UNIVERSITY OF LEICESTER

# THE MECHANISM OF PLASTICITY IN MESOILIMBIC DOPAMINE FUNCTION UNDERLYING BEHAVIOURAL AND NEUROCHEMICAL SENSITISATION

Thesis submitted for the degree of

Doctor of Philosophy

at the University of Leicester

By

**Athar Naser Hussein**

Department of Neuroscience, Psychology and Behavioural

University of Leicester

May 2018

# Abstract

**The Mechanisms of Plasticity in Mesolimbic DA Function Underlying Behavioural and Neurochemical Sensitisation-Athar Hussein**

**Background:** Repeated psychostimulant exposure results in progressive and enduring behavioural sensitisation modelling increased drug craving observed in human psychostimulant abusers.

**Aims**: This project aimed to evaluate mechanisms underlying sensitisation-related neuroadaptations during repeated nicotine or amphetamine administration, and to investigate whether behavioural sensitisation to each drug employed the same or different processes.

**Methods:** Behavioural sensitisation during repeated daily nicotine (0.6 mg/kg) or amphetamine (1 mg/kg) administration was measured, compared to saline control (1 ml/kg). Male Lister-hooded rats received five daily injections of drug or saline and locomotor activity was recorded. Ten days later, rats were challenged with the same doses of drug/saline and their locomotor activity was measured. Using immunohistochemistry, we measure expression of immediate early genes (IEG), activity-regulated cytoskeleton-associated protein (Arc) and *c-fos*, and of Methy1-CpG-binding-protein 2 (MeCp2) and brain-derived neurotrophic factor (BDNF), in nucleus accumbens (NAc) sub-regions, and ventral tegmental area (VTA). Finally, effects of drug pre-treatment on dopamine receptor gene expression in NAc and VTA, and on dopamine release in NAc shell and core subregions were investigated.

**Results:** Nicotine and amphetamine increased locomotor activity, during daily treatment and challenge ten days later. Behavioural sensitization, was accompanied by increased in Arc and MeCp2 expression in NAc, which differed between nicotine and amphetamine treatment, but no changes in either *c-fos*, or BDNF were observed. Although there was evidence of behavioural and immunohistochemistry cross-sensitisation from amphetamine to nicotine, no evidence for either behavioural or immunohistochemistry cross-sensitisation from nicotine to amphetamine was seen. Dopamine receptor gene expression increased in VTA after nicotine pre-treatment and NAc after amphetamine pre-treatment, but treatment history did not affect dopamine release in NAc shell or core.

**Conclusions:** Behavioural sensitization was accompanied by increases in IEG, MeCp2, and dopamine receptors gene expression, which differed between nicotine and amphetamine suggesting that sensitisation to the two drugs occurs through separate mechanisms, perhaps involving increased neuronal plasticity within different sub-regions of NAc and VTA.

## Acknowledgements

It is been an absolute gift to undertake this massive journey, a lot of work has gone into this thesis, and not only from me. May I first thank my wonderful supervisor Dr. Andrew Young for both practical and theoretical guidance of the very high quality, for always being up for discussion on any given subject and without him I cannot do anything. I would like to express my sincere gratitude to everyone who has repeatedly encouraged me and lifted my spirits through my PhD, especially during the first year. I am grateful to have had the opportunity to conduct this study, with special thanks to Dr Juan Canales. I would also like to thank and express my gratitude to the College of Life Sciences and all people working there and in Neuroscience, Psychology and Behavioural department at University of Leicester, for being great friends and for all their help, we have had the greatest times. I would like to thank, Dr Will Norton, Dr James McCutcheon, Dr Todor Gerdjikov for helpful discussions, and Dr Kees Straatman for his helping on immune images. Also, I would like to thank the Preclinical Research Facility team at Leicester University for their helping during the behavioural experiments. Thank you to all Andrew's and Will's lab members and students of bygone years, especially to Peters Kate and Tilley Ceinwen, who, showed me the way at the beginning and taught me the way of the lab. Personal and special thanks to my wonderful family and friends for their continued understanding and support. Thank for my Mum, sisters and brothers for their support, encouragement, I am forever grateful. Finally, I would express my thanks to my government, Iraq government and my University, Al-Qadisiyah University for giving me my an amazing opportunities, make me happy and live a full life.

**Contents**

# List of Tables



# List of Figures

# List of Abbreviations

| | |
|---|---|
| **ABC** | Avidin /Biotin complex |
| **AC** | Adenylcyclase |
| **aCSF** | Artificial cerebral spinal fluid |
| **ADHD** | Attention deficit hyperactive disorder |
| **AMPA** | α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid |
| **AMPH** | Amphetamine |
| **ANOVA** | Analysis of variance |
| **Arc** | Activity-regulated cytoskeleton-associated protein |
| **ATP** | Adenosine triphosphate |
| **BDNF** | Brain-derived neurotrophic factor |
| **$Ca^{2+}$** | Calcium |
| **cAMP-** | Cyclic aminomethyl-phosphate |
| **CDK-5** | Cyclic-dependent kinase-5 |
| **cDNA** | Complementary DNA |
| **CNS** | Central nervous system |
| **COMT** | Catechol O-methyltransferase |
| **CREP** | cAMP response element binding protein |
| **CRE** | cAMP response element |
| **CRF** | Corticotrophin releasing factor |
| **DA** | Dopamine |
| **DAB – 3** | , 3-diaminobenzidine tetrahydrochloride |
| **DAT** | DA transporters |
| **DNA** | Deoxyribonucleic acid |

| | |
|---|---|
| **DOPAC** | 3, 4-dihydroxyphenylacetic acid |
| **ERK1/2** | Extracellular-regulated kinase first/second |
| **FSCV** | Fast scans cyclic voltammetry |
| **GABA** | γ-aminobutyric acid |
| **GPCRs** | Gs-protein coupled receptors |
| **5HT- 5** | Hydroxytryptamine; serotonin |
| **$H_2O$** | Water |
| ***$H_2O_2$*** | *Hydrogen peroxide* |
| **HVA** | 3-methoxy-4-hydroxyphenylacetic acid |
| **IEGs** | Immediate early gene |
| **IgG** | Immunoglobin |
| **i.p** | Intraperitoneal |
| ***$K^+$*** | *Potassium* |
| **KCl** | Potassium chloride |
| **L-DOPA** | L-dihydroxyphenylalanine |
| **LDT** | Lateral dorsal tegmentum |
| **LHb** | Lateral habenula |
| **LH** | Lateral hypothalamus |
| **LTP** | Long-term potentiation |
| **MAPK** | Mitogen-activate protein Kinase |
| **MAO** | Monoamine oxidase |
| **MeCp2** | Methy1-CpG binding protein 2 |
| **mPFC** | Medial prefrontal cortex |
| **$Na^+$** | Sodium |

| | |
|---|---|
| **NAc** | Nucleus accumbens |
| **nAChRs** | Nicotinic acetylcholine receptors |
| ***NaCl*** | *Sodium Chloride* |
| **NGS** | Normal goat serum |
| **NIC** | Nicotine |
| **NMDA** | *N*-methyl – *D*-aspartatic acid |
| **RNA** | Ribonucleic acid |
| **PB** | Phosphate buffer |
| **PBS** | Phosphate buffer saline |
| **PFA** | Paraformaldehyde |
| **PFC** | Prefrontal cortex |
| **PKA** | Protein kinase A |
| **PP-1** | Protein phosphatase -1 |
| **PPT** | Pedunculo potine tegmentum |
| **PVN** | Paraventricular nucleus |
| **qPCR** | Quantitative Polymerase Chain Reaction |
| **SAL** | Saline |
| **TH** | Tyrosine hydroxylase |
| **TrkB** | Tropomyosin receptors Kinase B |
| **VMAT** | Vesicular monoamine transporter |
| **VTA** | Ventral tegmental area |

# Chapter 1: Introduction

## 1.1. Addiction

Drug addiction or drug dependence is a neuropsychiatric disorder thought to result from neural adaptation at the molecular, cellular and tissue levels following repeated drug exposure (Nestler, 2005). It has two major characteristics: a compulsive drug-seeking behaviour and a loss of control in limiting intake (Koob and Le Moal, 2001). Drug dependence are a profound public health concern, with significant costs for affected individuals and the economy as a whole Miller and Hendrie (2008). In 2005, drug dependence cost in U.S about $468 billion and this cost results from disease, crime, accident, child abuse, domestic violence, homelessness and lost wage (Rose, 2017). In 2013, 1.5 million Americans aged 12 and over were current cocaine users (Abuse, 2014). In addition to financial costs of addiction, drug addiction, alcohol abuse, and smoking cause one in four deaths and many other serious economic and social consequences (see Figure 1). Henschke *et al.* (2006) found that lung cancer in the U.S, due to tobacco smoking, accounted for more death than any other cancer, more even then the second and third cancer killer (breast and colon cancer) combined.

To begin our introduction, we operationally define tolerance due to that repeated use of drug usually, but not always, leads to tolerance. Tolerance is a phenomenon developed by certain drug such that progressively larger and larger doses are required to maintain initial drug effectiveness. Tolerance development is a process not necessarily in all cases connected with addiction. Tolerance production is a quality of a certain of drug which produce an addiction such as morphisms, while some drug produce an addiction with no developed tolerance such as cocaine addiction, and other drug produce a tolerance without recognized symptoms of addiction such as organic nitrites (Wise and Koob, 2014). Drug dependence typically involves the initial exposure to a stimulus such as motivational stimulus followed by behavioural patterns seeking to repeat the experience. After many repetitions of the behaviour stimulus sequences, the addiction become established as compulsive pattern of drug-seeking and drug-taking behaviour (West, 2001, Robinson and Berridge, 2008). Besides drug addiction, the term "addictive behaviour" also includes eating disorders, pathological gambling and sex addiction that have been shown to effect the same neuroadaptation in the brain as drug of abuse, suggested that brain function can be similarly activated by both drug of abuse and natural reward (Avena *et al.*, 2008). The major advanced genetic, molecular and cell biology research have focused on identification the neuroadaptations that develop with chronic drug

administration to understand the accurate relationship of these neuroadaptation with a clinical fact of addiction which is remains a major challenge (Everitt *et al*., 2001). The primary outcome measure of an effective treatment of addiction is the prevention or reduction of ongoing relapse vulnerability. Thus, the important challenge for neurobiological research is to understand what the brain mechanism contribute to the transition from voluntary drug taking to the compulsive use in addiction that will provide the keys for development of pharmacotherapies to treat drug addiction.

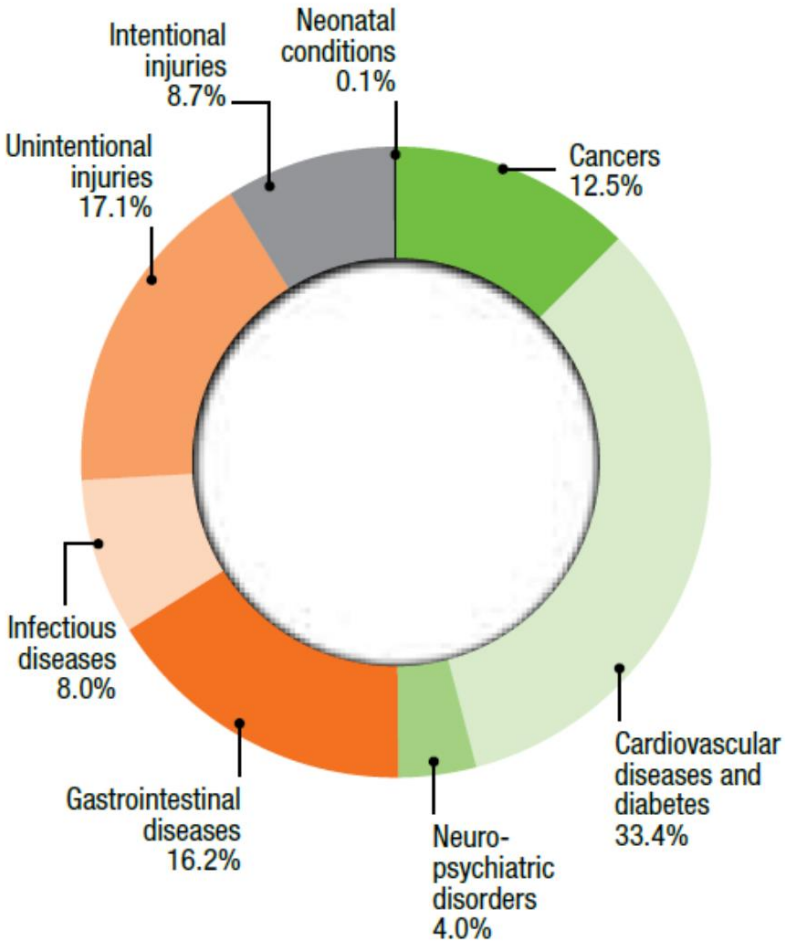



Figure 1: Distribution of alcohol-attributable deaths by disease or injury cause in 2012. Taken from World Health Organisation, 2014.

### 1.1.1Neurobiological theories of addiction

Robinson and Berridge in their review that was originally published in 1993, explained that to understand addiction, we need to understand the process by which voluntary drug-taking behaviour evolves into compulsive drug-taking behaviour. Thus, they identified three characteristic feature of addictive behaviour which need to be explained. The first is drug craving, the phenomenon that addicts intensely "want" drugs. The second is why such drug craving persists long after the discontinuation of drug use even without withdrawal symptoms such as physical or psychological distress syndromes (depression, and anhedonia). The third is that as craving for drugs increases, the pleasure derived from the taking drugs often decreases (Robinson and Berridge, 1993). A number of theories have been put forward to answer these questions and explain how and why addiction occurs.

The positive reinforcement theory of addiction (pleasure seeking or euphoria view) asserts the view that overall enjoyment of life and pleasure-seeking behaviour push drug abusers to use higher doses of drugs to improve mood and to cope with physical and emotional pain (Stewart *et al*., 1984). However, Robinson and Pritchard (1992) identified many problems that face this theory, the most important one, being that there is no clear relationship between the ability of individual drugs to produce pleasurable effect and their addictive potential. For instance, nicotine is considered highly addictive but it does not produce euphoria or other strong hedonic states: also many addictive drugs can produce strong dysphoric states especially with initial use. In addition, the euphoria view of addiction dose not explain drug craving or relapse elicited by environmental stimuli associated with drug taking.

An alternative view, the negative reinforcement theory of addiction (withdrawal avoidance), propose that drug taking is to avoid the aversive symptoms associated with withdrawal (Wise and Bozarth, 1987). In this case behaviours (drug taking and drug seeking behaviour) are reinforced, not because of the state the drugs produce, but because of the state they alleviate, in another words drug use is maintained to avoid aversive symptoms associated with withdrawal: this is termed negative reinforcement. In addition, previously neutral environmental stimuli associated with withdrawal can themselves come to elicit a withdrawal like syndrome. Thus drug may not only alleviate withdrawal syndrome but also alleviate withdrawal symptoms induced by exposure to drug-related stimuli. However, this theory also has many problems. For instance, there is high tendency to relapse even after a long period of abstinence from drugs, and after withdrawal symptoms have subsided (Robinson and Berridge,

2008). Lamb *et al*. (1991) reported that people formerly addicted to heroin pressed a lever continuously to receive a low-dose injection of morphine in the absence of withdrawal syndrome. Another problem with the negative reinforcement theory is that many drug that are used clinically, for example tricyclic antidepressants drugs (Invernizzi *et al*., 1991) produce withdrawal syndromes but are not self-administrated for non-medical purpose.

Another theory put forward by Torregrossa *et al*. (2011) termed aberrant learning and memory theory in addiction, posits that drug addiction does not occurs overnight, but rather it takes years to develop, and indeed is hypothesized to be learned. They found that strong association formed between addictive drugs, the environmental stimuli and the context in which they are grounded, it is appreciated that chronic drug exposure led to long lasting changes in the circuit underlying normal learning and memory process. Long lasting alteration in the brain function have been found in the neural circuit that are known to be responsible for normal learning and memory processes and it has been hypothesized that drugs of abuse enhance positive learning and memory about the drugs while inhibiting learning about negative consequences of drug use. Thus, over time certain cues in the environment become associated with drug use until the cues alone are sufficient to stimulate craving for the drug. However, Robinson and Bridge explained in their review in 2008 that it is important to emphasize that associative learning processes can modulate the expression of neural sensitisation in behaviour at particular places or times and not others. This is why behavioural sensitisation is often expressed in context in which the drugs have previously been experienced. However, it is important to note that learning per se is not enough for pathological motivation to take drugs and learning is only part of the processes and probably the one that contribute most to the pathological pursuit of drugs (Robinson and Berridge, 2008).

Although there are aspects of theories which fit the data, there are also aspects that do not. Robinson and Berridge (1993) took elements of each theory to develop their incentive sensitisation theory, which accounts for many of the unexplained features of the previous theories. The authors argue that the important way to explain addiction is to identify the brain changes by which drug-taking behaviours evolve to compulsive drug-taking behaviour.

### 1.1.2 The incentive sensitisation theory of addiction

The purpose of incentive sensitisation theory of addiction presented in 1993 by Robinson and Berridge was to explain a biopsychological feature of addiction. They posit that addictive behaviour is due to progressive and persistent neuroadaptation caused by repeated drug administration and manifest both neurochemically and behaviourally by the phenomena of sensitisation (Robinson and Berridge, 1993). In the incentive sensitisation theory Robinson and Berridge proposed that the progressive increase in drug wanting that characterize addiction is not accompanied by the increase in the pleasure derived from drugs, although it is usually assumed that addicts "want" drugs because they "like" drugs, and the more they like them the more they want them. The authors stated that wanting is not liking and the neural system responsible for wanting incentive is proposed separable from those responsible for liking incentive (for mediating pleasure) and repeated drug use does not sensitise the neural system responsible for pleasurable effects of drugs, only those responsible for incentive salience, transforming wanting into craving. Thus, with repeated drug use the act of drug taking and drug-associated stimuli gradually become more and more attractive and drug associated stimuli become more and more able to control behaviour because the neural system that mediate "wanting" become progressively sensitised (Robinson and Berridge, 1993).

Robinson and Berridge (1993) explained in their the incentive sensitisation theory of addiction, that repeated intermittent drug administration causes a progressive and persistent neuroadaptations in brain cells and circuits that normally regulate the attribution of incentive (motivational) salience to stimuli, a psychological process which drives motivated behaviour. The nature of these changes is to make these brain circuits hypersensitive ('sensitized') to the incentive motivational effect of the drug and of drug-associated cues which endure for long time even after the drug discontinuation. These sensitisation- related neuroadaptation have not been considered in the previous theories of addiction. The term sensitisation refers to an increase in the behavioural and neurochemical effect of drugs with repeated drug taking, and it is proposed that these changes lead, in many cases, to addiction. In addition, the authors explain that persistence of incentive sensitization makes the incentive motivation for drug stay for a long time even after the drug discontinuation and in many cases is permanent. This accounts for why addicts are hypersensitive to drugs and drug-related stimuli even after prolonged abstinence. Sensitized incentive salience can be manifest in behaviour via either implicit (unconscious) wanting or explicit (conscious) craving process and some of these

behavioural effect are increased by repeated intermittent drug administration, a phenomenon referred to as behavioural sensitisation.

## 1.2. Behavioural sensitisation

Behavioural sensitisation is a phenomenon typically characterized by a progressive, long lasting increase in locomotor activity, occurring with repeated administration of psychostimulant drugs. In contrast to tolerance which develops after psychostimulant administration in short intervals, behavioural sensitisation occurs after the administration of psychostimulant over a longer period of time. An increased response to the tested drugs can also be elicited by prior, repeated administration of different drugs: a phenomenon termed cross sensitisation. Most psychostimulant drugs exert characteristic effects on locomotor activity in laboratory animals which become progressively enhanced upon repeated treatment, making locomotor activity a suitable parameter to measure for assessment of behavioural sensitisation (Robinson and Becker, 1986, Kalivas and Stewart, 1991, Robinson and Berridge, 1993).

Behavioural sensitisation can be examined in two distinct temporal domains termed induction and expression. The induction phase is defined as the transient sequence of cellular and molecular events produced by repeated psychostimulants administration that causes changes in neural function responsible for behavioural augmentation. On the other hand, expression is defined as the enduring neural alteration arising from the induction process and directly mediating the behavioural response (Pierce and Kalivas, 1997). A variety of data show that these process are not only temporally but also anatomically distinct. Although both involve the mesolimbic dopamine (DA) pathway, projecting from ventral tegmental area (VTA) to the nucleus accumbens (NAc), the induction phase occurs at the cell bodies in the VTA, while behavioural expression is mediated in the terminal field in NAc (Vanderschuren and Kalivas, 2000). Thus, the neural events associated with expression are distributed among the interconnected nuclei of motivation circuits associated with NAc.

Although behavioural sensitisation is difficult to demonstrate in human subjects, there was a report showing enhanced responses to drugs of abuse such as D-amphetamine (AMPH) after chronic consumption (Steketee and Kalivas, 2011). Drug sensitisation is considered one of underlying mechanism responsible for increased vulnerability and relapse to substance addiction in drug dependent humans (Landa *et al*., 2014). Therefore, the development of animal models employing behavioural sensitisation is critical for investigating the mechanism

involved in development and persistence of drug addiction in human and for development of potential novel pharmacotherapies of addiction (Campbell and Carroll, 2000). To understand the neuropharmacological mechanism associated with the action of drug of abuse, we need to understand which neural circuits have a consequential role in translating the biologically relevant stimuli into adaptive behavioural responses.

### 1.2.1. Neurocircuitry underlying behavioural sensitisation

In the 1950's, James Olds and Peter Milner introduced electrodes in different brains regions of rats and subject them to a small current when they pressed a lever. In some brain regions, they found a remarkable effect: the rats would work to self-administer electrical currents (Olds and Milner, 1954). Interestingly, the animals omitted to eat or drink during the stimulations, they just pressed the lever repeatedly. It seemed that there was nothing better than this brain stimulation and they became "dependent" on the electrical stimulation. This was the first evidence for a specific "reward" centre in the brain (Olds and Milner, 1954). These brain areas were later anatomically mapped in more details and denominated the "reward system". The important role of these systems is to mediate the rewarding properties of natural stimuli vital for survival, such as foraging, drinking, breeding, and interaction with these systems is thought to underlie the action of addictive drugs (Schultz *et a*l., 1997). In support of this notion, Robinson and Berridge (1993) demonstrate that the reward systems have an important role in reward seeking due to their role in increasing the incentive value of motivational stimuli.

Initially, Wise and Rompré (1989) demonstrated that the mesocorticolimbic DA system is a common denominator of reward systems. This system consists of DAergic neurons originated from the VTA and projecting to limbic structure including NAc and amygdala (mesolimbic DA system) as well as to cortical areas, principally prefrontal cortex (mesocortical DA system). Growing numbers of studies using a variety of experimental techniques have indicated that the processes underlying induction and expression of behavioural sensitisation occur in distinct anatomical location within this reward system. First, psychostimulants act on DA cell bodies in the ventral mesencephalon including the VTA to initiate the sequence of cellular events which establishes behavioural sensitisation. Second, enhanced DA release and postsynaptic responsiveness to DA in the NAc and dorsal striatum mediated the expression of behavioural sensitisation (Kalivas and Stewart, 1991).

In addition, lesion studies have also implicated the medial prefrontal cortex (mPFC) (Del Arco and Mora, 2008), the hippocampus, amygdala, lateral-dorsal tegmentum (LDT) and the paraventricular nucleus (PVN) of the thalamus in the development of behavioural sensitisation. A recent study demonstrated that behavioural sensitisation to AMPH depends on enhanced activity of ventral hippocampal neurons that increase activity of VTA neurons (Lodge and Grace, 2008). Lesion of amygdala have been also reported to inhibit the conditioned responding for psychostimulant administration (Oliva and Wanat, 2016). Much of the study of the neural basis of behavioural sensitisation has involved evaluating changes in neurotransmission in the nuclei comprising the reward system. Once established, behavioural sensitisation is often accompanied by enhanced DA release in the NAc, a region of basal ganglia involved in many reward-related behaviours (Bromberg-Martin *et al*., 2010). Thus, the terminal regions of the mesolimbic DA system are clearly implicated in the long term expression of drug-induced sensitisation. Indeed, imbalance of DA control of plasticity in this system is a key factor in the occurrence of behavioural sensitisation. Thus, understanding the mechanism of the action of DA on VTA-NAc pathway neurons is important for understanding the mechanism underlying drug addiction, which may be lead to developing pharmacological approaches to prevent its development.

In addition to DA in NAc, it is now widely accepted that other neurotransmitters and neuromodulators are involved in behavioural sensitization to psychostimulant drug (see Figure 2). The changing in DA response in PFC was suggested to be a suitable animals model of psychotic disorders development (Steketee and Kalivas, 2011, Engel and Jerlhag, 2014). The mPFC is under DAergic modulation by the VTA and it is one of the main sources of excitatory glutamate projection to the NAc, thus it has been proposed that glutamatergic over activity in the NAc may underlie behavioural sensitisation (Casanova *et al*., 2013). In addition, behavioural sensitisation to AMPH is associated with an increase in the activity of neurons in the prelimbic cortex (Aguilar-Rivera *et al*., 2015). Moreover, the gamma-amino-butyric acid (GABA) projection between the NAc and the VTA is also implicated in the mechanism of behavioural sensitisation. Jacobson *et al*. (2016) stated that deletion of GABA receptor isoforms differentially regulates the behavioural response to cocaine. Thus, one aim of the present study was to develop and validate a method to obtain the whole mesolimbic DA pathway which will allow us to measure the effect of pharmacological manipulation of VTA on DA release in NAc.

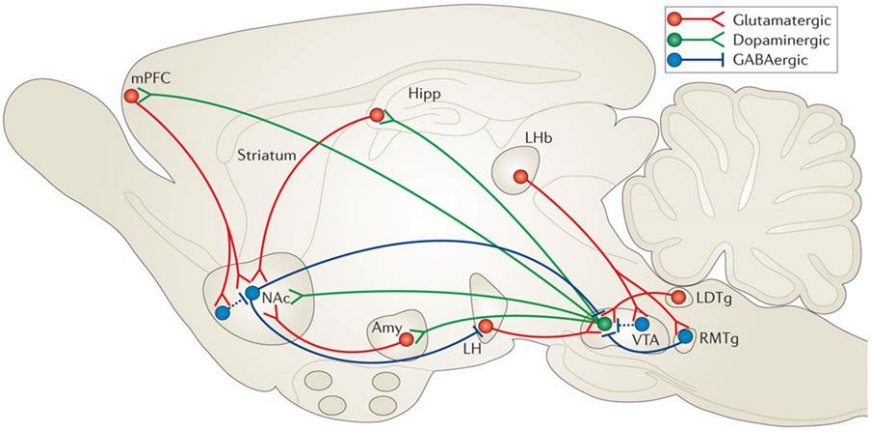



Figure 2: The brain's reward circuit involved in behavioural sensitisation. The primary reward circuit includes DAergic projections from the VTA to the NAc, mPFC, amygdala (Amy) and hippocampus (Hipp) which release DA in response to reward-related stimuli. There was also GABAergic projection from the NAc to the VTA. The NAc receives dense innervation from glutamatergic projections originating in mPFC, Amy as well as Hipp. The VTA receives such inputs from the lateral dorsal tegmentum (LDTg), lateral habenula (LHb) and lateral hypothalamus (LH) (Russo and Nestler, 2013).

### 1.2.2. Neurochemistry of behavioural sensitisation

#### 1.2.2.1- Glutamate:

Glutamate is the major excitatory neurotransmitters in the mammalian brain, and is known for its role in the neural brain functioning, including cognitive, emotion and reward, in addition to its role in neuro-development and synaptic plasticity in the central nervous system (CNS). Changes in glutamate neurotransmission are also implicated in pathological changes such as schizophrenia and drug addiction (van der Zeyden *et al*., 2008). It is responsible for the fast excitatory transmission mediated by binding of glutamate to extracellular regions of ligand-gated ion channels.

Experimental evidence has shown that glutamate efferent from PFC regulate the activity of VTA and NAc neurons. Excitatory projections from PFC to the VTA play an important role in regulating the activity of VTA neurons and extracellular level of DA within forebrain regions. Extracellular DA level within NAc and PFC are characterized by a tonic basal concentration on which phasic increases in DA level occurs in response to behaviourally relevant stimuli.

The phasic increases in extracellular DA are likely to be caused by increased burst firing of midbrain DA neurons which in turn leads to enhanced DA release in the terminals which is critically dependent on afferent input to VTA (Carr and Sesack, 2000). The DA release in the NAc is dependent, at least in part, on glutamate input from PFC to VTA, because blockade of glutamate receptors in the VTA region supresses this activity (Pehek and Hernan, 2015).

NAc neurons are regulated either directly through the excitatory input on GABA projection neurons in the NAc which has a direct connection with DA cell body in the VTA (Mora *et al*., 2008) or indirectly through the action on other neurons located in the pedunculo pontine tegmentum (PPT) and/or LDT which in turn stimulate DA neurons in the VTA projecting to the NAc. In fact, in the NAc, both DA inputs arising from VTA and cholinergic interneurons have a key modulatory action on the activity of GABA output projections neurons facilitating motor behaviour in response to different stimuli. In this context, dysfunctional regulation of DA and possibly acetylcholine release in the NAc by inputs coming from PFC could underlie behavioural abnormalities associated with psychiatric disease (Del Arco and Mora, 2008).

There are two type of glutamate receptor, ionotropic and metabotropic. Ionotropic glutamate receptors (iGluRs) include AMPA and NMDA receptor subtypes, are the main mediators of excitatory synaptic transmission in the CNS. Metabotropic glutamate (mGluRs) receptors include eight receptors, and are divided into 3 groups, both excitatory (group 1) and inhibitory (group 2 and 3) are generally play a modulatory role (Meyerson *et al*., 2016). Many studies support the idea that enhanced glutamate neurotransmission is associated with behavioural sensitisation to psychostimulant drugs. For example, repeated injection of AMPA (glutamate agonist) into the VTA enhanced cocaine sensitisation and caused increased DA and glutamate release in the NAc in addition to glutamate release in the VTA (Dunn *et al*., 2005). It is frequently reported that activity of NMDA receptors is involved in the process of behavioural sensitisation elicited by many drugs of abuse. Indeed, many articles demonstrate that administration of NMDA antagonists disrupted the development of behavioural sensitisation, e.g. (Cui *et al*., 2015, Pehek and Hernan, 2015).

### 1.2.2.2- GABA

GABA is the main inhibitory neurotransmitter in the mammalian central nervous system (CNS). Brain GABA content is greater than most other neurotransmitters being present in about 30% of all synapses (Charych *et al*., 2009). This neurotransmitter is abundant throughout the brain, including high concentration in substantia nigra, globus pallidus in the basal ganglia, hypothalamus and hippocampus. GABA regulates many neural process and is consequently involved in many neurological conditions (van der Zeyden *et al*., 2008).

Substantial evidence implicates GABA receptors in the modulation of DAergic neuro-transmission and reward. There are two types of GABA receptors: $GABA_A$ ionotropic and $GABA_B$ metabotropic receptors, which differ in term of composition, pharmacology and action. There are 19 different $GABA_A$ receptors subunits and these receptors are expressed either presynaptic/postsynaptic, extra-synaptic or both (Charych *et al*., 2009). The $GABA_A$ receptors differentially regulate the behavioural response to the drug of abuse. Morris *et al*. (2008) found that deletion of alpha-containing $GABA_A$ subunits from the mesolimbic DA pathway including NAc abolished behavioural sensitisation to cocaine in mice, while activation of these receptors led to induction of behavioural sensitisation. $GABA_B$ receptors are heterodimeric receptors consisting of $GABA_{B1}$ and $GABA_{B2}$ receptors subunit. $GABA_B$ receptors are coupled via G-protein to potassium and calcium channels as well as adenylate cyclase. The action of $GABA_B$ receptors is different depending on their location. Pre-synaptically, $GABA_B$ receptors facilitate release of neurotransmitter, including glutamate and GABA, while post-synaptically, they are more likely to generate inhibitory potential (Fatemi and Folsom, 2015)

### 1.2.2.3 Dopamine

Since the discovery of the DA by Carlsson and their colleagues in the mid of 1950s, this catecholamine neurotransmitter has received an enormous amount of attention. Important nuclei that contain DAergic neurons include the substantia nigra pars compacta and the VTA and these nuclei send projections to the neostriatum, the limbic cortex, and other limbic structures (Missale *et al*., 1998, Carlsson, 2001). DA is known to play an important role in many brain functions including voluntary movement, attention, affect, sleep, decision-making, reproductive behaviour and regulation of food intake (Missale *et al*., 1998, Rondou *et al*., 2010)

and it contributes to synaptic plasticity in brain regions such as the striatum and PFC (Jay, 2003). Dysfunction in various DAergic systems is known to be associated with various neurological and psychiatric disorders such as Parkinson's disease (PD), schizophrenia and drug addiction (Kienast and Heinz, 2006).

Due to the main role of DA in psychostimulant-induced behavioural sensitisation, it is important to explain the mechanisms of DA synthesis and metabolism. DA, like other catecholamine, is synthesised in neurons from the amino acid tyrosine. Tyrosine hydroxylase (TH), the rate-limiting enzyme of catecholamine synthesis, converts tyrosine to L-DOPA (L-dihydroxyphenylalanine), which in turn is converted to DA by the enzyme DOPA decarboxylase (or more generally aromatic amino acid decarboxylase). When the demand for neurotransmitter increases at a catecholaminergic synapse, TH is activated to make more L-DOPA which is transferred into the synaptic vesicles, after decarboxylation to DA, by the vesicular monoamine transporter (VMAT). In noradrenergic neurons, synthesis continues inside the vesicles by the action of DA-β-hydroxylase. The neurotransmitters are stored inside the vesicle until needed (Daubner *et al*., 2011). DA is metabolized by different ways including oxidative deamination catalyzed by monoamine oxidase (MAO), aldehyde reductase and aldehyde dehydrogenase, in addition to conjugation by sulfotransferases or glucuronidase and O-methylationby catechol O- methyl transferase (COMT). The main DA metabolites are 3-methoxytyramine (3-MT), 3, 4-dihydroxypheny acetic acid (DOPAC) and 3-methoxy-4-hydroxyphenylacetic acid (homouanillic acid: HVA) which is the terminal metabolite (Nagatsu and Stjärnet, 1997) (see Figure 3).

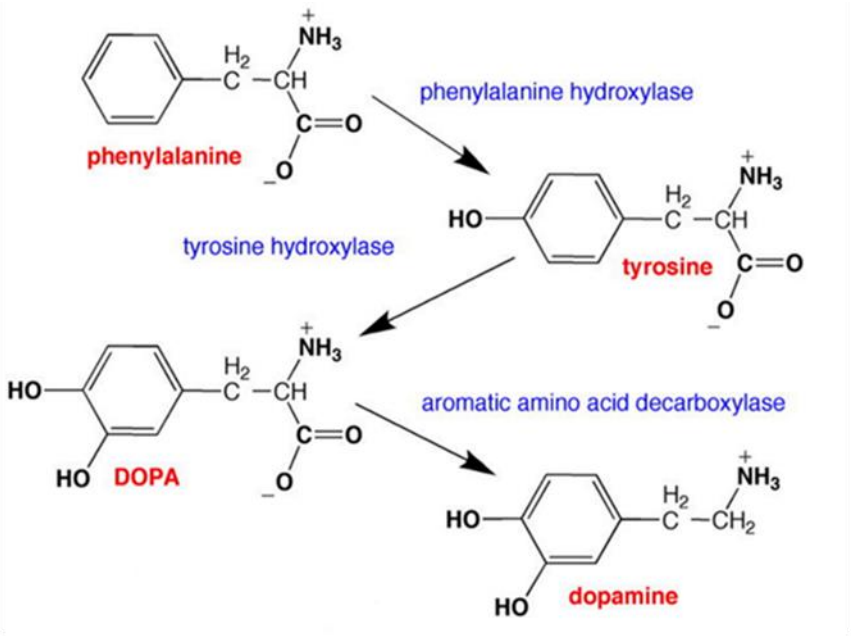



Figure 3: The DA neurotransmitters biosynthetic process. Phenylalanine hydroxylase converts phenylalanine to tyrosine which taken up from the blood into the neurons and in turn is converted to L-DOPA by tyrosine hydroxylase. L-DOPA is converted to DA by aromatic amino acid decarboxylase (Daubner *et al*., 2011).

### 1.2.2.4 Dopmaine receptors function and signaling

DA receptors signal through G protein-dependent cellular process. G proteins are comprised of alpha stimulatory or inhibitory (αs or αi), beta (β) and gamma (γ) subunit complex and upon agonist binding, DA receptors undergo conformation changes that activate Gαs or Gαi and dissociate the G protein β and γ subunits and that directly modulate downstream targets of DA receptors including second messenger and ion channels (McCudden *et al*., 2005). The physiological effects of DA are mediated by five DA receptors subtypes, divided into two major groups: the DA1-like group (DA1 and DA5) which are positively coupled to the Gs/olf and are excitatory, and the DA2-like group (DA2, DA3 and DA4) receptors, negatively coupled to Gi/o protein, which are inhibitory, (Neve *et al*., 2004). Although DA receptors are similar in structure, receptor subtypes differ in their affinity for DA, their ability to modulate the cyclic adenosine monophosphate (cAMP) pathway and their pharmacological properties. DA receptors subtypes are expressed differentially through the brain. DA1 receptors are the most abundant among DA1-like group in the mesolimbic and mesocortical area such as the striatum, substantia nigra, amygdala and frontal cortex and these receptors are mainly expressed

postsynaptically on DA target neurons such as medium spiny neurons (MSNs) in the striatum. DA5 receptor mRNA is found at low levels in the pyramidal neurons of the prefrontal cortex, substantia nigra, hypothalamus and hippocampus and at very low level in the MSNs of the striatum. The DA2 receptor are the most abundant receptor among the DA2-like group, and are expressed at highest levels in the striatum, and the olfactory tubercle, and are also abundant in the substantia nigra, and VTA and are expressed both postsynaptically on DA target neurons and presynaptically on DAergic neurons. DA3 and DA4 receptors have more limited pattern of distribution compared to DA2 receptors. DA3 receptors are expressed in the limbic area such as the shell of NAc and the striatum, substantia nigra and VTA where it is expressed both postsynaptically on DA target cells and presynaptically on DAergic neurons. The DA4 receptor has the lowest levels of expression in the brain, it's found in the NAc, frontal cortex, amygdala, hippocampus and substantia nigra pars reticulata (Missale *et al*., 1998, Romanelli *et al*., 2010).

The release of DA by natural stimuli or drugs of abuse produces changes in how neurons integrate excitatory or inhibitory neurotransmission (Kalivas and O'Brien, 2007). A significant body of evidence from pharmacological and genetic studies have focused on the functional role of DA receptors on locomotor activity. Missale *et al*. (1998) indicated that locomotor activity is controlled by DA1, DA2 and DA3. The activation of DA1 Gαs-coupled receptors expressed extensively on postsynaptic neurons has a moderate stimulatory effect on locomotor activity, while the role of DA2 and DA3 Gαi-coupled receptors are more complex because their function results from their action both pre and post-synaptically.

Generally, DA2-like autoreceptors, localized presynaptically provide an important negative feedback mechanism which regulates neural firing rate, and synthesis and release of neurotransmitters in response to extracellular level of these neurotransmitters, while, the receptors expressed postsynaptically exert a different action on locomotor activity through involvement of postsynaptic population (Missale *et al*., 1998). In addition, splice variants of DA2 receptors create isoforms differing in the insertion of a stretch of 29 amino acids in the third intracellular loop, long (DA2L) and short (DA2S) isoforms, that have a different neuronal distribution, DA2S are expressed mainly presynaptically causing a decrease in DA release and consequently a decrease in locomotor activity, while activation of DA2L which are expressed postsynaptically stimulates locomotor activity. Since DA4 and DA5 receptors have limited expression in the primary motor regions of the brain, they have minimal roles in the control of movement (De Mei *et al*., 2009).

The effect of DA receptor activation is complex and there are distinctions between activation of DA1-like vs DA2-like receptors present in pre-and-postsynaptic locations within the local circuitry controlling behaviour. Some keys events illustrate that direct activation of DA1 receptors is an important antecedent for developing the enduring changes in neural physiology that underlie establishment of additive behaviour. Importantly, these signalling cascades involve changes in gene transcription and chromatin remodelling which is thought to mediate the transition from social use to regulated and compulsive relapse (Kalivas and O'Brien, 2007, McClung and Nestler, 2008). Stimulation of DA1 receptors in the striatum and cortex stimulates the adenyl cyclase (AC), an enzyme that catalyses the conversion of adenosine triphosphate (ATB) to cAMP leading to activation of cAMP-dependent protein kinase (PKA). Since DA1-like agonist stimulate adenyl cyclase, PKA phosphorylates multiple downstream targets including the cAMP regulatory element-binding protein (CREB), cAMP-regulated phosphoprotein, 32kDa (DARPP-32), and various ion channels (Girault, 2012). Phosphorylation of CREB by PKA promotes the transcription of many genes involved in drug dependent such as *c-fos*, ΔFosB and preprodynorphine (Nestler, 2001a, McClung and Nestler, 2003b). Thus, Konradi *et al*. (1994) demonstrated that induction of the CREB factor may be an early nuclear event mediating long term neuroadaptation of administration of psychostimulants including AMPH.

The mechanism by which drugs of abuse regulate gene expression is very complex. Briefly, Romanelli *et al*. (2010) showed that activation of PKA, by DA1 receptor activation, causes phosphorylation of multiple downstream target, including CREB, DARPP-32, and various ion channels. DA1-like receptor mediated phosphorylation of CREB by PKA leads to enhance CREB-dependent transcription of numerous genes including immediate early gens (IEGs) in the NAc which is associated with synaptic plasticity and long-term changes in gene expression and synaptic function associated with drug addiction.

PKA- dependent phosphorylation of DARPP-32, a striatal-enriched phosphoprotein, at Thr34 leads to DARPP-32-dependent inhibition of protein phosphatase 1 (PP1) and inhibition of this protein is negatively regulated through cyclic-dependent kinase-5 (CDK5) induced phosphorylation of DARPP-32 at Th75, causing inhibition of PKA. In contrast, PP1 inhibition is augmented by PKA-dependent activation of phosphatase PP2A, which dephosphorylates DARPP-32 at Th75. Therefore, DARPP-32 is regulated by phosphorylation at multiple residues through complex positive and negative feedback system. DARPP-32 activates ΔFosB which regulate the expression of glutamate receptors and CDK5. PKA directly activates

voltage-ligand-gated channels or indirectly by phosphorylation of these channels through DARPP-32-mediated inhibition of PP1(Romanelli *et al*., 2010). Activation of D1-like receptors decreases $Na^+$ and $K^+$ inward rectifying current but increase L-type $Ca^{2+}$ and decreases N and P/Q type $Ca^{2+}$ channels activity (Kisilevsky *et al*., 2008, Robison and Nestler, 2011).

DA2-like receptors act oppositely to DA1-like receptors in the regulation the downstream of cAMP. DA2-like receptors inhibit adenylate cyclase through coupling to Gαi which decreases or prevents stimulation of cAMP and subsequently decreases DARPP-32 phosphorylation at Th34 and increase the DARPP-32 phosphorylation at Th75 thus causing inhibition of phosphoprotein. It should therefore be expected that this will lead to decreases CREB phosphorylation. However, in sagittal brain slices in many brain areas including neocortex, neostriatum, hippocampus and substantia nigra. DA2 receptor agonists such as quinpirole, stimulate the CREB phosphorylation presumably through activation of cAMP mechanism (Montmayeur *et al*., 1993), (see Figure 4). Thus, the role of DA receptors in behavioural sensitisation is poorly understood. Therefore, an aim of the studies reported here is to explain how the activation of DA receptors, after psychostimulant use, can induced long term neuroadaptation such as changes in gene expression. The present studies employed experiments to measure DA receptors gene expression in the NAc and VTA using real-time quantitative PCR (qPCR) after pre-treatment with nicotine or AMPH.

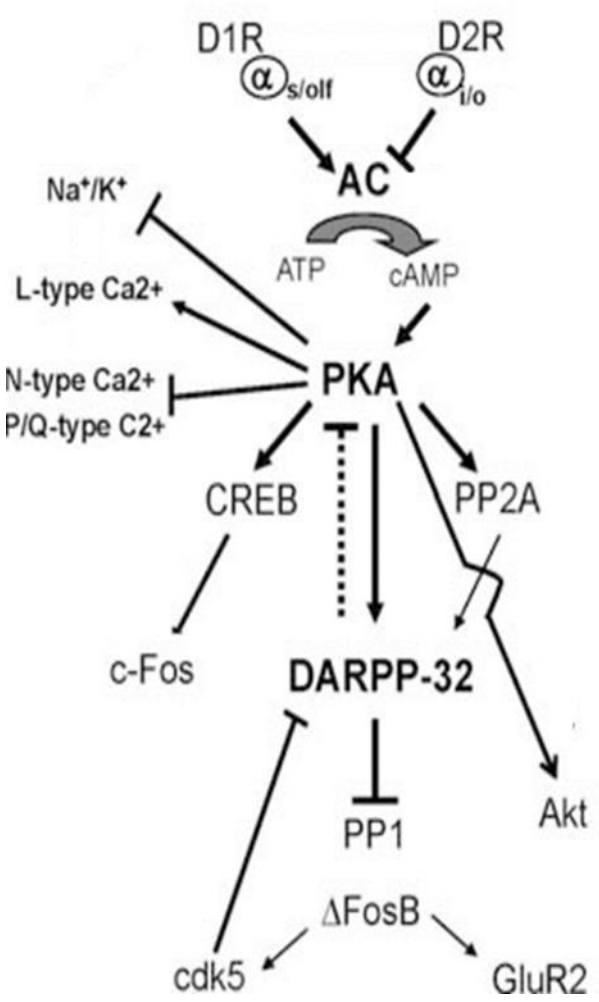



Figure 4: DA receptor G protein-mediated signalling. D1- and D2-like receptors activate multiple signalling pathways through G proteins, including the adenylate cyclase-PKA, DARPP-32 pathway, as well the extracellular-regulated kinase (ERK1/2) pathways. Although not depicted in the figure, many PKA-mediated responses involve inhibition of dephosphor-ylation via DARPP-32-mediated inhibition of PP1. The D1 receptor, in particular, influences the localization and function of AMPA- and NMDA-type glutamate receptors by both PKA-mediated phosphorylation and protein- protein interactions (Romanelli *et al*., 2010).

### 1.2.3 Molecular and cellular mechanism of behavioural sensitisation

To understand behaviour sensitisation, one must comprehend how the effect of drugs during an initial exposure leads to stable molecular and cellular changes after repeated drug exposure? Although drugs of abuse are chemically divergent molecules and they each employ distinct pharmacological mechanisms, in some cases involving transmitters system other than DA,

most of them share ability to increase extracellular DA in NAc and other target limbic regions (Kalivas and Stewart, 1991). This is therefore believed, to represent a critical step in the development of behavioural sensitisation and addiction. This increase is mediated either through increased firing of the ascending mesolimbic DA neurons consequent on activation in the VTA, leading to increase the DA release in the NAc and mPFC or by a direct action of drugs in the NAc to increase DA release. Thus, the primary action of nicotine is to activate mesolimbic neurons through cholinergic activation of the cell body in VTA (Kleijn *et al*., 2011), while the main action of AMPH is to increase release and decrease reuptake in the terminal region of the pathway in NAc (Degoulet *et al*., 2013)

Although drugs of abuse differ in their acute mechanism of action, the long-lasting nature of behavioural sensitisation suggests that chronic activation of these acute mechanism may induce some shared molecular adaptation in the mesocorticolimbic DA system and glutamatergic corticolimbic pathway (Robison and Nestler, 2011). Accumulating evidence suggests that neural plasticity changes, including synaptic plasticity, involving glutamate transmission and brain-derived neurotrophic factor (BDNF), play a critical role in psychostimulant-induced behavioural sensitisation: the robust increase in the excitability of DAergic neurons that may be attributable to persistently enhanced responsiveness in DA-innervated brain areas such as NAc and mPFC (Vanderschuren and Kalivas, 2000). For instance, Laplante *et al*. (2013) demonstrated that expression of psychostimulant-induced behavioural sensitisation is more likely to be due to postsynaptic upregulation of DAergic function. Thus, current research using microarrays and RNA- sequencing following acute and chronic exposure to drugs of abuse has demonstrated that the putative molecular basis of the behavioural sensitisation is through an alteration in expression of genes that may affect the key regions of reward (Nestler, 2014a). Thus, a key aim of the present study was to assess the importance of the DA neurotransmission for the associative components of nicotine or AMPH-induced locomotor sensitisation by employing experiments to measure the effects of pre-treatment with nicotine or AMPH on stimulated DA release *in vitro*.

### 1.2.3.1 Long term adaptation changes in behavioural sensitisation

The development of behavioural abnormalities produced by repeated drug administration reflects pre and post synaptic neuroadaptational changes, which, in many cases, persist for a long time after the final drug administration. This has led to suggestions that these neuroadaptation changes may be mediated through changes in intracellular signal transduction. A growing body of evidence indicates that psychostimulant-induced changes in gene expression occurs at an epigenetic level: that is through processes that mediate changes in gene expression by regulating chromatin structure without involved changes in DNA sequences, and that these changes contribute to the long-lasting effect of these drugs on behaviours (Nestler, 2014). Regulation of gene expression is one mechanism that should lead to stable changes within neurons. According to this scheme, repeated exposure to drug of abuse would lead to changes in nucleolar function and alteration of the transcription of target genes which in turn leads to alteration in the activity of the these neurons, ultimately causing changes in the neural circuit in which those neuron operate and subsequently to changes in behaviour (Nestler, 2001a). Much effort has been exerted to investigate the role of IEGs transcription in the mediation of psychostimulant-induced neural plasticity changes (Kodama *et al*., 1998, Yang *et al*., 2008).

The expression of IEGs is one of the first event that occurs sequentially when a cell is stimulated. These IEGs, whose transcrption is induced quickly and trainseintly within minutes of stimulation, can be classified into two groups. The first group includes genes which encode transcription factors, proteins which in response to cell signalling pathways, bind to specific sequences of DNA and regulate the expression of target genes. The second group includes genes which encode proteins which are distributed through the cytoplasm into peridendritic regions of cell and may directly modify cellular function (Morgan and Curran, 1986, Bartel *et al*., 1989). The regulation of gene expresion through the action of transcription factors has long been proposed as the classical mechnism contributing to neuroplasticity resulting from chronic exposure to drugs of abuse (Nestler, 2001a). Although neurons contain hundreds of transcriptional factors, studies of neuroadaptation induced by drugs of abuse have focused primarily on a small subset of cAMP and Gs- protein coupled receptor (GPCRs) related pathway transcription factors such as CREB and ΔFosB.

The transcription factor ΔFosB is a highly stable splice product of the IEGs FosB, and shares homology with other Fos family transcription factors (e.g. *c-Fos*, Fra-1 and Fra-2). It heterodimerized with the Jun family protein activator protein-1(AP-1) complex that binds to AP-1 sites in the target genes to regulate transcription. Although all the Fos family proteins are induced by acute exposure to drugs of abuse, chronic administration of these drugs induces the long-lasting expression specially of ΔFosB, a process that is most robust in reward-related brain areas including NAc, dorsal striatum and PFC (Perrotti *et al*., 2008). Thus, the early expression of *c-fos* encode the expression of ΔFosB. ΔFosB induction in the NAc by chronic drug exposure (McClung *et al*., 2004) and by chronic consumption of several natural rewards, such as food and sexual activity (Wallace *et al*., 2008) occurs only in MSN expressing DA1 receptors. This transcriptional factors has been linked directly to several dependence-related behaviours. For example, mice lacking *c-fos* in DA1 receptors-containing neurons in the NAc, show reduced behavioural sensitisation to cocaine (Zhang *et al*., 2006). In addition, Renthal *et al*. (2008) demonstrated that accumulation of ΔFosB in the striatum, in response to chronic AMPH feeds back to desensitize *c-fos* induction to subsequent drug doses. This mechanism may be important in regulating an animal's sensitivity to repeated drug exposure, thus, we focused in present study on *c-fos* expression in NAc and VTA, (see Figure 5).

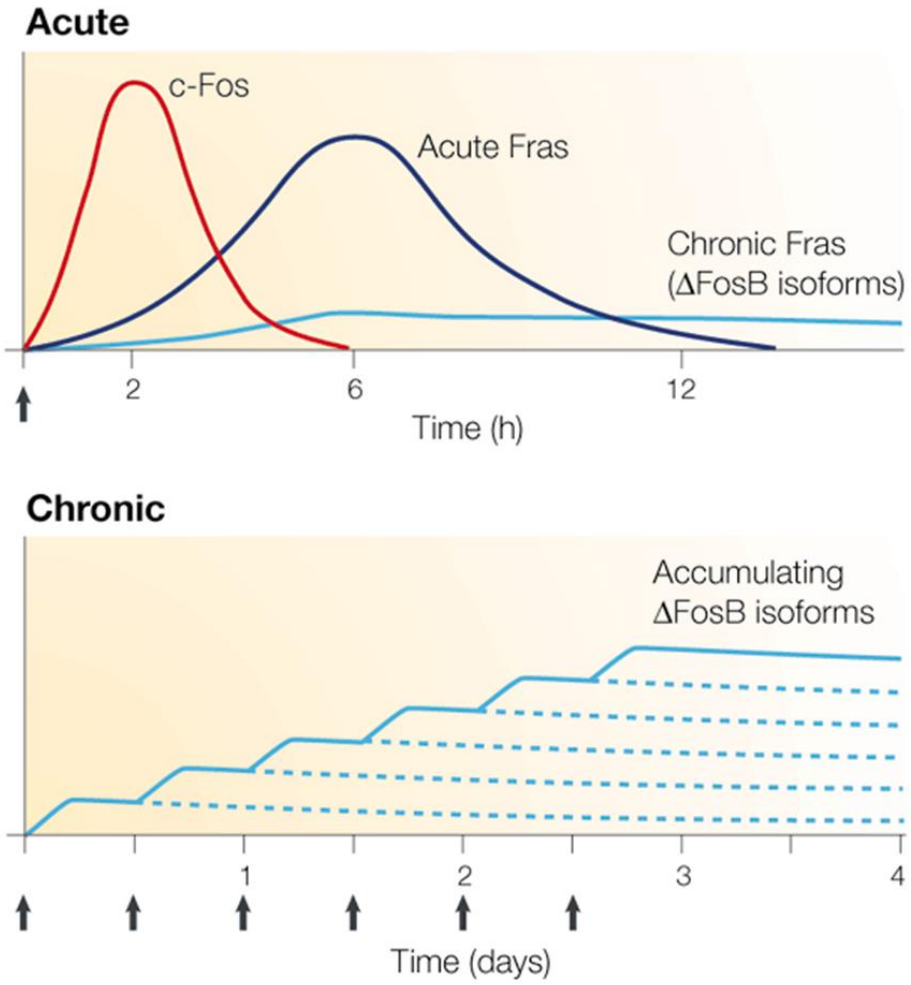



Figure 5: Scheme showing the gradual accumulation of ΔFosB versus the rapid and transiently induction of other Fos family protein in response to the drug of abuse. Acute administration of a drugs of abuse cause induction of Fos family protein in the NAc. These protein include *c-fos* and several Fras (Fos-related antigens; for example, FosB), and all of these protein of the Fos family are unstable. By contrast, isoforms of ΔFosB are highly stable therefor persist in the brain for long time after drug exposure (Nestler, 2001a).

