; Piazza, Deminière, Le Moal, & Simon, 1989; Schenk, Lacelle, Gorman, & Amit, 1987; Schenk, Robinson, & Amit, 1988). While several environmental and genetic manipulations have been shown to facilitate the acquisition of self-administration, one of the most robust effects is produced following pre-exposure to drugs under conditions that produce behavioural sensitisation.

Behavioural sensitisation. Acute administration of many drugs can produce dose-dependent increases in various behaviours. Following repeated drug exposure, some drug-produced behaviours may decrease (indicating tolerance), whereas other behaviours can increase (indicating sensitisation). The latter phenomenon was first reported in the 1930s and refers to the progressive and persistent increase in drug-produced behaviour that occurs as a function of repeated exposure to some drugs (Downs & Eddy, 1932; Seevers & Tatum, 1931). The most commonly measured drug-produced behaviour is locomotor activity, although sensitisation of several other behaviours including sniffing, head movements, and rearing has also been reported (Pierce & Kalivas, 1997; Post & Rose, 1976; T. E. Robinson, 1984; Stewart

& Badiani, 1993). Behavioural sensitisation has been observed following repeated exposure to several different psychostimulants including cocaine, amphetamine, methylphenidate, and MDMA, as well as other classes of drugs including opioids, nicotine, and ethanol (for reviews see Kalivas & Stewart, 1991; Pierce & Kalivas, 1997; T. E. Robinson & Becker, 1986; Vanderschuren & Kalivas, 2000).

A behavioural sensitisation procedure is characterised by two phases: the *development* of sensitisation and the *expression* of sensitisation. The development of sensitisation (also called the induction or initiation of sensitisation) typically involves a regimen of repeated, intermittent, experimenter-administered drug exposure followed by a withdrawal period of at least one day. The expression of sensitisation refers to the manifestation of the sensitised behavioural response produced by subsequent re-exposure to the drug. A moderate dose of the drug is typically used so as to observe changes in behaviour without ceiling or floor effects, although full dose-effect curves are sometimes produced. A greater behavioural response in drug pretreated animals compared to vehicle/placebo pretreated controls indicates the development of a sensitised behavioural response and a leftward shift in the dose-response curve.

The acquisition of drug self-administration is also dose-dependent; self-administration of higher doses of drug is generally acquired more readily than when lower doses serve as the reinforcer (Carroll & Lac, 1997; Schenk et al., 1993). Much like increasing the dose, pretreating animals to a regimen of drug exposure that results in behavioural sensitisation also sensitises the acquisition of self-administration (i.e. shifts the acquisition curve for self-administration to the left). For example, low doses of amphetamine and cocaine, that were usually subthreshold as a reinforcer, were reliably self-administered in rats pretreated with amphetamine (Piazza et al., 1990, 1989, 1991; Pierre & Vezina, 1997; Vezina, Lorrain, Arnold, Austin, & Suto, 2002) or cocaine, (Horger et al., 1990; Zhao & Becker, 2010), respectively. For less reinforcing drugs, such as ethanol or MDMA, pretreatment can speed the acquisition of self-administration and/or increase the proportion of animals that meet an acquisition criterion (Camarini & Hodge, 2004; Rodd-Henricks et al., 2002; van de Wetering & Schenk, 2017). Animals pre-exposed to drugs under conditions that produce behavioural sensitisation also worked harder in order to obtain amphetamine infusions under a PR schedule (Lorrain, Arnold, & Vezina, 2000; Mendrek, Blaha, & Phillips, 1998; Vezina et al., 2002) and showed an enhanced escalation of cocaine intake under extended access self-administration conditions (Ferrario & Robinson, 2007).

These findings suggest that pre-exposure to drugs under these conditions also results in the sensitisation of the reinforcing properties of drugs. As will be discussed in the next section, the development of behavioural sensitisation involves neuroadaptations in brain mechanisms that are also involved in reward, reinforcement, and incentive motivation. For these reasons, the behavioural sensitisation procedure has been a useful tool for efficiently examining potential neuroadaptations and manipulations that are relevant to drug-taking and self-administration, albeit without necessarily measuring drug-taking.

Neurocircuitry of addiction

In the 1950s, researchers discovered that laboratory animals would avidly perform tasks (e.g. lever pressing) in order to receive mild electrical stimulation of certain sub-cortical brain structures (Olds & Milner, 1954). These findings were later replicated in humans (Bishop, Elder, & Heath, 1963) and were the first of many to suggest that the activation of certain brain regions was rewarding and could positively reinforce behaviour. In the years following this discovery, the so-called ‘reward circuitry’ of the brain was extensively mapped using such procedures. The primary neural substrates driving reward were identified to be the dopamine (DA) neurons of the ventral tegmental area (VTA) and their efferent projections to the nucleus accumbens (NAcc) via the median forebrain bundle (Olds & Milner, 1954). The next question for some researchers was, could improper functioning in this brain system underlie pathological responses to rewarding stimuli such as drugs?

Ventral tegmental area and nucleus accumbens

Dopamine and reinforcement. A wealth of evidence indicates that drug-produced DA activity within this VTA→NAcc mesoaccumbal system plays an essential role in the acute rewarding/reinforcing effects of drugs. Firstly, nearly all drugs of abuse have the pharmacological capacity to increase DA activity within these brain regions, either by directly facilitating the release of DA in the NAcc (in the case of psychostimulants) or by activating DAergic VTA neurons (in the case of opioids and alcohol). This was clearly demonstrated by Di Chiara and Imperato (1988) who measured extracellular DA concentrations in the NAcc using *in vivo* microdialysis. Drugs of abuse, such as amphetamine, cocaine, nicotine, and morphine, increased extracellular concentrations of DA by up to 1000%, 330%, 225%, and 200%, respectively, whereas drugs that are not abused such as imipramine (anti-depressant), atropine (anti-muscarinic drug), and diphenhydramine (antihistamine), did not increase synaptic DA concentrations (Di Chiara & Imperato, 1988). The increase in synaptic DA produced by drugs of abuse has also been shown to directly relate to the pattern of responding

during self-administration. Laboratory animals initially responded rapidly, which greatly increased extracellular DA levels, after which sustained responding maintained these elevated DA levels (Hurd, Weiss, Koob, And, & Ungerstedt, 1989; Pettit & Justice, 1991; Ranaldi, Pocock, Zereik, & Wise, 1999; Wise et al., 1995).

Secondly, drugs of abuse are readily self-administered when intracranially administered directly into these brain regions (Bozarth, 1987; McBride et al., 1999; Routtenberg, 1972). Psychostimulants such as amphetamine are self-administered into the NAcc (Hoebel et al., 1983; A. G. Phillips, Mora, & Rolls, 1981), while opioids such as morphine are more readily self-administered into the VTA (Bozarth & Wise, 1981, 1982). Drugs that selectively facilitate DA neurotransmission in these brain regions are also self-administered suggesting that the DAergic action of drugs alone is reinforcing (Howell & Byrd, 1991; Nader & Mach, 1996; Ranaldi, Wang, & Woolverton, 2001; Self, Belluzzi, Kossuth, & Stein, 1996; Self & Stein, 1992; Weed & Woolverton, 1995; Woolverton, Goldberg, & Ginos, 1984; Yokel & Wise, 1978).

Thirdly, pharmacological manipulation of the DAergic system influences drug self-administration. Administration of DA agonists decreased self-administration of various psychostimulants, including cocaine, amphetamine, and methamphetamine, producing a leftward shift in the dose-effect curve for self-administration (Barrett, Miller, Dohrmann, & Caine, 2004; Caine & Koob, 1994a; Munzar, Baumann, Shoaib, & Goldberg, 1999; Yokel & Wise, 1978). Conversely, administration of DA antagonists, either systemically or intra-NAcc, produced an increase in responding and a rightward shift in the dose-effect curve for self-administration (Brennan, Carati, Lea, Fitzmaurice, & Schenk, 2009; Britton et al., 1991; Caine & Koob, 1994a; Corrigan & Coen, 1991a; Hubner & Edward Moreton, 1991; Koob, Le, & Creese, 1987; G. D. Phillips, Howes, Whitelaw, Robbins, & Everitt, 1994; G. D. Phillips, Robbins, & Everitt, 1994; Risner & Jones, 1976; Schenk, 2002; Yokel & Wise, 1976). This compensatory responding produced by DA agonists and antagonists is similar to what is seen when the self-administration infusion dose is increased or decreased, respectively (Goldberg, Hoffmeister, et al., 1971; Pickens & Harris, 1968; Weeks & Collins, 1964).

Finally, the destruction of mesoaccumbal DA neurons decreases both the acquisition and maintenance of self-administration. Lesions of the NAcc and VTA with 6-hydroxydopamine (6-OHDA) prevented the acquisition of amphetamine (Lyness, Friedle, & Moore, 1979) and heroin (Bozarth & Wise, 1986) self-administration, respectively. NAcc 6-OHDA lesions also greatly decreased responding maintained by self-administered nicotine and

amphetamine (Corrigall & Coen, 1991b; Lyness et al., 1979; Singer, Wallace, & Hall, 1982). Lesions of the both NAcc or the VTA with 6-OHDA similarly decreased the self-administration of cocaine (Caine & Koob, 1994b; D. C. S. Roberts, Corcoran, & Fibiger, 1977; D. C. S. Roberts, Koob, Klonoff, & Fibiger, 1980; D. C. S. Roberts & Koob, 1982). In these studies, the recovery of cocaine self-administration was also correlated with the level of DA depletion produced by the lesion, with rats with the greatest DA depletion failing to recover cocaine self-administration at all (D. C. S. Roberts & Koob, 1982; D. C. S. Roberts et al., 1980).

It is clear that the acute reinforcing effects of drugs are due to their effect on DA within the brain's 'reward' circuitry. Indeed, with few exceptions, this is the only pharmacological effect common to all drugs of abuse. Of course, the brain's 'reward' circuitry did not evolve to engender drug-taking behaviour, but rather to reinforce biologically essential behaviours such as feeding, drinking, sexual behaviour, maternal/paternal behaviour, and social interaction, all of which induce DAergic activity in these brain regions (Bassareo & Di Chiara, 1999a; Hansen, Bergvall, & Nyiredi, 1993; Manuel Mas, Fumero, & González-Mora, 1995; Pfaus et al., 1990; White & Milner, 1992). Drugs of abuse are capable of activating these brain regions to a far greater extent than natural rewards, however, and therefore can serve as incredibly powerful reinforcers that can direct behaviour towards the acquisition and consumption of drugs at the expense of other naturally reinforced behaviours.

Dopamine and incentive motivation. Given the vital role of mesoaccumbal DAergic mechanisms in the reinforcing effects of drugs and other rewards, it was initially presumed that DA mediated the subjective experience of pleasure (Olds, 1956; Wise, 1980, 1982). Early studies conducted by Robinson and Berridge suggested otherwise, however. DAergic manipulations had no effect on specific measures of pleasure but did have a significant impact on the motivation to obtain and consume rewards (Berridge & Valenstein, 1991; Berridge, Venier, & Robinson, 1989). This led to their suggestion that positive reinforcers/rewards are both 'liked' (referring to the subjective pleasurable effects of the drug) as well as 'wanted' (referring to the incentive motivational properties of the drug/drug-associated stimuli). Although both drug 'liking' and 'wanting' can influence how reinforcing a drug is, they argued that mesolimbic DA activity primarily serves to promote the latter (T. E. Robinson & Berridge, 1993).

Additional support for this idea has grown over the years (for recent reviews see Berridge & Kringelbach, 2015; Berridge & Robinson, 2016). For example, NAcc DA release was increased by the consummation of a food-reward but also by the anticipation of food

reward, which was potentiated by food-deprivation (Bassareo & Di Chiara, 1999b). NAcc DA release or DA neuron firing was also greatest when rewards were novel and when they were presented unexpectedly (Baldo & Kelley, 2007; Bassareo, De Luca, & Di Chiara, 2002; Bassareo & Di Chiara, 1997, 1999a; Schultz, Dayan, & Montague, 1997). These findings suggested a role of DA in incentive and learning, not just pleasure. Studies in human drug users have shown similar findings. Suppression of DA neurotransmission had no effect of subjective ratings of drug-produced euphoria but did significantly decrease the desire to consume drugs (Brauer & de Wit, 1997; Leyton, Casey, Delaney, Kolivakis, & Benkelfat, 2005). Further, increases in brain DA activity, as measured by neuroimaging, were more correlated with subjective ratings of drug or food ‘wanting’ than with pleasure ratings (C. T. Smith, Dang, Cowan, Kessler, & Zald, 2016; Volkow et al., 2002).

An important role of DAergic mechanisms in conditioned reinforcement has also been demonstrated, further evidencing the role of DA in ‘wanting’. For example, presentation of food or drug-associated stimuli increased the firing rate of VTA neurons and enhanced DA release in the NAcc core (Ito, Dalley, Howes, Robbins, & Everitt, 2000; P. E. M. Phillips, Stuber, Heien, Wightman, & Carelli, 2003; Schultz, 1998). Acquisition of conditioned approach behaviour towards reward-associated stimuli was also prevented by lesions of the NAcc core or the administration of DA antagonists (Di Ciano, Cardinal, Cowell, Little, & Everitt, 2001; Parkinson, Olmstead, Burns, Robbins, & Everitt, 1999). Conditioned reinforcement was similarly decreased by these DAergic manipulations (Hall, Parkinson, Connor, Dickinson, & Everitt, 2001; Parkinson et al., 1999). Further, drug-seeking induced by exposure to conditioned stimuli previously associated with drug-taking was either attenuated or potentiated by systemic or intra-accumbal administration of DA antagonists or agonists, respectively (Cervo, Carnovali, Stark, & Mennini, 2003; Crombag, Grimm, & Shaham, 2002; Di Ciano, Underwood, Hagan, & Everitt, 2003; Gál & Gyertyán, 2006; Gilbert et al., 2005; Pilla et al., 1999; Schenk, Gittings, & Colussi-Mas, 2011).

It is now, therefore, widely accepted that the primary role of mesoaccumbal DA is not to mediate reward pleasure or ‘liking’, but to imbue rewards and associated stimuli with incentive salience, making them ‘wanted’, and motivating-goal directed behaviour towards their acquisition (Berridge & Robinson, 2016; Everitt & Robbins, 2005; Kalivas & Volkow, 2005; Koob & Volkow, 2010; Wise, 2008). According to Robinson and Berridge’s popular incentive sensitisation theory of addiction, repeated drug exposure results in the sensitisation of this neural system and, therefore, the sensitisation of the incentive motivational properties

of drugs and drug-associated stimuli (Berridge & Robinson, 2016; T. E. Robinson & Berridge, 1993, 2000, 2001, 2008).

A large source of evidence in support of this theory comes from studies that have used the behavioural sensitisation procedure. The locomotor activating effects of drugs have long been known to also be mediated by central DAergic mechanisms (Carlsson, Lindqvist, Magnusson, & Waldeck, 1958; Costall & Naylor, 1979; Hornykiewicz, 1966; Ungerstedt, 1979). Extracellular concentrations of DA are positively correlated with drug-produced locomotor activity (Hurd et al., 1989; Kalivas & Duffy, 1990; Sharp, Zetterström, Ljungberg, & Ungerstedt, 1987; Steinpreis & Salamone, 1993) and manipulations that attenuate DAergic activity decrease locomotor activity (Garrett & Holtzman, 1994; Pijnenburg, Honig, & Van Rossum, 1975; Schindler & Carmona, 2002). It is not surprising, then, that behavioural sensitisation is accompanied by sensitised DAergic mechanisms (Kalivas & Stewart, 1991; Pierce & Kalivas, 1997; Vanderschuren & Kalivas, 2000). For example, repeated drug exposure under conditions that produced behavioural sensitisation also enhanced drug-produced NAcc DA release (Kalivas & Duffy, 1990; Kalivas, Duffy, & White, 1998; Parsons & Justice, 1993; Pettit, Pan, Parsons, & Justice, 1990; T. E. Robinson, Jurson, Bennett, & Bentgen, 1988), increased dendritic spine density in NAcc medium spiny neurons (MSN; Ball, Wellman, Fortenberry, & Rebec, 2009; Brown & Kolb, 2001; Yilin Li, Acerbo, & Robinson, 2004; Yilin Li, Kolb, & Robinson, 2003), and resulted in an upregulation of DA D₁ receptors (Henry & White, 1991; Yong Li et al., 1999; Wolf, White, & Hu, 1994).

The VTA has been demonstrated to be a crucial substrate involved in the *development* of behavioural sensitisation (Vanderschuren & Kalivas, 2000). Repeated intra-VTA, but not intra-NAcc, administration of various drugs including amphetamine, cocaine, or morphine, enhanced the locomotor activating effect produced by subsequent systemic or intra-NAcc drug exposure (Cador, Bjijou, & Stinus, 1995; Cornish & Kalivas, 2001; Hooks, Jones, Liem, & Justice, 1992; Kalivas & Duffy, 1987; Kalivas & Weber, 1988; Vezina, Kalivas, & Stewart, 1987). In contrast, NAcc activation appears to be crucial for the *expression* of behavioural sensitisation (Vanderschuren & Kalivas, 2000). Following repeated systemic or intra-VTA exposure of various drugs, intra-NAcc, but not intra-VTA, administration of psychostimulants or selective dopamine agonists resulted in the expression of sensitised locomotor activity (Abraham, Quadros, & Souza-Formigoni, 2011; Cador et al., 1995; Cunningham, Finn, & Kelley, 1997; Paulson & Robinson, 1991; Perugini & Vezina, 1994; Pierce & Kalivas, 1995; Vanderschuren, Schoffelmeer, Mulder, & De Vries, 1999; Vezina, 1996).

As previously mentioned, repeated exposure to drugs under conditions that produce behavioural sensitisation has been shown to also sensitise the reinforcing/incentive properties of drugs, as indicated by the facilitated acquisition of drug self-administration, higher break-points under PR schedules, and the more rapid escalation of drug-intake under extended-access conditions (Camarini & Hodge, 2004; Ferrario & Robinson, 2007; Horger et al., 1990; Lorrain et al., 2000; Mendrek et al., 1998; Piazza et al., 1990, 1989, 1991; Pierre & Vezina, 1997; Rodd-Henricks et al., 2002; van de Wetering & Schenk, 2017; Vezina et al., 2002; Zhao & Becker, 2010; but see Horger, Giles, & Schenk, 1992; Schenk, Snow, & Horger, 1991). Behavioural sensitisation has also been shown to enhance the reinforcing effects of drugs as determined by the CPP paradigm (Gaiardi et al., 1991; Lett, 1989; Shippenberg & Heidbreder, 1995). Evidence suggests that the development of sensitisation to both the locomotor-activating and reinforcing effects of drugs involves overlapping DAergic mechanisms (for reviews see Berridge & Robinson, 2016; Pierce & Kalivas, 1997; T. E. Robinson & Berridge, 1993, 2008; Vanderschuren & Pierce, 2010; Vezina, 2004). For example, manipulations that prevent the development of behavioural sensitisation also prevent the subsequent facilitation of drug-taking; treatment with a DA D₁ receptor antagonist blocked both the development of amphetamine locomotor sensitisation (Vezina, 1996; Vezina & Stewart, 1989) and the sensitised acquisition of amphetamine self-administration (Pierre & Vezina, 1998). These findings suggest that the sensitised mesoaccumbal DAergic mechanisms that underlie behavioural sensitisation also underlie the sensitisation of the reinforcing/incentive motivational properties of drugs.

The reinforcing/incentive motivational properties of conditioned stimuli similarly appear to be sensitised following regimens of repeated drug exposure that result in behavioural sensitisation. For example, a sensitising pretreatment regimen of amphetamine or cocaine exposure facilitated the acquisition of conditioned approach behaviour towards stimuli previously associated with reward (Harmer & Phillips, 1998; Taylor & Jentsch, 2001) and enhanced responding maintained by conditioned reinforcers (Cunningham & Kelley, 1992; Di Ciano, 2008; Mead, Crombag, & Rocha, 2004; Taylor & Horger, 1999; Wyvell & Berridge, 2001). Specific firing patterns of DA neurons produced by exposure to drug-conditioned stimuli were also potentiated following a sensitising regimen of amphetamine exposure (Tindell, Berridge, Zhang, Peciña, & Aldridge, 2005).

Although there is much evidence to suggest that repeated, intermittent exposure to drugs of abuse results in the sensitisation of the mesoaccumbal DA system and the incentive

motivational properties of drugs/drug-associated stimuli, sensitisation is not always a result of repeated drug exposure. If drug administration occurs relatively constantly, rather than intermittently, tolerance to both the locomotor and DA releasing effects of drugs can develop instead, especially if examined under shorter withdrawal periods (Pierce & Kalivas, 1997; Vanderschuren & Kalivas, 2000). The expression of sensitisation is also heavily context-dependent, often requiring the same environmental and contextual stimuli where the drugs were originally administered (e.g. Ball, Budreau, & Rebec, 2006; Stewart & Vezina, 1987; Vezina & Stewart, 1984). This has been suggested to explain the mixed findings from studies examining sensitisation in humans (T. E. Robinson & Berridge, 2008; Vezina & Leyton, 2009). While an extensive review of the incentive sensitisation theory of addiction is outside the scope of the present thesis, the important role of central DAergic mechanisms and incentive motivation in addiction is undisputed. It is also clear, however, that several other brain regions, circuits, neurotransmitters systems, and mechanisms also play crucial roles.

Amygdala

Like the NAcc, the basolateral amygdala (BLA) receives DAergic input from the VTA, while glutamatergic output neurons of the BLA innervate the NAcc as well as the prefrontal cortex (PFC). Acute drug administration has been shown to significantly increase extracellular DA in the BLA (Hurd, McGreggo, & Pontén, 1997; Young & Rees, 1998), as has presentations of conditioned stimuli previously associated with reward (Harmer & Phillips, 1999; Hori, Tanaka, & Nomura, 1993; Nomura, Izaki, Takita, Tanaka, & Hori, 2004; Weiss et al., 2000). The BLA has also been shown to modulate the increases in NAcc DA produced by drug exposure or conditioned stimuli (Hurd et al., 1997; Jones et al., 2010; Louilot, Simon, Taghzouti, & Le Moal, 1985).

An important role of the BLA in mediating the effects of stimuli previously associated with drug reward has been demonstrated. Excitotoxic lesions, or inactivation of the BLA with lidocaine, severely impaired the acquisition and maintenance of drug-seeking under second-order schedules of reinforcement (Alderson, Robbins, & Everitt, 2000; Kantak, Black, Valencia, Green-Jordan, & Eichenbaum, 2002; Whitelaw, Markou, Robbins, & Everitt, 1996). Bi-lateral infusion of a DA antagonist into the BLA similarly attenuated drug-seeking under a second-order schedule, while pharmacological disconnection of the glutamatergic connections from the BLA to the NAcc had the same effect (Di Ciano & Everitt, 2004). Lesions of the BLA, local inactivation with tetrodotoxin/lidocaine, or bi-lateral infusions of DA antagonists, also attenuated the reinstatement of drug-seeking behaviour produced by the presentation of

conditioned cues (Fuchs & See, 2002; Grimm & See, 2000; Kantak et al., 2002; McLaughlin & See, 2003; Meil & See, 1997; See, Kruzich, & Grimm, 2001). Interestingly, such manipulations had no effect on the acquisition or maintenance of self-administration or on the reinstatement of drug-seeking produced by a drug prime or stress, suggesting that the BLA may play a selective role in mediating the effects of conditioned-stimuli (Grimm & See, 2000; McFarland, Davidge, Lapish, & Kalivas, 2004; McFarland & Kalivas, 2001; Meil & See, 1997; See, Fuchs, Ledford, & McLaughlin, 2006; Whitelaw et al., 1996).

The central nucleus of the amygdala (CeA), in contrast, appears to play an important role in the acute, primary reinforcing effects of various drugs of abuse since lesions of the CeA, or local administration of various antagonists, decreased self-administration of cocaine, opioids, and alcohol (Caine, Heinrichs, Coffin, & Koob, 1995; Dyr & Kostowski, 1995; Heyser, Roberts, Schulteis, & Koob, 1999; Hyytiä & Koob, 1995; McGregor & Roberts, 1993; Möller, Wiklund, Sommer, Thorsell, & Heilig, 1997). The CeA also plays a major role in stress responses. Stress-induced reinstatement of drug-seeking requires the activation of norepinephrine (NE; noradrenalin), and the stress hormone, corticotrophin-releasing factor (CRF), in the CeA (see Shaham et al., 2003). Acute withdrawal from drugs also potentiates anxiety-like responses to acute stressors, which has similarly been shown to require CRF activity within the CeA (Koob, 2008). Neuroadaptations within the CeA and the CRF system following repeated drug exposure have been implicated in the development of long-term negative emotional states that have been suggested play a major role in negatively reinforcing drug-seeking and drug-taking (Koob et al., 2014; Koob & Le Moal, 2008a; Piazza & Le Moal, 1996).

Dorsal striatum

The dorsal striatum, which contains the caudate nucleus and putamen (C-P), has been identified as a key brain region involved in habitual, stimulus-driven behaviour that has been suggested to contribute to the maintenance and relapse of drug-taking (Everitt et al., 2008; Everitt & Robbins, 2005, 2016). Reinforcement learning, such as during self-administration, is thought to involve two processes: one associating the behaviour with the consequence (action-outcome), and one associating the stimuli with the behaviour (stimulus-response). These processes govern goal-directed and stimulus-driven behaviour, respectively (Balleine & O'Doherty, 2010). Initially, behaviours are goal-directed and performed with conscious regard for the outcome, but with extensive repetition, behaviour can become reflexive and habitual, driven primarily by the presentation of conditioned-stimuli (Adams, 1982; Tricomi, Balleine,

& O'Doherty, 2009). Such habitual behaviour is resistant to extinction and can persist even when the outcome/reward has been significantly devalued (Adams, 1982; Tricomi et al., 2009).

Considerable evidence suggests that drug-seeking behaviour can become habitual and stimulus-driven following repeated drug use (Everitt et al., 2008; Everitt & Robbins, 2005, 2016). For example, cocaine, nicotine, or ethanol-seeking on chained-schedules of reinforcement or under extinction conditions was decreased by reward devaluation when tested following a short history of self-administration, which suggests that drug-seeking under these conditions was goal-directed (Clemens, Castino, Cornish, Goodchild, & Holmes, 2014; Corbit, Nie, & Janak, 2012; Olmstead, Lafond, Everitt, & Dickinson, 2001; Zapata, Minney, & Shippenberg, 2010). Following a more extensive history of self-administration, however, drug-seeking was no longer affected by reward devaluation procedures, suggesting that drug-seeking had become habitual (Clemens et al., 2014; Corbit et al., 2012; Dickinson, Wood, & Smith, 2002; Miles, Everitt, & Dickinson, 2003; Olmstead et al., 2001; Zapata et al., 2010).

The dorsolateral striatum (DLS) in particular has been heavily implicated in stimulus-driven behaviour, whereas the dorsomedial striatum (DMS), like the ventral striatum (i.e. NAcc), appears to be more involved in goal-directed behaviour (Balleine & O'Doherty, 2010; Shiflett, Brown, & Balleine, 2010; Yin, Knowlton, & Balleine, 2004; Yin, Ostlund, Knowlton, & Balleine, 2005). This is consistent with differences in the anatomical connectivity of these regions; the DMS receives excitatory input primarily from the medial PFC, while the DLS receives more excitatory input from sensorimotor cortices (Crittenden & Graybiel, 2011; Voorn, Vanderschuren, Groenewegen, Robbins, & Pennartz, 2004). It has, therefore, been suggested that the development of habitual drug-seeking behaviour reflects the increasing involvement of the dorsal striatum, and in particular, the DLS (Everitt et al., 2008; Everitt & Robbins, 2005, 2016).

Accumulating evidence supports this idea. Well-established drug-seeking behaviour maintained by conditioned stimuli on a second-order schedule of reinforcement was accompanied by enhanced DA release in the dorsal, but not the ventral striatum (Ito, Dalley, Robbins, & Everitt, 2002), and was decreased by intra-dorsal striatal administration of DA or glutamate antagonists (Vanderschuren, Di Ciano, & Everitt, 2005). Inactivation of the DLS in particular, but not the DMS, decreased well-established drug-seeking maintained by conditioned stimuli whereas the reverse was found when drug-seeking was newly acquired (Murray, Belin, & Everitt, 2012). Similarly, well-established drug-seeking behaviour produced by conditioned stimuli that was shown to be insensitive to reward devaluation (i.e. habitual)

was decreased by intra-DLS administration, but not by intra-DMS administration, of DA antagonists (Corbit et al., 2012; Zapata et al., 2010). In contrast, newly-acquired drug-seeking behaviour that was sensitive to reward devaluation (i.e. goal-directed) was not decreased by intra-DLS administration but was decreased by intra-DMS administration of DA antagonists (Corbit et al., 2012; Zapata et al., 2010).

Habitual behaviour provides an efficient means of performing repetitive behaviour with little top-down, cognitive processing (Canales, 2005; Jog, Kubota, Connolly, Hillegaart, & Graybiel, 1999). However, habitual behaviour needs to be amenable by top-down cognitive process in case the habitual behaviour becomes maladaptive. Evidence suggests that habitual drug-taking/seeking behaviour is often associated with a deficit in prefrontal cortical top-down cognitive processes, which prevents adequate control over the maladaptive and habitual drug-seeking behaviour (Dalley, Everitt, & Robbins, 2011; Jentsch & Taylor, 1999; Robbins & Everitt, 1999).

Prefrontal cortex

The PFC receives DAergic afferents from the VTA, while excitatory glutamatergic efferents of the PFC innervate the NAcc, VTA, and the dorsal striatum. Thus, the positioning of the PFC allows for the direct regulation of important DAergic regions involved in incentive salience as well as both goal-directed and stimulus-driven behaviour. Neuroimaging studies in humans with a SUD have shown that drug administration or the presentation of drug-associated stimuli increases PFC activity, and this is correlated with subjective measures of drug-craving (for reviews see Goldstein & Volkow, 2011; Koob & Volkow, 2016). In rodents, anatomical and functional analogues of important human PFC brain regions include the orbitofrontal cortex (OFC) and the medial PFC, which contains the anterior cingulate, prelimbic, and infralimbic cortices (Dalley, Cardinal, & Robbins, 2004; Koob & Volkow, 2016; Uylings, Groenewegen, & Kolb, 2003). These brain regions are similarly activated by drug administration, or the presentation of drug-associated stimuli and evidence suggests that these brain regions play an important role in mediating drug-seeking behaviour (Koob & Volkow, 2010, 2016; Shaham et al., 2003; Weiss, 2005).

