Understanding striatal neuroadaptations in addiction-relapse vulnerability

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Bachelor of Biomedical Science (Hons)

A thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy

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July, 2016

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Rikki K. Quinn

Acknowledgements

Firstly, I would like to sincerely thank my supervisors Associate Professor Chris Dayas and Associate Professor Murray Cairns for their support and guidance throughout my PhD. I would also like to thank Dr Doug Smith for his assistance over the past few years.

I thank the members of the Dayas lab both past and present, for making my PhD an enjoyable experience. Without your friendship and support in the lab the last few years would have been much more difficult, and far less enjoyable.

I would also like to thank the Medical Science Building crew, thanks for making our building a great environment to work in, and for the sneaky bar lunches to help get through the tough weeks. To my work wife, Kristen, thank you for all the tea breaks and chats. You made the whole process so much more enjoyable.

Finally, thank you to my friends and family for their support and encouragement. Thank you to my sister, Carly for the chats, drinks and giving me Sydney escapes when I needed a break. To Mum and Dad, for the support, encouragement, proof reading and the free dinners! Without you I couldn't have gotten through it. Lastly to Luke, who has supported me throughout the entire process, motivated me, pushed me and celebrated with me, thank you for your patience and understanding.

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Abbreviations

4E-BP	Eukaryotic translation initiation factor-4E binding protein
AMPAR	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
Arc	Activity-regulated cytoskeletal-associated protein
AS	Antisense
BDNF	Brain derived neurotrophic factor
BMA	Bayesian Model Averaging
CAMKIIα	Ca2+/Calmodulin-dependent kinase II alpha
CBT	Cognitive Behavioural Therapy
CeA	Central Amygdala
CM	Contingency Management
Cplx1	Complexin-1
CPP	Conditioned Place Preference
CRA	Community Reinforcement Approach
CREB	cAMP response element-binding protein
DA	Drug Availability
DAT	Dopamine Transporter
DEPC	Diethylpyrocarbonate
DLS	Dorsolateral Striatum
DMS	Dorsomedial Striatum
DMSO	Dimethyl Sulfoxide
Drd1	Dopamine Receptor 1
DS	Dorsal Striatum
DSM	Diagnostics and Statistics Manual

FR	Fixed Ratio
GluA1	Ionotropic Glutamate Receptor 1
GluA2	Ionotropic Glutamate Receptor 2
HEK	Human embryonic kidney
HRein	High reinstator
i.c.v	Intracerebroventricular
i.p.	Intraperitoneal
i.v	Intravenous
Limk1	Lim-domain-containing protein kinase
LNA	Locked Nucleic Acid
LRein	Low reinstator
LTD	Long Term Depression
LTP	Long Term Potentiation
MCMC	Markov Chain Monte Carlo
MeCP2	Methyl-CpG Binding Protein 2
mGluR1	Metabotropic Glutamate Receptor 1
miRNA	microRNA
mPFC	Medial Prefrontal Cortex
MRI	Magnetic Resonance Imaging
MSN	Medium Spiny Neuron
mTOR	Mechanistic target of Rapamycin
mTORC1	mTOR Complex 1
mTORC2	mTOR Complex 2
NAC	Nucleus Accumbens

NACc	Nucleus Accumbens Core
NACsh	Nucleus Accumbens Shell
NDA	Non-drug Availability
NMDAR	N-methyl-D-aspartate Receptor
Nsf	N-ethylmaleimide Sensitive Fusion Protein
ODN	Oligonucleotide
OFC	Occipitofrontal Cortex
p70s6k	p70 ribosomal S6 kinase
PBS	Phosphate-buffered Saline
PFC	Prefrontal Cortex
PI3K	Phosphatidylinositol-3-kinase
PR	Progressive Ratio
PrL	Prelimbic
RISC	RNA-induced silencing complex
SA	Self-administration
SEM	Standard Error of the Mean
Syt1	Synaptotagmin-1
TBST	Tris-buffered Saline with Tween
THC	Tetrahydrocanabanoid
UTR	Untranslated Region
VS	Ventral Striatum
VTA	Ventral Tegmental Area

Abstract

Addiction is a cyclical disorder associated with rigid, habit like behaviour and a high propensity to relapse into drug seeking. Up to 90% of addicts will relapse during in their treatment, posing a substantial clinical hurdle to recovery. Currently, few pharmacotherapies are available to aid in recovery from drug seeking and prevention of relapse. The lack of effective treatment options is a product of our limited understanding of the persistent neuroadaptations that render individuals vulnerable to addiction and relapse. The striatum is a critical brain region involved in regulation of many facets of addiction-like behaviour, including reinforcement of conditioned stimuli and regulation of habit-like behaviour. Importantly, the striatum can be divided into four functionally heterogenous subregions: the nucleus accumbens shell (NACsh) and core (NACc) and the dorsomedial (DM) and dorsolateral (DL) striatum. A number of studies have identified changes in synaptic plasticity processes in these regions following illicit drug use. However, the molecular mechanisms that contribute to synaptic plasticity are yet to be fully elucidated. This thesis aimed to uncover the role of a number of critical synaptic plasticity associated genes in the development of addiction. Moreover, I aimed to understand the role of miRNA regulation of these genes, and how these changes in molecular substrates may contribute to addiction vulnerability.

I first investigated the role of the mTOR complex 1 (mTORC1) in regulation of cocaine taking, withdrawal and reinstatement of drug use. mTORC1 is a serine threonine kinase involved in the translation of several synaptic plasticity proteins, including AMPAR subunits and CAMKIIα, and has been previously implicated in addiction behaviour. I observed that withdrawal

from cocaine taking increased mTORC1 activity in the NAC. Moreover, inhibition of mTORC1 using rapamycin decreased the expression of addiction-like behaviour, while reducing markers of mTORC1 activity. Intra-cerebroventricular injection of rapamycin also decreased the motivation to consume cocaine measured using progressive ratio (PR) testing. Furthermore, inhibition of mTORC1 in the NACsh reduced PR responding as well as cue-induced reinstatement of drug seeking. These addiction behaviours were reduced by systemic administration of rapamycin. Critically, decreased expression of indices of mTORC1 activity were evident in the NAC, but not DS. Together these experiments demonstrate a key role for mTORC1 expression in the NAC but not DS in the regulation of addiction-like behaviour. Importantly, it has been shown that *mTOR* and other synaptic plasticity genes are altered in the striatum of rats that show evidence of addiction vulnerability. However, the mechanisms contributing to these changes are unclear. Thus, the second aim of this thesis was to assess the role of miRNA in the regulation of these critical synaptic plasticity genes.

miRNA are short, non-coding RNA molecules that negatively regulate gene expression. I assessed the expression of miRNA in the striatal subregions and their role in regulation of synaptic plasticity genes. Previous evidence suggests that there is a global downregulation of synaptic plasticity genes, including *mTOR*, *Arc* and *Drd1*, in the striatum of addiction-vulnerable animals compared to resilient controls. However, the results of my first study showed that decreased mTORC1 in the NACsh reduced indices of addiction. Critically, this demonstrates a temporal time course for expression of these genes. As such, I developed a novel method of identifying addiction

vulnerable animals throughout the course of the addiction cycle. I found that during the drug-taking phase of addiction cycle, cocaine self-administering rats had altered expression of key miRNA, including miR-101b, miR-137, miR-132 and miR-212 in the striatal subregions. However, there were few changes between vulnerability groups. In contrast, there was a significant increase in the expression of these miRNA in the striatal subregions of addiction vulnerable rats compared to resilient controls following cocaine reinstatement. Importantly, miR-212 has been implicated in the regulation of compulsive cocaine use. Moreover, miR-101b has been previously shown to regulate *mTOR*, and was found in the current thesis to regulate *Drd1* expression. These results suggest that miRNA are involved in the regulation of key synaptic plasticity genes involved in control of addiction relevant behaviour.

Overall, the results of this thesis demonstrate a critical role for synaptic plasticity genes including *mTOR*, *Arc* and *Drd1* and their miRNA regulators in the development of addiction. My findings have helped to elucidate the molecular changes in the striatal subregions that may contribute to addiction vulnerability and an increased propensity to relapse in addicted individuals.

Publications

CHAPTER TWO

James, Morgan H*., **Quinn, Rikki K***., Ong, Lin Kooi, Levi, Emily M., Charnley, Janine L., Smith, Doug W., Dickson, Phillip W., Dayas, Christopher V. (2014). mTORC1 Inhibition in the Nucleus Accumbens `Protects' Against the Expression of Drug Seeking and `Relapse' and Is Associated with Reductions in GluA1 AMPAR and CAMKIIα Levels. *Neuropsychopharmacology, 39*(7), 1694-1702. doi: 10.1038/npp.2014.16

*Co-first authors

CHAPTER THREE

James, Morgan H., **Quinn, Rikki K**., Ong, Lin Kooi, Levi, Emily M., Smith, Doug W., Dickson, Phillip W., Dayas, Christopher V. (2016). Rapamycin reduces motivated responding for cocaine and alters GluA1 expression in the ventral but not dorsal striatum. *European Journal of Pharmacology,* 784**,** 147-154.

CHAPTER FOUR

Quinn, Rikki K., James, Morgan H., Hawkins, Guy E., Brown, Amanda L., Heathcote, Andrew., Smith, Doug W., Cairns, Murray J., Dayas, Christopher V. (2016) Addiction phenotyping reveals temporally specific miRNA expression patterns in the dorsal and ventral striatum. In preparation for submission to *Biological Psychiatry*

CHAPTER FIVE

Quinn, Rikki K., Brown, Amanda L., Goldie, Belinda J., Levi, Emily M., Dickson, Phillip W., Smith, Doug W., Cairns, Murray J., Dayas, Christopher V. Distinct miRNA expression in dorsal striatal subregions is associated with risk for addiction in rats. *Translational Psychiatry* 2015, 5, e503.

CHAPTER ONE: Introduction

1.1 Addiction Overview

Drug addiction is a significant problem worldwide and is associated with substantial costs to both the individual and society as a whole. The cost of addiction has been estimated to be $55.2 billion dollars per year, with illicit drugs accounting for $8.2 billion dollars per year (1). This is associated with costs to the health system, cost relating to crime and loss of productivity (2). Addiction and substance abuse disorders are diagnosed using criteria outlined in the Diagnostics and Statistics Manual V (DSM V). The key characteristics of substance abuse disorders include uncontrollable drug use regardless of negative consequences such as loss of employment, relationship breakdown and financial problems (2). Importantly, addiction presents as a cyclical disorder in which many sufferers are unable to permanently recover from drug use despite multiple attempts to quit. The cycle of abuse typically begins with controlled, recreational drug use that can escalate to compulsive, uncontrollable use (3). Importantly, in the context of cocaine addiction, only a relatively small percentage (~16%) of users progress from controlled to ‘uncontrollable’ drug use and addiction - even though many people will experiment with drug use throughout their life (4, 5). Individuals suffering from addiction tend to make multiple attempts to abstain from drug use.

Depending on the class of drug, adverse physical symptoms develop from the acute withdrawal from the pharmacological effects of the drug. While some individuals will overcome these symptoms, up to 90% of addicted

individuals relapse within a year (5, 6). Indeed addiction is often defined as a chronically relapsing disorder (6). As such, relapse presents the greatest clinical hurdle in overcoming addiction. Addiction is a complex disorder and is exacerbated by a range of psychosocial factors. Whatever the underlying reasons leading to the addicted state, there are thought to be three primary triggers that precipitate relapsing into drug seeking behaviour after attempts to quit – re-exposure to the drug itself, exposure to discriminative or contextual cues previously associated with the drug and psychological stressors (7).

1.2 Treatments for Drug Addiction

Maintaining abstinence and preventing relapse to compulsive drug taking has been the primary goal of addiction treatment for some time (8). Despite this, the available options to suppress the relapsing nature of addiction remain largely ineffectual. Indeed, the majority of current addiction pharmacotherapies simply replace the addictive drug with analogues that help individuals cope with drug craving, such as methadone for opiate addiction and Sativex for cannabis use (9). While these strategies can curb some of the destructive behaviours caused by the primary addictive agent, these pharmacotherapies do not treat the root cause of relapse, the persistent neuroadaptations embedded in brain circuits that control normal reward-seeking behaviour. While many studies describe drug-induced synaptic plasticity in reward-seeking circuitry (10, 11), the persistent molecular neuroadaptations that underpin drug-related synaptic plasticity and relapse risk are less well understood (12).

1.2.1 Pharmacological Treatments

Pharmacological treatments for addiction generally aim to address short term reduction of withdrawal symptoms caused by abstaining from drug use, or the long term cravings and relapse risk associated with the disorder (13). They can be broadly separated into three categories: full agonists that directly stimulate the receptors in the brain; partial agonists that bind the receptors in the same way as full agonists, but which activate the receptor to a lesser degree; or antagonists that bind the receptor but do not activate it; rather, they simply block the site of action of drugs of abuse (13).

A number of pharmacological interventions are currently available to treat opioid addiction, targeting both short-term withdrawal symptoms as well as long-term relapse risk. Buprenorphine is a partial opioid receptor agonist that is primarily used in the treatment of opioid withdrawal symptoms (13). Similarly, Methadone, a complete opioid receptor agonist, is often used to aid in treatment of opioids such as heroin (14). Naltrexone, however, acts as an opioid receptor antagonist, blocking the receptor and thereby preventing opioid drugs from binding to and activating the receptor (15). Importantly, the most commonly used pharmacological treatments to prevent relapse act as replacement therapies, which aim to prevent opiates from exerting their actions on the brain. Inhibition of the rewarding effects of opioids is thought to reduce craving and thus decrease relapse to drug use, allowing individuals to return to work and resume normal social activities.

Importantly, similar replacement therapies are available to treat nicotine addiction. Nicotine replacement therapies such as nicotine gum or patches,

are generally used for short term cessation of smoking, and helps users to reduce nicotine consumption slowly, leading to improved treatments compared to abrupt withdrawal from nicotine use (16). In addition to nicotine replacement therapies, the partial nicotine agonist Varenicline can be used to treat nicotine addiction (17). Varenicline acts on nicotinic receptors to a lesser extent than nicotine, leading to decreased cravings in smokers. Moreover, it has a significantly longer half-life than nicotine, and therefore requires only 2 daily administrations in contrast to nicotine, which is usually administered multiple times per day, helping to improve patient compliance.

Similarly, a number of pharmacotherapies exist to treat alcohol addiction. Benzodiazepines are the most common pharmacotherapy used to treat short-term withdrawal symptoms. Withdrawal from alcohol can lead to increased heart rate and blood pressure, and anxiety (18). Benzodiazepines act on $GABA_A$ receptors, stimulating the binding of the inhibitory neurotransmitter GABA, which in turn acts to reduce heart rate, blood pressure and anxiety like symptoms (19). Importantly, however, long-term use of benzodiazepines can lead to physical tolerance, and therefore should be used only as a short-term treatment of withdrawal symptoms (19).

Disulfiram was a popular treatment for long-term maintenance of alcohol addiction. Disulfiram inhibits the enzyme responsible for metabolizing alcohol by breaking down acetaldehyde to acetate, aldehyde dehydrogenase (20). This leads to a build-up of acetaldehyde that interacts with Disulfiram to cause nausea, sweating, vomiting, hyperventilation and a number of other symptoms, leading to an aversion to alcohol through negative reinforcement.

However, a key constraint in the efficacy of disulfiram is patient compliance (20, 21). Naltrexone, an opioid receptor antagonist, is often used to reduce craving in alcohol dependence by preventing release of dopamine upon alcohol consumption and thus reducing the rewarding properties of the drug. naltrexone has been shown to not only reduce the frequency and intensity of drinking, but also reduce the risk of relapse (13). Importantly, however, evidence suggests naltrexone may have limited efficacy in severe cases of alcohol addiction (22). Similarly, acamprosate, a modulator of glutamate, can be used to decrease withdrawal and prevent relapse in alcohol abusers. Interestingly, studies from America have found little benefit to the use of acamprosate to treat alcohol addiction (23). However, studies from Europe have shown that the use of acamprosate in abstinent individuals leads to an increase in the number of people who remain abstinent from alcohol (24). As such, acamprosate appears to be most beneficial in the treatment of protracted withdrawal.

Importantly, although treatment options are available for a number of different drugs of abuse, no pharmacological treatments are available for use against psychostimulant addiction, including cocaine and amphetamines (13). A key constraint in identifying effective pharmacological treatments to aid in recovery from psychostimulant addiction is a lack of understanding about the long-term neurobiological changes that give rise to the persistent nature of the disorder. As such, treatment of psychostimulant addiction is currently limited to behavioural therapies that aim to change the behaviour of the individual to reduce relapse risk (25).

1.2.2 Behavioural Therapies

At the present time, frontline treatments for psychostimulant addictions are behavioural therapies. The primary forms of behavioural therapy used to combat drug addiction are Cognitive Behavioural Therapy (CBT), Community Reinforcement Approach (CRA) and contingency management (CM) (26). Generally behaviour therapies have limited success in the majority of individuals in the long term (8).

CBT aims to help individuals identify indicators of drug use, such as specific thoughts, feelings or events, and aid in the development of skills to cope (25). CBT is often applied through a structured 12-week course, usually consisting of 8-10 weekly 1-hour sessions. CBT has been shown to have some efficacy in the treatment of psychostimulant addiction including cocaine (27). Importantly, however, the efficacy of CBT appears to diminish over time, with limited effectiveness after 12 months (27).

CRA relies on the reinforcing capacity of natural social rewards (28). For example, positive behaviours such as abstaining from drug use will lead to positive improvements and support from social groups, such as family and friend. Similarly, CM is a behavioural therapy designed to loosely reflect operant conditioning, such that positive behaviours (e.g. abstinence) are rewarded with a reinforcing reward such as money (29). Evidence has shown that CM is somewhat effective in treatment of psychostimulant addiction. Importantly, the most beneficial results have been found using combination approaches incorporating both CRA and CM (28). However, while these behavioural therapies do have some efficacy in the treatment of addiction,

many individuals will still relapse into drug seeking behaviour after a prolonged period of abstinence. It is evident from the high rates of relapse associated with addiction that there are persistent changes in neuronal signalling that are yet to be fully elucidated. Without an understanding of these mechanisms we are unable to develop effective pharmacotherapies to aid in recovery from addiction.

Importantly, the current behavioural and pharmacotherapies have limited success in preventing relapse into drug seeking. Indeed, the current pharmacotherapies primarily function by activating or antagonising the receptors on which drugs of abuse act, and while these treatments are effective in treating short-term withdrawal symptoms following cessation of drug use, they fail to address the underlying maladaptions that contribute to the high rate of relapse seen in drug addiction. Improved pharmacotherapies would focus on reversing these long-term drug induced changes in neuronal processes ultimately leading to enduring drug abstinence and reduced relapse in these individuals. The lack of effective pharmacological treatment for relapse prevention stems from our incomplete understanding of the complex molecular neuroadaptations produced by drug taking.

1.2.3 Development of Novel Pharmacotherapies

Critically, attempts to improve our understanding of the neurobiology of addiction must consider a fundamental aspect of drug taking – that not all people who take drugs become addicted. It is therefore essential to understand the individual differences that lead these individuals to be susceptible to the development of addiction. Only by understanding the

underlying causes of the disorder can we develop targeted treatments to help reduce the substantial individual and societal costs associated with pathological drug use, as well as reducing drug related crime. Importantly, studies have shown that every dollar invested in addiction treatment yields up to $7 in savings from crime and healthcare costs (30). A major point of difference to the approach taken in this thesis is that any animal model of addiction used to identify potential therapeutic targets should recapitulate this individual vulnerability seen in drug addiction. The most effective method for studying the underlying neuromechanisms are preclinical animal studies, however, as will be discussed below, many previous approaches have significant limitations that have restricted our ability to tease out fundamental mechanisms and therefore to identify novel targets for pharmacological treatments for addiction.

1.3 Animal Models of Drug Addiction

As discussed above, the neurobiological mechanisms underpinning addiction are not well understood. Preclinical animal models are important tools to explore our understanding of the underlying mechanisms that contribute to the development of addiction. Importantly, there are a number of animal models commonly used to assess acute and chronic effects of drug use on addiction relevant behaviour and the brain. However, it is important to note that not all animal models mimic addiction *per se* and many studies fail to factor the pharmacological effects of cocaine exposure on putative molecular substrates involved in addiction.

1.3.1 Conditioned Place Preference

The conditioned place preference (CPP) model is a commonly used model for examining the rewarding properties of a drug. CPP involves placing an animal in an apparatus comprised of two or three chambers with distinct contextual stimuli/cues. Animals are placed in the apparatus and allowed to habituate, confirming whether the animals display a natural preference for either chamber (31). Following this habituation period, animals are administered drug via intraperitoneal (i.p.) injection and restricted to a single chamber, referred to the 'drug-paired' chamber. At a subsequent time, the animal is again placed in the CPP apparatus and allowed free access to both the drug-paired, neutral or saline chamber. The amount of time spent in the drug-paired chamber is considered to be an indication of the rewarding properties of a drug (31). Importantly, while this method is useful for examining the rewarding properties and associative learning mechanisms of drugs of abuse, CPP models lack face validity to the human condition.

The poor face validity of CPP is because the animals receive drug via experimenter administration, that is, they do not choose to self-administer the drug themselves. Furthermore, studies using CPP models, as with many other animal models, are often confounded by the amount of drug the animals are exposed to. For example, control animals are generally drug-naïve, and therefore the molecular and biochemical changes observed in these animals could be attributed to the acute pharmacological effects of the drug, rather than the underlying mechanisms leading to addiction. In summary, CPP is a useful procedure for examining proof of principle hypotheses regarding

contextual learning and reward processes but it lacks a key analogue of human drug taking - self-administration and regulation of drug in take.

1.3.2 Self-administration

Self-administration (SA) is considered the gold standard for studying drug taking, given that the animals are required to learn to lever press or nose-poke to receive an infusion of drug. To achieve this, animals are placed in an operant conditioning chamber and connected to a syringe pump via an indwelling intravenous catheter to allow self-administration. To receive an infusion of drug, animals are required to perform an operant task, such as pressing a lever, to activate the syringe pump. A key consideration in this procedure is the amount of drug consumed between experimental groups and the common misnomer that drug taking is synonymous with addiction. Indeed, many studies employing self-administration use a drug naïve control group, or short versus extended access exposure. Differences in drug consumption may confound results, such that the evoked changes reflect or mask underlying neurobiological mechanisms that contribute to addiction. Yoked control animals are often used in an attempt to control for these variables or the learning processes engaged during the act of self-administration. Yoking involves preparing animals with indwelling catheters as with test animals. These animals are partnered with a self-administration animal, and only receive an infusion of drug when their self-administration counterpart performs the operant task to receive drug. Thus these animals receive infusions of drug in a ‘yoked’ manner. Use of yoked animals allows experimenters to control for drug consumption, as these animals receive

identical amounts of drug as their self-administration counterpart, however unlike these animals, drug consumption is not contingent upon a specific behaviour (e.g. lever pressing). Yoked controls are an important way of controlling for drug consumption. *Importantly, we argue that further analyses are required to identify animals that show heightened vulnerability to addiction given our understanding that only around 15-30% of drug-takers meet criterion for an addiction diagnosis (4, 5).*

1.3.3 Addiction Phenotyping

As noted above, only a small percentage of individuals who experiment with drug actually develop full-blown addiction. Therefore, it is important to incorporate this individual variability into addiction models (5). One of the first to address this issue was Deroche-Gamonet and colleagues who applied a series of behavioural tests based on the DSM IV criteria for addiction. These focused on studying the inability to refrain from drug seeking during periods of drug unavailability, motivation to seek and consume drug, and persistent drug seeking despite negative consequences. Using this approach they showed that approximately 15% of animals express an addiction-like phenotype, consistent with data from human addiction studies (4, 5).

To assess addiction behaviours, animals were first trained to self-administer cocaine in once daily, 3 hour sessions. Once stable responding was achieved, the SA sessions were divided into periods of drug availability (DA), signalled by a distinct cue such as white noise, and periods of non-drug availability (NDA), signalled by a separate distinct cue such as illumination of a house light. Lever pressing during this NDA period was considered as an

index of the animals' inability to refrain from drug seeking during known periods of drug unavailability (5). This was factored as the first addiction score in their model.

The second behavioural test used in this phenotyping procedure was a progressive ratio (PR) test, designed to examine motivation or effort to seek and consume a drug. Animals were placed in the operant conditioning chamber, and the number of lever presses required on the operandum to receive a single infusion of drug was increased in a geometric progression. As the number of responses required to receive the reinforcer increases, animals reach a 'breakpoint' - the point at which they cease to perform the task to obtain drug. Animals that have a higher breakpoint are therefore considered to have exerted more effort or more highly motivated to seek and consume drug (5).

The third behavioural parameter they used assessed an important criterion of addiction; continued drug seeking despite negative consequence. To model this scenario in animals they paired drug infusions with a negative outcome, a mild foot shock (0.8mA for 2 seconds) delivered to the grid floor. Animals that continued to self-administer drug despite this aversive stimulus were considered compulsive drug-seekers (5).

Upon completion of these three behavioural tasks, addiction-like behaviour is assessed based on their scores. Animals in the top third of the distribution in a single test were classified as positive for that behaviour. As such, animals were divided into groups based on the number of positive

criteria reached (0-3 positive criteria). Those showing 3 positive responses for each behaviour were deemed to be ‘addict-like’ animals.

Importantly, as previously discussed, relapse is currently the biggest clinical hurdle in overcoming addiction (6). As such, this study further classified animals based on their propensity to relapse. Using both drug exposure and cue-induced reinstatement, the group demonstrated that rats that showed an increased propensity to relapse also showed significantly higher scores on each of the behavioural tasks assessed (5). Animals that scored in the top and bottom 40% of the distribution for reinstatement were classified as high (HRein) and low (LRein) reinstators respectively.