ΔFosB produces its effect on behavioural phenotypes in response to drug exposure by regulating numerous genes, and the neuroplasticity regulated by these genes may be extremely stable during drug abstinence. For example, an enduring increase in dendritic spine density has been reported in NAc MSN during extended abstinence from chronic psychostimulant administration (Li *et al.*, 2003). The genes regulated by ΔFosB include activity- regulated cytoskeleton-associated protein (Arc), as well as genes that are important

for glutamatergic synaptic function and plasticity including AMPA receptors subunit (McClung and Nestler, 2003). Thus, ΔFosB serves as the master control protein that governs this structural plasticity.

Few studies have looked at gene expression of Arc, an effector IEGs that encode a protein in synaptic site undergoing strong activity and directly regulating synaptic plasticity (Ujike *et al*., 2002). Arc protein plays an important role in the processes underlying the formation of long-term changes in the neuron function and synaptic modification and is required for other forms of synaptic plasticity including long-term potentiation (LTP) (Guzowski *et al*., 2000). The strong association of Arc with neural plasticity is supported by the fact that newly synthesized Arc is not only transported into dendrites but also accumulates specifically at synaptic sites that have experienced strong activity (Moro *et al*., 2007). Rearrangement and structural modification of neural networks and circuitry must develop for long lasting plasticity such as is required for behavioural sensitisation (Ujike *et al*., 2002). Various microscopic studies with Golgi-staining have demonstrated anatomical changes including the increases in the length of dendrites and the density of dendritic spines in NAc and PFC as a result of repeated exposure to psychostimulants, which corresponds to enhanced synaptic connectivity or transduction efficacy in the circuitry (Robinson and Kolb, 1997).

However, the molecular mechanisms responsible are not well understood. Ujike *et al*. (2002) have investigated a set of genes related to synaptogenesis including Arc, which act directly on cellular function associated with cytoskeleton. The authors found increase in Arc expression in the striatum after repeated exposure to methAMPH. Moreover, Salery *et al*. (2017) found that mice which were behaviourally sensitized to cocaine showed an increase in Arc mRNA expressed in the dendrites in the striatum. Due to that, Arc expression serves as an excellent marker of drug-induced neural activity leading to long term changes in the neural function. Therefore, in the present study, we measured the effect of chronic treatment with nicotine or AMPH on Arc gene expression in NAc and VTA and the relationship of this expression with drugs-induced behavioural sensitisation.

In addition to these IEGs involved in neural plasticity, there are other genes which have an important role in the neural activity including MeCp2 and BDNF. MeCp2 is a member of DNA-binding protein family that binds to methylated DNA at CpG sites. DNA methylation refers to addition of methyl group to the cytosine or adenine nucleotide in the DNA. It has been suggest that such DNA methylation may affect the transcription of genes in different

ways leading to a reduction in gene expression. In fact the inclusion of methyl group in DNA may block the binding of transcriptional protein to the gene (Nestler, 2014). However, one study, investigating the persistence of gene expression after drug withdrawal, found that global DNA methylation was increased in rat NAc and mPFC two week after AMPH treatment (Mychasiuk *et al*., 2013). The importance of MeCp2 in normal brain development is highlighted by the fact that in human mutation in MeCp2 causes autistic spectrum disorder such as Rett syndrome (RTT) (Cohen *et al*., 2011). MeCp2 has previously been shown to be essential in learning and memory and is now being explored as a key modulator of neural activity-regulated gene expression (Feng and Nestler, 2010).

Hutchinson *et al*. (2012) demonstrated that MeCp2 contributes to both neural and behavioural adaptation induced by psychostimulant exposure. Deng *et al*. (2010) investigated the effect of the psychostimulant AMPH on MeCp2 expression and they found that systemic administration of AMPH induced a restricted pattern of pMeCp2 induction in certain neurons of the NAc and PFC, that expressed DA1 receptor correlated with the degree of locomotor sensitisation. However, AMPH induces pMeCp2 selectively in GAD67- and parvalbumin-postive GABAergic interneurons and it fails to produce pMeCp2 in the MSNs in the NAc despite these neurons expressing high level of DA1 receptors. The authors (Deng *et al*) showed that loss of pMeCp2 prevented chronic AMPH increasing dendritic arborisation of NAc projection neurons, thought to reflect the point of excitatory synapses, but increased the number of GABAergic synapses on these cells. They concluded thatMeCp2 may alter the balance between excitatory and inhibitory transmission in the NAc.

Psychostimulants induced a rapid and robust phosphorylation of MeCp2 (pMeCp2) at a specific amino acid residues. However the exact mechanism by which psychostimulants exerts their effect on phosphorylation of MeCp2 is not well understood. In cultured neurons, MeCp2 phosphorylation is triggered by the release of glutamate at excitatory synapses, suggesting that synaptic activation may regulate MeCp2 function as a part of the adaptive response to neural stimulation (Zhou *et al*., 2006). The phosphorylation of MeCp2 has been suggested to play an important role in the neural activity-dependent induction of BDNF, a protein which promotes many aspect of experience-dependent synaptic development. Thus overexpression of MeCp2 may affect the development of neural dendrites and spines (Deng *et al*., 2014). Consistent with this possibility, the phosphorylation of MeCp2 in response to neural activity has also been suggested to reduce the binding of MeCp2 to methylated DNA leading to the hypothesis that synaptic activity regulates neuronal development by

decreasing the affinity of MeCp2 for methylated cytosine within regulatory regions of genes such as BDNF, resulting in a change in chromatin structure that is required for gene activation (Cohen *et al*., 2011). Thus, more study is needed to explain the role of MeCp2 induction in psychostimulant sensitisation.

BDNF is a member of the neurotrophic protein group, and is important in neuron growth, survival and differentiation during nervous system development (Hyman and Hofer, 1991). It is highly regulated in the NAc by several drugs of abuse (Russo et al., 2009). BDNF, a prominent CREB-regulated factor, has a major role in neuroplasticity and synaptogenesis. The two major sources of BDNF protein in the NAc originate from DAergic and glutamatergic afferent neurons arising in the VTA and PFC, respectively (Graham *et al*., 2007). BDNF action is associated with increased glutamatergic activity, LTP, dendritic protein synthesis and dendritic spine formation. It has also been shown that increased BDNF level in the VTA and NAc lead to behavioural changes associated with drug dependence (Simchon Tenenbaum *et al*., 2015). Thus, most attention to date has been given to explaining the role of BDNF in reward-related neuronal systems.

A DA-dependent increase in the BDNF appears to be important in establishing psychological as well as drug-induce neuroplasticity (Li and Wolf, 2015). BDNF induces its effect by binding to tropomyosin receptor Kinase B (TrkB), which is widespread in brain regions. Activation of these receptors, can promote several signalling cascades including activation of extracellular-regulated kinase (ERK), which is transmitted to the cell nucleus where it acts on transcription factors such as CREB as well as altering gene expression (Thomas *et al*., 2008). Several recent studies have examined the role of BDNF in the development of behavioural sensitization. For example, daily BDNF injection into VTA or chronic BDNF administration via osmotic minipumps into VTA or NAc potentiated the acute locomotor- activating effect of cocaine during the induction phase, but it had no effect on the expression phase of sensitisation after withdrawal from cocaine (Horger *et al*., 1999, Pierce *et al*., 1999). Thus, the degree to which BDNF-induced potentiation of psychostimulant-induced locomotor sensitisation is unclear and requires further study.

## 1.3 Drugs of abuse and behavioural sensitisation

### 1.3.1 Nicotine

Tobacco smoking is a major health problem associated with early mortality due to its causing many serious diseases such as myocardial infarction and lung cancer Henschke et al. (2006)Using tobacco spread worldwide from its origin in the Americas, where the native people chewed and smoked the leaves. In 1560's Jean Nicot de Villemain, the French ambassador to Lisbon, learned about the medicinal properties of tobacco and introduced the plant to the French court. Later his name was used for the plant tobacco, *Nicotiana tabacum* and for the addictive alkaloid nicotine. During the early 20th century, cigarette use spread worldwide, and became a socially acceptable pastime. However, later in the 20th century, epidemiological and experimental studies, (see Dani and Balfour (2011), for good review), led to scientific and government acceptance of link between the cigarette smoking and health problems such as lung cancer and cardiovascular dysfunction, resulting in a growing movement to reduce smoking. Balfour (1990) and Benowitz (2010) demonstrated that nicotine, a major compound found in tobacco, is the primary factor leading to addiction to smoking and many habitual smoker become dependent upon the tobacco.

#### 1.3.1.1 Mechanism of action of nicotine

Nicotine produces its effect via nicotinic acetylcholine receptors (nAchR) which are distributed in several areas of the mammalian CNS. These receptors normally respond to the neurotransmitter, acetylcholine, which is rapidly metabolised to choline and acetic acid in the synaptic cleft after release by the enzyme acetylcholinesterase (Dani and De Biasi, 2001). The nAChRs belong to the ligand gated ion channels family, which is permeable to $Na^+$, $Ca^{2+}$ and $K^+$, and these receptors are widely expressed in mammalian nervous system. There are several families of nAChRs subtype expressed in CNS. Briefly, there are several subunits and receptors can be made up of just one subunit such as α7 or different subunits such as α4β2 (Millar and Gotti, 2009). Like other psychostimulant drugs such as AMPH and cocaine, a substantial portion of the effect of nicotine on behaviour is attributable to actions upon the mesocorticolimbic DA systems, particularly on the mesolimbic pathway, which normally helps to reinforce rewarding behaviours. Nicotine increases the firing of midbrain DA neurons, through nAChRs which are widely expressed on the DA cell body as well as by non-DA interneurons. In addition it increases DA overflow in the NAc via its effect on nAChRs subunit on DA terminals (Vezina *et al*., 2007). Nicotinic receptors

operate in the brain using different mechanism depending on their location within the neurons but the most important mechanism is upregulation nAChRs and nAChRs currents and produces LTP of excitatory input to VTA DA neurons (Vezina *et al*., 2007)

Most nAChR subunits are found in VTA. A functional α7 nAChRs was found in DAergic and GABAergic neurones in VTA but was not found on DA terminals. However, there is some evidence for presence of α7 on DA terminals in the striatum (Grady *et al*., 2007). In addition, this α7 receptor subtype has also been identified in glutamatergic afferent projection to VTA and striatum (Livingstone *et al*., 2009). Presynaptic α7 nAChRs on glutamate afferents to the VTA participate in mechanisms of synaptic plasticity and influence the probability of burst firing in mesolimbic DA neurons, with consequent increases in accumbens DA release (Jones and Wonnacott, 2004). While the nAChRs receptors containing β2 subunit are present on the majority of DAergic cell bodies and GABAergic neuron in VTA, they are also found on DAergic neuron terminals in NAc, in particular as α4β2 and α6β2 heteromers (Jones and Wonnacott, 2004).

Vezina *et al*. (2007) stated that a number of conformational changes have been associated with nicotine binding nAChRs causing either opening or closing of the receptor ion channels leading to changes in the functional state of the receptors. From the resting state, with no agonist bound, the receptors undergo the first conformation change when an agonist binds. This comprises short term activation of the receptors by opening of the associated ion channel. If the agonist remains bound to the receptor the conformation quickly (< 5 milliseconds) converts to a second non-functional state caused by the ion channels closing: this state is termed the desensitised state. Normally the receptors recover rapidly from desensitised state and return to the resting state when the nicotine is removed. Repeated administration of nicotine causes dynamic changes in the total number of nAChRs which is believed to be a result of repeated desensitization and activation processes, and these changes are linked to alterations in the DA system that appear only during nicotine sensitisation after repeated exposure to nicotine but not during acute drug taking (Barik and Wonnacott, 2009).

Repeated intermittent nicotine injections in rodents are known to cause behavioural sensitisation as evidenced by an enhanced locomotor response to a subsequent injection of the drug. Considerable evidence suggests that the behavioural effect of nicotine may be mediated via the central DAergic system especially the mesolimbic system from VTA to

the NAc in which nicotine acts on nAChRs localized on DAergic cell bodies in VTA (Baker *et al*., 2013a) and on their nerve terminals in NAc (Kleijn *et al*., 2011). These finding suggest that stimulation of DA transmission in the mesolimbic system plays an important role in the stimulants effects of nicotine. Recently, it has been proposed that long-term genomic alteration in dorsal and ventral striatum may underlie the persistent nature of behavioural sensitisation which is expressed even weeks or months following repeated nicotine administration (Morud *et al*., 2016). This profile of nicotine effect suggests that nicotine induces long-term synaptic alteration at the level of gene expression. Mychasiuk *et al*. (2013) demonstrated that repeated administration of nicotine induced persistent changes in gene expression in the NAc. Systemic injection of nicotine has been shown to increase *c-fos* immunoreactivity in several brain areas that receive DAergic innervation from VTA including NAc (Shim *et al*., 2001). Thus, it is likely that the behavioural sensitisation to nicotine may be mediated through alteration in extracellular DA release or postsynaptic gene expression. An aim of the experiments presented here was to evaluate the genomic alteration by measuring expression of IEGs such as *c-fos* or Arc in the DA target neurons after repeated nicotine administration.

### 1.3.2 Amphetamine

Amphetamine (AMPH)-like drugs comprise a diverse subclass of chemical compounds that include synthetic and natural compounds which share a molecular site of action at monoamine transporters particularly the DA transporters (DAT) (White and Kalivas, 1998). AMPH and its substituted derivatives such as methamphetamine have an important medical and social effect in many cultures due to its effects on mental function and behaviour (Sulzer *et al*., 2005). Some plant-derived AMPH- like drugs were used for thousands of years, before AMPH was synthesized in 1887 at the University of Berlin. Until the mid-1960s, it was available on prescription for use by students and armed forces for increasing vigilance and keeping people awake, before its adverse properties, including its addictive potential were fully recognized. Subsequently, although AMPH addiction has been recognised as a serious public health problem in all countries throughout the world, it is still widely used illegally as a performance enhancer, for weight control and for its psychotogenic effect. In addition, AMPH and its derivatives are used as a treatment for attention deficit hyperactive disorder (ADHD) (for an excellent historical overview see Sulzer *et al*., 2005).

### 1.3.2.1 Mechanism of action of D-AMPH

Neurotransmitter transporters regulate the synaptic neurotransmitter concentrations through reuptake and recycling of released transmitter. DAT are expressed pre-synaptically in all DAergic neurons with a highest level in neurons originated from VTA and substantial nigra (Sulzer *et al*., 2005). As discussed previously (see section 1.2.3) drugs of abuse share the ability to increase extracellular DA in the NAc (Sulzer, 2011). In the case of AMPH, it has multiple mechanisms through which it increases DA. Firstly, it produces non-exocytotic stimulation- independent release of neurotransmitters via reversal of the transporter which does not required any neural activity. This mechanism includes the redistribution of neurotransmitters from synaptic vesicles to cytosol and reverse neurotransmitters transport through plasma membrane uptake carriers (DAT) (Sulzer *et al*., 2005, Sitte and Freissmuth, 2015). DAT is a major presynaptic terminal protein regulating extracellular DA concentration and is a presynaptic target for many drugs of abuse including AMPH.

AMPH is considered as an exogenous substrate of the DAT and vesicular monoamines transporters (VMAT1 & 2). Thus, AMPH blocks DA reuptake by binding with DAT. It enters the DAergic terminals and accumulates in acidic structure such as synaptic vesicles. The acidic pH gradient in secretory vesicles provides the energy to accumulate transmitters against their concentration gradient. After entering the synaptic vesicles AMPH bind to free protons and increased pH (i.e. reversing the acid gradients and creating instead an alkaline gradient) leading to a reduction in the energy needed for neurotransmitters accumulation and causing redistribution of vesicular DA to the cytosol. Subsequently, the increased cytosolic DA binds to the cytosolic face of DAT and is transported out of the cell, thus increasing the extracellular DA level (Howell and Kimmel, 2008) (see Figure 6). Secondly, another mechanism that may be important in regulating the extracellular level of DA is through inhibition of MAO, responsible for the enzymatic breakdown of DA, this further enhancing cytosolic DA. Thirdly, AMPH promotes DA synthesis by upregulating the activity of TH (Sulzer, 2011).

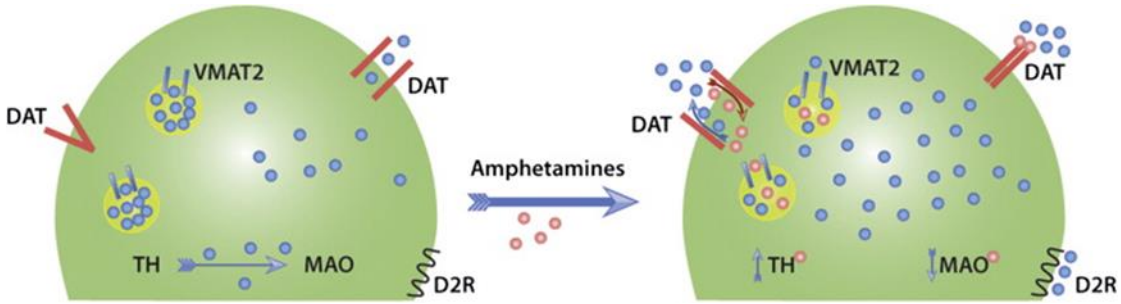



Figure 6: Mechanism by which AMPH enhances extrasynaptic DA levels independent of synaptic vesicle fusion and inhibits DA reuptake. Under normal conditions, DAT takes up extracellular DA into cytosol and transfers the DA from cytosol to synaptic vesicles via the vesicular monoamine transporter (VMAT2) where it accumulates to very high level using energy from an inward acidic gradient provided by a vesicular ATP. AMPHs are DAT substrate and act to release DA via reverse transport of from sysnaptic vesicles to cytosol. In the cytosol, AMPH increase cytosolic DA by activating TH ; inhibiting MAO and reditribution of vesicular DA to the cytosol by VMAT inhibition and / or eliciting DA reverse transport as VMAT substrate by collapsing the synaptic vesicles PH gradient. These effects on synaptic vesicles result in a decrease of the quantal size of neurotransmitters release per synaptic vesicles fusion event (Sulzer, 2011).

In rats, the repeated administration of psychomotor stimulants such as AMPH produces behavioural sensitisation which is expressed as enhanced hyper-locomotion. Once it is developed, following repeated AMPH administration, behavioural sensitisation remains for a long time, up to one year, even in the absence of further drug administration, as a form of long-term memory that contributes to the drug-seeking and drug-taking behaviour (Vezina, 2004). As mentioned above, AMPH is a substrate for DA, norepinephrine and serotonin transporters and through the action at these transporters, AMPH causes increases in synaptic levels of the associated neurotransmitters and therefore acts as an indirect agonist. AMPH exerts its rewarding effect through its ability to increases extracellular DA levels in the NAc (Di Chiara and Imperato, 1988). Although AMPH causes inhibition of DA uptake by competitively binding to DAT, it induces an increase in extracellular DA much larger than other DAT blocker such as cocaine due to its ability to enter DA terminals causing movement of DA out of vesicles and release of DA into extracellular space v ia DAT-mediated reverse transport (Sulzer, 2011). In addition to the effect of repeated administration of AMPH on DA releasing in NAc, the DA level in mPFC also has an effect on the

expression of behavioural sensitisation. Casanova *et al*. (2013) showed that an increase DA releasability in the mPFC in rats showing development and expression of behavioural sensitisation to AMPH.

The prolonged time course of behavioural sensitisation of psychostimulant dependence has raised the question of whether long-term drug-induced alteration in gene expression plays a critical role (Nestler, 1992). IEGs and their protein products are involved in different function of neurons. Acute administration of AMPH causes increases in DA release from presynaptic terminals and increases the level of mRNA of IEGs such as *c-fos,* which mediated between receptors-activated second messengers system and expression of several genes involved in regulation of functions of neurons such as ΔFosB and lead to long term changes in neural responsiveness (see above section 1.2.3.1). AMPH acts indirectly via increased DA activation of DA1 receptors to induce expression of IEGs such as *c-fos* in the striatum according to previous studies (Nguyen *et al*., 1992). DA1 receptors activate the cAMP pathway including cAMP-dependent protein kinase (PKA) which in turn regulate transcription factors such as CREB which have been shown to be involved in the transcriptional regulation of many genes including IEGs such as *c-fos* (Konradi *et al*., 1994) (see section 1.2.3.1). These behavioural changes as well as genomic changes induced by AMPH are mediated by the indirect action of AMPH on DA1 receptor on postsynaptic striatal neurons (Simpson *et al.*, 1995). Thus, it is likely that the behavioural sensitisation to nicotine and AMPH may be caused by alteration in extracellular DA release or postsynaptic gene expression. One way to evaluate the genomic alteration is to examine expression of IEGs such as *c-fos* or Arc by using immunohistochemistry of the protein they encode in the DA target neurons after repeated administration of drugs such as nicotine and AMPH, which we used in present study.

### 1.3.3 Drugs cross-sensitisation

There are several possible explanations for why people may abuse multiple substance. For example, one substance may enhance the positive objective effect of another: one substance may overcome certain unwanted or adverse effect of another or one may substitute for a preferred substance when it is unavailable. There is now considerable empirical support for each of these explanations and preclinical studies have applied a number of animal models to evaluate these potential interactive effects. For example, drug self-administration procedures are used to assess the drug-taking behaviour, a key feature of polysubstance use.

In drug self-administration studies, a drug (e.g., nicotine, cocaine, alcohol) serves as a reinforcing stimulus for operant performance and alteration in the drug's reinforcing effect by co-administration of/or previous exposure to, another drug are determined by the change in the rate of acquisition of self-administration (Le *et al*., 2003, Cortright *et al*., 2012). Another explanation of polysubstance use is that cross-sensitisation occurs between different drugs, by which pre-treatment with one drug results in sensitisation to the behavioural effect of another drug (Vanderschuren *et al*., 1999). In addition to drug-dependent effects, polysubstance use has a major effect on the health and social development. Laboratory-based studies of polysubstance use often report that the health consequences associated with combination of drugs far exceeds that of either drug alone. The combined use of multiple substances increases the use of both substances which leads to an increased incidence of acute or long-term toxicity-induced liver or kidney dysfunction, damage the cardiovascular and respiratory system (Wise and Koob, 2014).

Many studies focus on nicotine's interaction with other psychostimulants. The societal impact of nicotine polysubstance use is a major concern since chronic health problems associated with nicotine dependence, such as lung cancer and cardiovascular disease are amongst the most prominent causes of death around the world (Wise and Koob, 2014). A study by Richter *et al*. (2002) has indicated a high correlation between psychostimulant use and tobacco cigarette smoking in human substance abusers. The authors (Richter *et al*) found that individuals who reported previous participation in drug treatment had a three-fold greater chance of being a cigarette smoker compared to the general population. There have been several investigations into the behavioural basis of psychostimulant cross-sensitisation. In cross- sensitisation studies, AMPH-induced locomotor activity was significantly higher in nicotine-sensitized mice than in saline-pre-treated mice (Celik *et al*., 2006). Kim *et al*. (2011) found that acute nicotine injection, 2-4 h before challenge with AMPH enhanced locomotor behaviour: it also enhanced AMPH-induced DA efflux from slices of NAc. It is well understood that nicotine mediated effects following binding to nAChRs receptors which are expressed on either DA cell bodies in VTA or on terminals in NAc and the subsequent augmentation of DA release. The alteration of DAergic neurotransmission can result in differing behavioural effects, emphasising the link between nicotine sensitisation and DA function.

In addition to behavioral cross-sensitisation, psychostimulant also appear to interact within common pharmacological, neurochemical and molecular mechanism. Investigation into the neurochemical basis of cross-sensitisation have primarily focused on overlapping effects within the mesolimbic DA system particularly the NAc. The effect of psychostimulant drugs on extracellular DA is often described as additive which mirrors the degree of change in the most behavioural measures. Microdialysis studies have found that nicotine and psychostimulant drugs such as methAMPH and cocaine share the ability to increase extracellular DA level in the NAc (Di Chiara, 2000). In addition pre-treatment with nicotine potentiated AMPH–stimulated locomotor activity and DA overflow in NAc, and conversely, AMPH pre-treatment enhanced nicotine-simulated locomotor activity and DA overflow in NAc (Jutkiewicz *et al*., 2008). Levine *et al*. (2011) found that pretreatment of mice with nicotine increased the response to cocaine as assessed by both addiction-related behaviors and synaptic plasticity in the striatum. However, the behavioral and neurochemical effect of nicotine are not enhanced following cocaine exposure.

## 1.4 Summary of research questions

The behavioural studies such as those described by Wise and Bozarth (1987) and Vezina *et al*. (2007) provide substantial evidence that behavioural sensitisation is a long-lasting phenomenon that can be persist for at least a year. This phenomenon reflects neuroadaptative changes, which, in many cases, persist for a long time beyond the end of drug treatment and occur through both pre and postsynaptic mechanisms. This has led to suggestions that these neuroadaptative changes may be mediated through alterations in intracellular signal transduction that includes the regulation of gene expression. In recent years, several studies were published on the role of IEGs (Moro *et al*., 2007), MeCp2-(Deng *et al*., 2010) and BDNF (Thomas *et al*., 2008) in the psychostimulant-induced neural plasticity involved in their behavioural effect. Moreover, the presynaptic adaptation includes a persistent increase in the ability of drugs of abuse to enhance the release of DA in the striatum (Pierce and Kalivas, 1997). It is well known that most abused drugs share the ability to increase extracellular DA in NAc and other target limbic regions, which either regulate gene expression or increase the postsynaptic DA responsiveness leading to long-term behavioural sensitisation. Furthermore, Laplante *et al*. (2013) found that the increased behavioural response to AMPH more likely occurs due to postsynaptic DA receptors upregulation. Thus, taking all of this evidence into account, the first key question addressed by this study is what

are the mechanisms underlying sensitisation-related neuroadaptations that occur with each drug administration and after a drugs challenge? The second key question is whether the behavioural sensitisation caused by psychostimulant drugs with differing primary pharmacology involves the same or a different biochemical mechanism?

## 1.5 Aims, objectives and hypothesis

The overall aim of the studies presented here was to evaluate the mechanism underlying sensitisation-related neuroadaptations that occur with drug administration and after a drugs challenge. Specifically we hypothesised that behavioural sensitisation to nicotine or AMPH would be associated with an increase in post-synaptic neuron plasticity in the NAc, to change the DA level in pre-synaptic terminals neurons, in addition to post-synaptic DA receptors upregulation. Four key objectives were defined; to ascertain whether behavioural sensitisation to each drug was accompanied by changes in (1) epigenetic markers; (2) neurochemical function in the mesolimbic DA pathway; (3) DA neurotransmitter level; (4) DA receptors gene expression. Finally, we hypothesised that behavioural sensitisation to nicotine or AMPH may share a common neural mechanism in term of cross-sensitisation. To address these objectives complementary *in vivo* behavioural, *in vitro* and *ex vivo* methods were used to measure behavioural, neurochemical and epigenetic consequences of psychostimulant sensitisation. Behavioural experiment had been reported in previous experiments but we repeated it to form a basis regimen to the rest of experiments. However, the cross-sensitisation experiment is a novel experiment under the same conditions.

## 1.6. Methods for studying drugs sensitisation

To obtain the goals of this study, we used several methodological approaches. Firstly, we tested the ability of repeated treatment with nicotine or AMPH in adult rats to elicit a sensitized behavioural response after a challenge injection with the same drug 10 days after cessation of the initial treatment. Second, in an independent groups of rats, we investigated whether the behavioural sensitisation caused by different drugs involves the same or a different mechanism, using a cross-sensitisation protocol, measuring the locomotor activity of animals pre-treated with nicotine or saline and challenged with AMPH, and of those pre-treated with AMPH or saline and challenged with nicotine. Third, in order to investigate cellular changes occurring after the sensitisation and cross-sensitisation procedures, post-mortem histological analysis of brains tissue taken form animals that were used in

behavioural experiments was used to assess, the expression of the IEGs (Arc and *c-fos* ), MeCp2 and BDNF in the NAc sub regions (shell and core) and VTA using immunohistochemistry procedure. Fourth, we measured the effect of repeated treatment with either nicotine or AMPH on DA release after local perfusion with either nicotine or AMPH using fast scan cyclic voltammetry (FSCV) *in vitro*, in brain slices taken from animals pre-treated with either nicotine or AMPH. Finally, we measure, the effect of treatment with either nicotine or AMPH on DA receptors gene expression in the NAc and VTA in brain tissue taken from some animals used in behavioural experiments, using real-time qPCR procedure.

# Chapter 2: Behavioural sensitisation experiments

## 2.1 Introduction

Drug dependence is a chronically relapsing disorder that is defined by two major characteristics: a compulsion to take the drugs with a narrowing of the behavioural repertoire toward excessive drug intake and loss of control in limiting intake (Nestler, 2001b). Drug dependence has a detrimental effect not only on the health and behaviour of the individual but on the social cohesion and social development (Wise and Koob, 2014). The growing knowledge about the brain mechanisms underlying drug dependence offers a good opportunity to develop new treatments. However, studying the neurobiology of drug dependence is challenging due to its relapsing-remitting clinical course. Intermittent administration of a psychostimulants is known to induce progressive augmentation of behavioural activity in response to a given dose of drug of abuse, a phenomenon known as behavioral senstisation (Kalivas and Stewart, 1991, Robinson and Berridge, 1993, Robinson and Berridge, 2008).

Behavioural sensitisation comprises two distinct temporal domains termed induction and expression. The induction phase is the transient sequence of cellular and molecular events produced by psychostimulant administration that causes changes in neural function responsible for behavioural augmentation. On the other hand, the expression phase is the enduring neural alteration arising from the induction process and directly mediating the behavioural response (Robinson and Becker, 1986, Kalivas and Stewart, 1991, Pierce and Kalivas, 1996). Several observations indicate that both drug dependence and behavioural sensitisation are long-lasting phenomena, and may share similar neural/molecular adaptation processes (Robinson and Berridge, 1993). Thus, animal models employing behavioural sensitisation are critical for investigating the mechanism involved in development and persistence of drug dependence.

The present study focussed on the effect of the psychostimulants AMPH and nicotine, two drugs with substantial health and social impact on the community. Cigarette smoking is associated with diseases such as myocardial infraction and lung cancer leading to widespread chronic ill health and premature death among smokers around the world (Henschke *et al.* 2006). AMPH dependence is also a serious public health problem as it is widely used illegally as a performance enhancer and for weight control as well as clinically as a treatment for attention deficit hyperactive disorder (ADHD) (for an historical overview

see, Sulzer *et al*. (2005)). Notably, repeated administration of both drugs enhanced motor activity and produced an increase in extracellular DA level in the NAc (Wallace, 2012, Morud *et al*., 2015). Because locomotor activity is known to be related to mesolimbic DA system, it is possible that co-administration of these compounds modulate levels of DA release (Wallace, 2012, Quarta *et al*., 2007).

Both nicotine and AMPH are known to be psychoactive compounds, but acting through different pharmacological mechanisms. Nicotine is an agonist at nicotinic acetylcholine receptors that are widely distributed throughout the brain and are known to regulate release of DA especially in the NAc (Albuquerque *et al*., 2009). On the other hand, AMPH is an indirect DA agonist, which decreases the reuptake of DA and indirectly increases the neural release of DA by acting on the presynaptic DA transporters on the nerve terminals (Sulzer, 2011). There are known interactions between central nicotine acetylcholine receptors and DA receptors (Schoffelmeer *et al*., 2002, Quarta *et al*., 2007) which provide possible mechanism for the co-abused of AMPH and nicotine.

The aim of this research is to address one of the most important question which is: what is the mechanism underlying sensitisation-related neuroadaptations that occur with each drug administration and after a drug challenge? In addition, the research aims to assess whether the behavioural sensitisation that is caused by different drugs involves the same or a different mechanism, with a view to develop pharmacological approaches to reverse sensitisation and/or prevent its development. The answer to these questions was addressed through *in vivo* behavioural testing. First, we tested the ability of repeated daily injection with either nicotine or AMPH in adult rats to create a significantly sensitized behavioural response upon a challenge injection with the same drug, 10 days after cessation of nicotine or AMPH treatment (Drug-Drug vs SAL-Drug, challenged with the same drug that used in pre-treatment period). Second, in an independent group of rats, we investigated whether the behavioural sensitisation that is caused by different drugs involves the same or different mechanism. This was done using a cross-sensitisation regime (Drug-Drug vs SAL-Drug, challenge with opposite drug that used in pre-treatment period), by comparing the locomotor activity of animals pre-treated with nicotine and challenges with AMPH with those of acute AMPH (e.g., NIC-AMPH vs SAL-AMPH groups), or comparing those pre-treated with AMPH and challenged with nicotine with those of acute nicotine challenged (AMPH-NIC vs SAL-NIC groups). Thirdly, we wished to ascertain that the apparently drug-induced behavioural sensitisation is drug dependent rather than context dependent. This was done by

comparison the locomotor activity of rats pre-treated with drugs and challenged with saline, and those pre-treated with saline and challenge with saline, in an independent group of rats (Drug-SAL vs SAL-SAL, e.g. NIC-SAL vs SAL-SAL or AMPH-SAL vs SAL-SAL).

## 2.2 Materials and Methods

### 2.2.1 Animals and chemicals

Male Lister-hooded rats (n = 127) were obtained from Charles River (Cambridge, UK). The rats were matched for body weight (300-350 g) at the beginning of the experiment and in addition were weighed on each day of the experiments. The animals were randomly assigned to nine treatment groups ( n = 8-17 per group) depending on the treatment and challenge dose that would be taken during the experminets (see Figure 7, which presents the injection regimen for the nine treatment groups across the duration of the experiments). The rats were allowed one week to acclimatize before starting the experiments. During this week all of the animals were housed in groups of four per plexiglass cage in a colony room and maintained at a constant temperature of 20 ± 3 ℃ and a 12:12 h light / dark cycle (the light cycle was 7:00 am light on and 7:00 pm light off). The rats had *ad libitum* access to food and tap water except during experimental sessions. Daily care was provided to the rats by in-house animal technicians working within the Preclinical Research Facility, University of Leicester.

D-AMPH sulphate (prod. nr A-5880) and (-) - nicotine ([-]-1-methyl-2-[3- pyridyl] pyrrolidines) hydrogen tartrate salt (prod. nr N-5260) were purchased from Sigma-Aldrich (UK). The drug doses were calculated as salts and freshly prepared by dissolving them in physiological saline (0.9% NaCl) for intraperitoneal (i.p) injection in volumes of 1 ml / kg body weight. All procedure using animals were carried out with local ethical approved from the University of Leicester Animal Welfare and Ethical Review Body (AWERB) under the Animals (Scientific procedures) Act, 1989 and with the personal and project license (PPL, nr = 6004390).

### 2.2.2 Locomotor activity apparatus

The rats were brought daily in their home cage from the colony room to a separate room for locomotor activity mesurments. The behavioural testing was performed in a sound-attenuated testing room containing 4 clear plexiglas cages that were 24 cm in width, 46 cm in length and 22 cm in height so that four rats could be mointored simultaneously. Rat behaviour was recorded using a logitech C525 camera placed 50 cm above the floor of the boxes. The camera was connected to a computer equipped with the Any-maze software (San Diego Instruments, California, USA) for data acquistion. This software programe allows the measurement of different behaviour activities such as total distance moved (m), total time mobile and average speed of the animals in the activity chambers. By using this camera, digital videos were captured and stored on a computer for offline analysis of locomotor behaviour.

### 2.2.3 Behavioural studies

The behavioural testing process for locomotor sensitisation was adapted from previously described sensitisation schedule (Vanderschuren *et al*., 2002, Ostrander *et al*., 2003, Byrnes *et al*., 2011). Drug treatment started after an acclimatization period of at least 1 week. Three days prior to the experiments, rats were moved from the colony to the testing room where they remained in their home cage for a 10-30 min after which they were handled (20 min per day) in order to familiarize them with the experimental procedure. They were then habituated (3 days, 30 min per day) to the activity chamber to avoid environmental novelty-induced enhancement of the behavioural activating of drugs (Eilam and Golani, 1989, Badiani *et al*., 1995). Rats were randomly assigned to one of the nine experimental groups and underwent the following sensitisation regimen which took over 16 days (see Figure 7).

In this regimen, there were two phases: the induction phase (pre-treatment) which took five consecutive sessions. In this phase, (-) - nicotine (0.6 mg/kg, i.p, n = 42), D- AMPH sulphate (1 mg/ kg, i.p, n = 43) or its saline vehicle groups (1 ml/kg., i.p, n = 42) were given daily (one session on each day for five consecutive days), according to experimental group. Following each injection, the rats were placed in the test apparatus for 60 min, and activity was monitored continuously using Any-maze video tracking software (San Diego Instruments, California, USA). This procedure was carried out between 11 am and 3 pm and the animals were returned to their home cages after each testing day. Horizontal locomotor

activity was measured immediately following each injection for 60 minutes and locomotor sensitisation was evaluated by comparing movement (total distance travelled in meters) on the last day with that on the first day of treatment. Doses of nicotine or AMPH were chosen based on previous literature, which shows that repeated treatment with these doses significantly enhanced locomotor activity and below those required to elicit stereotypical behaviour in drug-naive animals (Di Chiara and Imperato, 1988, Morales-Mulia *et al*., 2007, Hall *et al*., 2008, Shen *et al*., 2015).

The expression phase (challenge), involved evaluating the intensity of nicotine or AMPH–induced behaviour in response to a challenge injection of (-) - nicotine or D- AMPH sulphate. All of the animals from the induction phase underwent ten days withdrawal, where the animals remained in the colony room, following the fifth pre-treatment session: this was the time point previously shown to be sufficient to induce the expression of locomotor sensitisation in response to nicotine and psychostimulant (Aguilar-Rivera *et al*., 2015, Morud *et al*., 2015). The first group of rats were tested to assess behavioural sensitisation to nicotine or AMPH: after 10 days withdrawal period. For this, on day 16 all of the animals from the NIC-NIC groups were challenged with (-) - nicotine (0.6 mg/kg, i.p) while the animals from AMPH-AMPH groups were challenged with a D-AMPH sulphate (1mg/kg, i.p) and their behavioural response was compared with animals that receive acute drug administration: that is, the animals from the SAL-NIC group which were pre-treated with saline and challenged with (-)-nicotine (0.6 mg/kg, i.p), and the animals from SAL-AMPH group which were pre-treated with saline and challenged with D-AMPH (1 mg/kg, i.p).

Because association between environment and drug administration is known to modulate the development and/or the expression of behavioural sensitisation (Robinson *et al*., 1998), it was important to ascertain that the apparently drug-induced behavioural sensitisation is drug dependent rather than context dependent. To this end, the second group of rats were pre-treated with either nicotine or AMPH and challenged with saline. The third group of rats were tested a cross-sensitisation phenomena: rats pre-treated with nicotine were given AMPH (NIC-AMPH group, n= 17) and those pre-treated with AMPH were given nicotine (AMPH-NIC group, n= 17). In the cross-sensitisation test, all rats received the same dose of the alternative drug from their pre-withdrawal treatment (see Figure 7). To determine the time course for studying the effect of drug treatment, the locomotor activity was measured at 10 min intervals over the course of a 60 min locomotor activity session (Schenk *et al*., 1991, Collins *et al*., 2004).

| Groups | Induction phase | Withdrawal period | Expression phase |
|---|---|---|---|
| 1 | NIC (0.6 mg/kg; n = 42) | 10 days | • NIC-NIC (n=17)<br>• NIC-AMPH (n =17)<br>• NIC-SAL (n = 8) |
| 2 | AMPH (1 mg/kg; n = 43) | 10 days | • AMPH-AMPH (n = 17)<br>• AMPH-NIC (n = 17)<br>• AMPH- SAL (n = 9) |
| 3 | SAL (1 ml/kg; n = 42) | 10 days | • SAL-NIC (n =14)<br>• SAL-AMPH (n =14)<br>• SAL-SAL(n = 14) |

Figure 7: Drug administration protocol and behavioural testing. Rats were assigned to one of three groups receiving different drug treatments over a 5-day period (induction phase) [NIC group (0.6 mg/kg, n = 42), AMPH group (1 mg/kg, n =43) or saline (SAL) group (1 ml/kg, n = 42)]. The rats were subsequently left undisturbed for 10 days in their home cages (withdrawal period). On the day after withdrawal period rats were challenged with either NIC, AMPH or SAL (Expression phase) using the same dose that was used in induction phase. A range of behavioural tests, focusing on distance travelled (m) were conducted during drug exposure and challenge day. All injection were given i.p. On challenge day and 2 h after recording locomotor activity, rats were humanely killed and the brain removed for histological analysis.

### 2.2.4 Data analysis

All behavioural data were analysed by using appropriate analysis of variance (ANOVA) using StatView software 5.0 (SAS Institute, NC, USA). The locomotor activity (distance travelled, m) data were analysed separately for pre-treatment sessions and challenge session. To determine the time course for studying the effect of drug treatment, the locomotor activity was measured at 10 min intervals over the course of 60 min locomotor activity session. The influence of pre-treatment of drugs on animals' activity during the induction phase (pre-treatment session) was analysed by using three-way ANOVA (5 × 6 × 3) analysis, using within-subjects factors of days [5 levels (day 1, day 2, day 3, day 4 and day 5)] and time bins [6 levels (10 min, 20 min, 30 min, 40 min, 50 min, 60 min)] and with a between-subject factor of drugs treatments [3 levels (nicotine, AMPH and saline)]. The expression phase of sensitization following challenge injection was analysed by using two- way ANOVA, using

within-subject factor of time bins and with a between-subject factor of drug conditions. Significant main effects or interaction were analysed using planned comparisons of acute effect of drug (SAL-SAL vs SAL-Drug), sensitisation effect (Drug-Drug vs SAL-Drug, challenge with the same drug that used in pre-treatment sessions) and cross-sensitisation (Drug-Drug vs SAL-Drug, challenge with the opposite drug that used in pre-treatment sessions) and this analysis were calculated manually.

## 2.3 Results

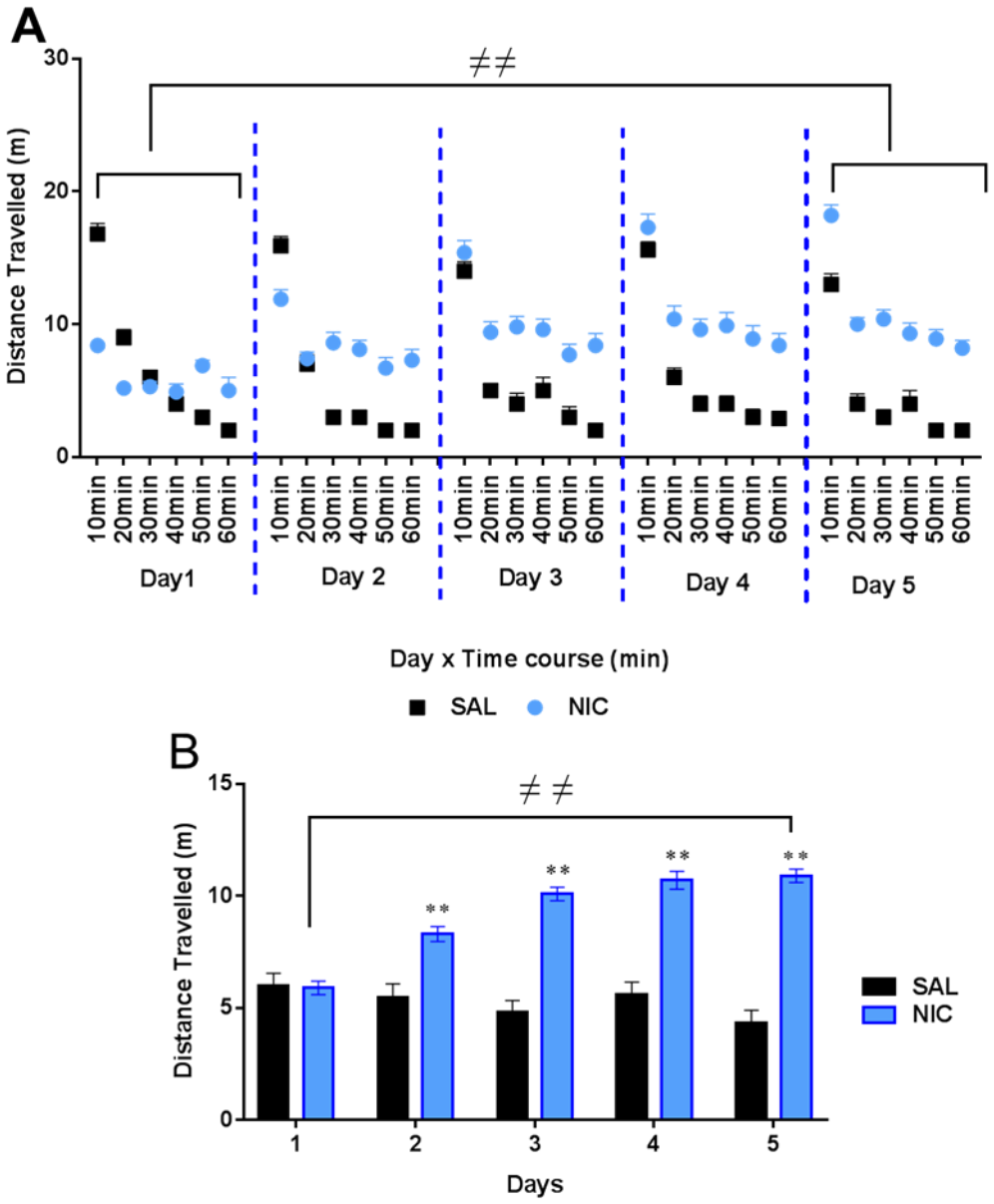
### 2.3.1 Sensitisation and cross-sensitisation to nicotine

The three-way ANOVA drugs treatment × days × time bins (3 × 5 × 6) revealed a significant main effect of drug pre-treatment [F (1, 82) = 25.50; p <.0001], day [F (4,328) = 6.30; p <.0001] and time [F (5, 410) = 345; p <.0001]. A two-way analysis revealed a significant interaction between drug pre-treatment × day [F (4, 328) =15.70; p < .0001], drug pre-treatment × time [F (5,410) = 36; p < .0001] and day × time [F (20, 1640) = 2.50; p <.01]. There was also a significant three-way interaction between drug pre-treatment × days × time [F (20, 1640) = 5.90; p < .0001]. On the first day of drug treatment (day1), acute administration of nicotine (0.6 mg/kg; i.p) does not affect the locomotor activity in naïve rats compared with rats receiving saline. After that the nicotine-induced activity increased during the entire test period. The following days the behavioural sensitisation to nicotine was evident during the test period and on day 5, the rats treated with nicotine showed a strong enhancement of activity compared with their activity in day 1 and compared with activity of the control rats (saline pre-treated) group (planned comparison, p <.01). These data suggest that the animals developed behavioural sensitisation to nicotine during the induction phase (Figure 8 A and B).

Following a withdrawal period of 10 days, animals received a challenge dose of either nicotine, AMPH or saline, and locomotor activity was measured (Figure 8 C). A two-way ANOVA (treatment × time) revealed a significant main effect of treatment [F (4, 65) = 2.80; p <.05], time [F (5, 325) = 59.8; p < .0001] and interaction between treatment and time [F (20,165) = 7.78; p < .0001]. Planed comparison confirmed that sub chronic intermittent nicotine administration resulted in a significant increase response to a subsequent nicotine challenge compared with saline pre-treated rats (NIC-NIC group vs SAL-NIC group) (p < 0.01). Another groups of nicotine pre-treated or saline-pre-treated rats were given a vehicle

challenge and post hoc analysis showed no significant effect of drug vs vehicle treated rats (NIC-SAL group vs SAL-SAL group) indicating that there was no effect of conditioning either to the injection or to the context on nicotine-induced behavioural senstisation.

To assess the effect of treatment history with AMPH on nicotine-induced activation (cross-sensitisation), planned comparison revealed a significant increase in activity produced by a nicotine injection in AMPH pre-treated rats compared with acute nicotine injection (AMPH-NIC group vs SAL-NIC group) (Figure 8 C). This means that a cross-sensitisation from D-AMPH to nicotine had been developed in term of behavioural sensitisation.



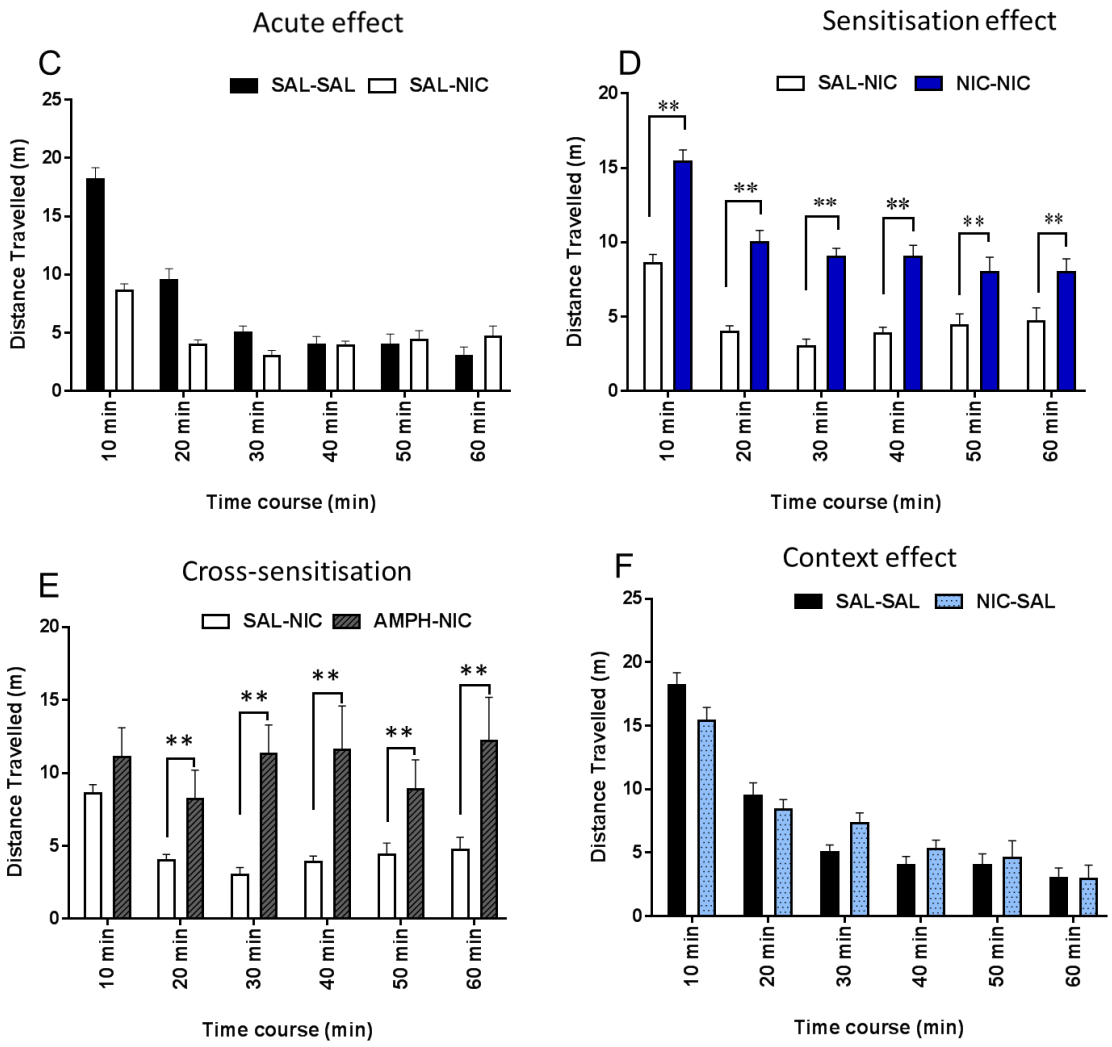



Figure 8: Behavioural sensitisation to nicotine. A) The time course (10 min bin) of locomotor activity during the five days injection that indicate a significant differences between nicotine day 1 vs day 5 as compared with control. B) five days (induction phase) nicotine injections elicits locomotor activity, although the locomotion was not significantly enhanced by the first nicotine injection. After 10 days of the last nicotine exposure (withdrawal period), nicotine challenge-induced expression of behavioural sensitisation was detected. C) Measure thee acute effect of nicotine, D) sensitisation effect, E) cross-sensitisation and F) the context effect on nicotine response. Data represent the mean ± SEM for distance travelled (m). ## = significantly differ NIC day 1 from NIC day 5 (Figure A and B). ** = significantly differ NIC from SAL (Figure B), NIC-NIC from SAL-NIC and AMPH-NIC from SAL-NIC (Figure D and E respectively), (P <.01).

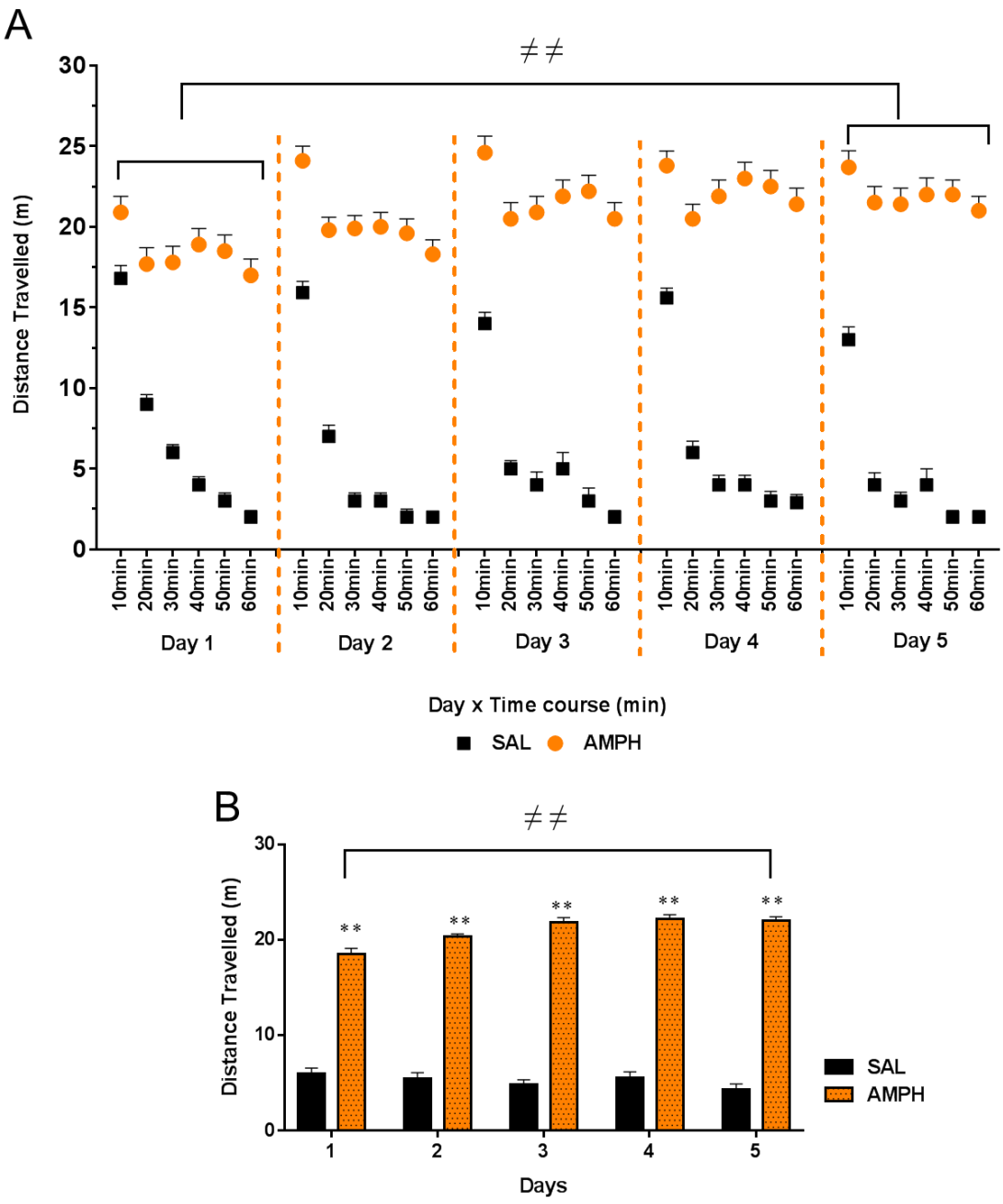
### 2.3.2 Sensitisation and cross-sensitisation to D-AMPH

Three-way ANOVA drug pre-treatment × days × time revealed a significant main effect of drug pre-treatment [F (1, 83) = 232; p <.0001] and a significant main effect of time [F (5,415) = 281; p <.0001] but there was no effect of day [F (4,332) = 2; p = .08]. However, two- way analysis with repeated measure on day revealed a significant interaction between drug pre-treatment × day [F (4, 332) = 6.10; p < .0001], drug pre-treatment × time [F (5,415) = 107; p < .0001], and day × time [F (20, 1660) = 2.90; p < .0001]. A three-way ANOVA (drug pre-treatment ×days × time), yielded a significant interaction between drug pre-treatment × days × time [F (20, 1660) = 1.80; p < .01]. Following injection on day 1, the activity scores of AMPH-treated rats were higher than for their saline-treated counterparts and this stimulatory effect grew with subsequent exposure. By day 5, AMPH produced an augmented locomotor response following the injection, compared with day 1 and with the control (saline pre-treated) group, which persisted throughout the test session (planned comparison, p <.01). This means that animals developed behavioural sensitisation to AMPH over repeated treatment during the induction phase (Figure 9 A and B).