Inactivation of the medial PFC (anterior cingulate or prelimbic cortex) or the OFC attenuated the reinstatement of previously extinguished drug-seeking behaviour produced by acute drug exposure, conditioned-stimuli, or stress (Capriles, Rodaros, Sorge, & Stewart, 2003; Fuchs, Evans, Parker, & See, 2004; McLaughlin & See, 2003; Park et al., 2002). DAergic activity in the PFC appears to be particularly important since local infusions of various DA

antagonists into these brain regions similarly attenuated drug-, cue-, or stress-induced reinstatement (Capriles et al., 2003; McFarland & Kalivas, 2001; Park et al., 2002). Moreover, local infusions of DA or cocaine into the medial PFC was sufficient to reinstate drug-seeking (Park et al., 2002). An important role of glutamatergic outputs from the PFC to the NAcc in mediating drug-seeking behaviour has also been demonstrated since drug-seeking reinstated by intra-medial PFC cocaine was blocked by local administration of glutamate antagonists into the NAcc (Park et al., 2002). Pharmacological disconnection of glutamate efferents from the PFC to the NAcc also blocked cocaine-induced reinstatement (McFarland & Kalivas, 2001). Further, cocaine-induced reinstatement was associated with PFC-mediated increases in NAcc glutamate (McFarland, Lapish, & Kalivas, 2003).

Although widespread PFC activity is associated with craving and drug-seeking produced by drugs or drug-associated stimuli, widespread hypoactivity of the PFC is apparent during times of withdrawal or during tasks that involve higher-order cognitive and emotional control (Goldstein & Volkow, 2011; Koob & Volkow, 2010, 2016). Humans with a SUD show impaired performance on PFC-dependant tasks involving attention, cognitive flexibility, reward valuation, inhibitory control, and decision making (Goldstein & Volkow, 2011; Koob & Volkow, 2010, 2016). These deficits have been associated with increased drug use, greater likelihood of relapse, and overall worse treatment outcomes (Aharonovich et al., 2006). Parallel studies in laboratory animals have shown similar results. Impairments in reversal learning (an OFC-dependant task) were observed following repeated cocaine exposure in both rats and monkeys (Calu et al., 2007; Jentsch, Olsson, de la Garza, & Taylor, 2002; Schoenbaum, Saddoris, Ramus, Shaham, & Setlow, 2004). Impairments have also been observed in working memory and sustained attention tasks (PFC-dependant tasks) following self-administration of cocaine under-extended access, but not limited-access conditions (Briand, Flagel, et al., 2008; Briand, Gross, & Robinson, 2008; George, Mandyam, Wee, & Koob, 2008). In one of these studies, these deficits were also associated with decreased DA D₂ receptor messenger RNA (mRNA) in the medial PFC and OFC (Briand, Flagel, et al., 2008), a finding that is consistent with those from human neuroimaging studies (see Goldstein & Volkow, 2011; Koob & Volkow, 2010, 2016).

It should be stated that the review on the neurocircuitry/neurobiology of addiction given here is by no means exhaustive, which would be outside of the scope of the present thesis. The brain regions and mechanisms covered here are arguably some of those that are most relevant to addiction, but also to the current thesis. For more in-depth reviews that include other relevant

brain regions and mechanisms see (Berridge & Robinson, 2016; Gardner, 2011; Goldstein & Volkow, 2011; Kalivas & Volkow, 2005; Koob & Volkow, 2010, 2016; Shaham et al., 2003; Vanderschuren & Pierce, 2010; Weiss, 2005).

MDMA

The behavioural and neurobiological effects of repeated exposure to most drugs of abuse such as cocaine, amphetamine, opioids, and alcohol, have been relatively well studied. The effects of repeated exposure to $\pm$ 3,4-methylenedioxymethamphetamine (MDMA), is less understood, however.

MDMA/ecstasy use disorders

MDMA (commonly referred to as ‘ecstasy’ or ‘molly’) is a popular recreational drug of abuse, particularly in New Zealand and Australia. Because MDMA is often adulterated with a variety of other compounds, the term ecstasy will be used throughout this thesis when outside of controlled clinical or pre-clinical settings. It is estimated that there are approximately 21 million ecstasy users worldwide (United Nations Office on Drugs and Crime [UNODC], 2019). For comparison, there are approximately 18 million cocaine users and 188 million cannabis users (UNODC, 2019). As is the case with other drugs of abuse, a proportion of ecstasy users develop symptoms consistent with a SUD. For example, some users reported: taking ecstasy in larger amounts or for longer periods than intended (Cottler, Womack, Compton, & Ben-Abdallah, 2001); wanting to cut down or stop using ecstasy but not managing to (Topp, Hall, & Hando, 1997); cravings and urges to use ecstasy (Hopper et al., 2006); continued usage, despite knowing they had a physical or psychological problem that could have been caused or exacerbated by ecstasy (Cottler, Leung, & Abdallah, 2009; Yen & Hsu, 2007); tolerance to the positive effects of ecstasy (Parrott, 2005; Peroutka, Newman, & Harris, 1988); and the development of withdrawal symptoms (Cottler et al., 2001; McKetin et al., 2014).

Ecstasy abuse has contributed to a number of deaths due to MDMA-induced hyperthermia, renal failure, hepatic toxicity, cardiac arrhythmia, rhabdomyolysis, and disseminated intravascular coagulation (Chadwick, Curry, Linsley, Freemont, & Doran, 1991; Dykhuizen, Brunt, Atkinson, Simpson, & Smith, 1995; Fineschi & Masti, 1996; Freedman, Johanson, & Tancer, 2005; García-Repetto et al., 2003; Khakoo, Coles, Armstrong, & Barry, 1995; Schifano et al., 2003; Screaton et al., 1992). The long-term consequences associated with the repeated use of MDMA may be greater than the risk of death from acute toxicity, however. Ecstasy abuse has been attributed to the onset of several severe psychopathologies including depression, anxiety, panic attacks, paranoid psychosis, mixed affective psychosis, and

hallucinations (Cassidy & Ballard, 1994; McGuire, Cope, & Fahy, 1994; Parrott, Sisk, & Turner, 2000; Series, Boeles, Dorkins, & Peveler, 1994; Topp, Hando, Dillon, Roche, & Solowij, 1999; Williamson, 1997; Yen & Hsu, 2007). Ecstasy abuse has also been associated with cognitive deficits in memory and decision-making (Halpern et al., 2004; Parrott, Lees, Garnham, Jones, & Wesnes, 1998; Roiser, Rogers, Cook, & Sahakian, 2006). As will be described further below, repeated ecstasy/MDMA use has also been associated with severe neurodegeneration in humans as well as laboratory animals, which might underlie some of the aforementioned cognitive and psychological consequences of ecstasy abuse. Given the popularity of MDMA/ecstasy as a drug of abuse, and the recent developments for the use of MDMA as an adjunct for the psychotherapy of post-traumatic stress disorder, there is a clear need for a better scientific understanding of the drug and the effect of repeated exposure on brain and behaviour.

MDMA self-administration

The reinforcing effects of MDMA have been less extensively studied than the reinforcing effects of other drugs of abuse. Nonetheless, like other drugs of abuse, MDMA is reliably self-administered by various laboratory animals (Aarde, Miller, Creehan, Vandewater, & Taffe, 2015; Ball et al., 2015; Ball, Walsh, & Rebec, 2007; Ball & Slane, 2012, 2014; Beardsley, Balster, & Harris, 1986; Bird & Schenk, 2013; Bradbury et al., 2013; Colussi-Mas, Wise, Howard, & Schenk, 2010; Creehan, Vandewater, & Taffe, 2015; Do & Schenk, 2013; Feduccia, Kongovi, & Duvauchelle, 2010; Gould et al., 2011; Oakly, Brox, Schenk, & Ellenbroek, 2013; Reveron, Maier, & Duvauchelle, 2010; Schenk & Bradbury, 2015; Schenk et al., 2007; Z. Wang & Woolverton, 2007). Once acquired, MDMA self-administration is comparable to the self-administration of other psychostimulant drugs of abuse such as cocaine or amphetamine. For example, the dose-effect curve for MDMA self-administration was in the shape of an inverted U (Daniela, Brennan, Gittings, Hely, & Schenk, 2004; Ratzenboeck, Saria, Kriebbaum, & Zernig, 2001; Schenk et al., 2003). Halving the MDMA infusion dose resulted in the doubling of the number of responses so that drug intake (mg/kg) remained relatively constant (Do & Schenk, 2013; Reveron et al., 2010; Schenk et al., 2012). Likewise, increasing in the FR response requirement resulted in the proportional increase in lever pressing (Daniela et al., 2006; Schenk et al., 2011). Replacing MDMA with its vehicle solution resulted in the extinction of the operant behaviour, which was subsequently reinstated by the reintroduction of MDMA (Daniela et al., 2006; Schenk et al., 2011). Further, extinguished drug-seeking behaviour following MDMA self-administration was reinstated by priming injections of

MDMA, cocaine, or yohimbine (stress inducer), or by the presentation of a light stimulus that was previously paired with MDMA infusions (Ball et al., 2015, 2007; Schenk et al., 2011; Schenk, Hely, Gittings, Lake, & Daniela, 2008). Like other drugs of abuse, stimuli that are paired with MDMA infusions can also develop conditioned reinforcing properties and play an important role in the maintenance of MDMA self-administration (Daniela et al., 2006).

The acquisition of MDMA self-administration, however, exhibits a profile that differs from that of other psychostimulants. Firstly, the acquisition of MDMA self-administration proceeds with a protracted-time course. Response rates for MDMA are initially low, and acquisition of reliable MDMA self-administration (1 mg/kg/infusion) typically requires around 15, 2-hour daily sessions (Schenk et al., 2012). In contrast, the self-administration of cocaine, amphetamine, or methamphetamine is usually reliably acquired within a few days (Carroll & Lac, 1997; Highgate & Schenk, 2018; Maan, Highgate, & Schenk, 2020; Schenk et al., 2007). Secondly, only about 50% of rats meet acquisition criteria for reliable MDMA self-administration under these conditions (Schenk et al., 2012) whereas virtually all animals acquire cocaine, amphetamine, or methamphetamine self-administration (Carroll & Lac, 1997; Do & Schenk, 2013; Highgate & Schenk, 2018; Maan et al., 2020). These differences might reflect the unique pharmacology of MDMA compared to other psychostimulants.

Pharmacology of MDMA

MDMA is a ring-substituted phenethylamine and is structurally similar to methamphetamine (psychostimulant) and mescaline (hallucinogen). Unless otherwise stated, all mention of MDMA in this thesis will refer to the racemic mixture (i.e. $\pm$ MDMA), which contains equal amounts of both S-(+)-MDMA and R-(-)-MDMA.

Pharmacokinetics. MDMA is primarily metabolised in the liver by various cytochrome P450 enzymes and catechol-*O*-methyltransferase (COMT) via two main pathways (Figure 1.1; de la Torre & Farré, 2004; de la Torre et al., 2004; Maurer, Bickeboeller-Friedrich, Kraemer, & Peters, 2000). In the first pathway, MDMA is *O*-demethylenated by P450 enzymes into 3,4-dihydroxymethamphetamine (HHMA), which is then *O*-methylated by COMT into 4-hydroxy-3-methoxymethamphetamine (HMMA). In the second pathway, MDMA is first *N*-demethylated by P450 enzymes into 3,4-methylenedioxyamphetamine (MDA), which is then *O*-demethylenated by P450 enzymes and subsequently *O*-methylated by COMT to form 3,4-dihydroxyamphetamine (HHA) and 4-hydroxy-3-methoxyamphetamine (HMA), respectively. The half-life of ~1-2 mg/kg of orally administered MDMA is approximately 7 hours in humans (de la Torre et al., 2000; Kolbrich et al., 2008; M. Mas et al., 1999) and 1 hour in rats (Baumann

et al., 2009). Higher doses have been shown to significantly increase the time for MDMA to be eliminated, however, indicating that the metabolising enzymes are saturable (Baumann et al., 2009; de la Torre et al., 2000). The metabolites of MDMA have much longer half-lives, and some of which are also bio-active (Baumann et al., 2009; de la Torre & Farré, 2004; de la Torre et al., 2004). MDA, in particular, has potent effects on the monoamine system, much like MDMA (Stone, Stahl, Hanson, & Gibb, 1986).

Figure 1.1. Primary metabolic pathways of MDMA. MDMA: 3,4-methylenedioxymethamphetamine; HHMA: 3,4-dihydroxymethamphetamine; HMMA: 4-hydroxy-3-methoxymethamphetamine; MDA: 3,4-methylenedioxyamphetamine; HHA: 3,4-dihydroxyamphetamine; HMA: 4-hydroxy-3-methoxyamphetamine.

Pharmacodynamics. MDMA is a potent releasing agent/reuptake inhibitor of the monoamine neurotransmitters: serotonin (5-HT), DA, and NE. This is accomplished via multiple, synergistic mechanisms. Acting as a substrate for the monoamine transporters, MDMA is able to enter the neuron where it inhibits/reverses vesicular monoamine transporter 2 (VMAT), which results in substantially increased concentrations of monoamines in the cytosol (Bogen, Haug, Myhre, & Fonnum, 2003; Eiden & Weihe, 2011; Fitzgerald & Reid, 1990; Fleckenstein et al., 2002; Gu & Azmitia, 1993; Rudnick & Wall, 1992; Sabol & Seiden, 1998; Schuldiner, Steiner-Mordoch, Yelin, Wall, & Rudnick, 1993; Sulzer & Rayport, 1990). MDMA also inhibits/reverses the monoamine transporters, possibly via agonistic activity at trace amine-associated receptor 1 (TAAR1; Berry, Gainetdinov, Hoener, & Shahid, 2017; Bunzow et al., 2001; Miller, 2011), which results in the substantial efflux of the already increased cytosolic concentrations of monoamines into the synapse while also preventing their reuptake (Battaglia, Brooks, Kulsakdinun, & De Souza, 1988; Berger, Gu, & Azmitia, 1992; Cleary & Docherty, 2003; Cole & Sumnall, 2003; Crespi, Mennini, & Gobbi, 1997; Fitzgerald & Reid, 1990; Gudelsky & Nash, 2002; Hekmatpanah & Peroutka, 1990; Hysek et al., 2011; Iravani, Asari, Patel, Wieczorek, & Kruk, 2000; Nash & Brodtkin, 1991; Rudnick & Wall, 1992). MDMA also inhibits monoamine oxidase, enzymes that are involved in the catabolism on DA, 5-HT, and NE (Leonardi & Azmitia, 1994; Scorza et al., 1997).

The end result is substantially increased synaptic concentrations of monoamines. Measurement of extracellular concentrations of neurotransmitters via *in vivo* microdialysis has shown that acute MDMA administration results in dose-dependent increases in extracellular concentrations of 5-HT, DA, and NE (Baumann, Clark, Franken, Rutter, & Rothman, 2008; Baumann, Clark, & Rothman, 2008; Kankaanpää, Meririnne, Lillsunde, & Seppälä, 1998; Kurling, Kankaanpää, & Seppälä, 2008; Nair & Gudelsky, 2004; O'Shea et al., 2005; Shankaran & Gudelsky, 1999). Because MDMA has the greatest affinity for the 5-HT transporter, these effects are primarily exerted on the 5-HT system (Battaglia, Brooks, et al., 1988). Depending on the dose and the brain region assessed, the effect of acute MDMA administration on extracellular concentrations of 5-HT is typically much greater than the effect on DA (see Schenk, 2011). As will be discussed below, this is not a pharmacological profile that is typical of a drug of abuse.

Role of serotonin in self-administration

As previously mentioned, considerable evidence indicates that DAergic mechanisms positively mediate the reinforcing effects of drugs and their self-administration by laboratory

animals. In contrast, several lines of evidence suggest that drug-produced increases in 5-HTergic neurotransmission is not reinforcing and is indeed inhibitory to self-administration. Firstly, humans do not abuse drugs that selectively increase synaptic concentrations of 5-HT, such as selective serotonin reuptake inhibitors (SSRI; a class of anti-depressant), nor are such drugs self-administered by laboratory animals (Götestam & Andersson, 1975; Howell & Byrd, 1995; D. C. S. Roberts et al., 1999; Tessel & Woods, 1975; Vanover, Nader, & Woolverton, 1992).

Secondly, for drugs that have both DAergic and 5-HTergic effects (i.e. most psychostimulants), those with both greater DAergic effects and smaller 5-HTergic effects are more efficacious reinforcers. For example, amphetamine or cocaine analogues that had greater binding affinities for the DA transporter relative to the 5-HT transporter were more effective at maintaining self-administration (Ritz & Kuhar, 1989; D. C. S. Roberts et al., 1999). Conversely, drugs that were more potent *in vivo* releasers of 5-HT, but had equal potencies as DA releasers, were less effective at maintaining self-administration (Wee et al., 2005).

Thirdly, manipulations that increase or decrease 5-HTergic activity have the opposite effect on self-administration. For example, increasing 5-HT levels by the administration of the synthetic precursor of 5-HT, L-tryptophan, decreased the self-administration of cocaine (Carroll, Lac, Asencio, & Kragh, 1990b; McGregor, Lacosta, & Roberts, 1993) and amphetamine (Lyness, 1983; J. E. Smith, Dianna, Smith, Leccese, & Lyness, 1986). Conversely, inhibition of 5-HT synthesis with p-chlorophenylalanine (pCPA), or 5-HT depletion achieved by 5,7-dihydroxytryptamine (5,7-DHT) lesions, facilitated the self-administration of amphetamine (Fletcher, Korth, & Chambers, 1999; Leccese & Lyness, 1984; Lyness, Friedle, & Moore, 1980), MDMA (Bradbury et al., 2013), ethanol (Lyness & Smith, 1992), and morphine (Dworkin, Guerin, Co, Smith, & Goeders, 1988; J. E. Smith, Shultz, Co, Goeders, & Dworkin, 1987). Administration of various 5-HT agonists also decreased the self-administration of cocaine (Carroll, Lac, Asencio, & Kragh, 1990a; Carroll et al., 1990b; Czoty, Ginsburg, & Howell, 2002; Peltier & Schenk, 1993; Richardson & Roberts, 1991), amphetamine (Dianna, Smith, Smith, & Lyness, 1986; Porrino et al., 1989; Wee & Woolverton, 2006), methamphetamine (Munzar et al., 1999), ethanol (Lyness & Smith, 1992), and heroin (Higgins, Wang, Corrigan, & Sellers, 1994; Y. Wang, Joharchi, Fletcher, Sellers, & Higgins, 1995). In one study, amphetamine was mixed with various doses of the 5-HT releasing agent, fenfluramine, and the rate of self-administration of the mixture was inversely correlated with the dose of fenfluramine (Wee & Woolverton, 2006).

5-HT projections originating from the dorsal and median raphe innervate several brain regions that have been implicated in addiction, including the NAcc, VTA, and PFC (Di Matteo, Di Giovanni, Pierucci, & Esposito, 2008). 5-HTergic mechanisms can modulate DA neurotransmission by direct activation of 5-HT receptors located on DA neurons or via inhibitory gamma-aminobutyric acid (GABA) and excitatory glutamate intermediary connections (Bankson & Cunningham, 2001; Di Matteo et al., 2008; Gudelsky & Yamamoto, 2008). 5-HT-mediated inhibition of DA release has been suggested to be one mechanism by which increased 5-HT attenuates the reinforcing effects of drugs since increasing synaptic 5-HT concentrations with an SSRI, administered at doses that decreased cocaine self-administration, also decreased cocaine-produced increases in extracellular DA in the caudate nucleus (Czoty et al., 2002). It should be noted that the relationship between 5-HT and DA is complicated, however. There are at least 14 distinct 5-HT receptor subtypes (Barnes & Sharp, 1999) and activation of some of these receptors has been shown to facilitate DA release (5-HT_{1a}, 5-HT_{1b}, 5-HT_{2a}, 5-HT_{2b}), whereas activation of others (5-HT_{2c}) has been shown to inhibit DA release (reviewed by Di Matteo et al., 2008).

Given the pharmacology of MDMA, and the apparent inhibitory role of 5-HT in the reinforcing effects of drugs of abuse, why then is MDMA self-administered by laboratory animals, and why is it abused by humans?

Effect of repeated MDMA exposure on serotonin and dopamine

The answer to this question lies in an understanding of the changes in both 5-HTergic and DAergic mechanisms that occur with repeated MDMA exposure. Repeated exposure to MDMA has long been known to produce substantial deficits in 5-HT neurotransmission. In rats, repeated experimenter-administered injections of MDMA (10 – 40 mg/kg, twice daily, for 4 days) produced long-lasting and dose-dependent reductions in 5-HT reuptake sites as well brain tissue levels of 5-HT and 5-hydroxyindoleacetic acid (5-HIAA), a metabolite of 5-HT (Battaglia, Yeh, & De Souza, 1988; Battaglia et al., 1987; Commins et al., 1987; O'Hearn, Battaglia, De Souza, Kuhar, & Molliver, 1988). Both baseline and MDMA-produced increases in extracellular striatal 5-HT were also decreased following repeated MDMA exposure (10 mg/kg, every 2 hours, for a total of 4 injections; Shankaran & Gudelsky, 1999). These effects were prevented by the co-administration of the SSRIs, citalopram (Battaglia, Yeh, et al., 1988), or fluoxetine (Schmidt, 1987), suggesting that MDMA-produced deficits are due to the potent effect of MDMA on the 5-HT system. Similar deficits have also been observed in non-human primates following repeated MDMA/ecstasy exposure (Insel, Battaglia, Johannessen, Marra,

& De Souza, 1989; Kleven, Woolverton, & Seiden, 1989; Ricaurte, DeLanney, Irwin, & Langston, 1988; Wilson, Ricaurte, & Molliver, 1989), and can take several years to fully recover (Ricaurte, Martello, Katz, & Martello, 1992).

The relevance of some of these findings to human MDMA/ecstasy use has been questioned, however, due to the use of relatively high doses of non-contingent MDMA (Baumann, Wang, & Rothman, 2007; de la Torre & Farré, 2004; Ricaurte, McCann, Szabo, & Scheffel, 2000). More recent studies investigating the effect of self-administered MDMA offer greater external validity and have shown similar results. In rats, lower densities of 5-HT reuptake sites (Schenk et al., 2007) and persistent decreases in 5-HT tissue levels (Do & Schenk, 2013) were found following MDMA self-administration (365 – 440 mg/kg over ~17 days). MDMA-produced increases in extracellular NAcc 5-HT were also decreased following 20 days of MDMA self-administration (~100 mg/kg over 20 days; Reveron et al., 2010). 5-HTergic deficits appear to be dependent on exposure levels, however, since self-administration of smaller amounts of MDMA failed to significantly decrease densities of 5-HT reuptake sites or 5-HT tissue levels in rats (Do & Schenk, 2013) or rhesus monkeys (Banks et al., 2008; Fantegrossi et al., 2004). Some 5-HT deficits have also been found in human ecstasy users (McCann, Ridenour, Shaham, & Ricaurte, 1994; McCann, Szabo, Scheffel, Dannals, & Ricaurte, 1998; Semple, Ebmeier, Glabus, O'Carroll, & Johnstone, 1999; Thomasius et al., 2003; Verkes et al., 2001), and these appear to persist for several years (Thomasius et al., 2006).

While it is clear that repeated MDMA exposure can result in various 5-HTergic deficits, some studies have found that repeated MDMA exposure can sensitise DAergic mechanisms. In rats, tissue levels of DA in the NAcc were increased four weeks after repeated MDMA exposure (20 mg/kg/day for 10 days; Mayerhofer, Kovar, & Schmidt, 2001). MDMA- and cocaine-produced increases in extracellular NAcc DA were also enhanced following repeated MDMA exposure (20 mg/kg/day for 4 days; Kalivas et al., 1998; Morgan, Horan, Dewey, & Ashby, 1997). Intermittent dosing appears critical to the development of these sensitised DAergic responses, however, as when similar amounts of MDMA was administered over a single day, there was no effect (Shankaran & Gudelsky, 1999). Rats that acquired MDMA self-administration (165 mg/kg over an average of 21 days) also showed enhanced MDMA-produced (10 mg/kg) increases extracellular striatal DA (Colussi-Mas et al., 2010). Self-administration of lesser amounts of MDMA (~100 mg/kg over 20 days) failed to impact MDMA-produced (3 mg/kg) increases in extracellular NAcc DA, however (Reveron et al., 2010). These results suggest that both the total amount of exposure and the pattern of exposure

mediate the development of sensitised DAergic mechanisms following repeated MDMA exposure.

Repeated intermittent MDMA exposure also has been shown to result in behavioural sensitisation (Ball et al., 2006; Bradbury, Gittings, & Schenk, 2012; Kalivas et al., 1998; Spanos & Yamamoto, 1989). Consistent with other psychostimulant drugs, MDMA-induced behavioural sensitisation appears to involve sensitised DAergic mechanisms. MDMA-induced behavioural sensitisation was accompanied by enhanced MDMA-produced increases in NAcc DA (Kalivas et al., 1998), increased dendritic spine density in DA terminal regions (Ball et al., 2009), and was prevented by co-treatment with the DA D₂ receptor antagonist, eticlopride (van de Wetering & Schenk, 2017). Cross-sensitisation to the behavioural effects of other DAergic drugs including cocaine, amphetamine, and the DA D₂ agonist, quinpirole, has also been demonstrated (Kalivas et al., 1998; Schenk & Bradbury, 2015).

Serotonin, dopamine, and MDMA self-administration

Like other drugs of abuse, increased 5-HT neurotransmission appears to be inhibitory to the reinforcing effects of MDMA and has been shown to limit the acquisition of MDMA self-administration. Rats with greater MDMA-produced increases in extracellular NAcc 5-HT were less likely to subsequently acquire MDMA self-administration (Bradbury et al., 2013). In contrast, manipulations that impaired 5-HT neurotransmission facilitated the acquisition of MDMA self-administration and rendered the acquisition profile more comparable to that of other psychostimulant drugs of abuse. For example, neurotoxic 5,7-DHT lesions (Bradbury et al., 2013) or a genetic mutation of the 5-HT transporter (Oakly et al., 2013) substantially facilitated the acquisition of self-administration, decreasing the latency to acquire and increasing the percentage of rats that met acquisition criteria. Once MDMA self-administration has been acquired, however, it appears 5-HT plays a limited role, since the administration of various 5-HT antagonists did not affect the maintenance of MDMA self-administration (Schenk et al., 2016).

Like other drugs of abuse, evidence suggests that the reinforcing effects of MDMA are mediated by DAergic mechanisms. In rhesus monkeys, the potency of the two isomers of MDMA, S(+)-MDMA and R(-)-MDMA, as well as the racemic mixture, (±)-MDMA, to function as reinforcers and maintain self-administration was positively correlated with the varying potency of each drug to stimulate DA release (Johnson, Hoffman, & Nichols, 1986; Z. Wang & Woolverton, 2007). In rats, treatment with various DA receptor antagonists produced a rightward shift in the dose-response curve for MDMA self-administration (Brennan et al.,

2009; Daniela et al., 2004). Moreover, rats that met acquisition criteria for MDMA self-administration displayed greater MDMA-produced increases in extracellular striatal DA compared to those that failed to acquire self-administration (Colussi-Mas et al., 2010). These results suggest that the development of a sensitised DAergic response might be crucial for the acquisition of MDMA self-administration. In support of this idea, pretreatment with MDMA under conditions that produced sensitisation to the locomotor activating effects of MDMA also facilitated the acquisition of MDMA self-administration, decreasing the latency to acquire and increasing the proportion of rats that met acquisition criteria (van de Wetering & Schenk, 2017).

Collectively, these findings suggest that repeated MDMA exposure can result in both 5-HTergic deficits and DAergic sensitisation. These neuroadaptations, for the reasons discussed above, would be expected to enhance the reinforcing efficacy of MDMA. Thus, it has been hypothesised that the acquisition of MDMA self-administration and the subsequent escalation of MDMA-intake progress as a function of these neuroadaptations, and with repeated exposure, the pharmacological and behavioural profile of MDMA becomes increasingly similar to other psychostimulant drugs of abuse such as amphetamine or cocaine (Schenk, 2011).

The current thesis

The purpose of the current research was to further investigate the neuroadaptations that develop with repeated MDMA exposure in rats. Initial experiments aimed to identify the specific brain regions that undergo behaviourally relevant neuroadaptive change, as measured by Δ FosB immunohistochemistry (IHC). Subsequent experiments aimed to investigate the more specific neurochemical changes within these brain regions, as measured by *in vivo* microdialysis and liquid chromatography-mass spectrometry (LC-MS).

Δ FosB and immunohistochemistry

Drug-craving and vulnerability to relapse can persist even after decades of abstinence. This suggests that the various drug-induced neuroadaptations that underlie drug-craving and relapse are equally persistent. Thus, an important focus of addiction research recently has been to investigate the mechanisms by which these maladaptive neuroadaptations are maintained over such long periods of time, even after drug exposure has ceased.

Recent evidence has highlighted the important role of changes in gene expression in mediating the brain changes induced by repeated drug exposure (Madsen, Brown, & Lawrence, 2012; Mews, Walker, & Nestler, 2019; Robison & Nestler, 2011). Gene expression is the

process by which the genetic information encoded on a sequence of DNA is synthesised into a functional cellular product, such as a protein. The first step of this process, termed transcription, is where a particular segment of DNA is copied into mRNA by the enzyme, RNA polymerase. In the next phase, termed translation, the messenger RNA is used as the blueprint to form an amino-acid chain that can be later folded into a functional protein.

One mechanism by which repeated drug exposure can influence gene expression is by increasing or decreasing the activity or number of various transcription factors (Madsen et al., 2012; Mews et al., 2019; Robison & Nestler, 2011). Transcription factors are specialised proteins or complexes of multiple proteins, that function to regulate the transcription process. They bind to target sequences of DNA and increase or repress the transcription of the encoded gene by respectively promoting or blocking RNA polymerase activity. Transcription factors are one of the mechanisms that ensure that genes are expressed at the right time, in the right amount, and in the right cell.

While several transcription factors have been implicated in drug addiction (see Mews et al., 2019; Nestler, 2012; Robison & Nestler, 2011), Δ FosB is one such transcription factor that, for the reasons discussed below, may play a particularly important role in the development and maintenance of drug addiction. Like other Fos family proteins, Δ FosB dimerises with Jun family proteins to form the active transcription factor complex, activator protein-1 (AP-1), which binds to AP-1 sites on the promoter regions of specific genes to regulate their transcription. All Fos family proteins are induced very rapidly in the brain in response to a wide variety of environmental stimuli such as drug exposure or stress (Mews et al., 2019; Nestler, 2008; Robison & Nestler, 2011). The induction of these proteins is typically transient, however, with protein levels returning to baseline within a few hours. Δ FosB is unique compared to other Fos family proteins, and indeed most other transcription factors, in that certain isoforms of the protein can persist in the brain for several weeks or even months due to their particularly high stability (Alibhai, Green, Potashkin, & Nestler, 2007; Carle et al., 2007; Chen, Kelz, Hope, Nakabeppu, & Nestler, 1997; Ulery, Rudenko, & Nestler, 2006). This allows Δ FosB (the stable isoforms of) to accumulate in very high levels following repeated drug exposure and to remain at these elevated levels for long periods of time after drug exposure has ceased. It has therefore been suggested that Δ FosB-mediated transcription is an important mechanism by which repeated drug exposure can produce persistent brain changes that underlie certain aspects of addiction (McClung et al., 2004; Nestler, 2008; Nestler, Kelz, & Chen, 1999).