Given the importance of identifying the neurobiological mechanisms that contribute to relapse risk, our laboratory has begun to study vulnerability to relapse. Our approach uses a modified version of the model developed by Deroche-Gamonet et al. 2004 that places greater emphasis on propensity to relapse. We train animals in a similar way for drug-available versus non-available responding periods, and tests animals on repeated PR tests before allowing animals to undergo extensive extinction of lever pressing training after the final cocaine self-administration session. Extinction mimics the process for drug self-administration in the operant chamber, except that drug cues are absent and the operant lever responding does not result in infusion of the cocaine. Animals remain on this extinction procedure until their responding returns to a pre-determined baseline level of ≤ 6 lever presses/hour. Once this criterion has been reached, animals are placed in the operant chamber and re-exposed to the discriminative cues that were present

during the DA period. Reinstatement of lever pressing on the drug-paired lever is considered a gauge of propensity to engage in drug-seeking or relapse-like behaviour. In our model we have defined relapse vulnerability as those animals that score in the top forty percent of the distribution for reinstatement (32). The other factors that are important for addiction vulnerability as described in detail above are factored differently from Deroche-Gamonet et al. Thus, for an animal to remain classified as relapse vulnerable, they are required to score in top thirty percent for the two addiction relevant behavioural test, whilst those that score in the bottom of the distribution for each behaviour are said to be addiction-relapse resilient (32).

Importantly, we find that there are no significant differences in the amount of drug consumed between phenotyped groups - although there are significant differences in the addiction behaviours expressed by vulnerable versus resilient animals (32). We have argued that the molecular differences identified between phenotyped groups is more likely to reflect underlying changes that contribute to the development and persistent nature of addiction, rather than simply the pharmacological effects of the drug on key brain regions. Accordingly, the use of this model in conjunction with molecular and biochemical studies should increase our understanding of the neural mechanisms underpinning the high risk of relapse associated with addiction and lead to the identification of therapeutic targets to aid in recovery.

1.4 Brain regions associated with reward and decision-making.

A number of brain regions have been implicated in playing a role in the development and maintenance of addiction. Importantly, many of the brain regions identified in animal studies are evolutionarily conserved, suggesting that the fundamental causes of addiction are similar across species. Historically, a significant number of studies have focused on the so-called brain reward circuit, comprising a number of key brain areas particularly the projection from A10 dopamine cells in the ventral tegmental area (VTA) to the nucleus accumbens (NAC) region of the ventral striatum (VS) (33, 34). Extended parts of the reward circuit include the amygdala, hypothalamus, prefrontal cortex (PFC) and hippocampus (35). This evolutionarily conserved system is thought to be responsible for the reinforcing actions that underpin survival functions, e.g. finding food and a reproductive partner (35). A long held view is that the powerful pharmacological actions of drugs 'hijack' the VTA→NAC pathway. Consistent with this hypothesis all drugs of abuse increase dopamine levels in the NAC; however, the mechanism via which different drugs produce these effects can differ considerably.

1.4.1 Nucleus Accumbens and Addiction

There is a substantial body of evidence to show the NAC is a central player in regulation of the rewarding and reinforcing properties of drugs of abuse. The NAC receives connections from a number of other regions involved in the learning and memory and the addiction process, including the PFC, hippocampus and amygdala (36). The NAC region of the VS can be

further divided into two subregions, the shell (NACsh) and core (NACc), which have distinct roles in regulation of addiction behaviour (33).

A study by Taylor et al. 1984 demonstrated that the NAC plays an important role in regulation of conditioned reinforcers (37). They demonstrated that following administration of D-amphetamine directly into the NAC, animals increased locomotor activity and responding in an instrumental task for a conditioned reinforcer (37). However, it has been shown that the NAC subregions play differing roles in the regulation of these behaviours. For example, Parkinson et al. (38) demonstrated that a functional lesion of the NACsh prevents D-methamphetamine-induced increases in operant responding and locomotor activity. In contrast, lesions of the NACc had no effect on operant responding or locomotor activity in response to intra-NAC infusions of D-methamphetamine (38). Importantly, NACc lesioned animals showed increased responding for both conditioned and non-conditioned stimuli. This key study demonstrated a central role for the NACsh, but not NACc, in enhancing the response for stimuli associated with drugs, such as cocaine and amphetamines, thus playing a role in regulation of the rewarding properties of drugs of addiction. In contrast, the NACc is important for integration of the conditioned stimuli associated with drugs of abuse, thereby regulating conditioned responding for such drugs.

Further supporting a role for the NAC in the development of addiction dopamine signalling is altered in response to drugs of abuse as well as drug-associated stimuli (39). Ito et al. 2000 demonstrated that dopamine release was increased in both the NACc and NACsh following self-administration of

cocaine (39). Importantly, dopamine release following exposure to drug associated stimuli was restricted to the NACc, further establishing a critical role for this region in regulation of associative responding in response to drug use.

Other work supporting the distinct roles of the NACc and NACsh in regulation of these specific addiction-relevant behaviours, Sellings et al. 2006 demonstrated that animals with selective lesions of the NACsh showed reduced CPP in response to intravenous cocaine administration (40). However, animals with lesions of the NACc showed no change in cocaine-induced CPP. Given that CPP models are effective at indicating the reinforcing properties of drugs of abuse, this study further highlights the importance of NACsh function in regulation of the rewarding effects of cocaine.

Interestingly, the NACc and NACsh have been shown to have distinct roles in reinstatement of drug seeking. An important study by Bossert et al., 2007 demonstrated that the NAC subregions are essential for drug context- and cue-induced reinstatement (41). The group found that injection of a dopamine receptor (DR) 1 antagonist into the NACsh, but not NACc attenuated context-induced reinstatement of drug seeking. In contrast, administration of this antagonist into the NACc, but not the NACsh, attenuated cue-induced reinstatement. This study provides evidence of the distinct functional roles of the NAC subregions in reinstatement of drug seeking.

Together these studies provide evidence of the distinct roles of the NAC subregions in the context of addiction. Importantly, they highlight the importance of the NACsh in controlling the reinforcing properties of a drug, and the NACc in regulation of conditioned responding for drugs of abuse. Importantly, the NAC has direct inputs to the dorsal region of the striatum, suggesting these regions may function in cooperation to contribute to the pathological drug seeking associated with addiction.

1.4.2 Dorsal Striatum and Addiction

Largely due the highly influential but sometimes controversial dopamine hypothesis of reward and addiction, the field has focused heavily on the mesostriatal dopamine projection from the VTA on the ventral striatum. However, a number of important studies published in the last decade have identified that nigro-striatal dopamine neurons also recruited during reward-seeking albeit over a different temporal course during the addiction process (35, 42-46). These studies have also highlighted a role for the dorsal striatum (DS) in regulation of behaviour critical to the development of addiction. Like the ventral striatum and NAC, the DS can be divided into a number of functionally heterogeneous subregions, the most relevant to this thesis being the dorsomedial (DMS) and dorsolateral striatum (DLS) (47).

Functional imaging studies also support a role for the DS in addiction. For example, Letchworth et al. (2001) found that chronic cocaine self-administration increased dopamine transporter (DAT) binding in the striatum of Rhesus monkeys. Interestingly, these changes were in contrast to acute cocaine administration, where decreases in DAT binding were observed (42).

These results highlight temporal changes in neuronal signalling over the course of cocaine use. Moreover, the increases observed with chronic intake were dose dependent, with greater increases evident following chronic exposure to high doses of cocaine. Importantly, these changes were evident in both the dorsal and ventral areas of the striatum.

Nader et al. 2002 found further evidence of a role for the dorsal striatum in addiction in a study. This group found that chronic cocaine self-administration increased the density of DR 1 and 2 in the DL caudate of monkeys, a region homologous to the DLS in rats. Interestingly, however, acute cocaine use did not alter the expression of either D1 or D2 receptors in the striatum (43). Moreover, a similar study added further evidence for a role of the DS in cocaine addiction. Porrino et al. (44) demonstrated using glucose metabolism, a measure of functional activity, that acute cocaine intake decreased glucose metabolism, and thus activity, of the ventromedial, central and DM striatum in monkeys. Further, they found that following chronic cocaine use glucose metabolism was decreased in these same regions as well as in the DL striatum (44). Critically, it has been hypothesized that extended cocaine use leads to a shift in control from the goal-directed DMS to the habitual DLS (48). These results lend support to this hypothesis by demonstrating that changes in DLS signalling are evident following chronic, but not initial, cocaine use.

An important study by Vandershuren et al. 2005 demonstrated a role for the DS in the control of cocaine seeking where a conditioned stimulus is needed. The study found that infusion of dopamine receptor or α-amino-3-

hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR) antagonists into the DS reduced responding for cocaine in rats trained to self-administer on a second-order schedule of reinforcement (45). Interestingly, however, intrastriatal infusion of the N-methyl-D-aspartate receptor (NMDAR) antagonist had no effect of responding on this schedule. This study provides evidence that dopamine receptor and AMPAR signalling in the DS is critical for the cue-induced cocaine seeking.

In a seminal study by Volkow et al. (46) they found that exposing abstinent human cocaine abusers to drug-associated cues led to a significant increase in dopamine release in the DS, with little change in dopamine release in the NAC. Further, this dopaminergic release was accompanied by self-reported cravings for cocaine in these individuals, demonstrating a role for the DS in regulation of compulsive drug seeking and addiction relevant behaviour (46).

It is evident from these studies that the DS is a critically important region in a number of addiction relevant processes. Moreover, a number of these studies identified dopamine as a critical neurotransmitter implicated in these processes. Importantly, dopamine signalling has often been demonstrated to be a critical neurotransmitter involved in the development of addiction (49). Dopamine signalling within the NAC has been shown to be essential for promoting the reinforcing properties of drugs of abuse. These results extend our understanding of the role that dopamine signalling in the DS plays in the development of addiction. However, though these results provide insight into the broad role of the DS in the development of addiction, it is important to

note that the DS can be divided into functionally distinct subregions. Important work has begun to identify complex cognitive functions for discrete regions of the dorsal striatum.

The DMS plays a role in goal-directed, or action-outcome, behavioural control (50). Goal-directed actions refer to behaviours that involve performing a specific task or action to obtain a specific goal, or outcome – for example, pressing a lever to obtain a food pellet (50). Critically, goal-directed behaviour is directly related to the value of the goal, such that outcome devaluation leads to a reduction in the task. The DMS receives input from a number of brain regions relevant to the addiction process, including the PFC, which has also been implicated as important for goal-directed learning (51, 52), as well as premotor regions of the cortex (53). A 2005 study presented the first evidence that the DMS is critical for regulation of action-outcome associations necessary for goal-directed behaviour (54). The study demonstrated that instrumental responding decreased in animals that received DMS lesions prior to training compared to controls. Moreover, lesioned animals were unable to adjust the rate of instrumental responding following devaluation of the reward (54). Outcome devaluation is an important animal model used to differentiate between goal-directed and habit like responding, which relies on the concept that flexible decision making associated with goal-directed behaviour is directly related to the value of the reward, such that changes in this value will ultimately affect the behaviour. An example of this phenomenon is evident in animals that have been trained to perform a task, such as lever pressing, to obtain a reward, such as a food pellet. However, if an animal is allowed to eat to satiety, therefore decreasing the value of the food reward,

lever-pressing behaviour will be reduced. Importantly, insensitivity to outcome devaluation is indicative of habit-like behaviour, indicating that behavioural control has devolved to a rigid, inflexible form, whereby the value of the outcome no longer influences the behaviour itself. Yin et al., 2005 demonstrated that after short periods of training, sham animals showed a decrease in responding following outcome devaluation, while those animals with DMS lesions showed no change in responding, suggesting a deficit in goal-directed behaviour and a premature shift toward habit-like control (54). Importantly, they subsequently demonstrated that animals that received DMS lesions following initial operant training also showed insensitivity to outcome devaluation in comparison to sham animals (54). This evidence strongly implicated the DMS in regulation of goal-directed behaviour and the acquisition of action-outcome associations.

Importantly, loss of sensitivity to outcome devaluation has shown that this devolution of control can be accelerated by exposure to stress, high-fat foods, and drugs of abuse. Critically, aspects of the addiction phenotype, including compulsive drug seeking, are reminiscent of habit-like behaviour. A key clinical feature of addiction is an inability of addicts to reduce drug-taking behaviour despite negative consequences, such as financial and social complications. This diagnostic feature of addiction is akin to the loss of sensitivity to outcome devaluation seen in habit-like behaviour tests, suggesting that addiction represents a pathological habit. Key to understanding the causes of these behavioural changes is a better understanding of the brain regions involved in the development and expression of habit-like behaviour. Whereas the DMS appears important for

action-outcome directed behavioural control, the DLS appears more important for stimulus-response, or habit-like behaviours. Under these circumstances, stimulus-response association comes to control the action outcome relationship (50). The transition from goal directed to habit like behaviour often occurs following periods of extended training, and is thought to reflect a devolution of control from goal-directed DMS control to habit-like DLS control. Yin et al., (55) found that the DLS is critical for regulation of these stimulus-response associations associated with habit-like behavioural control. For example, they found that following extended training paradigms, animals with DLS lesions continued to show sensitivity to devaluation tests, while sham animals did not (55). In a subsequent study they trained animals to respond on a lever for a food reward for an extended period of time. Animals then received an intra-DLS injection of muscimol or vehicle control and were trained on an omission paradigm, whereby the animal is now required to refrain from pressing the lever to obtain the reward. In this way the animal's habit versus goal-directed behavioural control was tested. Animals that received vehicle injections showed no ability to learn the omission schedule, indicating that in these animals behaviour was under habitual control. Conversely, animals that received muscimol injections to inhibit DLS function were able to learn the omission procedure, suggesting that these animals' behavioural control had reverted to goal-directed regulation.

Human studies also support a role for the DLS in habit based responding. For example, Tricomi et al., 2009 trained human participants either on a moderate or extended amount to respond for a food reward and assessed the neuronal regions that show activation in response to the reward (56).

Following training, the food reward was devalued by eating until satiety, and participants were placed in a magnetic resonance imaging (MRI) scanner. Following outcome devaluation, moderately trained participants significantly decreased responding for the food reward, while those that received extended training continued to respond for the reward (56). These findings are similar to the animal literature showing that overtraining of an instrumental tasks lead to habit formation (55). fMRI revealed that that during the devaluation test, overtrained participants displayed activation of the caudo-ventral putamen, the human homologue of the rodent DLS. These human and rodent studies provide strong evidence for the role of the DLS and its homologous regions in the regulation of rigid, inflexible behaviour.

It is evident from these studies that the DS is a critical region for control of behaviour, and is increasingly important in the context of addiction. Given that addiction is characterised by inflexible behaviour, it is considered to be a disorder of learning and memory (3). A recent hypothesis has suggested that addiction may in fact represent a devolution of control for the goal-directed DMS control to habit like DLS control. Specifically, it has been suggested that DMS deficits may be responsible for the development of many compulsive aspects of the disease – in particular drug-seeking and relapse. These DMS deficits cause impairments in goal-directed behaviour and lead to a premature shift in control and the development of habits. Further, DMS deficits may render individuals incapable of altering their behaviour, despite negative outcomes associated with drug addiction. As such, understanding the molecular profiles of these DS subregions is critical for our understanding of the development and long-term maintenance of the disease.

1.5 Synaptic plasticity

1.5.1 Overview of Synaptic Plasticity Processes

Synaptic plasticity processes are critical neural substrates of learning and memory. These processes, including long term potentiation (LTP) and long term depression (LTD), are responsible for strengthening and weakening synapses respectively. One consequence of impairments in synaptic plasticity are constraints on the capacity of the brain to adapt to new information and to learn new behavioural strategies. Importantly, synaptic plasticity impairments have been implicated in a number of neuropsychiatric disorders, including schizophrenia (57, 58). There is now a significant body of evidence to suggest that changes in the ability to evoke synaptic plasticity contributes to the lasting nature of addiction and the behavioural inflexibility seen in addiction.

1.5.2 Synaptic plasticity deficits in addiction

Synaptic plasticity refers to the strengthening or weakening of synaptic connections between neurons. Long-term strengthening of synapses is commonly referred to as LTP (59), while the process of weakening is referred to as LTD (60). Importantly, distinct molecular mechanisms have been shown to be responsible for these processes.

Key regions in the ‘brain reward circuit’ display long lasting changes in plasticity in response to drug use, including the NAC and DS (61-63). Moreover, persistent impairments in these processes have been linked with the risk of relapse in addiction (3, 10, 64-66).

An important study demonstrating plasticity deficits in addicted animals was performed by Kasanetz et al. 2010. They phenotyped animals into addiction vulnerability groups (67) and showed that during the early stages of the addiction cycle, cocaine exposure produced deficits in the ability to evoke LTD in the NAC of all animals. However, LTD deficits were only persistent in the NAC of rats that showed an 'addiction-like' phenotype using a procedure to identify addicted animals early in the addiction cycle (68).

Interestingly, other studies have identified a long-term impact of cocaine on ability of cell groups in reward centres to undergo plasticity. For example, Chen et al., 2008 demonstrated that cocaine self-administration led to enhanced LTP in the VTA compared to yoked-cocaine controls (69). Further, this potentiation was shown to be persistent, whilst LTP produced in response to natural rewards was transient.

As alluded to above, persistent impairments in the ability to evoke synaptic plasticity may contribute to deficits in the capacity to learn new behaviours, leading to the inflexible, habitual responding observed in drug addiction. To understand the potential molecular changes responsible for the impairments in plasticity in addiction vulnerable animals our group recently phenotyped a cohort of cocaine self-administering rats in addiction vulnerability groups. We found that animals phenotyped as addiction-relapse vulnerable showed deficits in a number of synaptic plasticity genes, including activity-regulated cytoskeletal-associated protein (*Arc)*, the mechanistic target of Rapamycin (*mTOR)* and dopamine receptor 1 (*Drd1*) (32). These genes have all been shown to play a role in regulation of addiction relevant

behaviour, and represent likely candidates for long-term adaptations underpinning addiction. In the following section I will outline recent evidence linking these molecules with the development and expression of addiction.

1.5.3 Mechanistic Target of Rapamycin

In the brain, mTOR acts as an important signalling node integrating synaptic activity with protein synthesis (70). mTOR is a serine threonine kinase involved in protein translation including in dendrites (71). This role extends to the production of proteins required for plasticity and behavioural tasks requiring new learning (72). mTOR combines with separate sets of proteins to form two distinct complexes: **mTORC1** and **mTORC2** (73). Importantly, these separate complexes have distinct effector signalling pathways and thus have very different effects on cellular function (74). To date, work in the brain has mostly focused on mTORC1, with studies showing that it regulates protein synthesis necessary for LTD and LTP (75). More recently, a role for mTORC2 in drug-induced structural plasticity has also emerged (76), but the possibility that this second complex leads to relapse-relevant changes in the NAC remains untested.

1.5.3.1 Role of mTOR in Synaptic Plasticity

mTORC1 controls translation through phosphorylation of two key signalling molecules, the S6 ribosomal kinase 1 (p70s6k) and eukaryotic translation initiation factor-4E binding protein (4E-BP)(77). Importantly, phosphorylation status of these downstream effector molecules can be used as a biochemical readout of mTORC1 activity in vivo. For example, phosphorylation of p70s6k at threonine-389 site is used as gauge of mTOR

activity. mTORC1 has also been shown to play a role in the regulation of a number of key cellular processes, including cellular metabolism, division and growth capacity (78). Notably, dysregulated mTOR signalling has been observed in a number of neurological diseases, and is known to play a role in neuronal development (78, 79). Within the brain, mTORC1 expression in dendrites has been found to contribute to LTP and LTD (80). These effects have been linked with its ability to promote local dendritic protein translation including those required for learning and memory (81). Dysregulated mTORC1 signalling may contribute to neuroadaptations that disrupt learning and promote the development of addiction.

Important to implicating mTOR in cellular processes including plasticity was the identification of the allosteric inhibitor rapamycin. Inhibition of mTORC1 in the medial PFC using rapamycin has been shown to impair long-term fear memory (82), while systemic rapamycin treatment led to deficits in retrieval of spatial memory (83). Moreover, mTORC1 inhibition in the hippocampus or amygdala of rats impaired novel object recognition (84) and long-term memory consolidation in an avoidance-learning task (85).

1.5.3.2 Role of mTOR in drug use

Importantly, a number of recent studies have assessed changes in mTOR activity in response to drugs of abuse, and how mTOR signalling may regulate synaptic plasticity processes and addiction relevant behaviour.

A key study by Mameli et al. 2007 found that mTORC1 plays a key role in cocaine-induced LTP in the VTA. They found that pharmacological activation of metabotropic glutamate receptor 1 (mGluR1) reversed cocaine-induced

LTP in the VTA, and further that this process was mediated by mTORC1 (86). Specifically, mTORC1 is required for synthesis and insertion of Ca2+ impermeable ionotropic glutamate receptor 2 (GluA2) AMPAR subunits. Importantly, potentiation of dopaminergic VTA neurons has been shown to be critical for subsequent synaptic plasticity in the NAC (62). Similarly, it has been shown that exposure to cues previously associated with cocaine use increased mTORC1 activity in the NAC (87). Further, these authors found that inhibition of mTORC1 in the NACc using rapamycin prevented cue-induced reinstatement of cocaine seeking in rats (87).

A study by Wu et al. 2011 found an increase in phosphorylated p70s6k protein levels in a number of key neuronal regions, including the NAC, VTA and cortex 1 hour after animals received an injection of cocaine. Further, these authors also showed that this effect can be reversed by prior administration of the selective mTORC1 inhibitor rapamycin (88). Similarly, Puighermanal et al., 2009 demonstrated that there was a significant increase in mTORC1 expression in the hippocampus of rats 30 minutes after exposure to tetrahydrocanabanoid (THC), and that this effect returned to baseline levels 4-6 hours following THC exposure (89). A subsequent study by the same group demonstrated that these changes in mTORC1 expression were also evident in the striatum, prefrontal cortex and the amygdala, suggesting an important role for mTORC1 signalling in a number of addiction relevant brain regions in response to THC (89). Similarly, it has been shown that similar effects on mTORC1 expression are evident in response to methamphetamine and morphine exposure, with increased phosphorylated p70s6k seen in the NAC 3 days post methamphetamine administration (90),

and increased VTA mTORC1 expression following morphine administration (91). Finally mTORC1 expression was increased in the NAC following acute exposure to alcohol. Importantly, these studies demonstrate that while the acute pharmacological effects of drugs of abuse differ, there appear to be similar mechanisms that may contribute to the neuroadaptations that ultimately lead to addiction.

A study by Cui et al. 2010 used a CPP model to outline the role of mTORC1 in regulation of specific addiction relevant behaviours and the reinforcing properties of these drugs. They found that rapamycin delivered directly to the hippocampus before morphine administration prevented the acquisition of CPP. Similarly, the study found and increase in mTORC1 expression in the hippocampus following re-exposure to a chamber linked with morphine exposure in animals that had previously acquired morphine CPP (92). However, though these studies provide evidence that mTORC1 expression is altered following exposure to drugs, the method of drug administration poorly mimics the human condition.

Neasta et al. 2010 used a self-administration paradigm to assess changes in mTORC1 expression associated with alcohol use. The authors found that excessive alcohol consumption led to an increase in mTORC1 expression in the NAC 24 hours after final alcohol administration. Furthermore, they found that systemic administration of rapamycin reduced the volume of alcohol consumed by both C57B/6J mice and Long Evans rats, while having no effect on a natural reward (93). Similarly, Barak et al. 2013 demonstrated an increase in mTORC1 activity following exposure to alcohol-

related cues in the central amygdala (CeA), and the prelimbic (PrL) and occipitofrontal (OFC) regions of the cortex. Further, infusions of rapamycin into the CeA after a period of abstinence impaired reconsolidation of alcohol related memories, which ultimately supressed relapse in rats (94). Critically, retrieval of alcohol-associated cue memories increased expression of mTORC1 and *Arc* in the amygdala, OFC and mPFC. *Arc* is a key regulator of synaptic plasticity and has also been implicated in playing a role in the propensity to develop addiction and relapse (32). The role of *Arc* in synaptic plasticity and the addiction process will be discussed in more detail below.

In summary, important studies have now implicated mTOR in regulation of synaptic plasticity and memory formation and reconsolidation. Moreover, there is a significant body of evidence to suggest the mTORC1 signalling is altered in response to drugs of abuse. Importantly, however, though these studies have shown a role for mTORC1 in regulation of addiction relevant behaviours, few studies have addressed the mechanisms by which these changes in mTORC1 signalling occur. Moreover, few studies have addressed the role of mTORC1 in the regulation of behavioural indices of addiction. As such, further research is needed to fully elucidate the role of mTORC1 signalling in the development and persistence of addiction.

1.5.4 Activity-Regulated Cytoskeletal-Associated Protein

As described above *Arc* is considered a ‘master regulator’ of synaptic plasticity. *Arc* is an immediate early gene that functions in the regulation of AMPAR endocytosis. Furthermore, it has been shown to play a role in synaptic strengthening through the stabilization of F-actin filaments, which is

in turn critical for localization of cellular machinery required for translation of new proteins (95, 96). Importantly, changes in *Arc* expression have been shown to occur in response to exposure to drugs of abuse, including cocaine, amphetamine and alcohol. These changes have been observed in a number of regions, including the DS and NAC.

Arc plays an important role in regulation of learning and memory processes by influencing the expression of LTP and LTD. Steward et al. 1998 demonstrated that exposure of hippocampal neurons to LTP-like stimuli, including high frequency stimulation, lead to subsequent induction of *Arc* expression (97). A subsequent study demonstrated that inhibition of *Arc* expression in the hippocampus using antisense (AS) oligonucleotides (ODNs) impaired consolidation of spatial memory (98). Moreover, hippocampal-targeted Arc AS-ODNs 1.5 hours prior to LTP induction lead to deficits in the maintenance of LTP in rats (98). In agreement with these findings, infusion of Arc AS-ODNs into the hippocampus of rats during early phase LTP led to a transient deficit in LTP, while infusion of *Arc* AS-ODNs 2 hours following LTP induction led to a reversal of LTP in hippocampal neurons (96). Interestingly, *Arc* knockout mice showed enhanced early phase LTP, 15 minutes post induction, while these animals also showed significant deficits in late phase LTP (99). The opposing effect of *Arc* inhibition on early phase LTP may reflect the differing role in *Arc* in neuronal regions. Importantly, these *Arc* knockout mice showed impaired consolidation of long-term memory, with no effect on short term memory (99). Demonstrating a role for *Arc* in memory processes, Ploski et al. 2008 showed that infusion of Arc AS-ODNs into the lateral amygdala of rats impaired consolidation of Pavlovian fear conditioning

(100). Park et al. 2008 demonstrated a role for *Arc* in LTD. Thus, *Arc* knockout mice failed to elicit normal mGluR-mediated LTD (101). As such, there is a substantial body of evidence demonstrating a role for *Arc* in the key neural substrates of learning and memory, LTP and LTD.