After the 10 days withdrawal period, animals were challenged with either nicotine, AMPH, or saline, and locomotor activity was measured over the subsequent hour. A two-way ANOVA (treatment × time) revealed a significant main effect of treatment [F (4, 65) = 24.8; p < .0001], time [F (5, 325) = 54; p < .0001] and interaction between these variables [F (20,330) = 6.2; p <.0001]. Pre-treatment with AMPH caused a clear-cut augmentation of the locomotor response to AMPH challenge. The animals of AMPH pre-treated group that were challenged with AMPH showed increase in activity compared to the animals which were pre-treated with saline and challenged with AMPH (AMPH-AMPH group vs SAL-AMPH group). Planned comparison confirmed that AMPH-pre-treated group demonstrated a higher AMPH-induced activity during challenge period (p < .01).

Both groups that were challenged with saline on the challenge day (AMPH-SAL and SAL-SAL groups, pre-treated with AMPH or saline, respectively) showed considerably less locomotor activity following the saline injection indicating that there was no effect of conditioning either to the injection or to the context on AMPH-induced locomotor activity. For the cross-sensitisation test from nicotine to AMPH, planned analysis compared the locomotor activity in groups pre-treated with nicotine and saline respectively and challenged with AMPH (NIC-AMPH and SAL-AMPH groups) showed no significant effect of

nicotine-pre-treatment on the response to AMPH challenge as compared with acute AMPH (i.e. AMPH challenge after saline pre-treatment, planned comparison, p = ns) Figure 9 C.



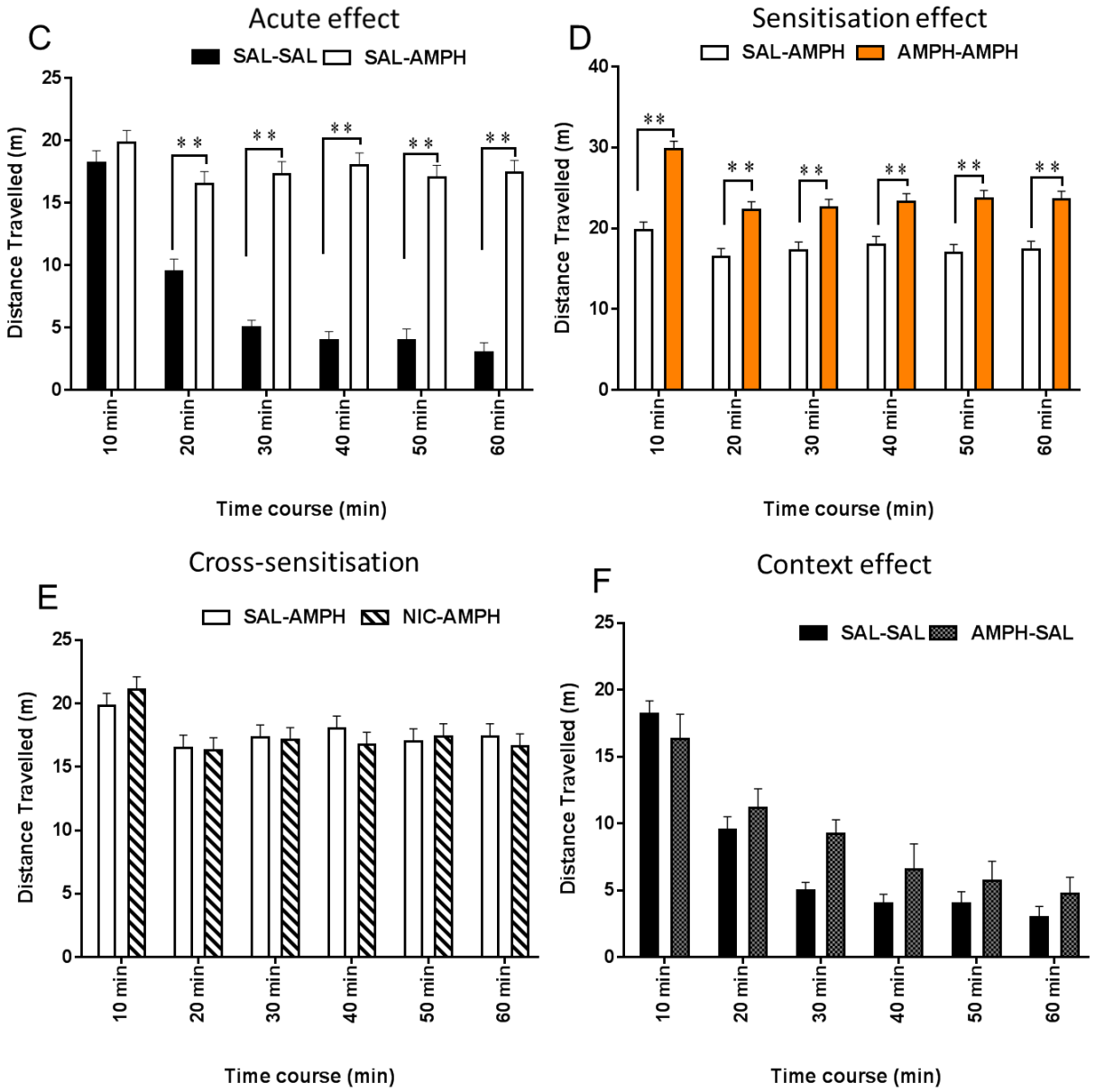



Figure 9: The behavioural sensitized response to AMPH. A) After consistent D-AMPH administration for five days (induction phase), distance travelled in meter was recorded for 1 h (A) and for 10 min time bins (B) immediately after AMPH injection. D-AMPH administration increase the locomotor activity across the indication phase. After 10 days discontinuous from the last injection, rats was challenged with D-AMPH and locomotor activity was recorded for 1 h. The behavioural sensitisation to D-AMPH was enhanced in rats treated with AMPH. C) Measure the acute effect of AMPH, D) the sensitisation effect, E) the cross-sensitisation and F) the effect of context on AMPH response. Data represent the mean ± SEM for distance travelled (in meter). ## = significantly differ AMPH day1 from AMPH day 5 (Figure A and B). ** = significantly differ AMPH from saline (Figure B), SAL-AMPH from SL-SAL and AMPH-AMPH from SAL-AMPH (Figure C and D respectively). (P <.01).

## 2.4 Discussion

The data presented here showed that repeated treatment with nicotine or AMPH (one injection per day for five consecutive days) facilitates the development of sensitisation in rats, and that 10 days of withdrawal is sufficient to express the sensitisation in these animals. In addition, there was no effect of condition either to the injection or to the context on nicotine or AMPH sensitise response. In term of cross –sensitisation, the present data shown that animals pre-treated with AMPH exhibit increase behavioural response to nicotine challenge as compared with acute nicotine injection. While, pre-treatment with nicotine does not effect on animal locomotor response to AMPH challenge. Thus, psychostimulant sensitisation can be induced in laboratory rodent via intermittent exposure to behavioural-activating dose, followed by a withdrawal period of days to weeks. Upon subsequent psychostimulant challenge with the same dose, animals exhibit an augmented behavioural response (behavioural sensitisation) compared to animals receiving the drugs for the first time (acute administration).

In the present work, we employed psychostimulant sensitisation to study the mechanism underling sensitisation in term of the effect of drugs on animals' behaviour and on neuronal plasticity in the mesolimbic pathway, projecting from VTA to NAc. It was hypothesized that repeated nicotine or AMPH administration would led to rapid development of locomotor sensitisation during either the pre-treatment period and upon drug challenge following a withdrawal period. In the present study, we tested this hypothesis using a 5-day, 0.6 mg/kg nicotine or 1 mg/kg AMPH pre-treatment regimen. In many rodent strains nicotine has immediate depressant effect which would hide/mask subsequent effects of stimulants (Schenk *et al*., 1991, Morud *et al*., 2016). Supporting this hypothesis we found that the initial effect of nicotine was to suppress activity on day1 as compared with saline treated rats. However, by the second injection (day 2), the suppressive effect was no longer apparent, instead an enhancement of activity occurred and this effect increased to a significant level by the fifth injection (day 5). For AMPH, on each of the five pre-treatment days, the drug produced marked hyperlocomotion.

It is frequently reported that the expression of psychostimulant sensitisation does not emerge during pre-treatment period but it is often revealed only after a period of withdrawal and subsequent challenge with a smaller dose of the sensitising agent (Kalivas and Stewart, 1991). Indeed, after 10 days withdrawal, animals in the present study showed sensitized

behavioural response to a challenge of the same dose that was used in pre-treatment period (0.6 mg/kg nicotine or 1 mg/kg, AMPH) compared to animals receiving the drugs for the first time, which is in line with previous studies ( e.g. (Byrnes *et al*., 2011, Hamilton *et al.*, 2012, Goutier *et al*., 2014).

A number of reports support a critical role for conditioned learning, particularly conditioning to the drug administration environment, in the expression of sensitisation and they concluded that the presence or absence of such stimuli during testing for sensitisation could significantly affect the results (Robbins and Everitt, 2002, Badiani and Robinson, 2004, Mattson *et al*., 2008). Anagnostaras and Robinson in 1996 showed that an environmental stimulus that comes to predict the presence of a drug can also acquire the ability to set the occasion for the sensitized response on the test day without the need to elicit an excitatory conditioned response of its own (Anagnostaras and Robinson, 1996). However, the contribution to environment-specific sensitisation is not clear. For example, Vezina and Leyton (2009) demonstrated that some exposure regimen such as local AMPH infusion into the VTA, do produce hyperlocomotion and sensitisation of DA release in NAc, but do not lead to the development of a condition response so that the expression of sensitisation is context independent. Similarly, *in vitro* striatal slice experiment, where behavioural context cannot have an influence, showed sensitisation of DA release. From this, Vezina and Leyton (2009) concluded that sensitisation is a non-associative phenomenon that can nonetheless come under environmental stimulus control. Moreover, Robinson *et al*. (1998) demonstrated that repeated psychostimulant treatment induced behavioural sensitisation in both a context-dependent and a context-independent manner. Moving from the findings above, our result showed that behavioural sensitisation to nicotine or AMPH occurred due to the drug effect rather than an effect of conditioning either to the injection or to the context.

Sensitised responses were only seen in animals that had received drug during the induction phase and at challenge. Had this been a conditioned sensitisation response then the sensitisation would have been present in drug pre-treated animals challenged with saline (NIC-SAL and AMPH-SAL groups)-that is the conditioned association between the injection and the previous experience of the drug would have been sufficient to produce a sensitised response. Since this was the case, we can conclude that the sensitisation was due to the drug, rather than due to conditioning. Similarly, animals pre-treated with saline and

challenged with saline (SAL-SAL group) does not show any sensitisation in either induction or challenge, indicating that the sensitisation was dependent on the drugs, rather than simply due to the process of receiving repeated injection.

To test whether behavioural sensitisation to different psychostimulants occurs as a consequnce of the same or different neuroadaptive phenomena, firstly the hypothesis on behavioural cross-sensitisation was tested by employing another experiments using the same sensitisation regimen above but with different challenge drug. The rats pre-treated with nicotine were challenged with AMPH while those pre-treated with AMPH will challenged with nicotine. In this regard, there was an effect of behavioural sensitisation, however, after 10 days of withdrawal, there was no evidence of the cross-sensitisation in rats treated with nicotine and challenge with AMPH, while we found evidence of the cross-sensitisation in rats treated with AMPH and challenged with nicotine. That is to say pre-treatment with nicotine, causing sensitisation to that drug did not also cause sensitisation when AMPH was given as the challenge dose. Indeed the locomotor response at the challenge for AMPH was similar whether they had been pre-treated with nicotine or with saline.

This result is similar to that reported by Collins *et al*. (2004), who studied the effect of repeated nicotine on AMPH challenge response. Their data revealed that rats pre-treated with nicotine for 7 days were not sensitized to the locomotor effect of AMPH challenged after 1 or 37 days withdrawal even though they were sensitize to nicotine itself. In addition, our present results confirmed the finding of Schenk *et al*. (1991), who found that different mechanisms mediate sensitisation to the different groups of drugs since pre-treatment with nicotine for 9 days did not appear to cause sensitisation of the response to other psych stimulant drugs such as cocaine after 1 day withdrawal. In contrast to nicotine pre-treated animals, rats pre-treated with AMPH showed increased locomotor response for nicotine as compared with saline pre-treated rats, which give us an evidence for cross-sensitisation. In similar way, Celik *et al.* (2006) indicated that caffeine and AMPH produced cross-sensitisation to nicotine-induced locomotor activity in mice which imply that similar mechanisms might play a role in the development of drug dependence to these substances.

# Chapter 3: Immunohistochemistry experiments

## 3.1 Introduction

The most important characteristic of behavioural sensitisation to psychostimulant drugs, such as AMPH and cocaine, is the very long lasting hypersensitivity to the drugs after sessions of exposure which is presumably caused by neuroplasticity changes in the circuit, at the cellular and molecular level mainly in the DArgic and glutamatergic systems (Nestler, 2005). To understand the molecular mechanism of behavioural sensitisation, it is important to analyse the expression of patterns of neuroplasticity-related genes. Several studies have proposed that these neuroadaptation require protein expression, the blockade of which inhibits behavioural sensitisation (Karler *et al*., 1993). Immediate early genes (IEGs) constitute a class of transcription factors, the expression of which can be elicited by diverse stimuli including pharmacological treatments. Amongst IEGs are Arc (activity-regulated cytoskeleton-associated protein) and *c-fos* and their production encodes the expression of late genes which effect of neuronal activity (Moro *et al*., 2007, Salery *et al*., 2017). In addition MeCp2 (Methy1-CpG binding protein 2) and BDNF (brain–derived neurotrophic factor) are believed to play an important role in the neuroplasticity (Ujike *et al*., 2002) and warrant investigation in the context of senstisation (for further discussion see chapter 1, section 1.2.3).

Behavioural sensitisation comprises two distinct temporal phases termed induction and expression that occur as cellular and molecular events produced by psychostimulant administration that cause changes in neural function responsible for behavioural augmentation. A variety of data show that these processes occur in distinct area within reward system (Robinson and Becker, 1986, Kalivas and Stewart, 1991, Pierce and Kalivas, 1996). Evidence suggests that both involve the mesolimbic DA pathway, projecting from VTA to the NAc, with the induction phase occurring at the cell bodies in the VTA, while behavioural expression is mediated in the terminal field in NAc (Vanderschuren and Kalivas, 2000). NAc has been divided anatomically into two distinct components, the central core region and the more medial and ventral shell region (Brog *et al*., 1993, Ikemoto, 2007). Although these regions have unique patterns of afferent and efferent connections, they both receive DAergic input cells from the VTA. In addition each region has been identified as participating in reward processing associated with drugs of abuse, but evidence

suggests that the two regions participate in distinct, complementary aspect of drugs reward (Boye *et al*., 2001, Sellings and Clarke, 2003).

The aim of this experiment was to evaluate the neuroadaptation underlying the development of behavioural sensitisation and cross-sensitisation in order to find the mechanism underlying behavioural sensitisation. To address this aim post-mortem histological and biochemical analysis was performed. Firstly, the behavioural sensitisation to nicotine or AMPH after 5 days treatment and in response to challenge dose after 10 days withdrawal period was measured (as shown in chapter 2). Secondly, using the same animals, further experiments were carried out to explore the cellular changes which have occurred after this sensitisation procedure, mainly through post-mortem histological analysis, using expression of the IEGs Arc and *c-fos* and of MeCp2 and BDNF expression in NAc and VTA using immunohistochemistry procedure.

## 3.2 Materals and Methods

### 3.2.1 Behavioral studies

After finishing the behavioural experiment as shown in Chapter 2 (behavioural sensitisation experiment section 2.2.3), some animals from different groups were chosen randomly for immunohistochemistry experiments. Briefly, as described in chapter 2 (section 2.2.3), following acclimatisation, handling and habituation, male Lister-hooded rats (n = 127) were injected with nicotine (0.6 mg/kg, i.p, n = 42), AMPH (1 mg/kg, i.p, n = 43) or saline (1 ml/kg, i.p, n = 42) for five consecutive days and their locomotor activity was measured immediately for 1h. After the last injection the rats were left for 10 days withdrawal period and in challenge day, the animals injected with either nicotine, AMPH or saline (using the same dose as in pre-treatment) and their locomotor activity was measured for 1 h.

### 3.2.2 Immunohistochemistry

Previous studies have shown that the optimum time for killing the animal after drug treatment, in order to measure changes of IEGs such as *c-fos* and Arc, and of BDNF, MeCp2 expression is 2 hours (Herdegen and Leah, 1998, Banerjee *et al*., 2009). Therefore, in the current experiments, one hour after the end of the challenge test sessions (i.e 2 h after the drug/saline injection), some animals from different groups (n = 5-7 per group) were deeply anesthetized with sodium pentobarbital (100 mg/kg, i.p) containing 2% lidocaine and fixed

by transcardic formaldehyde perfusion. Once the animal was deeply anesthetized trasncardic perfusion surgery was conducted according to (Gage *et al*., 2012). Brifly, a lateral incision (5-6 cm) was made through the skin and abdominal wall beneath the rib cage, then a small incision was made in the diaphragm by using curved, blunt scissor and continued along the entire length of the rib cage. Any tissues connecting to the heart was trimmed and the haemostat was placed over the animals head, to give a clear view of the heart and major blood vessels.

After clamping the descending aorta by using a haemostat to prevent the perfusion buffer going to the lower part of the animals body, a small incision to the posterior end of the left ventricles was made using iris scissors. A 15 gauge perfusion needle was passed through the cut ventricle into ascending aorta avoiding reaching to the aortic arch, and a haemostat was clamped on to the ascending aorta around the perfusion needle tip to prevent leakage until the perfusion procedure was finished. Finally, a small incision was made in the right atrium to create as large an outlet as possible without damaging descending aorta, at this point the animal was ready to be perfused (Gage *et al*., 2012) (see Figure 10). Each animal was perfused transcardially with 0.9% NaCl solution (5 min) followed by 4% paraformaldehyde (PFA) in 0.1 M sodium phosphate-buffered saline (PBS: pH 7.4) for 15 min, at a flow rate of 15 ml/min using variable-flow peristaltic pump.

The brains were removed and post-fixed in the same fixative (4% PFA) at 4 C° for 24 h and then the brains were rinsed with PBS (0.1 M; pH =7.4) 3 × 10 min and after washing with PBS the brains were cryoprotected in 15% sucrose in 0.1 M phosphate buffer for additional 24 h at 4℃ or until the tissue sink. When the brains had sunk, they were transferred to a 30% sucrose /PBS solution at 4 Cº and left in the solution until they sank again. Following sucrose infiltration, the brains were rapidly frozen in isopentane and stored at -80 Cº prior to sectioning. Using freezing microtome, cryostat (Thermo scientific cryostar NX50, Germany), 30 µm coronal sections were cut through the VTA and NAc, using the Paxinos and Watson (1998) atlas to identify selected brain regions by anatomical landmarks, and all sections were stored in 0.1 M PB and 0.1% sodium azide at 4 Cº until being processed for immunohistochemical analysis.

For immunohistochemistry, free floating sections from selected brain areas and from control and drug treated animals were processed according to a standard protocol (Jedynak *et al*., 2016). All floating sections were washed, at the same time, with 0.1 M PBS 3×5 min and

then blocked for 10 min with 3 % $H_2O_2$ in PBS to reduce endogenous peroxidase activity and rinsed again with PBS (2 ×10 min). The specific gene primary anti-body was then added and sections were incubated for 30 min in PBS with 5% normal goat serum (NGS) in order to block non-specific staining and followed by washing (3×5) min with 0.1 M PBS.

For all genes, the brain sections were incubated with appropriate concentration of primary antibodies (different antibody according to the gene under investigation: see section 3.2.3) diluted in 0.1 M PBS-1 % Triton X-100 containing 1% NGS for two night in the fridge with gentle shaking. Section were then washed with 1.0 M PBS (3×5 min), followed by 1 h incubation with biotinylated secondary goat anti-rabbit antibody IgG (Vector Laboratories, 1:500 dilution in 0.1 m PBS and 1 % NGS) at room temperature and rinsed again with 0.1 M PBS (3×5 min). A standard avidin-biotin procedure was then performed by incubating the brain sections with avidin- biotin complex (ABC; Elite Kit, Vector Laboratories) diluted to 1:200 (5 µl reagent A + 5 µl regent B in 1 ml PBS 0.1 M) for1 h. After washing the sections with 0.1M PB 3×5 min immunoreactivity were revealed by washing the sections with nickel- enhanced 3'3'diaminobenzidine (25 mg DAB/ 100 ml of 0.1 M PB, 80 mg/100 ml of 0.1 M PB nickel ammonium sulphate and 10 µl $H_2O_2$) for 5 min at room temperature and then sections were washed in 0.1M PB (3×5 min) before mounting. All washes and incubation were carried out on an orbital shaker. Sections were mounted on gelatine / chrome-alume coated slides, air-dried and dehydrated through graded ethanol (70 % for 5 min, 95% and 100% for 10 min) and finally with xylenes for 10 min to clear the tissue, and cover slipped with Depex ( BDH laboratory supplies, Pool, England ). If sections were not mounted straight away, 0.1 % sodium azide was added to sections and they were stored in the - 4 $C^o$ until mounting. It is important to note that, all the steps above are the same for all different genes which were measured (Arc, *c-fos*, MeCp2 and BDNF) and the only difference in procedure was that primary anti body and its concentration varied between different genes.

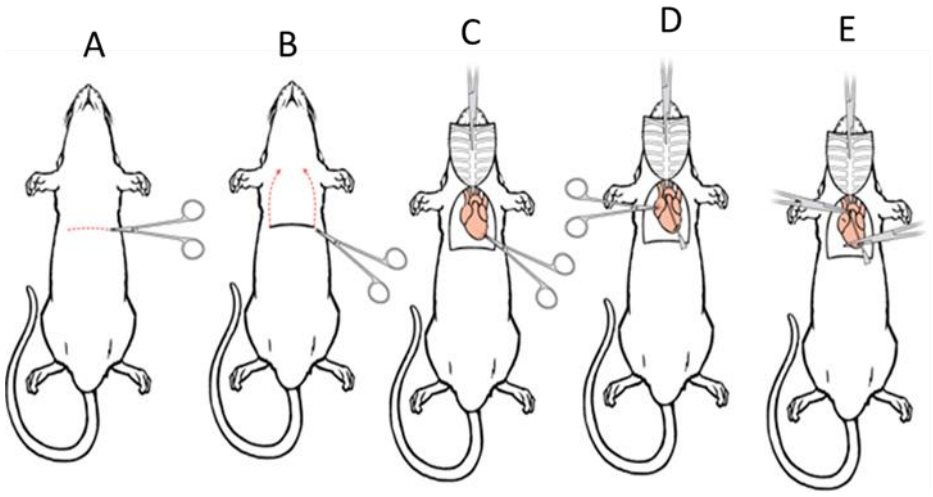



Figure 10: The steps of the transcardial prefusion surgery in rodent : **A**) Make a lateral incision through skin and abdominal wall. **B**) Make an incision through the diaphragim and cut the diaphragm exposing the heart and make an incision on either side of the ribs up to the collar bone. **C**) Clamp the tip of the sternum with haemostat and place the haemostat over the head and make the cut through the posterior end of the left ventricle. **D**) Pass the perfusion needle through the cut ventricle into ascending aorta and by using iris scissors make a small cut through the posterior end of the right atrium . **E**) Clamp the aorta with the tip of prefusion needle (Gage *et al*., 2012).

### 3.2.3 Antibodies:

For each of the antibodies used preliminary experiments were carried out to ascertain the optimum primary antibody concentration. Specifically, for antibodies to Arc, *c-fos* and MeCp2 we used 1:1000 concentration and we found that the concentration was too weak to express the genes. Further experiments were done with 1:2500 primary antibody concentration and we found that the staining was too dense, such that we could not recognize the neurons that express the antibodies. Thus, we found that the appropriate primary antibody concentration to use to carry on the immunohistochemistry experiments was 1:2000 for Arc, *c-fos*, and MeCp2. Similarly, for BDNF tested 1:3000, 1:2000 and 1:1500 dilutions and found that the best concentration was 1:1500 which is used in the subsequent experiments. In summary, the primary antibodies used were:

- Arc: 1:2000 dilution of rabbit polyclonal anti-ARC, C-Terminal antibody (Synaptic-Systems, Germany).
- *c-fos*: 1:2000 dilution of rabbit polyclonal anti- (c)-*fos* antibody (Santa Cruz Biotechnology, Santa Cruz, CA) (Brenhouse and Stellar, 2006).
- MeCp2: 1:2000 dilution of rabbit polyclonal anti- MeCp2 (Millipore EMD).
- BDNF: 1:1500 dilution of rabbit anti-BDNF (Millipore EMD) (Fanous et al., 2011), in 0.1M PBS-Triton X-100 containing 1% NGS.

### 3.2.4 Image analysis and quantification

Immunohistochemistry localisation of the Arc, *c-fos*, MeCp2 and BDNF resulted in nuclei that exhibited a different levels of labeling intensity. Thus, in order to count the protein-postive nuclei accurately, a counting threshold was chosen based on consistent and measurable target parameters such size, shape and stain density. Firstly, we used automated counting technique by using ImagJ software (U.S. National Institutes of Health, Bethesda, MD). When appropriate threshold parameters were employed, automated counting was faster and more easily transferred between the sections. However, we found that the ImagJ software could not distinguish between the cells and dark spots from remaining stains and it counted these spots as cells. Therefore, we switched to the second method which was to count the cells mannualymanually. Although, with manual counting cells by visual observation, it was dificult to find appropriate thresholds for counting and maintain consistency over large number of sections before starting manual counting, the images were thresholded at the standard RGB-scale level to allow detection of the nuclei stained with moderate to high intensity and avoid lightly stained nuclei by using ImagJ software. Slides from each rats were coded for analysis, such that the scoring was carried out blind to the experimental condition. For each animal, the four antigens were quantified in the core and shell subregions of the NAc and in VTA (see Figure 11).

Delineation of the NAc shell and core subregions in the sections stained for three antigens is difficult because no clear histological marker separate these two regions, thus, in order to overcome this obstacle, we used the Paxino and Watson (1998) atlas for the neuroanatomical characteristics and estimated the outline for each regions. Each area of interest was identified

at low magnification (4 x) using Nikon E600 microscope (Nikon Instruments Europe B.V. / United Kingdom) attached to a digital camera (Andor iXonEM+ EMCCD DU 885 camera). Then, under higher magnification (10 x) the images were captured and the cells counted manually.The cells in the shell and core NAc subregion and VTA in both hemispheres for all the sections (2- 3 sections per-brain for each pre-treted animal) were counted.

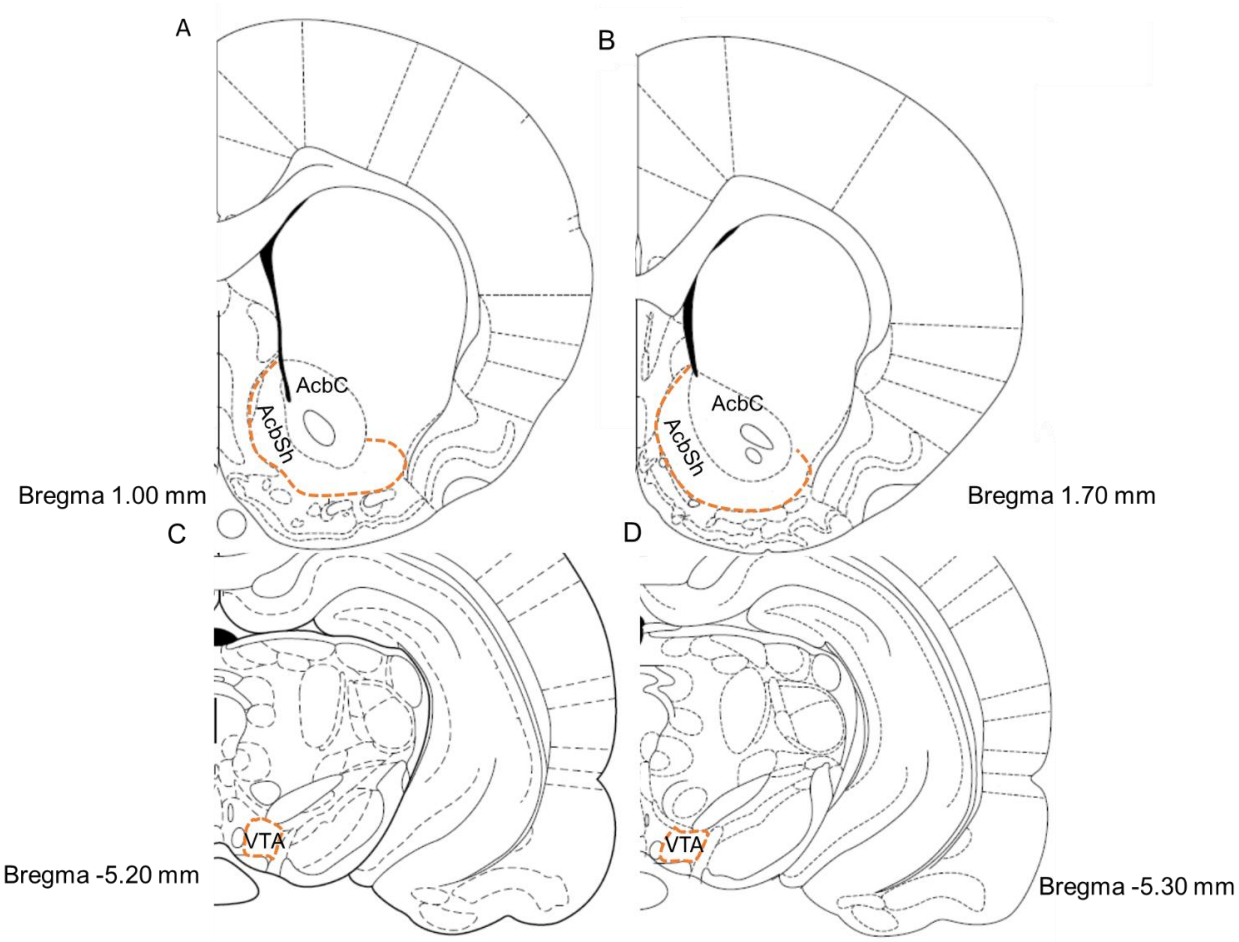



Figure 11: Schematic representation of brain regions (coronal sections) selected for gene expression analysis. NAc sub region (shell and core, A and B) VTA (C and D) from Paxinos and Watson (1998) atlas.

### 3.2.5 Data analysis

In immunohistochemistry experiments, we aimed at ascertaining the acute effect of drug (SAL-SAL vs SAL-Drug), senstisation effect (Drug-Drug vs SAL-Drug, challenge with the same drug t used in the pre-tretment peroid) and cross-senstisation (Drug-Drug vs SAL-Drug, challanged with the opposite drug that used in the pre-trerment period). In these experiments, data representing the density of the four antigens (the number of cells per surface area) in each area of interest were expressed as an average density of positive immunoreactivity nuclei per rat and the group averages were determined from the rat averages: thus the experimental unit is the rat. The individual density was obtained across multiple sections (2- 3 sections per treated rat) for shell and core subregions of NAc and for VTA and then averaged to find the density in each region for each rat in each treatment group. Differences in the densities of the four antigens immunoreactivity nuclei (Arc, *c-fos*, MeCp2 and BDNF proteins) between treatment groups (nicotine, AMPH or saline) were analysed using one-way ANOVA. Following confirmation of main effect by overall ANOVA analysis, planned comparisons of specific groups, ( acute group SAL-SAL vs SAL-Drug, senstisation Drug-Drug vs SAL-Drug challenge with the same drug that used in pre-tretemnt session, and cross-senstisation groups SAL-Drug vs Drug-Drug, challenge with the opposite drug that used in pre-tretemnt sessions), were done and statistical significance was set at $p < 0.05$. The data are expressed as mean ± SEM and all statistical analysis were performed using StatView 5.0 (SAS Institute, NC, and USA).

## 3.3 Result

### 3.3.1 Nicotine sensitisation

#### 3.3.1.1 Arc protein expression in the NAc and VTA

To evaluate long-term adaptation induced by 5 days of nicotine injection followed by 10 days of withdrawal, testing for Arc protein expression was performed in the NAc- shell and core sub regions. One-way ANOVA revealed a significant main effect of nicotine treatment in both NAc, shell [$F(4, 24) = 6.02$; $p < .01$] and core [$F(4, 24) = 11.34$; $p < .0001$]. Planned comparison revealed that Arc expression in the core was significantly increased after acute nicotine injection compared with saline control animals (no drug administration) (SAL-NIC vs SAL-SAL group, planned comparison, $p < .05$). In addition, Arc expression in the core,

but not in the shell, was significantly increase in nicotine sensitised rats compared with acute nicotine injection (NIC-NIC group vs SAL-NIC group) ($p < .05$), while this effect was enhanced in both core and shell in animals pre-treated with AMPH and challenged with nicotine compared with acute nicotine injection (AMPH-NIC group vs SAL-NIC group) (planned comparison, $p<.05$) Figure 12 A-H.

In the VTA, one-way ANOVA showed a significant main effect of treatment [$F(4, 21) = 8.68$; $p < .05$]. Post hoc comparison revealed that only acute nicotine showed an increased in Arc mRNA expression compared with control animals (SAL-SAL group vs SAL-NIC group, Fisher's LSD, $p < .01$), while animals pre-treated with either nicotine or AMPH showed no effect on challenge with nicotine as compared with saline pre-treated animals (planned comparison, p = n.s) Figure 12N-V.

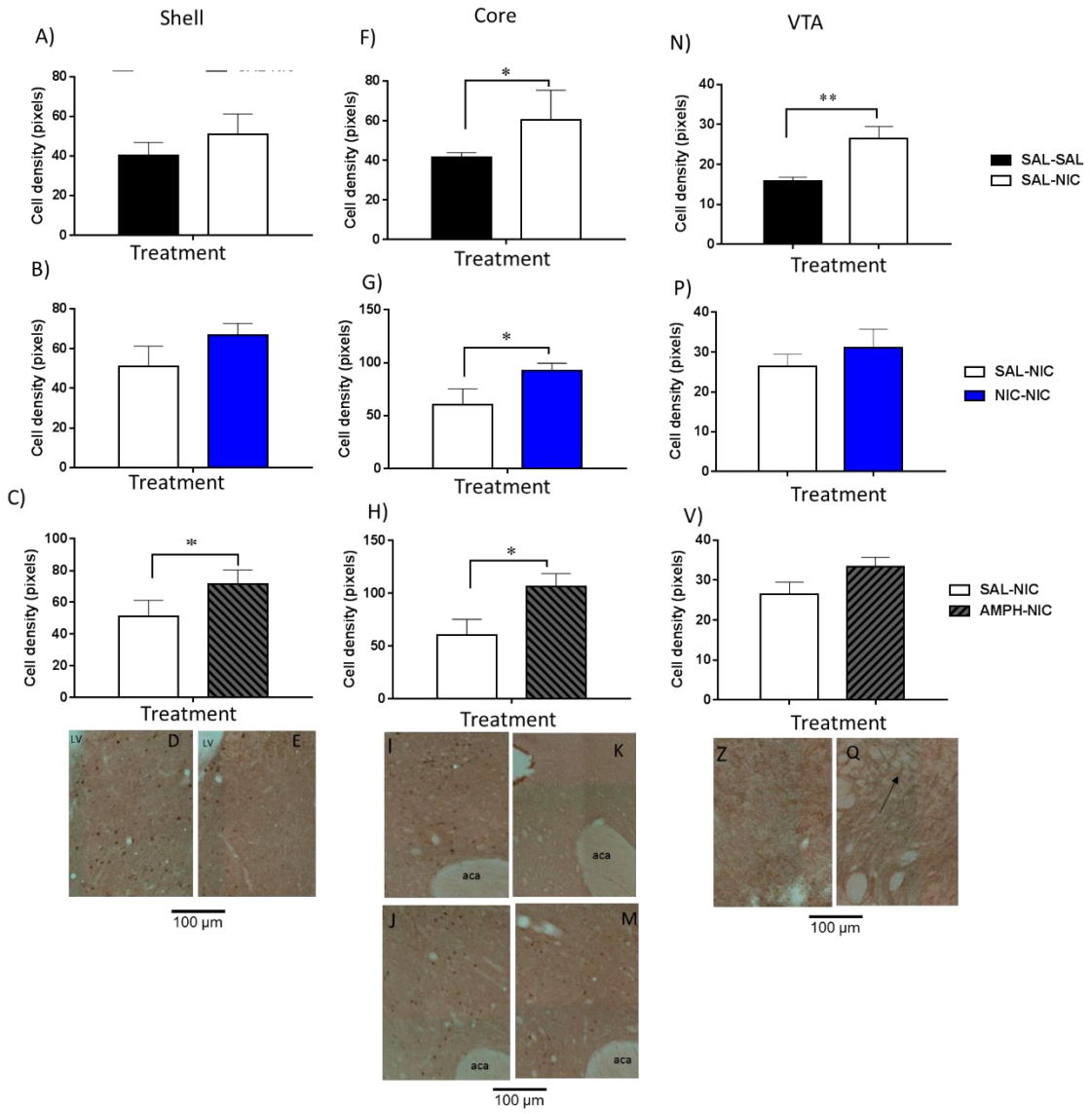



Figure12: Arc protein expression in the NAc- shell and core sub regions and VTA. A) There was a significant increase in Arc protein expression in the nicotine sensitise rats compared with animals receive acute nicotine. In addition the graph shows that treatment with AMPH increase Arc protein expression in the core (H) and shell (C) of NAc compared with control rats in response to nicotine challenge ( $p < .05$). In the VTA, there was a change in Arc expression in animals treated with saline and challenge with nicotine compared with control animals (N) ($p < .01$). Bright filed photomicrographs (10 x) showing Arc expression in sections through NAc, in NAc shell of rats treated with AMPH (D) or saline (E) and challenged with nicotine. Arc expression in the NAc core of rats treated with nicotine (I) or AMPH rats (J) and challenged with nicotine compared with acute nicotine administration (M) and saline control rats (K). Bright filed photomicrographs (10 x) showing Arc expression in sections through VTA of rats treated with saline and challenge with either saline (Z) or nicotine (Q). Data represent the mean ± SEM for cell density (2-3 sections per treated animal).

### 3.3.1.2 *C-fos* protein expression in the NAc and VTA

In the NAc-shell and core sub region, one-way ANOVA revealed a significant main effect of treatment in both NAc shell [$F(4, 23) = 3.71$; $p < .05$] and core [$F(4, 23) = 4.85$; $p < .05$]. However, planned comparison revealed that nicotine sensitised rats did not show any effect on *c-fos* protein expression compared with acute nicotine injection (NIC-NIC group vs SAL-NIC group) (planned comparison, p =n.s). Rather, acute nicotine had a significant effect on *c-fos* protein expression in animals treated with AMPH in the core, but not in the shell, compared with acute nicotine injection (AMPH-NIC group vs SAL-NIC group) (planned comparison, $p < .01$) Figure 13A-F.

In the VTA, one-way ANOVA indicate a significant main effect of treatment [$F(4, 24) = 9.50$; $p < .0001$]. Planned comparison revealed that only treatment with saline and challenged with nicotine induced a change in the *c-fos* protein expression in the VTA compared with control rats (SAL-NIC group vs SAL-SAL group) (planned comparison, $p < .05$) Figure 13I-K.

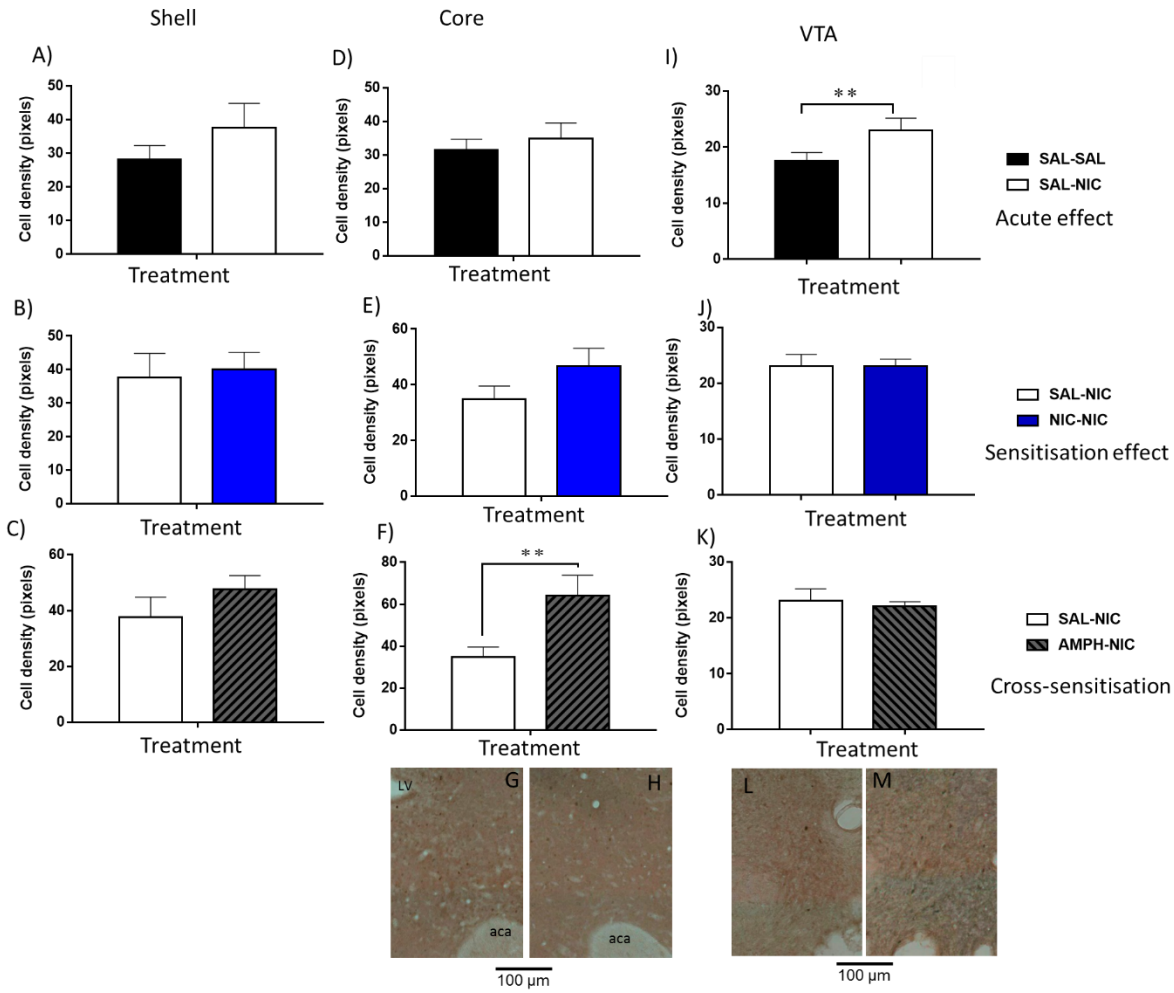



Figure 13: *C-fos* protein expression in the NAc shell and core sub regions and VTA. A) Treatment with AMPH has a significant effect on *c-fos* expression in the NAc core compared with saline treated animals in response to acute nicotine (F) ($p < .01$). In VTA, acute nicotine administration show a *c-fos* changed in animals pre-treated with saline compared with control rats (I) ($p < .05$). Bright field photomicrographs below (10×) show *c-fos* induction in NAc core of rats treated with AMPH (G) or saline (H) and challenged with nicotine. In VTA, bright field photomicrographs show *c-fos* expression in the rats treated with saline and challenged with either saline (L) or nicotine (M). Data represent the mean ± SEM for cell density (2-3 sections per treated animal).

### 3.3.1.3 MeCp2 protein expression in the NAc and VTA

One-way ANOVA indicated a significant main effect of pre-treatment on MeCp2 protein expression in both NAc shell [$F(4, 22) = 39.940$; $p < .0001$] and core [$F(4, 22) = 20.42$; $p < .0001$]. Planned comparison revealed that acute nicotine show a significant increase in the MeCp2 expression in the shell and core as compared with control rats (SA-NIC group vs SAL-SAL group, planned comparison, $p < .01$). In addition, planned comparison revealed that challenge with nicotine increased MeCp2 expression in the NAc depending on animals' drug history. In nicotine sensitised rats, nicotine challenge increase MeCp2 protein expression in the NAc core only but not the shell compared with acute nicotine injection (NIC-NIC group vs SAL-NIC group) (planned comparison, $p < .05$). In contrast, in animals receiving AMPH pre-treatment, nicotine challenge induced an increase in the MeCp2 expression in the core and shell compared with acute nicotine injection (AMPH-NIC group vs SAL-NIC group) ( planned comparison, $p < .05$) Figure 14 A-I.

In the VTA, one-way ANOVA revealed a significant main effect of treatment [$F(4, 23) = 7.95$; $p < .01$], and post hoc analysis indicated that MeCp2 changed only in the animal treated with saline and challenged with nicotine compared with control rats (SAL-NIC group vs SAL-SAL group) (planned comparison, $p < .01$) Figure 14 O-P.

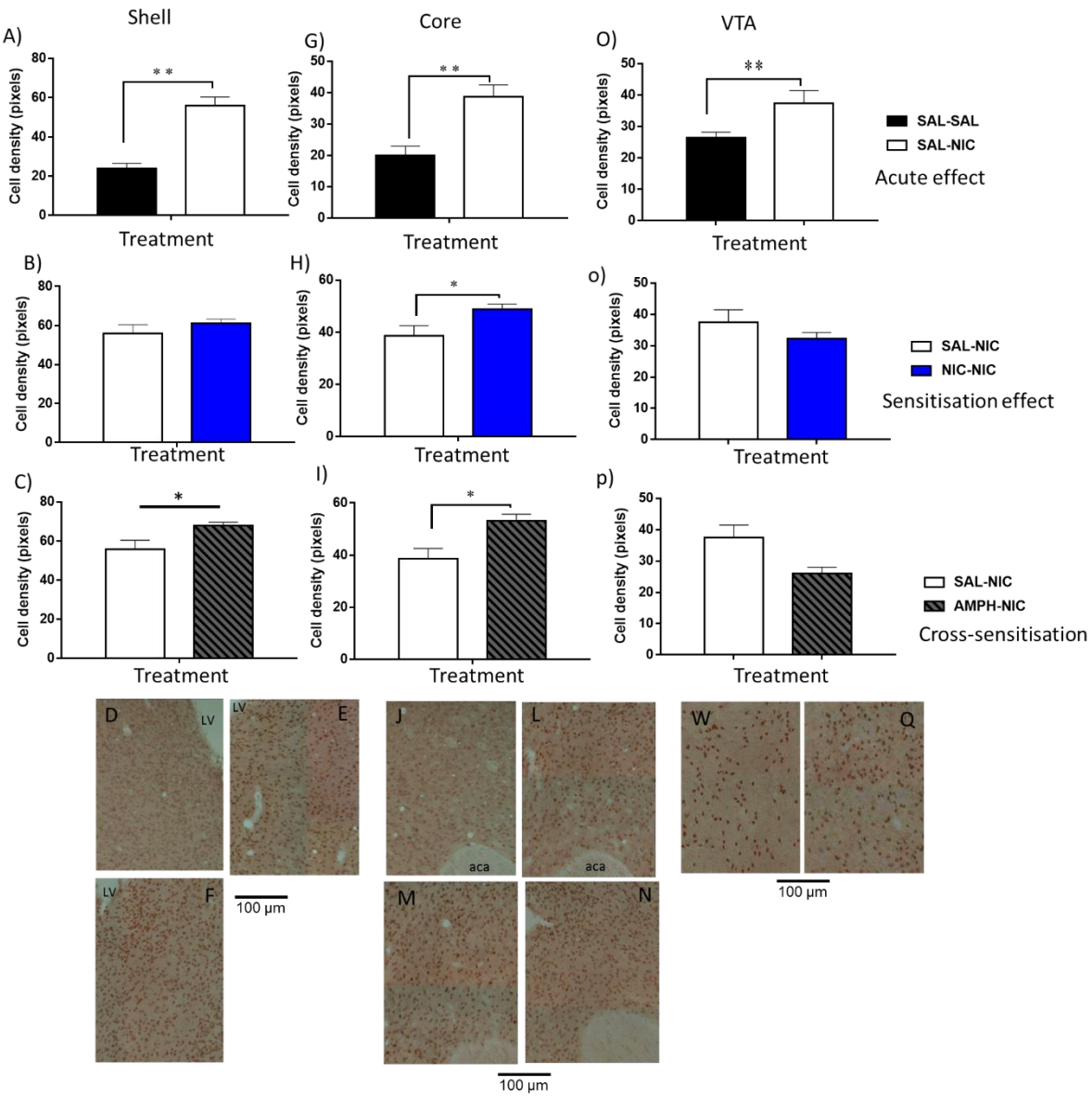



Figure14: MeCp2 protein expression in the NAc shell and core sub region and VTA. In NAc, nicotine sensitise rats show a significant increase in MeCp2 protein expression in the core (H) as compared with acute nicotine injection D-AMPH history treatment also had a significant effect on MeCp2 expression in the core (C) and shell (I), in response to nicotine challenge, compared with acute nicotine administration (, $p < .05$). In VTA, challenge with nicotine induce a significant response to MeCp2 expression in saline pre-treated (O) rats compared with control animals (, $p < .01$). Representative bright field photomicrographs showing detection of MeCp2 protein in the NAc core of rats treated with either nicotine (J) or AMPH (L) and challenged with nicotine compared with acute nicotine administration (M) and saline control rats (N) and in the NAc shell of rats treated with AMPH and challenged with nicotine (E) compared with acute nicotine administration (D) and saline control rats (F). In VTA, representative bright field

photomicrographs showing MeCp2 expression in rats treated with saline and challenge with either saline (Q) or nicotine (W). Data represent the mean ± SEM for cell density (2-3 sections per treated animal).

### 3.3.1.4 BDNF protein expression in the NAc and VTA

BDNF protein expression in the NAc sub region and in the VTA is shown in Figure 13. In the NAc, one- way ANOVA indicated a significant effect of treatment in both NAc shell [F (4, 23) = 6.96; p <.01] and core [F (4, 23) = 7.05; p <.01]. Planned comparison revealed that challenge with nicotine caused an increase in BDNF expression in the shell and core of saline pre-treated rats as compared with control animals (SAL-NIC group vs SAL-SAL group, p < .01). Challenge with nicotine in animals pre-treated with AMPH showed a higher expression in the shell, but it did not reach a statistical significance compared with acute nicotine administration (AMPH-NIC group vs SAL-NIC group). Following nicotine pre-treatment there was no effect of nicotine challenge on BDNF expression in the shell and core of NAc compared with acute nicotine injection (NIC-NIC group vs SAL-NIC group) (planned comparison, p = ns) Figure 15 A-H.

In VTA, one-way ANOVA revealed a significant effect of treatment [F (4, 20) = 7.81; p <.01]. Planned comparison revealed that only animals treated with saline and challenge with nicotine showed an increased in BDNF expression compared with control group (SAL-NIC group vs SAL-SAL group) (, p <.01) Figure 15 L-N.

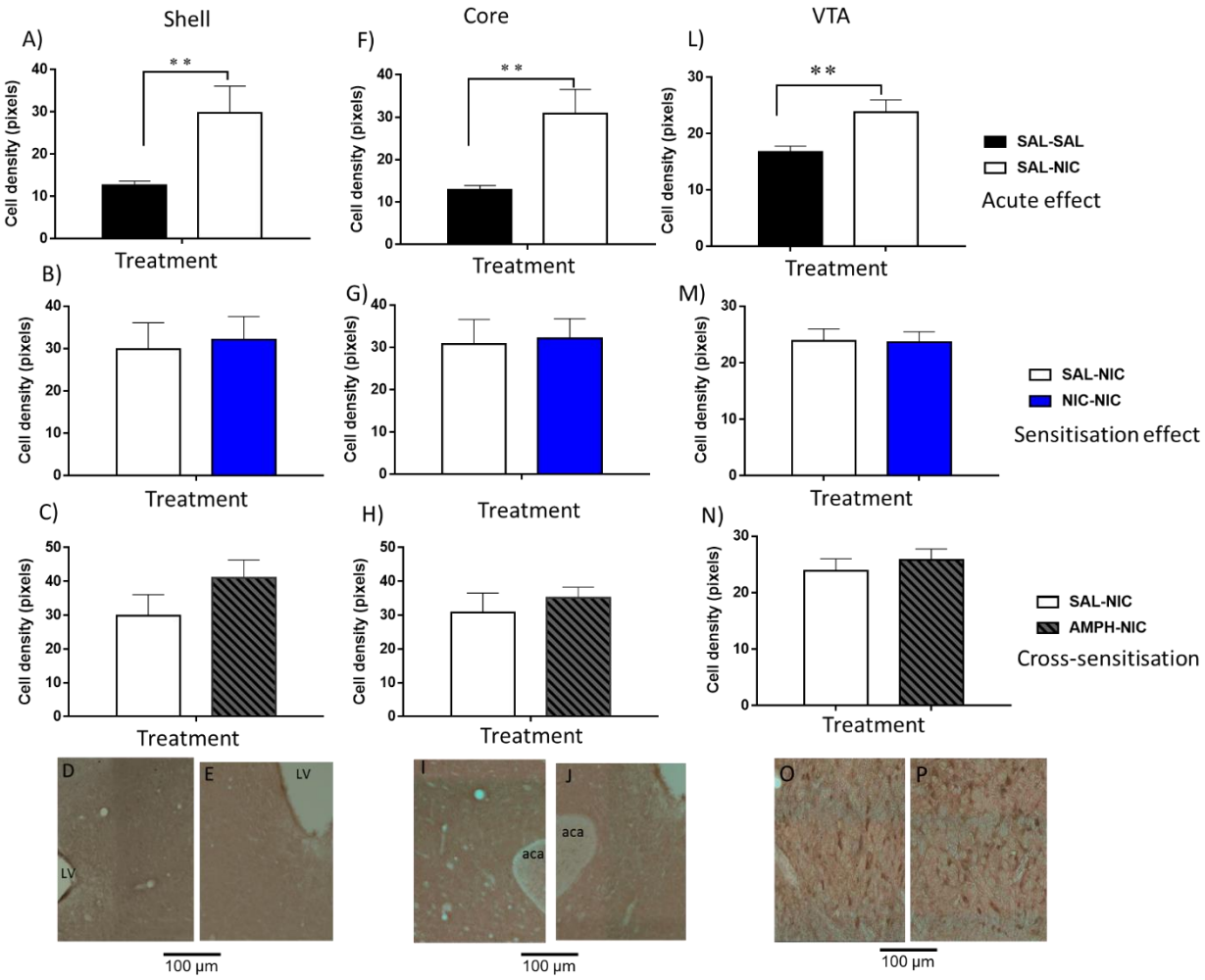



Figure 15: Shows the BDNF protein expression in the NAc shell and core sub region and VTA. In NAc, BDNF mRNA expression changed in the NAc shell in AMPH-NIC vs SAL-NIC groups but it does not reach a statistical significant, while in increase in rat challenge with nicotine as compared with saline control rats n shall (A)and core (F). In VTA, rats challenge with nicotine showed a change in BDNF protein expression compared with saline control animals (L) (, $p < .01$). Bright field photomicrographs showing induction of BDNF in the NAc shell of rats treated with saline and challenged with nicotine (D) compared with saline control (E) rats and in the core for acute nicotine administration (I) compared with saline control rats (J). In VTA, photomicrographs showing induction of BDNF in rats treated with saline and challenged with nicotine (O) as compared with saline control animals (P). Data represent the mean ± SEM for cell density (2-3 sections per treated animal).