Chronic exposure to various experimenter- (Brenhouse & Stellar, 2006; Conversi, Bonito-Oliva, Orsini, Colelli, & Cabib, 2007; De Pauli et al., 2014; Hope et al., 1994; Kaplan, Leite-Morris, Fan, Young, & Guy, 2011; Kaste, Kivinummi, Piepponen, Kiianmaa, & Ahtee, 2009; Y. Kim et al., 2009; Marttila, Raattamaa, & Ahtee, 2006; McDaid, Graham, & Napier, 2006; Moratalla, Elibol, Vallejo, & Graybiel, 1996; Muller, Unterwald, & Fujimoto, 2005; Nye, Hope, Kelz, Iadarola, & Nestler, 1995; Perrotti et al., 2005, 2008) or self-administered (Cornish, Hunt, Robins, & McGregor, 2012; Krasnova et al., 2013; Larson et al., 2010; Perrotti et al., 2005, 2008; Pich et al., 1997; Winstanley et al., 2007) drugs of abuse have been shown to produce pronounced increases in Δ FosB in various brain regions, as measured by Western blot or IHC. The most pronounced increases induced by repeated drug exposure are typically observed within D₁-type MSN of the NAcc and other parts of the striatum, brain regions that play crucial roles in mediating reinforcement. Several studies have also found increased Δ FosB following repeated drug exposure in numerous other brain regions implicated in addiction, including the PFC and amygdala.

The behavioural effects of increased Δ FosB within some of these brain regions has been demonstrated by studies that have manipulated Δ FosB expression using viral-mediated gene transfer or bitransgenic animals. Overexpression of Δ FosB in the NAcc and surrounding striatal areas increased both cocaine- (Kelz et al., 1999) and morphine- (Zachariou et al., 2006) produced CPP as well as cocaine-produced locomotor activity (Grueter, Robison, Neve, Nestler, & Malenka, 2013; Kelz et al., 1999). In contrast, local overexpression of Δ c-Jun, which antagonises Δ FosB-mediated transcription, reduced cocaine CPP (Peakman et al., 2003). Following repeated morphine exposure, overexpression of Δ FosB in the striatum also enhanced the development of physical dependence and withdrawal symptoms (Zachariou et al., 2006). Importantly, striatal overexpression of Δ FosB also facilitated the acquisition of cocaine self-administration and increased responding maintained by cocaine under PR schedules (Colby, Whisler, Steffen, Nestler, & Self, 2003). In the OFC, overexpression of Δ FosB mimicked the effect of repeated cocaine exposure on rodent tests of attention and decision making while overexpression of Δ JunD, which also antagonises Δ FosB-mediated transcription, prevented the effect of repeated cocaine exposure on these tests (Winstanley et al., 2007). Overexpression of Δ FosB in the OFC also sensitised the locomotor activating effects of cocaine (Winstanley et al., 2009). These findings indicate that Δ FosB expression alone can result in the development of several relevant behavioural phenotypes that are also consequences of repeated drug exposure (Nestler, 2008; Robison & Nestler, 2011).

Progress has been made in identifying the specific transcriptional mechanisms by which Δ FosB can induce long-term brain changes that lead to the development of these phenotypes. Δ FosB has been shown to mediate the transcription of several genes/proteins that regulate structural plasticity and dendritic spine formation of striatal MSN. These include activity-regulated cytoskeleton-associated protein (ARC), cyclin-dependent kinase 5 (CDK5), p35, nuclear factor-kappaB (NF- κ B), synaptotagmin, microtubule-associated proteins, actin-related proteins, and kinesin (Bibb et al., 2001; Maze et al., 2010; Nestler, 2008, 2012; Robison & Nestler, 2011; Winstanley et al., 2009). Indeed, overexpression of Δ FosB in the striatum has been shown to increase the number and density of dendritic spines in D₁-type MSN, and this was associated with sensitised behavioural responses to cocaine (Grueter et al., 2013). Δ FosB also regulates the transcription of several genes/proteins involved in glutamatergic synaptic function and plasticity including the α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor subunit, GluR2, as well as CDK5, p35, and Ca²⁺/calmodulin-dependent protein kinase II (CaMKII; Bibb et al., 2001; Kelz et al., 1999; Nestler, 2012). These genes have been implicated in the rewarding effects of psychostimulants (Kauer & Malenka, 2007; Kelz et al., 1999; T. E. Robinson & Kolb, 2004) and were upregulated by Δ FosB overexpression or repeated cocaine exposure, an effect that was blocked by Δ c-Jun overexpression (Bibb et al., 2001; Kelz et al., 1999; McClung & Nestler, 2003; Peakman et al., 2003). Δ FosB also mediates the expression of dynorphin, an opioid peptide, which regulates DAergic output from the VTA (Bruchas, Land, & Chavkin, 2010; Zachariou et al., 2006). Enhanced morphine dependence and withdrawal symptoms induced by Δ FosB overexpression were shown to be mediated by dynorphin (Zachariou et al., 2006). Finally, Δ FosB also regulates the expression of several other transcription factors, including NF- κ B, which has been implicated in dendrite formation as well as the neurotoxic effects of methamphetamine (Ang et al., 2008; Shah, Silverstein, Singh, & Kumar, 2012), and c-Fos, another Fos family protein (Renthal et al., 2008; Robison & Nestler, 2011).

It is clear that Δ FosB mediates an array of neural mechanisms that are known to be to be relevant to drug-taking and drug-seeking behaviour. The accumulation of Δ FosB in the brain has, therefore, provided a useful marker to identify brain regions that have undergone relevant neuroadaptive change following repeated drug exposure (Nestler, 2008; Robison & Nestler, 2011). The effect of repeated MDMA exposure on Δ FosB expression has not yet been determined, however. The first aim of the current research was, therefore, to map the accumulation of Δ FosB across the brain following chronic MDMA self-administration in rats.

Two procedures are typically used to measure Δ FosB in the brain; Western blot (immunoblotting) and IHC. For the purpose of mapping the accumulation of Δ FosB across the brain, IHC offers much greater spatial resolution, as Δ FosB can be measured in as many discrete brain regions on a tissue slice as needed. IHC involves the detection of target antigens (proteins) by labelling them with specific antibodies (Ramos-Vara, 2005). Visualisation of the antibody-antigen interaction can be achieved by labelling the antibody with an enzyme that can catalyse a colour producing reaction, or with a fluorescent fluorophore. Each coloured or fluorescent cell can then be counted under a microscope. Often, an un-labelled primary antibody is used to which one or more secondary antibodies are subsequently bound that can host larger labelling complexes in order to increase the intensity of the staining/fluorescence (Figure 1.2).

Figure 1.2. Basic principle of immunohistochemistry. The target antigen/protein is labelled with a specific primary antibody, to which a secondary antibody can be also be bound to improve detection. The secondary antibody is labelled with an enzyme that can catalyse a colour producing reaction or with a fluorescent fluorophore, which can then be viewed under a microscope.

Microdialysis and LC-MS

Mapping the expression of Δ FosB throughout the brain following repeated drug exposure can identify brain regions that have undergone neuroadaptive change. However, this procedure cannot provide any information on the specific drug-produced neurochemical adaptations that may take place within these brain regions. A number of pre-clinical procedures have been developed to quantify these neurochemical changes. Microdialysis is one such technique that allows for the collection of samples from extracellular fluid *in vivo* (Chefer, Thompson, Zapata, & Shippenberg, 2009; Ungerstedt & Pycock, 1974; Westerink & Cremers, 2007). A probe with a semipermeable membrane is inserted into the brain area of interest and perfused with artificial cerebrospinal fluid (aCSF; Figure 1.3A). Neurotransmitters, metabolites, and other small molecules diffuse across the membrane down their concentration gradient and into the perfusate (Figure 1.3B). The dialysate is then collected and can be subsequently analysed by various means (Figure 1.3C).

Microdialysis has the advantage of providing some temporal information since samples can be collected every ~5-30 min. Microdialysis is also advantageous compared to some other neurochemical assays in that only the extracellular fluid is sampled, and it is the extracellular concentration of neurotransmitters or metabolites that are of interest for neurotransmission. Importantly, microdialysis also permits neurochemical samples to be collected from awake, freely moving subjects, allowing for the concurrent collection of behavioural data. When coupled with appropriate behavioural and analytic procedures, microdialysis can provide an effective means of measuring the neurochemical correlates of drug-produced behaviour (Chefer et al., 2009; Davies, 1999; Westerink, 1995; Westerink & Cremers, 2007).

Figure 1.3. Basic principle of microdialysis. A) Perfusate consisting of artificial cerebrospinal fluid (aCSF) is pumped at a constant flow rate through a microdialysis probe implanted in the brain region of interest. B) At the tip of the probe, a semi-permeable membrane allows for the diffusion of molecules present in extracellular fluid into the perfusate. C) The perfusate, now dialysate, continues to flow into a collection vial where the contents can be subsequently analysed.

High-performance liquid chromatography (HPLC) methods are the most common procedures used to analyse dialysate samples collected via *in vivo* microdialysis (Davies, Cooper, Desmond, Lunte, & Lunte, 2000). HPLC involves passing solvents containing the dialysate sample through a separation column under high pressure (Figure 1.4A). Each compound/component of the sample interacts differently with the adsorbent material within the column, which leads to the physical separation of the sample components as they flow through the column over time (Figure 1.4B). Using various chromatographic detection methods, the relative amount of each component of the sample mixture can be determined according to the time at which it emerged from the column (retention time; Figure 1.4C). This produces a chromatogram with peaks representing the relative amounts of the various sample components (Figure 1.4D). To quantify a particular compound/sample component, the height or area of the chromatographic peaks and their retention times are compared to those produced by samples containing known amounts of the compound of interest (i.e. calibration standards).

The most common chromatographic detection methods involve electrochemical, ultra-violet, or fluorescence detection (Swartz, 2010). These detection methods can be limited by the fact that they often need to be specifically tailored for the analysis of a particular compound or class of compound. The right combination of solvents, column specifications, and other parameters must be chosen to ensure that the target compound(s) are adequately separated and detected. Thus, multiple samples may need to be collected, and the analysis may need to be re-run for each compound of interest. The duration of the analysis may also need to be very long in order to ensure that all detectable sample components are adequately separated and do not co-elute, which would prevent quantification

Figure 1.4. Basic principle of high-performance liquid chromatography (HPLC). A) The sample is mixed with various solvents under high pressure into a separation column. B) Each component of the sample mixture interacts differently with the adsorbent material within the column, which leads to the physical separation of the sample components as they flow through the column over time. C) Using various chromatographic detection methods, the relative amount of each component of the sample mixture is measured. D) This produces a chromatogram with peaks representing the relative amount of each sample component according to time at which it emerged from the column (retention time).

Another detection method for HPLC is mass spectrometry (MS; Niessen, 2006). The first step in MS detection involves the ionisation of the sample compounds as they elute from the column. The compounds, once ionised, are then further separated according to their mass-to-charge ratio (m/z). Lastly, the relative abundance of each ion is measured and recorded as a mass spectrum; a two-dimensional visualisation of the relative abundance of each ion according to their m/z (Figure 1.5A). Mass spectrum are typically recorded several times per second, and thus the total data set may include hundreds to thousands of sequential mass spectrum as a function of retention/acquisition time. The total ion chromatogram (TIC) represents the total abundance of all measured ions as a function of retention time (Figure 1.5B), while an extracted ion chromatogram (EIC) represents the abundance of ions of a particular m/z as a function of retention time (Figure 1.5C). To quantify a particular compound, an EIC is generated for the target ion and the peak height or area is compared to calibration standards. A three-dimensional representation of LC-MS data is shown in Figure 1.5D.

HPLC coupled with MS detection (LC-MS) has several advantages over other detection methods. Firstly, imperfections in chromatographic separation, such as multiple compounds having the same retention time, are less of an issue due to the further separation and measurement of compounds based on their m/z (see Figure 1.5D). This improves analytical selectivity, allowing for the quantification of several sample components in a single analysis, and leads to shorter analytic timeframes since adequate chromatographic separation can be achieved in shorter time periods. Secondly, MS detectors generally have much better sensitivity, providing lower detection limits, which allows for the use of smaller samples volumes and/or the analysis of compounds present in lower concentrations. Thirdly, analytical specificity can be increased by using tandem mass spectrometry (MS/MS), which predictably fragments ions into smaller structures in order to provide information that can be used to help determine the identity of a compound. Finally, due to the increased selectivity of LC-MS, the sample preparation and chromatography procedures can be much more inclusive, which allows for metabolomics analysis; an unbiased, global analysis of all small molecules/metabolites within a biological system/sample. This type of analysis is typically not hypothesis-driven, but rather hypothesis-generating, being able to potentially identify unexpected or novel compounds of interest. These advantages of LC-MS have yet to be fully exploited for the study of the neurobiology of drug addiction. The current research, therefore, aimed to develop and employ a novel procedure that exploits the combined advantages of microdialysis and LC-MS in order to study the effects of repeated MDMA exposure.

Figure 1.5. Data obtained by liquid chromatography-mass spectrometry (LC-MS). A) Example mass spectrum at 5.9 min acquisition time showing the relative response/abundance of ions as a function of their mass to charge ratio (m/z). B) Total ion chromatogram (TIC) shows the total response/abundance of all ions as a function of retention/acquisition time. C) Example extracted ion chromatogram (EIC) showing the response/abundance of 466.16 m/z as a function of retention/acquisition time. D) Three-dimensional representation of LC-MS data showing the TIC as well as an example mass spectrum and EIC.

Aims and objectives

MDMA/ecstasy is a popular recreational drug, and some users exhibit symptoms characteristic of a SUD. Despite this, the behavioural and neurobiological effects of repeated MDMA exposure are less well understood than other drugs of abuse. The purpose of the current research was to investigate the neuroadaptations that develop with repeated MDMA exposure in laboratory rats.

First, the brain regions that undergo significant neuroadaptive change following chronic MDMA self-administration were identified by measuring the accumulation of Δ FosB across multiple brain regions relevant to addiction using IHC. It was expected that MDMA self-administration would result in a significant accumulation of Δ FosB in the NAcc, dorsal striatum, amygdala, and PFC, as has been shown with other drugs of abuse.

Second, the behavioural relevance of the region-specific differences in Δ FosB accumulation was determined by examining the effect of locally infused MDMA into discrete brain regions on locomotor activity following a sensitising regimen of repeated MDMA exposure. It was predicted that behavioural sensitisation would be expressed when MDMA was infused into the same brain regions that also showed enhanced Δ FosB accumulation following repeated MDMA exposure.

Third, a targeted investigation of the more specific neurochemical changes within the identified brain regions of interest was carried out. A microdialysis and LCMS based procedure for the collection and quantification of dialysate samples was developed and validated. This procedure was then employed to determine the effect of a sensitising regimen of repeated MDMA exposure on MDMA-produced changes in dialysate concentrations of targeted neurochemicals, notably DA and 5-HT. It was predicted that MDMA-produced increases in dialysate concentrations of DA would be enhanced following a sensitising regimen of repeated exposure, while dialysate concentrations of 5-HT would be decreased.

Finally, a novel, untargeted metabolomics procedure that incorporated time-series metabolomics data as well as behavioural data was developed and validated. This procedure was then used to analyse microdialysis samples and examine the effect of a sensitising regimen of MDMA exposure on the wider metabolome in attempt to identify other neurochemicals of interest that are relevant to MDMA-produced behavioural sensitisation.

CHAPTER 2: Δ FOSB AND IMMUNOHISTOCHEMISTRY

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Introduction

There is evidence to suggest that certain transcription factors play an important role in maintaining drug-induced neuroadaptations over long periods of time (Mews et al., 2019; Nestler, 2012; Robison & Nestler, 2011). Δ FosB is one such transcription factor that has been shown to accumulate in very high-levels within various brain regions relevant to addiction following repeated exposure to drugs of abuse (Nestler, 2008; Perrotti et al., 2008). The effect of repeated MDMA exposure on Δ FosB has not yet been investigated, however, and was, therefore, the focus of the current research.

The effect of repeated experimenter-administered MDMA on c-Fos, another transcription factor, has been previously mapped across a wide range of brain areas using IHC (Colussi-Mas & Schenk, 2008). Induction of c-Fos in only a small sample of the brain regions was correlated with sensitised MDMA-produced behaviour, however. Further, the expression of c-Fos, like most transcription factors, is transient, lasting only a few hours. In contrast, Δ FosB (certain isoforms) has been demonstrated to be particularly stable, remaining at elevated levels for up to several weeks or months following repeated drug exposure (Alibhai et al., 2007; Carle et al., 2007; Chen et al., 1997; Ulery et al., 2006). It is for this reason that Δ FosB-mediated transcription has been suggested to be a particularly important mechanism by which repeated drug exposure might produce persistent brain changes that underlie certain aspects of addiction (McClung et al., 2004; Nestler, 2008; Nestler et al., 1999).

The effect of repeated drug exposure on brain Δ FosB is typically examined following repeated experimenter-administered drug. While some studies have measured brain Δ FosB following self-administered drugs, few have done so under the long-term and long-access conditions that have been shown to result in an escalation of drug intake and extensive drug-seeking behaviour, two measures that have been suggested to be particularly relevant to the study of SUDs (Ahmed, 2011; Edwards & Koob, 2013). Even fewer studies have mapped the accumulation of Δ FosB across numerous brain regions following repeated drug exposure under these conditions. Given these gaps in the literature, the current research aimed to map the accumulation of Δ FosB across several brain regions including the PFC, dorsal striatum, ventral

striatum, amygdala, VTA, and raphe, following an extensive history of long-access MDMA self-administration.

Methods

Subjects

Male Sprague-Dawley rats weighing 300 – 400 g were used. Rats were bred in the vivarium at Victoria University of Wellington and housed 2-3 per cage until they weighed 250 – 300 g. Thereafter, rats were housed individually. The housing room was kept at a constant temperature (21°C) and humidity (55%). A 12-hour light/dark cycle (lights on at 0700 hours) was in effect. Food and water were available *ad libitum* except during testing. All experimental protocols were approved by the Animal Ethics Committee of Victoria University of Wellington.

Surgical procedures

Deep anaesthesia was produced by an injection of a solution combining ketamine (90.0 mg/kg, intraperitoneal [i.p.], Phoenix Pharm, Auckland, New Zealand) and xylazine (9.0 mg/kg, i.p., Phoenix Pharm, Auckland, New Zealand). The anti-inflammatory analgesic, carprofen (5 mg/kg, subcutaneous [s.c.], Zoetis, Auckland, New Zealand) was then administered, and Lacrilube® was applied to both eyes to prevent corneal desiccation. The external jugular vein was isolated and tied off at the anterior end using sterile thread. A Silastic® catheter was then inserted, advanced towards the atrium, and secured in place with sterile thread. The distal end of the catheter, which was fitted with a 2 cm, sealable piece of 22-gauge stainless steel tubing, was routed subcutaneously to an exposed part of the skull and fixed in place using four small screws embedded in dental acrylic. Following surgery, Hartmann's solution (2 × 5 mL, s.c.) was administered to restore electrolyte balance, and carprofen (5.0 mg/kg, s.c.) was administered once daily for two days.

Apparatus

Self-administration testing was conducted in operant chambers equipped with two levers (Med Associates Inc., USA; model ENV-001) set within sound-attenuating boxes. A 20 mL syringe housed in a mechanical pump (Med Associates Inc., USA; model – PHM-100A) was connected through a swivel and tether apparatus to the i.v. catheter. Responses on the active lever resulted in a 0.1 mL infusion of drug delivered over 12 seconds and the concurrent illumination of a light located above the lever. Responses on the inactive lever were recorded but produced no programmed consequence. Experiments were conducted within a temperature-

controlled (19 – 21°C) darkroom. Drug delivery and data acquisition were controlled by the Med PC software (Med Associates Inc.).

Self-administration

For self-administration, $\pm$ MDMA HCl (BDG synthesis, New Zealand) was dissolved in a sterile 0.9% NaCl (saline) solution containing 3 IU/mL heparin. Daily 6-hour MDMA self-administration sessions were conducted six days per week. Prior to each self-administration session, catheters were flushed with 0.2 mL of sterile 0.9% NaCl solution containing heparin (30 IU/mL) and penicillin (250 000 IU/mL) to prevent blood coagulation and infection. Immediately after each self-administration session, catheters were flushed again with the heparin-penicillin solution. Every seventh day, catheter patency was confirmed by the immediate loss of the righting reflex following the administration of sodium pentobarbital (25 mg/kg, i.v.).

Each self-administration session commenced with an experimenter-administered infusion to clear the catheter of the penicillin-heparin solution. Thereafter, infusions of 1 mg/kg MDMA were delivered according to an FR-1 schedule of reinforcement until a total intake of 90 mg/kg had been self-administered (9 – 14 days). The MDMA infusion dose was then decreased to 0.5 mg/kg until a total MDMA intake of 350 mg/kg was reached (20 – 26 days). Thereafter, an additional nine daily self-administration sessions were conducted. Total MDMA intake ranged from 515 – 692 mg/kg over a 29 – 35-day period ($n = 4$). Control rats ($n = 4$) were handled in the same way and placed into the self-administration chambers for a number of days matched to each self-administration rat.

FosB-like immunohistochemistry

Twenty-four hours following the final self-administration session, rats were deeply anesthetised with sodium pentobarbital (100 mg/kg, i.p.) and transcardially perfused with 150 mL of 0.9% NaCl solution containing heparin (5 IU/mL) followed by 350 mL of 4% paraformaldehyde (PFA) solution in 0.1 M potassium phosphate buffer (PB; pH 7.4). Perfusions were conducted using a perfusion pump (EYLA micro tube pump MP-3, Tokyo Rikakikai Co. Ltd, Tokyo, Japan) at a flow rate of ~14 mL/min. Brains were then rapidly removed and placed in 4% PFA fixative overnight before being placed in a cryoprotective PB solution containing 0.9% NaCl (phosphate-buffered saline [PBS]) and 30% saccharose for 2-3 days. Brains were then frozen in isopentane at -30°C, which was gradually cooled to -50°C over 4 min, before being stored at -80°C until sectioning. Coronal sections (30 μ m) were cut

along the entire brain using a cryostat (CM3050S, Leica Biosystems), which were then immersed in PBS containing 0.1% sodium azide and stored at 4°C.

A one in six series of sections was processed to reveal neurons expressing FosB/ Δ FosB. Free-floating sections were washed three times in PBS (for 5 min each) containing 0.3% Triton X-100 (PBST) before being incubated for 10 min with 3% H₂O₂ and washed three times in PBST again. Sections were then incubated for 60 min with PBST containing 1% bovine serum albumin, washed three times in PBST, and then incubated overnight with the primary rabbit antibody (Fos B 102, sc-48, Santa Cruz) diluted 1/2000 in PBST. This antibody recognises both FosB and all isoforms of Δ FosB, but not other Fos family proteins (Perrotti et al., 2005, 2004, 2008). After three washes in PBST, sections were incubated for 120 min with the secondary, biotinylated goat anti-rabbit antibody (BA-1000, Vector Laboratories) diluted 1/1000 in PBST. After another three washes in PBST, sections were then incubated for 60 min with preformed avidin-biotin complex (Vectastain Elite Kit, PK-6200; Vector Laboratories) diluted 1/1000 in PBST and washed three times in PBST again. Visualisation of bound peroxidase was achieved by reaction in a solution of 50 mM Tris-HCl buffer containing 0.02% diaminobenzidine, 0.8% NiCl₂, and 0.003% H₂O₂, to yield a blue-black precipitate. The reaction was stopped by two washes in PBST after ~10 min. Sections were then mounted on gelatin-coated slides, stained with 1% neutral red, and cover-slipped with DPX mounting medium. A control was run without the primary antibody to ensure the absence of nonspecific immunostaining in the tissue.

Sections were analysed using an Olympus BX-51 light microscope, Lumenera Lt665R camera, and MBF Neurolucida® software. Coded slides were used to blind the observer to the group treatment, with the code only broken at the completion of the experiment. For each brain region, bilateral templates (unilateral for the raphe) were made according to the approximate shape and size of the region (Figure 2.1). All cells within the templates showing positive FosB-like staining were manually counted using Neurolucida from four coronal sections per region (three for the VTA), per rat, each 180 μ m apart. Data were averaged across hemispheres and sequential coronal sections for each rat. Final data were expressed as the mean number of FosB/ Δ FosB labelled cells/mm² in each brain region for both the control and MDMA self-administration conditions.

Figure 2.1. Schematic representation (adapted from Paxinos & Watson, 2005) of the regional templates used for quantification of FosB/ Δ FosB immunoreactivity. For clarity reasons, each template is represented only at one bregma level (displayed in mm below each section) on one hemisphere. OFC, orbitofrontal cortex; Cg1, Anterior cingulate; PrL, prelimbic cortex; IL, infralimbic cortex; DL, dorsolateral caudate-putamen; DM, dorsomedial caudate-putamen; VL, ventrolateral caudate-putamen; VM, ventromedial caudate-putamen; core, nucleus accumbens core; shell, nucleus accumbens shell; BLA, basolateral amygdala, CA, central nucleus of the amygdala; aVTA, anterior ventral tegmental area; pVTA, posterior tail of the ventral tegmental area; dorsal raphe; median raphe.

Statistical analysis

A one-way repeated-measures analysis of variance (ANOVA) was used to analyse the change in mean MDMA-intake (mg/kg) during the first 29-days of self-administration. Independent samples *t*-tests were used to compare the densities of FosB/ Δ FosB labelled cells in tissue from each brain region from the control and MDMA self-administration groups. Because there were 14 comparisons made here, the *p*-values from each *t*-test were adjusted using the Hochberg-Bonferroni correction in order to control the false discovery rate (Hochberg, 1988). The Hochberg-Bonferroni method is more powerful than the standard Bonferroni procedure and is, thus, a more appropriate correction to use when a larger number of means are being tested. The level of significance for all tests was $p < .05$. All analyses were conducted using the Statistical Package for Social Sciences (SPSS, v25, IBM).

Results

Figure 2.2 displays the mean total MDMA intake (mg/kg) over 29 daily sessions of 6-hour self-administration. MDMA intake changed significantly as a function of session, $F(28, 84) = 11.14$, $p < .001$, $\eta_p^2 = .788$, and a significant linear contrast indicated an escalation of MDMA intake over the 29-day period, $F(1, 3) = 76.47$, $p = .003$, $\eta_p^2 = .962$. Inactive lever responding and responding produced by control rats remained low as previously shown (Schenk et al., 2012), with preference for the active lever averaging above 80%.

Figure 2.2. Mean MDMA intake (mg/kg) as a function of daily 6-hour self-administration session ($n = 4$). Error bars represent standard error of the mean.

Figures 2.3 – 2.8 shows FosB/ Δ FosB immunoreactivity in various brain regions/subregions as a function of self-administration condition. For each figure, (A) displays conventional light microscopy images of coronal sections of rat brain with the regional templates used for quantification overlaid. (B) Displays higher magnification images of the shaded area presented in A from representative rats in the control and MDMA self-administration conditions. (C) Displays the mean densities of FosB/ Δ FosB labelled cells as a function of region and self-administration condition. A summary of the statistical analyses is shown in Table 2.1.

In the ventral striatum/NAcc, MDMA self-administration significantly increased densities of FosB/ Δ FosB labelled cells in the core, but differences in the shell were not significant after correction for multiple comparisons (Figure 2.3; Table 2.1). In the dorsal striatum/C-P (Figure 2.4; Table 2.1), MDMA self-administration significantly increased densities of FosB/ Δ FosB labelled cells in the dorsomedial and ventromedial regions, but not within the dorsolateral or ventrolateral regions. In the PFC (Figure 2.5; Table 2.1) and amygdala (Figure 2.6; Table 2.1), MDMA self-administration significantly increased densities of FosB/ Δ FosB labelled cells within all subregions. In the raphe, (Figure 2.7; Table 2.1), no FosB/ Δ FosB labelled cells were detected in the median raphe for either group while significantly greater densities of FosB/ Δ FosB labelled cells were found in the dorsal raphe of MDMA self-administering rats. In the VTA (Figure 2.8; Table 2.1), no FosB/ Δ FosB labelled cells were detected in the anterior region for either group while greater densities of FosB/ Δ FosB labelled cells were observed in the posterior tail of the VTA of MDMA self-administering rats, but this difference was not significant after correction for multiple comparisons.

Figure 2.3. Effect of MDMA self-administration on FosB/ Δ FosB immunoreactivity in the ventral striatum/nucleus accumbens (NAcc). (A) Conventional light microscopy image of a coronal section of rat brain with the regional templates used for quantification overlaid. For clarity reasons, each template is represented only at one bregma level (+1.68 mm), on one hemisphere. Scale bar is 1 mm. (B) Higher magnification images of the shaded areas in A displaying FosB/ Δ FosB immunoreactivity in each subregion for representative rats in the control and MDMA self-administration conditions. Scale bar is 100 μ m. (C) Mean FosB/ Δ FosB positive cells / mm² in each subregion for control versus MDMA self-administering rats. Symbols represent individual subject data. Core, NAcc core; shell, NAcc shell. * $p < .05$ compared to control.

Figure 2.4. Effect of MDMA self-administration on FosB/ΔFosB immunoreactivity in the dorsal striatum/caudate-putamen (C-P). (A) Conventional light microscopy image of a coronal section of rat brain with the regional templates used for quantification overlaid. For clarity reasons, each template is represented only at one bregma level (+1.68 mm), on one hemisphere. Scale bar is 1 mm. (B) Higher magnification images of the shaded areas in A displaying FosB/ΔFosB immunoreactivity in each subregion for representative rats in the control and MDMA self-administration conditions. Scale bar is 100 μm. (C) Mean FosB/ΔFosB positive cells / mm² in each subregion for control versus MDMA self-administering rats. Symbols represent individual subject data. DL, dorsolateral C-P; DM, dorsomedial C-P; VL, ventrolateral C-P; VM, ventromedial C-P. * *p* < .05 compared to control.

Figure 2.5. Effect of MDMA self-administration on FosB/ΔFosB immunoreactivity in the prefrontal cortex. (A) Conventional light microscopy image of a coronal section of rat brain with the regional templates used for quantification overlaid. For clarity reasons, each template is represented only at one bregma level (+3.24 mm), on one hemisphere. Scale bar is 1 mm. (B) Higher magnification images of the shaded areas in A displaying FosB/ΔFosB immunoreactivity in each subregion for representative rats in the control and MDMA self-administration conditions. Scale bar is 100 μm. (C) Mean FosB/ΔFosB positive cells / mm² in each subregion for control versus MDMA self-administering rats. Symbols represent individual subject data. OFC, orbitofrontal cortex; Cg1, anterior cingulate; PrL, prelimbic cortex; IL, infralimbic cortex. * $p < .05$ compared to control.