The molecular mechanisms by which *Arc* regulates these processes is thought to involve the regulation of AMPAR subunit trafficking and remodelling of dendritic spines. Modulation of AMPAR subunits are critical for LTD and LTP, which requires endocytosis and exocytosis of these receptors respectively (102). Importantly, overexpression of *Arc* in hippocampal cell culture has been shown to lead to downregulation of AMPAR and increased endocytosis of these receptors. Conversely, cultured *Arc* knockout hippocampal neurons showed increased expression of GluA1 AMPAR subtypes on the surface of the cells and significantly reduced AMPAR endocytosis (102). These cells also showed deficits in AMPAR scaling, thought to be a mechanism of homeostatic regulation of synaptic plasticity in neurons. It has been suggested that persistent increases in neuronal excitability lead to a compensatory increase in *Arc* expression, that subsequently leads to increased endocytosis of AMPARs to restore activity to basal levels. Conversely, persistent decreases in neuronal activity are suggested to lead to a concomitant decrease in *Arc* expression that acts to decrease endocytosis of AMPARs. Importantly, this homeostatic plasticity has been shown to occur in neuronal cultures (103).

In addition to regulation of AMPARs, *Arc* has also been implicated in regulation of dendritic spine structure through actin remodelling. LTP in the

hippocampus has been shown to be associated with increased F-actin content in the spine, synapse diameter and cofilin phosphorylation (104). Importantly, when LTP was reversed 2 hours following induction with high frequency stimulation using Arc AS-ODNs, the study found a dephosphorylation of cofilin and a loss of F-actin in the spines (96). Furthermore, when used in conjunction with an F-actin stabilizing drug, Arc AS-ODNs were unable to reverse LTP (96).

These studies help to highlight the complicated role of *Arc* in regulation of synaptic plasticity processes, which subsequently affect learning an memory. Given that addiction is often considered to be a disorder of learning and memory, a number of studies have focused on the role of *Arc* in addiction relevant processes.

Importantly, Fumagalli 2009 demonstrated that a single cocaine self-administration session led to an increase in *Arc* expression in the mPFC compared to yoked cocaine or saline animals (105). Similarly, self-administration of heroin has been shown to cause increased *Arc* expression in the mPFC, NAC and DMS (106). A key study by Hearing et al. 2008 demonstrated that Arc expression was increased in the DLS and NAC following re-exposure to an operant chamber associated with cocaine self-administration (107). Similarly, the same group also showed that Arc AS-ODN administration in the rat DLS impaired the induction of *Arc* following a single cocaine administration (108). However, they also found that Arc AS-ODN did not affect responding when animals were re-exposed to a cocaine paired environment, however, in subsequent extinction sessions, animals that

received Arc AS-ODN administration had significantly greater responding, suggesting that *Arc* expression is important for extinction learning.

Data reviewed above indicates that *Arc* is a key regulator of synaptic plasticity and dysregulated *Arc* signalling has been implicated in addiction. Critical synaptic plasticity processes including LTD have been shown to be disrupted following cocaine self-administration (68, 69). *Arc* is a critical regulator of synaptic plasticity processes, and is altered in animals following cocaine use (32), *Arc* likely represents an important player in the context of addiction. However, the mechanisms contributing to the deficits in *Arc* expression in these animals are poorly understood. Given that *Arc* is an immediate early gene and its expression is finely regulated, is has been suggested that post-transcriptional regulation by microRNA (miRNA) may reflect an important mechanisms by which these alterations in *Arc* signalling may contribute to the development of addiction.

1.6 microRNA

1.6.1 Overview of miRNA

Regulation of gene expression is critical in fine-tuning synaptic plasticity processes. miRNAs are important regulators of mRNA expression, and have been implicated in the control of a number of synaptic plasticity processes, and represent important candidates in the control of the neuroadaptations that lead to addiction.

miRNAs are short, non-coding RNA molecules approximately 22 nucleotides in length that regulate gene expression and thereby protein

translation. miRNAs are highly conserved across evolution (109, 110), with more than 1800 human miRNA identified in Release 20 of miRBase (111). Importantly, a recent study identified more than 3700 novel human miRNA (112), suggesting a critical role for miRNA in a vast array of biological processes. Since miRNA were first discovered in C. elegans, they have been shown to play key roles in the regulation of fundamental biological processes, such as cell differentiation and neurodevelopment, including dendritic spine morphology and synaptic plasticity (113). Further, it has been suggested that up to 60% of all human genes are regulated by miRNA (114). Moreover, given the role of miRNA in regulation of neuronal development and synaptic function, small non-coding RNA molecules have been implicated in the development of many neurological and psychiatric disorders, including schizophrenia, and more recently, addiction.

1.6.2 miRNA Biogenesis

The first stage of miRNA biogenesis involves transcription of the double stranded stem-loop miRNA precursor, referred to as the pri-miRNA (115). This transcription is performed primarily using the enzyme RNA polymerase II (110), however evidence has shown that RNA polymerase III is also responsible for the transcription of some pri-miRNA (116). Following transcription, the pri-miRNA is cleaved by the RNase III endonuclease *Drosha* to form the intermediate pre-miRNA (117). Following this the pre-miRNA is transported from the nucleus to the cytoplasm via the receptor Exportin-5 (117). Once in the cytoplasm, the pre-miRNA loop and terminal base pairs are cleaved by a second RNase III endonuclease termed Dicer to

leave two single RNA strands approximately 22 nucleotides in length (117). These strands are comprised of the mature miRNA, and the strand from the opposing arm of the stem loop precursor, referred to as the miRNA* (117). While this miRNA* strand is often degraded (118), evidence has shown that in some instances miRNA* strands have the capacity to function as mature miRNA molecules and regulate gene expression as well (117).

1.6.3 Mechanisms of miRNA regulation of gene expression

To regulate mRNA expression, the mature miRNA strand is incorporated into the RNA-induced silencing complex (RISC). RISC-bound mature miRNA can bind to the 3' untranslated region (UTR) of the target mRNA. The most critical region of the miRNA for this binding is nucleotides 2-7 at the 5' end, referred to as the seed region (119). To regulate expression of their mRNA targets the seed regions bind with perfect complementarity to the 3' UTR. There are two mechanisms by which RISC-incorporated miRNA can regulate gene expression – either via translational repression or cleavage and subsequent degradation of the mRNA target (120). Importantly, the mechanism by which miRNA regulate gene expression generally reflects the complementarity with which they bind to their target. Binding can occur via perfect complementarity, observed primarily in plant interactions, or imperfect base pairing as occurs more commonly with mammalian miRNA::mRNA interactions. The exact mechanisms by which miRNA prevent translation of mRNA are poorly understood (121). However, the majority of miRNA::mRNA interactions result in reductions in the amount of mRNA targets, suggesting that mRNA degradation may occur through prolonged miRNA repression

(122). This degradation may be a result of deadenylation of the mRNA target (118).

1.6.4 Identification of miRNA targets

A single miRNA can regulate a number of mRNA targets given the size of the seed region required for a miRNA to bind to its target mRNA. Similarly, a single mRNA may be regulated by a number of miRNA regulators. Accordingly, identification of miRNA::mRNA interactions is difficult. The primary method of identifying miRNA::mRNA interactions is to use computational algorithms to predict interactions. Confirmation of putative interactions is then performed using experimental techniques such as luciferase reporter gene assays. Complicating the identification of miRNA::mRNA interactions are the prediction algorithms and programs available resulting in the potential to identify many possible interactions. The huge number of potential interactions means that the majority of miRNA::mRNA interactions have yet to be assessed functionally.

In summary, miRNA represent an important epigenetic mechanism of posttranscriptional control. Given that miRNA also controls gene expression for synaptic plasticity-relevant molecules, including at the synapse, they represent potential substrates for the neuroadaptations that occur in addiction. A better understanding of the interactions between miRNA and their mRNA targets in the context of synaptic plasticity and compulsive drug taking will increase our understanding of the neurobiological mechanisms of addiction, which will in turn aid in the identification of improved therapeutic targets for treatment of relapse and addiction.

1.6.5 miRNA in synaptic plasticity

As previously discussed, impaired synaptic plasticity processes, important neural substrates of learning and memory, have been implicated in the development of addiction. However, the neural mechanisms that contribute to these deficits are poorly understood. There is an increasingly large body of evidence to suggest that miRNA play a role in regulation of the molecular and cellular processes required for the induction and maintenance LTD and LTP.

An important study demonstrating a broad role for many miRNA in learning and memory formation was conducted by Konopka et al. 2010. Introduction of a mutant form of Dicer, a critical miRNA processing molecule, into adult rat brains prevented maturation of miRNA (123). As a result of the lack of mature miRNA, this study observed a significant improvement in the performance of these animals in a number of tests designed to assess learning and memory. Furthermore, they found that animals possessing mutant Dicer showed alterations in dendritic spine size. As such, Konopka et al. 2010 suggested that lack of mature miRNA in the adult brain may alter synaptic protein translation, leading to the morphological changes in dendritic spines that facilitates synaptic remodelling and subsequently improves performance in learning and memory tests. This study provides evidence for a broad role of miRNA in regulation of synaptic transmission in the brain.

Activity dependent changes in miRNA expression support a role for these RNA species in plasticity. For example, Nudelman et al. 2010 found that miR-132 expression is increased in response to activity dependent plasticity (124). Specifically, they found pharmacological stimulation increased miR-132

expression in the hippocampus. Further, activation of olfactory neurons using odorant exposure increased miR-132 primary transcript expression (124). Moreover, Lambert et al. 2010 found that miR-132 (125) overexpression in hippocampal neurons compromised the ability of these neurons to evoke LTD (125). These studies support a role for miR-132 in regulation of synaptic plasticity processes in hippocampal neurons.

miR-134 is another miRNA implicated in structural plasticity through the regulation of dendritic spine growth (126). Thus, overexpression of miR-134 inhibited Lim-domain-containing protein kinase (Limk1) and decreased the width and volume of dendritic spines. Limk1 is a protein kinase involved in the regulation of actin filaments, and has been shown to affect dendritic spine morphology (127). Brain derived neurotrophic factor (BDNF) reversed the inhibitory actions of miR-134 and restored Limk1 function. Schratt et al. 2006 suggested that miR-134 regulates Limk1 repression until synaptic stimulation increases BDNF expression, which then inhibits miR-134, leading to a derepression of Limk1. This study provides strong evidence for the role of miR-134 in the formation and maturation of long-term synaptic plasticity.

A subsequent study by Gao et al., 2010 examining the role of miR-134 in synaptic plasticity processes found that it was critical for the expression LTP in hippocampal neurons (128). They found that miR-134 overexpression impaired LTP induction in the hippocampus via repression of cAMP response element-binding protein (CREB). miR-134 knockdown led to a derepression of CREB, and restored the ability of hippocampal neurons to evoke LTP. Given the importance of CREB, a transcription factor critical for synaptic

plasticity (129), miR-134 is likely to be an important player in regulation of these processes.

BDNF is a critical regulator of synaptic plasticity, and has been shown to interact with miRNA to regulate LTD and LTP (126). For example, Mellios et al. 2008 found that miR-30a-5p and related family group can regulate BDNF expression in the PFC (130). The bi-directional regulation involving BDNF and miRNA expression indicates the complex role these molecules play in the regulation of genes required for synaptic plasticity in the brain. Given the extensive evidence of a role for miRNA in synaptic plasticity processes, and that these processes are dysregulated in addiction, studies have begun to address the role of miRNA in the development of addiction.

1.6.6 miRNA in addiction

Given the relative recent discovery of the role of miRNAs in plasticity, understanding the role of non-coding RNAs play in addiction is an emerging field. One of the first to demonstrate a potential role for miRNA in the addiction process was Chandresekar et al., 2009. Using i.p. administered cocaine exposure, they found that several miRNA were differentially expressed in addiction relevant brain regions (131). For example, miR-124 and let-7d, were significantly down regulated in the VTA and hippocampus. The expression of let-7d was also decreased in the PFC. They also observed that the expression of miR-181 was significantly up-regulated in the hippocampus and NAC.

A subsequent study by the same group examined the functional role of these miRNA using the CPP model (132). Lentiviral mediated overexpression

of miR-124 and let-7d, or inhibition of miR-181a using a lentivirus silencing construct, was found to attenuate cocaine-induced CPP. Conversely, inhibition of both miR-124 and let-7d and overexpression of miR-181a enhanced cocaine-induced CPP. Moreover, they identified a number of key mRNA regulated by these miRNA, including BDNF, the glutamate receptor GRM5 and Methyl-CpG Binding Protein 2 (MeCP2). These results suggest that changes in expression of these miRNA in the NAC can alter the ability of cocaine to produce the rewarding effects that influence subsequent use of the drug.

As explained in Section 1.3.1 experimenter-administered cocaine injections do not closely mimic human drug taking. Therefore, although these studies provide interesting insight into the direct effects of cocaine administration on neuronal miRNA and the effect these miRNA have on the rewarding properties of cocaine, their role in the processes concerning compulsive cocaine taking are less clear. Indeed, the level of each miRNA was compared to a drug-naïve control, and so these data are potentially confounded by the direct pharmacological effect of cocaine on miRNA expression. Furthermore, these studies do not address individual differences in miRNA::mRNA interactions that contribute to the development of addiction.

In this regard, Hollander et al. 2010 conducted a seminal investigation into the role of miRNA in the regulation of addictive behaviours (133). They showed that animals given extended access to cocaine self-administration showed significantly higher levels of miR-212 in the DS compared to animals that had restricted access to cocaine or to naïve controls. To test the

functional relevance of this increase, Hollander et al., used a lentivirus to overexpress miR-212. This manipulation led to a substantial decrease in cocaine self-administration. Conversely, knockdown of miR-212 using a locked nucleic acid-modified (LNA) oligonucleotide increased cocaine intake (133). miR-212 knockdown also increased responding in time-out periods, where cocaine is not administered following cocaine infusions. Responding during time-out, where conditioned cues signal that drug is not available, is considered a measure of compulsive drug seeking. The authors hypothesised from their data that miR-212 signalling represents a protective homeostatic mechanism that is engaged to control the neuroadaptations produced by chronic cocaine consumption.

To further understand the signalling pathways through which miR-212 might mediate its protective effects, Kenny and colleagues performed further studies *in vitro*. Overexpression of miR-212 in human embryonic kidney (HEK) cells increased CREB; this increased CREB is linked with decreasing the motivational properties of cocaine. In support, miR-212 was unable to regulate a mutant version of the CREB gene. Further, it was shown that the positive effects of miR-212 on CREB signalling were inhibited when the CREB gene was engineered to harbour a mutation in the binding site for the essential co-activator of CREB TORC2. This study demonstrated a critical role for the CREB-TORC signalling pathway in the protective effects of miR-212 signalling on drug consumption.

Importantly, Kenny and colleagues also that found that inhibition of Raf1, necessary for the phosphorylation of adenylyl cyclase, impaired miR-212

regulation of the CREB-TORC2 signalling pathway, preventing amplification. Further, SPRED1, a negative regulator of Raf1, is under translational repression by miR-212. Continued investigation of the relationship between miR-212 and CREB-TORC found that repression of SPRED1 increased Raf1 signalling and CREB-TORC. TORC1 overexpression decreased cocaine consumption in extended access animals, supporting the role for miR-212 control of CREB-TORC signalling as a protective response to chronic cocaine self-administration (see Figure 1).

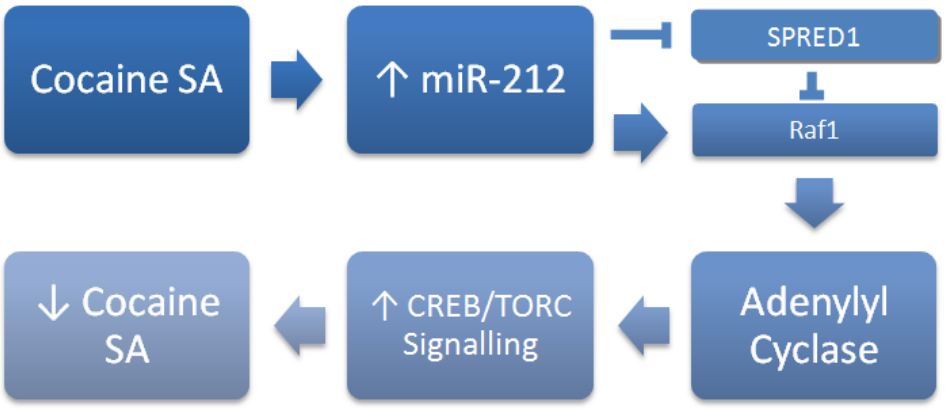

Figure 1: miR-212 signalling pathway. Consumption of cocaine increases miR-212 expression, which activates Raf1 and inhibits SPRED1, a Raf1 repressor. This activates adenylyl cyclase, which in turn activates the CREB/TORC signalling pathway decreasing cocaine intake.

In a subsequent study, Kenny and colleagues examined the link between miR-212 and MeCP2 (134). MeCP2 has been shown to bind to DNA affecting transcription by recruiting repressors including histone deacetylases to silence target genes (135), as well as increase BDNF expression. Importantly, BDNF expression in the DS has been shown to increase cocaine consumption (136). Similarly, decreased BDNF expression in the NAC can

decrease cocaine consumption (137). Im et al., 2010 found that inhibition of MeCP2 in the DS decreased cocaine consumption whereas the expression of miR-212 was increased. Consistent with this proposed interaction, overexpression of miR-212 led to a decrease in MeCP2. Thus, miR-212 and MeCP2 appear to are part of a negative homeostatic interaction important for regulation of cocaine intake.

Both studies highlight an important role for miR-212 in regulation of cocaine intake through interactions with CREB-TORC signalling pathways and MeCP2. However, it is important to note that while these studies clearly demonstrate a role for this miRNA in regulation of compulsive cocaine consumption, the role of miR-212 in regulation of other indices of addiction has not been addressed. It has been suggested that individual differences in miR-212 signalling may correlate with differences in the propensity to develop addiction and relapse into drug seeking behaviour.

The studies to date point to an important role for miRNA in cocaine-induced neuroadaptations, and further that specific miRNA play a role in regulation of behaviour relevant to addiction, including motivation, rewarding properties of drug and compulsive cocaine intake. Importantly though, and as made clear in early sections of this thesis, only a percentage of people who take drugs are considered drug addicted (138). As yet, there have been no studies investigating the expression of miRNA in brain regions relevant to addiction using models that assess individual differences in addiction vulnerability. Therefore, it remains unclear whether differences observed in miRNA expression are a consequence of cocaine-induced pharmacological

effects that would be observed in all drug-exposed individuals or whether they are indeed relevant to the neuroadaptations that contribute to the high rate of relapse associated with addiction.

1.7 Rationale

Addiction has been defined as a disorder of learning and memory. This stems from evidence showing that drug linked cues can powerfully motivate renewed drug-seeking and that new learning is required to extinguish level pressing for drugs. Consistent with this hypothesis, LTP and LTD, neuronal substrates responsible for learning and memory, are evoked by drug exposure and become dysregulated after chronic drug self-administration. In fact, there is an increasing body of evidence to suggest that synaptic plasticity deficits play an important role in the development and persistent nature of addiction. Critically, however, the molecular mechanisms that promote long-term plasticity are only now coming to light. Recent studies suggest that drug taking corrupts master regulators of synaptic protein production, trafficking and signalling. For example, synaptic plasticity related genes including *mTOR*, *Arc* and dopamine receptors are persistently altered in response to drugs of abuse. The current study aimed to examine the expression of these genes in neuronal regions critical to the development of addiction, and how manipulation of these genes affects addiction-relevant behaviour. Importantly, although evidence of changes in these and other synaptic plasticity genes have been previously implicated in addiction-relevant processes, little is know about how drug-induced changes in these key signalling pathways are sustained. The field of epigenetics has provided new insights into how gene expression can be persistently modified to influence activity dependent gene

expression. As outlined above, it is now clear that miRNA are an important level of epigenetic control at the synapse, however, only a small number of studies have investigated the role of miRNA in the regulation of plasticity genes that have previously been linked with addiction risk. As such, the current project aimed to examine the expression profiles of miRNA involved in regulation of the important synaptic plasticity genes *mTOR*, *Arc* and *Drd1* in the ventral and dorsal striatum. Studies in this thesis were aimed at identifying miRNA expression profiles at key phases of the addiction cycle, post-reinstatement (relapse) and early-mid phase during cocaine taking. To assess miRNA expression profiles at 'early-mid phase' I developed a computational model to identify addiction-relapse vulnerable animals prior to extinction and relapse. This allowed an assessment of how these synaptic plasticity genes and their miRNA regulators are altered throughout different stages of the addiction cycle. By understanding the mechanisms by which changes in key synaptic plasticity genes occur, as well as understanding the temporal changes that occur throughout different critical stages of the addiction cycle, we gain further insight into the addiction process and this in turn may lead to the identification of novel therapeutic targets to aid individuals in overcoming addiction.

1.7.1 Aim and Hypothesis

Aim 1: Investigate the effect of mTOR inhibition on addiction relevant behaviour.

Evidence has shown that mTOR and its downstream signalling pathway play a role in regulation of addiction. Deficits in *mTOR* gene expression have

been associated with cocaine addiction-relapse vulnerability in rats. Further, chronic alcohol administration has been shown to increase mTOR signalling in the striatum of rats, with additional effects on downstream molecules, including GluA1 AMPAR subunits. Given the changes in *mTOR* expression observed following exposure to drugs of addiction, the first aim of my project was to determine the effect of mTORC1 inhibition on addiction relevant behaviour. Additionally, I investigated the effect of mTORC1 inhibition on expression of downstream signalling molecules, including GluA1. Importantly, though this aim aids in the understanding of the role of mTORC1 in regulation of addiction-relevant behaviour, the mechanisms that contribute to altered mTOR expression following cocaine use are not well understood. This brings me to the second aim of my thesis, involving the investigation of how miRNA contribute to altered expression of *mTOR*, and indeed other synaptic plasticity genes, following cocaine use.

Aim 2: Investigate miRNA expression profile in the NAC of animals phenotyped as addiction vulnerable at early and late stages of the addiction cycle.

While there is a substantial body of evidence showing alterations in synaptic plasticity molecules including *mTOR*, *Arc* and dopamine receptors in the NAC of animals with a history of cocaine consumption, little is known about the molecular mechanisms that contribute to these deficits in the context of addiction. Evidence has demonstrated that miRNA play an important role in the regulation of a number of synaptic plasticity and learning and memory processes. As such, the current project aimed to investigate the

expression profile of miRNA in the NAC of animals shown to be addiction vulnerable, with a particular focus on miRNA involved in regulation of the key synaptic plasticity genes *mTOR*, *Arc* and *Drd1*. Further, the current study aimed to develop a computational model to identify addiction-relapse vulnerable animals during early stages of the addiction cycle so as to understand the temporal expression profile of these miRNA in addiction-relapse vulnerable animals.

Aim 3: Investigate miRNA and mRNA expression profile in the DS of animals phenotyped as addiction vulnerable at early and late stages of the addiction cycle.

As discussed previously, there is an emerging body of evidence to suggest that the DS plays a role in regulation of a number of addiction relevant behaviour. Moreover, the expression of the synaptic plasticity genes including *Arc*, *mTOR* and *Drd1* in the DS have been shown to play a role in addiction processes. Further, these genes have been shown to be altered in rats that display an ‘addicted’ phenotype. However, as with the NAC, the mechanisms contributing to these neuroadaptations are poorly understood. Therefore, the current project aimed to investigate to expression profile of synaptic plasticity related miRNA in the DS of addiction-relapse vulnerable animals at both early and late stages of the addiction cycle.

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CHAPTER TWO: mTORC1 inhibition in the nucleus accumbens 'protects' against the expression of drug seeking and 'relapse' and is associated with reductions in GluA1 AMPAR and CAMKIIα levels.

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July 2016

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Neuropsychopharmacology (2014), 1–9

www.neuropsychopharmacology.org

mTORC1 Inhibition in the Nucleus Accumbens 'Protects' Against the Expression of Drug Seeking and 'Relapse' and Is Associated with Reductions in GluA1 AMPAR and CAMKIIα Levels

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The mechanistic target of rapamycin complex 1 (mTORC1) is necessary for synaptic plasticity, as it is critically involved in the translation of synaptic transmission-related proteins, such as Ca^{2+}/Calmodulin-dependent kinase II alpha (CAMKIIα) and AMPA receptor subunits (GluAs). Although recent studies have implicated mTORC1 signaling in drug-motivated behavior, the ineffectiveness of rapamycin, an mTORC1 inhibitor, in suppressing cocaine self-administration has raised questions regarding the specific role of mTORC1 in drug-related behaviors. Here, we examined mTORC1's role in three drug-related behaviors: cocaine taking, withdrawal, and reinstatement of cocaine seeking, by measuring indices of mTORC1 activity and assessing the effect of intra-cerebroventricular rapamycin on these behaviors in rats. We found that withdrawal from cocaine self-administration increased indices of mTORC1 activity in the nucleus accumbens (NAC). Intra-cerebroventricular rapamycin attenuated progressive ratio (PR) break points and reduced phospho-p70 ribosomal S6 kinase, GluA1 AMPAR, and CAMKIIα levels in the NAC shell (NACsh) and core (NACc). In a subsequent study, we treated rats with intra-NACsh infusions of rapamycin (2.5 μg/side/day for 5 days) during cocaine self-administration and then tracked the expression of addiction-relevant behaviors through to withdrawal and extinction. Rapamycin reduced drug seeking in signaled non-drug-available periods, PR responding, and cue-induced reinstatement, with these effects linked to reduced mTORC1 activity, total CAMKIIα, and GluA1 AMPAR levels in the NACsh. Together, these data highlight a role for mTORC1 in the neural processes that control the expression and maintenance of drug reward, including protracted relapse vulnerability. These effects appear to involve a role for mTORC1 in the regulation of GluA1 AMPARs and CAMKIIα in the NACsh.