### 3.3.2. D-AMPH sensitisation

#### 3.3.2.1 Arc protein expression in the NAc and VTA

Expression of Arc protein in the NAc and in the VTA is shown in Figure 14. In the NAc, the overall one-way ANOVA analysis revealed a main effect of treatment on gene expression in the both shell [$F(4,21) = 4.373$; $p < .01$] and core [$F(4,21) = 7.571$; $p < .01$] sub region of NAc, planned comparison revealed that acute AMPH administration cause increase Arc expression in the NAc shell and core as compared with control rats (no drug administrated) (SAL-AMPH group vs SAL-SAL group, $P < .01$). In addition, repeated AMPH treatment resulted in increased Arc expression in the shell, but not in the core, which was only apparent after a challenge AMPH compared to acute administration of AMPH (AMPH-AMPH group vs SAL-AMPH group, $p < .05$). There was no effect of nicotine pre-treatment history on Arc protein expression in either NAc sub region (shell and core) compared with acute AMPH administration (NIC-AMPH group vs SAL-AMPH group) (, p = ns) Figure 16A-J.

In the VTA, one way ANOVA revealed a significant main effect of treatment on Arc protein expression [$F(4, 19) = 18.84$; $p < .0001$]. Planned comparison revealed that Arc expression was only changed in VTA in animals pre-treated with saline and challenged with AMPH compared with control rats (SAL-AMPH group vs SAL-SAL group) (, $p < .01$) Figure 16 M-O.

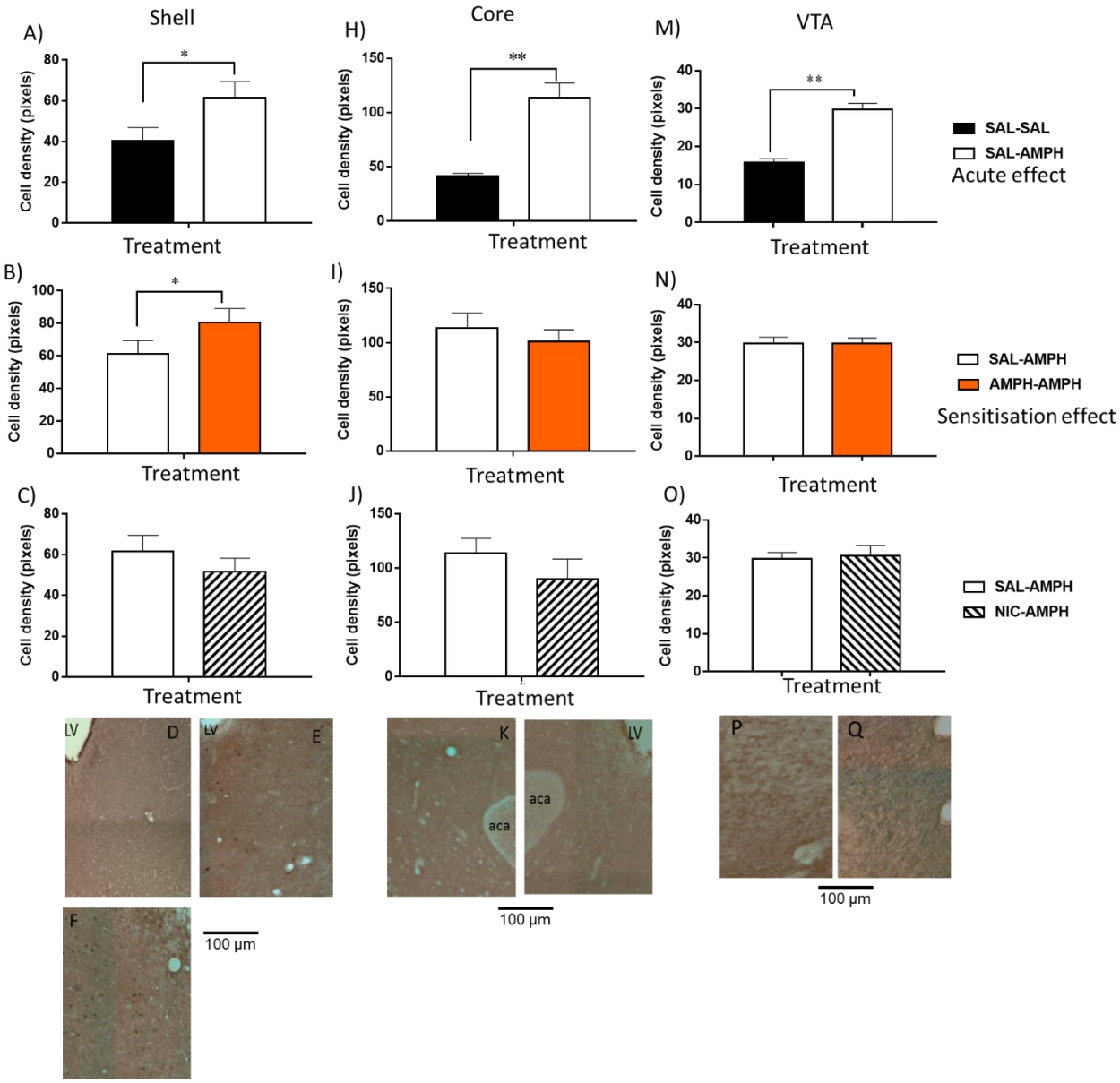



Figure 16: Density of Arc-positive cells in the sub region of the NAc and VTA. In NAc, AMPH challenge cause increase in Arc expression in the NAC shell (A) and core (H) of saline pre-treated rats as compared with saline control ($p < .05$). D-AMPH behavioural sensitisation was accompanied by increases in Arc expression in NAc shell compared with acute AMPH administration (B) (, $p < .05$). In VTA, AMPH treated animals show change in Arc mRNA expression after challenge with AMPH compared with control rats (M) (, $p < .01$). Bright field photomicrographs showing induction of Arc mRNA in the NAc shell of rats treated with AMPH and challenged with AMPH (F) compared with acute AMPH administration (E) and saline control rats (D) and in the core acute AMPH (K) compared with control rats (L). In VTA, photomicrographs showing induction of Arc in rats treated with saline and challenged with saline (P) or AMPH (Q). Data represent the mean ± SEM for cell density (2-3 sections per treated animal).

### 3.3.2.2 *C-fos* protein expression in the NAc and VTA

One way ANOVA indicate a significant main effect of treatment on *c-fos* expression in the shell [F (4, 20) = 3.19; p <.05] and in the core [F (4, 20) = 3.34; p <.05]. planned comparison indicated that acute administration of AMPH cause increased *c-fos* expression in the NAc shell and core as compared with saline control rats (SAL-AMPH group vs SAL-SAL group, , P < .01). In addition, AMPH sensitised rats did not show a significant effect on *c-fos* protein expression compared with acute AMPH administration (AMPH-AMPH group vs SAL-AMPH group) (p = ns) Figure 17A-H.

In the VTA, one-way ANOVA showed a significant main effect of treatment [F (4, 21) = 14.11; p <.0001] on *c-fos* protein expression. Planned comparison showed that challenge with AMPH significantly induced *c-fos* expression in the AMPH pre-treated animals compared with acute AMPH administration (AMPH-AMPH group vs SAL-AMPH group) ( p < .05). Figure 17 K-M.

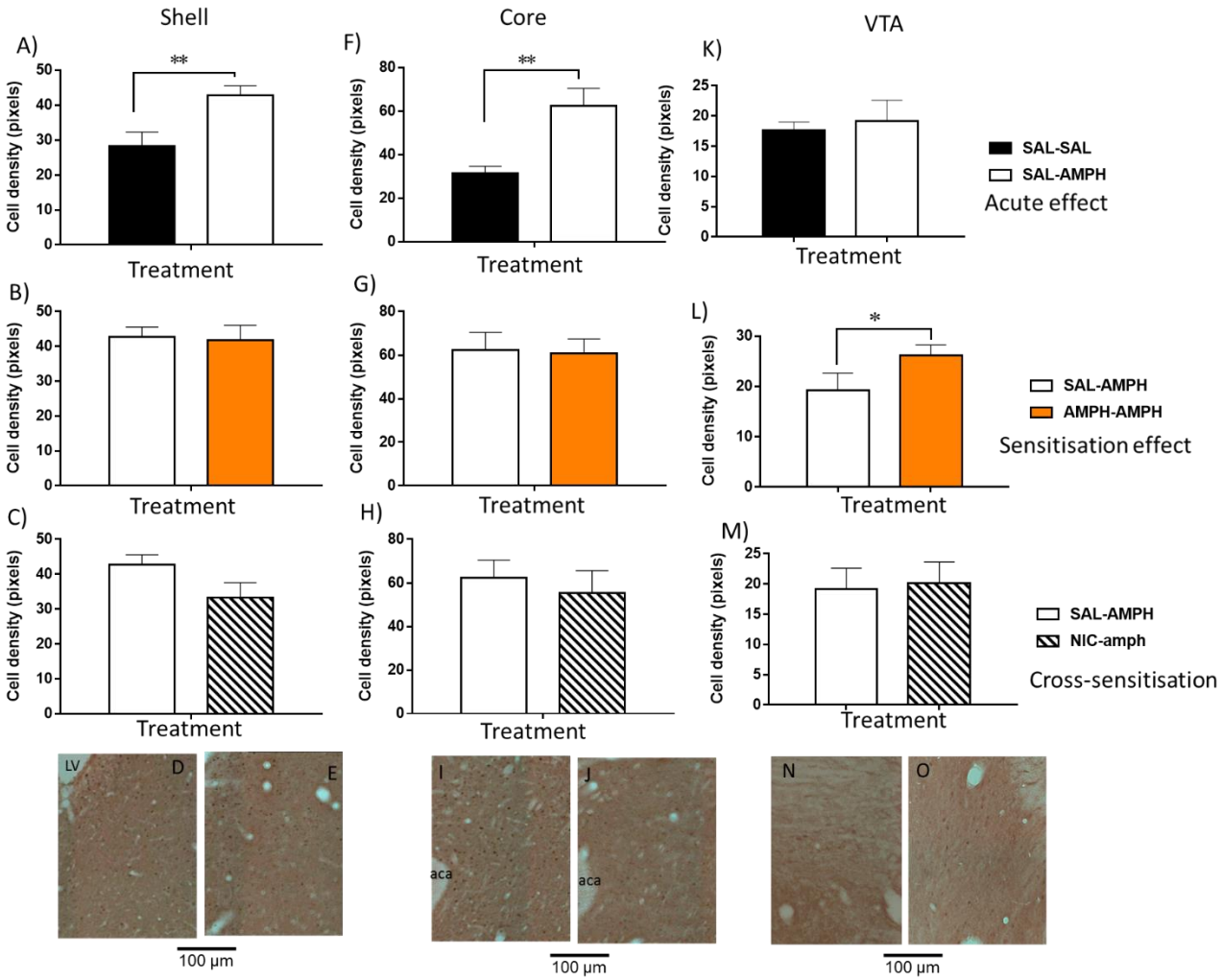



Figure17: Effect of sub chronic treatment with nicotine, AMPH or saline on *c-fos* protein expression in NAc shell and core sub region and VTA. In NAc, (planned comparison) indicated a significant increase in *c-fos* expression in the NAc core (F) and shell (F) of rat that received repeated saline treatment and AMPH at challenge day compared to control rats ($p < .01$). While there is no effect of *c-fos* mRNA expression in AMPH sensitise rats. In VTA, AMPH sensitised rats show a significant increase in *c-fos* mRNA expression compared with acute AMPH animals (L) ($p < .05$). Bright field photomicrographs showing induction of *c-fos* in the NAc shell and core of rats treated with saline and challenged with nicotine (D and I respectively) compared with saline control group (E and J respectively). In VTA, photomicrographs showing induction of *c-fos* in rats treated with either AMPH or saline and challenged with AMPH (O and N respectively). Data represent the mean ± SEM for cell density (2-3 sections per treated animal).

### 3.3.2.3 MeCp2 protein expression in the NAc and VTA

One- way ANOVA showed a significant main effect of treatment in the NAc shell [F (4, 21) = 10.54; P <.0001] and core [F (4, 21) = 10.51; p <.0001]. Only animals treated with saline and challenged with AMPH showed higher MeCp2 expression across the entire NAc (both in shell and core) compared with saline control animals (SAL-AMPH group vs SAL-SAL group) (planned comparison, all p value <.01). AMPH challenge failed to induce MeCp2 expression in either the AMPH or nicotine sensitised rats in either the shell or the core compared with acute AMPH administration animals (AMPH-AMPH group or NIC-AMPH group vs SAL-AMPH group) ( p = ns) Figure 18 A-H.

In the VTA, one-way ANOVA showed a significant main effect of treatment on MeCp2 labelled cells [F (4, 20) = 5.05; p <.01]. Planned comparison indicated that saline pre-treated rats showed a change in MeCp2 expression after challenge with AMPH compared with saline control rats (SAL-AMPH group vs SAL-SAL group) (p < .01) Figure 18 K-M.

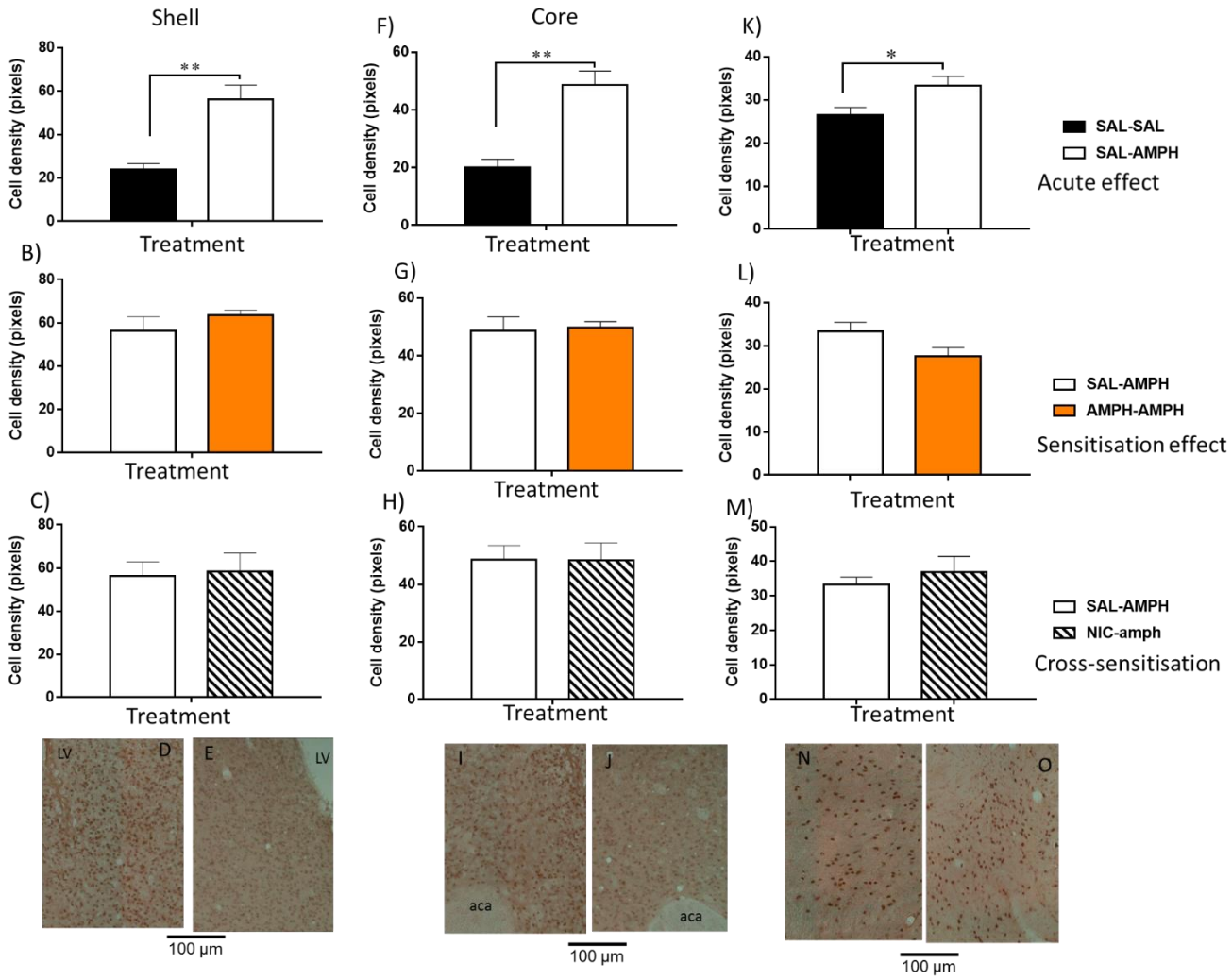



Figure18: Effect of sub chronic treatment with AMPH, nicotine or saline on MeCp2 protein expression in the NAc core and shell and VTA. In NAc, Sub chronic treatment with saline and challenge with AMPH caused a significant increase in MeCp2 expression in NAc shell (A) and core (F) compared to animals control animals ($p < .01$). In VTA, treated with saline and challenged with AMPH showed an increase in MeCp2 expression compared with saline control rats (K) ( $p < .01$). Bright field photomicrographs showing induction of MeCp2 in the NAc shell and core of rats treated with saline and challenged with AMPH (D and I respectively) compared with saline control group (E and J respectively). In VTA, photomicrographs showing induction of *c-fos* in rats treated with saline and challenged with either saline (O) or AMPH (N). Data represent the mean ± SEM for cell density (2-3 sections per treated animal).

### 3.3.2.4 BDNF protein expression in the NAc and VTA

One-way ANOVA revealed a significant main effect of treatment on BDNF protein expression in the NAc shell [F (4, 23) = 10.52; p <.0001] and core [F (4, 23) = 8.97; p <.01]. Planned comparison test revealed that on challenge day, acute administration of AMPH caused increase BDNF expression in the NAc shell and core as compared with saline control rats (SAL-AMPH group vs SAL-SAL group, p < .01). In addition, post hoc analysis revealed that AMPH challenge induced more BDNF positive cells in the core, of nicotine pre-treated animals compared with acute AMPH administration animals (NIC-AMPH group vs SAL-AMPH group) (p <.05) but not in the shell. However, this difference did not reach statistical significance in AMPH sensitised rats (AMPH-AMPH group vs SAL-AMPH group, p = ns) Figure 19A-H.

In the VTA, one-way ANOVA [F (4, 19) = 11.64; p <.0001] revealed a significant main effect of treatment. Planned comparison showed that BDNF protein expression was enhanced after challenge with AMPH only in saline pre-treated animals compared with saline control rats (SAL-AMPH group vs SL-SAL group) but did not reach statistical significant (p = n.s) Figure 19 L-N.

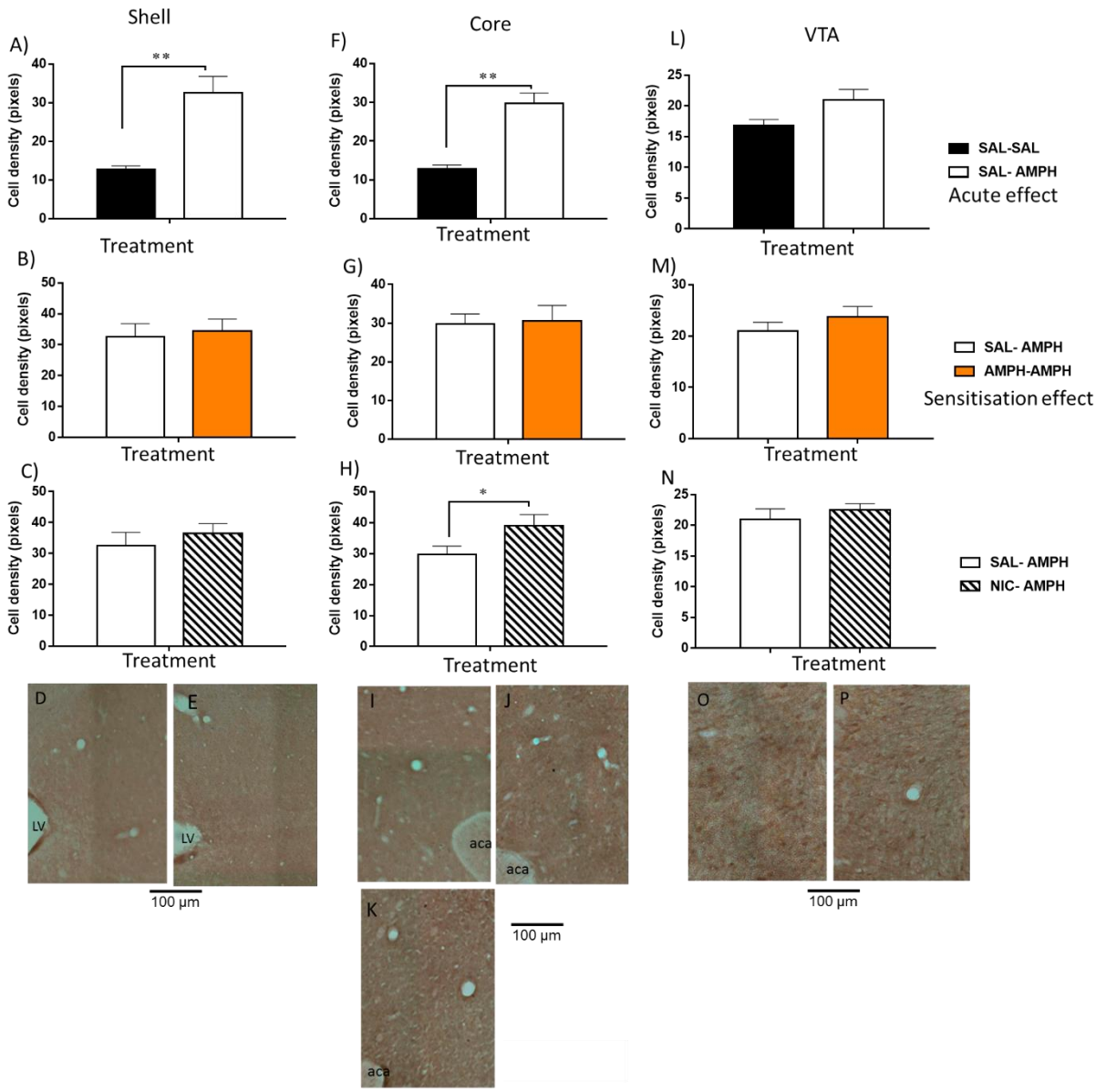



Figure19: Effect of sub chronic treatment with AMPH, nicotine or saline on BDNF protein expression in NAc shell and core sub region and VTA. In NAc, planned comparison indicate a significant increase in BDNF expression in the NAc shell (A) and core (B) of acute nicotine as compared with saline control, in the core of rat that received repeated nicotine treatment and AMPH at challenge day compared to acute AMPH administration (H) ($p < .05$). In VTA, challenge with AMPH change BDNF expression in the saline pre-treated rats as compared with control rats but does not reach statistical significant. Bright field photomicrographs showing induction of BDNF in NAc shell of rats treated with saline and challenged with AMPH (D) compared with saline control rats (e) and in the core rats treated with either nicotine or saline and challenged with AMPH (K, J) respectively compared with saline control rats (I) . In VTA, photomicrographs showing induction of BDNF in rats treated with saline and challenged with either saline (P) or AMPH (O). Data represent the mean ± SEM for cell density (2-3 sections per treated animal).

A

| Brain area | Genes/Drug conditions | Acute effect | Sensitisation effect | Cross-sensitisation effect |
|---|---|---|---|---|
| | | SS/SN | NN/SN | AN/SN |
| NAc shell | Arc | — | — | ↑ |
| | *c-fos* | — | — | — |
| | MeCp2 | ↑ | — | ↑ |
| | BDNF | ↑ | — | — |
| | | | | |
| NAc core | Arc | ↑ | ↑ | ↑ |
| | *c-fos* | — | — | ↑ |
| | MeCp2 | ↑ | ↑ | ↑ |
| | BDNF | ↑ | — | — |
| | | | | |
| VTA | Arc | ↑ | — | — |
| | *c-fos* | ↑ | — | — |
| | MeCp2 | ↑ | — | — |
| | BDNF | ↑ | — | — |

B

| Brain area | Genes/Drug conditions | Acute effect | Sensitisation effect | Cross-sensitisation effect |
|---|---|---|---|---|
| | | SS/SA | AA/SA | NA/SA |
| NAc shell | Arc | ↑ | ↑ | — |
| | c-fos | ↑ | — | — |
| | MeCp2 | ↑ | — | — |
| | BDNF | ↑ | — | — |
| | | | | |
| | | | | |
| NAc core | Arc | ↑ | — | ↓ |
| | c-fos | ↑ | — | — |
| | MeCp2 | ↑ | — | — |
| | BDNF | ↑ | — | ↑ |
| | | | | |
| | | | | |
| VTA | Arc | ↑ | — | — |
| | c-fos | — | ↑ | — |
| | MeCp2 | ↑ | — | — |
| | BDNF | — | — | — |

Table 1: Summarise Immunohistochemistry result, A) nicotine pre-treatment and B) AMPH pre-treatment.

## 3.4 Discussion

The present data represent that acute nicotine injection cause increase Arc and MeCp2 protein expression in the NAc core as compared with saline control. In addition, repeated treatment with nicotine or AMPH cause increase Arc and MeCp2 protein expression in the NAc core in response to acute nicotine injection as compared with saline pre-treated rats. In AMPH sensitisation experiments, our data found that acute administration of AMPH cause increase in the Arc protein expression in the NAc shell and core as compared with saline control rats. In addition, repeated treatment with AMPH cause increase Arc protein expression in the NAc shell as compared with saline pre-treated animals in response to AMPH injection.

Repeated exposure to the psychostimulant drugs, nicotine and AMPH induces changes in mesocorticolimbic reward circuit function that can lead to compulsive drug use (Hyman *et al.*, 2006). Psychostimulant-regulated gene transcription is thought to contribute to this

process by coupling drug intake with the expression of gene products that induce structural and functional plasticity of neurons in the mesocorticolimbic reward circuit. In particular in NAc, a brain region involved in the rewarding properties of these drugs, these changes occur even weeks or months following repeated administration (Hyman *et al*., 2006, Brunzell and Picciotto, 2008, Robison and Nestler, 2011). Thus, an important question to address here is whether the expression of nicotine or AMPH sensitisation involves changes in neuronal activity within NAc (shell and core sub regions) and VTA and whether these sensitisation processes involve the same or different neuroadaptive phenomena. One way to evaluate these genomic alterations is to examine expression of IEGs such as Arc and c-*fos* protein in addition to MeCp2 protein and BDNF protein by using immunohistochemical detection.

Repeated exposure to psychostimulants induces morphological changes in the mesolimbic target area such as an increase in the number of dendritic spines, changes in the morphology of dendritic spines and rearrangement of actin filaments. Many studies have demonstrated that repeated exposure to psychostimulants including nicotine (Gonzalez *et al*., 2006) or AMPH (Robinson and Kolb, 1997, Li *et al*., 2003) produce long-lasting changes in the structure of dendrites and dendritic spines on medium spiny neurons in the NAc at the site of DA postsynaptic interaction which may contribute to some of the persistent behavioural consequences of repeated exposure to psychostimulant drugs (Ujike *et al*., 2002). Apart from structural changes, there are well-known neurochemical changes, including changes in G protein subunit composition of receptors, increased adenylyl cyclase activity, cAMP and PKA in the NAc, increased TH enzyme activity, and increased ΔFosB, *c-fos* and AP-1 binding protein (Nestler, 2001a). However, Mychasiuk *et al*. (2013) demonstrated that the persistent epigenetic changes associated with exposure to nicotine and AMPH were region and drug dependent and differ from the transient changes that occur immediately after drug exposure.

Arc protein expression is a marker of neurons outgrowth and have been reported to be upregulated in the dorsal striatum after chronic cocaine in rats (Fumagalli *et al*., 2006). Arc may play an important role in synaptic plasticity underlying psychostimulant-induced adaptation changes including behavioural sensitisation (Kodama *et al*., 1998). Arc can be used to gain an understanding of the molecular mechanism of behavioural sensitisation for many reasons. First, Arc expression was elevated in the PFC and dorsal striatum after administration of psychostimulant drugs including AMPH (Moro *et al*., 2007) and cocaine in rats (Fumagalli *et al*., 2006). In addition, Arc has been implicated in neural plasticity

phenomena such as LTP (Guzowski *et al*., 2000) and neuritic elongation (Ujike *et al*., 2002). Second, newly synthesized Arc protein is transported into dendrites and accumulates at synaptic sites that have experienced strong activity in postsynaptic sites after psychostimulant administration (Steward *et al*., 1998).

The current results clearly demonstrate that repeated nicotine treatment produced an increase in Arc expression in the core of NAc which is consistent with other studies suggesting that repeated administration of nicotine promotes the activity of DA projection neurons in the NAc core while it diminishes the activity of DA projection to the shell (Di Chiara, 2002). In cross-sensitisation experiments, pre-treatment with AMPH cause increase in Arc protein expression to ward challenge with nicotine, in the NAc shell and core.

In AMPH sensitised rats, Klebaur *et al*. (2002) and Jedynak *et al*. (2016) demonstrated that repeated AMPH administration caused increased Arc mRNA expression in the shell of NAc which may be linked to persistent AMPA-type glutamate receptor plasticity 10-14 days following repeated AMPH supporting a role for Arc mRNA in directly modifying postsynaptic properties responsible for membrane excitation (Schiltz *et al*., 2005) which is in line with our result. In VTA, there was no effect of pre-treatment history with either or nicotine or AMPH on Arc expression in response to AMPH challenge. We did not found an effect of treatment with nicotine and challenge with AMPH on Arc protein expression in term of cross-sensitisation experiment.

The early gene, *c-fos* (which is rapidly induced within activated neurons) and its mRNA or protein products have long been used as markers of neural activation (Nestler, 2001a). *C-fos* have been the most commonly used as IEG markers of neural activity in addiction research (Cruz *et al*., 2015). Induction of *c-fos* is viewed as an early marker of neural activation since it is rapidly and transiently induced in response to acute administration of several types of psychostimulants in the NAc and other brain regions (Zhang *et al*., 2006, Kelz *et al*., 1999). *C-fos* exerts its effect on synaptic plasticity indirectly by regulating the transcription of other such as ΔFosB, so- ΔFosB called late response genes (Nestler, 2001a, Brenhouse and Stellar, 2006). By contrast, ΔFosB, is a member of Fos family of transcription factor, induced only slightly by acute drug exposure. However, it accumulates with repeated drug administration and its expression is associated with increased locomotor and rewarding responses to psychostimulants drugs (Kelz *et al*., 1999, Brenhouse and Stellar, 2006, Renthal *et al*., 2008, Deng *et al*., 2010). Thus, induction of *c-fos*, is important to regulate the expression of ΔFosB,

which is important for behavioural sensitisation in response to psychostimulant as mice lacking *c-fos* in DA1 receptor-containing neurons, reduced behavioural sensitisation to these drug (Zhang *et al*., 2006). Previous evidence suggested that behavioural sensitisation and gene expression changes such as *c-fos* stimulation are mediated by the indirect action of psychomotor stimulants on DA1 receptors expressed on postsynaptic striatal neurons (Simpson *et al*., 1995). Our data show that behavioural sensitisation observed in nicotine or AMPH sensitized rats does not affect by *c-fos* protein expression in the NAc (shell and core sub regions).

MeCp2 is required for the normal development, function and plasticity of neural circuits, and mutations causing loss of function in human lead to the neurodevelopment disorder Rett syndrome (Cohen *et al*., 2011). Conditional deletion of MeCp2 in developing hippocampal or cortical pyramidal neurons impairs excitatory synaptic transmission, and loss of MeCp2 during development disturbs the number and function of inhibitory GABAergic synapses (Deng *et al*., 2010, Zhang *et al*., 2010). MeCp2 is highly expressed in neurons of the adult brain including neurons within the mesocorticolimbic reward circuity (Deng *et al*., 2010). Deng *et al*. (2010) showed previously that manipulating in adult rats (or mice) the expression of MeCp2 NAc neurons by viral-mediated overexpression or knockdown of MeCp2 inversely regulated the rewarding properties of AMPH. This evidence led us to consider whether MeCp2 could contribute to circuit plasticity in psychostimulant-induce behavioural sensitisation. MeCp2 is an epigenetic factor that is rapidly phosphorylated (pMeCp2) in response to synaptic activity by administration of psychostimulant drugs suggesting a stimulus-dependent mechanism of Mecp2 regulation (Zhou *et al*., 2006).

The neuronal population in the NAc comprises two major classes: more than 90% of the cells are MSNs-GABAergic projection neurons that carry efferent signals from striatum. The remaining neurons: are local interneurons that use GABA or acetylcholine as a neurotransmitters and MeCp2 is expressed in both types of neurons (Kawaguchi *et al*., 1995). Thus to determine whether MeCp2 may contribute to psychostimulant-regulated behaviours, we quantified MeCp2 in the NAc (shell and core sub regions) and VTA, as MeCp2 is highly expressed in rat brain neurons within mesocorticolimbic reward circuitry (Deng *et al*., 2010). Analysis was performed in rats that had been behaviourally sensitised to nicotine or AMPH. We found that nicotine sensitized rats showed enhanced MeCp2 expression in the NAc core as the inhibition effect of acute administration of nicotine may be associated with an increase in the GABAergic density in saline pre-treated rats (Schenk

*et al*., 1991), while there was no effect on NAc shell. In cross-sensitisation experiment, we found increase MeCp2 protein expression in the rats pre-treated with AMPH and challenge with nicotine in the NAC shell and core.

One possible explanation for our result is that it is well known nicotine- induce their effect through activation of nAChR receptors that expressed on MSN neurons including GABAergic neurons. However, chronic nicotine treatment may altered the nAChR function in these neurons, and their response may be alter between chronic and acute nicotine administration (Fujii et al., 1999). The present study show that acute administration of nicotine cause increase MeCp2 expression in animals pre-treated with either nicotine, AMPH or saline however their inhibition effect on locomotor activity was differ depending on treatment history. These results suggest that the mechanism underlying the effect of acute nicotine exposure remains after chronic nicotine-induced changes to nAChRs, and that the effect of chronic nicotine treatment is mediated by a different mechanism. Leading us to suggest that MEcp2 expression in the animal pre-treated with either saline, nicotine or AMPH depending on acute effect of nicotine rather than on chronic treatment history, as Deng et al. (2010) suggested that pre-treatment with psychostimulant inhibit MeCp2 expression.

We found that AMPH sensitised rats showed no enhancement of MeCp2, which is in accord with to previous observations (Deng *et al*., 2014). These authors revealed that MeCp2 phosphorylation negatively regulates the expression of behavioural and neural plasticity in the mesocorticolimbic circuity. Further, Deng *et al*. (2010) observed a significant increase in the number of GABAergic synapses in the NAc of mutant MeCp2 mice which did not exhibit increases in locomotor activity compared with wild-type mice. The latter showed a progressive increase in locomotion after repeated injection of AMPH, indicating that the MeCp2 is important for activation of GABAergic synapses in NAc , subsequently, modulate behavioural responses.

Deng *et al*. (2010) showed that MeCp2 negatively regulated behavioural responses to pyschostimulant such as AMPH by engaging cellular and molecular process in the NAc that oppose psychostimulant-induce changed in neural function. To explain their findings, they demonstrated that AMPH-induced MeCp2 phosphorylation in specific population of NAc interneurons that express the lineage-specifying transcription factor Lhx6 (Deng *et al*., 2010) and co-express cytoplasmic GABA synthesizing enzyme GAD67, suggesting they are fast-

spiking interneurons (Gittis *et al*., 2010). Induction of MeCp2 in these neurons could increase the frequency of spontaneous inhibitory currents in neurons expression DA1-receptors expressing neurons. The present study showed that the animals that expressed behavioural sensitisation to AMPH showed no change in MeCp2 expression in the NAc which may have resulted in increased activation of NAc output pathway subsequent to a decrease in the activity of inhibitory GABAergic interneurons. No effect of pre-treatment with nicotine on MeCp2 expression on response to AMPH challenge in the NAc shell or core in term of cross-sensitisation.

BDNF is a member of the family of neurotrophic factors, produced as a pro-isoform and cleaved into its mature form: BDNF is involved in neural organisation and synaptic plasticity during brain development (Hyman and Hofer, 1991). Through activation of its high affinity receptor, TrkB, BDNF activates signalling cascades that affect gene transcription and increase glutamatergic activity, long-term potentiation, dendritic protein synthesis, and dendritic spine formation. It has been shown that increased BDNF levels in the VTA and NAc associated with the expression of substance dependence-related behaviours such as failure to reduce drug consumption and drug craving (Quintero, 2013). BDNF has become a key relevant factor in understanding the mechanisms underlying drug addiction (Schmidt *et al*., 2013).

Previous studies have demonstrated that BDNF can enhance psychostimulant-induced locomotor activity when injected into NAc or VTA (Horger *et al*., 1999). In contrast, Pierce and Bari (2001) found that when a withdrawal period was imposed following daily microinjection of BDNF into the VTA, the behavioural response to a challenge injection of cocaine or AMPH did not differ from control even through the animals exhibited behavioural activation to these drugs. Nicotine and AMPH treatments did not affect the BDNF expression in the two brain areas examined. Such lack of nicotine or AMPH treatment effects, either acute or chronic, on BDNF protein levels, in the NAc or VTA was also reported in previous studies. Li *et al*. (2013) examined BDNF mRNA levels after chronic cocaine self-administration and showed no alteration in the BDNF level in the NAc. Similarly, Warren *et al*. (2011) failed to demonstrate an alteration in BDNF mRNA level in the VTA following 15 days of methylphenidate administration which agrees with our results. In contrast to our negative result, Simchon Tenenbaum *et al*. (2015) showed that chronic

cocaine treatment led to an increase in BDNF mRNA in the NAc as we were not able to detect any changes in the BDNF expression in the nicotine or AMPH sensitised rats. Many differences in experiments regimens between the two studies may account for these differences: for example different (although pharmacologically fairly similar) drugs; different withdrawal period; different rat strains; and different procedure were used to detect the gene expression.

In VTA, all genes above had shown difference in their expression only after acute administration of drug as compared with control saline animals. However, pre-treatment with either nicotine or AMPH did not show any effect on genes expression as compared with acute drug administration.

In conclusion, the ultimate goal of epigenetic studies is to understand how repeated exposure to a drug of abuse changes the brain in sustained ways to cause the lasting syndrome of addiction. Such studies are also important to understand how an individual's life experience which then help determine that individual's vulnerability to the addiction-causing effects of subsequent drug exposure. The most important characteristic of the behavioural sensitisation phenomenon is the very long persistence of hyper-responsiveness to the drug. Rats previously sensitized to nicotine or AMPH remain hypersensitivity to the psychomotor activating and reward effect of a subsequent dose after at least 6 months of abstention. Various neurochemical adaptation have been found in the sensitisation phenomenon, including rearrangement and structural modification of neural network such as change in the length of dendrites and density dendritic spines in the NAc must involve in behavioural sensitisation.

In summary, animals treated with either nicotine or AMPH expressed behavioural sensitisation to drug challenge after 10 days withdrawal period. In addition, our results found that AMPH pre-treated animals had been shown cross-sensitisation to nicotine challenge. However, there was no evidence in our study for cross-sensitisation in rats pre-treated with nicotine in response to AMPH challenge. For immunohistochemistry results, nicotine sensitised animals showed an increase Arc and MeCp2 expression in the NAc core as compared with acute nicotine administration, which suggested increased neural plasticity in this area, while no alteration were observed for other genes expression (*c-fos* and BDNF). AMPH sensitised animals showed only increase Arc expression in NAc shell and had no effect on MeCp2, *c-fos* and BDNF gene expression.

Arc and MeCp2 have a major role in cellular plasticity during behavioural sensitisation, although these changes are not reflected in behavioural tests for AMPH sensitised rats, such alteration are primarily detected in the nicotine sensitised rats. Thus we suggest that the increase in Arc protein levels in nicotine and AMPH sensitised rats may be related to neuroplasticity effects of the sub-chronic treatment with these drugs leading to increased behavioural activity. Although MeCp2 is involved in neural plasticity, its expression was different depending on treatment history. We did not find any changes in the corresponding MeCp2 protein levels in AMPH sensitised rats, but we observed alteration in the expression of this genes in nicotine sensitised rats, which suggested that behavioural sensitisation for nicotine and AMPH-are induced by different mechanisms depending on treatment history. Nicotine induce its effect via nACRs in VTA, while AMPH produces its effect via DAT leading to increase DA release. Thus, activation of DA receptors in the NAc, or MeCp2 expression in the neurons following drug treatment and the resultant effect on behavioural activity occurs in a different way with the two drugs. Thus, it seems that the mechanism of behavioural sensitisation to nicotine and AMPH does not follow the same mechanism and depend on pre-treatment history. Also, the role of upregulation of MeCp2 expression in the behavioural effect of nicotine, merits further studies, at the brain level.

# Chapter 4: Measuring DA releasing in NAc in vitro using Fast Scan Cyclic Voltammetry (FSCV) experiments

## 4.1 Introduction

It is well established that both drug dependence and behavioural sensitisation following repeated intermittent exposure to psychostimulants are long-lasting phenomena, and may share similar neural adaptation processes, which may involve persistent enhanced responsiveness of mesolimbic DA neurons that innervate the NAc (Pierce and Kalivas, 1997, Vanderschuren and Kalivas, 2000). The experiments reported in the previous chapter investigated activity of gene markers for neural activity and plasticity. Such changes would be likely to be expressed through changes in DA release in both the development and maintenance of compulsive drug intake that characterises addiction (Robinson and Berridge, 2008). Experiments described in this chapter were aimed at measuring DA release in NAc directly by fast scan cyclic voltammetry (FSCV) in the brain slices, to ascertain whether sub chronic 'induction' treatment over 5 days (as described in section 2.2.3) affects the changes in stimulated release caused by acute treatment of the drugs. Initial experiments were aimed at developing a method for measuring from the whole pathway, such that the effect *in vitro* manipulations of the cell bodies in VTA on release in NAc could be measured. However, due to technical difficulties this was not possible, so subsequent experiments measured changes after local stimulation in NAc, but not VTA.

Techniques that allow for rapid measurement of DA release have revealed that despite different mechanisms, a broad class of abused drugs, psychostimulant and otherwise, including AMPH, cocaine, nicotine and ethanol, all increase DA transmission within the NAc and these better inform our understanding of the neurochemical consequences of drug intake (Di Chiara and Imperato, 1988, Daberkow *et al*., 2013, Singer *et al*., 2017). However, investigations of the potential mechanism that might account for this behavioural sensitisation have often but not always observed an augmentation of extracellular DA in the NAc following repeated psychostimulant (Segal and Kuczenski, 1992, Byrnes and Wallace, 1997) and nicotine (Nisell *et al*., 1996) administration. Research has focused on alteration in DA neural transmission as a mediator of drug-induced behavioural sensitisation. In this regard, most investigators focused on the mesolimbic DA system. Pierce and Kumaresan (2006) indicated that behavioural sensitisation of these drugs depends on their ability to elevate extracellular DA levels in the mesolimbic DAergic neurons that originate in the VTA

and project to the NAc. Although drug action in the DA terminal fields appears not to initiate behavioural sensitisation (Kalivas and Stewart, 1991), numerous studies of DAergic transmission have led to the conclusion that rats made behaviourally sensitised by repeated administration of drugs have augmented DA transmission to a subsequent drug challenge (Nakagawa *et al*., 2011, Degoulet *et al*., 2013).

Among the multiple DAergic terminal regions examined, the NAc is an important participant in reward-related motivated behaviour. The NAc receives heavy afferent projection from the VTA, hippocampus and the basolateral amygdala, and in turn, it sends efferent to several structures including the VTA, ventral pallidum and lateral hypothalamus. Thus, these interconnection are believed to be critical in supporting motivated behaviour associated with natural (Cacciapaglia *et al*., 2012) and drugs-related reward (Di Chiara and Imperato, 1988). Research using electrophysiological, pharmacological, neurochemical and lesion method provide support for NAc involvement in reward processing (Pennartz *et al*., 1994, Ikemoto *et al*., 1997, Ikemoto, 2007). Thus, abused drugs which induce behavioural sensitisation and drug- compulsive behaviour are associated with increased release of DA in the mesolimbic system.

NAc is divided anatomically into two distinct, yet functionally related components, the core which is the central region and the more medial and ventral shell region (Brog *et al*., 1993, Ikemoto, 2007). Although these regions have unique patterns of afferent and efferent connection, they both receive input from DAergic cells in the VTA. Each region has been identified to participate in reward processing associated with drugs of abuse, although there are a variety of studies which demonstrate that the two regions participate in distinct, complementary aspect of drugs reward (Boye *et al*., 2001, Sellings and Clarke, 2003). For instance, cocaine induces cue evoked increases in phasic DA within the NAc core but not in the shell, while morphine conditioned stimuli increase extracellular DA in the NAc shell but not in the core (Bassareo *et al*., 2007). The psychostimulant, AMPH acts to block the reuptake and promote the release of DA into synaptic cleft mainly through binding to and reversal of DAT resulting in both reuptake inhibition and release of DA (Pierce and Kalivas, 1995, Siciliano *et al*., 2014). It enters the DAergic terminals and accumulates in acidic structure such as synaptic vesicles. After entering the synaptic vesicles, AMPH binds to free protons and increased pH leading to a reduction in the energy needed for neurotransmitters accumulation and causing redistribution of vesicular DA to the cytosol. Subsequently, the increased cytosolic DA binds to the cytosolic face of DAT and is transported out of the cell,

thus increasing the extracellular DA level (Howell and Kimmel, 2008) (see chapter 1, section 1.3.1.1.). This action is confined to DA terminals fields, and is thought to mediate the acute locomotor stimulant effect of these drugs. On the other hand, nicotine appears to augment DA transmission primarily by stimulating DA neurons by binding to nAChRs located in the VTA and NAc (Pidoplichko *et al*., 1997, Zhang *et al*., 2012).

There are two main techniques used to measure extracellular level of DA, microdialysis and FSCV. *In vivo* microdialysis has been used to measure changes in extracellular DA level in brain tissue which reflects the balance between release and reuptake activity in the DA neurons over time. However, microdialysis suffers from relatively poor spatial and temporal resolution. FSCV is an electrochemical technique that was developed in the early 1980s by Julian Millar and colleagues in London (Millar *et al*., 1985). In 1990 Stamford described FSCV as a high speed, high spatial resolution detection method used for monitoring DA release and uptake in a "real- time" and by using this method it can measure DA release at sub-second time resolution (Stamford, 1990). Stamford mentions that one of the most important advantages of FSCV as a method for detection of real-time DA release, is its ability to separate DA from other sources of current such as release of other monoamines including 5-HT. Further, the changes in the extracellular concentration of DA can be monitored (Stamford, 1990).

FSCV involves applying a rapidly changing potential (-0.4 V to +1.3 V to – 0.4 V at a scan rate of 400 V/s: total scan time 8 ms) to carbon fibre recording electrode, and measuring the current flow. As the potential increases (oxidation sweep), at around 600 mV (the oxidation potential for DA) the DA around the tip of the electrode oxidises to form DA-o-quinone by losing two electrons that are recorded as current. As the potential decreases (reduction sweep) , DA-o-quinone is reduced back to DA by returning the electrons and producing current in the opposite direction (Phillips and Wightman, 2003) see Figure 20. The current generated by the oxidation and reduction (Faradiac current) are isolated by subtraction of the background (charging) current, and the concentration of DA is assessed by measuring the size of the oxidation peak in the background subtracted signal. FSCV can be combined with local stimulating of the NAc in brain slices to measure DA terminal properties and how DA level altered following various manipulation (Yorgason *et al*., 2014).

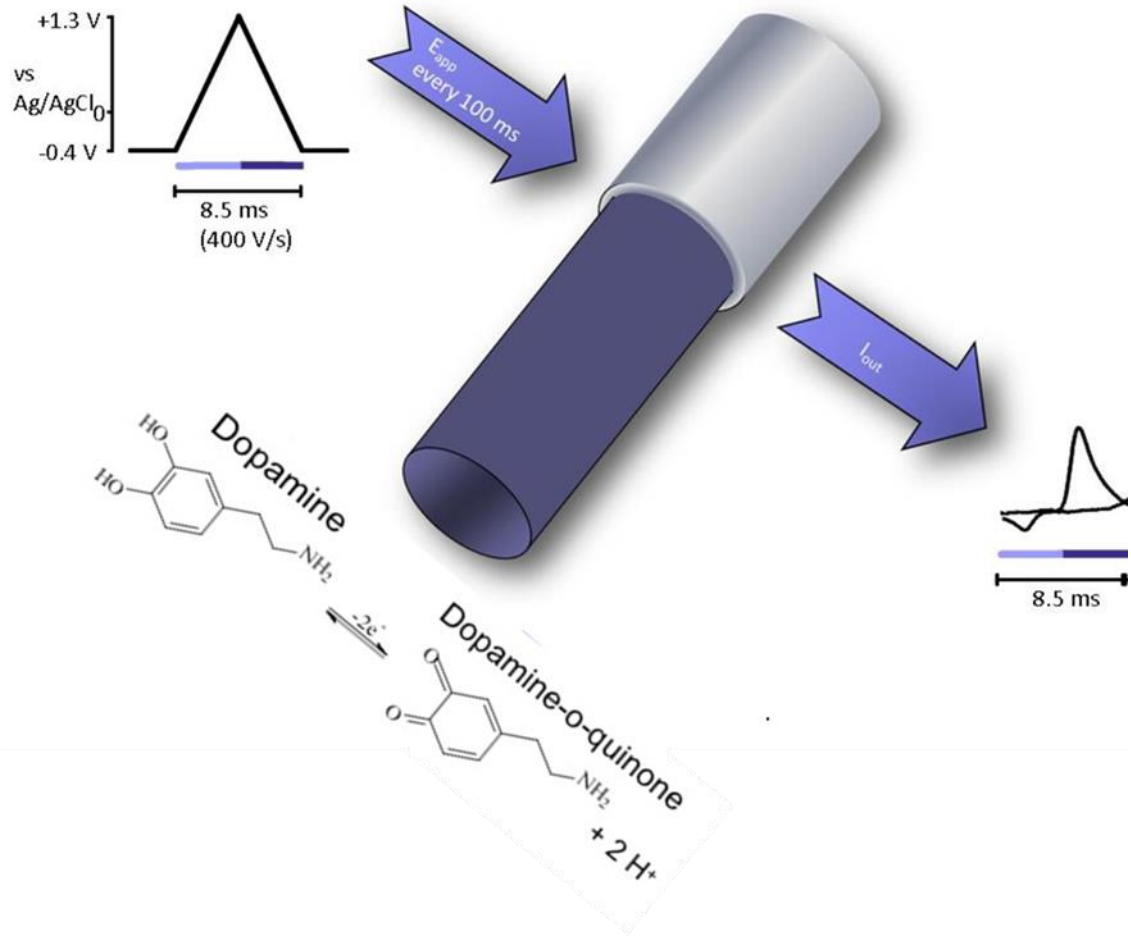



Figure 20: Voltammetry detection of DA. When sufficient potential from -0.4V to +1.3V is applied to working electrode, the DA is oxidized to DA-o-quinone by releasing two electrons that are detected as current. The DA-o-quinone that remaining at electrode surface is reduced to DA by absorbing these electrons, producing another current in the opposite direction (Phillips and Wightman, 2003).

The aim of experiments descriped in this chapter are to investigate changes in mesolimbic DA relaese by FSCV in brain slices *in vitro*. Initially, experiments were aimed at developing an experimental setup which would allow us to measure the effect of pharmacological manipulation of VTA on DA release in NAc. To obtain these targets, firstly, we aimed to develop and validate a method for horizontal or sagittal slicing of the brain to obtain the whole mesolimbic DA pathway and to record DA releasing in NAc after stimulation of cell bodies in VTA, *in vitro* with FSCV. In order to achieve this we examined 3D atlas, used several different orientations for cutting and stained with dextran tetramethyl-rhodoamine dye. This would allow us to measure the effect of pharmacological manipulation of VTA on

DA release in NAc. Due to a lack of success with developing this methodology, subsequent experiments used FSCV measurements in coronal slices containing NAc to investigate if the behavioural ssensitisation to the drugs is associated with increased release of DA in the mesolimbic system. Thus, using FSCV we examined the effect of acute and sub chronic administration of drugs on DA release in the NAc in the slices challenged with either nicotine or AMPH.

## 4.2 Material and Methods

### 4.2.1 *In vitro* electrochemistry

#### 4.2.1.1 Fast-Scan Cyclic Voltammetry (FSCV) technique:

Electrochemical recording of DA were performed using FSCV a high speed, high spatial resolution detection method used for monitoring DA release and uptake in a "real- time" (Stamford, 1990). Extracellular DA was monitored at the carbon fiber electrode using FSCV (Wightman *et al*., 1988) by applying a triangular waveform (-0.4 to +1.2 to -0.4 V vs Ag/AgC/ 400 V/s). At 10 Hz, DA release was evoked using either high potassium artificial cerebral spinal fluid ($K^+$ aCSF) stimulation (see table 2 for chemical component of high $K^+$ aCSF) or electrical stimulation applied with a bipolar stimulating electrode positioned on the surface of the slice in close proximity to the carbon fibre electrodes (working electrode).

#### 4.2.1.2 FSCV carbon fibre electrode

FSCV was used to measure the oxidation and reduction of DA at a carbon fibre microelectrode. These electrodes were constructed in the lab, as described by Clark *et al*. (2010). The working electrode preparation comprised three stages and took place over three days. Firstly, a 1.5 cm length of carbon fibre (7µm) was inserted into a 1 cm length vitreous silica capillary (OD 90 µm; ID 20 µm; CM Scientific, Cambridge, UK), in a petri dish filled with ethanol. They were then left overnight to allow the ethanol to evaporate away. Secondly, a small drop of two-component epoxy (Devcon, ITW Polymers, Danvers. MA) was applied to one end of the silica and the carbon fibre was gently drawn through the epoxy into the capillary, to form a seal: this was then left to dry overnight. Finally, a gold pin socket (MillMax, 0667; id 0.6 mm; Farnell Electronics, Leeds, UK) was connected to the opposite end of the fused silica using electrically conducting silver epoxy (Circuit works CW2400,

Farnell Electronics, Leeds, UK), ensuring electrical contact between the carbon fibre protruding from the capillary and the pin socket. The carbon fibre working tip was cut to a length of 120 µm (± 20 µm) with iris scissors. To create electrical insulation the connector was coated with two component epoxy (Devcon) (see Figure 21). Prior to use, each electrode was tested to ensure it produced an appropriate background signal, and that it gave a linear response to a physiological concentration of DA. All signals were recorded relative to an Ag/AgCl electrode, which provides a stable reference potential. The Ag/AgCl reference electrode was prepared by cutting a piece (7-10 mm) of sliver wire (Sigma- Aldrich, UK), which was inserted into a gold pin connector glued with sliver epoxy, and the surface coated with AgCl using a chloride unit (NPI Electronics, Germany, see appendix A for preparing Ag/AgCl reference electrodes).