Figure 2.6. Effect of MDMA self-administration on FosB/ Δ FosB immunoreactivity in the amygdala. (A) Conventional light microscopy image of a coronal section of rat brain with the regional templates used for quantification overlaid. For clarity reasons, each template is represented only at one bregma level (-2.16 mm), on one hemisphere. Scale bar is 1 mm. (B) Higher magnification images of the shaded areas in A displaying FosB/ Δ FosB immunoreactivity in each subregion for representative rats in the control and MDMA self-administration conditions. Scale bar is 100 μ m. (C) Mean FosB/ Δ FosB positive cells / mm² in each subregion for control versus MDMA self-administering rats. Symbols represent individual subject data. CA, central nucleus of the amygdala; BLA, basolateral amygdala. * $p < .05$ compared to control.

Figure 2.7. Effect of MDMA self-administration on FosB/ΔFosB immunoreactivity in the dorsal and median raphe. (A) Conventional light microscopy image of a coronal section of rat brain with the regional templates used for quantification overlaid. For clarity reasons, each template is represented only at one bregma level (-7.80 mm). Scale bar is 1 mm. (B) Higher magnification images of the shaded areas in A displaying FosB/ΔFosB immunoreactivity in each subregion for representative rats in the control and MDMA self-administration conditions. Scale bar is 100 μm. (C) Mean FosB/ΔFosB positive cells / mm² in each subregion for control versus MDMA self-administering rats. Symbols represent individual subject data. * $p < .05$ compared to control.

Figure 2.8. Effect of MDMA self-administration on FosB/ Δ FosB immunoreactivity in the ventral tegmental area (VTA). (A) Conventional light microscopy image of a coronal section of rat brain with the regional templates used for quantification overlaid. For clarity reasons, each template is represented only at one bregma level per region (-5.04 or -6.84 mm), on one hemisphere. Scale bar is 1 mm. (B) Higher magnification images of the shaded areas in A displaying FosB/ Δ FosB immunoreactivity in each subregion for representative rats in the control and MDMA self-administration conditions. Scale bar is 100 μ m. (C) Mean FosB/ Δ FosB positive cells / mm² in each subregion for control versus MDMA self-administering rats. Symbols represent individual subject data. aVTA, anterior VTA; pVTA, posterior tail of the VTA.

Table 2.1.

Summary of test statistics

Region / Subregion	<i>t</i> -test, Cohen's <i>d</i>	Corrected <i>p</i> -value [#]
Prefrontal cortex		
Orbitofrontal cortex	$t(6) = 3.85, p = .008, d = 2.72.$	$p = .045^*$
Anterior cingulate	$t(6) = 5.29, p = .002, d = 3.74.$	$p = .019^*$
Prelimbic cortex	$t(6) = 7.43, p < .001, d = 5.25.$	$p = .004^*$
Infralimbic cortex	$t(6) = 7.51, p < .001, d = 5.31.$	$p = .004^*$
Dorsal Striatum		
Dorsolateral caudate-putamen	$t(6) = 1.33, p = .231, d = 0.94.$	$p = .231$
Dorsomedial caudate-putamen	$t(6) = 3.79, p = .009, d = 2.68.$	$p = .045^*$
Ventrolateral caudate-putamen	$t(6) = 1.36, p = .224, d = 0.96.$	$p = .231$
Ventromedial caudate-putamen	$t(6) = 5.64, p = .001, d = 3.99.$	$p = .015^*$
Ventral Striatum		
Nucleus accumbens core	$t(6) = 19.48, p < .001, d = 13.78.$	$p < .001^*$
Nucleus accumbens shell	$t(6) = 3.01, p = .024, d = 2.13.$	$p = .094$
Amygdala		
Central amygdala	$t(6) = 4.07, p = .007, d = 2.88.$	$p = .048^*$
Basolateral amygdala	$t(6) = 4.47, p = .004, d = 3.16.$	$p = .034^*$
Ventral Tegmental Area		
Anterior	N/A	
Posterior	$t(6) = 2.40, p = .038, d = 1.87$	$p = .115$
Raphe		
Dorsal	$t(6) = 5.08, p = .002, d = 0.73$	$p = .020^*$
Median	N/A	

[#] Hochberg-Bonferroni correction (Hochberg, 1988), * $p < .05$.

Discussion

The transcription factor, Δ FosB, accumulates within the striatum and some other brain regions following repeated drug exposure (Brenhouse & Stellar, 2006; Conversi et al., 2007; Cornish et al., 2012; De Pauli et al., 2014; Kaplan et al., 2011; Larson et al., 2010; McDaid et al., 2006; Perrotti et al., 2005, 2008; Pich et al., 1997; Winstanley et al., 2007). Δ FosB has been identified as an important regulator of long-term neuroplasticity within these brain regions and has, therefore, been suggested to be relevant to addiction (Frankowska, Dudek, & Siwek, 2016; Mews et al., 2019; Nestler, 2008). In this study, the impact of chronic MDMA exposure on Δ FosB accumulation in numerous brain areas was demonstrated for the first time.

IHC was used to map the accumulation of FosB/ Δ FosB in 16 different brain regions that have been implicated in various aspects of addiction. This was carried out following a minimum of 29 long-access (6 hour) daily MDMA self-administration sessions that resulted in an escalation of drug intake and that has previously been shown to produce extensive drug-seeking behaviour (Schenk et al., 2016, 2011), two measures that have been suggested to be consistent with the development of drug addiction (Ahmed, 2011; Edwards & Koob, 2013).

Significant region-specific increases in the accumulation of FosB/ Δ FosB as a function of MDMA self-administration were observed. The pattern of FosB/ Δ FosB expression was largely similar to those seen following repeated experimenter- or self-administered exposure to other drugs of abuse. The antibody used in this study labels both FosB and all isoforms of Δ FosB, although it is likely that these results primarily reflect stable isoforms of Δ FosB. This is because these results were collected 24 hours following the final self-administration session and during these 24 hours, all non-stable forms of FosB would have degraded, leaving only Δ FosB, as has been previously demonstrated using double labelling procedures (Perrotti et al., 2005, 2004, 2008).

The most pronounced induction of Δ FosB across all the regions was observed within the striatal complex, particularly within the ventral striatum/NAcc. This was not surprising as virtually all drugs of abuse tested have been shown to induce Δ FosB expression in the NAcc following repeated exposure (Nestler, 2008; Perrotti et al., 2008). In the current study, Δ FosB expression was higher in both the NAcc core and shell following MDMA self-administration, but the difference in the shell was not significant following statistical correction for multiple comparisons.

An important role of accumbal Δ FosB in the reinforcing effects of drugs has been demonstrated by studies that have manipulated Δ FosB expression. Overexpression of Δ FosB

in the NAcc and surrounding striatal areas increased both cocaine- (Kelz et al., 1999) and morphine- (Zachariou et al., 2006) produced CPP. In contrast, local overexpression of a dominant-negative antagonist of Δ FosB-mediated transcription, Δ c-Jun, reduced cocaine CPP (Peakman et al., 2003). Importantly, Δ FosB overexpression also facilitated cocaine self-administration (Colby et al., 2003).

Pronounced increases in Δ FosB expression were also observed in subdivisions of the dorsal striatum/C-P following MDMA self-administration. There is considerable functional heterogeneity between the lateral and medial C-P. The former is crucial for the development of stimulus-response habits while the latter mediates action-outcome or goal-directed behaviour (Everitt & Robbins, 2016; H.-J. Kim, Lee, Yun, & Kim, 2017; Yin & Knowlton, 2006). This is consistent with differences in the anatomical connectivity of the medial and lateral C-P; the lateral portion receives excitatory input from the sensorimotor cortices whereas the medial portion receives excitatory input more so from the medial PFC (Crittenden & Graybiel, 2011; Voorn et al., 2004). Therefore, in the current study, the analysis of Δ FosB accumulation in the C-P was divided into the dorsolateral, dorsomedial, ventrolateral, and ventromedial portions.

Significant increases in Δ FosB accumulation following MDMA self-administration were only found in the two medial portions of the C-P, with the most pronounced increase in the ventromedial portion. A similar pattern of results has also been observed following repeated amphetamine exposure (Conversi et al., 2007) and following the self-administration of methamphetamine (Cornish et al., 2012), cocaine (Pich et al., 1997), and nicotine (Pich et al., 1997). These results suggest that repeated drug exposure results in the accumulation of Δ FosB in regions of the C-P involved in the acquisition and expression of goal-directed actions more so than regions involved in habitual, stimulus-driven instrumental behaviour.

MDMA self-administration produced significant increases in Δ FosB expression in several brain regions outside of the striatum, most notably within the medial PFC. Significant increases in Δ FosB expression were observed in the anterior cingulate, prelimbic, and infralimbic cortices, with the increase being more pronounced in ventral regions. Similar results have been observed across the medial PFC following repeated exposure to amphetamine (Conversi et al., 2007) and morphine (Kaplan et al., 2011), as well as following the self-administration of cocaine (Pich et al., 1997; Winstanley et al., 2007) and nicotine (Pich et al., 1997). Smaller, yet significant increases in Δ FosB expression were also found in the OFC akin

to those found following self-administration of methamphetamine (Cornish et al., 2012) and cocaine (Winstanley et al., 2007).

The PFC is involved in several cognitive and behavioural processes that become disrupted following repeated drug exposure (Goldstein & Volkow, 2011). This has been suggested to play a major role in the development and maintenance of drug addiction (Goldstein & Volkow, 2011). Drug-induced accumulation of Δ FosB within the PFC might contribute to the dysregulated functioning of the PFC since local overexpression of Δ FosB in the OFC mimicked the effect of repeated cocaine exposure on rodent tests of attention and decision making (Winstanley et al., 2007). In contrast, overexpression of Δ JunD, which antagonises Δ FosB-mediated transcription, prevented the effects of repeated cocaine exposure on these tests (Winstanley et al., 2007).

Significant increases in Δ FosB were also observed in both the CeA and BLA following MDMA self-administration. The increase was most pronounced in the BLA. Previous studies have found similar increases in Δ FosB expression following methamphetamine self-administration (Cornish et al., 2012), and following repeated exposure to morphine, cocaine, and nicotine (Kaplan et al., 2011; Perrotti et al., 2008).

The amygdala plays crucial roles in several aspects of addiction; the BLA is particularly critical for conditioned-reinforcement and cue-induced drug-seeking while the CeA is particularly important for stress-induced drug-seeking and emotional regulation (Everitt & Robbins, 2016; Koob & Volkow, 2016). Like other drugs of abuse, extinguished MDMA-seeking behaviour can be reinstated by a drug prime (Schenk et al., 2011), a stress inducer (Ball et al., 2015), and by the presentation of conditioned cues (Ball et al., 2007; Schenk et al., 2011). Also like other drugs, conditioned cues and conditioned reinforcement play crucial roles in MDMA self-administration (Daniela et al., 2006). Δ FosB-mediated transcription within the CeA and BLA might affect these processes.

Unlike other psychostimulant drugs of abuse that primarily impact the DAergic system, MDMA also dramatically increases synaptic 5-HT. DAergic and 5-HTergic cell bodies originate from the VTA and raphe, respectively, and innervate many of the previously discussed brain regions. For this reason, Δ FosB accumulation in the anterior/posterior VTA and the dorsal/median raphe was also measured.

No Δ FosB labelled cells were detected in the median raphe in either self-administration condition. In the dorsal raphe, MDMA self-administration resulted in small, but significant increases in Δ FosB expression, similar to those produced by chronic methamphetamine self-

administration (Cornish et al., 2012). Although the dorsal raphe is densely populated with 5-HTergic neuron cell bodies, further work would be needed to determine if these are indeed 5-HT neurons expressing Δ FosB. Δ FosB is typically only expressed within GABAergic neurons (Nestler, 2008), which are also present in the dorsal raphe (M. F. Belin et al., 1979, 1983; Charara & Parent, 1998; Nanopoulos, Belin, Maitre, Vincendon, & Pujol, 1982). Given the low density of dorsal raphe Δ FosB detected in the current study, these may be GABAergic raphe neurons expressing Δ FosB.

In the anterior VTA, previous studies have failed to find increases in Δ FosB expression following repeated exposure to various drugs of abuse (McDaid et al., 2006; Perrotti et al., 2005, 2008). It was, therefore, not surprising that the current study also failed to detect Δ FosB within this brain region following MDMA self-administration. In the posterior tail of the VTA, however, MDMA self-administration resulted in increased levels of Δ FosB that were approaching significance. It might be surprising that a stronger effect was not observed in this region since increased FosB-like expression in the posterior tail of the VTA has been previously observed when measured 3 hours following acute MDMA exposure (Kaufling et al., 2010). This may be due to the quantification of several other non-stable FosB-like proteins in addition to Δ FosB, however. In the current study, these non-stable isoforms of FosB would have degraded when the rats were perfused 24 hours following drug exposure, leaving only Δ FosB (Perrotti et al., 2005, 2004, 2008). Exposure to several other psychostimulant drugs has also been shown to induce Δ FosB expression in the posterior VTA (Cornish et al., 2012; Kaufling et al., 2010; Perrotti et al., 2005, 2008). In one study, posterior VTA Δ FosB expression was shown to be selective for GABAergic neurons and due to the DAergic action of the drugs since selective 5-HTergic and NAergic acting agents failed to have an effect (Kaufling et al., 2010). It is likely that the Δ FosB detected in the current study was similarly expressed within GABAergic posterior VTA neurons and due to the DAergic effects of MDMA.

In summary, MDMA self-administration produced an accumulation of Δ FosB in several of the same brain regions as repeated exposure to other drugs of abuse. These brain regions are known to play crucial roles in the development and maintenance of drug addiction. Striatal regions that are involved in goal-directed behaviour (NAcc, DMS) showed a particularly pronounced accumulation of Δ FosB. Brain regions involved in several other important aspects of addiction, such as executive functioning and conditioning also showed increased Δ FosB expression. These regions included the CeA, BLA, and most major

subdivisions of the PFC. It has been suggested that MDMA self-administration progresses as a function of neuroadaptive responses in the same brain regions that mediate the progression of self-administration of other drugs of abuse (Schenk, 2011). The present data are consistent with this idea and also suggest that Δ FosB accumulation in several brain regions that are relevant to addiction is a common consequence of repeated exposure to drugs of abuse.

CHAPTER 3: LOCAL MDMA INFUSION

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Introduction

Repeated intermittent, drug exposure can result in the sensitisation of various drug produced behaviours such as locomotor activity and stereotypy (Pierce & Kalivas, 1997; Post & Rose, 1976; T. E. Robinson, 1984; Stewart & Badiani, 1993). It has been suggested that the development of sensitisation to these behavioural effects of drugs is relevant to addiction since repeated drug exposure under these same conditions can also sensitise the reinforcing effects of drugs, as evidenced by the facilitated acquisition of self-administration, higher breakpoints on PR schedules, and increased CPP (Berridge & Robinson, 2016; Pierce & Kalivas, 1997; T. E. Robinson & Berridge, 1993, 2008; Vanderschuren & Pierce, 2010; Vezina, 2004). Further, the mechanisms underlying the development and expression of these sensitised responses appear to be similar and involve various neuroadaptations in brain regions such as the VTA, NAcc, dorsal striatum, and PFC (Berridge & Robinson, 2016; Pierce & Kalivas, 1997; T. E. Robinson & Berridge, 1993, 2008; Vanderschuren & Pierce, 2010; Vezina, 2004).

Like other psychostimulant drugs, acute administration of MDMA produces dose-dependent increases in locomotor hyperactivity (Brennan & Schenk, 2006; L. H. Gold, Koob, & Geyer, 1988; Spanos & Yamamoto, 1989). Also like other psychostimulant drugs, sensitisation of the locomotor activating as well as the reinforcing effects of MDMA was produced following repeated MDMA exposure (van de Wetering & Schenk, 2017). Sensitisation of the locomotor activating effects of MDMA has also been observed following MDMA self-administration (Schenk & Bradbury, 2015). Relatively little is known about the brain regions involved in the development or expression of these sensitised responses to MDMA, however.

The brain regions in which local MDMA administration result in the development or expression of MDMA behavioural sensitisation has not been previously determined. Indeed, the acute locomotor activating effect of locally administered MDMA has also not been previously determined. These were assessed in the current study. Rats were pretreated with a sensitising regimen of systemic, repeated MDMA exposure and the locomotor activating effect of MDMA administered directly into the dorsomedial (medial C-P), dorsolateral (lateral C-P),

and ventral (NAcc) striatum was determined. This study was guided by the results of the previous chapter where it was shown that repeated MDMA exposure increased Δ FosB accumulation within the ventral and dorsomedial, but not the dorsolateral areas of the striatum. Thus, the current research aimed to determine if these region-specific increases in striatal Δ FosB were relevant to the expression of behavioural sensitisation caused by repeated MDMA exposure.

Previous research has implicated an important role of Δ FosB in the development and expression of sensitised behavioural responses to other drugs of abuse. Enhanced Δ FosB levels in the ventromedial C-P and NAcc was associated with the development of methamphetamine behavioural sensitisation (Conversi et al., 2007; McDaid et al., 2006). Opiate-induced behavioural sensitisation was also accompanied by enhanced Δ FosB levels in the NAcc and C-P (Kaplan et al., 2011) while both cocaine- and nicotine-induced behavioural sensitisation were accompanied by enhanced Δ FosB levels in the NAcc (Brenhouse & Stellar, 2006; Marttila et al., 2006). Compellingly, experimenter-induced overexpression of Δ FosB in the NAcc using transgenic animals also sensitised cocaine-produced locomotor hyperactivity, facilitated the acquisition of cocaine self-administration, increased the motivation to self-administer cocaine on PR schedules, and increased both cocaine- and morphine-produced CPP (Colby et al., 2003; Grueter et al., 2013; Kelz et al., 1999; Zachariou et al., 2006). These findings suggest a causal relationship between striatal Δ FosB and the development of these sensitised responses. Given the role of striatal Δ FosB in the development and expression of behavioural sensitisation to other drugs of abuse, it was predicted that MDMA behavioural sensitisation would be expressed by local administration of MDMA into the same brain regions that also showed enhanced accumulation of Δ FosB following repeated MDMA exposure, as demonstrated in Chapter 2.

Methods

Subjects

Male Sprague-Dawley rats were bred and housed as described in Chapter 2. All experimental protocols were approved by the Animal Ethics Committee of Victoria University of Wellington.

Surgical procedures

Pre- and post-operative surgical procedures were carried out as described in Chapter 2. Rats were stereotactically implanted with stainless-steel, 22-gauge, bi-lateral guide cannula (Plastics One, Roanoke, Va., USA) aimed 1 mm above the NAcc, medial C-P, or lateral C-P.

Targeted stereotaxic coordinates were as follows: NAcc: +2.2 mm anteroposterior (AP), ± 1.5 mm mediolateral (ML), -6.0 mm dorsoventral (DV); medial C-P: +1.8 mm AP, ± 2.0 mm ML, -4.2 mm DV; lateral C-P: 1.5 mm AP, ± 3.5 mm ML, -4.2 mm DV; all coordinates relative to bregma/skull with incisor bar -3.9 mm from interaural line (Paxinos & Watson, 2005). Guide cannulae were lowered into position and secured in place with screws and dental acrylic. Following surgery, 28-gauge dummy cannulae (Plastics One, Roanoke, Va., USA) with a 0.5 mm projection were inserted into the guide cannula to prevent obstruction.

Apparatus

Locomotor activity was measured in clear plexiglass chambers ($42 \times 42 \times 30$ cm; Med Associates Inc., USA; model ENV-515) set in sound-attenuating boxes. Each chamber contained two sets of 16 infrared sensors that were spaced 2.5 cm apart, which produced a lattice of beams that was 1.7 cm above the floor of the chamber. The sequential interruption of three beams (the approximate size of the body of the rat) was recorded as one (horizontal) locomotor activity count. Another series of beams spaced 2.5 cm apart and located 15 cm above the floor of the chamber was used to measure vertical locomotor activity (i.e. rearing behaviour). Any interruption of at least one of these beams was recorded as one rearing count. A white noise generator was used to mask noise and the chambers were washed with VirkonTM S disinfectant after each session. All locomotor experiments were run in a temperature-controlled (21 °C) room illuminated by dim red lighting. Locomotor activity was recorded in 5 min bins. Data acquisition was controlled by Activity Monitor software (Med Associates Inc.).

Drugs

$\pm$ MDMA HCl (BDG synthesis, New Zealand) was dissolved in a sterile 0.9% NaCl solution for i.p. injections, and in aCSF (137 mM NaCl, 2.7 mM KCl, 1.2 mM CaCl₂, 0.5 mM MgCl₂, 8.1 mM Na₂HPO₄, 1.5 mM KH₂PO₄) for intra-striatal microinjections.

Pretreatment and intra-striatal microinjections

During each of 5 daily pretreatment sessions, rats were placed into locomotor activity chambers for 30 min prior to receiving MDMA (0.0 or 10 mg/kg, i.p.) and remained in the locomotor activity chambers for an additional 60 min thereafter. This pretreatment regimen was used because it has previously been shown to sensitise the locomotor activating effects of MDMA and facilitated the acquisition of MDMA self-administration (van de Wetering & Schenk, 2017).

Following two drug-free days, the locomotor activating effect of MDMA administered locally into the NAcc, medial C-P, or lateral C-P was determined. Rats were briefly removed from the chambers and loosely restrained while the dummy cannula was removed, and a 28-gauge stainless-steel bi-lateral infusion cannula (Plastics One, Roanoke, Va., USA) with a 1 mm projection was inserted. Infusion cannulae were connected to 10 μ L Hamilton syringes housed in a mechanical infusion pump (PHD 2000 infusion, Harvard Apparatus). MDMA (200 μ g/ μ L/side; total volume 1 μ L/side) was administered over 2 min with infusion cannulae remaining in place for 1 additional minute. Locomotor activity was recorded in 5 min bins for 30 min prior to and 60 min following the MDMA infusion. This dose was chosen based on the results of pilot studies that showed moderate levels of locomotor activity following this dose of MDMA when injected into the striatum (Figure 3.1).

At the completion of the experiment, animals were euthanised by CO₂ asphyxiation. Brains were rapidly removed and placed in 4% PFA fixative overnight before being frozen at -80°C until sectioning. Coronal sections (100 μ m) were cut along the striatum using a sliding microtome (HM-450 with KS-34 freezing unit, Thermo Scientific). Sections were then mounted on gelatin-coated slides, stained with 1% neutral red, and cover-slipped with DPX mounting medium. Light microscopy was used to verify the coordinates of the infusion cannula (Figure 3.2). Data from rats with placements outside of the target area were excluded from any analysis (see Figure 3.3 for final sample sizes).

Figure 3.1. Pilot study results showing mean (+ standard error of the mean) locomotor activity as a function of time and bilateral drug administration into the central striatum. Amphetamine (AMPH; 20 μ g/1 μ L/side), MDMA (200 μ g/1 μ L/side), or vehicle (artificial cerebrospinal fluid [aCSF]) was administered at 60 min. $n = 3$ per group.

Figure 3.2. Schematic representation (adapted from Paxinos & Watson, 2005) of the location of the tip of the cannula for local MDMA injections (200 µg/1 µL/side) into the nucleus accumbens (NAcc; ○, ●), medial caudate-putamen (C-P; △, ▲), and lateral C-P (□, ■,) following pretreatment with 0.0 (○, △, □) or 10.0 (●, ▲, ■) mg/kg i.p. MDMA. Numbers indicate distance in mm along the anteroposterior plane from bregma.

Statistical analysis

Separate 2 (pretreatment: MDMA 0.0 vs 10.0) × 18 (time: -30 – 60 min) mixed-measures ANOVAs were conducted to analyse locomotor activity and rearing produced by microinjections of MDMA into each brain region. Sphericity could not be assumed for these tests (and follow-up tests), and therefore, Greenhouse-Geisser corrections to degrees of freedom were applied (Greenhouse & Geisser, 1959). Significant interactions were followed up with simple main effect analyses. *A priori* pairwise comparisons (simple when following significant simple main effects) were conducted to compare MDMA-produced locomotor activity and rearing at each time point to the final time point of the 30 min habituation period (i.e. -5 min time-period). Separate independent samples *t*-tests were used to analyse the total locomotor activity and rearing produced during the 60 min following microinjections of MDMA into each brain region. The level of significance for all tests was $p < .05$. All analyses were conducted using SPSS (v25, IBM).

Results

Figure 3.3 and 3.4 displays mean locomotor activity and rearing, respectively, as a function of MDMA pretreatment during the 30 min prior to and 60 min following microinjections of MDMA into the NAcc, medial C-P, and lateral C-P. The inserts in each graph display the mean 60 min total of locomotor activity or rearing produced following MDMA microinjections. Both behavioural measures showed similar effects across brain regions and treatment groups. The pre-injection activity profile was mostly comparable for all groups; initial activity counts were high, and activity decreased during over 30 min period. Following MDMA administration, markedly different activity profiles were observed as a function of both pretreatment and brain region.

Microinjections of MDMA into the NAcc produced locomotor hyperactivity for both pretreatment groups, but greater hyperactivity was observed in the MDMA 10.0 group. There was a significant two-way interaction (pretreatment $\times$ time), $F(2.42, 33.77) = 4.542, p = .013, \eta_p^2 = .245$. Locomotor activity changed significantly as a function of time for both the MDMA 0.0, $F(2.43, 14.58) = 8.68, p = .002, \eta_p^2 = .591$ and MDMA 10.0, $F(2.12, 16.95) = 19.83, p < .001, \eta_p^2 = .713$ pretreatment groups. MDMA-produced locomotor hyperactivity was both of greater magnitude and longer duration for the MDMA 10.0 group (see Figure 3.3, $p < .05$). Total MDMA-produced locomotor activity was also significantly greater in the MDMA 10.0 group, $t(14) = 3.00, p = .009, d = 1.58$.

Microinjections of MDMA into the medial C-P only produced locomotor hyperactivity in rats pretreated with MDMA 10.0. There was a significant two-way interaction (pretreatment $\times$ time), $F(2.26, 22.56) = 8.96, p = .001, \eta_p^2 = .472$. Locomotor activity changed significantly as a function of time for the MDMA 10.0 group, $F(1.66, 6.67) = 10.14, p = .011, \eta_p^2 = .717$, but not the MDMA 0.0 group ($p > .05$). MDMA-produced locomotor hyperactivity 15 – 30 min following MDMA was significantly greater in the MDMA 10.0 group ($p < .05$). Total MDMA-produced locomotor activity was also significantly greater in the MDMA 10.0 group, $t(10) = 3.01, p = .013, d = 1.62$.

Microinjections of MDMA into the lateral C-P produced minimal locomotor hyperactivity regardless of pretreatment condition. The two-way interaction (pretreatment $\times$ time) was not significant ($p > .05$) and there was no main effect of pretreatment ($p > .05$). There was a significant main effect of time, $F(3.00, 20.97) = 6.25, p = .003, \eta_p^2 = .471$, with significant MDMA-produced locomotor hyperactivity produced 5 and 15 min following

MDMA. There was no significant difference in total MDMA-produced locomotor activity between the MDMA 0.0 and 10.0 groups ($p > .05$).

Microinjections of MDMA into the NAcc produced rearing behaviour in both groups. There was a significant two-way interaction (pretreatment $\times$ time), $F(4.78, 66.87) = 6.70, p < .001, \eta_p^2 = .324$. Rearing changed significantly as a function of time for both the MDMA 0.0, $F(3.50, 20.97) = 7.07, p = .001, \eta_p^2 = .574$ and MDMA 10.0, $F(3.50, 27.92) = 15.52, p < .001, \eta_p^2 = .660$ pretreatment groups. MDMA-produced rearing was both of greater magnitude and longer duration for the MDMA 10.0 group (see Figure 3.4, $p < .05$). Multiple small, but significant differences in baseline rearing activity were also observed (see Figure 3.4, $p < .05$). Total MDMA-produced rearing was significantly greater in the MDMA 10.0 group, $t(14) = 4.05, p = .001, d = 2.16$.

Microinjections of MDMA into the medial C-P only produced rearing in rats pretreated with MDMA 10.0. There was a significant two-way interaction (pretreatment $\times$ time), $F(3.39, 33.92) = 5.61, p = .002, \eta_p^2 = .360$. Locomotor activity changed significantly as a function of time for the MDMA 10.0 group, $F(1.96, 7.83) = 6.27, p = .024, \eta_p^2 = .610$, but not the MDMA 0.0 group ($p > .05$). MDMA-produced rearing 20 – 30 min following MDMA was significantly greater in the MDMA 10.0 group ($p < .05$). Differences between the groups in total MDMA-produced rearing behaviour were only approaching significance, however ($p = .060$).

Microinjections of MDMA into the lateral C-P produced minimal rearing regardless of pretreatment condition. The two-way interaction (pretreatment $\times$ time) was not significant ($p > .05$) and there was no main effect of pretreatment ($p > .05$) or time ($p > .05$). There was no significant difference in total MDMA-produced rearing between the MDMA 0.0 and 10.0 groups ($p > .05$).

Figure 3.3. Mean (+ standard error of the mean) locomotor activity produced by local MDMA infusions (200 µg/1 µL/side) into the nucleus accumbens (NAcc; ○, ●), medial caudate-putamen (C-P; △, ▲), and lateral C-P (□, ■) as a function of session time (5 min bins), following pretreatment with 0.0 (○, △, □) or 10.0 (●, ▲, ■) mg/kg i.p. MDMA. MDMA was administered at time = 0 min. Inserts display the mean total MDMA-produced locomotor activity from 0 – 60 min, with symbols representing individual subject data. * $p < .05$ compared to MDMA 0.0. # $p < .05$ compared to -5 min time point for the same group.

Figure 3.4. Mean (+ standard error of the mean) rearing behaviour produced by local MDMA infusions (200 μ g/1 μ L/side) into the nucleus accumbens (NAcc; ○, ●), medial caudate-putamen (C-P; △, ▲), and lateral C-P (□, ■,) as a function of session time (5 min bins), following pretreatment with 0.0 (○, △, □) or 10.0 (●, ▲, ■) mg/kg i.p. MDMA. MDMA was administered at time = 0 min. Inserts display the mean total MDMA-produced locomotor activity from 0 – 60 min, with symbols representing individual subject data. * $p < .05$ compared to MDMA 0.0. # $p < .05$ compared to -5 min time point for the same group.

Discussion

In this study, the effect of a sensitising pretreatment regimen of repeated systemic MDMA exposure on the subsequent locomotor activating effect of MDMA administered directly into the ventral, dorsomedial, and dorsolateral striatum was determined. Both horizontal and vertical (rearing) locomotor activity measures showed similar results; therefore, these findings will be discussed together. Local administration of MDMA into the ventral (NAcc), but not the dorsal striatum (medial C-P or lateral C-P) produced significant locomotor hyperactivity in drug naïve subjects, which is consistent with what has been found with other psychostimulant drugs (Carr & White, 1987; Delfs, Schreiber, & Kelley, 1990; Kelley, Gauthier, & Lang, 1989). In MDMA pretreated subjects, locomotor hyperactivity produced by local administration of MDMA into the NAcc was enhanced, and significant hyperactivity was produced by microinjections of MDMA into the medial, but not lateral, C-P. These results are consistent with those from previous studies that have demonstrated the expression of behavioural sensitisation following the local administration of amphetamine into the NAcc or dorsal striatum (Kolta, Shreve, & Uretsky, 1989; Paulson & Robinson, 1991). These findings suggest that similar brain regions are involved in the acute and sensitised locomotor activating effect of MDMA as other psychostimulant drugs.