Neuropsychopharmacology advance online publication, 19 February 2014; doi:10.1038/npp.2014.16

Keywords: mechanistic target of rapamycin (mTOR); rapamycin; nucleus accumbens; GluA1; CAMKIIα; cocaine

INTRODUCTION

Dysregulated signaling through a number of neuronal surface receptors on nucleus accumbens (NAC) medium spiny neurons (MSNs), including dopamine, ionotropic, and metabotropic glutamate receptors, as well as neurotrophin receptors, has been linked with expression of addiction-relevant behaviors (Brami-Cherrier *et al*, 2002; Dayas *et al*, 2012; Russo *et al*, 2009). These receptor systems can signal through the MAPK-ERK and PI3K/AKT pathway to influence gene transcription and protein translation, with subsequent effects on neural activity and behaviors (McGinty *et al*, 2008; Russo *et al*, 2009). Interestingly, recent studies indicate that these receptor systems can recruit the mechanistic target of rapamycin (mTOR; Hoeffer and Klann 2010), a serine-threonine kinase that, together with Raptor and other interacting proteins, forms the mechanistic target of rapamycin complex 1 (mTORC1; Hoeffer and Klann 2010). mTORC1 is expressed in both neuronal cell bodies and neuronal dendrites and controls key steps in protein translation initiation via the phosphorylation and activation of translation initiation factors such as the p70 ribosomal S6 kinase (p70s6k) and eukaryotic

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Received 28 February 2013; revised 14 January 2014; accepted 15 January 2014; accepted article preview online 28 January 2014

translation initiation factor-4E-binding protein (Hoeffer and Klann 2010). It is now believed that mTORC1 plays a role in the synaptic plasticity processes of LTP (Cammalleri *et al*, 2003) and LTD (Mameli *et al*, 2007). The role of mTORC1 in these mechanisms is linked to the function of this complex in regulating the translation of specific synaptic proteins, such as Ca^{2+}/Calmodulin-dependent kinase II alpha (CAMKIIα) and AMPAR receptor subunits (GluA1s; Schratt *et al*, 2004; Slipczuk *et al*, 2009).

The formation of mTORC1 can be blocked by rapamycin, an allosteric mTOR inhibitor that acts by displacing Raptor, inhibiting its capacity to phosphorylate mTOR substrates (Hoeffer and Klann, 2010). Studies using rapamycin have implicated mTORC1 in late phase (protein synthesis dependent) LTP and LTD (Cammalleri *et al*, 2003; Stoica *et al*, 2011), as well as associative learning processes, including inhibitory avoidance memory and fear conditioning (Blundell *et al*, 2008; Parsons *et al*, 2006). More recently, experiments using rapamycin have implicated mTORC1 signaling in addiction-relevant behaviors and in alcohol-related memory consolidation (Barak *et al*, 2013). Both acute systemic and intra-NAC injections of rapamycin attenuated alcohol-drinking behaviors and conditioned place preference (CPP) for alcohol (Neasta *et al*, 2010). Acute systemic rapamycin treatment also suppressed the expression of cocaine CPP, behavioral sensitization to cocaine (Bailey *et al*, 2012), and the sensitization of meth-amphetamine CPP (Narita *et al*, 2005). These findings indicate that mTORC1 may have a role in controlling the processes responsible for the strengthening of drug/cue associations. NAC mTORC1 inhibition also attenuated cue-induced reinstatement of cocaine seeking but, interestingly, had no effect on cocaine self-administration under a fixed-ratio 1 (FR1) schedule of reinforcement (Wang *et al*, 2010b).

In the present study, we investigated the role of mTORC1 in drug-related behaviors by first assessing the effects of cocaine withdrawal on indices of mTORC1 activity. Next, we determined the role of mTORC1 in drug seeking by examining the effects of central (intra-cerebroventricular; i.c.v.) mTORC1 inhibition on lever pressing for cocaine using a progressive ratio (PR) schedule of reinforcement, a procedure thought more likely to reveal the neural processes underlying drug-motivated behavior. In these experiments, we assessed changes in mTORC1-signaling pathway proteins in the NAC shell (NACsh) and core (NACc). Cocaine-motivated behaviors have been shown to require proteins that enhance glutamate signaling in NAC MSNs (Anderson *et al*, 2008; Conrad *et al*, 2008; Wolf, 2010), so we assessed GluA1 AMPAR subunit and CAMKIIα levels in these brain regions after rapamycin treatment. Importantly, given the potential for mTORC1 inhibition to prevent neuroadaptations that promote synaptic plasticity and memory (Barak *et al*, 2013; Lin *et al*, 2014), we also tested whether rapamycin delivered to the NACsh during the cocaine-taking phase might have protracted effects on the expression of addiction-relevant behaviors including relapse-like behavior. We then assessed changes in NAC mTORC1 signaling pathway and GluA1 and CAMKIIα proteins, as they have recently been found to also play a role in cocaine relapse (Anderson *et al*, 2008).

MATERIALS AND METHODS

General Methods

For details on intravenous and intracranial surgery, drug preparation and behavioral testing equipment, see Supplementary Material (S1).

Self-Administration Training

Rats were trained to respond on the right lever for a cocaine infusion (0.25 mg/0.1 ml i.v. delivered over 4 s; (James *et al*, 2011a, 2010)) on an FR1 schedule (3 h/day, 5 days/week). Infusions were followed by the illumination of a white cue light above the active lever signaling a 20-s timeout period. Rats were limited to earning a maximum of 20 infusions during these sessions to prevent overdosing. Once stable responding was established (±10% rewarded responses over three sessions), the 3-h daily sessions were divided into alternating 'drug-available' and 'non-drug-available' periods, as described elsewhere (Brown *et al*, 2011; Deroche-Gamonet *et al*, 2004; Kasanetz *et al*, 2010). Briefly, the 40-min drug-available periods were signaled by a constant 70 dB white noise, whereas the 20-min non-drug-available periods were signaled by constant illumination of the white house-light. Cocaine infusions were only possible during drug-available periods, whereas lever presses during non-drug-available periods were recorded but had no scheduled consequences. An FR1 schedule was employed during the initial drug-available sessions and was then increased to FR5 with unrestricted access to drug. During these sessions, responses on the left 'inactive' lever were recorded but did not result in a drug infusion. Following the FR5 discrimination training sessions, rats underwent intracranial surgery (see Supplementary Material S1). After 7 days recovery from surgery, rats underwent an additional 5 days of cocaine self-administration on the FR5 drug-available/non-drug-available program.

For saline control groups, rats were prepared with an intravenous catheter and received 'yoked' saline infusions over a similar period of time to rats trained to self-administer cocaine. The number and temporal pattern of saline infusions was calculated based on the same parameters averaged across cocaine self-administering rats.

PR Testing

PR testing was conducted under drug-available conditions using the schedule 5, 10, 17, 24, 32, 42, 56, 73, 95, 124, 161, 208, 268, 345, 445, 573, 737, 947, 1218, 1566, 2012, 2585 presses required for one infusion. PR testing was performed over 5 h, however, PR sessions were programmed to shut down if a lever press was not recorded within 45 min of a previous lever press.

Spontaneous Locomotor Activity Assay

Locomotor activity was assessed in cocaine-naive rats using previously published procedures (James *et al*, 2011b). Rapamycin or vehicle was administered 3 h before testing.

Extinction and Cue-Induced Reinstatement Testing

Lever pressing was extinguished in 2 h extinction sessions until responding reached $\leqslant 6$ active lever presses over three consecutive sessions. Discriminative stimuli were withheld, along with drug infusions. One day after the last extinction session, rats were tested for reinstatement of drug seeking for 1 h by re-exposing rats to the cues paired with drug availability during self-administration training. Lever presses during reinstatement tests did not result in cocaine infusions but did result in illumination of the white cue light above the active lever (20 s).

Experiment One

Effect of cocaine self-administration on mTORC1 activity in the NAC. A group of rats ($n=6$) were trained to self-administer cocaine for a period of approximately 5 weeks. A separate group of rats received yoked saline over the same period ($n=6$). Both groups of rats were killed 24 h after the final self-administration (or yoked) session, and brains were processed for markers of mTORC1 activity in the NAC.

Experiment Two

Effect of i.c.v. rapamycin on PR responding. Rats acquired stable self-administration behavior on an FR1 schedule before being tested on a PR schedule. On days 1–3 of PR testing, baseline PR scores were obtained. On days 4–6, rats received an infusion of rapamycin (12.5 μg/μl, i.c.v., 1 μl/min for 2 min; $n=10$) or vehicle ($n=9$) 3 h before PR testing through an injector (28 gauge; Plastics One, VA, USA), which protruded 1 mm below the guide cannulae. The timing and dose of rapamycin was based on previous studies (Cota *et al*, 2008; Neasta *et al*, 2010). The injector remained in place for 60 seconds to ensure diffusion of the injectate. Rats were killed 24 h after the final PR session and brains were processed for markers of mTORC1 activity.

The effect of i.c.v. rapamycin on locomotor activity was assessed in a separate cohort of cocaine-naive rats (Supplementary Material S1). Rats from this group were killed 24 h after the final locomotor test and brains were assessed for markers of mTORC1 activity.

Experiment Three

Effect of intra-NACsh-directed rapamycin infusions on addiction-relevant behaviors. Rats were trained to discriminate between periods of signaled drug availability and non-drug availability, and then underwent cranial surgeries, as described in Supplementary Material S1. To facilitate intra-NACsh infusions, rats were gently restrained, stylets were removed and an injector (20.2 mm; 28 gauge; Plastics One) was inserted into each of the two guide cannulae. Rapamycin ($n=17$) or vehicle ($n=17$) was infused at a volume of 2.5 μg/0.5 μl/side over 1 min. For both cannuale, the injector remained in place for an additional 60 s to ensure diffusion of the injectate, after which stylets were replaced. Infusions took place once per day for 5 consecutive days. As rapamycin may interfere with memory consolidation processes when administered before learning (Parsons *et al*, 2006), approximately half of the rats ($n=8$) received rapamycin or vehicle 3 h before testing, whereas the remaining half ($n=9$) received treatment immediately following testing (immediately after the 3 h cocaine self-administration session). Importantly, no differences were observed between these two treatment regimes across all parameters measured (non-drug available responding ($F_{1,14}=0.03$, $P=0.98$); PR responding: ($F_{1,14}=7.12$, $P=0.32$); cue-induced reinstatement: ($F_{1,9}=0.60$, $P=0.97$), data not shown), and as such, data were collapsed across both rapamycin treatment groups.

To assess the effects of intra-NACsh rapamycin treatment on addiction-like behavior, rats were assessed on three well-established behavioral indices of addiction vulnerability (Brown *et al*, 2011; Deroche-Gamonet *et al*, 2004; Kasanetz *et al*, 2010; for timeline, see Figure 4). First, across the 5 days of rapamycin treatment, rats were assessed for active lever responding during signaled periods of non-drug-availability. Second, on the three days following the last treatment session, rats were tested for motivation to obtain drug on a PR schedule identical to *Experiment 2*. Finally, in order to assess the long-term effects of rapamycin, we assessed reinstatement behavior after extinction training by re-exposing rats to cues paired with drug availability during self-administration training. Rats were killed 24 h after reinstatement testing and brains were processed for protein analysis.

We also sought to assess the effect of intra-NACsh treatment on the activity of the mTORC1 pathway in rats with no history of cocaine self-administration but that had similar exposure to the operant environment. To do this, a separate group of rats ($n=6$) received yoked saline infusions. Rats were killed 3 weeks after the final saline yoking session, which equated to the average time between the final self-administration session and kill in the cocaine-exposed group. Brains from the saline-yoked group were processed for markers of mTORC1 activity in a similar manner to cocaine-exposed rats.

Details of animal kill, western-blot analysis, and statistical analysis are outlined in Supplementary Material S1.

RESULTS

Experiment One

Withdrawal from cocaine self-administration increased indices of mTORC1 activity. Rats trained to self-administer cocaine consumed on average a total of 542.59 (±24.86) mg/kg of cocaine over the training period. We compared the levels of total and phosphorylated mTOR protein in the NACc and NACsh of all treatment groups 24 h after the last saline or self-administered cocaine infusion. Cocaine self-administration increased both total mTOR ($t(10)=-2.33$, $P=0.042$) and phosphorylated mTOR levels ($t(10)=-3.43$, $P=0.006$) in the NACsh. In contrast, there was no effect of cocaine on total or phosphorylated mTOR levels in the NACc (P's >0.05). Total p70s6k levels in the NACc were significantly elevated in cocaine self-administering rats compared with yoked saline controls ($t(10)=-4.69$, $P<0.001$). No differences were observed in phospho-p70s6k, GluA1, and CAMKIIα levels in either subregion (Figure 1).

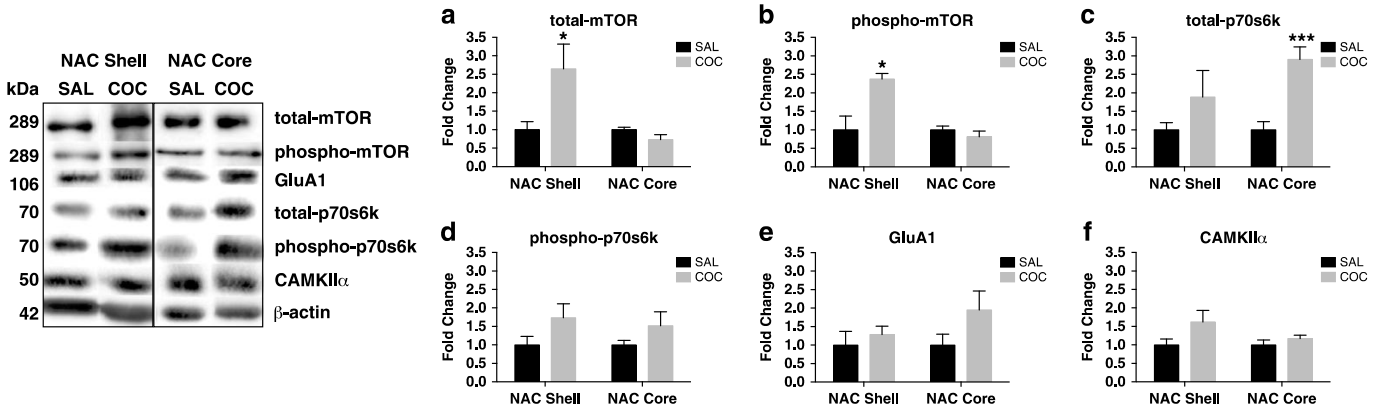

Figure 1 Withdrawal from cocaine self-administration increased indices of mTORC1 activity. Compared with saline controls, rats with a history of cocaine self-administration demonstrated increased total-mTOR (a) and phospho-mTOR (b) in the NACsh but not NACc. Cocaine-exposed rats also demonstrated an increase in total-p70s6k in the NACc (c). No significant changes were observed in phospho-p70s6k (d), GluA1 (e) or CAMKIIα in the NAC (f). Data represent means ± SEM. $^{*}P<0.05$; $^{***}P<0.001$. $n=6$/group.

Experiment Two

Effect of mTORC1 inhibition on motivation to obtain cocaine under PR reinforcement conditions. A comparison of treatment groups revealed no differences in FR5 responding ($P>0.05$; Figure 2a) or the number of cocaine infusions before rapamycin treatment ($P>0.05$; data not shown). The effect of i.c.v. rapamycin on motivation to obtain cocaine was then assessed using PR tests over 3 consecutive days. Rapamycin-treated rats exhibited significantly lower break points than vehicle-treated controls on days 2 ($F_{1,16}=5.03$, $P=0.043$) and 3 ($F_{1,16}=5.14$, $P=0.041$) of PR testing (Figure 2b). Of note, i.c.v. rapamycin treatment was associated with a modest decrease in body weight over the treatment period ($F_{1,16}=4.65$, $P=0.047$; Figure 2c).

Effect of mTORC1 inhibition on CAMKIIα and GluA1 levels. In i.c.v.-treated rats, rapamycin significantly reduced total mTOR levels in the NACsh ($t(10)=2.27$, $P=0.047$) but not the NACc ($P=0.13$; Figure 3a). Interestingly, no differences were observed between treatment groups in terms of total p70s6k levels in either NAC subregion (P's >0.05; Figure 3b), but significantly reduced phosphorylated p70s6k levels were observed in both the NACsh ($t(10)=11.13$, $P<0.001$) and NACc ($t(10)=4.209$, $P=0.002$) of rapamycin-treated rats (Figure 3c). We also assessed the effects of rapamycin treatment on the expression of synaptic proteins GluA1 and CAMKIIα. Rapamycin treatment reduced GluA1 levels in both the NACsh ($t(10)=9.05$, $P<0.001$) and NACc ($t(10)=3.66$, $P=0.004$; Figure 3d) and reduced total CAMKIIα levels in the NACsh ($t(10)=4.76$, $P<0.001$) and NACc ($t(10)=2.58$, $P=0.03$; Figure 3e).

For data outlining the effect of mTORC1 inhibition on CAMKIIα and GluA1 levels and locomotor activity in cocaine naive rats, see Supplementary Material S2.

Experiment Three

Effect of intra-NACsh mTORC1 inhibition on drug seeking in the signaled non-drug-available period and under PR conditions. Details of guide cannulae placement are described in Supplementary Material S3.

As with *Experiment 2*, rats were randomly allocated to treatment groups following self-administration training. Treatment groups did not differ in terms of overall cocaine self-administration ($P=0.83$, data not shown) or number of days taken to reach the treatment phase ($P=0.57$, data not shown). Importantly, rats from both groups were able to discriminate between drug-available and non-drug-available cues, as responding was significantly higher in drug-available sessions ($F_{1,26}=491.09$, $P<0.001$). Over the treatment period, rapamycin-treated rats did not differ to controls in terms of lever responding during drug-available periods ($P=0.58$; Figure 4a) nor amount of cocaine consumed ($P=0.84$, data not shown). However, rapamycin-treated rats exhibited significantly fewer active lever responses in the non-drug-available periods as compared with controls across the treatment period ($F_{1,26}=6.89$, $P=0.014$; Figure 4a). Throughout the treatment period, no differences were observed between treatment groups in terms of responding on the inactive lever ($P>0.05$; data not shown). In addition, rapamycin treatment had no effect on body weight over the treatment period ($P>0.05$, data not shown).

One day following rapamycin treatment, rats were assessed for motivation to obtain cocaine on a PR schedule. Rapamycin-treated rats exhibited a significantly lower average break point than vehicle-treated controls across the 3 days of PR testing (t $(27)=2.553$, $P=0.017$; Figure 4b). A similar analysis revealed no effect of treatment on inactive lever responding ($P>0.05$, data not shown).

Effect of intra-NACsh mTORC1 inhibition on cue-induced reinstatement of drug seeking. Two rapamycin-treated and two vehicle-treated rats did not complete the entire experimental protocol, precluding collection of extinction and reinstatement data for these rats. All other rats took an average of 20 days to reach the extinction criterion, and this did not differ between treatment groups ($F_{1,25}=1.841$, $P=0.188$; Figure 4c). All rats exhibited a significant reinstatement of responding on the active lever following presentation of the drug-available cue during reinstatement testing, as compared with extinction responding. Reinstatement on the active lever was significantly attenuated in

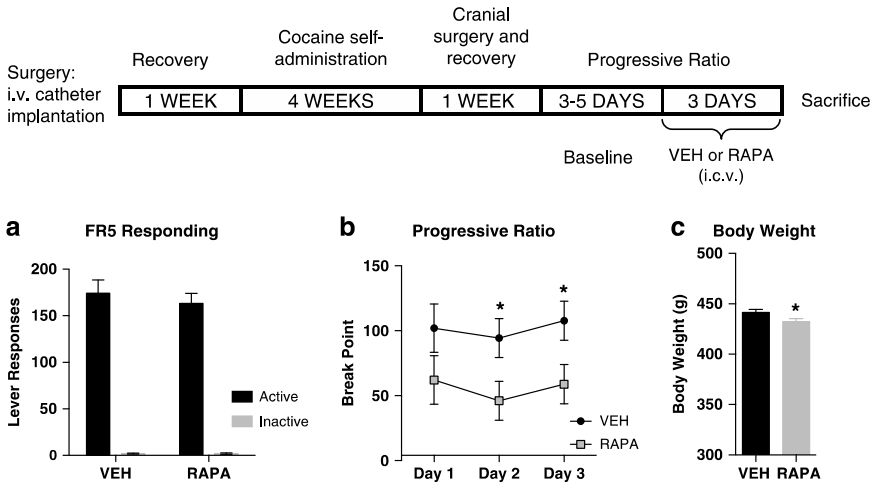

Figure 2 Effect of mTORC1 inhibition on motivation to obtain cocaine under progressive ratio reinforcement conditions. Vehicle- or rapamycin-injected rats did not differ in terms of active or inactive lever responding in cocaine self-administration sessions before treatment (a). Rats that received i.c.v. rapamycin exhibited significantly lower break points on days 2 and 3 of progressive ratio testing as compared with controls (b). i.c.v. administration of rapamycin was associated with modest decreases in body weight on the final day of treatment (c). Data represent means ± SEM. $^{*}P<0.05$; $^{***}P<0.001$. $n=9$–10/group.

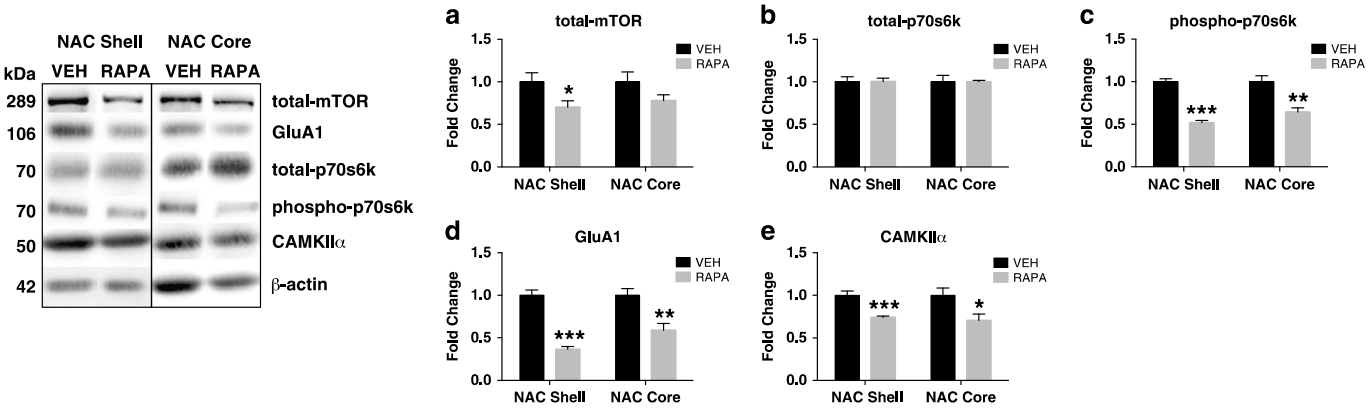

Figure 3 Effect of i.c.v. mTORC1 inhibition on CAMKIIα and GluA1 levels. i.c.v. rapamycin administration significantly attenuated total mTOR levels in the NACsh but not NACc (a). Rapamycin had no effect on total p70s6k levels (b), but significantly reduced phospho-p70s6k levels in both the NACsh and NACc (c). Similarly, rapamycin treatment was associated with reduced GluA1 (d) and CAMKIIα (e) expression in NACsh and NACc. Data represent means ± SEM. $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$. $n=6$/group.

rapamycin-treated rats, as compared with vehicle-treated controls ($F_{1,25}=5.929$, $P=0.023$; Figure 4d). No differences were observed in terms of responding on the inactive lever during reinstatement testing ($P=0.544$; data not shown).

Effect of intra-NACsh mTOR inhibition on mTORC1 signaling and GluA1 and CAMKIIα levels after reinstatement. Intra-NACsh rapamycin-treated rats displayed no changes in total mTOR or p70s6k levels in either NAC subregion (P's >0.05; Figure 5a and b). Importantly, however, in rapamycin-treated rats, significantly reduced levels of phosphorylated p70s6k within the NACsh were observed ($t(10)=3.34$, $P=0.008$; Figure 5c). Rapamycin treatment was also associated with significantly attenuated total GluA1 ($t(10)=2.91$, $P=0.016$) and total CAMKIIα ($t(10)=2.66$, $P=0.024$) levels in the NACsh, but had no effect on these parameters in the NACc (P's >0.05; Figure 5d and e).

For data outlining the effect of mTORC1 inhibition on CAMKIIα and GluA1 levels and locomotor activity in cocaine naive rats, see Supplementary Material S4.