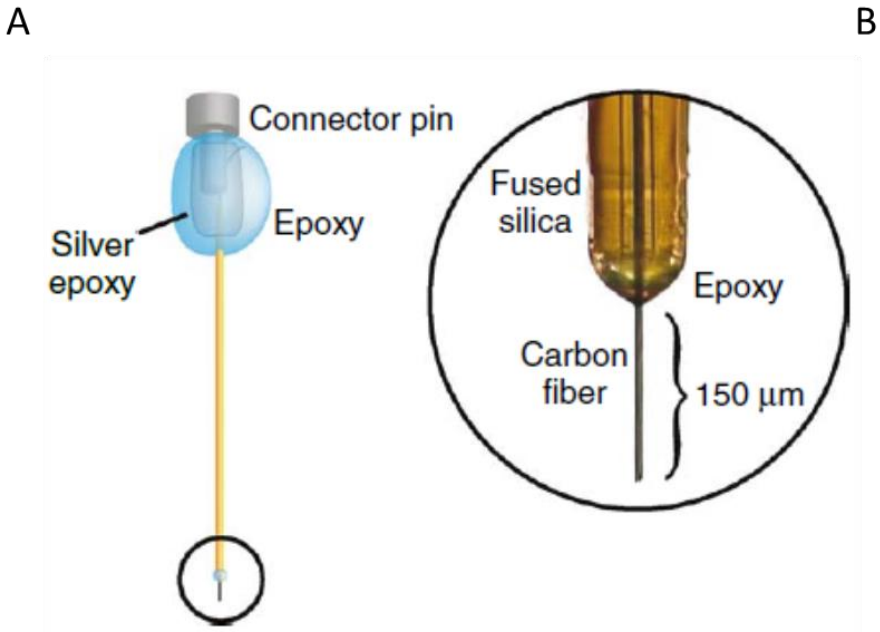



Figure 21: Carbon-fiber micro sensor electrode manufacturing. A- Diagram of the working electrode showing the attachment of the connector. B- Magnified diagram of the recording tip, showing the carbon fiber, protruding from the end of the capillary, and the epoxy seal (Clark *et al*., 2010).

### 4.2.1.3 The FSCV apparatus

The FSCV apparatus comprises a slice chamber that was continually superfused with oxygenated aCSF (mM) (see table 1 for chemical components of aCSF) at a flow rate of 1.5 ml/min. The aCSF was warmed by a thermostatically regulated peltier heater (University of Leicester, Biomedical Workshops) to temperature of 32 ± 2 °C. See figure 22 for slice setup in the slice chamber.

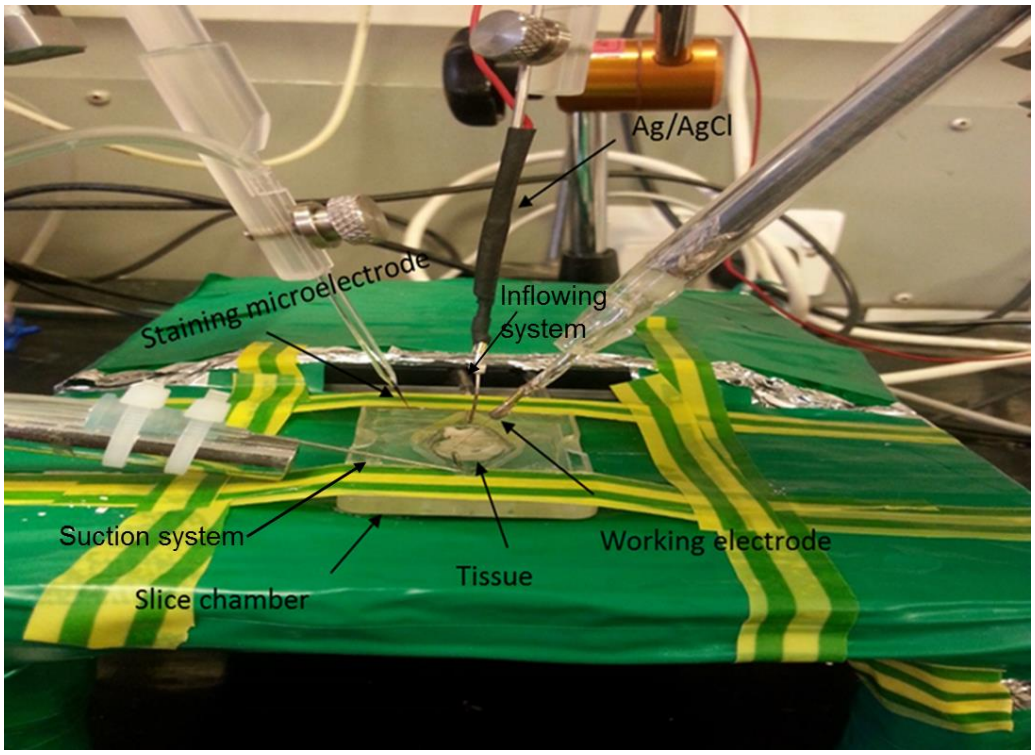



Figure 22: Slice setup in the FSCV chamber. After equilibration, the slice transferred to the slice chamber and superfused continuously with oxygenated aCSF. The bipolar stimulating electrodes and working electrode were placed in the NAc (core or shell sub region) and the reference electrode Ag/AgCl was placed on the slice chamber remote from the working electrode.

| Chemical Components | Normal aCSF | | High K+ aCSF | |
|---|---|---|---|---|
| | mM | g/Liter | mM | g/Liter |
| NaCl | 126 | 7.358 | 28 | 1.635 |
| KCl | 2 | 0.136 | 100 | 7.46 |
| KH2PO4 | 1.4 | 0.191 | 1.4 | 0.191 |
| MgSO4 | 2 | 0.493 | 2 | 0.493 |
| NaHCO3 | 26 | 2.187 | 26 | 2.187 |
| CaCl2 | 2.4 | 0.353 | 2.4 | 0.353 |
| (+)-Glucose | 10 | 1.802 | 10 | 1.802 |

Table 2: Chemical components of normal aCSF for *in vitro* fast scan cyclic voltammetry (FSCV) experiments and high $K^+$ aCSF solution that used to stimulate the DA release *in vitro* experiments.

#### 4.2.1.4 The DA calibration

Before using FSCV to measure the DA release, it is important to calibrate the working electrode to determine the concentration measured. The sensitivity of each working electrode was determined by using different DA concentration (1, 2, 3 and 4 µM) (see Figure 23 for calibration curve) and on the same day as the experiment the working electrode was tested for a good voltammetry signal by using 5 µM DA concentration at least 4 times (Kristensen *et al.*, 1986). Voltammetry scans were applied and the recordings were collected in real-time on a desktop computer using Demon voltammetry and analysis software (Yorgason *et al.*, 2011).

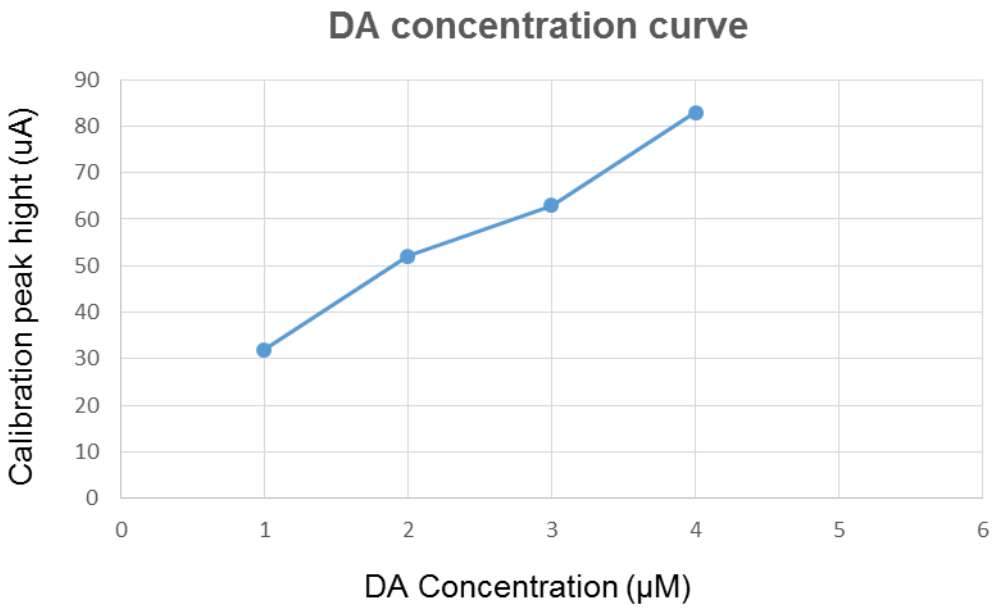



Figure 23: Example DA calibration in different DA concentration. Different dopamine concentrations (1, 2, 3, and 4 µM) were used to test the sensitivity of the working electrode before starting the FSCV experiments.

The potential was applied between (-0.4 to + 1.3 V at scan rate 400 V/s and 1 Hz) and when we obtained a stable baseline, a standard solution of DA (5 µM in aCSF) was applied in the medium for 1 min, after which the superfusate was returned to aCSF. Using the average current response (µA) of four such runs the calibration (µA/µM) was calculated for each electrode. When we got a good response we then started the experiment with slices, using either high $K^+$ stimulation or electrical stimulation (see below for details).

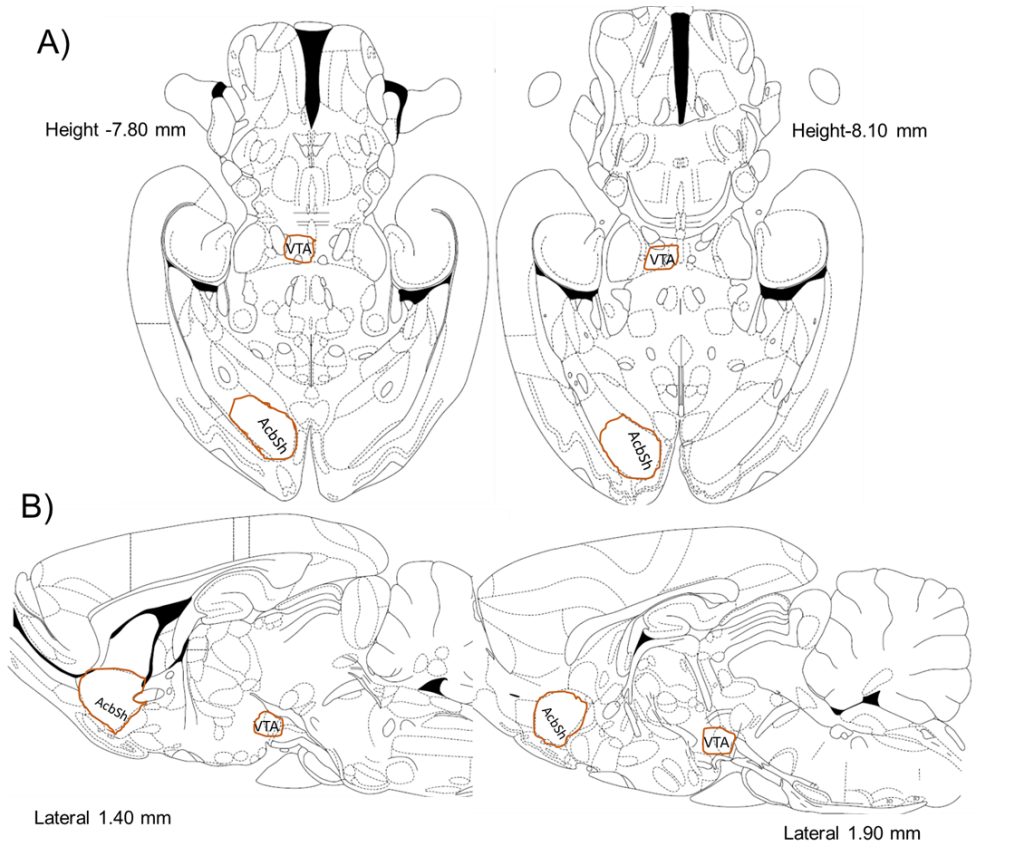
### 4.2.2 Experiment 1: Development and validation of method to obtain the whole mesolimbic DA pathway

#### 4.2.2.1 Animals culling and slice preparation

For experiment 1, non pre-treated, female Wistar rats (80-150g) (Division of Biomedical Service, University of Leicester) were humanely killed by cervical dislocation without anaesthesia (schedule 1). After that the skull was opened quickly using a bone cutter to expose the brain, which was rapidly removed and placed in ice-cold aCSF until the slicing procedure began. The brain was dissected using a single-edged carbon steel blade to remove the hindbrain. For experiments 1 we used several different slice orientations in either the horizontal or sagittal plane. In each case, the brain was kept cold during slicing, by adding crushed aCSF ice in addition to cold aCSF to the specimen chamber to get good slices.

For cutting sections in the horizontal plane: after separating the cerebellum from the cerebral (as mentioned above) a small portion of frontal cortex was removed and from this area the brain was fixed using cyanoacrylate glue to a vibroslicer (NVSLMI vibroslicer, World Precision Instruments) chuck. Then, the chuck was placed within a specimen chamber before being sectioned using the vibroslicer. In this slicing the brain was sliced from the ventral surface, NAc and VTA horizontal slices (440-520 µm thick) were cut and identified according to the rat atlas (Paxinos and Watson, 1998) (see figure 24 A).

For cutting sections in the sagittal plane: after separating the cerebellum from the brain attachment area, we separated the two brain hemisphere from midline and removed a small portion from a lateral edge of each hemisphere, then glued it onto the vibroslicer chuck to get the brain sagittal section (400 µm thick), then started slicing from brain midline after identifying the slices containing NAc and VTA according to the rat brain atlas (Paxinos and Watson, 1986) (see Figure 22 B). After slicing, the slices were transferred to a slice saver chamber comprising a wire mesh suspended in freshly prepared oxygenated aCSF and allowed to recover from the trauma associated with slicing at 21-23 °C for at least 1 h.




C)

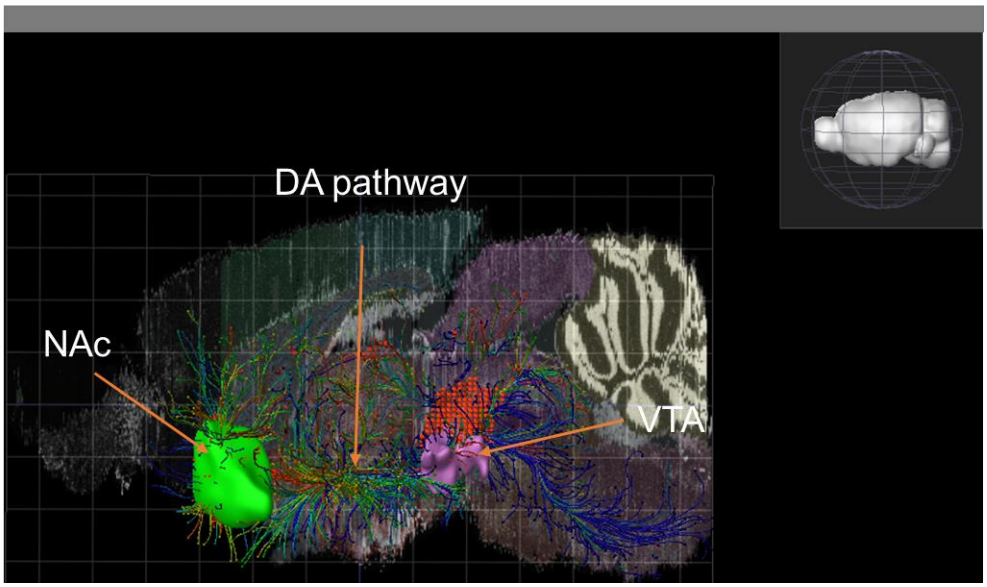



Figure 24: Horizontal (A) and sagittal (B) sections of rat brain showing the slices which used to find the mesolimbic DA pathway between VTA and NAC. A- taken from Paxinos and Watson, (1998). C) -3D images sagittal sections used to guide the slicing (Allen Institute for Brain Science, 2018).

### 4.2.2.2 The DA release in NAc shell using high $K^+$ stimulation

After cutting the slices into different sections (horizontal or sagittal) and after leaving them for at least 1h, they were transferred to the slice chamber for FSCV recording, and continuously superfused with oxygenated (32 ± 2℃) aCSF. The working electrode was placed using micromanipulator into the NAc shell, about 75 µm deep in the slice, using the rat brain atlas Paxinos and Watson (1998) as guide and under Olympus microscope. In addition, it was necessary to place the reference electrode (Ag/AgCl) in a convenient place in the slice chamber away from the working electrode to provide a stable reference potential.

After leaving the slice for 15-30 min in the slice chamber, the voltammetry scan was applied to working electrode (-0.4 to +1.3 V vs Ag/AgCl at scan rate 400 V/s) at 1 Hz and the recording began. After 10 s of recording, 1.5 ml of high-$K^+$ stimulant was applied to the tissue via the aCSF flow and this was continued for 60 s, after which the superfusate was returned to aCSF. 30 min later (at 1810 s), a second administration of high-$K^+$ stimulation was applied to a slice for 60 s. The high-$K^+$ stimulation enhanced the DA release in the NAc, which was detected as a current and referred to as S1 for first stimulation and S2 for second stimulation. See Figure 25 for stimulation protocol.

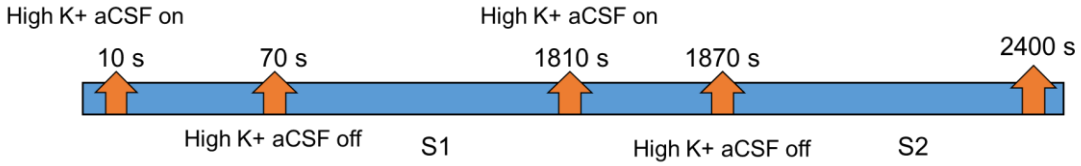



Figure 25: High K+ aCSF stimulation: after the slices were transferred from slice saver to slice chamber and leave it for 15-30 min, at 10 s the high $K^+$ stimulation was applied on slice chamber for 60 s. The DA stimulation (S1) was recorded for 30 min. the second high-$K^+$ stimulation was applied at 1810 s which was continued for 60 s when the flow was returned to aCSF for the reminder of the experiment (until 2400 s). The S2 was recorded starting from 1870 s until the end of the experiment (2400 s).

In this experiment, we simulated the whole slice including the mesolimbic DA pathway and recorded the DA releasing in the NAc shell. We found that the first and second DA release in the NAc shell were approximately equal between the different slices in different experimental days. In addition, it was clear that we could use the same slice for several high-$K^+$ stimulation, however, enough time should be given for the slice to recover and for DA reuptake which will effect on the S2:S1 ratio: in these experiments we found that 30 min between S1 and S2 was adequate to ensure that S1 and S2 were of similar magnitude (i.e. S1:S2 ration approximately 1.0). After obtaining a good DA recording in the NAc shell we used the same slice (horizontal or sagittal section) for the dye injection and electroporation of dextran tetramethyl-rhodamine dye.

Additional experiments were carried out using electrical stimulation. A bipolar tungsten stimulating electrode constructed in the lab as described by (Yavas and Young, 2017). Briefly, two 20 cm length of tungsten wire (0.075 mm) were inserted into a two-chamber (Θ- section) glass capillary, such that the two wires were electrically insulated by the septum running the length of the glass. The glass was then pulled to a narrow tip over a gas flame, and the tungsten wire was cut as it protruded from the glass, such that the tips were separated by approximately 0.5 mm. Electrical connectors were attached to the wires at the other end of the tubing, to enable connection to the stimulator (figure 26). The stimulating electrode was placed in the region of the VTA or median forebrain bundle, and electrical stimulation was delivered while recording in NAc. Several different stimulating electrode locations in the region of VTA and median forebrain bundle were used, and electrical stimulation (60 pules train, 4 ms pulses, 60 Hz and 120 uA) were applied (Wightman *et al*., 2007). Given the lack of responses to these electrical stimulations (see below), the stimulation electrodes were then placed in NAc, close to the recording electrode, and similar stimulation was delivered, in order to ascertain that the electrode was in a DA rich area. Further details of these experimental procedures are given in section 4.2.3.3.

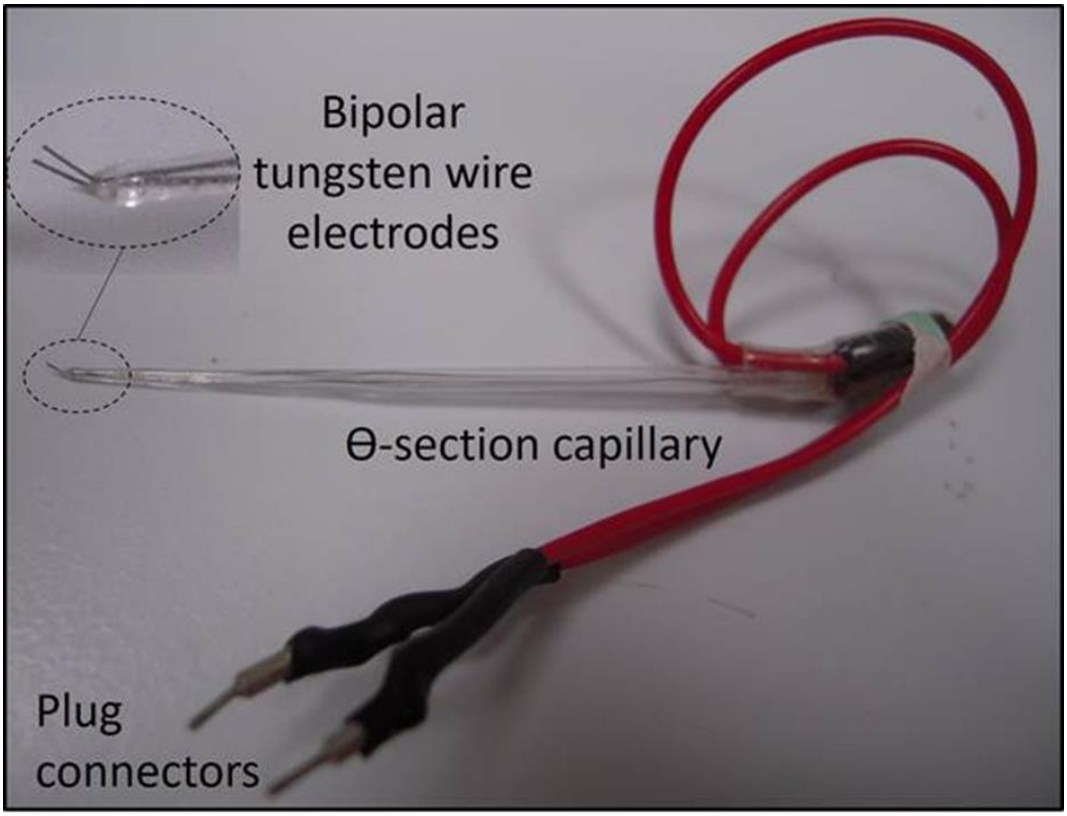



Figure 26: Bipolar tungsten stimulating electrode constructed in the lab as described by Yavas and Young (2017).

### 4.2.2.3 Histological identification of mesolimbic DA pathway using fluorescence dextran dye

Fluorescence dextran dye is a hydrophilic polysaccharide synthesized by Leu-conostoc bacteria and characterised by moderate to high molecular weight, good water solubility and low toxicity in addition to fast axonal diffusion and great access to other cell processes. It is available in different molecular weights starting from 300 to 2,000,000 Daltons, but for these experiments we used 3000 and 10000 molecular weight which were introduce to the cell via microinjection. Although, dextran with a high molecular weight up to 70,000 Dalton was used to trace the neuron projection and can work as an anterograde and retrograde tracer, 3000 and 10000 molecular weight may be preferred in many studies because they penetrate neuronal projection better and diffuse faster than other MW dextran. In addition, dextran dyes are used commonly in microscopy studies to monitor cell division and track the movement of live cells. Moreover, it is widely used in neurons as an anterograde and retrograde staining in live cells (Molecular ProbesTM, Invitrogen detection technologies).

#### 4.2.2.4 Procedure for applying the dye

After cutting the slices and leaving them in oxygenated aCSF for at least 1 hour, the slices were transferred to the slice chamber and the dextran tetramethyl-rhodamine dye was injected in slices that contained both NAc shell and VTA according to Paxinos and Watson (1998). Two methods of injection were used. The first method, was retrograde staining in which the dextran stain was injected into the NAc shell under a Olympus microscope 40 X (Japan) after stimulating this area with high-$K^+$aCSF (mM) solution. Then, when we obtained a good DA recording using FSCV, to make sure we placed the working electrode into the NAc shell we injected the dextran dye into the same area after removing the working electrode by using glass microelectrodes, which was pulled by an electrode puller (Narishige, Japan).

The second method of injection was anterograde staining, in which the dextran stain was injected into the VTA, under Olympus microscope, using the rat brain atlas to guide the position of injection. Several injections (3-6) were administrated to this area (VTA) in each slice containing NAc and VTA using glass microelectrodes. Immediately after injection, the brain slices were kept the dark and in oxygenated aCSF for at least 4-5 h and stored in a fridge (4 °C) overnight to take up the stain (Burger *et al*., 2005). When the staining procedure was completed the fluorescence was visualized in the brain slices using fluorescence microscope (Nikon, Labophot-2) to view the mesolimbic pathway. By using green fluorescence filter the emitted 'Texas red' and using a camera attached to the microscope, the images of slices were taken.

#### 4.2.2.5 Electroporation simulation

The dextran stain has a low rate of leakage inside the tissue and so to increase the uptake and local delivery of the dye, electroporation was used. This is a means of increasing uptake of exogenous macromolecules within a tissue by applying electrical current to the injection staining site. We used electroporation in two protocols. The first one is that according to (Barker *et al*., 2009) we delivered electrical stimulation to the injection site using bipolar tungsten electrodes, 5 ms square wave pulses and 100 Hz for 2-10 s with a 0.1µA current. The second protocol is that according to (Burger *et al*., 2005) we used 8 ms square wave pulses and current 500 µA at 10 Hz for 50 ms duration and repeated the same procedures three times.

### 4.2.3. Experiment 2: Altered DA release and re-uptake in coronal slices taken from pre-treated animals

Following the lack of success in recording release in NAc following stimulation of VTA in either the horizontal or coronal slices, putatively containing the whole mesolimbic pathway (experiment 1), it was decided to restrict the study to measure local effects in the terminal region, using coronal slices containing NAc (but not VTA).

#### 4.2.3.1. Animals and chemicals

Male Lister-hooded rats (n=18) were supplied by Charles River Laboratories (Cambridge, UK) and weighted between 250-300 g on arrival and housed as above (see section 2.2.1). Briefly, the rats were allowed one week to acclimatize before starting the experiments and were housed four per plexiglass cage in colony room 20 ± 3℃ (the light cycle was 7:00 am light on and 7:00 pm light off), with *ad libitum* access to food and tap water except during experimental sessions. The animals were randomly assigned to three groups (nicotine, AMPH and saline groups, n = 6 per group). For *in vivo* experiments, (-) - nicotine ([-]-1-methyl-2-[3- pyridyl] pyrrolidines) hydrogen tartrate salt and D-AMPH sulphate were purchased from Sigma-Aldrich (UK). The drug doses were calculated as salts and freshly prepared by dissolving them in physiological saline (0.9% NaCl) for i.p injection in volumes of 1 ml / kg body weight.

#### 4.2.3.2 Behavioural studies

All procedures involving animal handling and habituation were as described above (see section 2.2.3). Briefly, three day before starting the experiments, the animals were handled (20 min per day) and habituated to the activity boxes (46 × 24 × 22 cm) (30 min per day) to familiarize them with the experimental procedure. The day after handling and habituation, the animals were injected once a day with nicotine (0.6 mg/kg, i.p, n= 6), D-AMPH (1 mg/kg, i.p, n= 6) or saline (1 ml/kg, i.p, n= 6) for five consecutive days and locomotor activity was recorded immediately after each injection for 60 min. Ten days of withdrawal after the last injection, the animals were humanely killed, and the brain removed for *in vitro* study.

#### 4.2.3.3 *In vitro* FSCV recordings

Slice preparation and voltammetry was done as described previously (Melchior *et al*., 2015, Yavas and Young, 2017). All voltammetry experiments were conducted following final drug session (10 -14 day from the final session). Briefly, after ten days of withdrawal, animals were anesthetized with isoflurane, decapitated and the brain rapidly removed and cooled in ice-cold, pre-oxygenated (95% O2, 5% CO2) aCSF. After removal of the hindbrain, the posterior or caudal end of the sectioned brain was fixed using cyanoacrylate glue to a vibroslicer chuck, and coronal slices (400 µm thick) containing the NAc were cut, identified using atlas of (Paxinos and Watson, 1998), see Figure 27.

Multiple coronal slices including the NAc were prepared from each animals. Once sliced, brain slices were cut in half along the midline and transferred to the wire mesh slice saver containing freshly prepared oxygenated aCSF and allowed to recover at 21-23 °C for at least 1 h to provide 6 slices per brain. For recording, a slice was transferred to the tissue chamber and superfused with aCSF (31 ± 1 °C) for a 1 h equilibration period before recording (as mention in section 4.2.2.1). In each case, different drug conditions were used on each slice with the order of testing randomized across brains, meaning that the n-values quoted are the number of slices each from different brains (i.e. the experimental units is the animal).

A concentric bipolar stimulating electrode (125 µm outer diameter; CB-ARC75; FHC Inc, USA) was placed approximately 50 µm below the surface of the slices, within NAc shell or core. The working electrode was lowered into the tissue to a depth of approximately 120 µm to lie approximately 500 µm from the stimulating electrode and centrally located between the poles of the stimulating electrodes. Extracellular DA was monitored at the carbon fibre electrode every100 ms using FSCV (Wightman *et al*., 1988) by applying a triangular waveform (-0.4 to +1.2 to -0.4 V vs Ag/AgCl / 400 V/s) at a frequency of 10 Hz to induce the oxidation and reduction of DA at the surface of carbon fiber electrode. Electrical stimulation was delivered through a constant current stimulus isolator (Iso-Flex, AMP Instruments) as repeated pulse train (5 monophasic square-wave pluses) at 5 min intervals, under the control of the Demon voltammetry software (Yorgason *et al*., 2011).

The evoked background subtracted current was attributed to DA with the corresponding voltammogram featured a characteristic shape with peak oxidation and reduction potentials (vs. Ag/AgCl) at approximately + 600 and -200 mV, respectively. For control experiments (no drugs), 12 stimulus trains (5 pulses × 0.5 ms pulses; 300 µA, 20 Hz; offset by 10 ms from the FSCV waveform) were applied at 5 min intervals. Repeated 15 s current recordings were made, such that the stimulation occurred 5 s into each recording (see Figure 28 for FSCV example data). For experiments employing drug application, immediately after the completion of the fourth baseline stimulus train (20 min), the superfusion medium was switched to aCSF containing either nicotine (1 µM) or AMPH (2 µM) for a further 20 min (4 stimulations). Immediately after the eighth stimulation, the superfusion medium was returned to aCSF again for additional 20 min washout period (post-drug, 4 stimulations). The concentration of nicotine and AMPH used here is based on previous studies (Shim *et al*., 2001, Siciliano *et al*., 2014). Separate slices were used for each of the treatments, such that each slice received only 12 electrical stimulus trains, and a single drug treatment. See the Figure 29 for experimental timeline. In dopamine reuptake time experiments, we measure the half-life of dopamine which is the ration between dopamine increasing and dopamine reduction after 12 stimulation trains as explained above.

To summaries, stimulated DA release was measured over 12 repeated stimulations (S1 to S12), at 5 min intervals, and the effect of either nicotine or AMPH, given acutely by superfusion of the slices during S5 to S8, was compared to the stimulated release in slice receiving no drug (no drug control). Further, the effect of pre-treatment of either nicotine or AMPH was assessed in slices taken from animals repeatedly pre-treated (once per day for five days) with nicotine or AMPH, compared to animals pre-treated with saline.

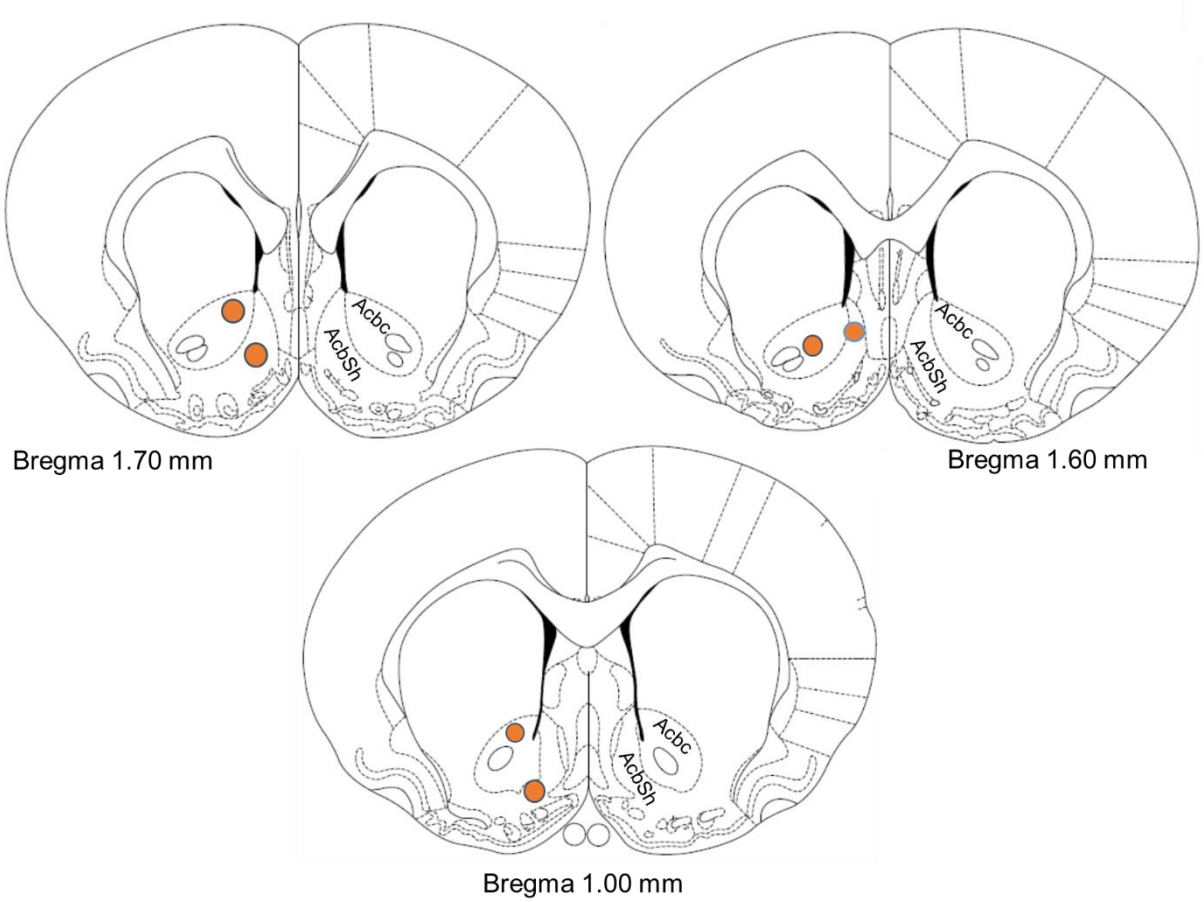



Figure 27: Coronal brain section used to measure the DA alteration in pre-treated rats after perfuse the slices with drugs *in vitro* FSCV experiments (Paxinos and Watson, 1998) and the small circle show the location of the working electrode in the NAc shell and core.

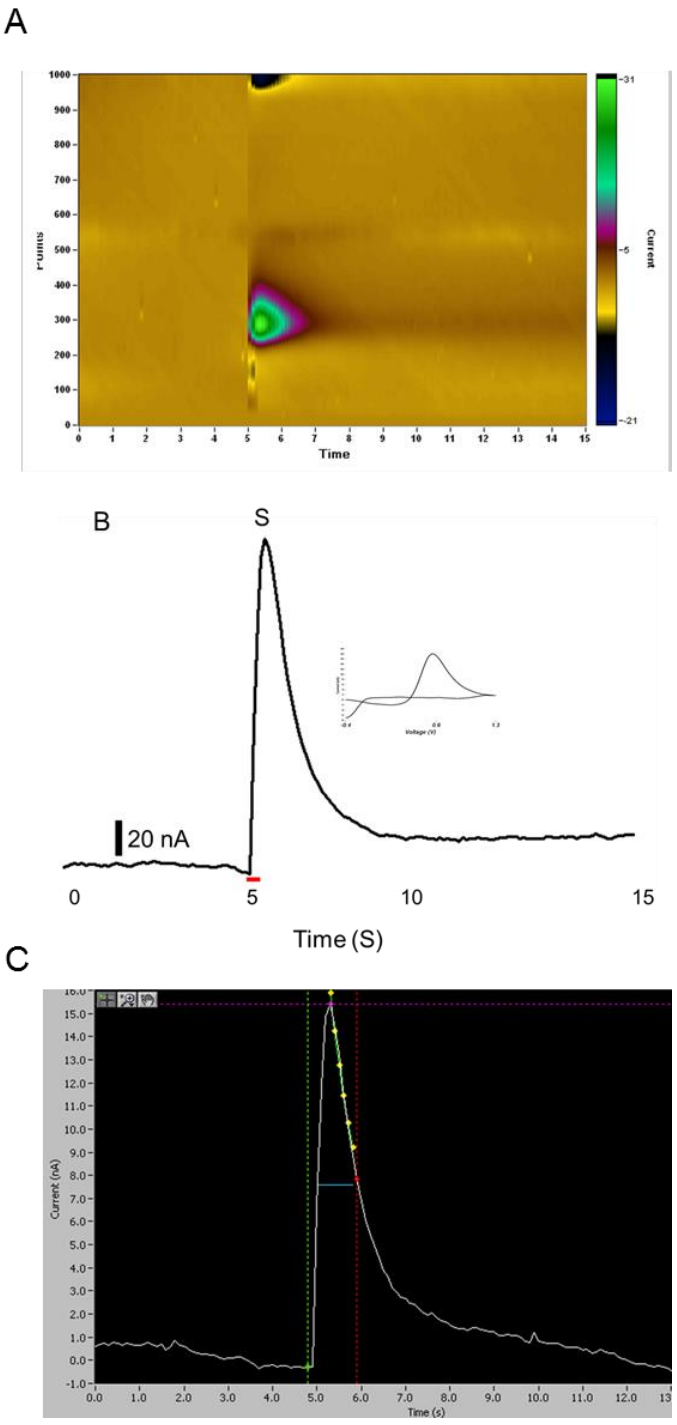



Figure 28: Example FSCV data from a single slice taking from control animals (pre-treated with saline and superfused with aCSF). A) Colour plot and B) current vs time plots of electrically stimulated (red bar) dopamine release during baseline period (without drugs), before the start of drug application. Inset shows current vs voltage plot (cyclic voltammogram), demonstrating the characteristic shape of the DA signal; C) Showing the current vs time of re-uptake (scale bar = 20 µA, T1/2 half live which is a time ratio between DA increase and decrease).

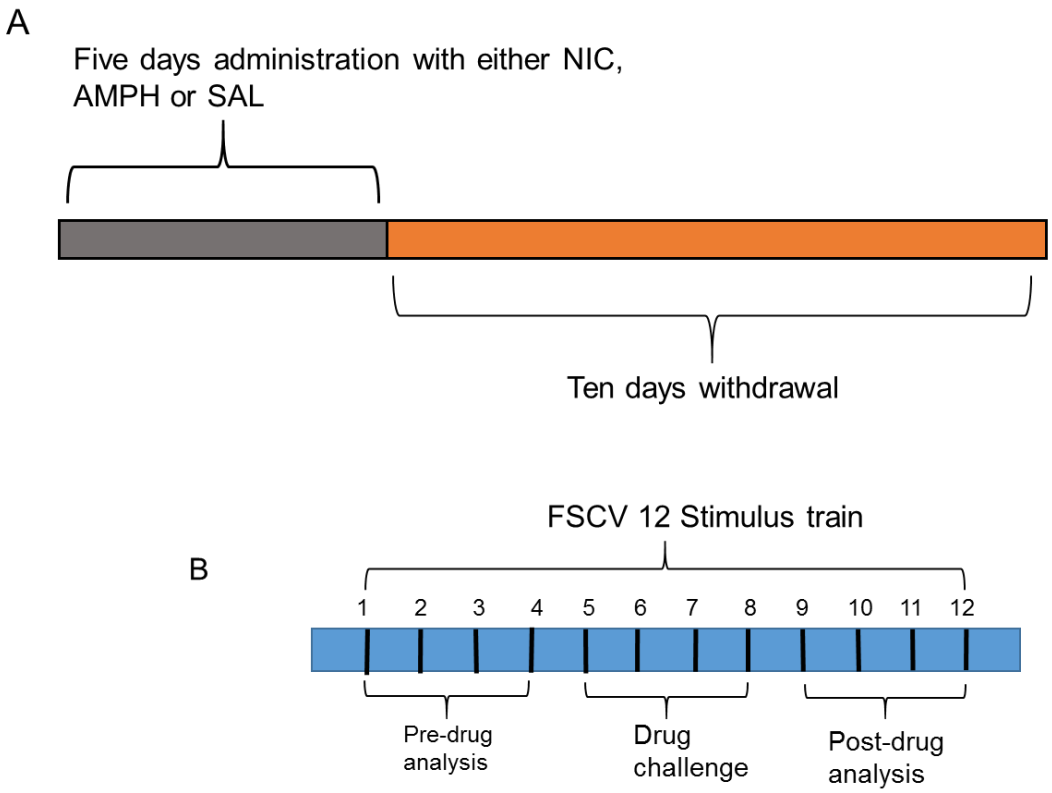



Figure 29: Experimental timeline for drug-exposure (A) and FSCV regordings (B). A) Animals were injected with either nicotine (0.6 mg/kg, i.p, n = 6), AMPH (1 mg/kg, i.p, n =6) or saline (1ml/kg, i.p, n = 6) once a day for five consuctive days and after 10 days withdrwal peroid, they were humanely killed, and the brain removed for in vitro FSCV recording. B) For recording 12 stimulus train were applied to the slice, 4 stimulus trains were applied before adding drug to the medium and another 4 stimulus trains were applied during perfusion of the tissue with the drug and another 4 stimulus train were applied after switching off the drug washout.

### 4.2.4. Data analysis

All behavioural data were analysed by using appropriate analysis of variance (ANOVA) using StatView software 5.0 (SAS Institute, NC, USA) and was similar to that previously described (section 2.2.4). To determine the time course for studying the effect of drug treatment, the locomotor activity was measured at 10 min intervals over the course of 60 min locomotor activity session. The influence of pre-treatment of drugs on animals' activity during the induction phase (pre-treatment session) was analysed by using three-way ANOVA (5 × 6 × 3), using within-subjects factors of days [5 levels (day 1, day 2, day 3, day 4 and day 5)] and time bins [6 levels (10 min, 20 min, 30 min, 40 min, 50 min, 60 min)]

and with a between- subject factor of drugs treatments [3 levels (nicotine, AMPH and saline)].

For the FSCV recordings the concentration of electrically stimulated DA release was calculated from the oxidation current using Demon voltammetry software by comparison with a DA standard calibration (5 µM). For each slice, the mean stimulated release over the four basline stimulation (S1 to S4) was calculated, and the changes measured at all 12 stimulus presentations were then expressed as the percentage of this mean baseline level. In addition, reuptake data were assessed as the same way using the Demon voltammetry software release kinetics function (figure 28 C). All data are shown as mean ± SEM percentage of baseline. Statistical analysis was conducting using two-way ANOVA between subject factor (treatment condition) and within subject factor (stimulating number) using Stat View 5.0 (SAS Institute, NC, and USA) and planned comparison that compare specific groups (acute groups, sensitisation and cross-sensitisation groups) that calculate manually.

## 4.3 Result

### 4.3.1 Experiment 1

Several different slice orientations used in order to try to include the whole pathway. Electrical stimulation of VTA, using various parameters did not show any stimulated DA release in NAc (data not shown). In each case, we verified that working electrode was in a DA rich area by applying local electrical stimulation and seeing DA release (data not shown). In an attempt to verify that the pathways was intact, staining was used. The result showed strong staining around the sight of injection, but little or no staining away from the injection sight, indicating that the pathway was probably not intact. Therefore we moved away from this approach and used coronal slices with local (terminal) stimulation, as is routine in the lab (Yavas and Young, 2017) for experiment 2. The possible reason why this approach did not work is that the topography of the pathway may mean that it is impossible to take a 400 µm slice without cutting the pathway. Future experiments might use thicker slices, but this has implication for the viability of the tissue. See Figure 30 for images of site of stain injection in NAc shell and VTA.

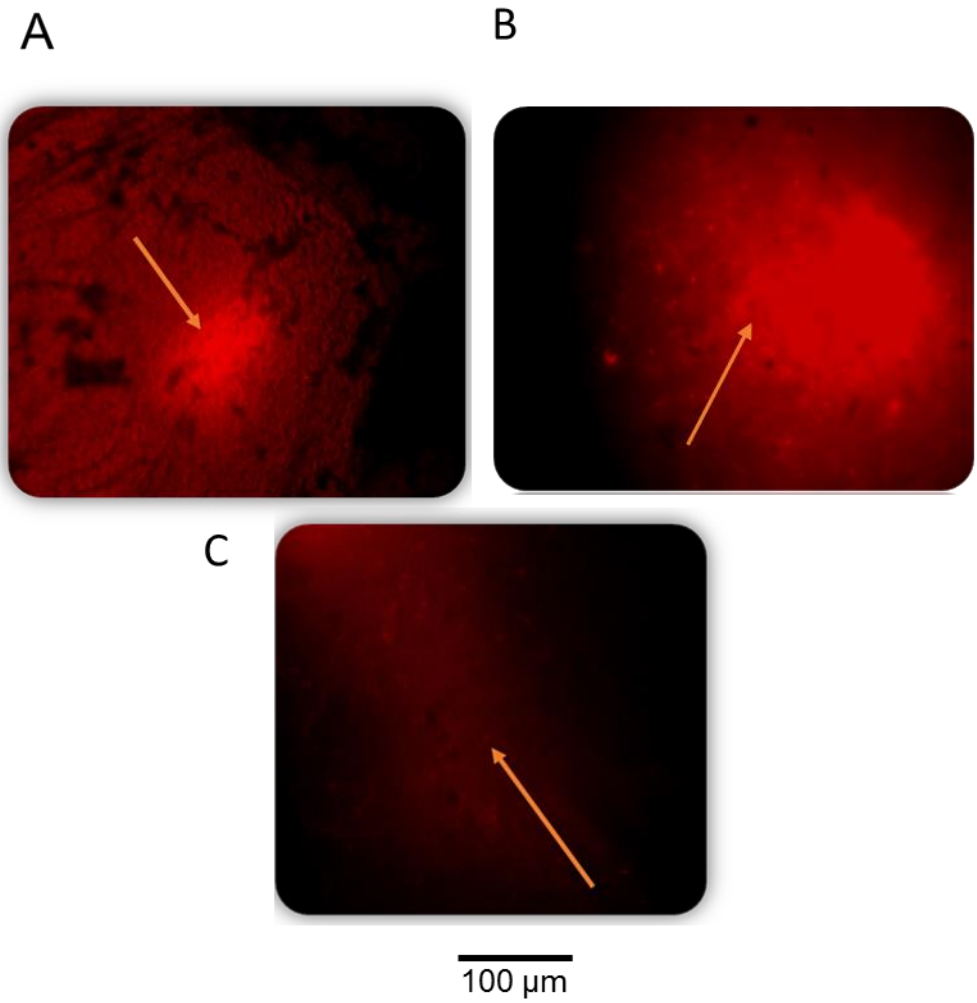



Figure 30: Dextran tetramethyl-rhodamine dye injection in the NAc. Image (A) refers to injection place of the dye in NAc shell after stimulated this area. Image (B) refers to dye injection place in the VTA, while image (C) refers to the dye diffusion extended from VTA injection sites down to NAc. However, the dye only extended a short distance from the injection site, and did not label the whole mesolimbic pathway.

### 4.3.2 Experiment 2

#### 4.3.2.1 Behavioural Studies

First, we investigated the effect of nicotine or AMPH on the development of behavioural sensitisation. As we showed before (section 2.3.1), rats pre-treated with nicotine (0.6 mg/kg, i.p) or AMPH (1 mg/kg, i.p) one injection for five consecutive day, exhibit increased locomotor activity compared with those pre-treated with saline, indicating that behavioural sensitisation to nicotine had developed. Three-way ANOVA (drug pre-treatment × days ×

time bins) (3 × 5 × 6) revealed a main effect of drug pre-treatment [F (2, 15) = 32.6; p < .0001], day [F (4, 60) = 26.5; p < .0001] and time [F (5, 75) = 57.6; p <.0001]. A two-way analysis using a day within-subject factor and time bins and a drug between- subject factor revealed a significant interaction between drug pre-treatment × day [F (8, 60) = 13; p < .0001], drug pre-treatment × time [F (10, 75) = 11.9; p <.0001] and no significant interaction between day × time [F (20, 300) = 1.5; p =.06].

A three-way ANOVA (drug pre-treatment × day × time) revealed no significant interaction between all three variables [F (40, 300) = 1.4; p = .057]. For nicotine pre-treated rats, post hoc Fisher's LSD revealed that on the first day of drug treatment (day1), acute administration of nicotine significantly decreased the locomotor activity in naïve rats compared with rats receive saline, while on the following days the behavioural sensitisation to nicotine was evident during the test period and on day 5 (Planned comparison, p <.01) see Figure 27. For AMPH pre-treated rats, statistical analysis showed that the significance of the sensitising effect of AMPH began at the first day after AMPH injection and continue during the experimental days compared with the rats who had a saline injection (p <.01, see Figure 31). These results essentially replicated those described in chapter 2, indicating that behavioural sensitisation developed after repeated injection with nicotine or AMPH.

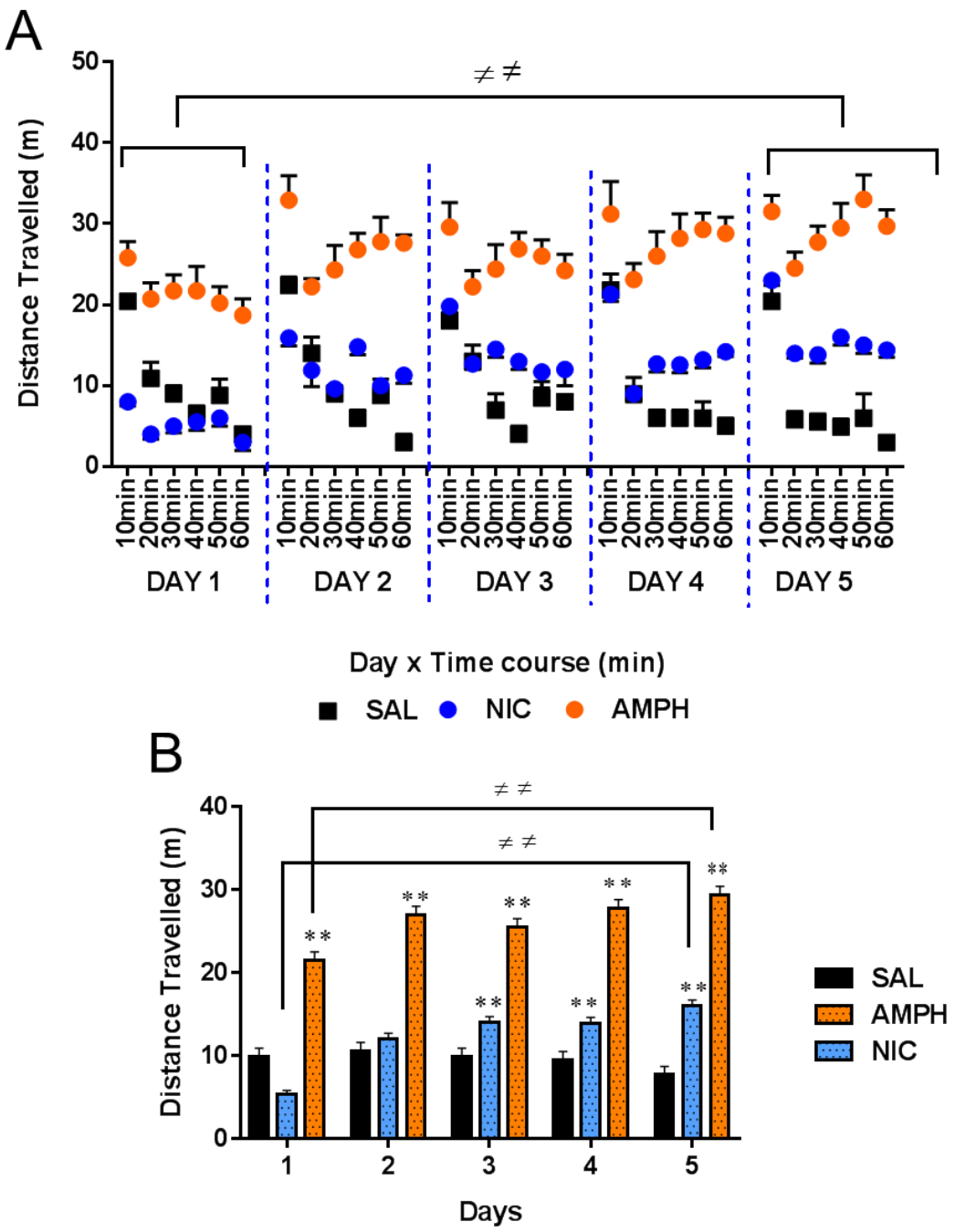



Figure 31: The effect of systemic injection of nicotine, AMPH or saline on the locomotor activity. Rats were pre-treated with nicotine, AMPH or saline once a day for five consecutive days. Distance travelled in meter was recorded for 10 min time bins (A) and for 1 h (B) immediately after drugs or saline administration. ## = significant differ NIC or AMPH day 1 from day 5 (Figure A and B). ** = significant differ NIC or AMPH from SAL (Figure B). Data represent the mean ± SEM for distance travelled (m).