The current study did not intend to extensively characterise the various brain regions involved in the expression of MDMA behavioural sensitisation. Rather, a few key brain regions were selected based on the results from Chapter 2 where it was demonstrated that the medial and ventral areas, but not the lateral areas of the striatum showed significant increases in Δ FosB levels following MDMA self-administration. As was predicted, local infusion of MDMA into these same brain regions also resulted in the expression of behavioural sensitisation. These results highlight the behavioural relevance of the region-specific increases in striatal Δ FosB observed following repeated MDMA exposure. Together, these findings suggest that MDMA-produced increases in Δ FosB within these brain regions might be involved in the development and/or expression of sensitised responses to MDMA following repeated exposure, as has been shown with other drugs of abuse (Brenhouse & Stellar, 2006; Colby et al., 2003; Conversi et al., 2007; Grueter et al., 2013; Kaplan et al., 2011; Kelz et al., 1999; Marttila et al., 2006; McDaid et al., 2006; Zachariou et al., 2006).

A handful of previous studies have similarly demonstrated an important role of striatal mechanisms in the development and expression of MDMA behavioural sensitisation. Repeated exposure to MDMA under conditions that produced behavioural sensitisation also increased

the firing rate of dorsal striatal neurons (Ball et al., 2006) and increased dendritic spine density in accumbal MSN (Ball et al., 2009). Both 5-HTergic and DAergic mechanisms within the striatum have also been implicated. Tissue levels of 5-HT in the striatum were decreased, while NAcc DA levels were increased following a sensitising regimen of repeated MDMA exposure (Mayerhofer et al., 2001). MDMA-produced increases in extracellular concentrations of DA in the NAcc were also increased following a sensitising regimen of repeated MDMA exposure (Kalivas et al., 1998). These findings support the results of the current study implicating an important role of these brain regions in the expression of MDMA behavioural sensitisation.

In summary, the current findings extend those of the previous chapter, highlighting the medial striatum (NAcc and DMS) as an important brain region impacted by repeated MDMA exposure and the relevance of this brain region to the expression of sensitised behavioural responses to MDMA.

Introduction

In Chapter 2, the impact of repeated MDMA exposure on the accumulation of the transcription factor, Δ FosB, in numerous brain regions was determined. A key finding was the region-specific differences within the striatum; repeated MDMA exposure produced significant increases in Δ FosB in the medial, but not the lateral regions of the striatum. The behavioural relevance of these findings was examined in Chapter 3; behavioural sensitisation following repeated systemic MDMA exposure was expressed following local infusions of MDMA into the medial, but not lateral striatum. In the current chapter, an investigation of the more specific neurochemical changes that occur within the medial striatum following a sensitising regimen of repeated MDMA exposure was carried out.

MDMA is a potent releaser and reuptake inhibitor of both 5-HT and DA (Battaglia, Brooks, et al., 1988; Baumann, Clark, Franken, et al., 2008; Baumann, Clark, & Rothman, 2008; Berger et al., 1992; Cole & Sumnall, 2003; Fitzgerald & Reid, 1990; Hekmatpanah & Peroutka, 1990; Iravani et al., 2000; Kankaanpää et al., 1998; Kurling et al., 2008; Nair & Gudelsky, 2004; Nash & Brodtkin, 1991; O'Shea et al., 2005; Rudnick & Wall, 1992; Shankaran & Gudelsky, 1999). Considerable evidence suggests that 5-HTergic neurotransmission becomes compromised following substantial exposure to MDMA. For example, decreased 5-HT tissue levels, decreased 5-HT re-uptake sites, and decreased synaptic overflow of 5-HT have all been observed following repeated MDMA exposure (Battaglia, Yeh, et al., 1988; Battaglia et al., 1987; Commins et al., 1987; Do & Schenk, 2013; Mayerhofer et al., 2001; O'Hearn et al., 1988; Reveron et al., 2010; Schenk et al., 2007; Shankaran & Gudelsky, 1999). These 5-HT deficits appear to be dependent on exposure levels, however, as repeated exposure to lesser amounts of MDMA failed to produce these deficits (Banks et al., 2008; Do & Schenk, 2013; Fantegrossi et al., 2004). It is not currently known whether such 5-HTergic deficits develop as a function of repeated exposure to MDMA under conditions that produce behavioural sensitisation.

In contrast, there is some evidence to suggest that repeated, intermittent MDMA exposure can sensitise DAergic neurotransmission, and that this might contribute to the development and expression of MDMA sensitisation. For example, tissue levels of DA were increased (Mayerhofer et al., 2001) and the capacity for MDMA to produce increases in NAcc DA was enhanced following a sensitising regimen of repeated MDMA exposure (Kalivas et

al., 1998). An extensive characterisation of both the DAergic and 5-HTergic correlates of sensitised MDMA-produced behaviour has not been previously carried out, however, and was, therefore, the primary aim of the current study.

This can be achieved using microdialysis and LC-MS. Microdialysis can be used to sample the constituents of extracellular fluid *in vivo*. Samples can be collected relatively frequently and importantly, can also be collected from awake, freely moving animals, allowing for the concurrent collection of behavioural data. With the appropriate sample preparation procedures, LC-MS can be used to detect and quantify numerous compounds of interest contained within microdialysate samples with good sensitivity, good selectivity, and all within in a single analysis, as has been previously demonstrated (Song, Mabrouk, Hershey, & Kennedy, 2012; Wong et al., 2016).

Initial attempts to develop in-house microdialysis and LC-MS procedures for the analysis of DA and 5-HT were previously performed by another lab member that had adapted a sample preparation method using benzoyl chloride (BzCl) derivatisation (Song et al., 2012; Wong et al., 2016). BzCl derivatises most small molecules present in microdialysis samples, reacting with both primary and secondary amines, phenols, and ribose-hydroxyl groups (Figure 4.1; Song et al., 2012; Wong et al., 2016). Derivatisation of dialysates with BzCl has been shown to increase detection sensitivity, decrease degradation, and render compounds more hydrophobic, which can improve reversed-phase chromatography (Song et al., 2012; Wong et al., 2016). This allows for the quantification of many small neurotransmitters, neuromodulators, and metabolites in a single LC-MS analysis, which is ideal for the current purposes (Song et al., 2012; Wong et al., 2016). Although initial testing using this procedure showed some promise, it was clear that many issues with the procedure needed to be addressed and optimised. The process of further developing and refining this procedure as well as the application of the procedure for the investigation of neurochemical changes following a sensitising regimen of repeated MDMA exposure is described in this chapter.

Figure 4.1. Benzoyl chloride derivatisation of serotonin and dopamine.

Methods 1

Subjects

Male Sprague-Dawley rats ($n = 25$) were bred and housed as described in Chapter 2. All experimental protocols were approved by the Animal Ethics Committee of Victoria University of Wellington.

Drugs and reagents

$\pm$ MDMA HCl (BDG synthesis, New Zealand) was dissolved in a sterile 0.9% NaCl solution for i.p. injections. aCSF (137 mM NaCl, 2.7 mM KCl, 1.2 mM CaCl_2 , 0.5 mM MgCl_2 , 8.1 mM Na_2HPO_4 , 1.5 mM KH_2PO_4 , pH 7.4) was used as dialysis perfusate and for standard dilutions. Standard stock solutions were made from DA HCl, 5-HT HCl, 3,4-dihydroxyphenylacetic acid (DOPAC), 5-HIAA, D2 DA HCl, and D2 5-HT HCl dissolved in H_2O at 1 mM and kept at -80°C in 1 mL aliquots. All chemicals used for the preparation of standards were purchased from Sigma Aldrich except for D2 5-HT, which was synthesised in-house. All water used for dialysis and LC-MS was distilled and deionized using a MilliQ system. Acetonitrile was LC-MS grade and purchased from Fisher Scientific. Ammonium formate, formic acid, sodium tetraborate, and BzCl were purchased from Sigma Aldrich.

Surgical procedures

Pre- and post-operative surgical procedures were carried out as described in Chapter 2. Intracerebral guide cannula containing dummy cannula (MAB 4.15.IC, Microbiotech, Sweden) were stereotaxically implanted in the medial striatum at the following coordinates; +1.4 mm AP, -2.0 mm ML, -3.6 mm DV. All coordinates were relative to bregma/skull with incisor bar -3.9 mm from the interaural line (Paxinos & Watson, 2005). Guide cannulae were secured in place with screws and dental acrylic.

Microdialysis

Testing was conducted within the home cage or the same locomotor activity chambers described in Chapter 3. Microdialysis probes (MAB 4.15.2.Cu, Microbiotech, Sweden) with a membrane surface area of 0.24 mm × 2 mm (diameter × length) were inserted into the guide cannula, and aCSF was perfused overnight at a flow rate of 1 µL/min using a microinfusion pump (PHD 2000 infusion, Harvard Apparatus). These probes had a relative recovery of approximately 15%, as determined *in vitro* at 1 µL/min. The following morning (approximately 12 hours later), baseline dialysis samples were collected at 20 min intervals for 1 hour within the home cage, 1 hour in the locomotor activity chambers, then for 2 hours following cumulative doses of MDMA (5.0, 10.0 mg/kg, i.p). A derivatisation procedure adapted from (Song et al., 2012; Wong et al., 2016) was used. To each 20 µL dialysate sample, 1.25 µL of internal standard (50 nM D2 DA/D2 5-HT), 10 µL of borate buffer (sodium tetraborate, 100 nM) and 10 µL of BzCl (diluted to 5% [v/v] in acetonitrile) was added, with mixing between each step. The sample mixture was then vortexed for 2 min and stored at -80 °C until analysed.

At the end of testing, animals were euthanised by CO₂ asphyxiation. Brains were rapidly removed and placed in 4% PFA fixative overnight before being frozen at -80°C until sectioning. Coronal sections (100 µm) were cut along the striatum using a sliding microtome (HM 450 with KS34 freezing unit, Thermo Scientific). Sections were then mounted on gelatin-coated slides, stained with 1% neutral red, and cover-slipped with DPX mounting medium. Light microscopy was used to verify the location of the probe membranes. Data from rats with placements outside of the target area were excluded from any analysis.

LC-MS

Analysis of microdialysate samples was carried out using an Agilent Technologies (Palo Alto) 6530 quadrupole time-of-flight (Q-TOF) LC-MS equipped with a Jet-Stream electrospray ionisation (ESI) source. Derivatised sample (40 µL) was injected by an auto-sampler kept at 4 °C onto a Poroshell 120 SB-Aq 2.7 µm column (2.1 x 100 mm; Agilent

Technologies) held at 36 °C. Eluent A was 99.9% water/0.1% ammonium formate. Eluent B was 99.9% acetonitrile/0.1% formic acid. Liquid chromatography was performed with the following gradient: 15% eluent B for 1 min, 40% at 3 min, 50% at 6 min, 97.5% at 7 min, held at 97.5% until 10 min, and reduced to 15% at 11 min. The flow rate was 0.45 mL/min until 7 min, increased to 0.5 mL/min from 8 – 10 min, and reduced back to 0.45 mL/min at 11 min. A post-run time was set to 3.5 min. Q-TOF ESI-MS parameters were as follows: positive ion mode; gas temperature, 275 °C; gas flow, 8 L/m; nebulizer, 30 psi; capillary voltage, 2750 V; nozzle voltage, 0 V; fragmentor voltage, 130 V; acquisition rate, 2 spectra/s; mass range, m/z 50 – 1100.

Data analysis

An external standard mixture was serially diluted (v/v) from the frozen stock solutions in aCSF at 0, 0.5, 1, 5, and 10 nM for DA/5-HT and 0, 5, 10, 50, and 100 nM for DOPAC/5-HIAA. External standards were prepared daily alongside the collection of each batch of dialysate samples and acted as pseudo quality controls. D2 DA and D2 5-HT were used as internal standards, and a mixture was diluted daily to 50 nM in aCSF and spiked in each standard/dialysate sample to a final concentration of 2.94 nM. Automated peak integration of the targeted analytes was performed by the MassHunter Workstation Quantitative software with visual inspection to ensure proper integration. Analyte concentration was determined by calibration curves made from external standards analysed in triplicate with the peak area response ratios (external standard / internal standard) plotted against the internal standard concentration ratios (expected external standard concentration / expected internal standard concentration) using a weighted ($1/SD^2$) linear model (Almeida, Castel-Branco, & Falcão, 2002; Moosavi & Ghassabian, 2018).

Results 1

Unfortunately, complete data were only obtained from a small proportion of the subjects that were tested. Several issues with the microdialysis equipment prevented the collection of dialysate samples from 9 subjects. These issues included probe/tubing disconnections, blockages, and tangles, which were remedied partway through the study with the development of new sampling equipment and procedures. Unexpected technical difficulties with the LC-MS and other analysis issues also prevented that analysis of samples from another 6 subjects. Thus, complete data were only obtained for 11 of the 25 subjects. Calculated concentrations of DA, 5-HT, DOPAC, and 5-HIAA for a representative subject are shown in Figure 4.2. DA and 5-HT increased dose-dependently following MDMA administration as

expected. In contrast, DOPAC decreased dose-dependently, while concentrations of 5-HIAA did not change, as previously demonstrated (Gough, Imam, Blough, Slikker, & Ali, 2006; Nash, 1990).

Figure 4.2. Calculated concentrations of DA, 5-HT, DOPAC, and 5-HIAA as a function of time/MDMA administration for a representative subject. Microdialysis samples were collected at 20 min intervals. MDMA 5.0 and 10.0 (mg/kg, i.p.) was administered at 120 min and 240 min, respectively.

Of the data that were collected and analysed correctly, it was clear that several other issues also needed to be addressed. Calibration curves for the standards were not acceptable, with low R^2 and poor repeatability. Dialysate concentrations of 5-HT and DA during baseline conditions were also incredibly low, and the peaks were well below the limits of detection (Figure 4.3). As can also be seen in Figure 4.3, a co-eluting peak of similar m/z to DA also prevented the accurate integration of DA.

Figure 4.3. Extracted ion chromatograms of derivatised serotonin (panels A and B) and dopamine (panels C and D) in rat brain microdialysate samples during baseline conditions (panels A and C) and following 10 mg/kg i.p. MDMA (panels B and D).

Method development 1

Chromatography gradient

The first issue that needed to be addressed was the co-eluting peak of similar m/z to DA. This peak was not present in standards or aCSF and was subsequently identified to be a possible contaminant from either the tubing, probes, or filters used during the collection and preparation of dialysate samples. Rather than trying to identify the exact source and eliminate the contaminant, the chromatography gradient was changed to alter the retention time of DA and the containment, while maintaining the adequate retention of the other targets. The new gradient that achieved this was as follows: 30% eluent B for 0.5 min, 97.5% at 9 min, held at 97.5% until 10 min, 50% at 10.5 min, 97.5% at 10.7 min, held at 97.5 until 11.5 min, 30% at 11.7 min. The flow rate was 0.45 mL/min until 10.7 min, increased to 0.5 mL/min from 10.7 – 11.7 min, and reduced back to 0.45 mL/min at 11.7 min.

BzCl concentration

The second issue that needed to be addressed was the low analytical sensitivity for DA and 5-HT. It was discovered that a lower concentration of BzCl used for sample derivatisation (2% instead of 5%) greatly improved the sensitivity for all target analytes, as indicated by a significant increase in the slope of the calibration curve (Figure 4.4). A validation procedure was subsequently carried out to quantify the detection limits and measurement repeatability/precision of the targeted compounds using the current procedures. Calibration standards of each target analyte were prepared and analysed in triplicate once daily over three subsequent days. 8-point calibration curves were made for each target analyte on each day, and the slope, R^2 , limit of detection (LOD), intra-assay relative standard deviation (RSD), and intra-day RSD were calculated.

Figure 4.4. 7-point calibration curves for 5-HT, DA, 5-HIAA, and DOPAC made from external standards analysed in triplicate at 0, 0.1, 0.5, 1, 5, 10, and 50 nM for 5-HT/DA and 0, 1, 5, 10, 50, 100, and 500 nM for 5-HIAA/DOPAC. Standards were derivatised with either 2% (◆) or 5% BzCl (●). The slope, intercept, and R^2 of each linear calibration curve are displayed in the legends.

During this process, it was realised that the internal standards were causing the calibration curves to be non-linear (Moosavi & Ghassabian, 2018; Tan & Awaiye, 2013). Internal standards are compounds that are added to all samples/standards. The data are then calculated as a ratio of the internal standard in order to compensate for analyte losses during any stage of sample preparation, storage, or analysis. A good internal standard will, therefore, need to have similar physicochemical properties to the target analyte and show similar behaviour throughout the entire procedure. Isotopically labelled compounds, such as those made with ^{13}C or deuterium, are ideal for this purpose. In the current study, D2 DA and D2 5-HT were used, but these are only two mass units greater than the target compounds. Thus, the +2 isotope of the DA and 5-HT targets was making a sizable contribution to the peak area of the internal standards, especially when the targets were at a high concentration (Figure 4.5). This caused the calibration curves to be non-linear and the calculation of dialysate concentrations to be inaccurate (Figure 4.6A).

Figure 4.5. Mass spectrum showing the actual (black lines) and predicted (grey outline) isotope pattern of derivatised dopamine (DA; 466.16 m/z ; 100 nM). Actual 468.17 m/z response is considerably higher than predicted due to overlap between the DA+2 isotope and the and D2 DA (5.56 nM) internal standard.

Figure 4.6. 8-point calibration curves for 5-HT made from external standards analysed in triplicate at 0, 0.1, 0.5, 1, 5, 10, 50, and 100 nM and D2 5-HT internal standard at 5.56 nM. A) Uncorrected data. B) Corrected data using R_x/R_y isotopic dilution.

To correct for this, the isotopic dilution feature in the Masshunter software was used, which requires the calculation of R_x and R_y where R_x = the ratio of target to isotope in pure analyte sample and R_y = the ratio of target to isotope in pure internal standard sample. The theoretical peak area contribution of the +2 isotopes of DA and 5-HT was calculated to be 6.06% and 5.24%, respectively (Loos, Gerber, Corona, Hollender, & Singer, 2015). Thus, for DA, R_x was $100/6.06 = 16.5016$ and R_y was $0/1 = 0$, while for 5-HT R_x was $100/5.24 = 24.0963$ and R_y was $0/1 = 0$. This procedure largely solved the issue and resulted in linear calibration curves (Figure 4.6B). Higher concentrations of internal standards (5.56 nM) were also used to decrease the relative contribution of the DA and 5-HT +2 isotopes (Moosavi & Ghassabian, 2018; Tan & Awaiye, 2013). Unfortunately, the software did not allow for this isotopic dilution procedure when an internal standard was being used for another compound (i.e. DOPAC and 5-HIAA). Thus, the DOPAC and 5-HIAA data were calculated without internal standards.

The corrected data from the aforementioned validation procedure is shown in Table 4.1. The R^2 and intra-assay/intra-day RSD for DA and 5-HT was sufficient and well within accepted limits (Araujo, 2009; Moosavi & Ghassabian, 2018). The results for 5-HIAA and DOPAC were more variable, likely due to the lack of appropriate internal standards. LODs were calculated from the peak area of 9 replicates of 0 nM blank standards and 1 nM (5-HT/DA) or 10 nM (5-HIAA/DOPAC) low concentration standards. The following equation was used: $LoD = LoB + 1.645(SD_{low\ concentration\ sample})$, where $LoB = Mean_{blank\ sample} + 1.645(SD_{blank\ sample})$ (Armbruster & Pry, 2008). The calculated LOD for benzoylated 5-HT and DA were equivalent to those calculated in (Wong et al., 2016) who used the same LOD calculation method. The LOD for 5-HIAA and DOPAC were considerably higher than in (Wong et al., 2016), but well below the detected levels in dialysate samples.

Table 4.1.

Validation results

Standard	R^2	LOD (nM)	Intra-assay RSD ($n = 3$)			Inter-day RSD ($n = 3$)		
			1 nM	10 nM	100 nM	1 nM	10 nM	100 nM
DA	0.9980	0.49	2.3%	2.1%	1.1%	5.6%	3.9%	1.3%
5-HT	0.9976	0.36	3.6%	1.5%	1.2%	4.7%	3.4%	2.2%
DOPAC* [#]	0.9911	24.4	6.5%	5.7%	3.7%	15.5%	8.9%	16.2%
5-HIAA* [#]	0.9950	25.7	2.4%	2.7%	2.5%	12.4%	12.9%	3.8%

*No internal standard used, [#]Concentration for $RSD \times 10$.

Probe implantation

A third issue that needed to be addressed was the high rate of subject/data attrition. A large proportion of the data loss from the initial experiment was due to disconnections, tangles, and blockages in the microdialysis sampling equipment. Although this issue was mostly solved by a redesign of the sampling equipment and procedure, the risk could be further reduced by minimising the time from probe implantation to testing. Currently, probes were implanted 12 hours prior to testing, as is commonly done (Baumann, Clark, & Rothman, 2008; Kalivas et al., 1998; Morgan et al., 1997). However, many studies have also used much shorter times such as 3 hours (Panos & Baker, 2010), 2 hours (Shankaran & Gudelsky, 1999), or even 1 hour (Benamar, Geller, & Adler, 2008). Probe implantation causes localised tissue damage, and thus, some time between the implantation of the probe is required for extracellular concentrations of neurochemicals to stabilise. If the probe is left implanted for too long, however (2+ days), gliosis or probe blockages can occur, preventing adequate sampling.

To assess this under the current conditions, rats ($n = 2$) were implanted with microdialysis probes, and dialysate samples were immediately collected every 40 min for 5 hours. MDMA (10 mg/kg i.p.) was administered after 3 hours to examine dose-dependent changes in dialysate concentrations. Figure 4.7 shows that dialysate concentrations were very high during the first hour but quickly stabilised within 2 hours. In accordance with this data and with previous studies, the time between probe implantation and testing was reduced to 3 hours.

Figure 4.7. Peak area of dopamine (DA; left) and serotonin (5-HT; right) as a function of time/MDMA administration. Microdialysis samples were collected every 40 min following the implantation of the probe at 0 min and the administration of MDMA (10.0 mg/kg, i.p.) at 180 min.

Method 2

Another large sample of rats ($n = 17$) was tested. The methods that were changed from Method 1 were as follows. Microdialysis probes were implanted 3 hours before testing. 2% BzCl was used for derivatisation. A new chromatography gradient was used. D2 DA/D2 5-HT (2.5 μ L) was spiked into each sample/standard to reach a final concentration of 5.56 nM. Dialysate concentrations following a 0.0 mg/kg i.p. dose of MDMA were also determined. Isotopic dilution was used to correct for isotopic 'bleed' from DA and 5-HT into the D2 internal standards. DOPAC and 5-HIAA concentrations were calculated without internal standards.

Results 2

Unfortunately, attrition rates were high once again. Dialysis issues prevented the collection of samples from 1 of the 17 subjects, but unexpected and uncontrollable issues with the LC-MS that were followed by a lengthy period of maintenance prevented the analysis of dialysate samples from another 9 subjects. Calculated concentrations of DA, 5-HT, DOPAC, and 5-HIAA for a representative subject are shown in Figure 4.8 and are consistent with that shown in previous tests and with previous studies.

Of the data that were able to be analysed correctly, there were still some issues that needed to be addressed. Firstly, calibration curves for DA and DOPAC did not have a large enough range, with the maximum concentrations being 10 and 100 nM, respectively. Maximum calculated concentrations of these compounds in dialysate samples were an order of magnitude higher than this and therefore may have been inaccurate. Secondly, the low dialysate concentrations of DA and particularly 5-HT were still difficult to detect, which prevented the accurate quantification of baseline values. Thirdly, there was large variation in the data. The lack of accurate baseline data prevented the transformation of the data to a percentage change from baseline, which might help account for the large between-subject variability and any small variations in probe recovery. The lack of appropriate internal standards for DOPAC and 5-HIAA was likely another contributing factor.

Figure 4.8. Calculated concentrations of dopamine (DA), serotonin (5-HT), DOPAC, and 5-HIAA as a function of time/MDMA administration for a representative subject. Microdialysis samples were collected at 20 min intervals. MDMA 0.0, 5.0, and 10.0 (mg/kg, i.p.) was administered at 120 min, 180 min, and 300 min, respectively.

Method development 2

Several aspects of the procedure were evaluated with the main goal of being able to detect and quantify the low concentrations of DA and 5-HT present in baseline dialysate samples. Unfortunately, further unexpected issues with the LC-MS and another lengthy period of maintenance prevented the collection of data from several tests, which then needed to be repeated.

BzCl concentration

Several tests were carried out examining the effect of different BzCl concentrations (0.25, 0.5, 1, 2, 4, 5%) used for derivatisation on analytical sensitivity. During this time, it was discovered that diluting fresh BzCl daily greatly decreased variation in the data. This suggests that hydrolysis or evaporation of BzCl in the solutions used for derivatisation may have

contributed to the variation of the data in prior work since the same BzCl solution was previously used for up to 1 week (Bentley, Carter, & Harris, 1984; V. Gold, Hilton, & Jefferson, 1954; Hall Jr, 1955; Hudson & Wardill, 1950).

The effect BzCl concentration on analytical sensitivity in standards consistently showed that lower concentrations of BzCl improved sensitivity for DA and particularly 5-HT. Larger peak areas and higher signal to noise ratios (S/N) were obtained for the same concentration of standard when derivatised with lower concentrations of BzCl (Figure 4.9). The slopes of calibration curves were also significantly steeper, and the detection limits were lower when using more dilute concentrations of BzCl (Figure 4.10). In contrast, DOPAC and 5-HIAA became very difficult to detect when using the lowest BzCl concentrations, eluting very early and often with split peaks (Figure 4.11). These results might be due to differences in sample pH and insufficient mixing and pH buffering of the large sample volumes by the mobile phase. Since samples derivatised with lower concentrations of BzCl have a higher pH, this might impact the retention and ionizability of the more acidic DOPAC and 5-HIAA differently than the basic DA and 5-HT. Changes to the sample or mobile phase buffers were not possible at this stage, and since DA and 5-HT were the primary targets of the analysis, it was decided to use lower concentrations of BzCl for the improved sensitivity of DA and 5-HT at the expense of being able to quantify 5-HIAA or DOPAC accurately.

Tests examining the effect of different BzCl concentrations used for derivatisation were also conducted in rats ($n = 12$) on several occasions, first using 1% BzCl then using 0.5% BzCl. Results mirrored those from using standards. The ability to detect baseline DA and 5-HT was improved when using lower BzCl concentrations, but 5-HIAA and DOPAC were no longer able to be reliably measured when using a BzCl concentration of 0.5%. Under these conditions, the lowest concentrations of DA were now able to be reliably detected and quantified, but baseline concentrations of 5-HT were still below detection/quantification limits (Figure 4.12).

Figure 4.9. Extracted ion chromatograms of serotonin (385.1547 m/z) from 0.5 nM external standard following derivatisation with 0.5%, 1%, or 2% BzCl. Signal to noise ratio (S/N) is shown for each peak.

Figure 4.10. 8-point calibration curves for serotonin made from external standards at 0, 0.1, 0.5, 1, 5, 10, and 50 nM following derivatisation with 0.5% (●), 1% (■), or 2% (▲) BzCl. The slope and intercept of each linear calibration curve are displayed in the legends.

Figure 4.11. Extracted ion chromatograms of 5-HIAA (left) and DOPAC (right) from 1000 nM external standards following derivatisation with 0.5% (top), 1% (middle), or 2% (bottom) BzCl.

Figure 4.12. Extracted ion chromatograms of derivatised serotonin (panels A and B) and dopamine (panels C and D) in rat brain microdialysate samples during baseline conditions (panels A and C) and following 10 mg/kg i.p. MDMA (panels B and D).

LC-MS injection volume

Several tests were carried out to determine the effect of HPLC injection volume in both external standards and rats ($n = 6$). As expected, increasing the injection volume resulted in a proportional increase in the peak area, but this also increased noise (Table 4.2). The S/N only improved marginally with larger injection volumes. Thus, any gain in detection limits was small and came with the sacrifice of temporal resolution since the microdialysis sampling interval would need to be increased to maintain the same sample volume. Baseline concentrations of 5-HT in dialysate samples were still difficult to detect reliably with even the largest injection volumes.

Table 4.2.

Effect of injection volume

5-HT (1 nM standards)		
Injection vol.	Peak Area	S/N
10 μ L	38771	1.25
30 μ L	107230	3.45
60 μ L	191553	3.80
100 μ L	254905	4.16

Microdialysis

Further increases in sensitivity did not seem achievable without major methodological changes, and thus it was decided to focus on improving analyte recovery during microdialysis. The concentration of the analyte recovered using microdialysis (the relative recovery) only represents a fraction of the concentration present in the extracellular fluid and is influenced by several factors (Chefer et al., 2009; Westerink & Cremers, 2007). Firstly, the flow rate of the perfusate through the dialysis probe is inversely proportional to relative recovery. At extremely low flow rates, relative recovery can approach 100%, but the volume of sample acquired per unit time will be greatly decreased, and thus the absolute recovery (total amount of analyte per sample) will be low. Conversely, higher flow rates decrease relative recovery while increasing absolute recovery and sample volume. It had already been determined that increasing the sample volume injected into the LC-MS produced marginal increases in the detection limits for 5-HT. It was therefore decided to test a lower flow rate in order to increase relative recovery,

at the expense of absolute recovery and sample volume. Halving the current flow rate to 0.5 $\mu\text{L}/\text{min}$ increased relative recovery of 5-HT standards *in vitro* from approximately 15% to 25%.

Another factor that influences the recovery is the probe dialysis membrane surface area. Probes with a greater membrane surface area have greater relative recovery as there is more area for analytes to diffuse across into the perfusate. The relative recovery of several probes from different manufacturers as well as custom-made probes with different size membranes was tested. Probes with a longer membrane (3 mm instead of 2 mm) increased relative recovery of the target analytes by approximately 5% at 1 $\mu\text{L}/\text{min}$. Probes with a larger diameter of 0.5 mm instead of 0.24 mm further increased recovery, and, when coupled with a 0.5 $\mu\text{L}/\text{min}$ flow rate, relative recovery of 5-HT was 45 – 50% *in vitro*. Using these dialysis procedures together with 0.5% BzCl derivation, baseline concentrations of 5-HT in microdialysate samples from rats were now reliably above detection limits (Figure 4.13). These dialysis procedures (larger probes, slower flow rates) are similar to those used by Baumann, Clark, and Rothman (2008), who also noted the difficulty in reliably detecting baseline 5-HT samples.