DISCUSSION

In the present study, we found evidence of increased mTORC1 activity in the NAC after withdrawal from cocaine self-administration. Further, we show that mTORC1 inhibition using rapamycin reduced the motivation to lever press for cocaine under PR conditions, with these effects being linked to a reduction in GluA1 AMPARs and CAMKIIα in the NAC. Importantly, in a separate experiment where we tracked the effect of mTORC1 inhibition on the expression of addiction-relevant behaviors over time, we found that intra-NACsh rapamycin did not alter cocaine self-administration under FR5 conditions. In contrast, in the week

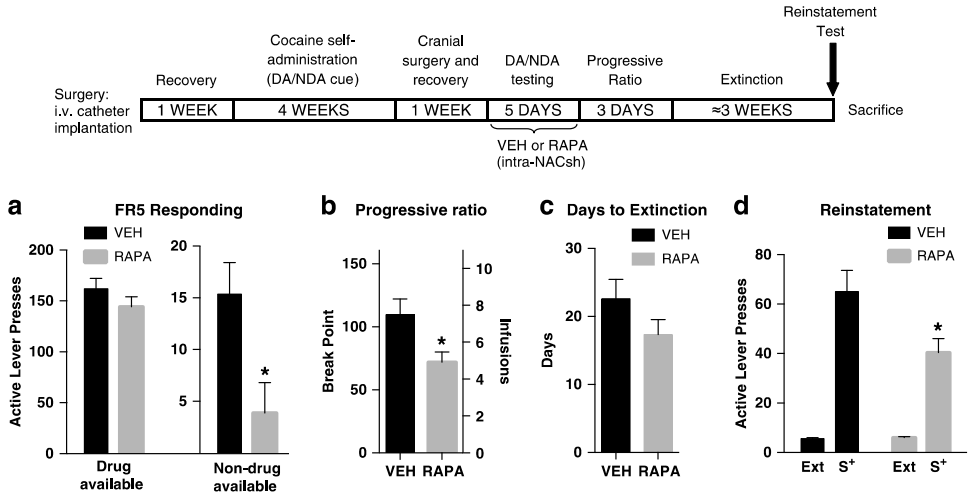

Figure 4 Effect of intra-NAC mTORC1 inhibition on drug-seeking and taking behaviors and progressive ratio responding. In both treatment groups, responding during periods of signaled drug availability was significantly higher than during periods of signaled non-drug availability. Treatment groups did not differ in their responding during periods of drug availability. However, intra-NACsh rapamycin treatment significantly reduced responding during periods of signaled non-drug-availability (a). In the week following treatment, rats that received intra-NACsh rapamycin exhibited significantly reduced break points (b). Treatment groups did not differ in terms of days taken to reach the extinction criterion (c). Rapamycin-treated rats exhibited significantly lower levels of cue-induced reinstatement of extinguished cocaine seeking, despite treatment occurring approximately 4 weeks prior (d). Data represent means ± SEM. $^{*}P<0.05$. $n=14$–15/group. DA, Drug-available; NDA, Non-drug-available; S^{+}, Presentation of 'drug-available' discriminative stimulus.

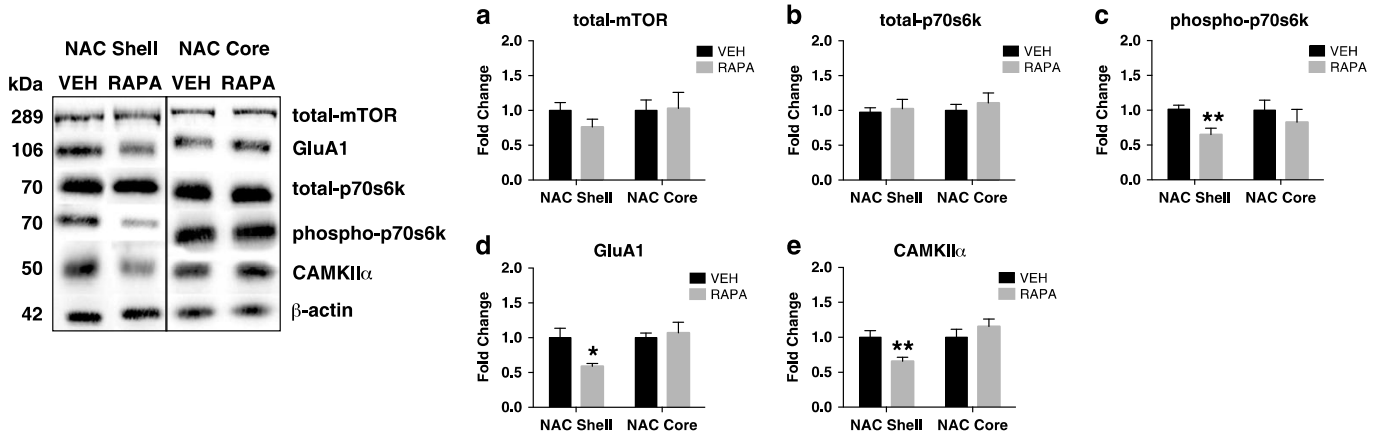

Figure 5 Effect of intra-NACsh mTORC1 inhibition on cue-induced reinstatement and CAMKIIα and GluA1 levels. Intra-NACsh vehicle- or rapamycin-treated rats did not differ in terms of total mTOR levels in the NAC (a). Similarly, total p70s6k levels did not differ between groups (b), but rapamycin treatment resulted in reduced phospho-p70s6k in the NACsh (c). Interestingly, rapamycin treatment resulted in reduced total GluA1 (d) and CAMKIIα (e) levels in the NACsh, but had no effect on these parameters in the NACc. Data represent means ± SEM. $^{*}P<0.05$; $^{**}P<0.01$. $n=6$/group.

following treatment, intra-NACsh rapamycin reduced drug seeking during periods of signaled non-drug-availability and attenuated motivation to lever press for cocaine on a PR schedule. Most notably, a 40% reduction in cue-induced reinstatement of drug seeking was observed in rapamycin-treated rats, despite the fact that treatment ceased up to 4 weeks before testing. The protracted rapamycin-induced reductions in drug-seeking behavior were accompanied by biochemical evidence of reduced mTORC1 activity and reduced total GluA1 and CAMKIIα levels. These data suggest that mTORC1 reduces the translation of synaptic plasticity proteins in the NAC, an effect that 'protects' against the expression of drug-seeking behavior.

Effect of Cocaine Withdrawal on Indices of mTORC1 Activity

Based on changes in the levels of phospho-mTOR, our study indicates that after 24 h of cocaine withdrawal, mTORC1 activity is increased in the NACsh. These findings are highly consistent with the report of Neasta *et al* (2010), who showed that markers of mTORC1 activity were increased after withdrawal from alcohol. It is possible that changes in mTORC1 activity manifest on a different time scale in the NACsh versus NACc. Further work will be required to understand the significance of these findings given the different roles ascribed to NAC core in shell in drug-motivated behavior (Ito *et al*, 2004).

Intra-cerebroventricular mTORC1 Inhibition Reduces PR Responding and GluA1 and CAMKIIα Levels

In accordance with findings of Lu and colleagues (Wang *et al*, 2010b), we found that cocaine self-administration (FR5) was unaffected by intra-NAC rapamycin injections (*Experiment 3*). However, i.c.v. rapamycin reduced lever pressing for cocaine when PR conditions were required to obtain cocaine infusions. To date, data supporting the role for mTORC1 in psychostimulant reinforcement has been limited to CPP and sensitization studies (Bailey *et al*, 2012; Narita *et al*, 2005). However, mTORC1 inhibition has been reported to reduce low-effort alcohol drinking in mice and FR1 alcohol self-administration in rats (Neasta *et al*, 2010). With respect to biochemical changes induced by i.c.v. rapamycin, we found significant reductions in indices of mTORC1 activity and GluA1 AMPARs and CAMKIIα within the NAC. The magnitude of these effects was similar to previous studies (Barak *et al*, 2013; Neasta *et al*, 2010). These findings are interesting given that dopamine D1-like agonists increase AMPAR insertion into NAC MSNs through a process involving CAMKIIα (Anderson *et al*, 2008; Sun *et al*, 2008) and that both dopamine and CAMKIIα have been shown to be important for the expression of psychostimulant sensitization and drug-seeking behavior (Licata and Pierce, 2003; Loweth *et al*, 2008; Pierce *et al*, 1998). In fact, lentiviral-mediated knockdown of CAMKIIα in the NACsh reduced motivation to self-administer cocaine on a PR schedule (Wang *et al*, 2010a). Collectively, these data support a hypothesis in which mTORC1 acts as a key effector in the molecular pathway controlling the dynamic regulation of synaptic proteins within the NAC that are required for the expression and maintenance of drug reward. However, we cannot rule out effects of mTORC1 inhibition after i.c.v. rapamycin in other parts of the reward circuit, including the VTA (Mameli *et al*, 2007).

Inhibition of mTORC1 in the NACsh Reduces Drug-Seeking Behavior Including Reinstatement

We found that mTORC1 inhibition in the NACsh significantly reduced lever-pressing behavior during periods of signaled non-drug-availability—a putative measure of compulsive drug seeking. Further, in the week following rapamycin, treated rats demonstrated reduced PR break points. These findings are in line with our i.c.v. rapamycin data and support a role for mTORC1 in the neural processes that are invoked to elevate motivational performance. Importantly, after extinction training, rapamycin-treated rats demonstrated close to a 40% reduction in reinstatement of cocaine seeking. Notably, the time to reach the extinction criterion did not differ between vehicle and treatment groups, indicating that there was no impairment in extinction learning produced by prior rapamycin injections.

The exact mechanism responsible for the reduction in reinstatement behavior exhibited after rapamycin treatment is unclear. However, our data showing reduced synaptic protein levels are interesting on several levels. Cocaine seeking has been shown to require an increase in translation, trafficking, and signaling through GluA1 AMPARs in the NAC, and CAMKIIα has a well-established role in this process (Anderson *et al*, 2008; Conrad *et al*, 2008; Ferrario *et al*, 2011; Lisman *et al*, 2012; Opazo *et al*, 2010). Given the functional link between these glutamate signaling molecules and reinstatement, the protective effects of intra-NACsh rapamycin on drug seeking might be mediated by a disruption in withdrawal-induced increases in synaptic GluA1s that increase the propensity for drug seeking (Anderson *et al*, 2008; Conrad *et al*, 2008; Mameli *et al*, 2007; Wolf, 2010). An important additional consideration is the possibility that rapamycin treatment disrupted associative memory processes during the drug-taking phase. For example, an important recent study identified a role for mTORC1 in alcohol-cue memory reconsolidation (Barak *et al*, 2013). Further, intra-amygdala or hippocampal rapamycin suppresses the consolidation and reconsolidation of fear memories (Blundell *et al*, 2008; Parsons *et al*, 2006) and mTORC1 is required for cocaine-induced plasticity in VTA dopamine neurons (Mameli *et al*, 2007).

Effect of mTORC1 Inhibition in Cocaine Naïve Rats

To assess the effects of rapamycin on mTORC1 signaling in cocaine naive rats, we performed biochemistry on NAC tissue in rats that received i.c.v. or intra-NAC rapamycin at equivalent time points to cocaine-trained rats. Although subtle differences in mTORC1 signaling were observed between i.c.v.- and intra-NAC rapamycin-treated cocaine naive controls, in general these experiments revealed that rapamycin treatment produced a generalized perturbation in mTORC1 activity and consequently GluA1 and CAMKIIα levels.

A particularly interesting observation in the present study was the effect of rapamycin on mTORC1 activity seen more than 1 month after treatment. These effects were more pronounced in cocaine-treated rats. The apparent long-term effects of rapamycin on mTORC1 activity might involve rapamycin-induced reductions in mTOR complex 2 and consequently AKT activity or other compensatory changes in this signaling pathway produced by sub-chronic rapamycin (Huang *et al*, 2013). Indeed, acute but not chronic rapamycin is known to reduce mTOR complex 2-induced phosphorylation of AKT at Ser473 (Huang *et al*, 2013). However, because cocaine appears to upregulate mTORC1 activity, a more likely explanation for the reductions in mTORC1 activity and addition-relevant behaviors after rapamycin treatment is a restoration of *normal/baseline* function in this signaling pathway.

Notably, rapamycin treatment did not affect locomotor activity or produce other non-specific effects on baseline motivational performance (ie, FR5 cocaine responding), findings that support recent demonstrations that rapamycin does not affect responding for a natural rewards (Neasta *et al*, 2010; Wang *et al*, 2010b). Thus, reductions in baseline mTORC1 in drug naive rats may be insufficient to alter general motivation status.

CONCLUSIONS

The present study extends our understanding of the role of mTORC1 in the neural processes that control the expression and maintenance of drug reward, including protracted

vulnerability to reinstatement. These findings are significant given the recent demonstrations that mTORC1 effectively reduces alcohol-motivated behaviors in rats (Barak *et al*, 2013; Neasta *et al*, 2010) and that acute systemic rapamycin suppressed cue-induced drug craving in abstinent heroin addicts (Shi *et al*, 2009). Although it is important to note the need to directly test the hypothesis that the rapamycin-induced reductions in mTORC1 activity and glutamate signaling molecules suppressed cocaine-seeking behavior, the present study indicates that mTORC1 inhibition can have long-lasting protective effects against drug-seeking behavior.

FUNDING AND DISCLOSURE

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

We acknowledge the assistance of Mr Cameron Adams and Mr Jonathan Cvetanoski for their technical assistance with the behavioral component of the i.c.v. experiment. We also thank Mrs Helen Carpenter and Mrs Michelle McConachy for their technical assistance with the western-blot analyses. These studies were supported by funding from the Australian National Health and Medical Research Council, the Hunter Medical Research Institute, and the University of Newcastle through project grants to C.V.D.

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Supplementary Information accompanies the paper on the Neuropsychopharmacology website (http://www.nature.com/npp)

2.2 Supplementary Material

2.2.1 General Methods

2.2.1.1 Animals and Ethics Statement

Male Sprague-Dawley rats (Animal Resources Centre, WA, AUS) weighing 200-250g upon arrival were housed two per cage on a reverse 12-hour light/dark cycle (lights off 0700h) with *ad libitum* access to food and water. All procedures were carried out in strict accordance with protocols approved by the University of Newcastle Animal Care and Ethics Committee, New South Wales Animal Research Act and Regulations and the Australian Code of Practice for the care and use of animals for scientific purposes.

2.2.1.2 Intravenous Surgery

Upon arrival, rats were handled daily for one week before undergoing intravenous catheter surgery as described previously by our laboratory (1-3).

2.2.1.3 Intracranial surgery

To place guide cannulae into the lateral ventricle (*Experiment 2)* or NACsh *(Experiment 3)*, animals were anaesthetized with isofluorane and then placed in a stereotaxic frame (Stoelting, USA). Craniotomies were made into the skull and stainless-steel guide cannulae (26-gauge, Small Parts, USA) were lowered to the level of either the lateral ventricle (AP:-0.9;ML:+1.5;DV:-4.5; unilateral) or the NACsh (AP:+1.7;ML:+/-4.2(19°);DV:-7.2; bilaterally), according to the Paxinos & Watson (4) atlas. Guide cannulae were secured to four stainless-steel jewellers' screws (Mann Optics, Australia) with dental

cement (Henry Schein, Australia) and stainless-steel stylets were inserted to maintain patency (33-gauge, Small Parts, USA).

2.2.1.4 Drugs

Cocaine hydrochloride (Johnson Matthey, Edinburgh, UK) was dissolved in sterile physiological saline (2.5mg/mL) (1, 5). Rapamycin (Cat #R-5000, LC Laboratories, USA) was dissolved in 100% DMSO according to previous studies (6, 7). To ensure that DMSO had no effect on behavioural responding, we compared DMSO-treated rats with another cohort of animals treated with saline. No differences were observed between these two groups across any of the addiction-relevant parameters assessed (non-drug available responding (p = 0.53); progressive ratio responding: (p = 0.67); cue-induced reinstatement: (p=0.22), data not shown).

2.2.1.5 Behavioural Testing Equipment

Behavioural procedures were conducted in standard operant conditioning chambers (Med Associates, VT, USA). Chambers were equipped with two retractable levers (6 cm above the floor), a white cue light above each lever, two speakers to deliver auditory stimuli, and a white house light at the top of the chamber wall opposite the levers. Auditory stimuli were produced by a white noise generator connected to a speaker that produced a 70dB white noise adjacent to the house light (Med Associates, VT, USA), and a tone source located in the top left corner (Sonalert with volume control, Med Associates, VT, USA), that was connected to a speaker that produced an intermittent tone (0.5 Hz at 70dB). A syringe pump (5 rpm motor, Med Associates, VT, USA) located on the outside of the cubicle delivered the IV

cocaine. Behavioural testing equipment was controlled by a Windows-based PC, using MED-PC IV software (Med Associates, VT, USA).

2.2.1.6 Animal Sacrifice and Tissue Harvesting and Processing

Rats were decapitated and their brains were rapidly removed and cooled in ice-cold diethylpyrocarbonate (DEPC)-treated PBS. Brains were blocked into forebrain and hindbrain regions on an ice-cold stage and the blocks were snap frozen in dry-ice chilled isopentane (Sigma-Aldrich). Tissue was stored at -80°C until required. Brains were cryosectioned into 100µm sections, allowing for verification of injection sites. The NACsh and NACc were then macrodissected from sections (bregma levels 1.32-2.28) of 6 vehicle- and 6 rapamycin-treated animals exhibiting behavioural responding representative of group effects. Punches were made using a 0.8mm diameter tissue punch and kept frozen until homogenisation. Tissues were sonicated in 100µL of homogenizing buffer (50mM Tris/HCl pH 7.5, 1mM EGTA, 1 x complete protease inhibitor cocktail tablet, 1mM DTT, 80µM ammonium molybate, 1mM sodium pyrophosphate, 5mM β-glycerophosphate, 1mM sodium orthovandate, 2µM microcystin, final concentration) with a microsonicator (UP50H, Hielscher Ultrasonics GmbH, Teltow, Germany) for 3 x 10 second pulses at 4°C until clear. 10% SDS was added to a final concentration of 2.5% and the samples were boiled for 5 minutes then centrifuged at 15000 rpm for 10min at 25°C. The clear supernatants were collected and protein concentrations determined by Pierce BCA protein assay kit (Thermo Fisher Scientific) as per the manufacturer's instructions. Samples were diluted with homogenizing buffer to equalize protein concentrations and kept frozen at -80°C until required.

2.2.1.7 SDS-Page and Western Blot Analysis

Western blotting were performed essentially as previously described (8). Briefly, 15µg of each sample was mixed with sample buffer (1% SDS, 10% glycerol, 0.5% DTT, 0.1% bromphenol blue, final concentration) subjected to SDS–PAGE gel electrophoresis before being transferred to nitrocellulose (Hybond ECL, GE Health Care). Membranes were then stained with Ponceau S (0.5% Ponceau in 1% acetic acid) to assess the efficacy of the transfer. Membranes were then washed in Tris-buffered saline with Tween (TBST) (150mM sodium chloride, 10mM Tris, 0.075% Tween-20, pH 7.5) and blocked in 5% Skim Milk Powder in TBST for 1 h at 25°C. Membranes were washed in TBST and incubated with anti-mTOR (1:500 Cell Signalling Technologies #2972), anti-p70s6k (1:500 Cell Signalling Technologies #9202), anti-phospho-p60s6k (Thr389), anti-CAMKIIα (1:1000 Millipore #05-532), anti-GluA1 (1:1000 Millipore #AB1504) and anti- β-actin-peroxidase(1:20 000 Sigma Aldrich A3854) overnight at 4°C. Membranes were washed in TBST and incubated with horse-radish peroxidase-linked anti-IgG secondary specific antibodies for 1 h at 25°C. Membranes were visualized on Fujifilm Las-3000 imaging system (Fuji, Stamford, CT, USA) using detection reagents (Luminata Forte Western HRP substrate, Millipore). The density of bands was measured using a MultiGauge V3.0 (Fuji, Stamford, CT, USA). Total mTOR, p70s6k, CAMKIIα and GluA1 protein levels were normalized to β-actin, while phospho-p70s6k was normalized to total-p70s6k. All results were expressed as a fold change relative to the VEH control group. p70s6k is phosphorylated at Thr-389 solely by mTORC1 and therefore phospho-Thr-389 p70s6k levels are used as a readout of mTOR activity (9, 10).

2.2.1.8 Statistical Analyses

In all experiments, self-administration behaviour prior to testing and protein levels were compared using independent Student's *t*-tests. For *Experiment 2,* the break points of each PR test over treatment days were compared across treatment groups using ANCOVA with baseline data used as the covariate. Body weight and locomotor activity were compared across groups on the final day of treatment using ANCOVA, with baseline data used as the covariates. For *Experiment 3*, responses on the active lever during non-drug-available periods was averaged across treatment days and compared using ANCOVA with baseline responding values used as the covariate. PR break points were averaged across all sessions and compared using a Student's *t*-test. Reinstatement scores were also compared using a Student's *t*-test. Data from all ANCOVA analyses are presented as estimated marginal means. An alpha value of 0.05 was adopted for all statistical tests.

2.2.2 Results

2.2.2.1 Effect of mTORC1 inhibition on CAMKIIα and GluA1 levels and locomotor activity in cocaine naïve rats

There was no effect of i.c.v. rapamycin on total mTOR, p70s6k or GluA1 levels in either subregion of the NAC. However, rapamycin was associated with a significant decrease in phosphorylated p70s6k in the NACsh ($t(9)=4.53$, $p=0.001$) and a significant decrease in CAMKII in the NACc ($t(8)=2.64$, $p=0.030$; *see Figure S1*). There was no effect of i.c.v. rapamycin treatment on spontaneous locomotor activity ($p>0.05$; *Figure S2*).

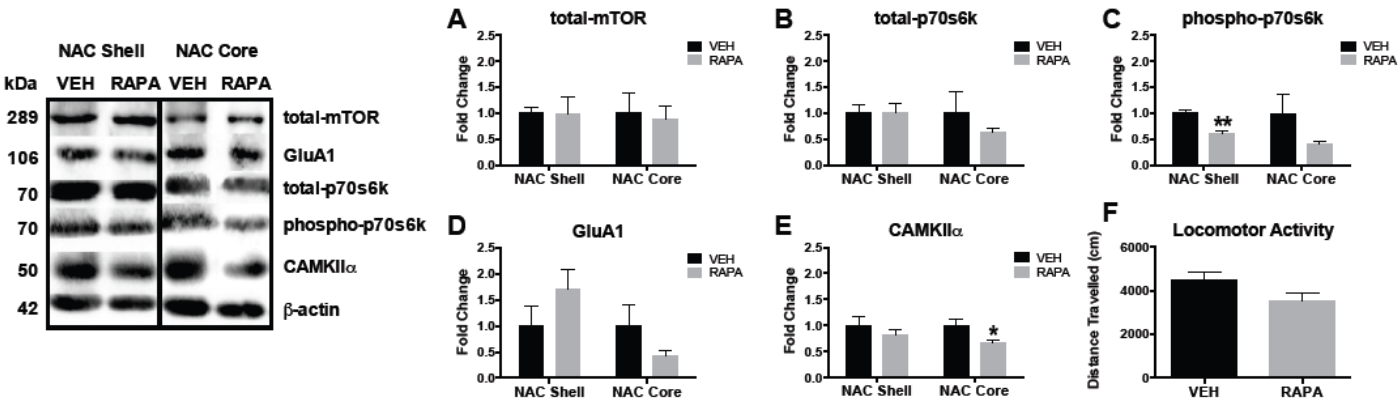

Figure S2

Figure S2: Effect of i.c.v. mTORC1 inhibition in cocaine naïve rats. Compared to vehicle-treated controls, i.c.v. rapamycin-treated rats demonstrated no changes in total mTOR (A) or total p70s6k levels in cocaine naïve rats (B). However, rapamycin significantly reduced phospho-p70s6k levels in the NACsh (C). Similarly, rapamycin treatment was associated with reduced CAMKIIα in the NACc (E). i.c.v. rapamycin administration had no effect on locomotor activity (F). Data represent means ± SEM. *p<0.05; **p<0.01. n=4-6/group

2.2.2.2 Effect of intra-NACSh mTORC1 inhibition on mTORC1 activity in cocaine naïve rats

We also assessed the effect of intra-NACsh rapamycin treatment on the activity of the mTORC1 pathway in animals with no history of cocaine self-administration (saline yoked controls). Rapamycin-treated saline controls displayed no changes in total mTOR or total and phosphorylated p70s6k levels in either the NACsh or NACc in these animals (*p*'s>.05). However, significantly reduced GluA1 levels ($t(8)=2.39$, $p=0.044$), and a trend towards CAMKIIα levels ($t(9)=2.171$, $p=0.058$), in the NACsh was observed in rapamycin treated animals. No significant effect on these parameters was seen in the NACc (*see Figure S4)*.

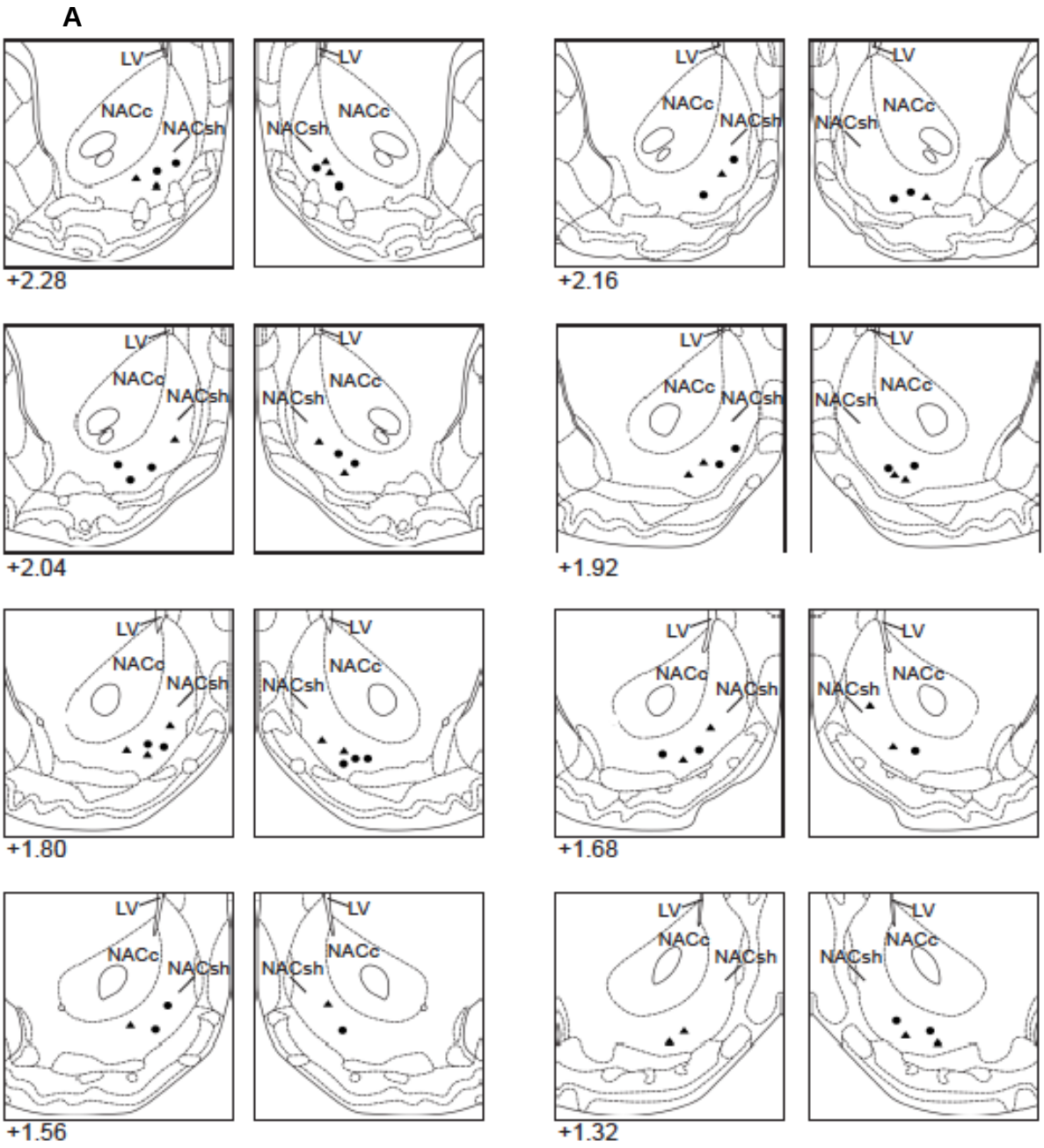

Figure S3

Figure S3: *(A) Location of intra-NACsh injections. Circles* represent microinjections of rapamycin (*n*=15). *Triangles* represent microinjections of vehicle (*n*=14). LV: lateral ventricle, NACc: nucleus accumbens core, NACsh: nucleus accumbens shell. Numbers represent the approximate rostrocaudal distance from bregma. Figures adapted from Paxinos and Watson (2007). (B) *Photomicrograph of representative intra-NACsh injector placement.* White outline denotes end of guide cannula. Black arrow denotes end of injector.