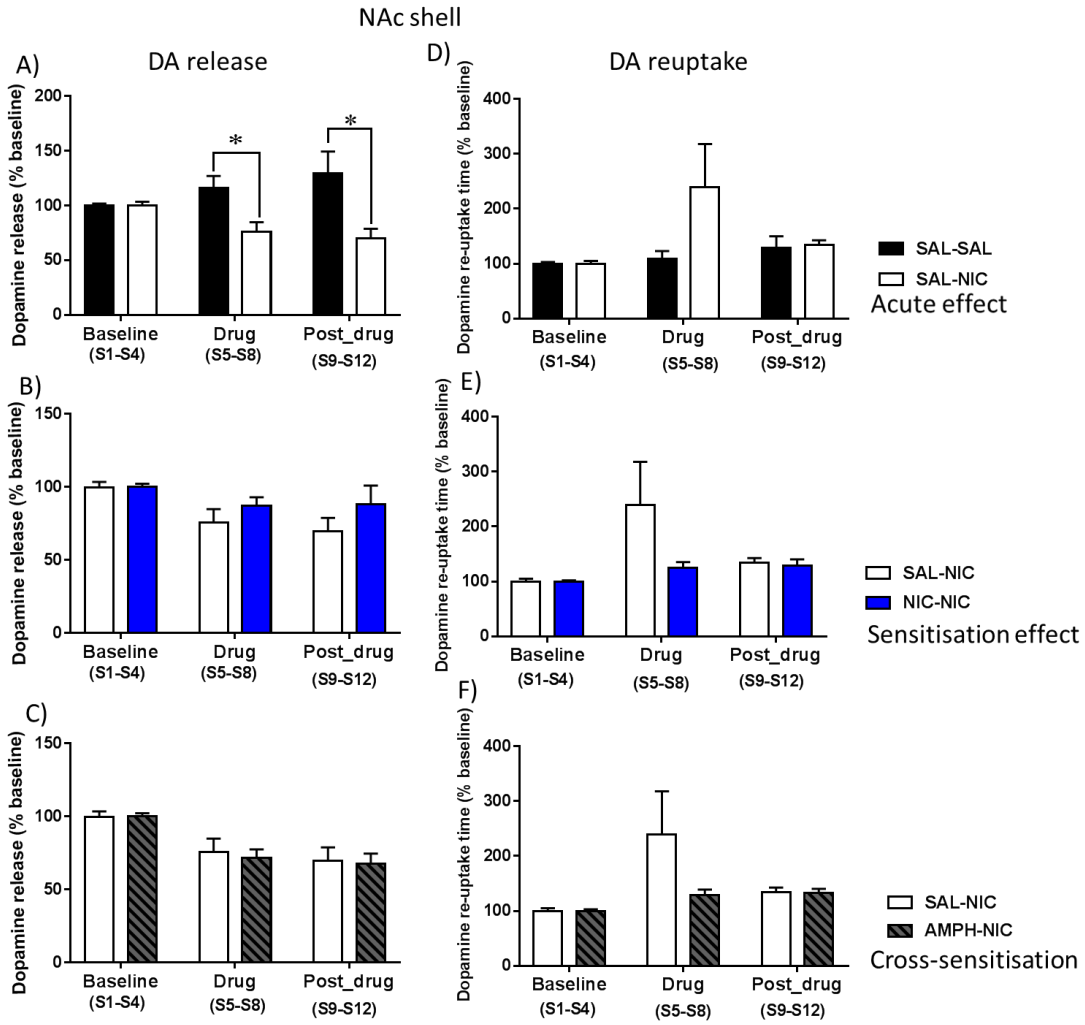
#### 4.3.2.2. Dopamine release in the NAc

Electrical stimulation elicited a rapid increase in DA response in both the shell and core of the NAc. Because DA release in the NAc is known to play a critical role in behavioural sensitisation, in the first part of experiment, we investigated the effect of pre-treatment with either nicotine or AMPH on the acute action of nicotine on electrically stimulated DA release in the NAc shell and core of coronal tissue slices, by comparing the effect of bath applied nicotine (S5 to S8) on electrically stimulated DA release in slices taken from saline pre-treated animals, thus confirming that any changes in stimulated DA levels observed after drug treatment were attributable to drug action, rather than changes due to multiple stimulation over time.

Two-way ANOVA (treatment conditions × stimulus number) was conducted on the data from NAc shell and core to measure the effect of treatment on DA release. In the shell there was a significant main effect of treatment [$F(3, 18) = 5.01$; $p < .05$] and of stimulus [$F(2, 36) = 3.94$; $p < .05$], and a significant treatment × stimulus interaction [$F(6, 36) = 4.33$; $P < .05$]. Planned comparison revealed increase DA release over repeated stimulation in the control slices (receiving no drug) as compared with acute nicotine application (1 µM) (Fisher's LSD, $p < .05$). No change in stimulated DA release was seen during the acute nicotine superfusion (1 µM), until the completion of the recording (S12), in slices taken from rats pre-treated with either nicotine or AMPH as compared with saline pre-treated rats (see figure 32 A -C).

In the core there was a significant main effect of treatment [$F(3, 18) = 5.59$; $p < .05$] and of stimulus [$F(2, 36) = 18.85$; $p < .0001$], and a significant treatment × stimulus interaction [$F(6, 36) = 5, 47$; $p < .05$]. As in the shell, planned comparison revealed a significant change over repeated stimulation in the control slices (no drug) as compared with acute nicotine application (1 µM), as expected. However, there was no change in stimulated DA release during the acute nicotine (1 µM) superfusion in tissue from nicotine or AMPH pre-treated rats as compared with saline pre-treated animals. In the both NAc sub region, the reduction in stimulated release caused by nicotine continued throughout the washout stimulations (post-drug stimulation, S9 to S12) as compared with baseline stimulation (S1 to S4), with little sign of returning to baseline levels (see Figure 32 G-I ).

To determine whether the effect of pre-treatment with drug on DA release after acute challenge was caused by changing in the DA reuptake. We examine the possible role of changes in DA reuptake in the sensitized tissue, by measuring the time of reuptake after drug challenge. In nicotine sensitisation and cross-sensitisation experiments, two-way ANOVA revealed in the shell a significant main effect of stimulus [F (2, 36) = 5.33; P <.05], but no significant main effect of either treatment [F (3, 18) = 2.04; p = .14] or treatment × stimulus interaction [F (6.36) = 2.16; p = .07], (see figure 32 D-F). In the NAc core, there was a significant main effect of stimulus [F (2, 36) = 4.30; p <.05], but no significant main effect of either treatment [F (3, 18) = .64; p = .59], or treatment × stimulus interaction [F (6, 36) = .57; p = 74] see Figure 32 J-L. As there was no interaction between treatment × stimulus, we did not do another analysis.



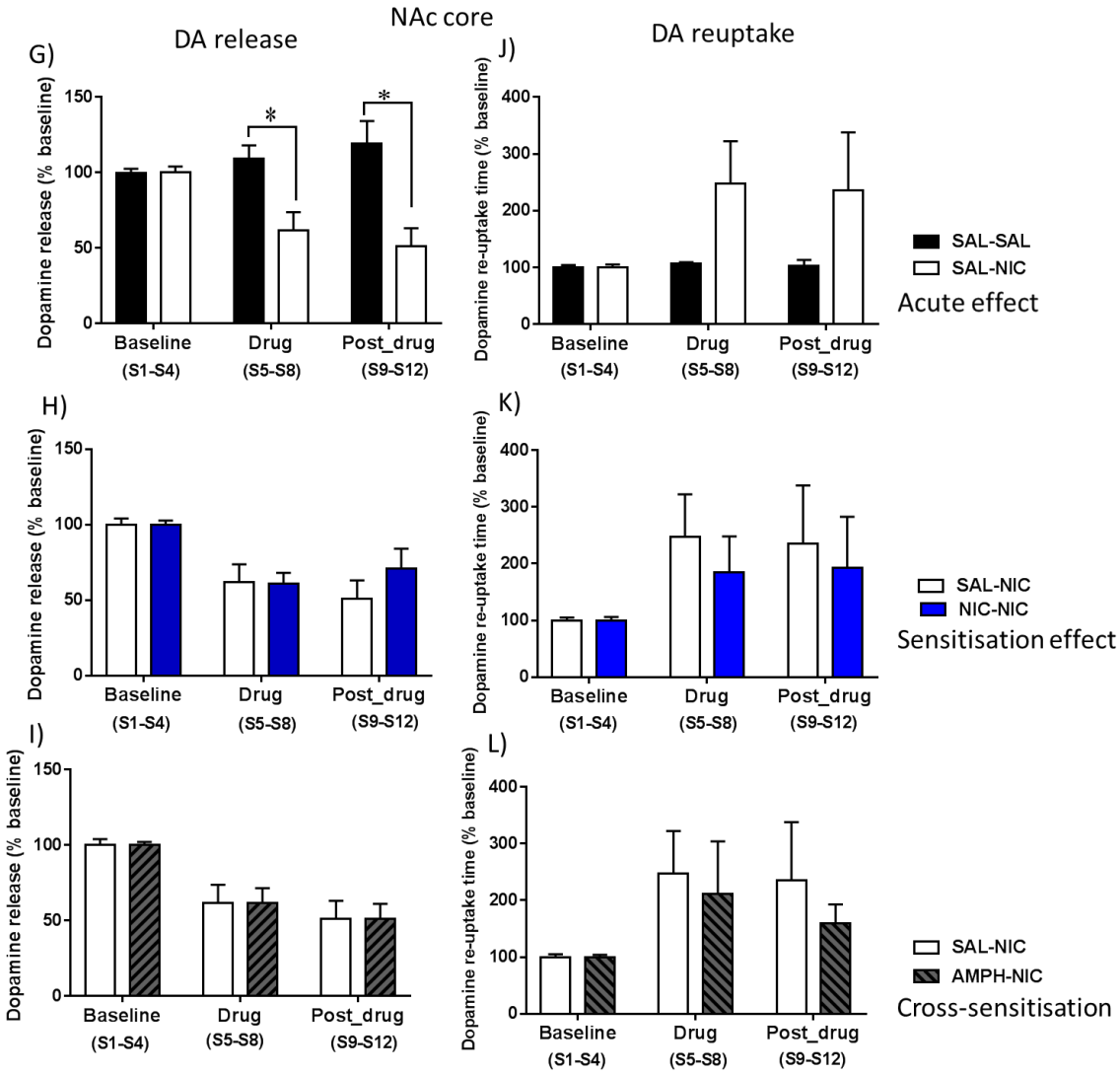



Figure 32: Effect of acute nicotine on stimulated DA release and uptake in the NAc shell (A-F) and core (G-L). Acute application of nicotine caused a decrease in DA release in tissue slices in the shell (A -C) and core (G-I) compared with control tissue. No significant effect of treatment was observed following acute application of nicotine compared to their control. In addition, nicotine application had no significant effect on DA reuptake in the shell (D-F) and core (J-L) on either treatment groups or their respective control (two-way ANOVA, p = .06 and .7 receptively). Data are mean SEM. * p < .05, based on significant interaction from ANOVA; n = 5-6 per group.

The second part of experiment sought to determine the effect of pre-treatment with either AMPH or nicotine on acute AMPH application on DA release that electrically stimulated in the NAc shell and core and compare this effect with acute AMPH application (S5 to S8). In the NAc shell, two way ANOVA revealed that there was a significant main effect of stimulus [F (2, 38) = 4.97; p <.05] but no significant main effect of treatment [F (3, 19) = 3.01; p =

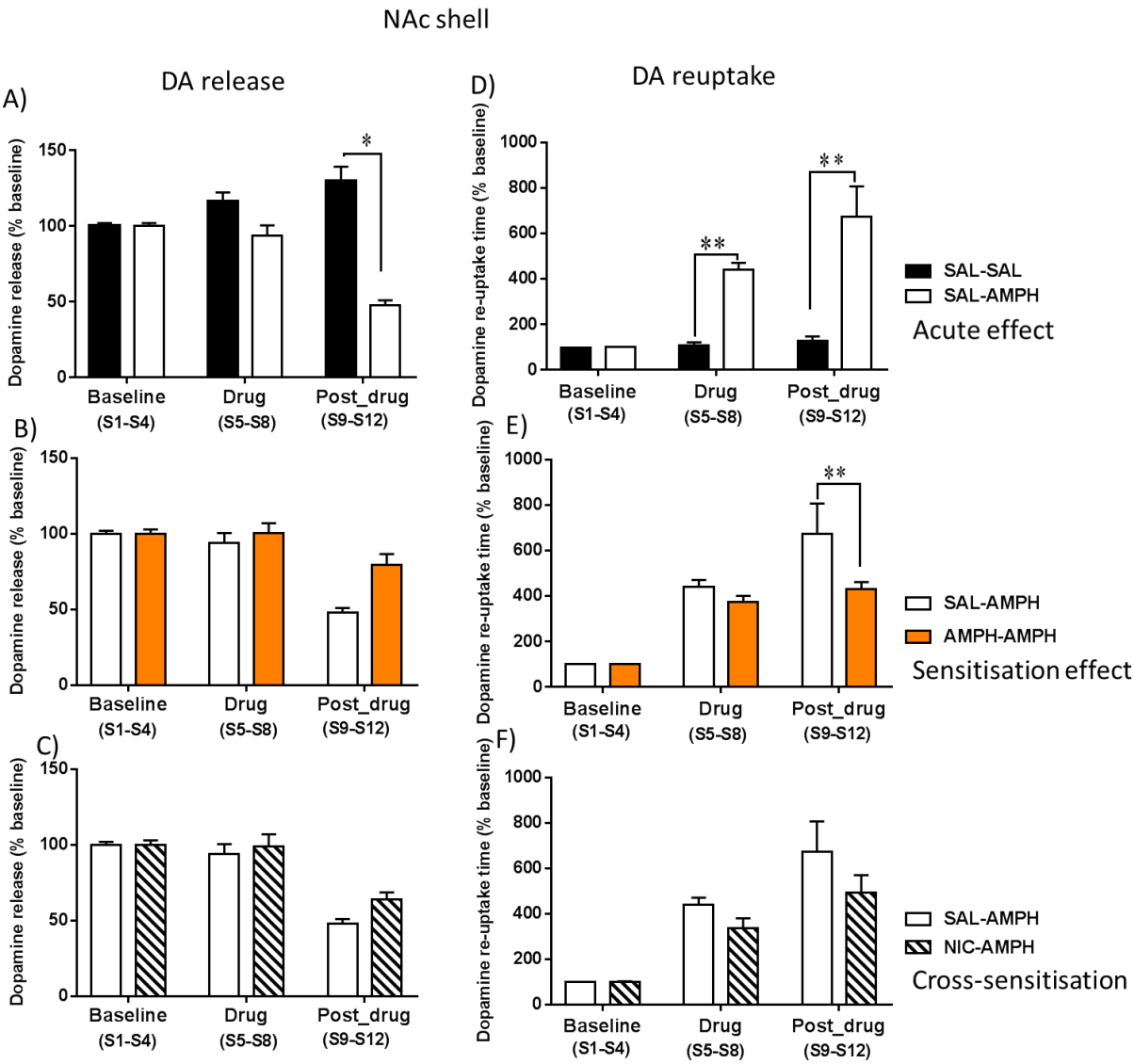
.06] and a significant main effect of treatment × stimulus interaction [F (6, 38) = 4.97; p <.05]. In the core, there was a significant main effect of stimulus [F (2, 38) = 20.32; p <.0001] but no significant main effect of treatment [F (3, 19) = 1.82; p =.17], and a significant main effect of treatment × stimulus interaction [F (6, 38) = 4.79; p <.05].

Planned comparison revealed as before, in the absence of any drug treatment, stimulated release was increased across the 12 stimulation as compared with acute AMPH application (2 µM) in the shell and core ( p<.05). Application of AMPH in the superfusate (2 µM for 20 min) in both shell and core did not cause any immediate change in stimulated DA release as compared with acute AMPH application. However, after 10 min (shell, S6) or 15 min (core, S7) of application, the stimulated DA release began to decrease, and effect which continued throughout the washout period, until the completion of the recording (S 12) in slices taken from animals pre-treated with either AMPH, nicotine or saline (Figure 33 A -C for shell and G-I for core).

To address whether acute perfusion of AMPH (2 µM) altered DA reuptake in animals pre-treated with either AMPH or nicotine. In the NAc shell, two-way ANOVA was conducted and revealed a significant main effect of treatment [F (3, 19) = 4.16; p < .05] and of stimulus [F (2, 38) = 33.58; p <.0001], and a significant treatment × stimulus interaction [F (6, 38) = 3.83; P < .05]. planned comparison revealed a significant increase in time of uptake (decreased in DA reuptake) after AMPH superfusion (2 µM) in slices taken from saline pre-treated animals, as compared with control slices (receiving no drug) and this changes continued throughout washout period as well (p <.01). AMPH application does not effect on DA reuptake time in slices taken from AMPH or nicotine pre-treated animals as compared with saline pre-treated animals. Pre-treatment with AMPH or nicotine caused decrease time of DA uptake (increase in DA reuptake) which occurred only in the washout period as compared with acute AMPH application (p <.01) (see Figure 33 D-F).

While in the core, there was a significant main effect of treatment [F (3, 19) = 18.64; p <.0001] and of stimulus [F (2, 38) = 52.32; p <.0001], and a significant treatment × stimulus interaction [F (6, 38) = 10.26; p <.0001]. As in the shell, post hoc analysis revealed a significant increase in time of reuptake (decrease in DA reuptake) in slices taken from saline pre-treated rats after AMPH application as compared with control slices (receiving no drug) that continued throughout the washout period (p <.05). Pre-treatment with nicotine cause increase time of DA reuptake (decrease in DA reuptake) as compared with acute AMPH

application which continued throughout the washout period, until the completion of the recording (S 12) ($p < .01$). While pre-treatment with AMPH does not change the reuptake time as compared with saline control slices after acute AMPH application and only cause increase the time of reuptake (decrease in DA reuptake) during washout period ($p < .01$) (see Figure 33 J-L for core).



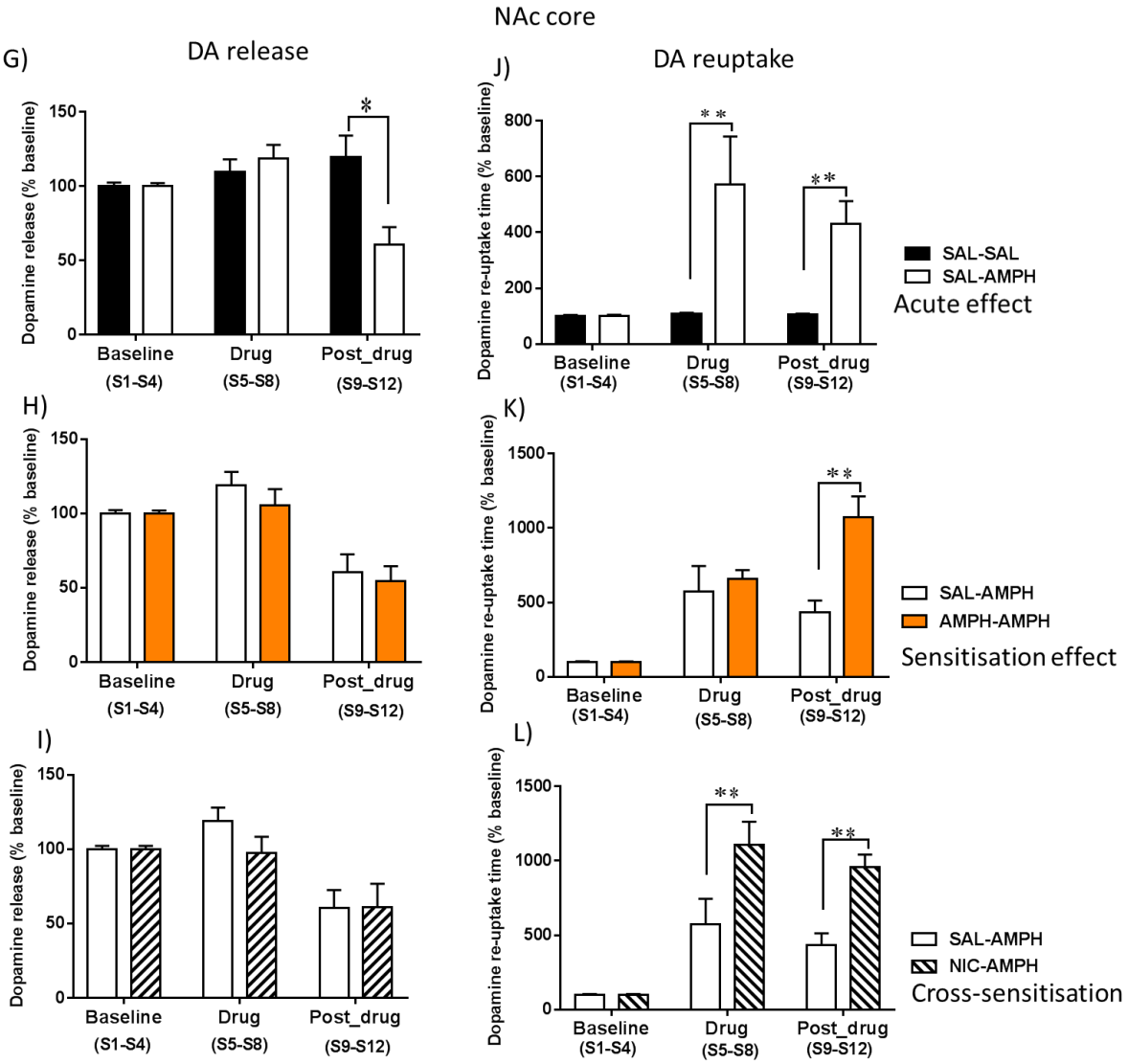



Figure 33: Effect of acute AMPH application on DA release and uptake in the NAc shell (A-F) and core (G-L). A - I representative DA release from NAc shell, while G -I from core respectively. Acute application of AMPH cause decrease the DA release in the saline pre-treated rats compared with control rats (without drug) in washout period only ($p < .05$), while pre-treatment with either AMPH or nicotine had no effect. D-F and J-L representative DA reuptake in the NAc shell and core respectively. Acute application of AMPH increased the reuptake time of saline pre-treated rats as compared with control rats, while treatment with drugs had a significant effect in shell throughout washout period only, while in the core treatment with AMPH or nicotine increase the time of reuptake which continue until the completion of recording for nicotine pre-treated rats only, while the pre-treatment with AMPH effect on reuptake time (increase the reuptake time) only in washout period as compared with saline control. Data are mean SEM. ** = significant differ AMPH or nicotine pre-treated slices vs saline pre-treated slices (Figure 33J-L), * $p < .05$, ** $p < .01$, based on significant interaction from ANOVA; n = 6 per group.

## 4.4 Discussion

In experiment 1 we attempted to develop a method which would allow measurement from NAc, while stimulating VTA and/or manipulating VTA pharmacologically. However, we were unable to obtain slices which would allow such measurements, and therefore the remainder of the experiments described in this chapter used coronal slices containing NAc core and shell but not VTA, to asses if the behavioural sensitisation to drugs is associated with increased release of DA in the mesolimbic DA system. Nicotine, applied acutely in the tissue superfusate caused decrease in stimulated DA release, which was unaffected by pre-treatment with either nicotine, AMPH or saline. Pre-treatment with either nicotine or AMPH had no effect on DA reuptake as compared with acute nicotine application. For AMPH experiments, acute application of AMPH caused a decrease in stimulated DA release, which was unaffected by pre-treatment history. In DA reuptake experiment, pre-treatment with either nicotine or AMPH does not effect on reuptake time after AMPH application as compared with acute saline pre-treated slices, while this effect appear significantly in washout period where the time of reuptake was decrease in in the nicotine or AMPH pre-treated slices in the NAc shell only. In the core, pre-treatment with nicotine caused increase the reuptake time after AMPH application which continued throughout washout period, while pre-treatment with AMPH increase reuptake time in the washout period only.

Mesocorticolimbic DA system are critically involved in the development and expression of drug-induced behavioural sensitisation and the brain regions involved in the long term behavioural effect of drugs have been studies in detail. Although glutamate input from mPFC and amygdala are thought to be involved in the induction of behavioural sensitisation, while the long-term expression of psychostimulant-induced behavioural sensitisation is likely to particularly involve persistent changes in the DA and glutamate neurotransmission in the NAc (Pierce and Kalivas, 1997, Vanderschuren and Kalivas, 2000). It was believed that activation of the DA system is required for sensitisation to occur, thus, most investigators using different methods, such as microdialysis and FSCV have reported that behavioural sensitisation to psychostimulant such as AMPH (Siciliano *et al*., 2014) or nicotine (Shim *et al*., 2001) is accompanied by an increase DA response in DA projection areas, including NAc .

However, while these observation suggest that enhanced DA may underline sensitisation, other investigators have noted that behavioural sensitisation to both AMPH (Kuczenski *et al*., 1997) and nicotine (Balfour *et al*., 1998) can be obtained in the absence of an enhanced DA response in NAc. It was believed that sensitisation may be mediated by an enhanced DA response only under some circumstances such as pre-treatment dose of drugs, duration of withdrawal (Kalivas and Duffy, 1993) and animals strain (Iyaniwura *et al*., 2001), which can affect the DA response to the subsequent drugs challenge. If this is the case then the integrity of the mesolimbic pathway might be a critical determinant of sensitised function, since it relies on activation of VTA. Measuring in slices containing the whole pathway (i.e. experiment 1) would have allowed us to investigate this idea. Another explanation is suggested by data from, Kuczenski *et al*. (1997), who found that under different experimental conditions, behavioural sensitisation was observed in the absence of an enhanced DA response in NAc. In addition, Laplante *et al*. (2013) found that increased behavioural response to AMPH was accompanied by decreased levels of DA in the NAc in adult rats suggesting AMPH-induced behavioural effects are more likely due to postsynaptic mechanism such as DA receptor upregulation rather than increased presynaptic DA release.

Our initial experiments aimed at producing slices containing intact mesolimbic DAergic pathway in order to develop an experimental setup which will allow us to measure the effect of pharmacological manipulation of VTA on DA release in NAc. Firstly, we have tried stimulation of the pathway by applying electrical stimulation in VTA and record the DA release in NAc (Wightman *et al*., 2007). We found that even where local stimulation for NAc did evoke DA release, electrical stimulation of VTA slices did not, suggesting that the pathway is not intact. There could be a number of reasons why VTA stimulation did not evoke DA release in NAc? Precise location of the electrodes, the stimulus parameters used, the pathway was not intact. However, local stimulation in NAc did evoke DA release, so at least the recording electrode was in a DA-rich area. This does not discount the possibility that, due to topography of the projection, the cells being recorded from are not the cells begin stimulated. However, the stimulation parameters used were based on those previously described *in vivo* as able to evoke release throughout NAc after VTA stimulation. Injection of dextran dye in the NAc or VTA after recording DA release showed that the dye only diffused for a short distance. Therefore the most likely explanation for the failure of VTA stimulation to evoke DA release in NAc in these experiments is that the pathway was not intact. Although we used several different planes of slicing, and different slice thicknesses,

it is likely that, due to the rout taken by the pathway, we did not manage to cut slices which maintained an intact pathway.

For this reason an alternative approach adopted was to use our well validated approach of measuring the effect of drugs on repeated electrically stimulated accumbal DA release in coronal slices (Yavas and Young, 2017) containing NAc but not VTA. This allowed us to determine local changes in the terminal field, but the influence of VTA mechanisms were precluded. Thus, the present study aimed to measure DA release in slices from animals which had received multiple drugs injection either nicotine or AMPH. The work presented here provide the characterization and comparison of acute and repeated nicotine or AMPH effect on DA release in the NAc shell and core. To this end, stimulated DA release was measured and the effect of either nicotine or AMPH superfused acutely in the aCSF, was compared to the stimulated release in slices receiving no drug (No drug control). Further, the effect of pre-treatment of either nicotine or AMPH was assesses by comparison with slices from animals pre-treated with saline.

It was recently been reported that nicotine-induced increases in DA release in the NAc, can be considered as the first step leading to behavioural sensitisation (Shim *et al*., 2001), however, many lines of evidence suggest that the mechanism by which nicotine exerts its behavioural effect is not fully defined. Whereas several studies showed that repeated injection of nicotine sufficient to produce behavioural sensitisation resulted an increase of extracellular DA release in the NAc (Shim *et al*., 2001), others reported that no significant changes were observed after acute (Benwell and Balfour, 1992) or chronic nicotine administration (Morud *et al*., 2016). It is well established that nicotine produces its effect via binding to nAChRs and these receptors are involved in a number of physiological and behavioural process which are implicated in dependence and neurodegenerative diseases. Thus, activation of nAChRs on non-cholinergic nerve terminals leads to enhanced release of several neurotransmitters including DA, and local infusion of nicotine into the NAc of the sensitised rats produced a more pronounced increase of DA release then that seen in the non-sensitised rats (Shim *et al*., 2001). The authors (Shim *et al*) suggest that intermittent exposure to nicotine may produce the nAChRs upregulation in DA terminal regions which may explain the expression of behavioural sensitisation to nicotine. However, Baker *et al*. (2013b) have shown that activation of nAChRs on the DA cell bodies in the VTA but not on terminals in the NAc is essential for expression of the sensitised locomotor response and

long-term potentiation of excitatory inputs to these cells and increased nicotine-evoked DA overflow in the NAc.

In contrast to what has previously been reported nicotine (1 µM) in present study attenuated the stimulated DA release in the NAc, an effect which persisted throughout the nicotine application and into the washout period when the tissue was perfused again with drug-free aCSF. A plausible explanation for this nicotine effect might be that prior exposure of nAChRs to nicotine resulted in a long-lasting decrease in nicotine-stimulated neurotransmitters release from rat striatum which occurs due to increase nAChRs sensitivity to the desensitisation effect of nicotine (Rowell and Duggan, 1998). Another possible explanation is that repeated administration of nicotine increased DA release which intern enhanced presynaptic D2-like receptor responsiveness and caused a decrease in input/output function in the NAc (Morud *et al*., 2015), thus adding nicotine to the superfusate caused activation of D2-like receptors which in turn inhibited DA release (Escobar *et al*., 2015).

Nashmi *et al*. (2007) suggested that chronic nicotine administration causes upregulation of nAChRs on midbrain GABAergic neurons without changes in the nAChRs number or function at the presynaptic terminals of DAergic axons. Thus, in consequences, GABAergic neurons from chronically nicotine-treated mice had a higher basal firing rate and responded more strongly to nicotine (Nashmi *et al*., 2007). If this were the case, due to increased inhibition, DAergic neurons would have lower basal firing rate and a decreased response to nicotine in our study. Another alternative explanation for the reduction in stimulated DA release after *in vitro* application of nicotine is that nAChRs effects on the DA uptake. It was reported that nicotine activates nAChRs on DA terminals to evoke DA release which subsequently is taken back up into terminals via DAT. A study by Middleton *et al*. (2004) demonstrated that nAChRs can modulate DAT function in striatum. The authors (Middleton *et al*) found that local injection of nicotine in the NAc caused a decrease in extracellular DA through enhancing DA uptake by DAT and this ability was inhibited by pre-treatment with mecamylamine, a nonselective nAChRs antagonist. Thus, these results suggest that nicotine stimulation of nAChRs increases clearance of DA by DAT in the striatum. Alternatively, nicotine may indirectly modulate DAT function through activation of nAChRs at the level of cell body to modulate DAT function at the terminal, since a study by Sziráki *et al*. (2002) found that the effect of peripheral administration of nicotine to increase DA release in the NAc was inhibited by local administration of mecamylamine into VTA. Thus, it seems plausible that in the current study, pre-treatment with nicotine may be acting at nAChRs at

the level of cell body to modulate the DAT function at the terminals. However, since the slices used only contained the terminal regions in NAc, it was not possible to investigate this further in the present study.

Turning to the effect of AMPH, it is widely accepted that the effect of the drug on extracellular DA is mainly attributed to its binding to and reversal of DA transporter (DAT) function, resulting in both reuptake inhibition and release of DA by vesicular DA depletion *(*Siciliano *et al*., 2014). Velázquez-Sánchez *et al*. (2013) found that AMPH induced the behavioural effect through this effect on DAT. The authors (Velázquez-Sánchez *et al*) found that using DAT inhibitor, JHW 007, did not produce a sensitised behavioural response and prevented the AMPH-induced DA release when given as a pre-treatment. Many possible explanations of our result can be considered. The first explanation is that depletion of vesicular DA. AMPH-induced depletion of DA vesicles resulting in decreased DA has been shown repeatedly *in vitro* studies (Fleckenstein *et al*., 2007, Sulzer, 2011). It was demonstrated that DA uptake by DAT plays a role in replenishing intracellular DA stores, particularly during prolonged DA release. However, Benoit-Marand *et al*. (2000) found that the rate of DA synthesis was doubled in mice lacking the DAT, leading the authors (Benoit-Marand *et al* ) to conclude that DA release is more dependent on DA synthesis than reuptake by DAT. This leads us to suggested further experiment with tyrosine present in the superfusate to confirm this issue. Moreover, it has been reported that alteration of DA synthesis may contribute to the augmentation in DA transmission produced by repeated administration of psychostimulant.

A study by Brock *et al*. (1990) conducted more than 10 days after discontinuing repeated cocaine injection reported reduction in DA release in the NAc caused by reduction in tyrosine hydroxylase activity. *In vivo* voltammetric studies demonstrated that the clearance of electrically stimulated DA release in NAc which is achieved mainly by re-uptake was reduced after 10 days of withdrawal after cocaine treatment (Cass *et al*., 1993). The data presented here are consistent with this explanation, since the attenuation of stimulated release observed was not immediate. If the change were due to a direct pharmacological action on DA release it would be expected to be visible in S5. However, in practice the change did not occur until S6 (shell) or S7 (core), which would be consistent with the effect being due to depletion of vesicular (releasable) DA. In addition, the fact that the release showed no sign of recovery during this washout period is consistent with this idea.

Another possible explanation of our result is, through activation DA2-like autoreceptors. In normal animals DA release is controlled by local feedback. Pre-synaptic feedback inhibition of DA release likely plays a major role in regulating DA transmission (Ford, 2014). The high extracellular DA level stimulate DA2 autoreceptors and inhibit further DA release (Laplante *et al*., 2013). Moreover, Escobar *et al*. (2015) indicated that repeated activation of DA2 receptors with quinpirole (QNP) decreased DA release in the NAc of animals who displayed locomotor sensitisation. Thus, we proposed that repeated AMPH exposure leads to underlying neurobiological changes in the mesolimbic DA system that modulate DA response to subsequent AMPH exposure where rapid DA overflow is governed more strongly by uptake. The fact this potentiation was not observed with acute exposure suggested that these underlying neurobiological changes emerge only with longer AMPH exposure.

Recently, Ingram *et al*. (2002) has shown that DAT, in addition to uptake, also elicits ion channel-like current in response to compounds like AMPH. Thus, under low DA concertation, depolarization due to DAT activity facilitates DA release while higher DA extracellular concentration will activate the inhibitory effect of DA2-like autoreceptors that will suppressed further release by DAT. Thus, it is likely that DA release for the first 20 min electric stimulation causes activation of DA2-like receptors in the NAc which leads in turn to a reduction in further activation of DA by electrical stimulation in our study.

# Chapter 5: DA receptors gene expression

## 5.1 Introduction

Animal studies have revealed a host of cellular neuroadaptation elicited by repeated administration of drug of abuse that are likely to contribute to the neural adaptation seen in addictive behaviours (Vanderschuren and Kalivas, 2000, Hyman *et al*., 2006). The critical involvement of DA transmission in drug dependence and reward has been recognised for many years (Koob and Nestler, 1997). Although the role of DA has been debated, DA is thought to be a key factor in both the development and the expression of behavioural sensitisation (Robinson and Berridge, 1993, Pierce and Kalivas, 1997, Steketee and Kalivas, 2011). The DA projection that is often linked to behavioural sensitisation is the mesolimbic projection from VTA to NAc: however, the DA projection from VTA to PFC and amygdala have also been implicated (Pierce and Kalivas, 1997).

The molecular site of action for a drug of abuse determines the starting neural adaptation that elicited as behavioural sensitisation. Although different sites of action indicate distinctions in neural adaptation between drugs, it is generally believed that certain neural changes such as activation of DA receptors appear to be held in common (Vanderschuren and Kalivas, 2000). Medium spiny neurons (MSNs) in the NAc are critically involved in drug-induced synaptic modification that are considered to be an important basis for drug-associated behaviour (Lobo and Nestler, 2011). MSNs are divided into two major population of neurons which may segregate with the projection target: the direct pathway MSNs which express DA1 receptors and project directly to midbrain DA area and VP, and the indirect pathway MSN which express DA2 receptors and project to the VP (Kreitzer and Malenka, 2008, Kupchik *et al*., 2015). Because 90% of striatal neurons are GABAergic, thus, activation of MSNs neurons will inhibit their downstream target which is GABAergic also, thus, activation of DA1-MSN will inhibit midbrain DA neurons by exciting inhibitory effect of GABA neurons in VTA which then regulate reward-related behaviour (Bocklisch *et al*., 2013) (see figure 2 section 1.2.1).

Interestingly, stimulation of DA receptors is necessary for both induction and expression of behavioural sensitisation (Vezina, 1996). Perhaps the most robust release of DA by psychostimulant engages DA-dependent neuroadaptations that are more critical to the development of sensitisation. The long-lasting hyper-responsiveness of mesencephalic DAergic pathways, including the mesolimbic and nigrostriatal pathway, is the most

prominent and consistent, thus, pre-exposure to psychostimulant has been shown to induce long-term hypersensitivity of these pathways (Kalivas and Stewart, 1991). Given that behavioural sensitisation includes augmented locomotor activity, hyper-responsiveness of accumbens and striatal DAergic nerve terminals is likely to contribute to the expression of psychostimulant sensitization. Many studies have suggested a direct involvement of DAergic neurotransmission in the expression of psychostimulant sensitisation. These studies showed that the ability of psychostimulant drugs to increase extracellular DA level in the NAc are augmented during long-term sensitisation. For example, there is a critical involvement of DA receptors activation in both AMPH sensitisation (Shi and McGinty, 2011) and in nicotine sensitisation (Di Chiara, 2000). The blockade of DA1-like receptors in VTA during repeated AMPH administration has been shown to prevent the development of behavioural sensitisation to AMPH, presumably by interfering with the effect of presynaptic DA1 receptors on glutamate and GABA terminals neurons (Vezina, 1996), indicating a permissive role of DA1 receptor involvement in sensitisation . In contrast, there was no such role for DA2 receptors in the development of drug-induced addictive behaviour. Indeed they may inhibit the development of sensitisation, since Vezina (1996) found that DA1 but not DA2 receptors blockade prevented the development of locomotor-activating effect of AMPH administration. Moreover, Kang *et al*. (2017) stated that activation of DA2 receptors does not affect the induction of cocaine-induced behavioural sensitisation. Thus, it seems to be that DA2 receptors activation is not necessary for the induction of locomotor sensitisation to psychostimulants.

However, the expression of psychostimulant sensitisation can occur through DA-independent mechanism, since DA1 receptors knoc-kout mice do exhibit behavioural sensitisation to AMPH albeit to lesser degree then wild-type mice did (Crawford *et al*., 1997). Although the role of DA2-like receptors in the induction of behavioural sensitisation is not clear, accumbens DA2-like receptor density and function seem to be involved in expression of psychostimulant sensitisation. Vanderschuren *et al*. (1999b) indicated that animals pre-exposed to AMPH became hypersensitive to the locomotor stimulant effect of the DA2 agonist (quinpirole), while Shi and McGinty (2011) showed that the DA2 receptor antagonist (eticlopride) decreased behavioural activity induced by AMPH challenge in AMPH sensitised rats. Further, Kang *et al*. (2017) found that photo-inhibition of DA2 receptors on MSN in NAc using optogenetic manipulation during withdrawal period did not affect the expression of cocaine-induced behavioural sensitisation. Thus, it seemed to be that

there are distinct mechanism mediating the augmentation of behavioural-dependent and independent DA receptors produced by repeated psychostimulant administration.

Polymerase Chain Reaction (PCR) is an enzymatic assay which produces an amplification of specific DNA fragments from a complex pool of DNA, enabling sufficient nucleic acid product to be produced to enable quantitative measurement (Taylor et al., 2010, Postollec *et al*., 2011, Garibyan and Avashia, 2013). Dr Kary Mullis, who discovered the PCR assay, in the mid of 1980s, stated that "lets you pick the piece of DNA you're interested in and have as much of it as you want" (Mullis, 1990). PCR can be performed using DNA from a variety of tissues including blood, skin, hair, saliva and important for this study, brain, and only a small amount of DNA is needed to generate millions of copies in a few hours. There are many advantage of PCR but the most important is that it is relatively simple technique to understand and use and it is highly sensitive with the potential to produce millions copies of a specific products for sequencing, cloning and analysis, in a short time. In addition, PCR can be used to analyse alterations of gene expression levels in disease conditions such as tumours, and microbes and in animal models of diseases. Although PCR is a valuable technique, it does also have a number of limitations in its use. Because the sensitivity of PCR relies on developing multiple copies of the gene in question any form of contamination of the samples by even trace amount of DNA can produce misleading results. In addition in order to design primers for PCR, some prior sequences data is needed, thus PCR can only be used to identify the presence or absence of an important gene (Taylor *et al*., 2010, Postollec *et al*., 2011, Garibyan and Avashia, 2013).

PCR-based methods, are used by scientific community in a variety of applications including gene expression, pathogen detection, genetic testing and disease research. Each PCR assay requires the presence of template DNA, a primer, nucleotides and DNA polymerase. The DNA polymerase is the enzyme that links individual nucleotides together to form the PCR product. The nucleotides include the adenine, thymine, cytosine and guanine (A, T, C & G) that are found in the DNA and act as the building blocks that are used by the DNA polymerase to create the resultant product, while the primers are short DNA fragment with a defined sequences complementary to the target DNA that is to be detected and amplified. The above PCR component (template DNA, primer, nucleotides and DNA polymerase) are mixed and then placed in a thermal cycler which allows repeated cycles of DNA amplification to occur in three steps, achieved through regulation of the temperature during the different program steps. Firstly, for denaturation, the reaction solution is heated

above the melting point of the two complementary DNA strands of the tract DNA causing the strands to separate. Secondly, for hybridisation (or annealing) the temperature is lowered to allow the specific primers to bind to the target DNA segments. Hybridisation between the primers and the target DNA occurs only if they are complementary in sequences (e.g A binding to G). Finally the temperature is raised again at which time the DNA polymerase is able to extend the primers by adding nucleotide to the developing DNA strand. Within each repetition of these three steps the number of copied DNA molecules doubles.

There are two methods of visualizing the PCR products: the first one is staining of the amplified DNA product with a chemical dye such as ethidium bromide which interacts with the two strands of the mixture. The second one is labelling the PCR primers or nucleotides with fluorescent dyes prior to PCR amplification. The most widely used method for analysing the PCR products is the use of agarose gel electrophoresis which separates DNA product on the basis of size and charge. This is the easiest way to analyse the product once stained/ labelled. It allows for determination of the presence and size of PCR product PCR can be used either qualitatively or quantitatively. Qualitative PCR is used to detect the presence or absence of specific DNA product and is a good technique when PCR is performed for cloning purposes or to identify a pathogen or gene. On the other hand, quantitative PCR (qPCR) provides information beyond mere detection of DNA and indicates how much DNA for a specific gene is present in the sample. Quantitative PCR allows for both detection and quantification of the PCR product in real time while it is synthesized. The real time PCR can be combined with reverse transcription which converts messenger RNA (mRNA) to cDNA (i.e. reverse transcription) after which quantification of the cDNA is performed using real-time quantitative PCR (qPCR). The qPCR has become a definitive technique for quantifying differences in gene expression level between samples see figure 31 for qPCR steps.

In summary, qPCR consists of a succession of amplification cycles in which the template nucleic acid is denatured, annealed with specific oligonucleotide primers, and extended to generate a complementary strand using a thermos-stable DNA polymerase. This results in an exponential increase of amplification products that can be monitored at every cycle in real time using fluorescent reporter. The increase in fluorescence is plotted against the cycle number to create the amplification curve, from which a quantification cycle Cq (often describe as Ct for cycle threshold) value can be determined. The Cq corresponding to the number of cycles for which the amount of fluorescence is significantly higher than the

background fluorescence. Therefore, the Cq value can be linked to the initial concentration of the target nucleic acid and serve as a basis for absolute or relative template quantification (Postollec *et al*., 2011).

Absolute quantification is based on comparison of Cq values with a standard curve generated from amplification of a known amount of the target gene and this method require similar amplification efficiency (presence of inhibitors, nucleotide variability) for all samples and standards. Relative quantification is used to estimate changes in gene expression based on the use of an external standard or reference sample that must be include in every run. The qPCR can be performed in one-step within a single tube or in two steps with reverse transcription performed independently of qPCR. The one-step protocol minimizes the risk of DNA contamination and the risk of experimental variation, but the risk of RNA degradation is increased. Therefore the two-step protocol is considered to be better (Wong and Medrano, 2005) and the study reported here used the two-step protocol.

In the present study, we aimed to determine the regulation of the DA receptors by chronic pre-treatment with nicotine or AMPH, considering that this may be one of the neurobiological substrates that mediates sensitisation, important in addiction. We applied repeated intermittent injections of nicotine, AMPH or saline daily for 5 days, according to regimen that elicited long-term behavioural sensitisation (see section 2.2.3). We planned our study in two stages: first, we investigated the effect of nicotine or AMPH exposure on induction and expression of behavioural sensitisation. Based on our findings, in the second stage, we studied the possible effects of intermittent drug treatment on DA receptors gene expression in rats NAc and VTA using qPCR.

## 5.2 Materials and Methods

### 5.2.1 Behavioural studies

The sensitisation procedure was the same previously described (see section 2.2.3). Briefly, three days before starting the experiments, male Lister hooded rats (n = 127) (285-300 g, at the beginning of experiments) were handled ( 20 min per day) and habituated to the activity boxes (24 cm in width, 46 cm in length and 22 cm in height) (30 min per day) to familiarize them with the experimental procedure. The day after handling and habituation, the animals were injected once a day with nicotine (0.6 mg/kg, i.p), D-AMPH (1 mg/kg,

i.p) or saline (1 ml/kg, i.p,) for five consecutive days and locomotor activity was recorded immediately after each injection for 60 min. Following ten days of withdrawal after the last injection, the animals were challenged with either nicotine or AMPH and their locomotor activity was recorder.

Two hour after the challenge injection, 56 animals from behavioural experiments were deeply anaesthetized with isoflurane and humanely killed by cervical dislocation (schedule 1, Animals Scientific Procedures Act, 1986) and used for qPCR experiments. The brains were removed and dissected according to visual anatomical landmarks and the atlas of (Paxinos and Watson, 1998). Slices were taken using a brain matrix, then the regions of interest dissected out. NAc and VTA were dissected immediately using a surgical blade and then snap-frozen in liquid nitrogen and transferred to -80 °C for storage until RNA extraction and qPCR analysis. All procedure involving animals were carried out under appropriate personal and project license authority, and with approved on the University of Leicester Animal Welfare and Ethics Review Body (AWERB).

### 5.2.2. qPCR studies

To minimize handling time during the RNA extraction procedure, it is recommended that samples should be processed in relative small batches of 10-20 (Seear *et al*., 2014). The first step of qPCR experiments is the RNA extraction procedure. RNA was isolated and precipitated from the whole brain using an RNAase Plus Mini Kit (Qiagen GmbH, Hilden, Germany) following manufacturer's instruction (see figure 34 for qPCR steps). Briefly when working with RNA, care must be taken to avoid degradation by RNases, which are extremely stable and active. Intracellular RNases are released during the lysis step of the RNA isolation procedure and must be rapidly and thoroughly inactivated to obtain high-quality RNA. Beta-mercaptoethanol (ß-ME) is a reducing agent that will irreversibly denature RNases by reducing disulfide bonds and destroying the native conformation required for enzyme functionality. At this stage the RNA can be used for cDNA synthesis immediately or stored at -20 °C until use. RNA was treated with DNAase 1 (Invitrogen), by adding 2.5 µl Turbo DNase into each 50 µl RNA sample, to degrade any genomic present in the sample.

For the second step it was vital to ensure that only high purity RNA (no contaminants) and high integrity (not degraded) sample was used: it is one of the most critical point in the qPCR experimental workflow. The quality and quantity of RNA was assessed using a Nanodrop 2000 (Thermo Scientific). The purity of the sample with respect to protein contamination

was assessed spectrophotometrically by measuring the $OD_{260/280}$ ratio using NanoDrop spectrophotometer (Lab-Tech International, Lwes, UK). An $OD_{260/280}$ of 1.8-2.0 indicates good quality RNA that is devoid of protein contamination. RNA integrity can be assessed using several methods but the methods chosen for these studies was visual inspection after electrophoresis on a agarose gel in the presence of the fluorescent dye, ethidium bromide. 1 µg of total RNA was electrophoresed on a non-denaturing 1.5% (w/v) agarose gel to check for RNA degradation. Observation of two sharp bands for the large and the small band subunits of ribosomal RNAs (rRNA) with the intensity of the lager band about twice that of the smaller band was indicative of intact RNA. We quantifying the intensity of the rRNA bands using imager with densitometry scanning. While the value of the 28s/18s ration can vary between different samples, a ratio between 1 and 2 is indicative of an intact RNA sample (Seear *et al*., 2014) see figure 36 A).

Third step was cDNA synthesis. When a batch of RNA samples had successfully met the standard quality control criteria, it was immediately converted into the much more stable cDNA form by reverse transcription (Taylor *et al*., 2010). This avoided the risk of RNA sample degradation from multiple freeze/thaws before conversation to cDNA. cDNA was synthesised from 0.5 µg of total RNA using a Quantitect Reverse Transcription Kit (Qiagen GmbH). RedTag ReadyMix (Sigma-Aldrich, Gillingham, UK) was used to amplify partial sequences of DA receptor cDNA with five set of primers and designed against a region conserved between all members of the DA receptors family (see table 3 PCR primers).

| Primer name | Sequence |
|---|---|
| RAT Rp18_F | AAGCCTTCCACTATCCGAAGAGAT |
| RAT Rp18_R | GTACAGTTTTGGTTCCACGTAGCC |
| RAT Drd1_F | TAGCTAAGCCACTGGAGAAGCTGT |
| RAT Drd1_R | TGTGTGTGACAGGTTGGATCTTTT |
| RAT Drd2_F | CAGAAGGAGAAGAAAGCCACACAG |
| RAT Drd2_R | ATATTCAGGATGTGCGTGATGAAG |
| RAT Drd3_F | ACGTGGCTAGGCTATGTGTGAACAGT |
| RAT Drd3_R | ACAGGATCTTGAGGAAGGCTTTG |
| RAT Drd4_F | CTATGTCAACAGTGCCCTCAACC |
| RAT Drd4_R | CTTGCGGAAGACACTTCGAAACT |
| RAT Drd4_F2 | AAAGAGAGGCGCCAAGATCACT |
| RAT Drd4_R2 | GAAGAAAGGCGTCCAACACATC |
| RAT Drd5_F | CACATGTCTCAAATCTCTCCAACG |
| RATDrd5_R | GAAACCTCTTCCTCACAGTCAAGC |

Table 3: PCR primers used in the qPCR experiments.

The final step was the qPCR procedure. The qPCR reaction mixture consisted of 10 µl SYBR Green Jump-Start Taq ReadMix (Sigma-Aldrich), 250 nM of forward and reverse primer, 1 µl diluted cDNA and sterile water in a total volume of 20 µl. The qPCRs were performed in duplicated on a Chromo 4 qPCR thermocycler (BioRad Laboratories, Hercules, CA) with the cycling conditions, as shown in figure 35. The melting curve step (50-95°C) was then performed to ensure that only a single product had been amplified in each reaction. Result were normalised to the expression level of the reference gene L8. Standard curves were performed for each primer pair on the same plate as the experimental samples with a dilution series of cDNA (see Figure 36 B for amplification and melt curve).

For each gene, cDNA from each of the treated animals and control animals provided material for qPCR analyses to be run in duplicate. “No template” and “no reverse transcriptase” controls were also performed for each primer pair and cDNA, respectively. The reference genes were used as controls to normalize the data by correcting for differences in quantities of cDNA used as template. Thus, a perfect reference gene is one that does not exhibit changes in expression between samples from various experimental conditions or time points (Taylor *et al*., 2010). The gene for ribosomal protein L8 was considered to be the most stable reference gene *(*Seear *et al*., 2014) by geNorm software and was used to normalize the data.

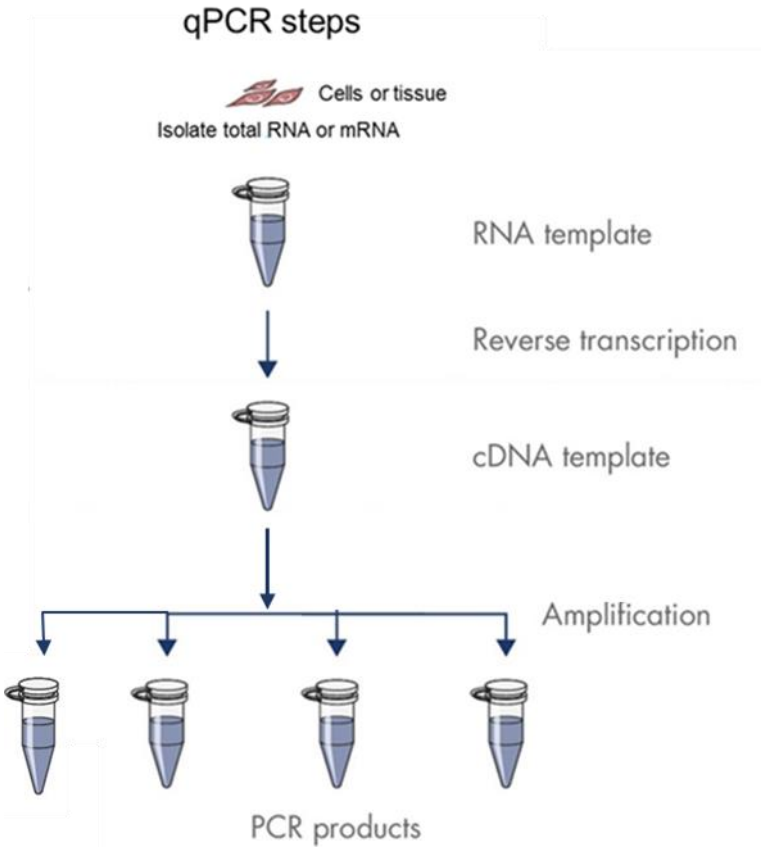



Figure 34: qPCR steps: after collecting the samples RNA was extracted from the whole brain tissue. RNA samples has successfully met the standards quality control, it was immediately converted into the much more stable cDNA by reverse transcription. Then qPCR was performed on 8 brains per genotype with 4 replicates for each brain.

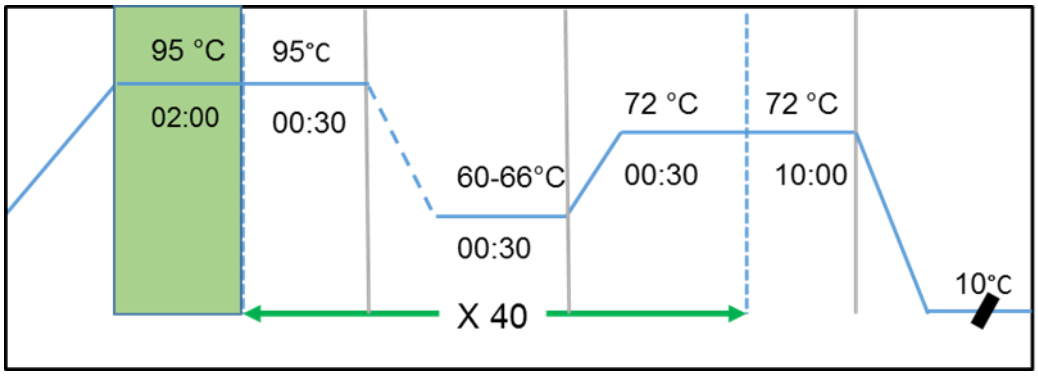



Figure 35: The temperature changes used in DNA amplification in a thermal cycler. For denaturation, the solution is heated to 95 °C for 3 min, followed by 95°C for 30 sec. For hybridisation the temperature was lowered to 60-66 °C for 30 sec and these processes were repeated for 40 cycles, and finally the temperature was raised again to 72 °C for 30 sec, with a final extension of 72 °C for 10 min.