Figure 4.13. Extracted ion chromatograms of derivatised serotonin in rat brain microdialysates during baseline conditions (left) and after 10 mg/kg i.p. MDMA (right).

Calculated dialysate concentrations of DA and 5-HT from a test subject are shown in Figure 4.14. Baseline dialysate concentrations of DA and 5-HT were approximately 5 nM and 0.5 nM, respectively. DA concentrations peaked at 24 nM (380% increase) following 5 mg/kg MDMA and ~100 nM (1900% increase) following 10 mg/kg MDMA. 5-HT concentrations peaked at 2.7 nM (440% increase) following both 5 and 10 mg/kg MDMA. These concentrations and percentage increases are within the range of what is expected based on previous studies (Freezer, Salem, & Irvine, 2005; Gough et al., 2006; Gudelsky & Nash, 1996; Hegadoren, Martin-Iverson, & Baker, 1995; Kankaanpää et al., 1998; Nair & Gudelsky, 2004; Schmidt, Sullivan, & Fedayal, 1994).

Figure 4.14. Calculated concentrations of dopamine (DA), serotonin (5-HT), and MDMA as a function of time/MDMA administration for a representative test subject. Microdialysis samples were collected at 30 min intervals. MDMA 0.0, 5.0, and 10.0 (mg/kg, i.p.) was administered at 120 min, 240 min, and 360 min, respectively.

Quantification of MDMA

Calibration curves and external standards were also prepared for MDMA, allowing for the quantification of MDMA in microdialysate samples (Figure 4.14). Following the adjustments to the microdialysis procedure, however, the concentrations of MDMA recovered were very high. Concentrations in dialysate samples following MDMA administration (5 or 10 mg/kg i.p.) ranged from 1000 – 8000 nM. These concentrations appeared to saturate the MS detector and were well above the linear range of the calibration curves (Figure 4.15). Dilution of the samples was not viable, while a separate analysis run for MDMA was not possible due to time constraints. Thus, a best estimate for the concentration of MDMA under these conditions was carried out using a non-weighted quadratic calibration curve for MDMA using external standards at 1000, 5000, and 10000 nM (Figure 4.15; Almeida et al., 2002; Moosavi & Ghassabian, 2018). D2 5-HT was used as the internal standard, and the R_x/R_y isotopic dilution for MDMA was calculated manually.

Figure 4.15. Linear and quadratic calibration curves for MDMA made from external standards analysed in triplicate at 0, 10, 50, 100, 500, 1000, 5000, and 10000 nM and D2 5-HT internal standard at 14.3 nM.

Methods 3

A final sample of rats ($n = 24$) was tested. Dialysis and analytical methods that were changed from Method 2 were as follows. Larger diameter and length guide cannula (9.14.IC, Microbiotech, Sweden) and microdialysis probes (MAB 9.14.3, Microbiotech, Sweden) were used. The membrane area of these probes was 0.5 mm (diameter) $\times$ 3 mm (length). A slower dialysis flow rate of 0.5 μ L/min was used, and samples were collected at 30 min intervals. To each 15 μ L dialysate sample, 2.5 μ L of internal standard at a higher concentration (100 nM D2 DA/D2 5-HT; final concentration 14.3 nM), 7.5 μ L of borate buffer (100 nM), and 7.5 μ L of freshly diluted BzCl (0.5% in acetonitrile) was added, with mixing between each step. The volume of derivatised sample injected by the HPLC was 30 μ L. Calibration curves were made from external standards at 0, 0.1, 0.5, 1, 5, and 10 nM for 5-HT and 0, 1, 5, 10, 50, and 100 nM for DA. DOPAC and 5-HIAA were no longer quantified. Non-weighted quadratic calibration curves for MDMA were made from 1, 5, and 10 μ M MDMA prepared in the same way as the other external standards.

MDMA pretreatment and locomotor sensitisation

The effect of a sensitising regimen of repeated MDMA exposure on locomotor activity and analyte concentration was also determined. Pretreatment with MDMA (0.0 [0.9% saline] or 10 mg/kg/day, i.p., for 5 days) was carried out in locomotor activity chambers as previously described in Chapter 3. Following two drug-free days, microdialysis probes were implanted and, 3 hours later, testing began. Dialysate samples were collected at 30 min intervals for 2 hours prior to and following the administration of ascending doses of MDMA (0.0, 5.0, 10.0 mg/kg i.p.). Measurement of locomotor activity was carried out simultaneously as described in Chapter 3, and the data were summed into 30 min bins to match the microdialysis sampling interval.

Statistical analysis

Separate 2 (pretreatment: MDMA 0.0 vs 10.0 mg/kg) $\times$ 16 (time: 30 – 480 min) mixed-measures ANOVAs were conducted to analyse locomotor activity and analyte concentrations. The average baseline 5-HT and DA concentrations were calculated for each rat, and the percentage change from baseline was also calculated. An independent samples *t*-test was used to compare average baseline analyte concentrations between pretreatment groups. Separate 2 (pretreatment: saline vs 10.0 mg/kg) $\times$ 12 (time: 150 – 480 min) mixed measures ANOVAs were conducted to analyse the DA and 5-HT percentage change data. Greenhouse-Geisser corrections to degrees of freedom (Greenhouse & Geisser, 1959) were applied when the

assumption of sphericity was not met, as determined by Mauchly's Test of Sphericity. Significant interactions were followed up with simple main effect analyses. The level of significance for all tests was $p < .05$. All analyses were conducted using SPSS (v25, IBM).

Results 3

Locomotor and dialysate data (5-HT and MDMA) were obtained from 21 of the 24 rats. Two subjects did not complete the pretreatment phase while one subject had an unacceptable probe placement. Probe placements for these 21 final subjects are shown in Figure 4.16. Quantified dialysate concentrations of DA were only successfully obtained from the first 6 subjects, however. DA data from subsequent analyses were very unreliable, with the DA peaks becoming difficult to detect and quantify accurately, even at high concentrations.

Figure 4.16. Schematic representation (adapted from Paxinos & Watson, 2005) of microdialysis probe placement in rat medial striatum as a function of MDMA pretreatment (0.0 or 10.0). Black bars represent the location of the probe membranes (0.5 mm x 3 mm) for each rat. Numbers represent the distance from Bregma in mm. Sample sizes are displayed in the legend.

Figure 4.17 displays locomotor activity as a function of MDMA pretreatment and time/MDMA administration. There was a significant two-way interaction (pretreatment $\times$ time), $F(2.76, 52.50) = 4.19, p = .012, \eta_p^2 = .181$. Locomotor activity changed significantly as a function of time for both the saline, $F(15, 150) = 11.16, p < .001, \eta_p^2 = .527$, and MDMA 10.0, $F(15, 135) = 8.28, p < .001, \eta_p^2 = .479$ pretreatment groups. Locomotor activity was significantly greater in the MDMA 10.0 pretreatment group at the 300, 330, 390, 420, and 450 min time points, $p < .05$.

Figure 4.18 shows mean MDMA concentrations as a function of MDMA pretreatment and time. A two-way ANOVA failed to produce a significant interaction, $p > .05$. A significant main effect of time indicated that MDMA concentrations increased as a function of time/MDMA administration, $F(15, 285) = 97.66, p < .001, \eta_p^2 = .837$. There was no main effect of pretreatment indicating that there was no difference in overall MDMA concentrations as a function of MDMA pretreatment, $p > .05$.

Figure 4.19A displays mean 5-HT concentrations as a function of MDMA pretreatment and time. A two-way ANOVA failed to reveal a significant interaction, $p > .05$. A significant main effect of time indicated that 5-HT concentrations increased as a function of time/MDMA administration, $F(3.45, 65.56) = 90.26, p < .001, \eta_p^2 = .826$. There was no main effect of pretreatment, indicating that there was no difference in overall 5-HT concentrations as a function of MDMA pretreatment, $p > .05$. The average baseline concentration of 5-HT was marginally higher in the MDMA 10.0 pretreatment group (mean = 0.48 nM, SD = 0.30) compared to the saline group (mean = 0.34 nM, SD = 0.15), but this was not significant, $p > .05$. Figure 4.19B shows the percentage change from baseline in mean 5-HT concentrations as a function of MDMA pretreatment and time. A two-way ANOVA failed to reveal a significant interaction, $p > .05$. A significant main effect of time indicated that the percentage change in 5-HT concentrations increased as a function of time/MDMA administration, $F(1.93, 36.60) = 38.41, p < .001, \eta_p^2 = .669$. A marginal decrease in overall percentage change in 5-HT concentrations was observed in the MDMA 10.0 pretreatment group, but this main effect was not significant, $p > .05$.

Figure 4.20A displays mean DA concentrations as a function of MDMA pretreatment and time for the first 6 subjects, while Figure 4.20B shows the same data converted to a percentage change from baseline. Given the sample sizes were incomplete, no statistical analyses were run on these data, and thus the results are tentative. Data from the first 6 subjects show that DA increased dose-dependently with MDMA administration for both pretreatment

groups. A trend of increased MDMA-produced increases in DA concentrations was observed in the MDMA 10.0 pretreatment group. The baseline concentrations of DA were also marginally higher in the MDMA 10.0 pretreatment group (mean = 4.95 nM, SD = 1.31) compared to the saline group (mean = 3.78 nM, SD = 1.33).

Figure 4.17. Mean locomotor activity (30 min bins) as a function of time/MDMA administration following pretreatment with MDMA 0.0 (○) or MDMA 10.0 (●). MDMA 0.0, 5.0, and 10.0 (mg/kg, i.p.) was administered at 120 min, 240 min, and 360 min, respectively. Error bars represent standard error of the mean. Sample sizes are displayed in the legend. * $p < .05$.

Figure 4.18. Mean MDMA concentrations as a function of time/MDMA administration following pretreatment with MDMA 0.0 (○) or MDMA 10.0 (●). Microdialysis samples were collected at 30 min intervals. MDMA 0.0, 5.0, and 10.0 (mg/kg, i.p.) was administered at 120 min, 240 min, and 360 min, respectively. Sample sizes are displayed in the legend.

Figure 4.19. Mean serotonin (5-HT) concentrations (A) and mean % change from baseline (B) as a function of time/MDMA administration following pretreatment with MDMA 0.0 (○) or MDMA 10.0 (●). Microdialysis samples were collected at 30 min intervals. MDMA 0.0, 5.0, and 10.0 (mg/kg, i.p.) was administered at 120 min, 240 min, and 360 min, respectively. Error bars represent standard error of the mean. Sample sizes are displayed in the legend.

Figure 4.20. Mean dopamine (DA) concentrations (A) and mean % change from baseline (B) as a function of time/MDMA administration following pretreatment with MDMA 0.0 (○) or MDMA 10.0 (●). Microdialysis samples were collected at 30 min intervals. MDMA 0.0, 5.0, and 10.0 (mg/kg, i.p.) was administered at 120 min, 240 min, and 360 min, respectively. Error bars represent standard error of the mean. Sample sizes are displayed in the legend.

Discussion

In the current study, a procedure for the quantification of extracellular concentrations of 5-HT, DA, and MDMA was developed. Samples were collected using *in vivo* microdialysis from the medial striatum, derivatised using BzCl, and analysed using HPLC coupled with Q-TOF ESI-MS. This procedure was then applied to determine neurochemical correlates of sensitised MDMA-produced behaviour following repeated, intermittent exposure.

Acute MDMA administration produced dose-dependent increases in locomotor activity, which was significantly sensitised following repeated exposure, as has been previously shown (Ball, Klein, Plocinski, & Slack, 2011; Bradbury et al., 2012; Kalivas et al., 1998; van de Wetering & Schenk, 2017). MDMA-produced locomotor activity was mirrored by dose-dependent increases in extracellular concentrations of MDMA. Correcting for probe recovery, the extracellular concentration of MDMA peaked at approximately 8 μ M and 17 μ M following the i.p. administration of 5 and 10 mg/kg MDMA, respectively, which is consistent with that previously found following similar doses of i.p. MDMA (Esteban et al., 2001). Importantly, MDMA pretreatment had no effect on extracellular concentrations of MDMA following acute administration, suggesting that any behavioural or neurochemical consequences of repeated MDMA exposure in the current study were not due to differences in MDMA uptake.

Acute MDMA exposure also produced dose-dependent increases in extracellular concentrations of 5-HT, although these increases were non-linear and perhaps not what might be expected given a doubling of the MDMA dose. This has also been observed in previous studies (Gough et al., 2006; Kankaanpää et al., 1998; Nair & Gudelsky, 2004) and can be explained by the pharmacology of MDMA. MDMA is an incredibly potent 5-HT releasing agent, and near-saturated effects of MDMA-produced 5-HT release or 5-HT transporter binding in rat brain tissue slices can be observed with extracellular concentrations of MDMA in the low μ M range *in vitro* (Battaglia, Brooks, et al., 1988; Green, Mehan, Elliott, O'Shea, & Colado, 2003; Rudnick & Wall, 1992; Schmidt, Levin, & Lovenberg, 1987). Given the measured extracellular concentrations of MDMA in the current study were near the top-end of the dose-effect curves from these studies, further substantial increases in 5-HT concentrations following the second, higher dose of MDMA would not be expected. The lack of a further substantial increase in extracellular 5-HT also suggests that other mechanisms are primarily driving the acute MDMA-produced locomotor response, which did increase substantially with the second, higher dose of MDMA.

In support of this idea, the sensitised MDMA-produced locomotor response following repeated MDMA exposure was not accompanied by significant changes in MDMA-produced extracellular 5-HT. MDMA pretreatment had no effect on the absolute extracellular concentrations of 5-HT following the MDMA challenge. However, there were non-significant trends for higher baseline concentrations of 5-HT and lower MDMA-produced increases in 5-HT when calculated as a percentage change from baseline. Previous studies have shown that substantial repeated MDMA exposure resulted in various 5-HTergic deficits such as decreased 5-HT tissue levels, decreased 5-HT transporter densities, and decreased MDMA-produced 5-HT release (Battaglia, Yeh, et al., 1988; Battaglia et al., 1987; Commins et al., 1987; Do & Schenk, 2013; Mayerhofer et al., 2001; O'Hearn et al., 1988; Reveron et al., 2010; Schenk et al., 2007; Shankaran & Gudelsky, 1999). Repeated exposure to lesser amounts of MDMA have failed to produce such deficits, however (Banks et al., 2008; Do & Schenk, 2013; Fantegrossi et al., 2004), as was observed in the current study. This suggests that a certain amount of exposure or pattern of exposure is required to produce significant 5-HTergic deficits. The role of 5-HT in the development and expression of MDMA sensitisation has not been well studied. The present findings indicate that changes in MDMA-produced 5-HT release in the medial striatum are not an important mechanism by which repeated MDMA exposure results in behavioural sensitisation.

In contrast to 5-HT, several previous studies have observed increases in DAergic neurotransmission as a function of repeated MDMA exposure and an important role of striatal DAergic mechanisms in the development and expression of MDMA sensitisation has been demonstrated. Repeated exposure to MDMA under conditions that produced behavioural sensitisation also resulted in elevated DA tissue levels (Mayerhofer et al., 2001) and enhanced MDMA-produced DA release (Kalivas et al., 1998). Increases in striatal neuron firing (Ball et al., 2006) and increased dendritic spine density in accumbal MSN (Ball et al., 2009) were also observed following a sensitising regimen of repeated MDMA exposure. Further, administration of the DA D₁ receptor antagonist, SCH-23390, prevented the expression of MDMA behavioural sensitisation (Ramos, Goñi-Allo, & Aguirre, 2004) while co-administration of the DA D₂ antagonist, eticlopride, during pretreatment prevented the development of MDMA behavioural sensitisation (van de Wetering & Schenk, 2017).

Unfortunately, the DA results from the current study were incomplete and not able to be statistically analysed since reliable data were only obtained from the first 6 subjects of the study. DA data from the other subjects were unreliable; DA peaks were difficult to detect and

quantify accurately, even at high concentrations. The samples from these subjects were analysed after a long period (3 months) of storage at -80 °C and following a lengthy period of LC-MS maintenance, which may have impacted the ability to accurately detect and quantify of DA in these samples (Chefer et al., 2009; Suominen et al., 2013).

Nevertheless, the tentative DA results of the current study are consistent with what would be expected based on previous studies. Acute MDMA administration produced dose-dependent increases in extracellular concentrations of DA. These increases were also non-linear, but in contrast to 5-HT, even greater increases on DA were observed than might be expected following a doubling of the MDMA dose. This too has been previously observed by studies that have used similar, relatively high doses of MDMA (Freezer et al., 2005; Gudelsky & Nash, 1996; Hegadoren et al., 1995; Schmidt et al., 1994). This might be due to dose-dependent interactions between 5-HTergic and DAergic neurotransmission since increased 5-HT neurotransmission has been shown to be inhibitory to DA release under some conditions (Bankson & Cunningham, 2001; Czoty et al., 2002; Di Matteo et al., 2008). Following repeated MDMA exposure, the current results would suggest that extracellular concentrations of DA at baseline and following acute-MDMA administration were enhanced. These results, although tentative, are consistent with that found previously using a similar dosing regimen of repeated MDMA exposure (Kalivas et al., 1998) and with the general documented role of DAergic mechanisms in behavioural sensitisation (for reviews see Pierce & Kalivas, 1997; T. E. Robinson & Berridge, 1993; Vanderschuren & Pierce, 2010; Vezina, 2004).

To summarise, in this chapter, a procedure for the sampling and targeted quantification dialysate sample components using microdialysis and LC-MS was developed and validated. This procedure was then employed to determine some of the specific neurochemical correlates of MDMA-induced behavioural sensitisation. The results partially supported predictions in that the MDMA-produced DAergic response appeared to become sensitised with repeated exposure. The MDMA-produced 5-HT response, at least extracellularly, and within the medial striatum, was not impacted, however. These findings add support to the idea that repeated MDMA exposure induces many of the same neuroadaptations that result from repeated exposure to other drugs of abuse (Schenk, 2011).

CHAPTER 5: UNTARGETED METABOLOMICS

Introduction

One of the advantages of MS-based sample analysis that the current research aimed to exploit is the ability to carry out metabolomics (Dettmer, Aronov, & Hammock, 2007). Metabolomics involves the global, unbiased analysis of the metabolome (all small molecules/metabolites within a biological system) and is typically implemented in order to identify differences between the metabolite profile of a control and test group that might be relevant to specific biological conditions (Fiehn, 2002; Oliver, Winson, Kell, & Baganz, 1998). Metabolomics is one of the more recent additions to the ‘omics’ field. Compared to the other ‘omics’ approaches such as genomics or proteomics, which involve the analysis of the genome and proteome respectively, metabolomics can be more directly relevant to a particular phenotype of interest since metabolites are downstream products of gene transcription and translation (Dettmer et al., 2007; Fiehn, 2002; Patti, Yanes, & Siuzdak, 2012). The metabolome is much more complex, however, and less well characterised than the genome or proteome (Gromski et al., 2015). The diverse physiochemical properties of metabolites also add to the complexity of metabolomics. No single analytical method is currently able to measure the entire metabolome simultaneously and, thus most metabolomics studies will focus on a certain class of metabolites such as lipids or amino acids (Shulaev, 2006; Zhang, Sun, Wang, Han, & Wang, 2012).

There are two main approaches in metabolomics; targeted and untargeted (Patti et al., 2012; Shulaev, 2006; Zhou, Xiao, Tuli, & Ransom, 2012). Targeted approaches are designed to quantitatively measure the absolute concentration of a limited number of known metabolites. Targeted metabolomics analyses are, therefore, partially hypothesis-driven, and are carried out in a similar manner as described in Chapter 4, but with an extensive list of target analytes and external standards (e.g. Song et al., 2012; Wong et al., 2016). The limitation of targeted metabolomics is that the metabolites of interest must be known *a priori*, which prevents the discovery of novel or genuinely unexpected metabolites that might be relevant. Untargeted approaches, in contrast, are truly hypothesis-generating and aim to semi-quantitatively measure the relative concentration all metabolites within a biological sample.

There are several stages to an LC-MS-based untargeted metabolomics procedure (for reviews see Dettmer et al., 2007; Goodacre et al., 2007; Zhou et al., 2012). The first three stages include sample collection, preparation, and analysis. Various samples such as tissue, plasma,

urine, or cerebrospinal fluid can be collected and prepared accordingly (e.g. internal standard spike, derivatisation, extraction) for analysis by LC-MS or other means. These procedures are generally designed to be as inclusive as possible so as to provide the largest amount of information on the metabolome. The next stage is data processing. Compared to targeted approaches, where analyte information such as retention time and m/z are already known, untargeted data processing is much more complex since the chromatographic peaks of each metabolite need to be correctly distinguished from one another and from chemical or analytical noise automatically. LC-MS data processing can include several steps such as mass detection, chromatogram building, chromatogram deconvolution/peak integration, alignment, and gap-filling, each of which can require considerable optimisation. The next stage is data analysis. Both univariate and multivariate approaches can be used to identify the metabolites that differ between the treatment groups (Bartel, Krumsiek, & Theis, 2013; Goodacre et al., 2007; Worley & Powers, 2013). In the final stage, metabolites of interest can be subsequently annotated (tentatively identified) or identified (Sumner et al., 2007). Annotation can be achieved by comparing the exact mass, physicochemical properties, and fragmentation patterns of the metabolite of interest to databases/libraries, while identification can be achieved only with the use of chemical reference standards (Sumner et al., 2007).

The aim of the current research was to expand the targeted findings of the previous chapter and identify novel or unexpected behaviourally relevant compounds that are impacted by repeated MDMA exposure. Untargeted metabolomics analysis was carried out on the microdialysis samples collected from the MDMA vs saline pretreated subjects in Chapter 4. Importantly, these samples were collected over a period of time following the administration of ascending doses of MDMA during which behavioural data (locomotor activity) were also collected. The combination of group and time/dose metabolomics data, as well as behavioural data, makes the current data set unique. This is particularly true within the addiction field, where metabolomics approaches have yet to gain much traction (Ghanbari & Sumner, 2018; Mussap, Loddo, Fanni, & Fanos, 2020). Given this unique combination of group, time/dose, and behavioural data, a relatively novel data statistical approach needed to be developed.

Metabolomics studies are typically carried out as a simple two-class comparison (i.e. control vs treated) at a single time point (Bartel et al., 2013; Broadhurst & Kell, 2007; Gromski et al., 2015). As previously mentioned, univariate tests (multiple t-tests, ANOVAs, etc.) can be carried out to examine class differences. However, due to the extensive number of variables (each metabolite) in an untargeted metabolomics study, this comes with the high risk of making

Type I errors (Broadhurst & Kell, 2007). Correction methods such as Bonferroni are typically not appropriate for such large data sets, and even after such corrections, the data can still be difficult to interpret (Broadhurst & Kell, 2007). Thus, multivariate methods such as principal components analysis (PCA) or partial least squares discriminant analysis (PLS-DA; also known as projection to latent structures) are often used to first summarise the data and identify variables/metabolites of interest prior to any univariate statistics (Gromski et al., 2015; Worley & Powers, 2013).

PCA reduces the dimensionality of the data, grouping multiple correlated variables/metabolites into a far fewer number of new variables called components that explain the majority of variance in the data set (Jolliffe, 2011; Worley & Powers, 2013). The first component generated by PCA will always explain the most variance in the data, while the second component will explain the second most variance in the data, and so on. PCA allows for the interpretation of complex multivariate data where patterns, groupings, or outliers can be visualised in 2 or 3 dimensions. If a grouping between different classes is observed and due to one particular component, the contribution that each of the original variables had in generating that component (loadings) can then be examined, and the variables/metabolites that drive the observed grouping can be identified. Because PCA is an unsupervised method that aims to explain the maximum variance in the data, a difference between two classes will only be revealed if the between-class variance exceeds the within-class variance (Worley & Powers, 2013). This is often not the case with metabolomics data, however, where biological variation, small methodological variations, and variations due to chemical or analytical noise can easily overshadow any experimentally-induced variation in the small number of relevant metabolites (Álvarez-Sánchez, Priego-Capote, & Castro, 2010; Álvarez-Sánchez, Priego-Capote, & Luque de Castro, 2010; Worley & Powers, 2013).

In this situation, supervised methods such as PLS-DA can be much more effective at identifying the drivers of any class differences (Gromski et al., 2015; Wold, Sjöström, & Eriksson, 2001; Worley & Powers, 2013). PLS-DA is a regression-based model that similarly reduces the dimensionality of the data into a few components but aims to maximise the covariance of the *X* variables (i.e. the metabolites) with a designated categorical *Y* variable that is coded as class membership (e.g. control vs treated). Thus, in regards to metabolomics, PLS-DA will aim to maximise the separation between two classes based on any metabolomic differences. Orthogonal PLS-DA (OPLS-DA) is a more recent variant of PLS-DA that includes a orthogonal signal correction (Bylesjö et al., 2006; Trygg & Wold, 2002; Wold, Antti,

Lindgren, & Öhman, 1998) and generates a single component that is predictive of the *Y* variable while all other components are orthogonal to *Y* (i.e. independent). This eases the interpretability of the model and essentially splits the data into that which is predictive of *Y* and representative of between-class variability, and that which is orthogonal to *Y* and representative of within-class variability. The variables that load strongly onto the predictive component are, therefore, the variables/metabolites most relevant to the differences between the classes (Galindo-Prieto, Eriksson, & Trygg, 2014). Variables that load strongly onto orthogonal components may also be of interest for identifying methodological discrepancies or contaminants in the analysis. PCA, PLS-DA, and OPLS-DA have all been used to effectively separate two treatment groups based on their metabolomic profile and identify potential metabolites of interest (e.g. Carrola et al., 2011; H.-J. Kim et al., 2011; Uarrota et al., 2014; X. Wang, Yang, Sun, & Zhang, 2012).

There is an increasing recent interest in monitoring dynamic changes in the metabolite profile over time in response to various treatments or interventions (Rusilowicz, Dickinson, Charlton, O'Keefe, & Wilson, 2018; Smilde et al., 2010). While a time-series metabolomics design can be much more powerful and yields much more information, it is also considerably more complex. A single analysis is not capable of fully describing group $\times$ time metabolomics data, but various combinations of methods or models can and have been used effectively (Antti et al., 2002; Boccard & Rudaz, 2014; Costello & Martin, 2018; Dai et al., 2016; Galindo-Prieto, Eriksson, & Trygg, 2015; Guo et al., 2016; Rantalainen et al., 2008; Rusilowicz et al., 2018; Smilde et al., 2010; Williams, Lenz, Rantalainen, & Wilson, 2006). One such method that is applicable for the current research is the use of standard PLS/OPLS regression models where the *Y* variable is continuous and representative of time (or other time-related variable of interest). Time-based patterns can then be visualised, and variables that are most predictive and correlated with *Y* can be identified. These findings can then be used to filter variables for more direct-class based comparisons. Alternatively, time-based models can be generated for both control and treatment groups, which can then be compared in various ways in order to identify differences (Galindo-Prieto et al., 2014, 2015; Wiklund et al., 2007).

The primary goal of the current study was to identify further neurochemical correlates of sensitised MDMA-produced behaviour that were not examined in Chapter 4. A combination of the aforementioned statistical methods including PCA, OPLS, and OPLS-DA, with follow-up univariate statistics, were used in order to analyse the time-series metabolomics data collected using microdialysis and LC-MS from MDMA pretreated and control rats. The goal of the analysis was to identify metabolites that 1) are relevant to time/MDMA administration,

2) are relevant to behaviour, and 3) are relevant to MDMA pretreatment. The process of optimising the initial data processing steps are also described in this chapter.

Methods

Sample collection, preparation, and analysis

LC-MS data from 336 microdialysis samples collected from the 21 subjects in the final study of Chapter 4 were used. To summarise the study design, subjects were pretreated with either saline ($n = 11$) or MDMA 10 mg/kg/day ($n = 10$) for 5 days. On the 8th day, microdialysis samples were collected every 30 min over a period of 8 hours (16 samples per rat). During this time, and following an initial 2-hour baseline period, subjects received ascending doses of MDMA (0.0, 5.0, 10.0, mg/kg, i.p.) every 2 hours. Locomotor activity was also measured. Microdialysate samples were derivatised with BzCl and analysed by HPLC coupled with ESI Q-TOF MS detection.

Data processing

A variety of software packages are capable of processing LC-MS-based metabolomics data. MZmine (version 2.51) was used in the current study as it is one of the most-effective and most cited open-source options designed for this purpose (Z. Li et al., 2018; Myers, Sumner, Li, Barnes, & Du, 2017a; Pluskal, Castillo, Villar-Briones, & Orešič, 2010; Samra, 2015). An advantage of MZmine is that each data processing step has a number of available modules/algorithms and each step is also able to be performed independently, which allows for more effective optimisation of the various parameters. The ideal parameters for the current data set were optimised by adjusting these parameters in a step-wise fashion in order to ensure the accurate separation and integration of some known target analytes (i.e. the internal standard [D2 5-HT], 5-HT, and MDMA) while minimising the number of false peaks. The goal was to produce a list of features, where each feature ideally represented a unique ion/metabolite (m/z -retention time pair) that has been correctly integrated (peak area). A summary of all final MZmine data processing parameters is shown in Table 5.1, while the process of optimising these parameters is described below.

Table 5.1.

MZmine data processing parameters

Mass detection (centroid):	Noise level: 10^4
Chromatogram builder (ADAP):	Minimum group size in # scans: 3 Group intensity threshold: 10^4 Minimum highest intensity: 10^4 m/z tolerance: 0.02 m/z
Deconvolution (local minimum search):	m/z centre calculator: median Chromatographic threshold: 35% Search minimum in RT range: 0.01 min Minimum relative height: 5% Minimum absolute height: 10^4 Minimum ratio of peak top/edge: 2 Peak duration range: 0.02 – 0.5 min
Isotope peak grouper:	m/z tolerance: 0.005 m/z RT tolerance: 0.01 min absolute Monotonic shape: yes Maximum charge: 2 Representative isotope: lowest m/z
Alignment (join aligner):	m/z tolerance: 0.02 m/z Weight for m/z: 3 RT tolerance: 0.25 min absolute Weight for RT: 2
Gap-filling (same RT and m/z range):	m/z tolerance: 0.01 m/z

Mass detection. The raw data produced by LC-MS contains thousands of successive scans per sample that cover the duration of each analysis (see Figure 1.5 for review). These scans can be visualised as a histogram (mass spectrum) that contain the peak intensity (centroided data) of all the different ions detected and measured by the mass spectrometer. After importing this raw data into MZmine, the mass detection step generates a list of the detected ions/masses and their intensities from each scan for each sample. A noise filter can be applied to exclude ions that do not reach a designated intensity/peak height threshold. This was set at 10^4 following the manual inspection of numerous low-intensity peaks under this threshold that would likely constitute instrumental or chemical noise (Figure 5.1). This threshold was also just below the lowest peak heights of 5-HT in baseline dialysate samples (see Figure 4.13), which is known from previous work in Chapter 4 to be near the lower limit of what is accurately quantifiable with the current sampling and LC-MS methods.