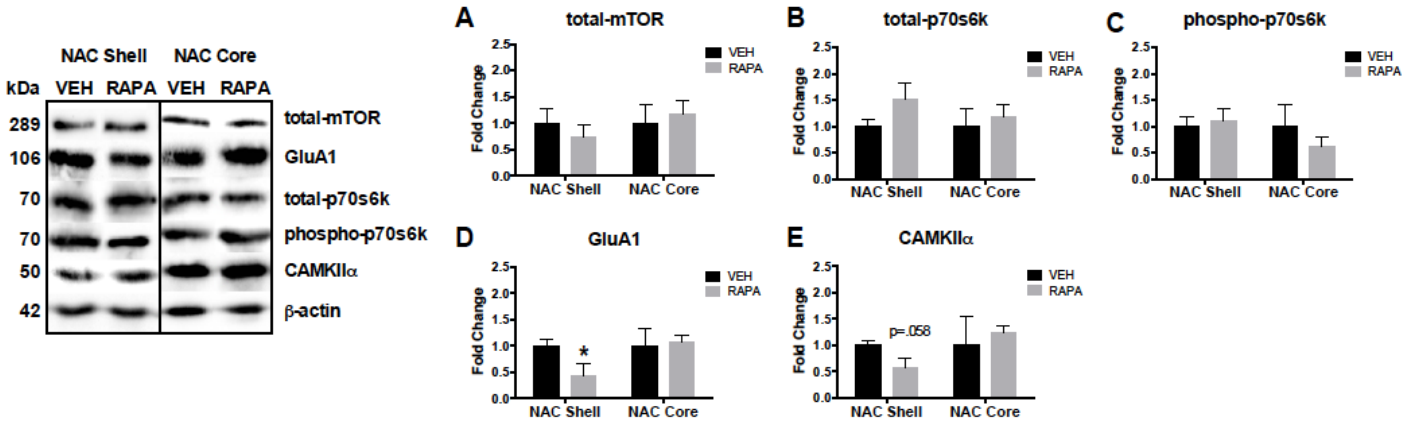

Figure S4

Figure S4: *Effect of intra-NAC mTORC1 inhibition in cocaine naïve rats.* Compared to vehicle-treated controls, no changes in total mTOR levels (A), total p70s6k (B) or phospho-p70s6k (C) were observed in the NAC after intra-NACsh rapamycin. Interestingly, rapamycin treatment resulted in reduced total GluA1 (D) and a trend towards reduced CAMKIIα (E) levels in the NACsh. Data represent means ± SEM. *p<0.05. n=4-6/group

2.2.3 References

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CHAPTER THREE: Rapamycin reduces motivated responding for cocaine and alters GluA1 expression in the ventral but not dorsal striatum.

James, Morgan H., **Quinn, Rikki K**., Ong, Lin Kooi, Levi, Emily M., Smith, Doug W., Dickson, Phillip W., Dayas, Christopher V. (2016). *European Journal of Pharmacology,* 784**,** 147-154.

Author contributions to this manuscript

Morgan H James	Designed and performed research, analyzed data and wrote the manuscript	
Rikki K Quinn	Designed and performed research, analyzed data and wrote the manuscript	
Lin Kooi Ong	Analyzed data and wrote the manuscript	
Emily M Levi	Performed research, analyzed data	
Doug W Smith	Wrote the manuscript	

Phillip W Dickson	Wrote the manuscript	
Christopher V Dayas	Designed the research and wrote the manuscript	

July 2016

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European Journal of Pharmacology 784 (2016) 147–154

Contents lists available at ScienceDirect

European Journal of Pharmacology

journal homepage: www.elsevier.com/locate/ejphar

Behavioural pharmacology

Rapamycin reduces motivated responding for cocaine and alters GluA1 expression in the ventral but not dorsal striatum

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ARTICLE INFO

Article history:
Received 13 November 2015
Received in revised form
26 April 2016
Accepted 10 May 2016
Available online 12 May 2016

Keywords:
Cocaine
Striatum
mTOR
Nucleus accumbens
Rapamycin
Progressive ratio

ABSTRACT

The mechanistic target of rapamycin complex 1 (mTORC1) regulates synaptic protein synthesis and therefore synaptic function and plasticity. A role for mTORC1 has recently been demonstrated for addiction-related behaviors. For example, central or intra-accumbal injections of the mTORC1 inhibitor rapamycin attenuates several indices of cocaine-seeking including progressive ratio (PR) responding and reinstatement. These behavioral effects are associated with decreased mTORC1 activity and synaptic protein translation in the nucleus accumbens (NAC) and point to a possible therapeutic role for rapamycin in the treatment of addiction. Currently, it is unclear whether similar behavioral and biochemical effects can be achieved by administering rapamycin systemically, which represents a more clinically-appropriate route of administration. Here, we assessed the effects of repeated, systemic administration of rapamycin (10 mg/kg, i.p.) on PR responding for cocaine. We also assessed whether systemic rapamycin was associated with changes in measures of mTORC1 activity and GluA1 expression in the ventral and dorsal striatum. We report that systemic rapamycin treatment reduced PR breakpoints to levels comparable to intra-NAC rapamycin. Systemic rapamycin treatment also reduced phosphorylated p70S6K and GluA1 AMPARs within the NAC but not dorsal striatum. Thus, systemic administration of rapamycin is as effective at reducing drug seeking behavior and measures of mTORC1 activity compared to direct accumbal application and may therefore represent a possible therapeutic option in the treatment of addiction. Possible caveats of this treatment approach are discussed.

1. Introduction

The mechanistic target of rapamycin (mTOR) is a serine-threonine kinase expressed ubiquitously in all eukaryotic cells, including neurons. mTOR forms two active complexes by binding to scaffolding proteins Raptor and Rictor, as well as several other components, to form either the mTOR complex 1 (mTORC1) or mTOR complex 2 (mTORC2). Studies in acute brain slices utilizing the allosteric inhibitor of mTORC1 activity rapamycin indicate that this system controls activity-dependent translation of proteins required for synaptic plasticity. This process involves phosphorylation of several intracellular targets, including p70 S6 kinase (p70S6K) and eukaryotic initiation factor 4E-binding proteins (4EBPs); (Hay and Sonenberg, 2004; Raught et al., 2001). Through this signaling cascade, mTORC1 regulates the translation of a number of plasticity-related proteins, including Ca^{2+}/Calmodulin-dependent kinase II alpha (CAMKIIα), AMPA and NMDA receptor subunits, including GluA1 (Dayas et al., 2012; Hou and Klann, 2004; Mameli et al., 2007). Given the well-characterized changes in these proteins in response to drug exposure, it has been suggested that dysregulated mTORC1 signaling may play a key role in modulating drug seeking and addiction.

Acute and chronic exposure to drugs of abuse is associated with increased mTORC1 activity in reward-relevant brain regions, particularly the nucleus accumbens (NAC) (James et al., 2014; Neasta et al., 2010; Wu et al., 2011). Further, accumbal levels of S6K and 4EBP are enhanced following exposure to drug-associated cues and contexts, suggesting that the ventral striatum is a key site at which changes in mTORC1 activity may modulate drug-seeking behavior. To this end, several studies have examined the effect of intra-accumbal infusions of the mTORC1 inhibitor rapamycin on

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http://dx.doi.org/10.1016/j.ejphar.2016.05.013
0014-2999/

drug seeking behavior. For example, we recently showed that repeated (5d) intra-accumbal infusions of rapamycin during self-administration training had no effect on cocaine responding on an FR1 schedule, but significantly attenuated breakpoints on a progressive ratio schedule (James et al., 2014). This treatment regime also significantly reduced responding on a subsequent discriminative cue-induced reinstatement test up to 6 weeks later, as well as levels of GluA1 AMPAR levels in the NAC (James et al., 2014). These findings are generally consistent with studies showing that acute intra-accumbal rapamycin infusions are effective at reducing psychostimulant reinstatement (Wang et al., 2010) and sensitization (Narita et al., 2005) behavior.

Whilst these findings point to a potential therapeutic role for rapamycin in the treatment of addiction, there is a need for a more complete understanding of the effects of rapamycin when delivered systemically – a more clinically relevant route of administration. Although a small number of studies have utilized systemic rapamycin treatment regimes, they have been largely restricted to either alcohol seeking (Barak et al., 2013) or paradigms that involve non-operant administration of psychostimulants, such as conditioned place preference (Wu et al., 2011; Bailey et al., 2012). As such, the effect of systemic rapamycin administration on psychostimulant self-administration behavior remains unclear.

Accordingly, we assessed the effects of rapamycin on progressive ratio responding for cocaine, a sensitive test of motivated responding for drug reinforcement. We chose a repeated (3d) systemic dosing regime to explore the effectiveness of subchronic rapamycin treatment on this behavior. To assess whether systemic rapamycin treatment is effective at producing physiologically-relevant reductions in mTORC1 signaling and AMPAR subunit levels within the NAC, we assessed mTORC1 activity, as well as GluA1 levels following treatment. To evaluate the specificity of these biochemical changes, we also assessed rapamycin-induced changes in mTORC1 activity in the dorsal striatum, another region known to be important for drug-seeking behaviors (Balleine et al., 2009; Corbit et al., 2014a, 2014b; Everitt and Robbins, 2013).

2. Materials and methods

2.1. Animals and ethics statement

Male Sprague-Dawley rats weighing 200–250 g upon arrival (Animal Resources Centre, WA, Australia) were housed two per cage on a reverse 12 h light/dark cycle (lights off 0700 h) with *ad libitum* access to food and water. All procedures were carried out in strict accordance with protocols approved by the University of Newcastle Animal Care and Ethics Committee, New South Wales Animal Research Act and Regulations and the Australian Code of Practice for the care and use of animals for scientific purposes.

2.2. Intravenous surgery

Upon arrival, rats were handled daily for one week before undergoing intravenous (IV) catheter surgery as described previously (James et al., 2011b; Yeoh et al., 2012). Briefly, animals were anaesthetised with isoflurane (1–3% with a flow rate of 2 l/min) and, using aseptic procedures a Silastic catheter was surgically implanted into the right jugular vein. Prior to surgery, rats were injected intramuscularly with .3 ml of a broad-spectrum antibiotic (150 mg/ml procaine penicillin, 112.5 mg/ml benzathine penicillin; Norbrook Laboratories, UK) and subcutaneously with .2 ml of a 50 mg/ml solution of carprofen (Norbrook Laboratories, UK). Post surgery, catheters were flushed daily with .3 ml of 50 mg/ml cephazolin (Mayne Pharma, Australia) and .2 ml of 50 unit heparinized saline to maintain catheter patency.

2.3. Drugs

For cocaine self-administration, cocaine hydrochloride (Johnson Matthey, Edinburgh, UK) was dissolved in sterile physiological saline (2.5 mg/ml), as per previous studies (James et al., 2010). Rapamycin, (Cat #553210, Calbiochem, USA) was dissolved in dimethyl sulfoxide (James et al., 2014; Wang et al., 2010). The dose of rapamycin (10 mg/kg) was based on previous reports where rapamycin was administered systemically (acutely and chronically) in drug-seeking paradigms (Bailey et al., 2012; Lin et al., 2014; Mazei-Robison et al., 2011; Neasta et al., 2010). Rapamycin was administered 3 h prior to behavioral testing because peak concentrations are achieved 1–3 h following systemic administration (Manufacturers' Product Information Sheet; also see manufacturers details for Rapamune (sirolimus)) and based on previous studies (James et al., 2014; Neasta et al., 2010).

2.4. Behavioral testing equipment

Behavioral procedures were conducted in Med-Associates self-administration chambers (Med Associates, VT, USA). Chambers were equipped with two retractable levers (6 cm above the floor), a white cue light above each lever, two speakers to deliver auditory stimuli, and a white house light at the top of the chamber wall opposing the levers. Auditory stimuli were produced by a white noise generator/speaker adjacent to the house light (Med Associates, VT, USA) that produced a 70 dB white noise, and a tone source located in the top left corner (Sonalert with volume control, Med Associates, VT, USA) that was connected to a speaker that produced an intermittent tone (0.5 Hz at 70 dB). A syringe pump (5 rpm motor, Med Associates, VT, USA) located on the outside of cubicle delivered the IV cocaine. Data acquisition and behavioral testing equipment were controlled by a Windows-based PC, using MED-PC IV (Med Associates, VT, USA).

2.5. Self-administration training

Animals were trained to respond on the right lever for a cocaine infusion (0.1 ml/i.v.) on an FR1 schedule (3 h/day, 5d/week). Infusions were followed by the illumination of a white cue light above the active lever signaling a 20 s timeout period. Animals were limited to earning a maximum of 20 rewards during initial training sessions to prevent overdose. Once stable responding was established (animals were required to obtain the maximum number of rewards per session, +10%, over three sessions)

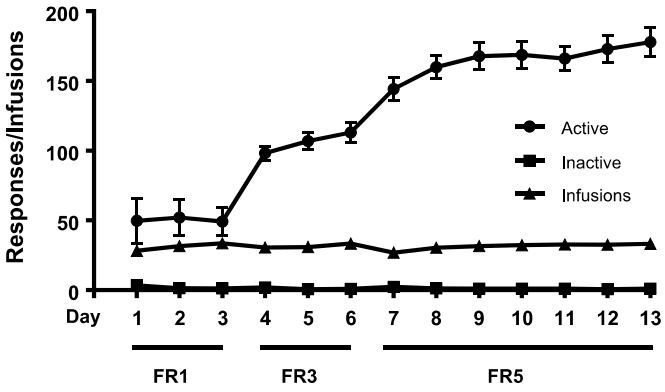

Fig. 1. Rats increased active lever responding across FR1, FR3 and FR5 self-administration training. Animals were initially trained to self-administer cocaine on a FR1 schedule, before progressing to FR3 and FR5 schedules. Rats readily exhibited increased responding on the active lever following changes in reinforcement schedules. In contrast, inactive lever responses and the number of rewarded responses (drug infusions) remained stable.

animals progressed to 3 h daily self-administration sessions as described elsewhere (James et al., 2011a, 2014; Yeoh et al., 2012). During these 3 h sessions, rats were given unrestricted access to cocaine on an FR1 schedule (3d), FR3 schedule (3d) and then an FR5 schedule (7d; Fig. 1). During all sessions, responses on the left 'inactive' lever were recorded but did not result in a drug reward.

2.6. Progressive ratio testing

Following the final day of self-administration on the FR5 schedule, animals were tested on a progressive ratio schedule, as described previously (e.g. Brown et al., 2011; James et al., 2014). Over three consecutive test days, animals were treated systemically with either rapamycin (10 mg/kg; i.p., n=11) or vehicle (equivolume, n=8), and then returned to their home cage which was placed in the self-administration testing room. Three h later, animals began testing on the PR schedule. During these sessions, cocaine was available for the entire session and the number of lever responses required to receive a cocaine reward was progressively increased, using the schedule 5, 10, 17, 24, 32, 42, 56, 73, 95, 124, 161, 208, 268, 345, 445, 573, 737, 947, 1218, 1566, 2012, 2585 presses required for one infusion. PR testing was performed over 5 h, however, PR sessions were programmed to shut down if a lever press was not recorded within 45 min of a previous lever press. Following the final PR test, animals were returned to their home cage and killed 24 h later as described below.

2.7. Animal sacrifice and tissue harvesting and processing

As in our previous study (James et al., 2014), animals were decapitated 24 h after the final PR test, and their brains were rapidly removed and cooled in ice-cold diethylpyrocarbonate (DEPC)-treated PBS. Brains were blocked into forebrain and hindbrain regions on an ice-cold stage and the blocks were snap frozen in dry-ice chilled isopentane (Sigma-Aldrich, MO, USA). Tissue was stored at −80 °C until required. Brains from six representative animals from both treatment groups were cryosectioned into 100 μm sections and the NAC core (NACc), NAC shell (NACs), dorsolateral striatum (DLS) and dorsomedial striatum (DMS) were then macrodissected from the sections using a .8 mm diameter tissue punch and kept frozen until homogenisation. Tissues were homogenised using a sonicator (Soniprep 150, MSE, London, UK) in homogenisation buffer (1 mM EDTA, 50 mM Tris, pH 6.8, .5% DTT, 1 mM Microcystin). SDS was added to a final concentration of 2.5% and the samples were boiled for 5 min then centrifuged at 20,000 × *g* for 10 min at 25 °C. The clear supernatants were collected and mixed with sample buffer (1% SDS, 10% glycerol, .5% DTT and .1% Bromophenol blue).

2.8. SDS-page and western blot analysis

Samples were subjected to SDS–polyacrylamide gel electrophoresis before being transferred to nitrocellulose (Jarvie and Dunkley, 1995). Membranes were then stained with Ponceau S (0.5% Ponceau in 1% acetic acid) to assess the efficacy of the transfer. Membranes were then washed in Tris-buffered saline with Tween (TBST) (150 mM NaCl, 10 mM Tris, .075% Tween-20, pH 7.5) and blocked in 5% Skim Milk Powder in TBST for 1 h at 25 °C. Membranes were washed in TBST and incubated with anti-mTOR (1:500 Cell Signaling Technologies #2972), anti-p70s6k

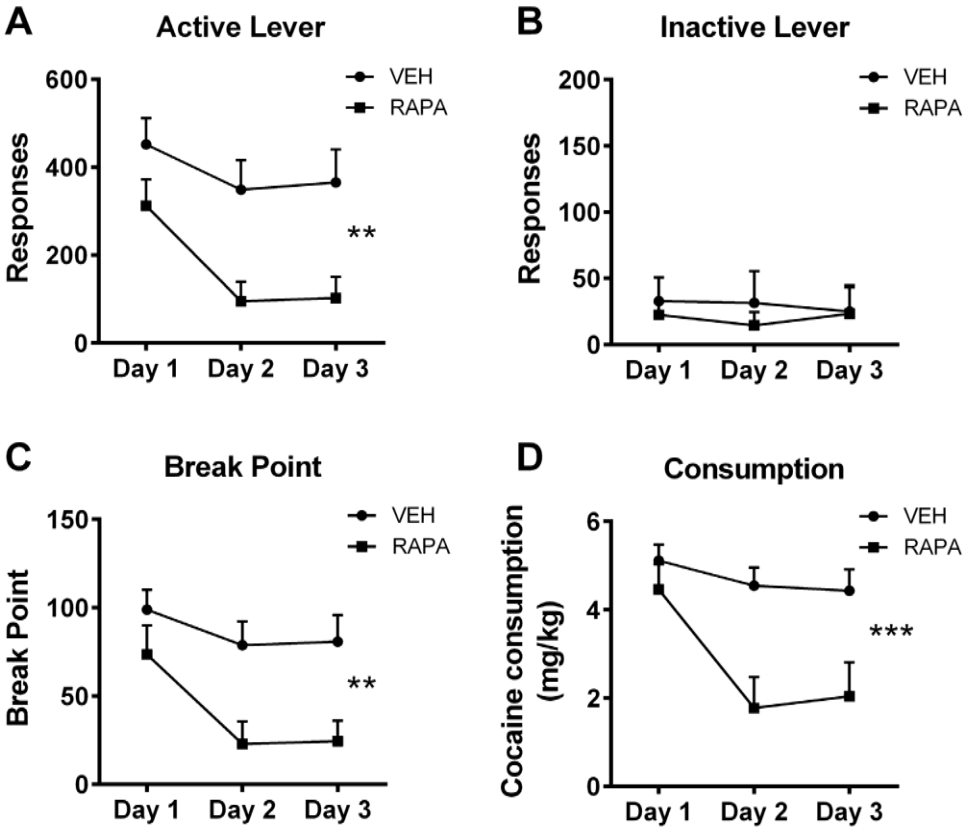

Fig. 2. Repeated systemic rapamycin treatment reduced drug-seeking on a progressive ratio schedule. Following 7d of stable responding on an FR5 schedule, we assessed the effect of repeated systemically administered rapamycin on progressive ratio responding. Animals treated with rapamycin (10 mg/kg; n=11) showed significant reductions in the average number of active lever responses across the treatment period as compared to saline treated controls (n=8; A). There was no effect of rapamycin on inactive responding (B). Rapamycin treatment was also associated with a reduction in progressive ratio break points (C) and cocaine consumption as a function of body weight (D) over the treatment period. **p < .01.

(1:500 Cell Signaling Technologies #9202), anti-phospho-p60s6k (Thr389; 1:500 Cell Signalling Technology #9205), anti-GluA1 (1:1000 Millipore #AB1504) and anti-β-actin (1:20 000 Sigma Aldrich A3854) primary antibodies overnight at 4 °C. Membranes were washed in TBST and incubated with horse-radish peroxidase-linked anti-IgG secondary specific antibodies for 1 h at 25 °C. Membranes were visualized on Fujifilm Las-3000 imaging system (Fuji, Stamford, CT, USA) using ECL plus detection reagents. The density of bands were measured using MultiGauge V3.0 (Fuji, Stamford, CT, USA). Total mTOR, total and phosphorylated p70S6k and total GluA1 levels were assessed. Total mTOR, p70S6k and GluA1 protein levels were expressed as the ratio of total protein to β-actin. Phosphorylated p70S6k (p-p70S6K) levels were expressed as a ratio relative to total p70S6k protein to account for variability between samples. Finally, protein levels in the rapamycin-treated group were expressed as a fold change relative to protein levels in the vehicle-treated group.

2.9. Statistical analyses

Changes in active lever responses, inactive lever responses, break point and body weight during progressive ratio testing were assessed using a 3 'treatment day' (d1, d2, d3) × 2 'treatment' (vehicle, rapamycin) mixed model ANOVA and subsequent post hoc Tukey comparisons. Protein data (calculated as fold change relative to vehicle) in each subregion (DMS, DLS, NACc, NACs) were compared across treatment groups (vehicle, rapamycin) using individual student *t*-tests. An alpha value of .05 was adopted for all statistical tests, except where multiple comparisons were conducted, in which case the alpha value was adjusted according to the bonferonni correction.

3. Results

3.1. Animals from both treatment groups displayed similar self-administration behavior prior to rapamycin administration

All animals acquired stable self-administration behavior on the training FR1 schedule in 7 days or less. As shown in Fig. 1, responding on the active lever was significantly greater than responding on the inactive lever. Animals were assigned to progressive ratio testing groups so as to ensure that each treatment group did not differ significantly in terms of active/inactive lever responding as well as overall cocaine consumption throughout the entire self-administration training period (P's > .05, data not shown).

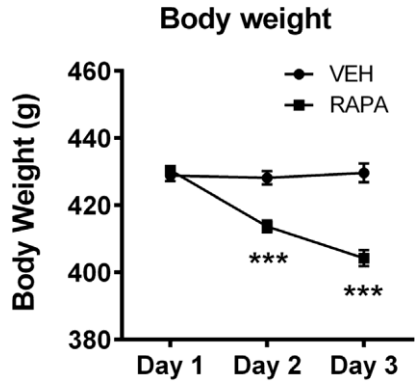

Fig. 3. Repeated systemic rapamycin treatment was associated with reductions in body weight in cocaine self-administering rats. Compared to vehicle-treated animals, animals that received rapamycin (10 mg/kg) exhibited modest, but significant reductions in body weight on days 2 and 3 of progressive ratio testing. Importantly, reductions in body weight were not correlated with changes in progressive ratio responding. ***p < .001.

3.2. Rapamycin reduces responding for cocaine on a progressive ratio schedule

To assess the effect of repeated rapamycin on cocaine taking behavior, we treated animals with rapamycin (10 mg/kg; i.p.) over three days of progressive ratio testing (Fig. 2). Across the three days of testing, there was a significant main effect of 'treatment' on active lever responding ($F(1,17)=7.55$, $P=.014$). In contrast, there was no significant main effect of 'treatment day', nor a significant 'treatment' × 'treatment day' interaction (P's > .05). There was no effect of rapamycin on inactive lever responding (P's > .05; Fig. 2 (B)), which is commonly regarded as an indicator of non-specific drug seeking and/or general motor activity. With respect to break points reached throughout the PR sessions, there was a significant main effect of 'treatment' ($t(17)=3.17$, $P=.006$) but no main effect of 'treatment day' and no interaction between the 'treatment' and 'treatment day' variables (Fig. 2(C)). Because systemic rapamycin treatment is known to reduce body weight (see below), we also compared cocaine consumption (mg/kg) across the three treatment days. This revealed significant main effects of both 'treatment' ($F(1,51)=14.26$, $P=.0004$) and 'treatment day' ($F(2,51)=4.27$, $P=0.0193$). The 'treatment' x 'treatment day' interaction was not significant ($F(2,51)=1.615$, $P=.2089$).