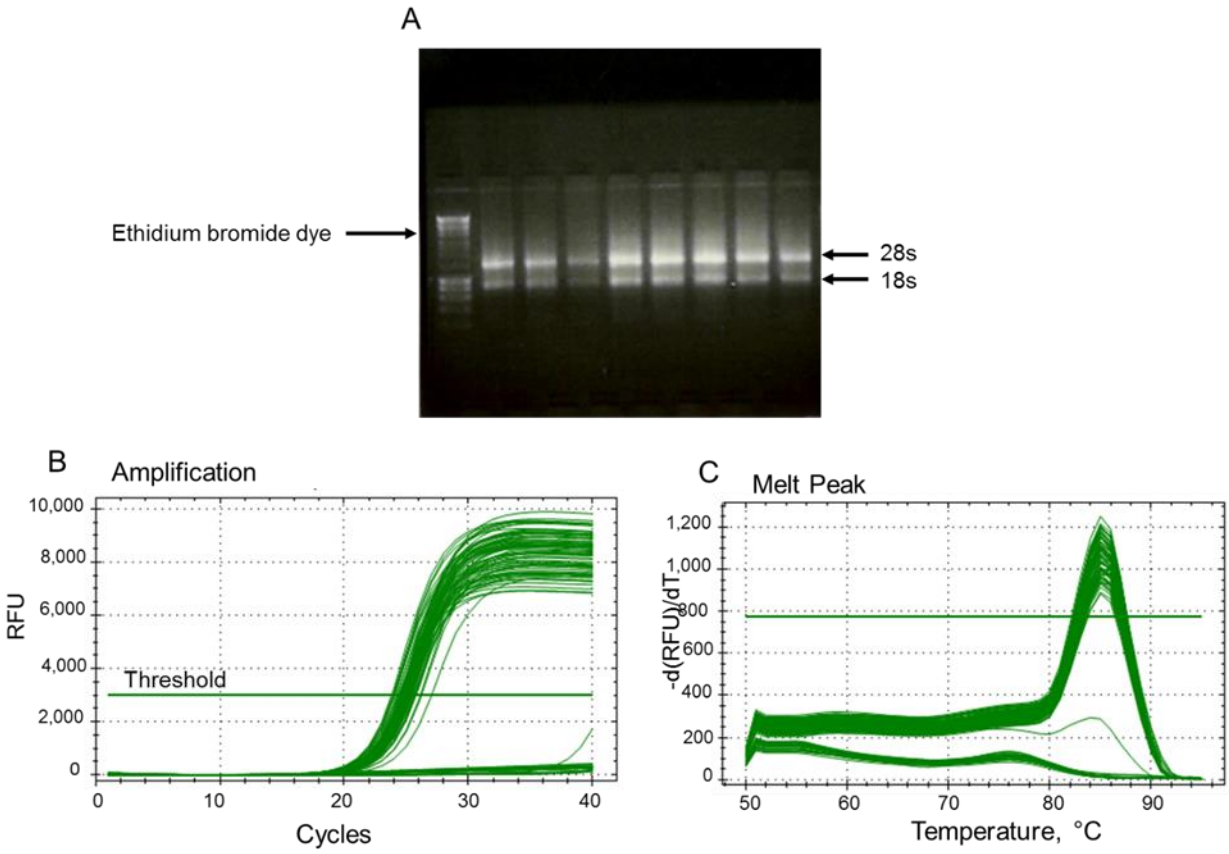



Figure 36: Highly specific real-time qPCR images. A) Ethidium bromide-stained agarose gel showing PCR products from NAc. B) Amplification profile. C) Melting curve analysis showed a single peak, indicating high specificity.

### 5.2.3 Data Analysis

All behavioural data were analysed by using appropriate analysis of variance (ANOVA) using StatView software 5.0 (SAS Institute, NC, USA) and planned comparison, as described in section 2.2.4. Briefly, the locomotor activity data were analysed separately for pre-treatment sessions and challenge session. The influence of pre-treatment of drugs on animals' activity during the induction phase (pre-treatment session) was analysed by using three-way ANOVA (5 × 3 × 6), using within-subjects factors of days [5 levels (day 1, day 2, day 3, day 4 and day 5)] and time bins [6 levels (10 min, 20 min, 30 min, 40 min, 50 min, 60 min)] and with a between- subject factor of drugs treatments [3 levels (nicotine, AMPH and saline)]. The expression phase of sensitization following challenge injection was analysed by using two- way ANOVA, using within-subject factor of time bins and with a between-subject factor of drug treatments (see section 2.2.4).

For the analysis of the DA receptors gene expression data, we calculated the qPCR data using the following equations:

$$\textbf{Calibrator = Mean } (C_T \textbf{ target}) - \textbf{Mean } (C_T \textbf{ reference})$$

$C_T$ = threshold cycle: the cycle at which the amplification plot crosses the threshold. The threshold is adjusted to a value above background and significantly below the plateau of an amplification plot.

For each sample:

- First, normalize the CT of the target gene to that of the reference gene:

$$\Delta C_T = C_T \text{ target} - C_T \text{ reference}$$

- Second, normalize the $\Delta C_T$ of the test sample to the $\Delta C_T$ of the calibrator:

$$\Delta\Delta C_T = \Delta C_T - \text{Calibrator}$$

- Finally, calculate the expression ratio:

$$2^{-\Delta\Delta CT} = \text{Normalized target gene expression level in sample.}$$

This method assumes that both target and reference genes are amplified with efficiencies near 100% and within 5% of each other.

$\Delta C_T$ value: The value describes the difference between the $C_T$ value of the target gene and the $C_T$ value of the reference genes.

$\Delta\Delta C_{T\ value:}$ The value describes the differences between the average $\Delta C_T$ value of the sample of interest and the average of $\Delta C_T$ value of a references sample which is known as the calibrator.

We then used ANOVA on the $2^{-\Delta\Delta CT}$ value to determine the effects of the sub-chronic and acute treatment with drugs on the expression of five DA receptors genes.

## 5.3 Result

### 5.3.1 Behavioural results

First, we investigated the effect of nicotine or AMPH on the development of behavioural sensitisation. Since the animals used to supply tissue for qPCR were a subset of those undergoing behavioural testing described in chapter 2, the result from the behavioural testing are shown in that chapter (section 2.3.1), and are not reproduced here. Briefly, rats treated (one injection for five consecutive days) with nicotine (0.6 mg/kg, i.p) or AMPH (1 mg/kg, i.p) exhibiting increased locomotor activity compared with those pre-treated with saline (1ml/kg, i.p), indicating that behavioural sensitisation to both nicotine and AMPH had developed. Second, after a 10 days withdrawal period, animals who developed behavioural sensitisation expressed behavioural sensitisation to nicotine or AMPH in response to challenge administration with nicotine or AMPH respectively, as compared with animals treated with saline. The animals which were pre-treated with AMPH had shown behavioural response for nicotine challenge as compared with acute nicotine, indicating a cross-sensitisation, however, there was no evidence of behavioural cross-sensitisation in nicotine pre-treated animals to AMPH challenge.

### 5.3.2 qPCR results

#### 5.3.2.1 Nicotine sensitisation and DA receptor gene expression

To determine the role of DA receptors in the initiation and expression of nicotine-induced behavioural sensitisation, we analysed the effect of sub-chronic treatment with nicotine or

AMPH on DA receptors gene expression of rats with experimental schedule (Figure 7) as compared with saline treated animals in response to nicotine challenge. In the NAc, two-way ANOVA revealed a significant main effect of drug treatment [$F(3, 27) = 3.83$; $p < .05$], DA receptors gene expression [$F(4,108) = 8.83$; $p < .0001$] and a significant interaction between drug treatment and DA receptor gene expression [$F(12,108) = 3.87$, $p < .0001$]. Planned comparison revealed that acute administration of nicotine did not affect DA receptor gene expression in the NAc as compared with control saline animals (without drug). Pre-treatment with nicotine or AMPH did not affect DA receptor gene expression at challenge nicotine administration as compared with saline pre-treated animals in response to acute nicotine administration (planned comparison, p = n.s) see figure 37 A-C.

In VTA, two-way ANOVA (drug treatment x DA receptors gene expression) revealed a significant main effect of drug treatment [$F(3, 27) = 3.17$; $p < .05$], DA receptors gene expression [$F(4,108) = 26.88$; $p < .0001$] and a significant interaction between treatment and DA receptors gene expression the VTA [$F(12,108) = 14.22$, $p < .0001$]. Planned comparison revealed that acute administration of nicotine did not affect DA receptor gene expression the VTA as compared with control animals (without drugs). Pre-treatment with nicotine had a significant effect on DA (DA1, DA3, DA4 and DA5) receptors gene expression as compared with acute nicotine administration ($p < 0.05$). See figure 37 D-F.

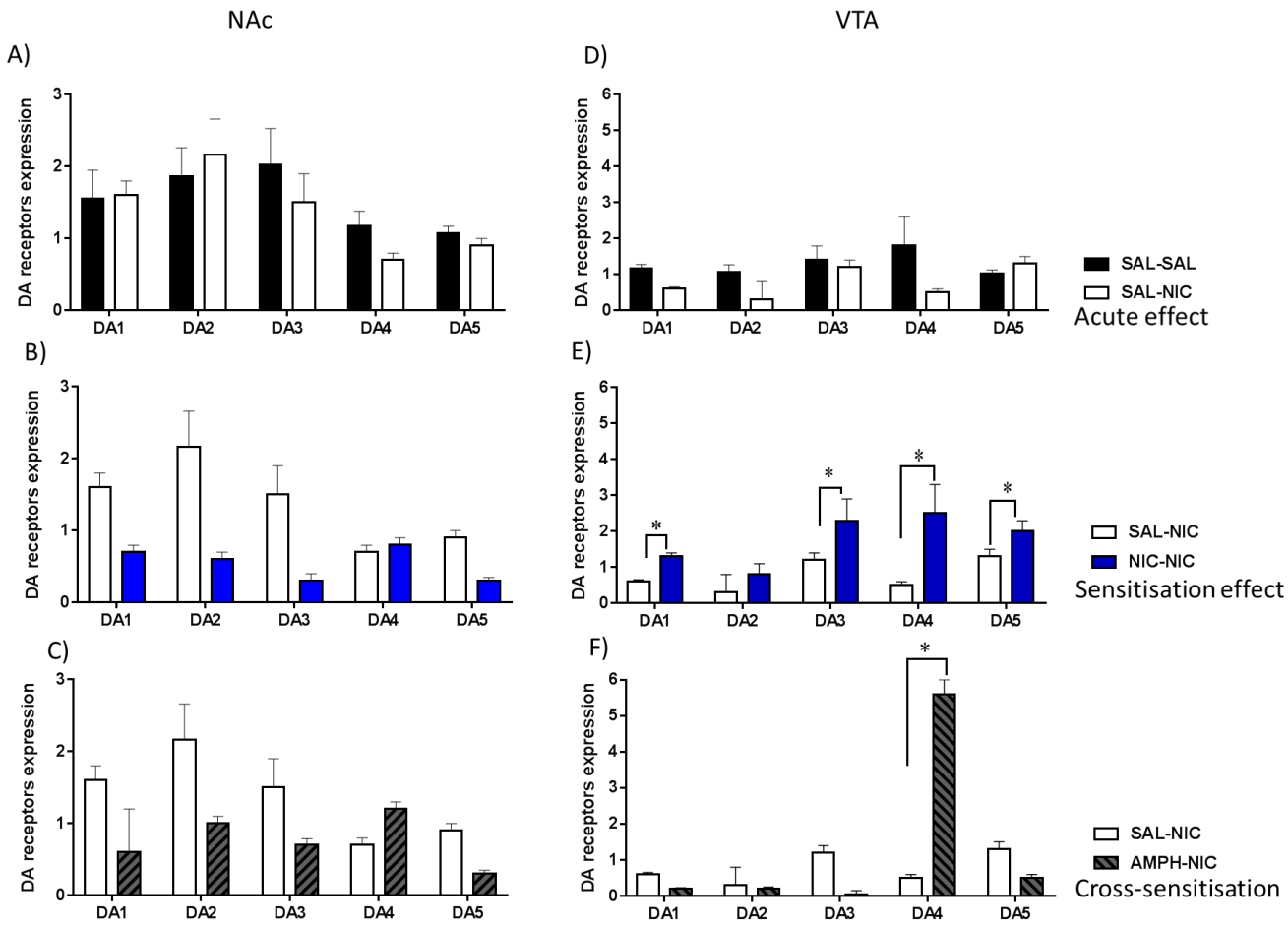



Figure 37: DA receptor gene expression in the nicotine treated animals. Data analysis revealed there was no effect of pre-treatment with nicotine or AMPH on response at nicotine challenge in the NAc (A-C). While pre-treatment with nicotine had a significant effect on DA receptors gene expression in the VTA (D-F) as compared with saline treated animals. Data are mean SEM. * $p < .05$, planed comparison, based on significant interaction from ANOVA; n = 6-8 per group.

### 5.3.2.2 AMPH sensitisation and DA receptor gene expression.

To determine the role of DA receptors on the initiation and expression of behavioural sensitisation to AMPH, we investigate the effect of five days treatment with nicotine or AMPH on DA receptors gene expression in response to AMPH challenge after 10 days withdrawal period using the experimental schedule (Figure 7). In NAc, two-way ANOVA (drug treatment x DA receptors gene expression) revealed a significant main effect of drug treatment [$F (3, 25) = 4.52$; $p <.05$], DA receptors gene expression [$F (4,100) = 19.45$; $p <.0001$] and a significant interaction between treatment and DA receptors gene expression

[F (12,100) = 4.03, p <.0001]. As compared with control animals (without drugs), acute administration of AMPH caused an increase in expression of DA1 receptors gene only (p <.05), while there was no effect on another DA receptors gene. In AMPH pre-treated animals, there was a significant increase in the DA3 and DA4 receptor gene expression level above the naïve control level at a challenge AMPH administration (p <.01), while pre-treatment with nicotine had no effect on DA receptor gene expression as compared with saline pre-treated rats in response to AMPH challenge, see figure 38 A-C.

In the VTA, although two-way ANOVA revealed a significant main effect of drug treatment [F (3,25) = 14.20; p <.0001], DA receptors gene expression [F (4, 100) = 30,49; p <.0001] and a significant interaction between treatment and DA receptors gene expression [F (12,100) = 10.43, p <.0001)], planned comparison revealed no significant effect of acute AMPH administration as compared with control saline animals (no drugs) (p = n.s). Pre-treatment with AMPH only increase expression of DA4 receptors gene expression as compared with acute AMPH administration (p <.01). While pre-treatment with nicotine caused increase DA (DA2, DA3, DA4 and DA5) receptors gene expression as compared with saline pre-treated rats on response to acute AMPH administration (, p < .01) see figure 38 D-F.

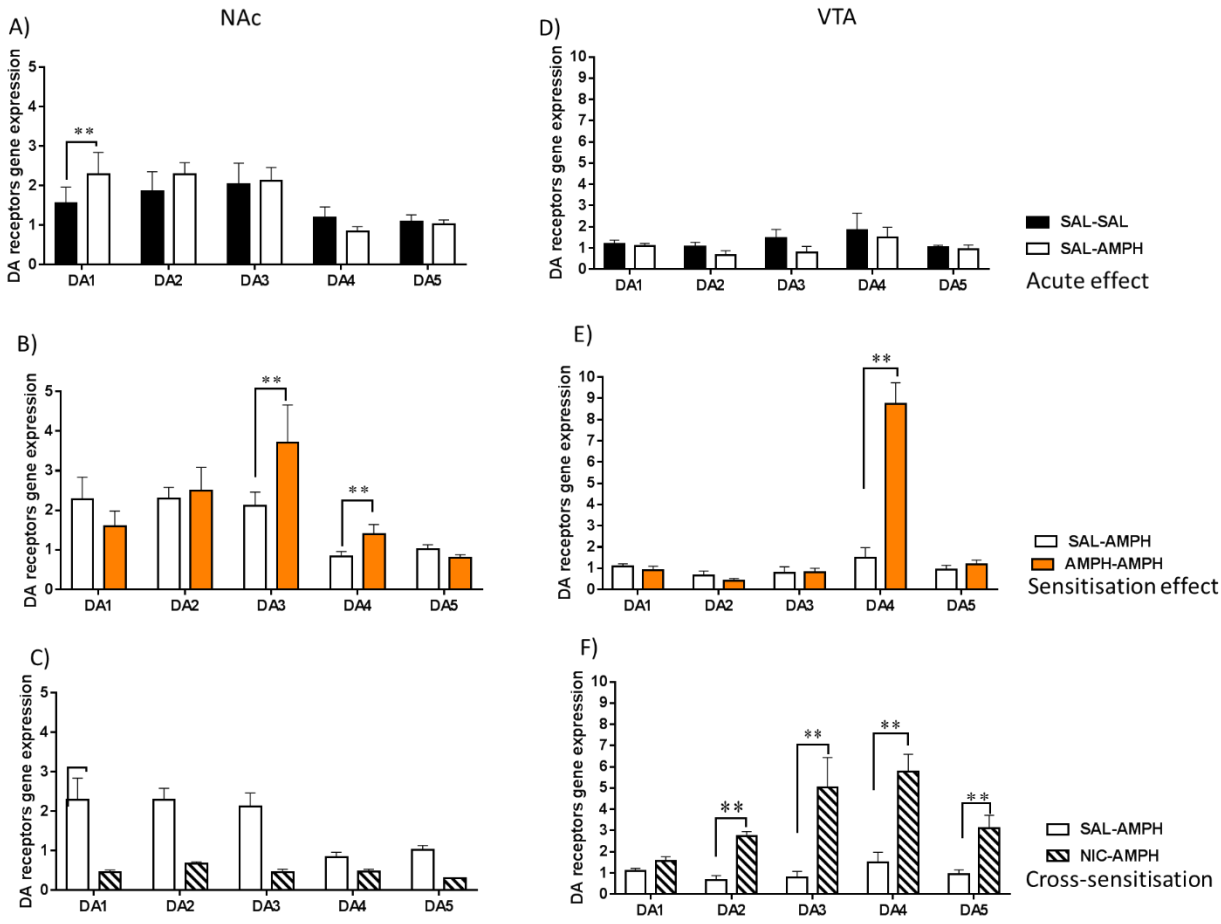



Figure 38: DA receptor gene expression in the AMPH treated animals. In the NAc, acute administration of AMPH increase DA1 gene expression as compared with control saline animals, while pre-treatment with AMPH increase DA3 and DA4 receptors gene expression as compared with acute AMPH administration ($p < .01$) A-C. In the VTA, repeated administration of AMPH increase DA4 gene expression only as compared with acute AMPH administration ($p < .01$) D-F. While treatment with nicotine cause increase DA receptors gene (DA2, DA3, DA4 and DA5) expression as compared with saline treated animals in response to acute AMPH administration ($P < 01$). Data are mean SEM. * $p < .05$ or ** $p < .01$, planned comparison, based on significant interaction from ANOVA; n = 6-8 per group.

## 5.4 Discussion

Our result demonstrate that pre-treatment with nicotine had no effect on dopamine receptors gene expression in the NAc as compared with saline pre-treated rats in response to acute nicotine injection, while it had a significant effect in VTA. In term of cross-sensitisation our data shown that there was no effect of treatment history with nicotine or AMPH on DA receptors gene expression in response to acute nicotine challenge. In AMPH sensitisation experiments, we found that pre-treatment with AMPH cause increase DA receptor gene expression in the NAc as compared with saline pre-treated rats in response to AMPH challenge, while it had no effect in VTA. In VTA, nicotine pre-treated animals had shown increase in DA receptors gene expression as compared with animals that received saline in response to AMPH challenge in term of cross-sensitisation.

Considerable evidence indicates that psychostimulants induced behavioural sensitisation is associated with enhanced DAergic transmission in the mesolimbic system comprising the VTA and NAc. In particular, the expression phase of behavioural sensitisation is characterised by a persistent drug hyper-responsiveness to drug challenge even after a long period of abstinence, which is associated with cascade of adaptation mechanisms that could contribute to compulsive drug craving (Robinson and Berridge, 1993, Steketee and Kalivas, 2011). Introduction of specific molecular and cellular events, signalling or synaptic modification mediated by chronic drug exposure may alter DA receptors expression which can be an important parameter for the final translation into addictive behaviours.

In present study, we investigated the effect of sub-chronic treatment with nicotine on DA receptor gene expression in the NAc and VTA. Our results argue that the nicotine sensitised rats did not show any changes in expression of DA receptors gene as compared with acute nicotine administration in the NAc. Together with a recent study in NAc (Le Foll *et al*., 2003), our result reveals that no significant changes were noticed in the DA1 and DA2 receptors gene expression in NAc following sub-chronic nicotine administration. For DA3 receptors, Smith *et al*. (2015) demonstrated that DA3 receptors have no effect on the expression of behavioural sensitisation to nicotine, which is agreement with our result.

Contrary to this, Le Foll *et al*. (2003) found an increase in DA3 expression in the NAc after nicotine behavioural sensitisation. Critical differences in the experimental conditions, including the duration of withdrawal period and the number of the nicotine exposure, may have accounted for this differences in the result of these studies. In general, DA-related

neurochemical changes in NAc, such as increased DA receptors responsiveness and up regulation of cAMP signal transduction that accompany sensitisation, are persistent and required a withdrawal time interval to be manifest (Richtand *et al*., 2001). Thus, the short withdrawal period used by Le Foll and his colleagues, compared with long withdrawal period that used by Smith *et al* and our study, may have caused a transient increase in the autoreceptors (Wolf *et al*., 1993) which would explain the difference between the data sets. An additional possibility is that surface exposure and /or binding affinity of these receptors for DA has also been changed over the nicotine exposure, therefore in spite of a potential increase in DA3 receptors gene expression it is possible that the receptors have decreased functionality or that fewer receptors are expressed on the cell surface (Smith *et al*., 2015) which may explain the decrease in the DA3 receptors gene expression in the NAc that was found in our study.

Considerable evidence indicates that nicotine, like other drugs of abuse, produces its effect on behaviour by activating the mesolimbic dopamine system projecting from VTA to the NAc. Our result showed increase DA receptors gene expression in the VTA more than their expression in the NAc, which is conducted with other result. Govind *et al*. (2009) stated that the action of nicotine in the VTA is more important for behavioural sensitisation then its role in the NAc, and they demonstrated that in experimental animals, nicotine increased the firing of midbrain DA neurons and increased dopamine overflow in the NAc and that these actions are likely to have mediated nicotine's locomotor activating effects. Nicotine receptors in the VTA are particularly important, as selectively blocking these receptors, prevents nicotine-induced NAc dopamine release, as well as locomotion (Vezina *et al*., 2007) . In addition, sub chronic treatment with nicotine upregulated nAChR and nAChR currents and produced LTP of excitatory input to midbrain DA neurons (Vezina *et al*., 2007).

It is well know that the gene expression in NAc related to postsynaptic receptors on (mostly) medium spiny GABA neurons in NAc. Thus, changes in presynaptic receptors in NAc would not show up as a change in gene expression in the NAc, but rather as changes in VTA, since this is where the genes for receptors in mesolimbic cells are located. So changes in gene expression in VTA indicate changes in postsynaptic receptors distribution in the VTA and presynaptic receptor distribution in NAc. This may be explain why pre-treatment with nicotine had an effect on DA receptor gene expression in the VTA rather than in the NAc. However, our data could not confirm this to be the case, and further experiments would be

required to ascertain whether the change in gene expression reflect changes in presynaptic (NAc) or postsynaptic (VTA) receptor expression or both.

In the present study, we have shown that pre-treatment with AMPH had no effect on DA1 and DA2 receptor gene expression, while it causes an increase in DA3 and DA4 receptor gene expression in the NAc and an increase in DA4 receptor gene expression in the VTA. It has been suggested that psychostimulant-induced alterations in the molecular, cellular and behavioural plasticity within the NAc, in association with DA receptors signalling in MSNs, can regulate drug-mediated addictive behaviours (Song *et al*., 2014). Recent studies using genetically-engineered animals have revealed roles for DA1-MSNs or DA2-MSNs in psychostimulant addictive behaviour. Kai *et al*. (2015) found that ablation of DA1 containing neurons in the NAc result in delayed initiation of behavioural sensitisation to psychostimulant administration. In contrast, the ablation of DA2-containing neurons in the NAc result in an increase in the development of sensitisation in the rats. However, other studies found the opposite result. For instance, McDougall *et al*. (2005) stated that repeated administration of AMPH cause an increase in locomotor activity in the DA1-deficient mice. However, Li *et al*. (2000) reported that cocaine-induced behavioural sensitisation (locomotor activity) could be reversed by DA receptor agonists acting at both DA1 and DA2 receptors which is in the line with our result.

In addition, an optogenetic study by Kang *et al*. (2017) found that photo-inhibition of DA2-MSNs of NAc after each administration of cocaine affected neither the initiation nor the expression of cocaine-induced behavioural sensitisation. Similarly, photo-inhibition of DA2-MSNs during a 14 day withdrawal period after cocaine administration did not affect the expression of behavioural sensitisation. Moreover, the study by Jung *et al*. (2013) suggested that DA2 receptors in the NAc are not involved in cocaine induced behavioural sensitisation. Furthermore, an *in vitro* study by Muscat *et al*. (1993) suggested that the sensitisation of axon terminal DA2 autoreceptors did not produce behavioural sensitisation to AMPH which may refer to that behavioural sensitisation to AMPH induce by different mechanism at different sites.

Projection neurons from the NAc can be separated into two subtype of MSNs; one containing DA1 that projects to the VTA and VP and other, containing DA2 receptors that projects to VP only (Kupchik *et al*., 2015). Despite the simple organisation of these two population of MSNs, MSNs receive multiple inputs and have different output to other brain

area as well as forming local circuits with other classes of interneurons. Thus, the resulting output of DA1 and DA2-MSNs is highly complex and can result in a range of different molecular, cellular and behavioural consequences (Song *et al*., 2014). The majority of projection neurons from NAc to the VTA are GABAergic which synapse largely onto GABAergic VTA neurons. Disinhibition, the removal of an inhibitory brake on neural activity firing, may affect circuit function in several parts of the brain. Disinhibition of VTA dopamine neurons has been implicated in drug effect when addictive drugs shut down VTA GABA neurons (Bocklisch *et al*., 2013). DA1 receptors expressed on the MSNs, that project to the VTA most of which are GABAergic neurons, thus GABA release from the axonal terminals is reduced in the DA1 cell-ablated animals. Therefore the reduction of GABA release may disinhibit, the removal of an inhibitory brake on neural firing, VTA GABAergic neurons that make contact with DA neurons, mediating promotion of addictive behaviour (Song *et al*., 2014). Thus, treatment with AMPH may cause a decrease DA1 receptors gene expression in the GABA neurons which may disinhibit VTA GABAergic neurons cause reduction in the GABA release mediating addictive behaviour, since Bocklisch *et al*. (2013) demonstrated that only DA1-MSNs project directly to the midbrain.

Persistent changes in DA3 receptors function could, in combination with DA1 receptors or other pre-and post-synaptic mechanism, contribute to the expression of behavioural sensitisation (Richtand *et al*., 2001). We found a selective increase in the DA3 and DA4 receptors gene expression in the NAc of AMPH-sensitised rats, which is likely to be related to an upregulation of DA3 and DA4 receptor genes, since it also occurred at expression level. Repeated AMPH administration were necessary to induce DA3 and DA4 overexpression in the NAc, since an acute AMPH administration had no effect. A study by Payer *et al*. (2014) showed that brain DA3 levels increased in cocaine dependence while the DA2 level was reduced. The mechanism underlying the upregulation of DA3 (contrasting with DA2 downregulation) is poorly understand. However, a possible explanation is that DA3 receptor induction may take place in DA1 receptors-harbouring GABA neurons expressing dynorphine and/or striatonigral substance P (SP) neurons, in contrast with DA1 and DA2 receptors that normally coexist in other substance P-containing neural population or MSN. After using the sensitisation protocol in the present study, transcription factors are markedly activated such as IEGs expression and the cAMP-responsive element-binding protein is phosphorylated in the same neurons and such factors may regulate positively DA3 receptor gene expression (Bordet *et al*., 1997).

However, DA3 receptors are both reciprocal autoreceptors and heteroreceptors and it is unknown whether the DA3 upregulation occurs on DAergic neurons or on GABAergic neurons. Thus, it is seemed to be the case that increases in the DA3 expression was parallel with the appearance of locomotor sensitisation to the psychostimulants through increased inhibition of GABAergic neurons on VTA, which is consistent with our data. Bocklisch *et al*. (2013) demonstrated that cocaine-induced inhibitory plasticity is DA-dependent and is expressed presynaptically in the VTA, and, as a consequence, increased VTA DA neuron firing which facilitates the induction of locomotor sensitisation. Then after several days of withdrawal, excitatory transmission in the NAc also adapts. The drug-evoked synaptic plasticity in back-projecting DA1-MSN emerges as a crucial step in circuit remodelling. In this context, it is worthy of note that DA1-MSN undergo presynaptic and postsynaptic changes, resulting in an overall strengthening of their inhibitory effect and enhanced locomotor sensitisation, whereas inhibition of DA1-MSN attenuated this behaviour (Chandra *et al*., 2013).

In contrast to this role of NAc dopaminergic mechanism in expression of psychomotor stimulation, the development of behavioural sensitisation appears to be mediated through changes in VTA. Regarding this issue, Vezina (1996) stated that using DA1 antagonist but not DA2 antagonist prevented the induction of sensitisation induced by injection of AMPH into the VTA. In addition, Wolf *et al*. (1993) stated that autoreceptor sensitivity in VTA has a transition effect which may be related to the development of behavioural sensitisation and it develops during the early withdrawal period but not after a long withdraw period. Thus the development of autoreceptor sensitivity may be one of the earliest steps in a cascade of neuronal events which lead to behavioural sensitisation. In term of cross-sensitisation pre-treatment with nicotine cause increase DA (DA2, DA3, DA4 and DA5) receptors gene expression in the VTA as compared with saline pre-treated rats after acute AMPH administration indicated that the action of nicotine in the VTA is more important for behavioural sensitisation then its role in the NAc.

# Chapter 6: General discussion

Among the most important characteristics of drug dependence is the recurring desire to take drugs even after many years of abstinence. The persistent vulnerability to relapse is a primary feature of the drug dependence and has been identified as a point where pharmalogical treatment may be most effectively employed. In order to achieve rational pharmacotherapies it is necessary to understand the neurobiological basis of the drug dependence (Kalivas and Volkow, 2005), and in the last decade there has been significant advance toward achieving this goal. Major studies have focused on the primary targets of addictive drugs to understand the neuroadaptation that develop with chronic drug administration, but understanding the relevance of these neuroadaptations to the clinical reality of drug dependence in humans remains a major challenge (Everitt *et al*., 2001).

Robinson and Berridge tried to explain the mechanism underlying drug dependence by putting forward their incentive sensitisation theory of addiction, in which they explain that with repeated drug use the act of drug taking and drug-associated stimuli, gradually become more and more attractive as a result of neural sensitisation and these changes are manifest both neurochemically and behaviourally (Robinson and Berridge, 1993).This later phenomenon has been referred to as behavioural sensitisation which is produced by the repeated administration of many different psychomotor stimulants including AMPH, however, it is not limited to psychomotor stimulants only. Other drugs which are not traditionally considered psychomotor stimulants also produced psychomotor activation, and produce behavioural sensitisation: these include nicotine, ethanol and phencyclidine (Kalivas and Stewart, 1991, Robinson and Berridge, 1993). They indicate that both drug dependence and behavioural sensitisation are long-lasting phenomena, in some case they may be permanent, and may share similar neural/molecular adaptation processes (Robinson and Berridge, 1993). Thus, animal models employing behavioural sensitisation are critical for investigating the mechanism involved in development and persistence of drug dependence.

Finally, it is hypothesised that the neural system (s) that normally attribute salience to incentive stimuli and become sensitized by addictive drugs, is the mesocorticolimbic dopamine system (Franken, 2003). Sensitisation results in an increase in the responsiveness of the DA system to activating stimuli, such that activating stimuli produce a greater increase in DA neurotransmission in sensitised than in non-sensitised individuals. Many drugs of

abuse, including AMPH, cocaine and nicotine while having very different primary molecular targets, all have the common action of increasing DA transmission the NAc (Robinson and Berridge, 1993). Some studies have shown that although AMPH sensitisation is not accompanied by changes in the extracellular concentrations of dopamine (Kuczenski *et al*., 1997), it is associated with an enhanced dopamine responses to drug challenge (Laplante *et al*., 2013). Thus, sensitisation produced by psychostimulant drugs is also accompanied by changes in the transduction of dopamine receptors-mediated events. This commonality of action has led to the widely held view that the mesolimbic dopamine system has a general role in the rewarding effect of drugs (Nestler, 2001a).

Repeated intermittent treatment with an addictive drugs not only produces sensitisation to that drug, but may also produce cross-sensitisation to other drugs. Cross-sensitisation has been reported between drugs in the same class (e.g. AMPH and cocaine) (Liu *et al*., 2007) and between drugs in different classes (e.g. psychomotor stimulants and nicotine) (Celik *et al*., 2006). Thus, the aim of this project was to examine the neurochemical mechanism underling behavioural sensitisation to drugs of abuse, and to examine whether drugs of different classes caused similar neurochemical changes. In the first experiment, we applied the sensitisation protocol, which we used to obtain behavioural sensitisation, in which the animals (n =127) were first treated daily with nicotine (0.6 mg/kg) AMPH (1 mg/kg) or saline (1 ml/kg) for five consecutive days. After a 10 day withdrawal period they each received a challenge dose of the drugs, and the effect of treatment on locomotor activity was measured. In another experiments, we used the same animals from the behavioural experiments to determine the neuroadaptation that developed during treatment sessions with a view to understanding the neurochemical and neurophysiological processes underlying the expression of behavioural sensitisation. To the best of our knowledge this has not been previously examined in the same experiments under the same treatment conditions.

Intermittent treatment with a current dose of nicotine and AMPH caused expression of behavioural sensitisation, a result we have shown *in vivo* (see chapter 2). To determine whether behavioural sensitisation is caused by changes in the neural plasticity in the NAc and VTA, we used immunohistochemistry to determine whether a group of IEGs that cause changes in the neural activity are involved in behavioural sensitisation after intermittent drug administration. In this experiment, we provided evidence indicating that pre-treatment with nicotine or AMPH, sufficient to produce behavioural sensitisation, potentiated resulting expression of IEGs, including Arc, *c-fos*, MeCp2 and BDNF which has been shown to

increase plasticity in the NAc, a brain area that has been implicated in the expression of behavioural sensitisation (see chapter 3).

To assess the contribution of DA to behavioural sensitisation the effect of acute and chronic treatment with nicotine or AMPH on stimulated DA release was measured. In this experiment, brain slices taken from animals pre-treated with nicotine or AMPH were challenged with these drugs after 10-14 days withdrawal period, and dopamine release was recorded *in vitro* using FSCV. We found that acute administration of either nicotine or AMPH to the slice during recording attenuated the electrically stimulated release, but that sub chronic pre-treatment with these drugs did not change either the stimulated DA level in the NAc, or the effect of the acutely applied drugs (see chapter 3). It is well documented that accumbal DA is involved in all types of reward-related behaviour, including reward learning, seeking and intake. However, dopamine release influences function through dopamine receptors that are located either pre-synaptically or post-synaptically. Pre-synaptic DA2-like autoreceptors mediate feedback mechanism and so affect the release of dopamine, while activation of DA1 like receptors that are located post-synaptically also affect the DA release through activation of MSN in the NAc. Finally, in the last experiment, we used qPCR to determine whether the behavioural sensitisation to nicotine or AMPH treatment was caused by changes in DA receptor gene expression in the NAc and VTA. We found that treatment with nicotine did not have effect on the DA receptors gene expression in the NAc, while it cause increase dopamine receptor gene expression in the VTA. While, treatment with AMPH caused increased expression of DA expression in the NAc (see chapter 4).

For nicotine sensitisation, the present study confirmed that behavioural sensitisation to the locomotor stimulating effect of nicotine may be induced by a schedule of intermittent presentation which was almost without effect in saline-pre-treated rats at the dose of nicotine used (0.6 mg/kg), and no evidence for cross-sensitisation to AMPH. In addition, our data suggest that there are both presynaptic and post-synaptic mechanism are involved in sensitised response to nicotine. In terms of presynaptic mechanisms, enhancement of DA release has been proposed as a neural mechanism underlying behavioural sensitisation to nicotine (Pierce and Kumaresan, 2006), but has not been consistently demonstrated in sensitised rats (Nisell *et al*., 1996). Balfour *et al*. (1998) have reported that repeated nicotine administration caused behavioural sensitisation to a subsequent challenge, but basal

extracellular DA, monitored during behaviour sensitisation, was reduced when compared with control.

Despite the increase the behavioural response to nicotine, there were no changes in the stimulated DA release with nicotine challenge in our *in vitro* FSCV study. This lack of change in stimulated DA release may be consistent with a downregulation of DAT with nicotine administration, which is surprising result since stimulation of nAChRs results in depolarization of the plasma membrane which generally decreases DA transport velocity, however this was not the case with nicotine (Hart and Ksir, 1996, Middleton *et al*., 2004). The mechanism underlying loss of DAT with chronic exposure to nicotine is unclear. Indeed, DAT downregulation has been shown to enhance DA clearance in NAc (Hart and Ksir, 1996). The current results are in agreement with the work by above studies, as they have demonstrated that DA reuptake time was increased in the nicotine pre-treated rats as compared with saline-treated rats after challenged with nicotine, although it did not reach a statistical significant.

In term of post-synaptic mechanism, there is a growing body of evidence for synaptic plasticity at dendritic sites having a significant role in the expression of nicotine sensitisation (Nestler *et al*., 2001). In our study, behavioural sensitisation to nicotine was noticed on both horizontal locomotor activity and increase the neural plasticity in the NAc through increase the expression of Arc and MeCp2 and decrease the expression of *c-fos* that may be indicating an increase in the expression of Δ FosB in the post-synaptic population in the NAc (as discussed in chapter 3) and decrease in BDNF as well. Similarly, in a previous study, sub chronic exposure to nicotine caused an increase in Arc (Kodama *et al*., 1998) and a decrease in *c-fos* expression (Nestler, 2001a) indicating increase neural plasticity in the NAc. In addition, another mechanism through increased DA receptors sensitivity could account for behavioural sensitisation to nicotine, similar to the increase in DA2 but not DA1 receptor density in the NAc which has been reported following repeated administration of cocaine (Li *et al*., 2000). However, in the current study we found that, in the nicotine sensitised rats, the measure of DA receptor gene expression was lower in NAc than in acute nicotine, while the DA receptors gene expressed in the VTA were higher than in the acute nicotine. This suggested distinct neuroadaptation response to sub chronic nicotine exposure between presynaptic and postsynaptic markers. Thus, our current results, together with findings from prior studies, indicate that the exaggerated behavioural response to nicotine coincides almost exactly with decreasing the level of NAc DA and decreasing the expression of DA receptors

gene in the NAc, while the DA receptors expression was increased in the VTA (Vezina, 2004).

The current result may be explained by the fact that the interaction between DAergic and cholinergic system are well known, and several investigator such as Shannon *et al*. (1999) have demonstrated that nAChs are involved in the modulation of the mesocorticolimbic DAergic system, suggesting a role for the brain stem cholinergic system in the pathophysiology of brain disorder such as schizophrenia. As such, the result imply that ACh normally plays a permissive, if not essential role in the intra-accumbens DA release. A number of studies are consistent with this notion. For example, microdialysis studies have shown that the systemic administration of a muscarinic receptor agonist (oxotremorine) inhibits AMPH-induced DA release in the NAc (Ichikawa *et al*., 2002). It has been suggested that nAChs agonists, including those devoid of appreciable affinities for DA2 receptors have anti-DAergic action (Shannon *et al*., 1999). The nature of ACh/DA interaction, however, is highly complex and involves several receptor subtypes. Even if ACh normally facilitates presynaptic DA release, it has been suggested that ACh opposes the DA action at postsynaptic sites in the NAc. A reduction in such a cholinergic modulation may render postsynaptic DAergic action relatively "unopposed" (Hoebel *et al*., 2007). In addition to possible local interaction effects, ACh in the NAc has also been suggested to be involved in the feedback regulations of DAergic neurons in the VTA, which may occur through either direct or indirect pathways. These findings strongly suggested that the cholinergic deficits in the NAc lead to a reduced phasic activation of VTA DAergic activity in both the mesolimbic and mesocortical systems via altered feedback pathway to the VTA (Laplante *et al*., 2012). It is well know that nicotine is a nACRs agonist like ACh, thus, the mechanism by which nicotine affect DA release may be the same mechanism of ACh in NAc and in the VTA. Further research is still necessary to determine the interaction between the DA release and ACh in the VTA during nicotine administration and after withdrawal period. Indeed-combined treatment for drug relapse that target DA dynamics in the VTA, which could be a potential target for the focus of further research.

Data from the current study confirm that intermittent administration of AMPH were necessary to induce behavioural sensitisation in the rats, since the acute AMPH administration does not produce a sensitised effect, in addition to cross-sensitisation to nicotine. Moreover, our data suggest that there are both presynaptic and post-synaptic mechanism involved in differential response to AMPH. In term of pre-synaptic mechanisms,

some prior reports showed that animals who exhibit AMPH sensitisation had a higher DA level in the NAc then control (Di Chiara and Imperato, 1988, Daberkow *et al*., 2013, Singer *et al*., 2017), whereas other reports demonstrated that AMPH sensitisation had no effect on DA levels and did not differ from controls as assessed with their baseline measure (Segal and Kuczenski, 1992, Byrnes and Wallace, 1997). The data in current study clearly indicate that pre-treatment with AMPH did not change the stimulated DA level in the NAc as compared with acute drug administration. However, our data cannot address the main question about the effect of pre-treatment with drugs on dopamine level in the NAc using FSCV technique because FSCV is limited by the fact that the basal levels cannot be measured, as they are incorporated in the background signal. It is important to point out that even though there are no differences between dopamine levels in the AMPH-sensitised rats as compared with acute administration, the percent changes in DA reuptake between control and AMPH challenge is slower in AMPH-sensitised rats then in the control animals, confirming that the effect of AMPH on DA release is mainly attributed to their binding to and reversal of DAT function, resulting in both reuptake inhibition and release of DA by vesicular DA depletion (Siciliano *et al*., 2014).

Although, AMPH application did not change the stimulated extracellular DA level in AMPH sensitised rats as compared with control animals, presynaptic mechanisms are important but cannot completely explain the different behavioural effect of AMPH treatment. Compensations in postsynaptic neurons in rats are profound. Post-synaptically, in the present study, we used activation of IEGs such as Arc and *c-fos*, in addition to MeCp2 and BDNF in the NAc and VTA, in addition to DA receptors expression in these areas to assess directly the response in postsynaptic neurons at cellular level. Our work demonstrated that behavioural activity was associated with increased Arc expression and decreased the *c-fos,* MeCp2 and BDNF expression in the NAc. In addition, our work demonstrated that there was increased in the expression of genes for DA3 and DA4 receptors in the NAc of AMPH sensitised rats and increased expression of genes for DA2, DA3, DA4 and DA5 in the VTA.

In term of cross-sensitisation, nicotine's interaction with other psychostimulants such as AMPH has received considerable attention. Many studies found that although both, nicotine and AMPH are psychoactive compounds, they act through different pharmacological mechanisms. Nicotine is an agonist at nAchRs which are known to regulate release of DA especially in the NAc (Vezina *et al*., 2007). On the other hand, AMPH is an indirect DA agonist acting on the presynaptic DAT on the nerve terminals, which decreases the reuptake

of DA (White and Kalivas, 1998). There are known interactions between central nAchRs and DA receptors which provide possible mechanism for the co-abuse of AMPH and nicotine. Previous studies have shown that pre-treatment with nicotine potentiated AMPH–stimulated locomotor activity and DA overflow in NAc, and conversely, AMPH pre-treatment enhanced nicotine-simulated locomotor activity and DA overflow in NAc (Jutkiewicz *et al*., 2008). Levine *et al*. (2011) found that pre-treatment of mice with nicotine increased the response to cocaine as assessed by both addiction-related behaviors and synaptic plasticity in the striatum. Therefore, the psychomotor activating effect of nicotine and the addictive potential of nicotine are thought to be mediated through dopamine system, and the hypothesis was that the sensitisation to nicotine was through similar adaptation to DA system as occurs with AMPH. If this were the case then we would expect to see cross-sensitisation between them. On the basis of these studies we hypothesised that nicotine and AMPH share a common mechanism to produce behavioral sensitisation, and aimed to assess this through cross-sensitisation experiments.

However, in the current experiments and using the same behavioural protocol as previous studies, animals which were treated with AMPH and challenged with nicotine had shown cross-sensitisation behavioural response, while, those pre-treated with nicotine did not show cross-sensitised behavioural response to the challenged drugs (AMPH challenge), suggesting that different mechanisms are involved. However, this conclusion is contrary to other studies.

Mychasiuk *et al*. (2013) demonstrated that the persistent epigenetic changes associated with exposure to nicotine and AMPH were region and drug dependent and differ from the latent epigenetic changes that occur immediately after drug exposure. In addition, Govind *et al*. (2009) stated that the action of nicotine in the VTA is more important for behavioural sensitisation than the role of it in the NAc. For AMPH, it is well hypothesized that the effect of AMPH is mainly attributed to their binding to and reversal of DAT function in the NAc, resulting in both reuptake inhibition and release of DA by vesicular DA depletion (Siciliano *et al*., 2014). Thus, in current project, we found that pre-exposure to nicotine increased the neural plasticity in the NAc core while pre-exposure to AMPH increased neural plasticity in the NAc shell. In addition, pre-exposure of nicotine caused an increase in DA receptor gene in the VTA rather than in the NAc, while pre-treatment with AMPH caused an increase DA receptors gene expression in the NAc and in the VTA. Thus, in current study and using the same animals in post-mortem experiments and *in vitro* experiments suggested that

behavioural sensitisation produced by sub chronic exposure to nicotine or AMPH did not follow the same mechanism

In addition to psychostimulant cross-sensitisation, much evidence indicates that there is also a cross-sensitisation between these drugs and stress: that is chronic exposure to stress increases the behavioural and neural responsiveness to commonly abused drugs. For instance, Miczek *et al*., (2008) explained that while animals can habituate to predictable stressors, chronic exposure to unpredictable stressors such as social stress, leads to behavioural sensitization of commonly abused drugs. The authors explained that sensitization of the mesocorticolimbic DA system has been implicated in stress cross-sensitization to commonly abused drugs. Similarly, to drugs, chronic stress also increases DA levels in the NAc and VTA. For example, Cruz *et al*., (2012) demonstrated that chronic exposure to stress in adolescent and adult rats induced increases in their locomotor activity after challenged with AMPH, which was associated with increased DA levels in the NAc and VTA in both age groups when exposed to stress. The original aim of this project was to extend the studies to look also neuroadaptation occurring during repeated exposure to stress and to examine whether the behavioural sensitisation that is caused by different drugs involve the same or a different mechanism, with a view to developing pharmacological approaches to reverse sensitization and/or prevent its development. However, time constraints meant that, we were unable to pursue this line. It does, however, present an attractive line for further research to ascertain whether the neurochemical changes we observed after nicotine or AMPH also occur after repeated stress.

Taken together, postsynaptic mechanism could be the main contribution to the differential behavioural response to nicotine and AMPH. Nevertheless, presynaptic changes in DA signalling mechanism could play a role as well in nicotine's and AMPH's locomotor activity. It may prove possible, therefore, to prevent relapse in human addicts by systemically administration of drugs that diminish the motivational properties of drugs. Our data suggested that the lack of changes in DA release and increased DA receptors expression in the NAc and VTA in the nicotine and AMPH sensitisation rats is therapeutically relevant as it indicates that nicotine and AMPH sensitisation will be sensitive to medication that rely on DA receptors especially DA3 and DA4 receptors since their genes were both expressed in the nicotine and AMPH sensitised rats. They can diminish nicotine and AMPH – behavioural sensitisation, but it remains unclear whether the receptor effects on behavioural sensitisation come from action expressed in the MSN in the NAc or it's action as

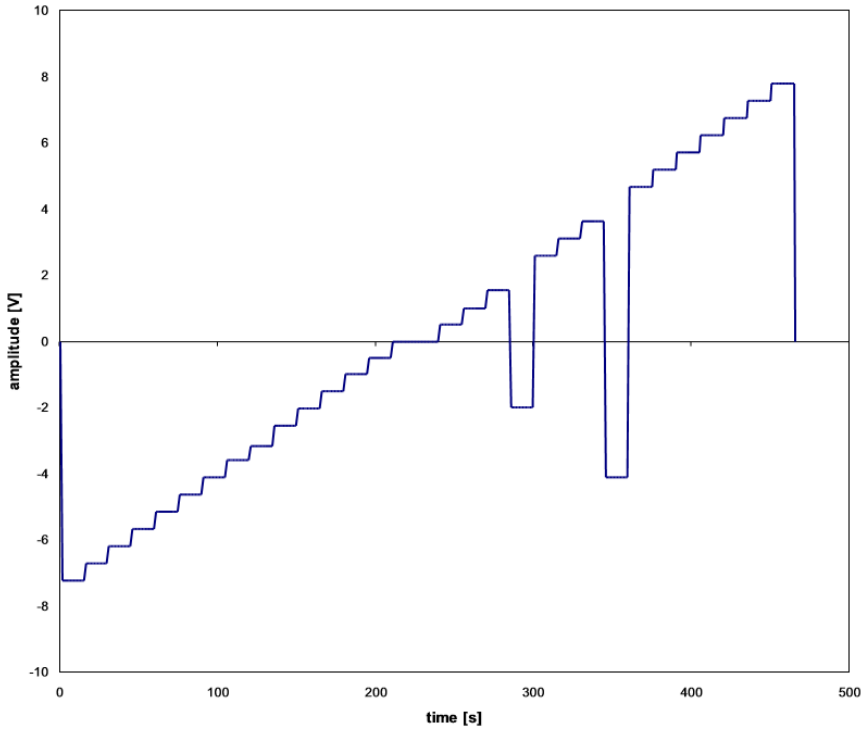
autoreceptors in the mesolimbic dopamine themselves. Finally, it is widely agreed that sensitisation with direct or indirect DA agonists is mediated by interactions in the DA system of the basal ganglia. What is now becoming clear is that one single mechanism or site of action within this DAergic circuitry cannot account for behavioural sensitisation induced by the diverse range of drugs. Since evidence points to there not being a common mechanism, it is unlikely that a single pharmacological approach will be effective for treatment of all addictive drugs.

In addition, studies in different labs have used different protocols, which may account for some of the discrepancies seen. Thus for example, different rat strains have been employed, different procedures used such as microdialysis or *in vitro* FSCV, and different doses have been used. In contrast to *in vivo* preparation that investigate global process of release, we aimed to develop an *in vitro* slice preparation containing the whole mesolimbic DA pathway within the slics, using different slice orientation (horizontal and sagittal slicing) so we could measure the effect of drugs application in the VTA on DA release in the NAc. However, because we were unable to evoke release in the NAc through electrical stimulation of VTA, we employed an alternative approach using coronal slices. While recordings of stimulated DA release in these slices are routine in the lab, they have the disadvantage that only local circuitry remains intact, making it impossible to investigate wider network regulation. In addition, it is likely that the mechanism of action by which AMPH or nicotine induce behavioural sensitisation may involve other receptors located in DAergic terminals and/or interneurons in the NAc such as NMDA receptors (Degoulet *et al*., 2013). Classical studies show that chronic nicotine exposure cause upregulation of nAChRs receptors, however it is hypothesis that the most of numerically upregulated receptors are actually desensitized so that the upregulation would be masked or distorted by decrease in receptors sensitivity. Shannon *et al*. (1999) stated that increase of nicotine reward is unlikely to be due to increases of monoamine in reward and cognitive areas. Thus, indirect evidence in the present study, and more direct evidence from work in other laboratories, suggested that future studies need to investigate whether such effect may be mediated by the abnormal development of the another neurons within NAc such as glutamate and/or GABA projections, in addition to looking at the ventral striatal cholinergic system. Depending on this conclusion, other experiments may need to measure the effect of pre-treatment with nicotine or AMPH on glutamate and GABA neurotransmitters level in the NAc and VTA.

Appendix A:

## Silver – Silver Chloride Reference Electrode

### *NPI Electronics, Tamm, Germany*




Acl-01 working scheme

Graphical representation of the in-built voltage ramp sequence applied to the silver wire, to electrolytically coat the surface with AgCl, using an optimised chlorinating procedure developed at the Max-Planck Institute in Göttingen, Germany (NPI Electronics, Germany).

Silver wire was dipped in 2 M KCl solution and cleaned by applying a negative voltage, starting at a potential of -7 V. The voltage then stepped in 0.25 V increments at 15 sec intervals as far as 0 V, where the voltage remained at 0 V for 30 sec. The electrolytic coating then started by applying a regularly incrementing positive voltage (0.25 V every 15 sec), up to a potential of 8 V, with two brief reversals of the potential (- 2 V for 15 sec and -4 V for 15 sec). The potential was held at 8 V for 15 sec, and then returned to 0 V to complete the coating process.

*Details taken from:*

*http://www.npielectronic.de/fileadmin/files/Literature_Brochures/Miscellaneous/ACl-01/Acl01_ad.pdf*

# References


ABUSE, S. 2014. Mental Health Services Administration (SAMHSA) Results from the 2013 National Survey on Drug Use and Health: Summary of National Findings, NSDUH Series H-48, HHS Publication No.(SMA) 14–4863. *Rockville, MD: Substance Abuse and Mental Health Services Administration***,** 13-4795.

AGUILAR-RIVERA, M. I., CASANOVA, J., GATICA, R., QUIRK, G. & FUENTEALBA, J. 2015. Amphetamine sensitization is accompanied by an increase in prelimbic cortex activity. *Neuroscience,* 288**,** 1-9.

ANAGNOSTARAS, S. G. & ROBINSON, T. E. 1996. Sensitization to the psychomotor stimulant effects of amphetamine: modulation by associative learning. *Behavioral neuroscience,* 110**,** 1397.

AVENA, N. M., RADA, P. & HOEBEL, B. G. 2008. Evidence for sugar addiction: Behavioral and neurochemical effects of intermittent, excessive sugar intake. *Neuroscience & Biobehavioral Reviews,* 32**,** 20-39.

BADIANI, A. & ROBINSON, T. 2004. Drug-induced neurobehavioral plasticity: the role of environmental context. *Behavioural pharmacology,* 15**,** 327-339.

BAKER, L. K., MAO, D., CHI, H., GOVIND, A. P., VALLEJO, Y. F., IACOVIELLO, M., HERRERA, S., CORTRIGHT, J. J., GREEN, W. N. & MCGEHEE, D. S. 2013a. Intermittent nicotine exposure upregulates nAChRs in VTA dopamine neurons and sensitises locomotor responding to the drug. *European Journal of Neuroscience,* 37**,** 1004-1011.

BAKER, L. K., MAO, D., CHI, H., GOVIND, A. P., VALLEJO, Y. F., IACOVIELLO, M., HERRERA, S., CORTRIGHT, J. J., GREEN, W. N., MCGEHEE, D. S. & VEZINA, P. 2013b. Intermittent nicotine exposure upregulates nAChRs in VTA dopamine neurons and sensitizes locomotor responding to the drug. *The European journal of neuroscience,* 37**,** 1004-1011.

BALFOUR, D. J., BENWELL, M. E., BIRRELL, C. E., KELLY, J. & AL-ALOUL, M. 1998. Sensitization of the mesoaccumbens dopamine response to nicotine. *Pharmacology Biochemistry and Behavior,* 59**,** 1021-1030.