Figure 5.1. An example portion of a raw LC-MS spectrum scan at 5 min retention time showing numerous low-intensity peaks below the 10^4 intensity noise filter (dashed line).

Chromatogram builder. For each sample, the chromatogram builder step connects the detected masses across each consecutive spectrum scan, building EICs for each. The automated data analysis pipeline (ADAP) chromatogram builder module was used (Myers, Sumner, Li, Barnes, & Du, 2017b), as this gave the best results. Several settings were adjusted to optimise this step for the current data set. The minimum group size in number of scans was set to 3 and group intensity threshold was set to 10^4 . These settings determine the number of consecutive scans a mass must be present and above the intensity threshold to be considered a feature. Higher settings prevented small narrow peaks such as 5-HT from being included, but lower settings included too many extremely narrow peaks that might constitute noise. The minimum highest intensity was set to 10^4 to match the noise threshold in the mass detection step. The m/z tolerance setting determines the maximum difference between two m/z values to be considered as separate features. Some variation in measured m/z values can exist for the same analyte. Thus lower tolerance values would sometimes result in peak splicing and create duplicates of the same ion (Figure 5.2A and B). In contrast, higher values would combine masses that are actually unique together, which is especially problematic when they also have overlapping retention times. This parameter was set to a relatively wide 0.02 m/z, which was necessary to detect the internal standard in all samples without peak splicing or duplicates (Figure 5.2C).

Figure 5.2. Extracted ion chromatograms of the detected derivatised D2 serotonin internal standard peaks using the ADAP chromatogram builder in MZmine. Narrow m/z tolerances (0.005 m/z) produced duplicate peaks for the internal standard due to peak splicing (A and B), which was remedied by widening the tolerance setting to 0.02 m/z (C).

Deconvolution. The deconvolution step detects and integrates peaks within each EIC, producing a feature list for each sample where each feature ideally represents a unique peak/ion. Since many unique metabolites can have a similar m/z , their peaks will be present within a single EIC, and thus, these peaks need to be separated and individually integrated (Figure 5.3). This is done based on peak retention time, intensity, shape, and other characteristics by various algorithms offered by MZmine. Local minimum search gave the best results with the current data set. The settings for local minimum search were altered in order to correctly detect and integrate the internal standard in as many samples as possible while minimising the number of false peaks. The chromatographic threshold was set at 35%, while the minimum relative height was set at 5%. These settings determine the relative thresholds for reducing noise based on the data points in each EIC. The minimum absolute peak height was set at 10^4 to match previous absolute thresholds in the mass detection and chromatogram builder steps. The search minimum in retention time (RT) range was set at 0.01 minutes and the peak duration range was set to 0.02 – 0.5 minutes. These settings determine the minimum retention difference between two peaks to be considered separate features and the range of acceptable peaks lengths/widths, respectively. The minimum ratio of peak top/edge setting was set to 2.0. This setting had the largest impact on the deconvolution results. Lower ratios would generate features from noise (Figure 5.4A), whereas higher values would only detect larger peaks with ideal shapes (Figure 5.4B). The settings chosen were quite conservative in order to minimise the integration of false peaks and ensure accurate peak integration. This did prevent the integration of internal standard peaks from a small number of samples where an HPLC issue caused peak tailing (Figure 5.4C), but this was remedied in a subsequent analytical step.

Isotopic peaks grouper. This step identifies and removes isotopes from each feature list. A relatively narrow m/z tolerance of 0.005 was set to avoid removing unique features that may overlap with an isotope. The RT tolerance was set to 0.01 min, which determines the maximum difference a potential isotope can differ from the parent compound. A monotonic shape was required, while the maximum charge to be identified was +2 and the representative isotope was set to be that with the lowest m/z . Thus, when ions following an isotope pattern were identified, they would be removed, and only the parent compounds with the lowest m/z would be kept. This step removed approximately 23% of features.

Figure 5.3. Extracted ion chromatograms of m/z 387.1673 – 387.1752 before (A) and after deconvolution (B and C). Two unique peaks of similar m/z were included in a single EIC from the chromatogram builder step (A) but were separated and integrated individually after deconvolution (B and C).

Figure 5.4. Extracted ion chromatogram of 118.08 m/z (A) showing MZmine deconvolution results with a low minimum ratio of peak top/edge setting of 0.5, which generated false peaks (shaded areas) from noise. Extracted ion chromatograms of the derivatised D2 serotonin internal standard (387.16 m/z ; B and C) showing MZmine deconvolution results with a higher minimum ratio of peak top/edge setting of 2.00, which would only integrate peaks with a good shape (B) and not those with tailing or other issues (C).

Alignment. This step matches all detected features from each of the samples together, generating a single feature list for all samples. The join aligner method was found to be the most effective. The m/z tolerance was set to 0.02 m/z in order to prevent the generation of duplicate features due to variations in peak m/z between samples. The RT tolerance was set to 0.25 min, which was large enough to cover the maximum variation in peak retention times between samples. A weighting of 3 and 2 were given to the m/z and retention time settings, respectively. Like the tolerance settings in previous steps, narrower tolerances would result in duplicate features while wider tolerances would combine unique features together. The settings used were the narrowest tolerances required in order to correctly align the internal standard and other targets across all samples without any duplicates.

Gap-filling. This step detects and integrates features that were not detected in every sample. For example, if feature A was only detected in samples 1 and 3, but not 2, gap-filling would re-analyse sample 2 in an attempt to detect and integrate the missing peak. The same RT and m/z range method was used with an m/z tolerance of 0.02 m/z. This setting was the minimum required in order to correctly integrate the missing internal standard peaks from the deconvolution step (Figure 5.4C) without any peak splicing or merging. Gap-filling does not have a noise filter threshold, however. Thus, many of the gap-filled peaks were very small and may constitute noise. These features were removed in a subsequent filtering step.

Validation. The current aligned and gap-filled feature list contained 7423 features. To validate the procedure, the peak areas of benzoylecgonine, 5-HT, and MDMA, as detected and integrated by MZmine in all 336 samples, were compared to the peak areas of these analytes as determined by the targeted integration of these analytes as carried out in Chapter 4. Viewing this data as on a scatter plot revealed a group of outliers where 5-HT was incorrectly integrated (merged peaks) in a small group of samples. This was resolved by reducing the m/z tolerance in the gap-filling step to 0.01 m/z. The final methods resulted in a linear relationship between the peak areas of the three compounds, as detected by each method (D2 5-HT: $R^2 = 0.983$, 5-HT: $R^2 = 0.988$, MDMA: $R^2 = 0.999$).

Filtering and final feature list. In order to filter out bad peaks, noise, and other non-relevant features, features that were not detected in at least 50% of samples were removed. Further, features that were initially detected in a small number of samples, but subsequently gap-filled in many other samples were removed, as determined by a detected:gap-filled ratio of 0.33. After filtering, the final feature list contained 737 features.

Data analysis

All processed metabolomics data were normalised to the internal standard (peak area), log-transformed in order to achieve a normal distribution, and Pareto scaled so as to avoid highly abundant features such as MDMA from dominating the statistical models, as is recommended for MS metabolomics data (Livera et al., 2015; Sysi-Aho, Katajamaa, Yetukuri, & Orešič, 2007; van den Berg, Hoefsloot, Westerhuis, Smilde, & van der Werf, 2006; Veselkov et al., 2011; Wheelock & Wheelock, 2013). The goal of the data analysis was to identify metabolites of interest based on three criteria. 1) The metabolite should be relevant to MDMA administration, and its relative concentration should change in a dose-dependent manner. 2) The metabolite should be relevant to behaviour, and its concentration should correlate in some way with locomotor activity. 3) The metabolite should be relevant to MDMA pretreatment, and its concentration should change in some way as a function of repeated MDMA exposure. This was achieved by using a combination of PCA, OPLS, and OPLS-DA models as well as follow-up univariate statistics.

First, an overview of the data was provided by PCA, which was carried out using all 737 variables (detected features/metabolites) and all 336 samples (observations). PCA data was evaluated by examining score plots for potential groupings and component loadings to identify relevant variables.

Criteria 1 was assessed by two separate OPLS regression models with the Y variable as MDMA response (peak area) on all samples collected from either the saline or MDMA pretreated rats. Score plots were examined to determine grouping based on acute MDMA administration (baseline, 0.0, 5.0, 10.0 mg/kg). Loading plots were examined, and a ranked list of variables with a predictive Variable Importance in Projection (VIP) score of > 1 (Galindo-Prieto et al., 2014, 2015) was generated, which represented the variables/metabolites that were most correlated and predictive of the MDMA response.

Criteria 2 was similarly assessed by two separate OPLS regression models, but with the Y variable as locomotor activity for all samples collected from either the saline or MDMA pretreated rats. Score plots were examined to determine grouping based on acute MDMA administration (baseline, 0.0, 5.0, 10.0 mg/kg). Loading plots were examined, and a ranked list of variables with a predictive VIP score of > 1 was generated, which represented the metabolites that were most correlated and predictive of locomotor activity.

Criteria 3 was assessed in two ways. First, was by examining Shared and Unique Structures (SUS) plots, where the predictive loadings of variables from two models (i.e. saline

vs MDMA pretreated) are plotted against each other (Wiklund et al., 2007). SUS plots were generated for the pair of OPLS models generated for assessing criteria 1 (MDMA response) and for the pair of OPLS models generated for assessing criteria 2 (locomotor activity). Variables that were differentially predictive of the MDMA response or locomotor activity in either pretreatment group were noted. Second, three separate OPLS-DA models with dummy *Y* variables coded as pretreatment group (saline vs MDMA) were generated for the baseline/0.0 samples, the MDMA 5.0 samples, and the MDMA 10.0 samples. Score plots were examined to determine grouping based on pretreatment group. Ranked lists of variables/metabolites that were relevant to the separation of saline and MDMA pretreated rats from these three models were generated based on predictive VIP scores that were > 1 .

Finally, to determine variables that meet all three criteria, the variables that were relevant to class separation (criteria 3) were filtered by those that were also relevant to the MDMA response (criteria 1) and locomotor activity (criteria 2). Univariate statistics (*t*-tests, ANOVAs) on remaining variables of interest were carried out. All multivariate analyses were carried out using the Soft Independent Modelling of Class Analogies software (SIMCA; v16, Umetrics) while univariate analyses were carried out using SPSS (v25, IBM). All multivariate models were autofitted by SIMCA, and the default K-fold cross-validation procedure was performed. The quality of the model was determined by examining the cumulative R^2X (close to 1), which represents the explained variation in *X*, R^2Y (close to 1), which represents the explained variation in *Y*, and Q^2 (above 0.5), which represents the predicted variation in *Y* (Szymańska, Saccenti, Smilde, & Westerhuis, 2012; Triba et al., 2015; Wheelock & Wheelock, 2013). The level of significance for all tests was $p < .05$.

Metabolite annotation and MS/MS

The variables/metabolites of interest were tentatively annotated by examining the mass of all observable adduct ions ($M+H$, $M+Na$, $M+NH_4$ etc.) and any fragments generated by in-source fragmentation (Gabelica & Pauw, 2005; Sumner et al., 2007; Xu, Lu, & Rabinowitz, 2015). Because most metabolites were derivatised, the mass of suspected metabolites following derivatisation was calculated and were compared to the results from two studies that have previously characterised the BzCl derivatisation and primary MS/MS fragments of several metabolites from microdialysis samples (Song et al., 2012; Wong et al., 2016).

To provide a more confident annotation (Sumner et al., 2007), targeted MS/MS was carried out on selected metabolites of interest and fragmentation patterns were compared to the METLIN database (C. A. Smith et al., 2005). Following the initial LC-MS analysis, remaining

volumes of microdialysate samples were pooled across all subjects into baseline, MDMA 0.0, MDMA 5.0, and MDMA 10.0 fractions. Triplicate 10 μ L injections from each pooled sample were then injected using the same HPLC procedure as described in Chapter 4. Targeted Q-TOF ESI-MS/MS was carried out with the following parameters: positive ion mode; gas temperature, 275 $^{\circ}$ C; gas flow, 8 L/m; nebulizer, 30 psi; capillary voltage, 2750 V; nozzle voltage, 0 V; fragmentor voltage, 130 V; acquisition rate, 2 spectra/s; mass range, m/z 50 – 500; collision-induced dissociation energy, 10, 20, and 40 eV.

Results

An overview of the data was provided by PCA on the metabolite profile (737 features/variables) from all 336 samples, $R^2X = 0.90$, $Q^2 = 0.85$. The PCA was visualised by plotting the component scores of each individual sample. Figure 5.5A shows the scores plot of each sample for component 1 versus component 2. Some outliers were present as determined by the ellipse representing the 95% confidence interval for Hotelling's T^2 test. Samples that cluster together represent those with a similar metabolomic profile. No grouping of the data was observed based on class/MDMA pretreatment group or time/acute MDMA dose (colour-coding not shown in graph). The most apparent grouping was based on individual subjects/analysis batches, suggesting the primary variation in the data is uninduced and stems from sampling, preparation, or analytical variation. Figure 5.5B shows the scores plot of each sample for component 1 versus component 3. Some outliers were also present in this plot. Grouping was observed based on time/acute MDMA dose, with MDMA 5.0 and 10.0 samples being separated from the baseline and MDMA 0.0 samples by the 3rd component. Figure 5.5C is a histogram displaying the loading of each variable (i.e. metabolite) on component 3 as a function of retention time. Several variables had a strong negative loading on component 3 and were driving the observed separation based on MDMA dose. These included MDMA itself (and several adducts/fragments), 5-HT, and what was suspected to be 3-methoxytyramine (3-MT), MDA, HHMA, HMMA, and HMA (see Figure 5.12 for a summary of the various variables/metabolites of interest and explanatory examples referred to throughout this results section).

Figure 5.5. Results of principal components analysis on the metabolite profile of all 336 microdialysis samples. A) Score plot of component 1 versus 2 showing colour-coded groupings based on subject/analytical batch. B) Score plot of component 1 versus 3 showing colour-coded grouping based on acute MDMA dose (baseline [BL], 0.0, 5.0, 10.0 mg/kg). C) Histogram showing variable loadings for component 3 as a function of retention time. Various annotated metabolites of interest are labelled.

Identification of metabolites that were relevant to MDMA administration (criteria 1) was achieved by separate OPLS models for each treatment group with the Y variables as the measured MDMA response, saline: $R^2Y = 0.98$, $Q^2 = 0.97$; MDMA: $R^2Y = 0.98$, $Q^2 = 0.96$. Figure 5.6 shows the score plots for these models with $t[1]$ being the predictive component and $t[2]$ being the orthogonal component plotted on the x- and y-axis, respectively. Grouping of samples was observed based on acute dose of MDMA. Figure 5.7 shows the loading plots of these two OPLS models with each variable plotted as a function of their predictive ($p[1]$) and orthogonal ($pso[1]$) loadings on the x- and y-axis, respectively. Variables/metabolites that are plotted high on the x-axis represent variables that were most positively predictive of the MDMA response such as the adducts, fragments, and metabolites of MDMA, 5-HT, and 3-MT. Variables that are plotted low on the x-axis represent variables that were most negatively predictive of the MDMA response such as 387.17 m/z. Variables plotted high or low on the y-axis represent variables that had high variability that was orthogonal to the MDMA response and thus likely not of interest, such as 307.17 m/z. DOPAC had both a moderately negative predictive and orthogonal loading. Variables plotted near the centre represent variables with little variation in any direction and were similarly not of interest, such as 301.14 m/z. 155 variables had a predictive VIP score of > 1 .

Figure 5.6. Score plots generated from two OPLS models with Y as MDMA response for microdialysate samples collected from saline (left) and MDMA (right) pretreated subjects. Colour-coded grouping based on dose of acute MDMA administration (baseline [BL], 0.0, 5.0, 10.0 mg/kg) is shown.

Figure 5.7. Loading plots generated from two OPLS models with Y as MDMA response for microdialysate samples collected from saline (top) and MDMA (bottom) pretreated subjects. Various annotated metabolites of interest and other examples are labelled.

Identification of metabolites that were relevant to behaviour (criteria 2) was achieved by separate OPLS models for each pretreatment group with the Y variables as locomotor activity, saline: $R^2Y = 0.45$, $Q^2 = 0.29$; MDMA: $R^2Y = 0.61$, $Q^2 = 0.48$. The quality of both models was borderline, and less than the previous models using the MDMA response as Y, but the results were largely similar. Some small differences were observed, however, such as 3-MT having a greater predictive loading for locomotor activity relative to 5-HT. Figure 5.8 shows the score plots for these models with grouping based on the acute dose of MDMA. Figure 5.9 shows the loading plots for these models with similar results to the previous models using MDMA response. 156 variables had a predictive VIP score of > 1 .

Figure 5.8. Score plots generated from two OPLS models with Y as locomotor activity for microdialysate samples collected from saline (left) and MDMA (right) pretreated subjects. Colour-coded grouping based on dose of acute MDMA administration (baseline [BL], 0.0, 5.0, 10.0 mg/kg) is shown.

Figure 5.9. Loading plots generated from two OPLS models with Y as locomotor activity for microdialysate samples collected from saline (top) and MDMA (bottom) pretreated subjects. Various annotated metabolites of interest are labelled.

Identification of metabolites that were relevant to MDMA pretreatment (criteria 3) was assessed in two different ways. First, SUS plots were examined, which compared the predictive variable loadings for the MDMA or locomotor activity response between the saline and MDMA pretreatment groups. Figure 5.10 shows these SUS plots with the predictive correlational loadings (pcorr) for the MDMA group plotted on the x-axis and the saline group plotted on the y-axis. Variables in the upper right quadrant are those that were positively predictive of the MDMA or locomotor activity response in both the saline and MDMA pretreatment groups while those in the lower-left quadrant were negatively predictive in both groups. Variables that deviate from the linear diagonal pattern are those that are predictive of the MDMA/locomotor activity response in one model (i.e. pretreatment group), but not the other. Neither plot showed any such variables, however, suggesting that any group differences were subtle. To further investigate any group differences, three separate OPLS-DA models with dummy *Y* variables coded as MDMA pretreatment group (saline vs MDMA pretreatment) were generated for baseline/0.0, MDMA 5.0, and MDMA 10.0 samples, baseline/0.0: $R^2Y = 0.94$, $Q^2 = 0.71$; MDMA 0.0: $R^2Y = 0.99$, $Q^2 = 0.86$; MDMA 10.0: $R^2Y = 0.92$, $Q^2 = 0.68$. Figure 5.11 shows the resulting score plots with a clear separation between the pretreatment groups on the x-axis (predictive component) and large within-group variation on the y-axis (orthogonal component). 218, 195, and 222 variables had a VIP score > 1 at the baseline/0.0, MDMA 5.0, MDMA 10.0 timepoints/doses, respectively.

Finally, variables that meet all three assessment criteria were identified. The variables that were most relevant to MDMA pretreatment at the baseline/0.0, MDMA 5.0, MDMA 10.0 timepoints/doses (criteria 3) were filtered by the variables that were also most relevant to the MDMA response (criteria 1) and to locomotor activity (criteria 2) based on VIP scores that were > 1 . This left 40, 44, and 61 variables of interest at the baseline/0.0, MDMA 5.0, MDMA 10.0 timepoints/doses that met all three criteria. Many of these variables represented adducts or fragments of the same metabolite, however. Thus, any fragments or adducts that were identified were removed. Some of the variables on this list also had high orthogonal loadings, with highly variable responses between the subjects, and thus, these variables were also removed. A summary of the few remaining unique variables/metabolites/features of interest as well as other notable metabolites discovered throughout the analysis and the assessment criteria that each variable met is shown in Figure 5.12. Follow-up univariate statistics revealed that there were significant increases in relative HMA concentrations at two time points as a function

of MDMA pretreatment (see Figure 5.12, $p < .05$). All other follow-up univariate tests were not significant, $p > 0.5$.

Figure 5.10. Shared and unique structures (SUS) plots comparing predictive correlational loadings (pcorr) for the MDMA response (left) and locomotor activity (LMA; right) between the saline (y-axis) and MDMA pretreated groups (x-axis). Some annotated metabolites of interest are labelled.

Figure 5.11. Score plots generated from three OPLS-DA models at baseline [BL]/0.0 (left), MDMA 5.0 (centre), and MDMA 10.0 (right) time-points/doses with a dummy Y variable coded as pretreatment group (saline vs MDMA). Colour-coded grouping based on pretreatment group is shown.

Figure 5.12. Summary of the metabolites of interest and other explanatory examples. Mean relative concentration (or locomotor activity [LMA]; + standard error of the mean) is plotted as a function of time/acute MDMA administration for subjects pretreated with saline (dashed line) and MDMA (solid line). MDMA 0.0, 5.0, and 10.0 mg/kg i.p. was administered at 120, 240, and 360 min, respectively. The m/z and annotation of each derivatised metabolite is shown at the top of each graph. Legends represent whether the metabolite was positively (+), negatively (-), or neutrally (·) predictive of the MDMA response (criteria 1), LMA (criteria 2), or MDMA pretreatment (criteria 3). * $p < .05$.

Metabolite annotation

298.14 m/z, 385.15 m/z, and 394.12 m/z were known to be benzoylated MDMA, 5-HT, and DOPAC, respectively, from the use of external standards as described in Chapter 4. Targeted MS/MS was used to annotate other selected features of interest. 384.12 m/z was confidently annotated as benzoylated MDA. The fragmentation patterns of MDA were very similar to MDMA (Figure 5.13) and to those recorded on the METLIN database. 376.15 m/z was annotated as benzoylated 3-MT, giving the same primary fragment of 151.07 m/z as that recorded in METLIN as well as 105 m/z, which is the characteristic fragment of all benzoylated metabolites, including 3-MT (Wong et al., 2016). Less conclusive annotation was given to the other features. 404.18 m/z, 494.19 m/z, and 390.17 m/z were suspected to be benzoylated HMMA, HHMA, and HMA respectively, based on the m/z of the multiple detected ions/adducts. Their MS/MS fragmentation patterns are not recorded in METLIN, but HMMA gave a primary fragment of 269.12 m/z, which would be expected if it fragmented in the same location as MDMA. No prominent fragments other than 105 m/z were observed for the other suspected MDMA metabolites, however. The proposed fragmentation of selected annotated metabolites is shown in Figure 5.14. 320.09 m/z has the same mass as benzoylated vanillylmandelic acid (VMA; Wong et al., 2016), an end-stage metabolite of catecholamines, but no fragmentation data for this metabolite or any of the other metabolites of interest were obtained.

Figure 5.13. Q-TOF ESI MS/MS spectra of benzoylated MDMA (left) and MDA (right) at 10 (top), 20 (middle), and 40 (bottom) eV collision energies.

Figure 5.14. Proposed fragmentation of selected metabolites of interest.

Discussion

In the current study, an untargeted metabolomics procedure was developed to evaluate behaviourally relevant changes in the neuro-metabolomic profile of rats as a function of repeated MDMA exposure. Microdialysis samples were collected from the medial striatum at 30 min intervals following the administration of ascending doses of MDMA from subjects pretreated with either saline or a behaviourally sensitising regimen of repeated MDMA exposure. During this time, locomotor activity was also measured. Metabolites of interest were assessed by PCA, OPLS, and OLPS-DA based on three criteria. 1) The metabolite was relevant to acute MDMA administration, and its concentration changed dose-dependently over time. 2) The metabolite was relevant to MDMA-produced behaviour, and its concentration was predictive of locomotor activity. 3) The metabolite was relevant to repeated MDMA exposure, and its concentrations at baseline or following acute MDMA administration changed as a function of MDMA pretreatment. While numerous metabolites met some of these criteria, very few met all three.

Several metabolites were found to be relevant to acute MDMA administration, and their concentration changed dose-dependently over time (criteria 1). Unsurprisingly, the metabolites that were most predictive of the measured MDMA response were the adducts and fragments of MDMA, MDA, and what was suspected to be HMMA, HHMA, and HMA. Other metabolites that were dose-dependently impacted by acute MDMA administration included 5-HT, DOPAC, and 3-MT, as has been previously demonstrated in mice or rats (Baumann, Clark, Franken, et al., 2008; Baumann, Clark, & Rothman, 2008; Górska & Gołembiowska, 2015; Gough et al., 2006; Kankaanpää et al., 1998; Kurling et al., 2008; Nair & Gudelsky, 2004; O'Shea et al., 2005; Shankaran & Gudelsky, 1999). Several unknown metabolites were also dose-dependently impacted by acute MDMA administration including 320.09 m/z, 345.19 m/z, 296.13 m/z, and 387.17 m/z. One of these unknown metabolites (320.09 m/z) may have been VMA, an end-stage metabolite of catecholamines. Another one of these metabolites (387.17 m/z) decreased in a remarkably dose-dependent manner with acute MDMA administration. This, however, might be an artefact of ion suppression since this metabolite co-eluted with the highly abundant MDMA at 4 min.

Several metabolites were also determined to be predictive of MDMA-produced locomotor activity (criteria 2). Unsurprisingly, these metabolites were similar to those that met criteria 1 since locomotor activity also increases dose-dependently with MDMA administration. MDMA and its metabolites were most strongly predictive of locomotor activity, followed by 3-MT and 5-HT. MDMA has long been known to produce dose-dependent increases in locomotor activity, as was demonstrated in Chapter 4. 5-HT has also been previously implicated in the acute locomotor activating effects of MDMA (Berger et al., 1992; Callaway, Rempel, Peng, & Geyer, 1992; Callaway, Wing, & Geyer, 1990; Hekmatpanah & Peroutka, 1990; Kehne et al., 1996), while 3-MT is the primary metabolite of DA, which is well known to be an important driver of MDMA and other drug-produced locomotor activity (Ball, Budreau, & Rebec, 2003; Bubar, Pack, Frankel, & Cunningham, 2004; Daniela et al., 2004; L. H. Gold, Hubner, & Koob, 1989; Kehne et al., 1996). It should be noted that in pilot metabolomics analyses of samples collected from smaller populations of rats throughout the method development described in Chapter 4 (when DA was able to be reliably measured), DA was consistently one of the metabolites most predictive of MDMA-produced locomotor activity (data not shown).

The concentration of numerous metabolites was also predictive of repeated MDMA exposure. The relevance of many of these metabolites to the development and expression of

behavioural sensitisation was questionable, however. Metabolites that were not also impacted by acute MDMA administration and predictive of MDMA-produced locomotor activity were unlikely to be relevant. Thus, these metabolites were excluded, and only those that met all three assessment criteria were examined further.

The final results suggest potential changes in MDMA metabolism. Although the concentration of MDMA itself did not change as a function of repeated MDMA exposure, the concentration of some of the metabolites of MDMA did change with repeated exposure and some met all three assessment criteria (see Figure 1.1 for review on MDMA metabolism). All detected metabolites, MDA, HMA, HHMA, and HMMA, increased dose-dependently with acute MDMA administration (criteria 1), as would be expected, and were positively predictive of locomotor activity (criteria 2). Concentrations of MDA, one of two primary metabolites of MDMA, were higher following 10 mg/kg MDMA in pretreated rats, while concentrations of HMA, a metabolite of MDA, were also higher following 5 and 10 mg/kg MDMA in pretreated rats (criteria 3). These results were mirrored, although non-significantly, by marginal decreases in HHMA, the second primary metabolite of MDMA, and HMMA, a metabolite of HHMA, in MDMA pretreated rats. These results might suggest an increase in one MDMA metabolism pathway relative to the other as a function of repeated MDMA exposure. MDA is a particularly potent psychostimulant in itself and has been shown to be more effective at stimulating locomotor activity than equivalent doses of MDMA (20 mg/kg i.p.; Bexis & Docherty, 2006). Thus, increased extracellular concentrations of MDA might contribute to the sensitised behavioural response observed in the current study. HMA and the other metabolites of MDMA have been previously shown to have a minimal direct effect on locomotor activity, however (Schindler, Baumann, Thorndike, Blough, & Goldberg, 2012; Yeh & Hsu, 1991). The pharmacokinetics of MDMA and its metabolites (MDA, HHMA, HMMA) in rat serum have been previously examined prior to, and following, the self-administration of a moderate amount of MDMA (Bradbury et al., 2013). Although there were no significant differences in any of the measures, the authors did note a tendency for greater values in rats that acquired MDMA self-administration on some measures. Further research with a targeted approach would be needed to elucidate the current findings and determine the role of changes in MDMA metabolism in behavioural sensitisation.

The final results also suggest potential changes in DA metabolism. Concentrations of 3-MT increased dose-dependently with acute MDMA administration, were positively predictive of locomotor activity, and were marginally higher following the administration of

10 mg/kg MDMA in MDMA pretreated rats. This might suggest a sensitised DAergic response following MDMA pretreatment, as was suggested in Chapter 4, and as has been previously demonstrated following sensitising regimens of repeated MDMA exposure (Kalivas et al., 1998; Morgan et al., 1997). In contrast, concentrations of DOPAC decreased dose-dependently with acute MDMA administration, were negatively predictive of locomotor activity, and were decreased at baseline and following MDMA administration in MDMA pretreated rats. It should be noted that the sample preparation procedures used in the current study were not ideal for measuring DOPAC, however, as was described in Chapter 4, and large variation was observed in DOPAC measurements. Nevertheless, these results are consistent with what was found throughout the method development phase and with previous studies (Gough et al., 2006; Nash, 1990).

Another metabolite (345.19 m/z) also met all three criteria. The concentration of this metabolite increased dose-dependently with acute MDMA administration, was positively predictive of locomotor activity, and was decreased during baseline/0.0 conditions in MDMA pretreated rats. This metabolite was not identified or annotated, however. Of course, it needs to be stated that most of the final results discussed here were not significant with follow-up univariate statistics, and thus, these findings should be interpreted with caution. Nonetheless, the current research serves as a proof of concept and demonstrates the potential power of a microdialysis and LC-MS-based time-series metabolomics procedure that incorporates behavioural measures.

CHAPTER 6: GENERAL DISCUSSION

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MDMA/ecstasy is a popular recreational drug of abuse. Like other drugs of abuse, a proportion of MDMA/ecstasy users develop symptoms characteristic of a SUD. Despite this, the behavioural and neurobiological effects of repeated MDMA exposure are relatively less well understood than other drugs. The purpose of the current research was to investigate behaviourally relevant neuroadaptations that develop with repeated MDMA exposure in rats.

Summary of findings

In Chapter 2, the accumulation of the transcription factor, Δ FosB, following chronic self-administered MDMA, was mapped across numerous brain regions using IHC. MDMA self-administration produced an accumulation of Δ FosB in several of the same brain regions as repeated exposure to other drugs of abuse. These brain regions are known to play crucial roles in the development and maintenance of drug addiction. Striatal regions that are involved in goal-directed behaviour (NAcc, DMS) showed a particularly pronounced accumulation of Δ FosB. Brain regions involved in several other important aspects of addiction, such as executive functioning and conditioning also showed increased Δ FosB expression. These regions included the CeA, BLA, and most major subdivisions of the PFC. It has been suggested that MDMA self-administration progresses as a function of neuroadaptive responses in the same brain regions that mediate the progression of self-administration of other drugs of abuse (Schenk, 2011). The present data are consistent with this idea and also suggest that Δ FosB accumulation in several brain regions that are relevant to addiction is a common consequence of repeated exposure to drugs of abuse.