3.3. Repeated systemic rapamycin treatment in cocaine self-administering animals is associated with a reduction in body weight

As there is a well-documented effect of systemic rapamycin administration on body weight gain (Chang et al., 2009; Deblon et al., 2012; Hebert et al., 2014), we also monitored changes in body weight over the rapamycin treatment period in cocaine-trained rats. Consistent with previous reports, there was a significant main effect of 'treatment' over the three days of testing ($F(1,51)=56.70$, $P<.0001$), a significant main effect of 'treatment day' ($F(2,51)=19.47$, $P<.0001$), and a significant 'treatment' × 'treatment day' interaction ($F(2,51)=21.35$, $P<.0001$; Fig. 3). Subsequent post-hoc comparisons indicated that rapamycin-treated animals exhibited significantly lower body weights on day 2 ($t(51)=4.932$, $P<.0001$) and day 3 ($t(51)=8.648$, $P<.0001$) of treatment. This equated to approximately 4% and 6% of weight loss relative to baseline, on days 2 and 3, respectively. Importantly, individual changes in body weight were not significantly correlated with individual changes in PR scores ($R^2=.15$, $P>.05$) over the treatment period, suggesting that changes in these two indices were unrelated.

3.4. Repeated systemic rapamycin treatment is associated with reductions in p-p70S6K and GluA1 levels in ventral but not dorsal striatum

To understand molecular mechanisms contributing to these changes in cocaine self-administration behavior, we measured a number of indices of mTOR activity in different subregions of the ventral and dorsal striatum (Fig. 4 A, B). Rapamycin treatment had no effect on total mTOR levels in either ventral or dorsal striatum (Fig. 4 (C), P's > .05). p70S6K is a key target of mTORC1, therefore, we assessed total and phosphorylated levels of p70S6K. Rapamycin treatment was associated with a small but significant increase total p70S6K levels only in the in NAC core ($t(10)=2.27$, $P=.047$), despite a similar (non-significant) trend in other striatal subregions (Fig. 4(D)). Rapamycin also significantly reduced levels of p-p70s6k in both the NAC core ($t(5.20)=3.43$, $P=.017$) and shell ($t(10)=3.98$, $P=.003$), but had no effect on p-p70S6K levels in either subregion of the DS (Fig. 4(E), P's > .05). Finally, because mTORC1 regulates translation of synaptic proteins, including AMPARs, we assessed total GluA1 levels in the striatum. Rapamycin treatment was associated with a significant

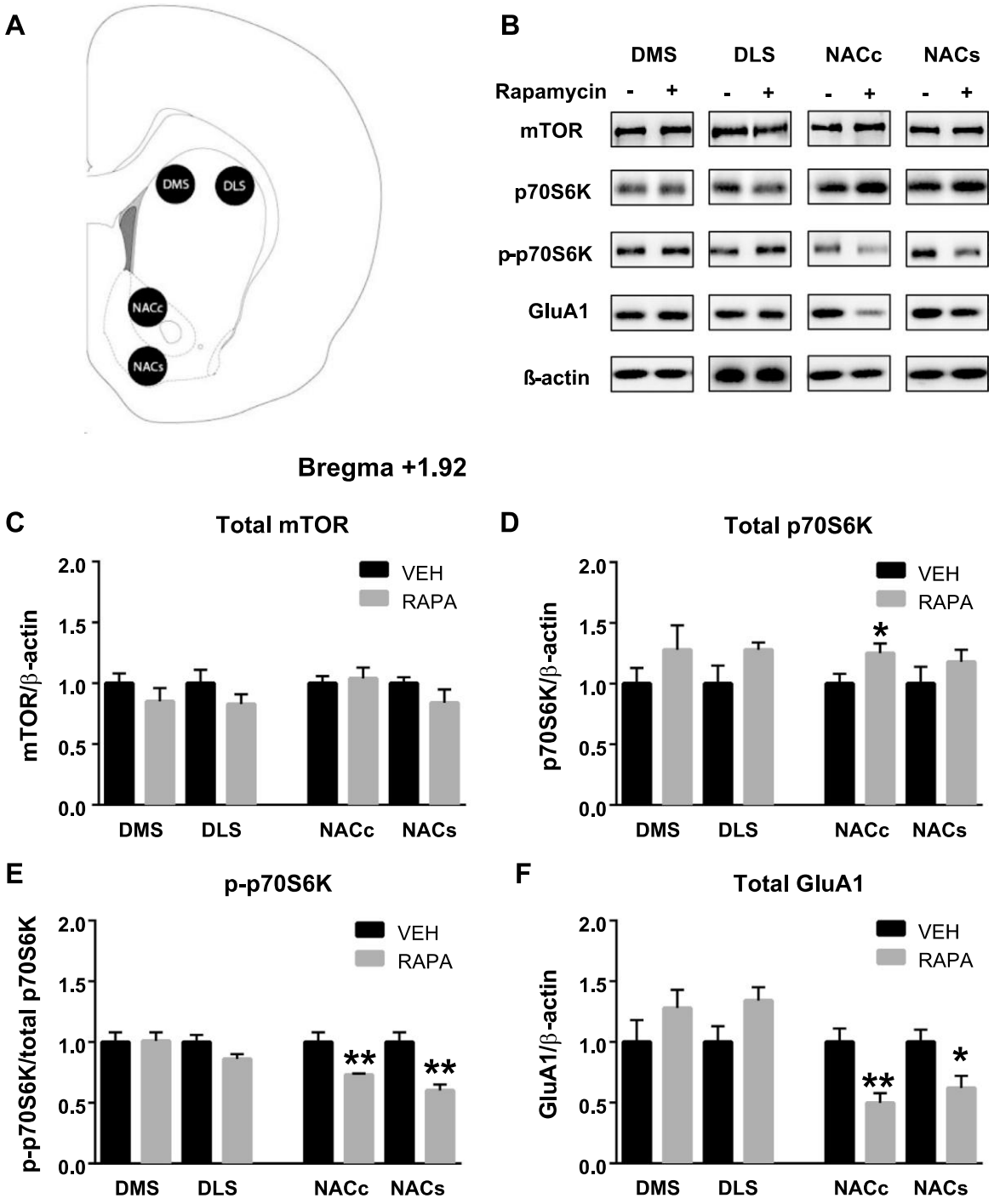

Fig. 4. Rapmycin-induced reductions in progressive ratio responding were associated with reduced mTORC1 activity and GluA1 expression in ventral, but not dorsal, striatum. Tissue punches were taken from 100 μm coronal sections from dorsomedial striatum (DMS), dorsolateral striatum (DLS), nucleus accumbens core (NACc) and nucleus accumbens shell (NACs). These sites are depicted in panel (A) – adapted from Paxinos and Watson (1997). Representative Western blots from vehicle- and rapamycin-treated animals across all proteins of interest in ventral and dorsal striatal regions are presented in panel (B). Rapamycin treatment had no effect on total mTOR levels in either dorsal or ventral striatum (C). Similarly, rapamycin had no effect on total p70S6K levels in DMS, DLS or NACs, but was associated with a significant increase in total p70S6K levels in NACc (D). Whilst rapamycin had no effect on p-p70S6K or total GluA1 levels in either subregion of dorsal striatum, it was associated with significant reductions in p-p70S6K and total GluA1 levels in both NACc and NACs (E, F). $^{*}p < .05$. $^{**}p < .01$.

reduction in total GluA1 levels in both the NAC core ($t(10) = 3.78$, $P = .004$) and shell ($t(10) = 2.59$, $P = .027$) subregions, but had no effect on this measure in either subregion of the DS (Fig. 4(F)).

4. Discussion

In the present study we report that repeated systemic rapamycin treatment attenuated responding for cocaine on a progressive ratio schedule of reinforcement, a behavioral measure of motivational responding for drug reinforcement. This effect is similar to that achieved by intra-NAC delivery of rapamycin in our previous study (James et al., 2014). We also show that systemic rapamycin treatment reduced levels of phosphorylated p70S6K – a marker of mTORC1 activity – in the core and shell subregions of the ventral striatum. Consistent with the role of mTORC1 in synaptic protein translation, rapamycin treatment also significantly reduced GluA1

AMPAR subunit levels in the NAC. Interestingly, these effects appeared to be specific to the ventral component of the striatum, as we saw no evidence of changes to mTORC1 signaling in the dorsomedial or dorsolateral striatum. These observations are important given previous demonstrations by our group (James et al., 2014) and others (Barak et al., 2013; Wang et al., 2010) that the NAC is as a key site for mTORC1 signaling-induced changes in drug seeking behavior. Together, these data indicate that systemic rapamycin delivery is equally effective at producing both behavioral and biochemical changes achieved by direct infusion of rapamycin into the NAC, and therefore highlight the potential therapeutic potential of rapamycin for the treatment of addiction.

In a recent study, we reported that repeated intra-NAC infusions of rapamycin during self-administration training reduced break points for cocaine on a subsequent progressive ratio test (James et al., 2014). Other studies have reported that intra-NAC delivery of rapamycin decreases binge drinking and sustained consumption of alcohol in rats (Neasta et al., 2010, 2014). Here, we extend these findings by showing that repeated systemic treatment with rapamycin, a more clinically-relevant dosing regimen, is also effective at reducing progressive ratio responding. Interestingly, the effect of rapamycin on self-administration of cocaine appears to be specific to higher effort schedules of reinforcement, as rapamycin treatment has no effect on cocaine seeking under lower effort schedules (Wang et al., 2010; James et al., 2014). These findings may suggest that mTORC1 signaling has a limited role in modulating the acute reinforcing properties of psychostimulants. In support, systemic rapamycin treatment did not block the acquisition of cocaine locomotor sensitization and conditioned place preference, but prevented the expression of these behaviors in mice (Bailey et al., 2012). Similarly, intra-NAC infusions of rapamycin had no effect on the acquisition of methamphetamine CPP in rats (Narita et al., 2005). Further, the expression, but not the acquisition, of cocaine-induced behavioral sensitization is associated with increased levels of phosphatidylinositol-3-kinase (PI3K), an upstream regulator of mTORC1 activity, in the NAC (Izzo et al., 2002). These effects may be specific to psychostimulants, as systemic and intra-NAC injections of rapamycin reduce alcohol drinking on low-effort schedules of reinforcement (Neasta et al., 2010, 2014).

We also show that repeated systemic rapamycin treatment was associated with decreased phosphorylated p70S6K, the most commonly used read out of mTORC1 activity, and decreased GluA1 levels in both the NAC core and shell. This finding is important given previous studies that have established the NAC as a critical site for drug-associated mTORC1 signaling. For example, exposure to cocaine and alcohol potently activates the mTORC1 signaling pathway in the NAC, as evidenced by increased levels of phosphorylated S6K, 4E-BP and PI3K proteins (Cozzoli et al., 2009; Neasta et al., 2010; Goulding et al., 2011; Wu et al., 2011; James et al., 2014). These effects were associated with increased translation of synaptic proteins in the NAC that contribute to drug reward and long-term relapse vulnerability. The reductions in mTORC1 signaling observed here may reflect a normalization of cocaine-induced changes in this system, however further studies that include a yoked-saline control group are needed to confirm this.

The GluA1 subunit of the AMPA receptor is among the synaptic proteins known to be regulated by mTORC1 (Slipczuk et al., 2009) and increased translation and synaptic trafficking of Ca^{2+} permeable GluA1 AMPARs has consistently been linked with increased risk of drug-seeking behavior (Churchill and Kalivas, 1999; Lu et al., 2003; Anderson et al., 2008; Conrad et al., 2008; Wolf, 2010; Ferrario et al., 2011). In the present study, we found that rapamycin treatment was associated with a reduction in NAC GluA1 subunit levels. Thus, translation of Glu1A AMPARs in NAC medium spiny neurons may be necessary for effort-based drug seeking.

We also investigated the effects of rapamycin treatment on mTORC1 signaling in the dorsal striatum, as together with the NAC, this region makes up a key component of the corticostriatal network that is important in the development of addiction. Indeed, the medial portion of dorsal striatum plays a key role in mediating action-outcome controlled drug seeking behavior, whereas compulsive drug seeking is thought to devolve to and depend on the lateral dorsal striatum (Balleine et al., 2009; Corbit et al., 2014a, 2014b). Interestingly, we observed no molecular changes in either the medial or lateral components of the dorsal striatum. This finding is consistent with previous findings from our laboratory that show that mTOR signaling in the NAC is more transcriptionally sensitive than the DS (Brown et al., 2011). It is also possible that mTORC1-related signaling in the dorsal striatum is engaged at a different phase of the drug-seeking to that measured here (24 h withdrawal). Regardless, the changes we observed in the NAC are likely to be relevant to the overall function of dorsal striatum given the known links between these regions through descending projections to the substantia nigra (Haber et al., 2000; Haber, 2003).

Importantly, systemic administration of rapamycin was associated with a modest decrease in body weight over the treatment period. This effect is unsurprising given that systemic rapamycin administration is associated with reduced weight gain and decreased fat mass in laboratory rats and mice (Chang et al., 2009; Deblon et al., 2012; Hebert et al., 2014; Yang et al., 2012; Cota et al., 2006). Acute, systemic rapamycin treatment (10 mg/kg) is sufficient to decrease food intake for five days, with differences in body weight gain persisting at least 10 weeks later (Hebert et al., 2014). Importantly, these effects appear to reflect specific metabolic changes – rather than general malaise – as systemic rapamycin administration does not produce conditioned taste aversion or conditioned place aversion, which are robust and sensitive tests of drug-induced malaise or illness (Barak et al., 2013; Hebert et al., 2014). Consistent with these data, the magnitude of body weight reductions in the present study were not correlated with changes in progressive ratio responding, suggesting that the behavioral effects we observed were not the result of non-specific effects of rapamycin administration. Nevertheless, it is important that the effects of rapamycin on physiological processes (e.g. glucose intolerance, insulin resistance) are fully examined before the potential therapeutic efficacy of rapamycin for addiction can be considered (Chang et al., 2009; Deblon et al., 2012).

5. Conclusion

In summary, we show that similar to intra-NAC delivery of rapamycin, systemically administered rapamycin attenuates responding for cocaine on a progressive ratio schedule of reinforcement. This finding builds upon observations from previous studies showing that rapamycin treatment does not affect responding for cocaine under low effort schedules, and suggests a unique role for mTORC1 signaling in effort-based motivational responding. We also report that systemic rapamycin treatment resulted in a downregulation of the mTORC1 signaling pathway and GluA1 expression in the ventral component of the striatum, suggesting that mTORC1-mediated translation of GluA1 AMPARs in the NAC might be critical to the expression of motivated responding for cocaine. Together, these findings demonstrate that systemic rapamycin treatment produces similar behavioral and biochemical effects as achieved with intra-NAC rapamycin. Importantly, potential off-target effects of systemic rapamycin delivery must be comprehensively assessed before the therapeutic

benefits of rapamycin for the treatment for addiction can be considered.

6. Authors contributions

MHJ, DWS and CVD were responsible for the study concept and design. MHJ and EML contributed to the acquisition of animal data. EML, RKQ and LKO performed the protein analyses. MHJ, RKQ, EML, LKO, PWD and CVD assisted with data analysis and interpretation of findings. MHJ and CVD drafted the manuscript. MHJ, CVD, PWD and DWS provided critical revision of the manuscript for important intellectual content. All authors approved the final version for publication.

Acknowledgements

This work was supported by project grants from the National Health and Medical Research Council (NHMRC) of Australia and an NHMRC CJ Martin Fellowship (1072706) to M.H.J. We would like to thank Ms Janine Charnley and Ms Jessica Buck for their assistance with the acquisition of animal data and Ms Helen Carpenter for her assistance with protein analyses.

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CHAPTER FOUR: Addiction phenotyping reveals temporally specific miRNA expression patterns in the dorsal and ventral striatum

Quinn, Rikki K., James, Morgan H., Hawkins, Guy E., Brown, Amanda L., Heathcote, Andrew., Smith, Doug W., Cairns, Murray J., Dayas, Christopher V. Submitted to *Molecular Psychiatry*

Author contributions to this manuscript

Rikki K Quinn	Designed and performed research, analyzed data and wrote the manuscript	
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Guy E Hawkins	Performed research, analyzed data and wrote the manuscript	
Amanda L Brown	Designed and performed research, analyzed data	

Andrew Heathcote	Wrote the manuscript	
Doug W Smith	Wrote the manuscript	
Murray J Cairns	Wrote the manuscript	
Christopher V Dayas	Designed the research and wrote the manuscript	

July 2016

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Addiction phenotyping reveals temporally specific miRNA expression patterns in the dorsal and ventral striatum

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4.2 Abstract

Background: miRNA within the ventral (VS) and dorsal (DS) striatum have been shown to regulate addiction relevant behaviours, including conditioned place preference and compulsive drug taking.

Method: To understand the temporal profile of addiction relevant miRNA expression across the addiction cycle, we developed a Bayesian Model Averaging paradigm to identify addiction-relapse vulnerable rats during the drug-taking phase of the addiction cycle. We then examined the expression of miRNA in striatal subregions of these vulnerable animals at distinct stages across the addiction cycle.

Results: We found distinct patterns of miRNA expression within the nucleus accumbens core (NACc) and shell (NACsh) across the addiction cycle. Cocaine taking preferentially altered miRNA expression within the NACc at early time points in all animals whereas, between vulnerable animals, miRNA expression was generally increased in the NACsh. While fewer miRNA changes were observed in the DS at early addiction time points, the expression of the schizophrenia risk-associated miR-137 was found to be sensitive to the expression of cocaine. At this time point, the expression of the protective miR-212 was engaged in the DMS of vulnerable rats – a process

that appears to become exhausted across the course of addiction cycle and is associated with increased relapse-risk based on previous work.

Conclusion: This study identifies temporally-specific changes in miRNA expression consistent with the engagement of distinct striatal subregions across the course of the addiction cycle. Dysregulated miRNA expression in the NAC and DS is likely to be a key substrate of ongoing drug-seeking and habit-based addiction.

4.3 Introduction

The ventral striatum (VS) is a key component of the brain reward-seeking system. Traditionally, the nucleus accumbens (NAC) of the VS has been a primary focus in the study of drug reward, and can be divided into functionally distinct subregions including the shell (NACsh) and core (NACc) (1). Importantly, recent evidence has implicated the dorsal striatum (DS) in addiction based on its role in decision-making and habit learning (2). Like the NAC, the DS is highly heterogeneous. With respect to addiction, most studies focus on the dorsomedial (DM) and dorsolateral (DL) subregions.

Abused drugs present a major challenge to the normal dynamic range of glutamatergic and dopaminergic synapses in the NAC and DS (3-6). Repeated intake/withdrawal cycles promote fluctuations in glutamate and dopamine neurotransmitter concentrations within the striatum (7-12). These frequent and dynamic fluctuations result in structural and cellular changes that can impair the ability to elicit long-term depression (LTD) and potentiation (LTP) (13-17). Notably, functional synaptic changes in addiction models have focused primarily on the NAC. However, changes in molecules required for

the induction and maintenance of synaptic plasticity are seen in both the NAC and DS (18-19).

Like humans, not all animals exposed to drugs exhibit behaviours that resemble addiction. Models that incorporate the identification of ‘addicted’ rats have identified important differences in the synaptic and molecular profiles that are linked with addiction vulnerability (13, 20). Importantly these changes are often more subtle than what is observed between drug-exposed versus drug-naïve animals but are arguably more relevant to human addiction (18). For example, Kasanetz et al., 2010 found that only animals classified as ‘addicted’ displayed evidence of ongoing impairments in synaptic function (13). Further, we showed that there was a modest but widespread reduction in plasticity-associated genes, including dopamine receptor 1 (*Drd1*) and mammalian target of Rapamycin (*mTOR*), in the NAC and DS of animals phenotyped as addiction vulnerable versus resilient (18). The pattern of suppression amongst synaptic plasticity associated genes led us to test the hypothesis that these changes might be sustained by an epigenetic mechanism such as microRNA (miRNA).

MiRNA are primarily negative regulators of mRNA expression and have been implicated in the neuroadaptations associated with addiction. Most notably, altered miR-212 expression in the DS has been implicated in the regulation of compulsive cocaine seeking (21, 22). In parallel studies where we assessed gene expression changes at the end of the addiction cycle (after relapse testing), we found that miR-212 expression was lower in the DMS of vulnerable animals compared to resilient controls, consistent with a

hypothesis that increased miR-212 in the DS is protective against cocaine-seeking (19).

Critically, a comprehensive understanding of the temporal profile of miRNA expression across the addiction cycle is lacking. Furthermore, in our study, phenotyped groups did not differ in levels of cocaine consumption indicating that specific miRNA changes may develop in vulnerable animals only irrespective of the levels of drug consumed. It is also unclear whether miR-212 changes develop in the VS and follow the same pattern of changes as in the DS.

This information is highly relevant to guide an improved understanding of the temporal changes that drive habit-based addiction. Therefore, to better understand the temporal expression of miRNA across the addiction cycle, we used a Bayesian Model Averaging (BMA) to facilitate selection of vulnerable animals at early phases of the addiction cycle. This involved applying behavioural 'predictor variables' to index future relapse vulnerability. We then examined the expression of candidate miRNA in vulnerable rats at two key time points, immediately following drug taking and after reinstatement, in VS and DS subregions.

4.4 Methods

4.4.1 General Methods

For details on intravenous surgery, behavioural testing equipment, tissue dissection and qPCR see Supplementary Material.

4.4.2 Experiment 1 – Effect of cocaine on miRNA expression in the striatal subregions 24h after drug taking

To assess the effect of cocaine on miRNA expression, rats were implanted with jugular catheters and randomized to either cocaine (n=16) or yoked-saline (n=7) groups. Cocaine rats were trained to lever press for cocaine (0.25mg/0.1mL i.v.) on a fixed ratio (FR) 1 schedule. Yoked-saline rats were placed in the operant chamber but lever presses had no scheduled consequence. Saline rats received non-contingent infusions of saline when their yoked-cocaine counterpart received an infusion of cocaine. Once stable responding for cocaine was established, rats were introduced to cocaine-associated cues reflecting drug availability (DA, white noise) and non-drug availability (NDA, illumination of house light). Rats then progressed to an FR3 and then FR5 schedule of reinforcement, and were tested for their inability to refrain from drug seeking during the NDA period (Figure S1a). Finally, rats were tested for motivation to consume drug on a progressive ratio (PR) test. Rats were sacrificed 24 hours after the final PR session. Brains were snap frozen and stored at -80°C for subsequent analysis of miRNA expression.

4.4.3 Experiment 2 – miRNA expression in the striatal subregions 24h after drug taking in addiction vulnerable rats

4.4.3.1 BMA Model

To identify animals early in the addiction cycle that displayed a future propensity for reinstatement at a drug taking point in the addiction cycle, we developed a model to identify early behavioural indices that can predict addiction-relapse vulnerability. To account for uncertainty in model selection

in a principled framework, we used BMA, allowing us to simultaneously test many candidate models that each contains a different combination of predictor variables. The influence or 'weight' of a candidate model is proportional to that model's ability to predict the outcome variable. The predicted outcome of a BMA analysis is therefore a weighted average of the predictions of the set of candidate models under consideration (23).

The outcome variable was the 'addiction-reinstatement' score described previously (18). Behavioural data for the candidate predictor variables were obtained from FR1, FR3, FR5 and PR sessions of a previously published cohort of rats (n=45) trained to self-administer cocaine (see Supplementary Methods) (18).

4.4.3.2 Prediction of relapse vulnerability

The BMA model developed was used to obtain a 'predicted reinstatement score' for rats (n=63) for experiment 2 (i.e., out of sample prediction). These rats included the cocaine group from experiment 1. Predicted reinstatement scores were used to phenotype rats as addiction vulnerable or resilient using the criteria previously described (18). Briefly, rats that scored in the top or bottom 40% of the distribution for predicted reinstatement as well as the top or bottom 30% of the distribution for NDA and PR are phenotyped as addiction vulnerable or resilient, respectively.

Rats were trained to self-administer cocaine and tested for addition-relevant behaviour as described for experiment 1 (Figure S1a). Twenty four hours after the final PR session, animals were sacrificed and brains snap frozen and stored at -80°C for molecular analysis.

4.4.4 Experiment 3 - miRNA expression in addiction vulnerable rats post-relapse

In this experiment, rats (n=45) were tested for addiction-relevant behaviours and phenotyped as addiction vulnerable or resilient as described previously (18). Briefly, rats were trained to self-administer cocaine on a FR1 and then FR3 schedule of responding (Figure S1b) before being tested for addiction-relevant behaviours – inability to refrain from drug seeking during a signalled period of NDA on an FR5 schedule of responding, motivation to consume drug using repeated PR tests, and cue-induced reinstatement of drug seeking. Twenty four hours after the reinstatement test, rats were sacrificed and their brains snap frozen and stored at -80°C for subsequent analysis. Behavioural data (18) and DS miRNA expression (19) from this cohort has been published previously.

4.5 Results

4.5.1 Selection of candidate miRNA involved in the development and expression of addictive behaviours

We selected candidate miRNAs previously implicated in drug taking, including miR-212 and the closely related miR-132 (21, 22). MiR-137 was selected because of its involvement in pre and post-synaptic plasticity and neuronal development and maturation (24). MiR-137 is also associated with schizophrenia (25), which has significant comorbidity with addiction (26). miRNA with the potential to regulate *Drd1* and *mTOR*, were also chosen because of the association of these synaptic plasticity genes with addiction-

relapse vulnerability (18). The target prediction algorithms miRanda and TargetScan which identified miR-101b as a potential regulator of *Drd1* and *mTOR* expression.

4.5.2 Experiment 1

4.5.2.1 Cocaine self-administering rats show greater active lever responding than saline controls

Cocaine rats showed significantly higher active lever responding in both FR5 DA (Mann-Whitney U=0, p<0.0001) and NDA (Mann-Whitney U=1, p<0.0001) periods as well as PR sessions (Mann-Whitney U=0, p<0.0001) compared to yoked-saline animals (Figure S2).