BANERJEE, P. S., ASTON, J., KHUNDAKAR, A. A. & ZETTERSTRÖM, T. S. C. 2009. Differential regulation of psychostimulant-induced gene expression of brain derived neurotrophic factor and the immediate-early gene Arc in the juvenile and adult brain. *European Journal of Neuroscience,* 29**,** 465-476.

BARIK, J. & WONNACOTT, S. 2009. Molecular and cellular mechanisms of action of nicotine in the CNS. *Nicotine Psychopharmacology.* Springer.

BARTEL, D. P., SHENG, M., LAU, L. F. & GREENBERG, M. E. 1989. Growth factors and membrane depolarization activate distinct programs of early response gene expression: dissociation of fos and jun induction. *Genes & Development,* 3**,** 304-313.

BASSAREO, V., DE LUCA, M. A. & DI CHIARA, G. 2007. Differential impact of pavlovian drug conditioned stimuli on in vivo dopamine transmission in the rat accumbens shell and core and in the prefrontal cortex. *Psychopharmacology,* 191**,** 689-703.

BENOIT-MARAND, M., JABER, M. & GONON, F. 2000. Release and elimination of dopamine in vivo in mice lacking the dopamine transporter: functional consequences. *European Journal of Neuroscience,* 12**,** 2985-2992.

BENOWITZ, N. L. 2010. Nicotine addiction. *New England Journal of Medicine,* 362**,** 2295-2303.

BENWELL, M. E. & BALFOUR, D. J. 1992. The effects of acute and repeated nicotine treatment on nucleus accumbens dopamine and locomotor activity. *British journal of pharmacology,* 105**,** 849-856.

BOCKLISCH, C., PASCOLI, V., WONG, J. C. Y., HOUSE, D. R. C., YVON, C., DE ROO, M., TAN, K. R. & LÜSCHER, C. 2013. Cocaine Disinhibits Dopamine Neurons by Potentiation of GABA Transmission in the Ventral Tegmental Area. *Science,* 341**,** 1521-1525.

BORDET, R., RIDRAY, S., CARBONI, S., DIAZ, J., SOKOLOFF, P. & SCHWARTZ, J.-C. 1997. Induction of dopamine D3 receptor expression as a mechanism of behavioral sensitization to levodopa. *Proceedings of the National Academy of Sciences,* 94**,** 3363-3367.

BOYE, S. M., GRANT, R. J. & CLARKE, P. B. 2001. Disruption of dopaminergic neurotransmission in nucleus accumbens core inhibits the locomotor stimulant effects of nicotine and D-amphetamine in rats. *Neuropharmacology,* 40**,** 792-805.

BRENHOUSE, H. & STELLAR, J. 2006. c-Fos and ΔFosB expression are differentially altered in distinct subregions of the nucleus accumbens shell in cocaine-sensitized rats. *Neuroscience,* 137**,** 773-780.

BROCK, J. W., NG, J. P. & JUSTICE, J. B. 1990. Effect of chronic cocaine on dopamine synthesis in the nucleus accumbens as determined by microdialysis perfusion with NSD-1015. *Neuroscience Letters,* 117**,** 234-239.

BROG, J. S., SALYAPONGSE, A., DEUTCH, A. Y. & ZAHM, D. S. 1993. The patterns of afferent innervation of the core and shell in the "Accumbens" part of the rat ventral striatum: Immunohistochemical detection of retrogradely transported fluoro-gold. *Journal of Comparative Neurology,* 338**,** 255-278.

BROMBERG-MARTIN, E. S., MATSUMOTO, M. & HIKOSAKA, O. 2010. Dopamine in motivational control: rewarding, aversive, and alerting. *Neuron,* 68**,** 815-834.

BRUNZELL, D. H. & PICCIOTTO, M. R. 2008. Molecular mechanisms underlying the motivational effects of nicotine. *The Motivational Impact of Nicotine and its Role in Tobacco Use.* Springer.

BYRNES, J. J., BRIDGES, R. S. & BYRNES, E. M. 2011. Amphetamine sensitization in reproductively experienced female rats. *Neuroscience letters,* 502**,** 168-172.

BYRNES, J. J. & WALLACE, L. J. 1997. Amphetamine-induced sensitization and release of dopamine in slices from the ventral tegmental area of rats is enhanced following administration of cholera toxin into the ventral tegmental area. *Neuroscience letters,* 223**,** 45-48.

CACCIAPAGLIA, F., SADDORIS, M. P., WIGHTMAN, R. M. & CARELLI, R. M. 2012. Differential dopamine release dynamics in the nucleus accumbens core and shell track distinct aspects of goal-directed behavior for sucrose. *Neuropharmacology,* 62**,** 2050-2056.

CASANOVA, J. P., VELIS, G. P. & FUENTEALBA, J. A. 2013. Amphetamine locomotor sensitization is accompanied with an enhanced high K+-stimulated Dopamine release in the rat medial prefrontal cortex. *Behavioural brain research,* 237**,** 313-317.

CASS, W. A., ZAHNISER, N. R., FLACH, K. A. & GERHARDT, G. A. 1993. Clearance of exogenous dopamine in rat dorsal striatum and nucleus accumbens: role of metabolism and effects of locally applied uptake inhibitors. *Journal of neurochemistry,* 61**,** 2269-2278.

CELIK, E., UZBAY, I. T. & KARAKAS, S. 2006. Caffeine and amphetamine produce cross-sensitization to nicotine-induced locomotor activity in mice. *Progress in Neuro-Psychopharmacology and Biological Psychiatry,* 30**,** 50-55.

CHANDRA, R., LENZ, J., GANCARZ, A., CHAUDHURY, D., SCHROEDER, G., HAN, M.-H., CHEER, J., DIETZ, D. & LOBO, M. K. 2013. Optogenetic inhibition of D1R containing nucleus accumbens neurons alters cocaine-mediated regulation of Tiam1. *Frontiers in Molecular Neuroscience,* 6.

CHARYCH, E. I., LIU, F., MOSS, S. J. & BRANDON, N. J. 2009. GABAA receptors and their associated proteins: Implications in the etiology and treatment of schizophrenia and related disorders. *Neuropharmacology,* 57**,** 481-495.

CLARK, J. J., SANDBERG, S. G., WANAT, M. J., GAN, J. O., HORNE, E. A., HART, A. S., AKERS, C. A., PARKER, J. G., WILLUHN, I., MARTINEZ, V., EVANS, S. B., STELLA, N. & PHILLIPS, P. E. M. 2010. Chronic microsensors for longitudinal, subsecond dopamine detection in behaving animals. *Nat Meth,* 7**,** 126-129.

COHEN, S., GABEL, H. W., HEMBERG, M., HUTCHINSON, A. N., SADACCA, L. A., EBERT, D. H., HARMIN, D. A., GREENBERG, R. S., VERDINE, V. K. & ZHOU, Z. 2011. Genome-wide

activity-dependent MeCP2 phosphorylation regulates nervous system development and function. *Neuron,* 72**,** 72-85.
COLLINS, S. L., MONTANO, R. & IZENWASSER, S. 2004. Nicotine treatment produces persistent increases in amphetamine-stimulated locomotor activity in periadolescent male but not female or adult male rats. *Developmental Brain Research,* 153**,** 175-187.
CORTRIGHT, J. J., SAMPEDRO, G. R., NEUGEBAUER, N. M. & VEZINA, P. 2012. Previous Exposure to Nicotine Enhances the Incentive Motivational Effects of Amphetamine via Nicotine-Associated Contextual Stimuli. *Neuropsychopharmacology,* 37**,** 2277-2284.
CRUZ, F. C., JAVIER RUBIO, F. & HOPE, B. T. 2015. Using c-fos to study neuronal ensembles in corticostriatal circuitry of addiction. *Brain Research,* 1628**,** 157-173.
DABERKOW, D. P., BROWN, H. D., BUNNER, K. D., KRANIOTIS, S. A., DOELLMAN, M. A., RAGOZZINO, M. E., GARRIS, P. A. & ROITMAN, M. F. 2013. Amphetamine Paradoxically Augments Exocytotic Dopamine Release and Phasic Dopamine Signals. *The Journal of Neuroscience,* 33**,** 452-463.
DANI, J. A. & BALFOUR, D. J. K. 2011. Historical and Current Perspective on Tobacco use and Nicotine Addiction. *Trends in neurosciences,* 34**,** 383-392.
DANI, J. A. & DE BIASI, M. 2001. Cellular mechanisms of nicotine addiction. *Pharmacology Biochemistry and Behavior,* 70**,** 439-446.
DAUBNER, S. C., LE, T. & WANG, S. 2011. Tyrosine hydroxylase and regulation of dopamine synthesis. *Archives of biochemistry and biophysics,* 508**,** 1-12.
DEGOULET, M., ROSTAIN, J. C., ABRAINI, J. H. & DAVID, H. N. 2013. Short-term development of behavioral sensitization to amphetamine requires N-methyl-D-aspartate-and nicotinic-dependent mechanisms in the nucleus accumbens. *Addiction biology,* 18**,** 417-424.
DEL ARCO, A. & MORA, F. 2008. Prefrontal cortex–nucleus accumbens interaction: in vivo modulation by dopamine and glutamate in the prefrontal cortex. *Pharmacology Biochemistry and Behavior,* 90**,** 226-235.
DENG, J. V., RODRIGUIZ, R. M., HUTCHINSON, A. N., KIM, I.-H., WETSEL, W. C. & WEST, A. E. 2010. MeCP2 in the nucleus accumbens contributes to neural and behavioral responses to psychostimulants. *Nature neuroscience,* 13**,** 1128-1136.
DENG, J. V., WAN, Y., WANG, X., COHEN, S., WETSEL, W. C., GREENBERG, M. E., KENNY, P. J., CALAKOS, N. & WEST, A. E. 2014. MeCP2 phosphorylation limits psychostimulant-induced behavioral and neuronal plasticity. *The Journal of Neuroscience,* 34**,** 4519-4527.
DI CHIARA, G. 2000. Role of dopamine in the behavioural actions of nicotine related to addiction. *European journal of pharmacology,* 393**,** 295-314.
DI CHIARA, G. 2002. Nucleus accumbens shell and core dopamine: differential role in behavior and addiction. *Behavioural brain research,* 137**,** 75-114.
DI CHIARA, G. & IMPERATO, A. 1988. Drugs abused by humans preferentially increase synaptic dopamine concentrations in the mesolimbic system of freely moving rats. *Proceedings of the National Academy of Sciences,* 85**,** 5274-5278.
ENGEL, J. A. & JERLHAG, E. 2014. Alcohol: mechanisms along the mesolimbic dopamine system. *Progress in brain research,* 211**,** 201.
ESCOBAR, A. P., CORNEJO, F. A., OLIVARES-COSTA, M., GONZÁLEZ, M., FUENTEALBA, J. A., GYSLING, K., ESPAÑA, R. A. & ANDRÉS, M. E. 2015. Reduced dopamine and glutamate neurotransmission in the nucleus accumbens of quinpirole-sensitized rats hints at inhibitory D2 autoreceptor function. *Journal of neurochemistry,* 134**,** 1081-1090.
EVERITT, B. J., DICKINSON, A. & ROBBINS, T. W. 2001. The neuropsychological basis of addictive behaviour. *Brain Research Reviews,* 36**,** 129-138.
FANOUS, S., LACAGNINA, M. J., NIKULINA, E. M. & HAMMER, R. P. 2011. Sensitized activation of Fos and brain-derived neurotrophic factor in the medial prefrontal cortex and ventral tegmental area accompanies behavioral sensitization to amphetamine. *Neuropharmacology,* 61**,** 558-564.

FENG, J. & NESTLER, E. J. 2010. MeCP2 and drug addiction. *Nature neuroscience,* 13**,** 1039-1041.
FLECKENSTEIN, A. E., VOLZ, T. J., RIDDLE, E. L., GIBB, J. W. & HANSON, G. R. 2007. New insights into the mechanism of action of amphetamines. *Annu. Rev. Pharmacol. Toxicol.,* 47**,** 681-698.
FORD, C. P. 2014. The role of D2-autoreceptors in regulating dopamine neuron activity and transmission. *Neuroscience,* 282**,** 13-22.
FRANKEN, I. H. 2003. Drug craving and addiction: integrating psychological and neuropsychopharmacological approaches. *Progress in Neuro-Psychopharmacology and Biological Psychiatry,* 27**,** 563-579.
FUJII, S., JI, Z., MORITA, N. & SUMIKAWA, K. 1999. Acute and chronic nicotine exposure differentially facilitate the induction of LTP. *Brain Research,* 846**,** 137-143.
FUMAGALLI, F., BEDOGNI, F., FRASCA, A., DI PASQUALE, L., RACAGNI, G. & RIVA, M. A. 2006. Corticostriatal up-regulation of activity-regulated cytoskeletal-associated protein expression after repeated exposure to cocaine. *Molecular pharmacology,* 70**,** 1726-1734.
GAGE, G. J., KIPKE, D. R. & SHAIN, W. 2012. Whole animal perfusion fixation for rodents. *J Vis Exp,* 65**,** 3564.
GITTIS, A. H., NELSON, A. B., THWIN, M. T., PALOP, J. J. & KREITZER, A. C. 2010. Distinct roles of GABAergic interneurons in the regulation of striatal output pathways. *Journal of Neuroscience,* 30**,** 2223-2234.
GONZALEZ, C. L. R., GHARBAWIE, O. A. & KOLB, B. 2006. Chronic low-dose administration of nicotine facilitates recovery and synaptic change after focal ischemia in rats. *Neuropharmacology,* 50**,** 777-787.
GOUTIER, W., KLOEZE, M. & MCCREARY, A. C. 2014. Nicotine-induced locomotor sensitization: pharmacological analyses with candidate smoking cessation aids. *Addiction biology*.
GOVIND, A. P., VEZINA, P. & GREEN, W. N. 2009. Nicotine-induced upregulation of nicotinic receptors: Underlying mechanisms and relevance to nicotine addiction. *Biochemical Pharmacology,* 78**,** 756-765.
GRADY, S. R., SALMINEN, O., LAVERTY, D. C., WHITEAKER, P., MCINTOSH, J. M., COLLINS, A. C. & MARKS, M. J. 2007. The subtypes of nicotinic acetylcholine receptors on dopaminergic terminals of mouse striatum. *Biochemical Pharmacology,* 74**,** 1235-1246.
GRAHAM, D. L., EDWARDS, S., BACHTELL, R. K., DILEONE, R. J., RIOS, M. & SELF, D. W. 2007. Dynamic BDNF activity in nucleus accumbens with cocaine use increases self-administration and relapse. *Nat Neurosci,* 10**,** 1029-1037.
GUZOWSKI, J. F., LYFORD, G. L., STEVENSON, G. D., HOUSTON, F. P., MCGAUGH, J. L., WORLEY, P. F. & BARNES, C. A. 2000. Inhibition of activity-dependent arc protein expression in the rat hippocampus impairs the maintenance of long-term potentiation and the consolidation of long-term memory. *Journal of Neuroscience,* 20**,** 3993-4001.
HAMILTON, K. R., STAROSCIAK, A. K., CHWA, A. & GRUNBERG, N. E. 2012. Nicotine behavioral sensitization in Lewis and Fischer male rats. *Experimental and clinical psychopharmacology,* 20**,** 345.
HART, C. & KSIR, C. 1996. Nicotine effects on dopamine clearance in rat nucleus accumbens. *Journal of neurochemistry,* 66**,** 216-221.
HENSCHKE, C. I., YIP, R. & MIETTINEN, O. S. 2006. Women's susceptibility to tobacco carcinogens and survival after diagnosis of lung cancer. *JAMA,* 296**,** 180-4.
HERDEGEN, T. & LEAH, J. 1998. Inducible and constitutive transcription factors in the mammalian nervous system: control of gene expression by Jun, Fos and Krox, and CREB/ATF proteins. *Brain Research Reviews,* 28**,** 370-490.
HOEBEL, B. G., AVENA, N. M. & RADA, P. 2007. Accumbens dopamine-acetylcholine balance in approach and avoidance. *Current Opinion in Pharmacology,* 7**,** 617-627.

HORGER, B. A., IYASERE, C. A., BERHOW, M. T., MESSER, C. J., NESTLER, E. J. & TAYLOR, J. R. 1999. Enhancement of locomotor activity and conditioned reward to cocaine by brain-derived neurotrophic factor. *Journal of Neuroscience,* 19**,** 4110-4122.

HOWELL, L. L. & KIMMEL, H. L. 2008. Monoamine transporters and psychostimulant addiction. *Biochemical pharmacology,* 75**,** 196-217.

HUTCHINSON, A. N., DENG, J. V., ARYAL, D. K., WETSEL, W. C. & WEST, A. E. 2012. Differential regulation of MeCP2 phosphorylation in the CNS by dopamine and serotonin. *Neuropsychopharmacology,* 37**,** 321-337.

HYMAN, C. & HOFER, M. 1991. BDNF is a neurotrophic factor for dopaminergic neurons of the substantia nigra. *Nature,* 350**,** 230.

HYMAN, S. E., MALENKA, R. C. & NESTLER, E. J. 2006. Neural mechanisms of addiction: the role of reward-related learning and memory. *Annu. Rev. Neurosci.,* 29**,** 565-598.

IKEMOTO, S. 2007. Dopamine reward circuitry: two projection systems from the ventral midbrain to the nucleus accumbens–olfactory tubercle complex. *Brain research reviews,* 56**,** 27-78.

IKEMOTO, S., GLAZIER, B. S., MURPHY, J. M. & MCBRIDE, W. J. 1997. Role of dopamine D1 and D2 receptors in the nucleus accumbens in mediating reward. *Journal of neuroscience,* 17**,** 8580-8587.

INGRAM, S. L., PRASAD, B. M. & AMARA, S. G. 2002. Dopamine transporter-mediated conductances increase excitability of midbrain dopamine neurons. *Nature neuroscience,* 5**,** 971.

INVERNIZZI, R., POZZI, L. & SAMANIN, R. 1991. Release of dopamine is reduced by diazepam more in the nucleus accumbens than in the caudate nucleus of conscious rats. *Neuropharmacology,* 30**,** 575-578.

IYANIWURA, T. T., WRIGHT, A. E. & BALFOUR, D. J. 2001. Evidence that mesoaccumbens dopamine and locomotor responses to nicotine in the rat are influenced by pretreatment dose and strain. *Psychopharmacology,* 158**,** 73-79.

JACOBSON, L. H., SWEENEY, F. F., KAUPMANN, K., O'LEARY, O. F., GASSMANN, M., BETTLER, B. & CRYAN, J. F. 2016. Differential roles of GABA B1 subunit isoforms on locomotor responses to acute and repeated administration of cocaine. *Behavioural brain research,* 298**,** 12-16.

JAY, T. M. 2003. Dopamine: a potential substrate for synaptic plasticity and memory mechanisms. *Progress in Neurobiology,* 69**,** 375-390.

JEDYNAK, J., HEARING, M., INGEBRETSON, A., EBNER, S. R., KELLY, M., FISCHER, R. A., KOURRICH, S. & THOMAS, M. J. 2016. Cocaine and amphetamine induce overlapping but distinct patterns of AMPAR plasticity in nucleus accumbens medium spiny neurons. *Neuropsychopharmacology,* 41**,** 464-476.

JONES, I. W. & WONNACOTT, S. 2004. Precise localization of α7 nicotinic acetylcholine receptors on glutamatergic axon terminals in the rat ventral tegmental area. *The Journal of neuroscience,* 24**,** 11244-11252.

JONES, S. R., O'DELL, S. J., MARSHALL, J. F. & WIGHTMAN, R. M. 1996. Functional and anatomical evidence for different dopamine dynamics in the core and shell of the nucleus accumbens in slices of rat brain. *Synapse,* 23**,** 224-231.

JUNG, E.-S., LEE, H. J., SIM, H.-R. & BAIK, J.-H. 2013. Cocaine-induced behavioral sensitization in mice: effects of microinjection of dopamine d2 receptor antagonist into the nucleus accumbens. *Experimental neurobiology,* 22**,** 224-231.

JUTKIEWICZ, E. M., NICOLAZZO, D. M., KIM, M. N. & GNEGY, M. E. 2008. Nicotine and amphetamine acutely cross-potentiate their behavioral and neurochemical responses in female Holtzman rats. *Psychopharmacology,* 200**,** 93-103.

KAI, N., NISHIZAWA, K., TSUTSUI, Y., UEDA, S. & KOBAYASHI, K. 2015. Differential roles of dopamine D1 and D2 receptor-containing neurons of the nucleus accumbens shell in behavioral sensitization. *Journal of neurochemistry,* 135**,** 1232-1241.

KALIVAS, P. W. & DUFFY, P. 1993. Time course of extracellular dopamine and behavioral sensitization to cocaine. I. Dopamine axon terminals. *The Journal of neuroscience,* 13**,** 266-275.

KALIVAS, P. W. & STEWART, J. 1991. Dopamine transmission in the initiation and expression of drug- and stress-induced sensitization of motor activity. *Brain Research Reviews,* 16**,** 223-244.

KALIVAS, P. W. & VOLKOW, N. D. 2005. The neural basis of addiction: a pathology of motivation and choice. *American Journal of Psychiatry,* 162**,** 1403-1413.

KANG, B. J., SONG, S. S., WEN, L., HONG, K. P., AUGUSTINE, G. J. & BAIK, J. H. 2017. Effect of optogenetic manipulation of accumbal medium spiny neurons expressing dopamine D2 receptors in cocaine-induced behavioral sensitization. *European Journal of Neuroscience*.

KARLER, R., FINNEGAN, K. T. & CALDER, L. D. 1993. Blockade of behavioral sensitization to cocaine and amphetamine by inhibitors of protein synthesis. *Brain research,* 603**,** 19-24.

KAWAGUCHI, Y., WILSON, C. J., AUGOOD, S. J. & EMSON, P. C. 1995. Striatal interneurones: chemical, physiological and morphological characterization. *Trends in neurosciences,* 18**,** 527-535.

KELZ, M. B., CHEN, J., CARLEZON, W. A., WHISLER, K., GILDEN, L., BECKMANN, A. M., STEFFEN, C., ZHANG, Y.-J., MAROTTI, L. & SELF, D. W. 1999. Expression of the transcription factor ΔFosB in the brain controls sensitivity to cocaine. *Nature,* 401**,** 272-276.

KIM, M. N., JUTKIEWICZ, E. M., ZHANG, M. & GNEGY, M. E. 2011. The sensitizing effect of acute nicotine on amphetamine-stimulated behavior and dopamine efflux requires activation of β2 subunit-containing nicotinic acetylcholine receptors and glutamate N-methyl-D-aspartate receptors. *Neuropharmacology,* 60**,** 1126-1134.

KLEBAUR, J. E., OSTRANDER, M. M., NORTON, C. S., WATSON, S. J., AKIL, H. & ROBINSON, T. E. 2002. The ability of amphetamine to evoke arc (Arg 3.1) mRNA expression in the caudate, nucleus accumbens and neocortex is modulated by environmental context. *Brain research,* 930**,** 30-36.

KLEIJN, J., FOLGERING, J., VAN DER HART, M., ROLLEMA, H., CREMERS, T. & WESTERINK, B. 2011. Direct effect of nicotine on mesolimbic dopamine release in rat nucleus accumbens shell. *Neuroscience letters,* 493**,** 55-58.

KODAMA, M., AKIYAMA, K., UJIKE, H., SHIMIZU, Y., TANAKA, Y. & KURODA, S. 1998. A robust increase in expression of arc gene, an effector immediate early gene, in the rat brain after acute and chronic methamphetamine administration. *Brain Research,* 796**,** 273-283.

KONRADI, C., COLE, R. L., HECKERS, S. & HYMAN, S. E. 1994. Amphetamine regulates gene expression in rat striatum via transcription factor CREB. *The Journal of neuroscience,* 14**,** 5623-5634.

KRISTENSEN, E. W., WILSON, R. L. & WIGHTMAN, R. M. 1986. Dispersion in flow injection analysis measured with microvoltammetric electrodes. *Analytical Chemistry,* 58**,** 986-988.

KUCZENSKI, R., SEGAL, D. S. & TODD, P. K. 1997. Behavioral sensitization and extracellular dopamine responses to amphetamine after various treatments. *Psychopharmacology,* 134**,** 221-229.

KUPCHIK, Y. M., BROWN, R. M., HEINSBROEK, J. A., LOBO, M. K., SCHWARTZ, D. J. & KALIVAS, P. W. 2015. Coding the direct/indirect pathways by D1 and D2 receptors is not valid for accumbens projections. *Nature neuroscience,* 18**,** 1230-1232.

LAMB, R., PRESTON, K., SCHINDLER, C., MEISCH, R., DAVIS, F., KATZ, J., HENNINGFIELD, J. & GOLDBERG, S. 1991. The reinforcing and subjective effects of morphine in post-addicts: a dose-response study. *Journal of Pharmacology and Experimental Therapeutics,* 259**,** 1165-1173.

LANDA, L., MACHALOVA, A. & SULCOVA, A. 2014. Implication of NMDA receptors in behavioural sensitization to psychostimulants: a short review. *European journal of pharmacology,* 730**,** 77-81.

LAPLANTE, F., DUFRESNE, M. M., OUBOUDINAR, J., OCHOA-SANCHEZ, R. & SULLIVAN, R. M. 2013. Reduction in cholinergic interneuron density in the nucleus accumbens attenuates local extracellular dopamine release in response to stress or amphetamine. *Synapse,* 67**,** 21-29.
LE, A., WANG, A., HARDING, S., JUZYTSCH, W. & SHAHAM, Y. 2003. Nicotine increases alcohol self-administration and reinstates alcohol seeking in rats. *Psychopharmacology,* 168**,** 216-221.
LE FOLL, B., DIAZ, J. & SOKOLOFF, P. 2003. Increased dopamine D3 receptor expression accompanying behavioral sensitization to nicotine in rats. *Synapse,* 47**,** 176-183.
LEVINE, A., HUANG, Y., DRISALDI, B., GRIFFIN, E. A., POLLAK, D. D., XU, S., YIN, D., SCHAFFRAN, C., KANDEL, D. B. & KANDEL, E. R. 2011. Molecular Mechanism for a Gateway Drug: Epigenetic Changes Initiated by Nicotine Prime Gene Expression by Cocaine. *Science translational medicine,* 3**,** 107ra109-107ra109.
LI, X., DEJOSEPH, M., URBAN, J. H., BAHI, A., DREYER, J.-L., MEREDITH, G. E., FORD, K. A., FERRARIO, C. R., LOWETH, J. A. & WOLF, M. E. 2013. Different roles of BDNF in nucleus accumbens core versus shell during the incubation of cue-induced cocaine craving and its long-term maintenance. *Journal of Neuroscience,* 33**,** 1130-1142.
LI, X. & WOLF, M. E. 2015. Multiple faces of BDNF in cocaine addiction. *Behavioural brain research,* 279**,** 240-254.
LI, Y., KOLB, B. & ROBINSON, T. E. 2003. The location of persistent amphetamine-induced changes in the density of dendritic spines on medium spiny neurons in the nucleus accumbens and caudate-putamen. *Neuropsychopharmacology,* 28**,** 1082.
LI, Y., WHITE, F. J. & WOLF, M. E. 2000. Pharmacological reversal of behavioral and cellular indices of cocaine sensitization in the rat. *Psychopharmacology,* 151**,** 175-183.
LIU, Y., MORGAN, D. & ROBERTS, D. C. S. 2007. Cross-sensitization of the reinforcing effects of cocaine and amphetamine in rats. *Psychopharmacology,* 195**,** 369-375.
LIVINGSTONE, P. D., SRINIVASAN, J., KEW, J. N., DAWSON, L. A., GOTTI, C., MORETTI, M., SHOAIB, M. & WONNACOTT, S. 2009. α7 and non-α7 nicotinic acetylcholine receptors modulate dopamine release in vitro and in vivo in the rat prefrontal cortex. *European Journal of Neuroscience,* 29**,** 539-550.
LODGE, D. J. & GRACE, A. A. 2008. Amphetamine activation of hippocampal drive of mesolimbic dopamine neurons: a mechanism of behavioral sensitization. *Journal of Neuroscience,* 28**,** 7876-7882.
MATTSON, B. J., KOYA, E., SIMMONS, D. E., MITCHELL, T. B., BERKOW, A., CROMBAG, H. S. & HOPE, B. T. 2008. Context-specific sensitization of cocaine-induced locomotor activity and associated neuronal ensembles in rat nucleus accumbens. *European Journal of Neuroscience,* 27**,** 202-212.
MCCLUNG, C. A. & NESTLER, E. J. 2003. Regulation of gene expression and cocaine reward by CREB and DeltaFosB. *Nat Neurosci,* 6**,** 1208-15.
MCCLUNG, C. A., ULERY, P. G., PERROTTI, L. I., ZACHARIOU, V., BERTON, O. & NESTLER, E. J. 2004. DeltaFosB: a molecular switch for long-term adaptation in the brain. *Brain Res Mol Brain Res,* 132**,** 146-54.
MCDOUGALL, S. A., REICHEL, C. M., CYR, M. C., KARPER, P. E., NAZARIAN, A. & CRAWFORD, C. A. 2005. Importance of D1 receptors for associative components of amphetamine-induced behavioral sensitization and conditioned activity: a study using D1 receptor knockout mice. *Psychopharmacology,* 183**,** 20-30.
MELCHIOR, J. R., FERRIS, M. J., STUBER, G. D., RIDDLE, D. R. & JONES, S. R. 2015. Optogenetic versus electrical stimulation of dopamine terminals in the nucleus accumbens reveals local modulation of presynaptic release. *Journal of neurochemistry,* 134**,** 833-844.
MIDDLETON, L. S., CASS, W. A. & DWOSKIN, L. P. 2004. Nicotinic receptor modulation of dopamine transporter function in rat striatum and medial prefrontal cortex. *Journal of Pharmacology and Experimental Therapeutics,* 308**,** 367-377.

MILLAR, J., STAMFORD, J. A., KRUK, Z. L. & WIGHTMAN, R. M. 1985. Electrochemical, pharmacological and electrophysiological evidence of rapid dopamine release and removal in the rat caudate nucleus following electrical stimulation of the median forebrain bundle. *European journal of pharmacology,* 109**,** 341-348.

MILLAR, N. S. & GOTTI, C. 2009. Diversity of vertebrate nicotinic acetylcholine receptors. *Neuropharmacology,* 56**,** 237-246.

MILLER, T. & HENDRIE, D. 2008. Substance abuse prevention dollars and cents: A cost-benefit analysis. *Center for Substance Abuse Prevention (CSAP), SAMHSA. DHHS Pub*.

MORA, F., SEGOVIA, G. & DEL ARCO, A. 2008. Glutamate–dopamine–GABA interactions in the aging basal ganglia. *Brain Research Reviews,* 58**,** 340-353.

MORGAN, J. I. & CURRAN, T. 1986. Role of ion flux in the control of c-fos expression.

MORO, H., SATO, H., IDA, I., OSHIMA, A., SAKURAI, N., SHIHARA, N., HORIKAWA, Y. & MIKUNI, M. 2007. Effects of SKF-38393, a dopamine D 1 receptor agonist on expression of amphetamine-induced behavioral sensitization and expression of immediate early gene arc in prefrontal cortex of rats. *Pharmacology Biochemistry and Behavior,* 87**,** 56-64.

MORUD, J., ADERMARK, L., PEREZ-ALCAZAR, M., ERICSON, M. & SÖDERPALM, B. 2015. Nicotine produces chronic behavioral sensitization with changes in accumbal neurotransmission and increased sensitivity to re-exposure. *Addiction biology*.

MORUD, J., ADERMARK, L., PEREZ-ALCAZAR, M., ERICSON, M. & SÖDERPALM, B. 2016. Nicotine produces chronic behavioral sensitization with changes in accumbal neurotransmission and increased sensitivity to re-exposure. *Addiction biology,* 21**,** 397-406.

MUSCAT, R., PATEL, J., TROUT, S., WIECZOREK, W. & KRUK, Z. 1993. Dissociation of the effects of amphetamine and quinpirole on dopamine release in the nucleus accumbens following behavioural sensitization: an ex vivo voltammetric study. *Behavioural pharmacology,* 4**,** 411-418.

MYCHASIUK, R., MUHAMMAD, A., ILNYTSKYY, S. & KOLB, B. 2013. Persistent gene expression changes in NAc, mPFC, and OFC associated with previous nicotine or amphetamine exposure. *Behavioural brain research,* 256**,** 655-661.

NAKAGAWA, T., SUZUKI, Y., NAGAYASU, K., KITAICHI, M., SHIRAKAWA, H. & KANEKO, S. 2011. Repeated exposure to methamphetamine, cocaine or morphine induces augmentation of dopamine release in rat mesocorticolimbic slice co-cultures. *PloS one,* 6**,** e24865.

NASHMI, R., XIAO, C., DESHPANDE, P., MCKINNEY, S., GRADY, S. R., WHITEAKER, P., HUANG, Q., MCCLURE-BEGLEY, T., LINDSTROM, J. M. & LABARCA, C. 2007. Chronic nicotine cell specifically upregulates functional α4* nicotinic receptors: basis for both tolerance in midbrain and enhanced long-term potentiation in perforant path. *Journal of Neuroscience,* 27**,** 8202-8218.

NESTLER, E. J. 1992. Molecular mechanisms of drug addiction. *J Neurosci,* 12**,** 2439-2450.

NESTLER, E. J. 2001a. Molecular basis of long-term plasticity underlying addiction. *Nature reviews neuroscience,* 2**,** 119-128.

NESTLER, E. J. 2001b. Molecular neurobiology of addiction. *Am J Addict,* 10**,** 201-17.

NESTLER, E. J. 2005. Is there a common molecular pathway for addiction? *Nat Neurosci,* 8**,** 1445-1449.

NESTLER, E. J. 2014. Epigenetic mechanisms of drug addiction. *Neuropharmacology,* 76**,** 259-268.

NESTLER, E. J., BARROT, M. & SELF, D. W. 2001. ΔFosB: a sustained molecular switch for addiction. *Proceedings of the National Academy of Sciences,* 98**,** 11042-11046.

NEVE, K. A., SEAMANS, J. K. & TRANTHAM-DAVIDSON, H. 2004. Dopamine receptor signaling. *Journal of receptors and signal transduction,* 24**,** 165-205.

NGUYEN, T., KOSOFSKY, B., BIRNBAUM, R., COHEN, B. & HYMAN, S. 1992. Differential expression of c-fos and zif268 in rat striatum after haloperidol, clozapine, and amphetamine. *Proceedings of the National Academy of Sciences,* 89**,** 4270-4274.

NISELL, M., NOMIKOS, G. G., HERTEL, P., PANAGIS, G. & SVENSSON, T. H. 1996. Condition-independent sensitization of locomotor stimulation and mesocortical dopamine release following chronic nicotine treatment in the rat. *Synapse,* 22**,** 369-381.

OLDS, J. & MILNER, P. 1954. Positive reinforcement produced by electrical stimulation of septal area and other regions of rat brain. *Journal of comparative and physiological psychology,* 47**,** 419.

OLIVA, I. & WANAT, M. J. 2016. Ventral tegmental area afferents and drug-dependent behaviors. *Frontiers in psychiatry,* 7.

PAXINOS, G. & WATSON, C. 1998. Rat Brain in Stereotaxic Coordinates (Deluxe Edition). Academic press.

PAYER, D. E., BEHZADI, A., KISH, S. J., HOULE, S., WILSON, A. A., RUSJAN, P. M., TONG, J., SELBY, P., GEORGE, T. P., MCCLUSKEY, T. & BOILEAU, I. 2014. Heightened D(3) Dopamine Receptor Levels in Cocaine Dependence and Contributions to the Addiction Behavioral Phenotype: A Positron Emission Tomography Study with [(11)C]-(+)-PHNO. *Neuropsychopharmacology,* 39**,** 321-328.

PENNARTZ, C. M., GROENEWEGEN, H. J. & LOPES DA SILVA, F. 1994. The nucleus accumbens as a complex of functionally distinct neuronal ensembles: an integration of behavioural, electrophysiological and anatomical data. *Progress in neurobiology,* 42**,** 719-761.

PERROTTI, L. I., WEAVER, R. R., ROBISON, B., RENTHAL, W., MAZE, I., YAZDANI, S., ELMORE, R. G., KNAPP, D. J., SELLEY, D. E., MARTIN, B. R., SIM-SELLEY, L., BACHTELL, R. K., SELF, D. W. & NESTLER, E. J. 2008. Distinct patterns of DeltaFosB induction in brain by drugs of abuse. *Synapse,* 62**,** 358-69.

PHILLIPS, P. E. & WIGHTMAN, R. M. 2003. Critical guidelines for validation of the selectivity of in-vivo chemical microsensors. *TrAC Trends in Analytical Chemistry,* 22**,** 509-514.

PIDOPLICHKO, V. I., DEBIASI, M., WILLIAMS, J. T. & DANI, J. A. 1997. Nicotine activates and desensitizes midbrain dopamine neurons. *Nature,* 390**,** 401-404.

PIERCE, R. C. & BARI, A. A. 2001. The role of neurotrophic factors in psychostimulant-induced behavioral and neuronal plasticity. *Reviews in the neurosciences,* 12**,** 95-110.

PIERCE, R. C. & KALIVAS, P. W. 1995. Amphetamine produces sensitized increases in locomotion and extracellular dopamine preferentially in the nucleus accumbens shell of rats administered repeated cocaine. *Journal of Pharmacology and Experimental Therapeutics,* 275**,** 1019-1029.

PIERCE, R. C. & KALIVAS, P. W. 1997. A circuitry model of the expression of behavioral sensitization to amphetamine-like psychostimulants. *Brain research reviews,* 25**,** 192-216.

PIERCE, R. C. & KUMARESAN, V. 2006. The mesolimbic dopamine system: the final common pathway for the reinforcing effect of drugs of abuse? *Neuroscience & biobehavioral reviews,* 30**,** 215-238.

PIERCE, R. C., PIERCE-BANCROFT, A. F. & PRASAD, B. M. 1999. Neurotrophin-3 contributes to the initiation of behavioral sensitization to cocaine by activating the Ras/mitogen-activated protein kinase signal transduction cascade. *Journal of Neuroscience,* 19**,** 8685-8695.

QUARTA, D., CIRUELA, F., PATKAR, K., BORYCZ, J., SOLINAS, M., LLUIS, C., FRANCO, R., WISE, R. A., GOLDBERG, S. R. & HOPE, B. T. 2007. Heteromeric nicotinic acetylcholine–dopamine autoreceptor complexes modulate striatal dopamine release. *Neuropsychopharmacology,* 32**,** 35-42.

QUINTERO, G. C. 2013. Role of nucleus accumbens glutamatergic plasticity in drug addiction. *Neuropsychiatric Disease and Treatment,* 9**,** 1499-1512.

RENTHAL, W., CARLE, T. L., MAZE, I., COVINGTON, H. E., TRUONG, H.-T., ALIBHAI, I., KUMAR, A., MONTGOMERY, R. L., OLSON, E. N. & NESTLER, E. J. 2008. ΔFosB mediates epigenetic desensitization of the c-fos gene after chronic amphetamine exposure. *The Journal of Neuroscience,* 28**,** 7344-7349.

RICHTAND, N. M., WOODS, S. C., BERGER, S. P. & STRAKOWSKI, S. M. 2001. D3 dopamine receptor, behavioral sensitization, and psychosis. *Neuroscience & Biobehavioral Reviews,* 25**,** 427-443.

ROBBINS, T. W. & EVERITT, B. 2002. Limbic-striatal memory systems and drug addiction. *Neurobiology of learning and memory,* 78**,** 625-636.

ROBINSON, J. H. & PRITCHARD, W. S. 1992. The meaning of addiction: reply to West. *Psychopharmacology,* 108**,** 411-416.

ROBINSON, T. E. & BECKER, J. B. 1986. Enduring changes in brain and behavior produced by chronic amphetamine administration: a review and evaluation of animal models of amphetamine psychosis. *Brain Research Reviews,* 11**,** 157-198.

ROBINSON, T. E. & BERRIDGE, K. C. 1993. The neural basis of drug craving: an incentive-sensitization theory of addiction. *Brain research reviews,* 18**,** 247-291.

ROBINSON, T. E. & BERRIDGE, K. C. 2008. The incentive sensitization theory of addiction: some current issues. *Philosophical Transactions of the Royal Society B: Biological Sciences,* 363**,** 3137-3146.

ROBINSON, T. E., BROWMAN, K. E., CROMBAG, H. S. & BADIANI, A. 1998. Modulation of the induction or expression of psychostimulant sensitization by the circumstances surrounding drug administration. *Neuroscience & Biobehavioral Reviews,* 22**,** 347-354.

ROBINSON, T. E. & KOLB, B. 1997. Persistent structural modifications in nucleus accumbens and prefrontal cortex neurons produced by previous experience with amphetamine. *Journal of neuroscience,* 17**,** 8491-8497.

ROBISON, A. J. & NESTLER, E. J. 2011. Transcriptional and epigenetic mechanisms of addiction. *Nature reviews neuroscience,* 12**,** 623-637.

ROMANELLI, R. J., WILLIAMS, J. T. & NEVE, K. A. 2010. Dopamine receptor signaling: intracellular pathways to behavior. *The dopamine receptors.* Springer.

ROWELL, P. P. & DUGGAN, D. S. 1998. Long-lasting inactivation of nicotinic receptor function in vitro by treatment with high concentrations of nicotine. *Neuropharmacology,* 37**,** 103-111.

RUSSO, S. J., MAZEI-ROBISON, M. S., ABLES, J. L. & NESTLER, E. J. 2009. Neurotrophic factors and structural plasticity in addiction. *Neuropharmacology,* 56**,** 73-82.

RUSSO, S. J. & NESTLER, E. J. 2013. The brain reward circuitry in mood disorders. *Nature Reviews Neuroscience,* 14**,** 609-625.

SALERY, M., DOS SANTOS, M., SAINT-JOUR, E., MOUMNÉ, L., PAGÈS, C., KAPPÈS, V., PARNAUDEAU, S., CABOCHE, J. & VANHOUTTE, P. 2017. Activity-Regulated Cytoskeleton-Associated Protein Accumulates in the Nucleus in Response to Cocaine and Acts as a Brake on Chromatin Remodeling and Long-Term Behavioral Alterations. *Biological Psychiatry,* 81**,** 573-584.

SCHENK, S., SNOW, S. & HORGER, B. A. 1991. Pre-exposure to amphetamine but not nicotine sensitizes rats to the motor activating effect of cocaine. *Psychopharmacology,* 103**,** 62-66.

SCHILTZ, C. A., KELLEY, A. E. & LANDRY, C. F. 2005. Contextual cues associated with nicotine administration increase arc mRNA expression in corticolimbic areas of the rat brain. *European Journal of Neuroscience,* 21**,** 1703-1711.

SCHMIDT, H. D., MCGINTY, J. F., WEST, A. E. & SADRI-VAKILI, G. 2013. Epigenetics and psychostimulant addiction. *Cold Spring Harbor perspectives in medicine,* 3**,** a012047.

SCHULTZ, W., DAYAN, P. & MONTAGUE, P. R. 1997. A neural substrate of prediction and reward. *Science,* 275**,** 1593-1599.

SEEAR, P. J., HEAD, M. L., TILLEY, C. A., ROSATO, E. & BARBER, I. 2014. Flow-mediated plasticity in the expression of stickleback nesting glue genes. *Ecology and evolution,* 4**,** 1233-1242.

SEGAL, D. S. & KUCZENSKI, R. 1992. In vivo microdialysis reveals a diminished amphetamine-induced DA response corresponding to behavioral sensitization produced by repeated amphetamine pretreatment. *Brain research,* 571**,** 330-337.

SELLINGS, L. H. & CLARKE, P. B. 2003. Segregation of amphetamine reward and locomotor stimulation between nucleus accumbens medial shell and core. *Journal of Neuroscience,* 23**,** 6295-6303.

SHANNON, H. E., HART, J. C., BYMASTER, F. P., CALLIGARO, D. O., DELAPP, N. W., MITCH, C. H., WARD, J. S., FINK-JENSEN, A., SAUERBERG, P. & JEPPESEN, L. 1999. Muscarinic receptor agonists, like dopamine receptor antagonist antipsychotics, inhibit conditioned avoidance response in rats. *Journal of Pharmacology and Experimental Therapeutics,* 290**,** 901-907.

SHIM, I., JAVAID, J. I., WIRTSHAFTER, D., JANG, S.-Y., SHIN, K.-H., LEE, H.-J., CHUNG, Y.-C. & CHUN, B.-G. 2001. Nicotine-induced behavioral sensitization is associated with extracellular dopamine release and expression of c-Fos in the striatum and nucleus accumbens of the rat. *Behavioural brain research,* 121**,** 137-147.

SICILIANO, C. A., CALIPARI, E. S., FERRIS, M. J. & JONES, S. R. 2014. Biphasic mechanisms of amphetamine action at the dopamine terminal. *Journal of Neuroscience,* 34**,** 5575-5582.

SIMCHON TENENBAUM, Y., WEIZMAN, A. & REHAVI, M. 2015. The Impact of Chronic Early Administration of Psychostimulants on Brain Expression of BDNF and Other Neuroplasticity-Relevant Proteins. *Journal of Molecular Neuroscience,* 57**,** 231-242.

SIMPSON, J., WANG, J. & MCGINTY, J. 1995. Repeated amphetamine administration induces a prolonged augmentation of phosphorylated cyclase response element-binding protein and Fos-related antigen immunoreactivity in rat striatum. *Neuroscience,* 69**,** 441-457.

SINGER, B. F., BRYAN, M. A., POPOV, P., ROBINSON, T. E. & ARAGONA, B. J. 2017. Rapid induction of dopamine sensitization in the nucleus accumbens shell induced by a single injection of cocaine. *Behavioural Brain Research,* 324**,** 66-70.

SITTE, H. H. & FREISSMUTH, M. 2015. Amphetamines, new psychoactive drugs and the monoamine transporter cycle. *Trends in pharmacological sciences,* 36**,** 41-50.

SMITH, L. N., BACHUS, S. E., MCDONALD, C. G. & SMITH, R. F. 2015. Role of the D3 dopamine receptor in nicotine sensitization. *Behavioural brain research,* 289**,** 92-104.

SONG, S. S., KANG, B. J., WEN, L., LEE, H. J., SIM, H.-R., KIM, T. H., YOON, S., YOON, B.-J., AUGUSTINE, G. J. & BAIK, J.-H. 2014. Optogenetics reveals a role for accumbal medium spiny neurons expressing dopamine D2 receptors in cocaine-induced behavioral sensitization. *Frontiers in behavioral neuroscience,* 8.

STAMFORD, J. A. 1990. Fast cyclic voltammetry: measuring transmitter release in 'real time'. *Journal of neuroscience methods,* 34**,** 67-72.

STEKETEE, J. D. & KALIVAS, P. W. 2011. Drug wanting: behavioral sensitization and relapse to drug-seeking behavior. *Pharmacological reviews,* 63**,** 348-365.

STEWARD, O., WALLACE, C. S., LYFORD, G. L. & WORLEY, P. F. 1998. Synaptic activation causes the mRNA for the IEG Arc to localize selectively near activated postsynaptic sites on dendrites. *Neuron,* 21**,** 741-751.

STEWART, J., DE WIT, H. & EIKELBOOM, R. 1984. Role of unconditioned and conditioned drug effects in the self-administration of opiates and stimulants. *Psychological review,* 91**,** 251.

SULZER, D. 2011. How addictive drugs disrupt presynaptic dopamine neurotransmission. *Neuron,* 69**,** 628-649.

SULZER, D., SONDERS, M. S., POULSEN, N. W. & GALLI, A. 2005. Mechanisms of neurotransmitter release by amphetamines: a review. *Progress in neurobiology,* 75**,** 406-433.

SZIRÁKI, I., SERSHEN, H., HASHIM, A. & LAJTHA, A. 2002. Receptors in the ventral tegmental area mediating nicotine-induced dopamine release in the nucleus accumbens. *Neurochemical research,* 27**,** 253-261.

TAYLOR, S., WAKEM, M., DIJKMAN, G., ALSARRAJ, M. & NGUYEN, M. 2010. A practical approach to RT-qPCR—publishing data that conform to the MIQE guidelines. *Methods,* 50**,** S1-S5.

THOMAS, M., KALIVAS, P. & SHAHAM, Y. 2008. Neuroplasticity in the mesolimbic dopamine system and cocaine addiction. *British journal of pharmacology,* 154**,** 327-342.

TORREGROSSA, M. M., CORLETT, P. R. & TAYLOR, J. R. 2011. Aberrant learning and memory in addiction. *Neurobiology of learning and memory,* 96**,** 609-623.
UJIKE, H., TAKAKI, M., KODAMA, M. & KURODA, S. 2002. Gene expression related to synaptogenesis, neuritogenesis, and MAP kinase in behavioral sensitization to psychostimulants. *Annals of the New York Academy of Sciences,* 965**,** 55-67.
VANDERSCHUREN, L. J. & KALIVAS, P. W. 2000. Alterations in dopaminergic and glutamatergic transmission in the induction and expression of behavioral sensitization: a critical review of preclinical studies. *Psychopharmacology,* 151**,** 99-120.
VANDERSCHUREN, L. J., SCHMIDT, E. D., DE VRIES, T. J., VAN MOORSEL, C. A., TILDERS, F. J. & SCHOFFELMEER, A. N. 1999. A single exposure to amphetamine is sufficient to induce long-term behavioral, neuroendocrine, and neurochemical sensitization in rats. *The journal of neuroscience,* 19**,** 9579-9586.
VELÁZQUEZ-SÁNCHEZ, C., GARCÍA-VERDUGO, J. M., MURGA, J. & CANALES, J. J. 2013. The atypical dopamine transport inhibitor, JHW 007, prevents amphetamine-induced sensitization and synaptic reorganization within the nucleus accumbens. *Progress in Neuro-Psychopharmacology and Biological Psychiatry,* 44**,** 73-80.
VEZINA, P. 1996. D1 dopamine receptor activation is necessary for the induction of sensitization by amphetamine in the ventral tegmental area. *Journal of Neuroscience,* 16**,** 2411-2420.
VEZINA, P. 2004. Sensitization of midbrain dopamine neuron reactivity and the self-administration of psychomotor stimulant drugs. *Neuroscience & Biobehavioral Reviews,* 27**,** 827-839.
VEZINA, P. & LEYTON, M. 2009. Conditioned cues and the expression of stimulant sensitization in animals and humans. *Neuropharmacology,* 56**,** 160-168.
VEZINA, P., MCGEHEE, D. & GREEN, W. 2007. Exposure to nicotine and sensitization of nicotine-induced behaviors. *Progress in Neuro-Psychopharmacology and Biological Psychiatry,* 31**,** 1625-1638.
WALLACE, D. L., VIALOU, V., RIOS, L., CARLE-FLORENCE, T. L., CHAKRAVARTY, S., KUMAR, A., GRAHAM, D. L., GREEN, T. A., KIRK, A. & INIGUEZ, S. D. 2008. The influence of ΔFosB in the nucleus accumbens on natural reward-related behavior. *Journal of Neuroscience,* 28**,** 10272-10277.
WALLACE, L. J. 2012. Effects of amphetamine on subcellular distribution of dopamine and DOPAC. *Synapse,* 66**,** 592-607.
WARREN, B. L., INIGUEZ, S. D., ALCANTARA, L. F., WRIGHT, K. N., PARISE, E. M., WEAKLEY, S. K. & BOLAÑOS-GUZMÁN, C. A. 2011. Juvenile administration of concomitant methylphenidate and fluoxetine alters behavioral reactivity to reward-and mood-related stimuli and disrupts ventral tegmental area gene expression in adulthood. *Journal of Neuroscience,* 31**,** 10347-10358.
WEST, R. 2001. Theories of addiction. *Addiction,* 96**,** 3-13.
WHITE, F. J. & KALIVAS, P. W. 1998. Neuroadaptations involved in amphetamine and cocaine addiction. *Drug and alcohol dependence,* 51**,** 141-153.
WIGHTMAN, R., AMATORH, C., ENGSTROM, R., HALE, P., KRISTENSEN, E., KUHR, W. & MAY, L. 1988. Real-time characterization of dopamine overflow and uptake in the rat striatum. *Neuroscience,* 25**,** 513-523.
WIGHTMAN, R. M., HEIEN, M. L., WASSUM, K. M., SOMBERS, L. A., ARAGONA, B. J., KHAN, A. S., ARIANSEN, J. L., CHEER, J. F., PHILLIPS, P. E. & CARELLI, R. M. 2007. Dopamine release is heterogeneous within microenvironments of the rat nucleus accumbens. *European Journal of Neuroscience,* 26**,** 2046-2054.
WISE, R. A. & BOZARTH, M. A. 1987. A psychomotor stimulant theory of addiction. *Psychological review,* 94**,** 469.
WISE, R. A. & KOOB, G. F. 2014. The development and maintenance of drug addiction. *Neuropsychopharmacology,* 39**,** 254-262.

WISE, R. A. & ROMPRÉ, P.-P. 1989. Brain dopamine and reward. *Annual review of psychology,* 40**,** 191-225.

WOLF, M. E., WHITE, F. J., NASSAR, R., BROODERSON, R. J. & KHANSA, M. R. 1993. Differential development of autoreceptor subsensitivity and enhanced dopamine release during amphetamine sensitization. *Journal of Pharmacology and Experimental Therapeutics,* 264**,** 249-255.

YANG, M. H., JUNG, M.-S., LEE, M. J., YOO, K. H., YOOK, Y. J., PARK, E. Y., CHOI, S. H., SUH, Y. J., KIM, K.-W. & PARK, J. H. 2008. Gene expression profiling of the rewarding effect caused by methamphetamine in the mesolimbic dopamine system. *Molecules and cells,* 26**,** 121-130.

YAVAS, E. & YOUNG, A. M. 2017. N-methyl-D-aspartate modulation of nucleus accumbens dopamine release by metabotropic glutamate receptors: fast cyclic voltammetry studies in rat brain slices in vitro. *ACS chemical neuroscience,* 8**,** 320-328.

YORGASON, J. T., ESPAÑA, R. A. & JONES, S. R. 2011. Demon voltammetry and analysis software: analysis of cocaine-induced alterations in dopamine signaling using multiple kinetic measures. *Journal of neuroscience methods,* 202**,** 158-164.

YORGASON, J. T., FERRIS, M. J., STEFFENSEN, S. C. & JONES, S. R. 2014. Frequency-Dependent Effects of Ethanol on Dopamine Release in the Nucleus Accumbens. *Alcoholism: Clinical and Experimental Research,* 38**,** 438-447.

ZHANG, J., ZHANG, L., JIAO, H., ZHANG, Q., ZHANG, D., LOU, D., KATZ, J. L. & XU, M. 2006. c-Fos facilitates the acquisition and extinction of cocaine-induced persistent changes. *The Journal of neuroscience,* 26**,** 13287-13296.

ZHANG, L., DONG, Y., DOYON, W. M. & DANI, J. A. 2012. Withdrawal from chronic nicotine exposure alters dopamine signaling dynamics in the nucleus accumbens. *Biological psychiatry,* 71**,** 184-191.

ZHANG, Z.-W., ZAK, J. D. & LIU, H. 2010. MeCP2 Is Required for Normal Development of GABAergic Circuits in the Thalamus. *Journal of Neurophysiology,* 103**,** 2470-2481.

ZHOU, Z., HONG, E. J., COHEN, S., ZHAO, W.-N., HO, H.-Y. H., SCHMIDT, L., CHEN, W. G., LIN, Y., SAVNER, E. & GRIFFITH, E. C. 2006. Brain-specific phosphorylation of MeCP2 regulates activity-dependent Bdnf transcription, dendritic growth, and spine maturation. *Neuron,* 52**,** 255-269.