One of the key findings from Chapter 2, was the region-specific increases in striatal Δ FosB; significant increases in Δ FosB levels were observed within the ventral and dorsomedial regions, but not the dorsolateral regions. In Chapter 3, the idea that these region-specific increases in striatal Δ FosB were relevant to the sensitisation of MDMA-produced behavioural responses following repeated exposure was determined. Following repeated, systemic MDMA exposure, local administration of MDMA into the ventral or dorsomedial striatum, but not the dorsolateral striatum resulted in the expression of behavioural sensitisation. These results mirrored those from Chapter 2. Together, these findings suggested that MDMA-produced

increases in Δ FosB within these brain regions might be involved in the development and/or expression of sensitised responses to MDMA following repeated exposure, as has been shown with other drugs of abuse (Brenhouse & Stellar, 2006; Colby et al., 2003; Conversi et al., 2007; Grueter et al., 2013; Kaplan et al., 2011; Kelz et al., 1999; Marttila et al., 2006; McDaid et al., 2006; Zachariou et al., 2006).

In Chapter 4, a targeted investigation of the more specific neurochemical correlates of sensitised MDMA-produced behaviour was carried out. A procedure for the quantification of extracellular medial-striatal concentrations of 5-HT, DA, and MDMA was developed that utilised microdialysis and LC-MS. Repeated MDMA exposure had no effect on extracellular concentrations of MDMA following acute systemic administration, suggesting that behavioural sensitisation and any resulting neurochemical changes were not due to differences in MDMA uptake. Baseline or MDMA-produced 5-HT was not significantly impacted by repeated MDMA exposure and did not correlate well with sensitised locomotor activity suggesting that MDMA-produced 5-HT release in the medial striatum is not an important mechanism by which repeated MDMA exposure results in behavioural sensitisation. In contrast, extracellular concentrations of DA at baseline and following MDMA administration were enhanced following repeated exposure and correlated well with sensitised locomotor activity. Unfortunately, these DA results were incomplete and were only collected from a small number of subjects. Nonetheless, these tentative findings are consistent with those found previously using a similar dosing regimen of repeated MDMA exposure (Kalivas et al., 1998) and with the general documented role of DAergic mechanisms in behavioural sensitisation to other drugs of abuse (for reviews see Pierce & Kalivas, 1997; T. E. Robinson & Berridge, 1993; Vanderschuren & Pierce, 2010; Vezina, 2004).

In Chapter 5, untargeted metabolomics was used in an attempt to identify novel or unexpected compounds that are relevant to MDMA behavioural sensitisation. A procedure for the analysis of behavioural data in combination with time-series metabolomics data collected via microdialysis was developed. The goal was to identify metabolites that 1) were dose-dependently impacted by acute MDMA administration, 2) were relevant to MDMA-produced behaviour, and 3) were impacted by repeated MDMA exposure. The results primarily served as a proof of concept, highlighting the potential power of a microdialysis and LC-MS-based time-series metabolomics procedure that incorporates behavioural measures. Nonetheless, some potentially interesting results were obtained. Potential changes in one pathway of MDMA metabolism relative to the other were observed. Potential changes in DA metabolism were also

observed, which would be consistent with the tentative findings of Chapter 4 and with previous literature (Colussi-Mas et al., 2010; Kalivas et al., 1998; Morgan et al., 1997; Schenk, 2011; van de Wetering & Schenk, 2017). Some potentially interesting unknown metabolites were also identified.

Relevance and value of findings

Self-administration and Δ FosB

Repeated exposure to drugs of abuse produces a plethora of neuroadaptations that, in ways not yet fully understood, foster continued and escalating drug-seeking/taking behaviour. Identification of these neuroadaptations and a comprehensive understanding of how they underlie such addictive behaviour is a crucial step towards the development of therapeutic interventions that can prevent or reverse these maladaptive neuroadaptations and restore normal behaviour. Like most other research with this overarching goal, the current research employed animal models to investigate the neuroadaptations that develop with repeated MDMA exposure.

Long-access self-administration sessions and an escalation of drug-intake have been suggested to be of particular importance for the comprehensive and valid modelling of a SUD since these conditions have been shown to exacerbate, or are often necessary for, the development of several features that are characteristic of a SUD (Ahmed, 2011; Ahmed & Koob, 1998; Ahmed et al., 2000; Edwards & Koob, 2013; Kitamura et al., 2006; O'Dell et al., 2007; A. J. Roberts et al., 2000). It is for these reasons that the first experiment of the current research employed a minimum of 29 long-access (6 hour) MDMA self-administration sessions to identify brain regions that have undergone significant neuroadaptive change, as measured by Δ FosB accumulation. These self-administration conditions resulted in a significant escalation of MDMA-intake and have been previously shown to produce extensive drug- and cue-produced MDMA-seeking (Schenk et al., 2016, 2011).

The current research demonstrated, for the first time, the impact of repeated MDMA exposure on the accumulation of Δ FosB in a number of brain regions that have been implicated in addiction. Δ FosB has been suggested to be particularly relevant to addiction for a number of reasons (for reviews see McClung et al., 2004; Mews et al., 2019; Nestler, 2008; Nestler et al., 1999; Robison & Nestler, 2011). To summarise, the protein is induced in the brain by repeated exposure to virtually all drugs of abuse (now also including MDMA). Because Δ FosB is highly stable, it can persist in the brain for many weeks or even months following stimulus exposure, rather than a few hours like most transcription factors. This allows the Δ FosB to accumulate at

very high levels following repeated drug exposure and have a long-lasting impact on gene transcription. Further, Δ FosB has been shown to be both sufficient and necessary for the development of multiple behavioural phenotypes that are relevant to addiction, such as sensitisation. Lastly, Δ FosB has also been identified as an important, and in some cases, a crucial mediator of many relevant neuroadaptations produced by repeated exposure to drugs of abuse.

There is evidence to suggest that this last point may also hold true for MDMA. For example, repeated MDMA exposure increased dendritic spine density and spine count on MSN in the NAcc and increased spine density on distal dendrites of output neurons in the mPFC; an effect that was accompanied by sensitised behavioural responses to MDMA (Ball et al., 2009). These effects might be mediated by Δ FosB since Δ FosB has been shown to mediate the transcription of numerous genes involved in dendritic spine formation and structural plasticity (Bibb et al., 2001; Maze et al., 2010; Nestler, 2008, 2012; Robison & Nestler, 2011; Winstanley et al., 2009) and increased Δ FosB was observed within these brain regions following repeated exposure in the current research.

Other neuroadaptive responses produced by repeated MDMA exposure may also be mediated by Δ FosB. Repeated, intermittent, MDMA exposure upregulated the expression of GluR2, which codes for an AMPA receptor subunit, in the cortex and caudate as well as CaMKII, which phosphorylates various glutamate receptors, in the hippocampus (Kindlundh-Högberg, Blomqvist, Malki, & Schiöth, 2008; Moyano, Del Río, & Frechilla, 2005; Moyano, Frechilla, & Del Río, 2004). Glutamate receptors have been implicated in the rewarding effects of MDMA (García-Pardo, Miñarro, & Aguilar, 2018; García-Pardo, Miñarro, Llansola, Felipo, & Aguilar, 2019) as well as other psychostimulant drugs (Kauer & Malenka, 2007; Kelz et al., 1999; T. E. Robinson & Kolb, 2004) and the expression of both of these proteins has been shown to be positively mediated by Δ FosB (Bibb et al., 2001; Kelz et al., 1999; McClung & Nestler, 2003; Peakman et al., 2003). Repeated MDMA exposure also produced changes in the expression of the opioid peptide, dynorphin, in various brain regions including the striatum and VTA (Di Benedetto et al., 2011; Di Benedetto, D'Addario, Candeletti, & Romualdi, 2006). Dynorphin regulates DAergic output from the VTA and the gene that encodes dynorphin is similarly mediated by Δ FosB (Bruchas et al., 2010; Zachariou et al., 2006). These findings, together with those from the current research, suggest that Δ FosB might mediate several neuroadaptive responses to repeated MDMA exposure that are relevant to sensitisation and reinforcement.

Behavioural sensitisation and the acquisition of self-administration

It has long been known that repeated exposure to some drugs of abuse can result in the persistent sensitisation of their behavioural effects such as locomotor activity (Downs & Eddy, 1932; Seevers & Tatum, 1931). Evidence implicating the relevance of this phenomenon to addiction has accumulated over the years and can be summarised by three main points (for reviews see Berridge & Robinson, 2016; Pierce & Kalivas, 1997; T. E. Robinson & Berridge, 1993, 2008; Vanderschuren & Pierce, 2010; Vezina, 2004). First, repeated drug exposure under conditions that result in behavioural sensitisation can also sensitise the reinforcing effects of drugs, as evidenced by the facilitated acquisition of self-administration, higher breakpoints on PR schedules, and increased CPP. Second, manipulations that prevent the development of behavioural sensitisation also prevent the sensitisation of the reinforcing effects of drugs. Third, the neural mechanisms underlying the development and expression of these sensitised responses appear to overlap considerably and involve various neuroadaptations in brain regions such as the PFC, VTA, NAcc, and dorsal striatum.

The subsequent experiments of the current research utilised a behavioural sensitisation procedure to further investigate some of the brain regions that differentially expressed Δ FosB following MDMA self-administration. The pretreatment regimen of repeated MDMA exposure used to induce behavioural sensitisation in the current research has been previously shown to also sensitise the reinforcing effects of MDMA, as indicated by the facilitated acquisition of self-administration (van de Wetering & Schenk, 2017). It would, therefore, be expected that the results of the current research are also relevant to the acquisition of MDMA self-administration.

The current research demonstrated that local injections of MDMA into the dorsomedial and ventral striatum, but not the dorsolateral striatum, resulted in the expression of behavioural sensitisation following repeated systemic exposure. These results would suggest that the ventral and dorsomedial striatum are also important for the sensitised acquisition of MDMA-self-administration. This might be expected given the documented role of these brain regions in goal-directed behaviour (Corbit et al., 2012; Ito et al., 2002; Murray et al., 2012; Vanderschuren et al., 2005; Zapata et al., 2010). As previously mentioned, Δ FosB expression within these brain regions may also be important and might mediate neuroadaptive responses such as changes in dendritic spine density that are associated with the development of these sensitised responses (Ball et al., 2009). This has been previously demonstrated with other drugs of abuse. An increase in both the locomotor activating and reinforcing effects of cocaine was

observed following striatal or accumbal overexpression of Δ FosB (Colby et al., 2003; Kelz et al., 1999), and this was accompanied by an increase in the number and density of dendritic spines in D₁-type MSN (Grueter et al., 2013).

The current research also provided some insight on the neurotransmitter systems that are involved in the development and expression of sensitised responses to MDMA. Baseline or MDMA-produced medial-striatal 5-HT concentrations were not impacted by repeated MDMA exposure, nor did they correlate well with sensitised locomotor activity. These results would suggest that 5-HT is similarly not important for the sensitised acquisition of MDMA self-administration. The role of 5-HT in the development and expression of sensitised responses to MDMA has not been previously examined. However, there is evidence for an inhibitory role of 5-HT in the normal acquisition of MDMA self-administration. Rats with a greater NAcc 5-HT response to MDMA (3 mg/kg, i.v.) were less likely to subsequently meet acquisition criteria for MDMA self-administration (Bradbury et al., 2013) while 5,7-DHT lesions (Bradbury et al., 2013) or a genetic mutation of the 5-HT transporter (Oakly et al., 2013) greatly facilitated the acquisition of MDMA self-administration. Given that repeated MDMA exposure can produce various 5-HTergic deficits, deficits that would be expected to facilitate the acquisition of MDMA sensitisation, the results of the current research were somewhat surprising. One explanation is that the challenge doses used in the current study, although ideal for assessing behavioural sensitisation, were not ideal for assessing changes in MDMA-produced 5-HT or the reinforcing effects of self-administered MDMA. As previously discussed, these doses used were near the top end of the dose-effect curve for MDMA-produced 5-HT release and transporter binding (Battaglia, Brooks, et al., 1988; Green et al., 2003; Rudnick & Wall, 1992; Schmidt et al., 1987). These doses are also much higher than what is typically received during self-administration (0.5 or 1.0 mg/kg/infusion). Thus, a lower dose may have been more appropriate for revealing any potential 5-HT deficits that are relevant to changes in the reinforcing efficacy of self-administered MDMA. Another, more parsimonious explanation is that the regimen of exposure in the current study simply had no significant effect on extracellular medial-striatal 5-HT and other mechanisms underlie the previously observed sensitised acquisition of MDMA self-administration (van de Wetering & Schenk, 2017).

One such likely mechanism is the sensitisation of central DAergic mechanisms, which have been demonstrated to underlie the sensitisation of the reinforcing effects of other drugs of abuse following repeated exposure (Cador et al., 1995; Kalivas & Weber, 1988; Pierre & Vezina, 1998; Vezina, 1993, 1996; Vezina et al., 2002; Vezina & Stewart, 1989). Like other

drugs of abuse, DAergic mechanisms play an important role in the acquisition of MDMA self-administration. For example, rats that met acquisition criteria for MDMA self-administration displayed greater MDMA-produced extracellular striatal DA compared to rats that failed to acquire self-administration (Colussi-Mas et al., 2010). This suggests that the development of a sensitised DAergic response to MDMA might contribute to the acquisition of MDMA self-administration. In support of this idea, previous studies have shown that MDMA-produced DA release (Kalivas et al., 1998) and tissue levels of DA (Mayerhofer et al., 2001) were increased following repeated exposure. Cross-sensitisation to both the reinforcing (Fletcher, Robinson, & Slippoy, 2001) and DA-releasing (Kalivas et al., 1998; Morgan et al., 1997) effects of cocaine has also been observed following repeated MDMA exposure. In the current research, DA concentrations at baseline as well as following MDMA administration appeared to be enhanced following repeated exposure and correlated well with sensitised locomotor activity. Additional changes in multiple DA metabolites were also apparent. These tentative results would suggest that the previously observed sensitised acquisition of MDMA self-administration (van de Wetering & Schenk, 2017) might be similarly due to sensitised DAergic mechanisms, as has been shown with other drugs of abuse (Berridge & Robinson, 2016; Vanderschuren & Pierce, 2010; Vezina, 2004).

Metabolomics

Only a handful of previous studies have used untargeted metabolomics to examine the effect of repeated exposure to drugs of abuse (see Table 5.2 for a summary). In one study, the effect of withdrawal from repeated morphine treatment was examined using nuclear magnetic resonance (NMR; Deng et al., 2012). Several changes in metabolites related to energy or neurotransmission (glutamate/GABA) were observed, and compellingly, some of these changes were reversed following methadone or clonidine intervention. The same research group also found several changes in metabolites related to energy or neurotransmission (glutamate/GABA/acetylcholine/tryptamine) following a regimen of nicotine exposure that resulted in CPP (H. Li et al., 2014). Another lab group used gas-chromatography MS (GC-MS) to examine the effect of repeated heroin (Zheng et al., 2013) or methamphetamine (Zheng et al., 2014) exposure. In these studies, multiple samples were collected during and after drug treatment in order to provide some temporal information on any metabolomic changes. Following repeated heroin exposure, changes in the concentration of metabolites associated with the tricarboxylic acid cycle were found as well as changes in 5-HT and tryptophan, which had restored to baseline following 4 days of withdrawal (Zheng et al., 2013). Following

repeated methamphetamine exposure, multiple changes in metabolites related to energy metabolism and neurotransmission (glutamate/aspartate) were also found, most of which had similarly restored to baseline following 2 days of withdrawal (Zheng et al., 2014). Another study examined the effect of chronic alcohol vapour exposure on the metabolome using direct injection MS (Meinhardt et al., 2015). Notable results included changes in levels of DA and Met-enkephalin. In a recent study, the effect of self-administered heroin on the metabolomic profile of serum samples collected after a drug-induced reinstatement session was examined (Ning, Leng, Chen, Ma, & Gong, 2018). Several metabolites, including those related to the tricarboxylic acid cycle, keto bodies, and neurotransmitter precursors (choline, glutamine, and phenylalanine), were impacted compared to drug-naïve controls. In another study, the effect of repeated morphine, methamphetamine, or cocaine exposure, under conditions that produced CPP, was examined (Zaitsu et al., 2014). Consistent with other studies, they found changes in the concentrations of metabolites related to the tricarboxylic acid cycle following morphine treatment. The concentration of several other metabolites (e.g. 3-hydroxybutyric acid, L-tryptophan, cystine) was also impacted depending on the drug.

Table 5.2.

Summary of metabolomics studies

Citation	Subjects	Drug	Sampling	Analysis
Deng et al. (2012)	Rhesus monkey	Morphine	Tissue from hippocampus and PFC	NMR
Li et al. (2014)	Mouse	Nicotine	Tissue from hippocampus, PFC, and striatum	NMR
Zheng et al. (2013)	Rat	Heroin	Serum and urine	GC-MS
Zheng et al. (2014)	Rat	Methamphetamine	Serum and urine	GC-MS
Meinhardt et al. (2015)	Rat	Ethanol	Tissue from PFC and striatum	MS
Ning et al. (2018)	Rat	Heroin	Serum	NMR
Zaitsu et al. (2014)	Rat	Morphine Methamphetamine Cocaine	Serum and urine	GC-MS

These studies illustrate the advantages of the procedure developed in the current research. In all of these studies, a complete lack or very poor temporal information was obtained due to the sampling procedures used (e.g. brain tissue or anaesthetised serum collection). When some temporal information was provided, multiple class-based comparisons (i.e. PLS-DA or OPLS-DA) were carried out at each time-point, and the temporal information was rarely incorporated into the analyses. Further, few studies took behavioural measures, and for those that did (e.g. CPP and reinstatement), these data were not factored into the analyses.

The use of microdialysis as a sampling procedure for untargeted metabolomics analysis, as was done in the final experiment of the current research, is a novel approach and can provide important and relatively high-resolution temporal information. Further, microdialysis samples can be collected from awake, freely moving subjects, allowing for the concurrent collection of behavioural data. This provides the means to examine both metabolomic and behavioural changes with reasonable temporal resolution in response to acute drug or other stimulus exposure (e.g. stress, conditioned cues, etc.). Given appropriate statistical analyses, both time-series metabolomics data, as well as behavioural data, can be used to help to identify metabolites that are the most relevant to the phenotype of interest (e.g. MDMA-produced behavioural sensitisation), as was demonstrated in the current research. Although the current procedures still needed further development, the current results do serve as a proof of concept. The current approach is unique not only in the addiction field, but also in other fields, and may be useful for examining both temporally and behaviourally relevant changes in the metabolome in response to various disease or treatment conditions.

Limitations

One possible limitation of the current research is that a yoked control group was not included in the Δ FosB study. This group would receive the same amount of i.v. MDMA exposure as MDMA self-administering rats, but non-contingently. This would allow any differences in Δ FosB expression as a function of contingent vs non-contingent drug exposure to be examined. It is unsure whether this would have been expected to have much of an effect, however, based on previous studies. No differences in Δ FosB accumulation in the NAcc core, shell, the C-P, or the medial PFC were observed in cocaine self-administering rats compared to yoked controls (Larson et al., 2010; Perrotti et al., 2008; Winstanley et al., 2007) with the only exception being a more pronounced increase in Δ FosB accumulation in the OFC (Winstanley et al., 2007).

It should also be mentioned that some of the experimental groups in the current research had a low sample size, notably within Chapters 2 and 3. While the sample sizes in Chapter 2 are indeed low, they are not uncommon and many comparable studies have had similar sample sizes (e.g. Cornish et al., 2012; Hope et al., 1994; Y. Kim et al., 2009; Perrotti et al., 2005; Pich et al., 1997). Importantly, the variation in both the independent (MDMA self-administration) and dependent (Δ FosB) variables is small, as is clearly shown in the figures. The effects are also large and significant in most cases, even after correction for multiple comparisons. In Chapter 3, some of the experimental groups also had a low samples size. Again, the variability in these data are small. The results are also supported by two different behavioural measures. Therefore, while it should still be acknowledged, this represents is a minor limitation.

Another possible limitation of the current research is the limited time periods at which neurochemical and behavioural data were collected. Behavioural sensitisation was assessed, and microdialysis samples were collected following 2 days of withdrawal from the MDMA pretreatment regimen. This time period was chosen in order to keep the procedures consistent and relevant to previous research where the same pretreatment regimen and withdrawal period was used to assess the effect of repeated MDMA exposure on the reinforcing effect of MDMA (van de Wetering & Schenk, 2017). If such neurochemical and behavioural assays were carried out after longer withdrawal periods, it might be expected that both neurochemical and behavioural sensitisation would become more pronounced, as has been shown with other drugs of abuse (Kalivas & Duffy, 1993b, 1993a; Paulson & Robinson, 1991, 1995; Vanderschuren et al., 1999). It also might be expected that different neuroadaptations may develop with protracted withdrawal, which may also be of interest (Pierce & Kalivas, 1997; Vanderschuren & Kalivas, 2000; Wolf, 1998).

There are also some limitations related to the use of microdialysis as a sampling procedure, as was done in Chapters 4 and 5. The temporal resolution of microdialysis (minutes) is worse compared to *in vivo* fast-scan cyclic voltammetry (seconds), which can also be used to measure extracellular concentrations of target analytes (D. L. Robinson, Venton, Heien, & Wightman, 2003). This procedure cannot be used to measure multiple analytes simultaneously, however, which would make it unsuitable for the current purposes. Another limitation of microdialysis is that very few brain regions can be sampled simultaneously. This was why the brain region sampled from in the current research was chosen based on the regions of interest identified in Chapters 2 and 3. Other procedures, such as IHC or tissue analysis, could be used to investigate multiple brain regions simultaneously. Of course, these other procedures have

their own limitations, such as a complete lack of temporal resolution. These procedures do provide much higher concentrations of analytes, however, which would make the analysis of some low concentration compounds easier (e.g. 5-HT).

In Chapter 4, a procedure for the quantification of multiple neurochemical targets was developed given the available resources, equipment, and time. There were several limitations to this procedure, however, and several improvements could still be made. First, the use of D2 internal standards was not ideal due to the isotopic overlap with the target analytes. D5 or $^{13}\text{C}_6$ BzCl is widely available and could be used to derivatise regular chemical standards to produce physiochemically similar internal standards for most target analytes, as has been done previously (Song et al., 2012; Wong et al., 2016). This would prevent the need to purchase or produce separate internal standards for each target analyte and also prevent the need for any isotopic dilution calculations. Second, refinement of the sample and/or the mobile phase buffers might help optimise the pH conditions for the derivatisation, retention, and ionisation of more target analytes, such as DOPAC and 5-HIAA. Although these analytes were originally able to be quantified, they were not able to be reliably detected using the final procedures, which were ultimately optimised for the detection of 5-HT. Adjusting the buffers as well as the BzCl percentage used for derivatisation may also help with the stability of the target analytes such as DA (Song et al., 2012; Wong et al., 2016). Finally, the targeted analyses may have been better suited to a triple quadrupole MS (QQQ), which are generally more appropriate for the quantification of known target analytes than Q-TOF MS. QQQs have greater analytical sensitivity and would have less trouble detecting and quantifying the low concentration of 5-HT. Additionally, QQQs have a greater linear range, which may have prevented the issues in the current study where concentrations of 5-HT were close to the detection limits, but the concentrations of MDMA were too high and saturating the MS detector. It should be noted that Q-TOF MS has very accurate mass detection, however, and are the preferred instrument for untargeted approaches such as those carried out in Chapter 5.

There are also some limitations and improvements that could be made to the untargeted metabolomics procedures. First and foremost, the extensive optimisation of the sample preparation and analytical procedures, as was carried out in Chapter 4, was focused on the targeted quantification a few metabolites, namely 5-HT, and not untargeted metabolomics. As a result, the ‘metabolomic net’ that was cast with the current procedures was very small. Numerous metabolites that may have been of interest that should be detected within benzoylated microdialysis samples such as glutamate or GABA (Song et al., 2012; Wong et

al., 2016), were not detected in the current samples. Given more inclusive sample preparation and HPLC methods, more metabolites could have been detected and analysed, and a more comprehensive analysis of the neuro-metabolome could have been carried out. Second, although BzCl has several advantages for targeted procedures (Song et al., 2012; Wong et al., 2016), it does make the identification of unknown metabolites more complicated, since there are no databases of benzoylated metabolites to compare the results with. Lastly, there was sizeable uninduced variation in the metabolomic profile in the current study. This was evidenced by the results of the PCA and the large orthogonal separation in the OPLS models. Interestingly, these models separated samples that were analysed prior to, and following, a lengthy period of LC-MS maintenance and sample storage at -80°C, which may have been the cause for the loss of the DA data in Chapter 4. These results suggested that the majority of the variation in the data was due to variations in the sample preparation, storage, or analytical procedures. Thankfully, the statistical models used in the current procedure are capable of dealing with such variation.

Future directions

One of the most interesting findings of the current research was the region-specific increases in Δ FosB in the striatum following MDMA self-administration. Significant increases in Δ FosB were observed in brain regions that are known to be involved in goal-directed behaviour, but not in brain regions that have been implicated in stimulus-driven behaviour (i.e. habitual behaviour). This might suggest one of two things; either Δ FosB does not mediate neuroadaptive responses related to the development of habitual behaviour or that MDMA self-administration in the current research was not habitual.

The latter has not been directly assessed and could be investigated with the use of reward devaluation procedures. A significant decrease in MDMA-taking or seeking behaviour following reward devaluation would suggest that the behaviour is not habitual, but still goal-driven. In the current study, over 29 sessions of 6-hour MDMA self-administration were carried out, with approximately 1300 reinforced responses made per subject. Given that habitual self-administration of other drugs of abuse has been demonstrated with lesser amounts of training (Clemens et al., 2014; Corbit et al., 2012; Dickinson et al., 2002; Miles et al., 2003; Olmstead et al., 2001; Zapata et al., 2010), it would be expected that MDMA self-administration in the current study would be similarly habitual.

If this were indeed the case, then the current results would suggest that Δ FosB does not mediate neuroadaptive responses related to the development of habitual behaviour. Δ FosB is

capable of mediating neuroadaptive changes within these brain regions that would be expected to influence both goal-directed and habitual behaviour, however (Nestler, 2008; Robison & Nestler, 2011). Thus, to elucidate the role of Δ FosB in the development of habitual drug-taking/seeking behaviour, future research could correlate Δ FosB accumulation in these brain regions with the degree to which subjects are susceptible to reward devaluation following drug self-administration. Alternatively, the effect of selective overexpression of Δ FosB in these brain regions on habitual drug-taking or seeking behaviour, as assessed by reward devaluation, could be examined.

Another valuable avenue for future research would be to continue the development of the untargeted metabolomics procedures and apply them to identify both behaviourally and/or temporally relevant changes in the metabolome that are associated with various treatment or disease conditions. Regarding drug addiction, these procedures could be used to identify metabolites that are relevant to drug-seeking responses, for example, or to identify relevant differences in the metabolite profile of drug self-administering subjects versus yoked controls. Because untargeted metabolomics is a relatively novel analytical approach in the addiction field, there are multiple interesting avenues for future research using such procedures.

Further optimisation of the procedures would be needed, however, particularly to the sample preparation and analysis procedures. As previously mentioned, this would include the optimisation of the sample and mobile phase buffers, the percentage of BzCl used for derivatisation, the chromatography gradient, and the type of column used in order to increase the number of metabolites able to be detected and analysed. This would provide a much more comprehensive analysis and the casting of a much larger ‘metabolomic net’. Another improvement could be to use more than one internal standard. D2 5-HT may not be a useful internal standard for some metabolites with different physiochemical properties. D5 or $^{13}\text{C}_6$ BzCl could be used to make a range of internal standards to provide better normalisation methods for a wider range of diverse metabolites (Sysi-Aho et al., 2007). Improvements to the data analysis procedure could also be made. For example, subtracting the response from blank samples (aCSF) from the dialysate samples and including more replicates may help to clean up the data. Further, the adducts and in-source fragments of highly abundant compounds could be identified and filtered prior to statistical analysis. This could be achieved using MZmine and algorithms such as CAMERA (Kuhl, Tautenhahn, Böttcher, Larson, & Neumann, 2012) or with subsequent time-series cluster analyses (Rusilowicz et al., 2018).

Conclusions

Drug addiction is a chronic, relapsing disorder characterised by compulsive drug-seeking and drug-taking behaviour despite adverse consequences. Drug addiction exacts an enormous medical, financial, and emotional toll on society and there is an unmet need for new prevention and treatment strategies. In order to develop these strategies, however, a better understanding of the various factors underlying harmful drug use and the development of SUDs are required.

Given the persistent and relapsing nature of SUDs, an important focus of addiction research recently has been to investigate the mechanisms by which maladaptive, drug-induced neuroadaptations are maintained over such long periods of time, even after drug exposure has ceased. Recent evidence has highlighted the important role of the transcription factor, Δ FosB, in mediating the development of such persistent drug-induced neuroadaptations involved in perpetuating addictive behaviour (Robison & Nestler, 2011). The findings of the current research add to this growing body of evidence, indicating that repeated, chronic exposure to MDMA, like other drugs of abuse, induces a persistent accumulation of Δ FosB in several brain regions implicated in addictive behaviour. Future research may extend these fundamental findings to develop novel treatments for addiction that target transcriptional-related mechanisms such as Δ FosB. As has been previously suggested, the presence of Δ FosB in certain brain regions may also prove to be a useful marker of addiction and could be used for monitoring the progress of treatment in the future (Nestler, 2008).

The results of the current thesis suggest that repeated MDMA exposure induces many of the same neuroadaptations that result from repeated exposure to other drugs of abuse. These include increases in levels of Δ FosB in several relevant brain regions as well as potential changes in DAergic mechanisms. It has been suggested that MDMA self-administration progresses as a function of neuroadaptive responses in the same brain regions that mediate the progression of self-administration of other drugs of abuse (Schenk, 2011). The present data are consistent with this idea. The present data also support the idea that repeated MDMA exposure induces neuroadaptations that render both the pharmacological and behavioural profile of MDMA increasingly similar to that of other psychostimulants such as methamphetamine (Schenk, 2011). This results in a drug that has far greater reinforcing efficacy (van de Wetering & Schenk, 2017), and thus, greater abuse liability with repeated use. This has important implications given the popularity of MDMA as a recreational drug of abuse and the more recent use of MDMA as a pharmacological adjunct to post-traumatic stress disorder psychotherapy.

Finally, the application of untargeted metabolomics in the addiction field has exciting potential as a method for generating new research avenues and identifying novel therapeutic targets. The research carried out in this thesis represents the first step of this process. The use of microdialysis as a sampling procedure combined with sophisticated multivariate statistical methods allows for the identification of neurochemicals that are both temporally and behaviourally relevant to changes in disease state. This novel approach may also be of value for other preclinical research disciplines and certainly warrants future development.

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Cheers.