4.5.2.2 Altered miRNA expression in the NACsh and NACc following cocaine self-administration

The expression of miRNA were examined 24 hours after the final cocaine self-administration session and compared to yoked-saline controls. Cocaine rats displayed significantly lower expression of miR-101b (t_{20}=2.68, p=0.014), miR-137 (t_{20}=2.88, p=0.009) and miR-132 (t_{20}=2.75, p=0.012) in the NACc compared to saline controls. No changes were observed in the NACsh (Figure 1a, c). miR-212 expression approached significance in the NACsh (t_{19}=-2.01, p=0.059) but no changes were observed in the NACc of cocaine versus saline rats.

4.5.2.3 Altered miRNA expression in the DMS and DLS following cocaine self-administration

We examined the effect of cocaine use on the expression of these miRNA in the DMS and DLS. Cocaine rats displayed significantly lower miR-137

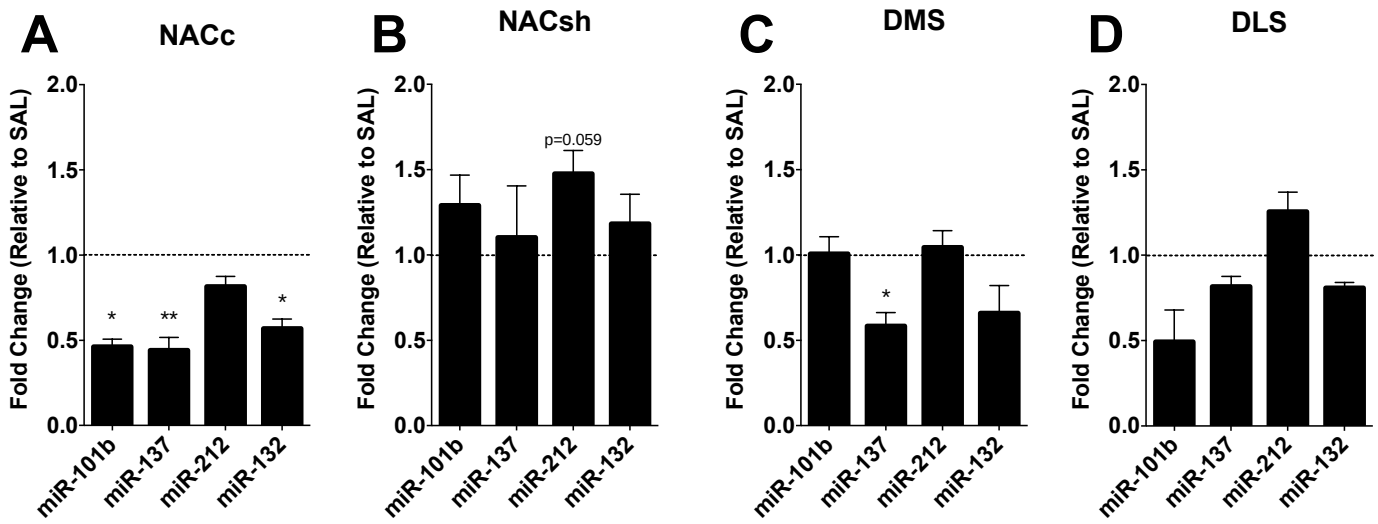

Figure 1

Figure 2: *Changes in miRNA expression in striatal subregions with cocaine use*. miRNA expression is altered in the NACc (A) and DMS (C) but not NACsh (B) or DLS (D) of cocaine (n=16) versus saline rats (n=7). *p<0.05; **p<0.01. Data presented +/- SEM.

expression (t_{20}=2.25, p=0.036) in the DMS, but not DLS, of cocaine versus yoked-saline animals. No changes in miR-101b, miR-212 or miR-132 were observed in either the DMS or DLS at this time point (Figure 1c, d).

4.5.3 Experiment 2

4.5.3.1 Early addiction-related behaviours can predict future propensity to relapse

The relationship between observed and BMA-predicted relapse scores is shown in Figure 2b. The BMA model accounted for almost 50% of the variance in relapse scores. Importantly, the predicted relapse scores perfectly discriminated the animals classified as vulnerable (squares) from those

classified the resilient (triangles; i.e., a horizontal line at the middle of the *y*-axis leads to perfect classification of the two target groups).

A summary of the BMA model is shown in Figure 2a. Overall, the strongest predictor variables were from sessions closer in time to the reinstatement session (i.e., PR, FR5) than earlier sessions (FR1). There was considerable uncertainty in the best set of predictors, with the largest posterior model probability reaching only 0.03. This highlights that there was considerable correlation among predictor variables, which can cause instability problems in standard regression approaches. Together, this demonstrates the utility of the BMA approach: If we had selected a single best-performing model, rather than averaging predictions over many models, we would use a single predictor – NDA responding in the FR5 session (i.e., leftmost column of Figure 2a). Predictions of this single-predictor model accounted for approximately half as much variance in relapse scores as the BMA model (25%).

To test whether the BMA model was over-fitting the data, we performed out-of-sample validation with an additional sample of rats (n=6) that served as vehicle controls in a previously published experiment (27) but otherwise experienced the same protocol as described above. The BMA model predicted the out-of-sample data well, explaining 55% of the variance in out-of-sample relapse scores. Although this relationship is somewhat unstable given the small sample size, it is of reasonable magnitude and in the expected direction.

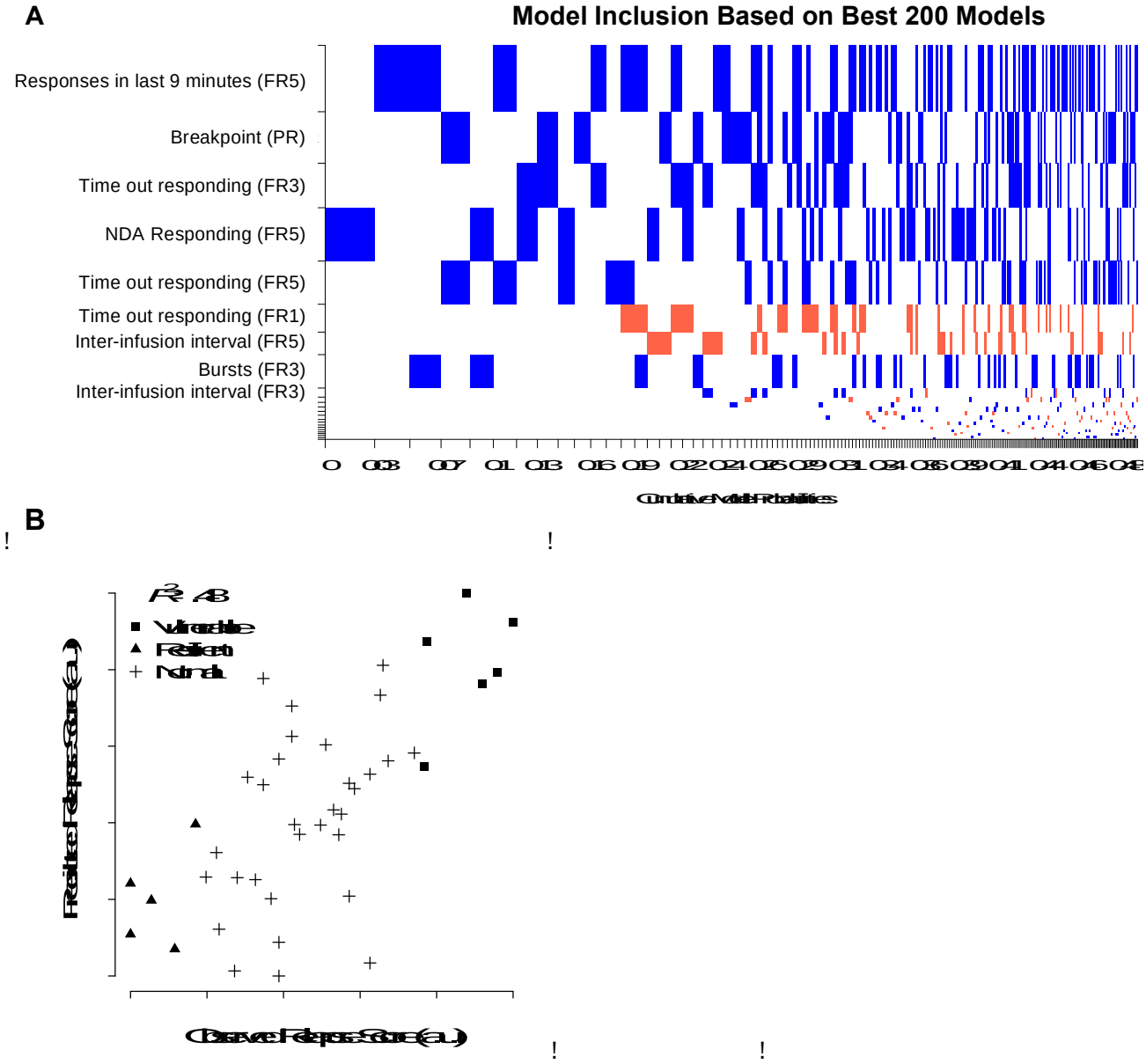

Figure 2

Figure 3: *Overview of the Bayesian Model Averaging analysis in experiment 2.* (A) The *y*-axis shows the 23 predictor variables where the vertical space occupied by each predictor represents the posterior inclusion probability (PIP) for the variable, so a larger space represents a better predictor. The *x*-axis shows the cumulative model probability, where the leftmost model has the largest weight sorted to lower weights on the right. Coloured squares code which predictors (*y*-axis position) were included in each model (*x*-axis position). Blue and red fill respectively represent positive and negative regression coefficients in a model. For simplicity, only the 10 highest PIP predictors (*y*-axis) and the 200 highest ranked models (*x*-axis) are shown. (B) Observed versus predicted relapse scores (*x*- and *y*-axis, respectively). Rats phenotyped as vulnerable and resilient are plotted with square and triangle symbols, respectively. Remaining rats are plotted with crosses.

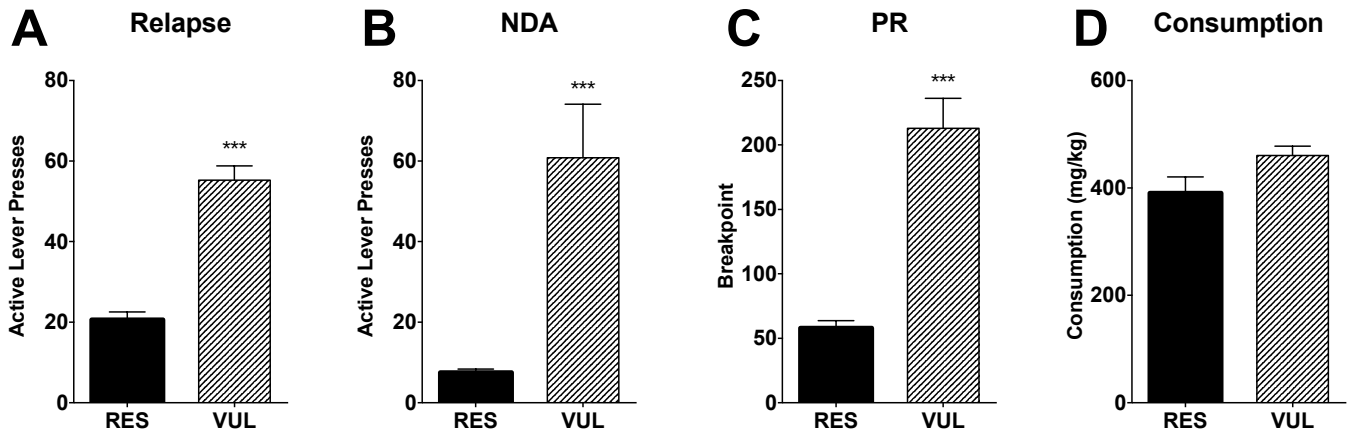

Figure 3

Figure 4: *Reinstatement and addiction-like behaviours.* Using our BMA model we identified animals that showed addiction vulnerability. Animals classified as addiction vulnerable showed significantly higher predicted relapse scores (A), greater active lever pressing in non-drug available periods (B) and higher progressive ratio breakpoint (C) indicating a higher motivation to seek and consume drug compared to addiction resilient rats. No changes in cocaine consumption were observed between groups. ***p<0.001. Data presented +/- SEM. n=8/group

4.5.3.1 Early behavioural phenotyping identified rats that show addiction-relapse vulnerability

Using the BMA model we generated a 'predicted reinstatement score' (Table S3) for the rats in experiment 2. Using this predicted reinstatement score, as well as the scores from NDA and PR tests, we phenotyped rats as addiction-relapse vulnerable or resilient (Figure 3). Addiction-relapse vulnerable rats showed significantly higher predicted reinstatement scores (t_{14}=8.608, p<0.001), NDA responding (Mann-Whitney U=0, p=0.0002) and PR breakpoints (Mann-Whitney U=0, p=0.0002) than resilient rats. Importantly, no change was observed in cocaine consumption between the

groups (p>0.05). These results indicate that the BMA model reliably classified rats as addiction-relapse vulnerable or resilient, despite the rats not having a reinstatement session.

4.5.3.1 miRNA expression in the NACc and NACsh of addiction vulnerable rats 24h after cocaine self-administration

We assessed the expression of candidate miRNA in the NAC subregions of animals that show early behavioural indices of addiction vulnerability. We found no change in miRNA expression between vulnerable and resilient animals in either region (p>0.05) (Figure 4a, b).

4.5.3.2 miRNA expression in the DMS and DLS of addiction vulnerable rats 24h after cocaine self-administration

We next assessed the expression of these miRNA in the DS subregions of addiction vulnerable and resilient animals using our BMA modelling approach. We found a significant increase in miR-212 in the DMS (Mann-Whitney U=11, p=0.028), but not DLS, of addiction vulnerable versus resilient rats (Figure 4c, d). No change was for other miRNA assessed in either the region (p>0.05).

4.5.4 Experiment 3

4.5.4.1 miRNA expression in the NACc and NACsh in addiction vulnerable rats post-reinstatement

Following relapse testing, miR-101b expression was significantly higher in the NACsh of addiction vulnerable versus resilient controls ($t_{5.323}$=2.852, p=0.03) (Figure 5). A trend towards increased miR-101b expression was also observed in the NACc (t_{10}=2.181, p=0.054). Addiction-relapse vulnerable

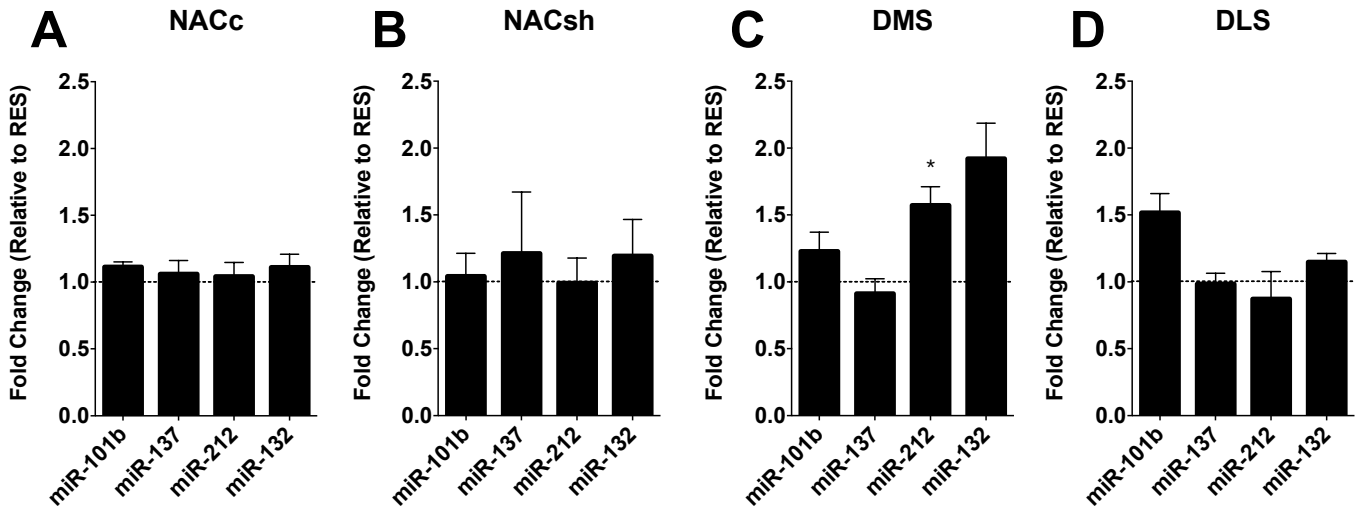

Figure 4

Figure 5: *Changes in miRNA expression in addiction vulnerable versus resilient rats during drug taking***.** Animals phenotyped as addiction vulnerable using the BMA model displayed altered miR-212 expression the DMS (C) compared to addiction resilient controls. *p<0.05 Data presented +/- SEM. n=8/group

animals exhibited significantly higher levels of miR-212 (t_{10}=4.574, p=0.001) and the closely related miR-132 (t_{10}=2.97, p=0.014) in the NACsh, with no change in the NACc. Interestingly, the expression of schizophrenia risk gene miR-137 was significantly higher in the NACsh (t_{10}=2.535, p=0.03) but not NACc of addiction-relapse vulnerable animals.

4.5.4.2 miRNA expression in the NACc and NACsh in addiction vulnerable rats post-reinstatement

We have previously shown that a number of miRNA have increased expression in the DMS, but not DLS, of addiction vulnerable rats following relapse (19). However, we have not yet assessed the expression of miR-137 in the DS subregions post-relapse. Given that change in miR-137 observed

following drug taking in the DMS, we assessed its expression in the DS subregions post-relapse in addiction vulnerable rats. We found increased miR-137 expression in the DLS (t_6=2.38, p=0.05) of addiction vulnerable rats, with no change in the DMS (Figure 5c, d).

4.5.4.3 miR-101b regulates Drd1 expression in vitro

To validate a potential functional interaction between miR-101b and *Drd1,* we used a luciferase reporter assay. Relative luciferase activity from the construct containing the *Drd1* MRE was increased by 17% when transfected with AS-101b compared to AS control (t_5=2.05, p=0.048)(Figure 5e). These data suggest that miR-101b has the potential to regulate its cognate recognition elements in *Drd1*.

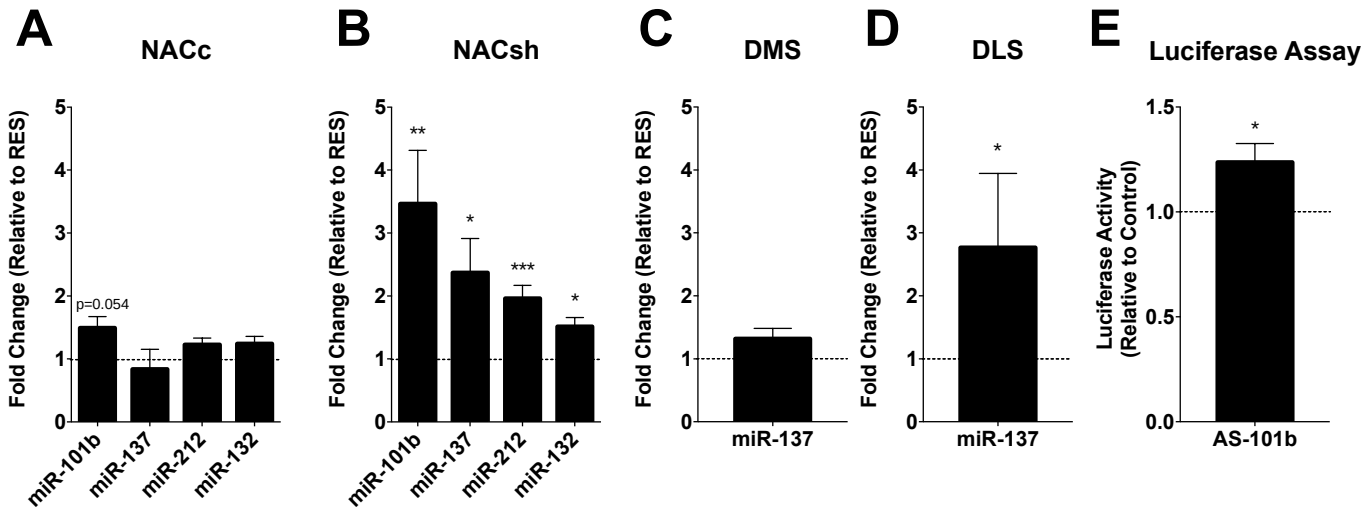

Figure 5

Figure 6: *Changes in miRNA expression in addiction vulnerable versus resilient rats following reinstatement***.** Following reinstatement, rats phenotyped as addiction-relapse vulnerable show changes in miRNA expression in the NACsh (B) compared to addiction resilient controls, with no change in the NACc (A). miR-137 expression was found to be altered in the DLS (D), but not DMS (C) of addiction vulnerable rats following reinstatement. *p<0.05; **p<0.01; ***p<0.001 Data presented +/- SEM. n=6/group

4.6 Discussion

Our aim was to examine the temporal profile of miRNA expression in the context of their contribution to plasticity deficits that develop in the striatum across the addiction cycle. We had previously identified changes in plasticity-associated miRNA, including miR-212 and miR-101b, in the DS of addiction-relapse vulnerable rats post-relapse (19). To achieve our aims here, we developed a phenotyping approach using BMA, a method that utilizes early behavioural indices to predict reinstatement behaviour in rats without the need to test this behaviour. Using this approach we found distinct patterns of miRNA expression within the NACc and NACsh across the addiction cycle. Cocaine taking appeared to preferentially alter miRNA expression within the NACc at early time points in all animals whereas, between vulnerable animals, miRNA expression was generally increased in the NACsh. Interestingly, ours is the first study to identify changes in miR-212 expression in the ventral NACsh, with DS miR-212 expression being associated with addiction vulnerability. While fewer miRNA changes were observed in the DS at early addiction time points, the expression of the schizophrenia risk-associated miR-137 was found to be sensitive to the expression of addiction-like behaviour. Interestingly, at this time point, the expression of the protective miR-212 was engaged in the DMS of vulnerable rats – a process, based on previous work, that appears to exhaust across the course of addiction cycle and is associated with increased relapse-risk.

4.6.1 Relapse vulnerability is associated with early behavioural indices of addiction

Previous studies have shown that behavioural traits including impulsivity (28, 29) and pattern of drug use (30) can predict drug craving and addiction-like behaviour in rats. Here we demonstrate that relapse vulnerability can be predicted using early behavioural indices of addiction. To identify animals that showed a propensity for reinstatement at an early time point in the addiction cycle, we developed a BMA model to identify early behavioural indices that can predict vulnerability. Standard approaches for predicting an outcome (such as reinstatement) typically involve selecting a subset of predictor variables from a larger set of candidate variables, and then proceeding as if the selected subset generated the observed data. This approach ignores uncertainty in model selection; that is, the uncertainty associated with selecting a specific subset of predictors. This leads to overconfident predictions (23). Critically, we were able to identify addiction vulnerable animals at this early stage using our BMA model, and at a late, post-reinstatement phase using the model previously described (18, 20). Identification of addiction-relapse vulnerable rats at both the early and late stages of the addiction allowed us to gain insight into the temporal expression of key miRNA within the striatum.

4.6.2 Differential miRNA expression in the NACc and NACsh across the addiction cycle

One of the most significant findings of the present study was the presumptive sensitivity of NACc miRNA to the effects of cocaine at the early

phenotyping time point. Indeed, cocaine appeared to decrease miRNA expression in the core compared to saline animals. Interestingly, miRNA expression changes were evident between vulnerable and resilient groups in the NAC but only at a later stage of the addiction cycle; i.e, in animals that had been tested for reinstatement. At this time point, the changes were primarily observed in the NACsh rather than in the NACc.

Cellular and molecular studies support time dependent changes in NACc and NACsh function, however it is difficult to draw strong conclusions from much of this work given the very different experimental designs employed, for example, differences in whether animals self-administered cocaine or received experimenter-administered injections, or differences in extinction versus home cage abstinence, etc. (13, 16, 27, 31-34). Generally, studies supporting distinct NACc and NACsh functions support a transfer of information from the shell to the core – signals that may then gain access to the DS through the core's descending projections to the midbrain dopamine cells (9, 35). However, core to shell information flow has also been predicted based on studies using protein synthesis inhibitors in the core - which prevents increased spine density formation in the NACsh (36).

Data indicate that synaptic changes occur in the NACc and NACsh on different time scales (31, 32, 36-39). However, after cocaine self-administration and withdrawal, most studies in the NAC support molecular and cellular changes that promote increased synaptic strength in the NACsh (16, 27, 40, 41). Importantly, we have demonstrated a significant temporal profile in miRNA expression changes in the NACc during early drug taking, to

the NACsh following reinstatement of drug seeking. Thus, our results are in alignment with studies suggesting distinct roles for the NACc and NACsh in the addiction process; e.g. primary reinforcement versus context-associations with reward critical in the development of addiction (33, 42).

4.6.3 Differential miRNA expression in the DMS and DLS across the addiction cycle

MiRNA in the DS subregions also followed a temporal expression profile across the addiction cycle. Here we show that changes in miRNA expression are centred on the DMS during early phases of the addiction cycle. Interestingly, in previous work we found that miRNA expression is generally decreased in the DMS following relapse (19). In that study we did not examine the expression of the schizophrenia-associated miR-137. We report that miR-137 expression is increased in the DLS following reinstatement compared to resilient controls. These changes are interesting given the distinct roles of the DS subregions in regulation of behaviour. Thus the DMS has been shown to be involved in goal-directed behaviour (43), while the DLS regulates habit behaviour (44, 45). The development of habit-based addiction has been hypothesized to require a transfer from goal-directed to habit-based responding. Not only has this been thought to require a shift from ventral to dorsal striatum, but also a transfer from the DMS to DLS. Lending support to the hypothesis that addiction manifests in part due to a devolution in control from the DMS to DLS in particular are the temporal changes in miR-212 and miR-137 expression seen in both this and in our previous study (19).

4.6.4 Dysregulation of miR-212 may reflect an impaired protective mechanism against relapse vulnerability

Previous studies have implicated miR-212 in compulsive cocaine seeking. Overexpression of miR-212 reduced compulsive cocaine taking, whereas its knockdown promoted drug seeking (21). Consistent with these findings we found that animals expressing early markers of addiction vulnerability display increased miR-212 expression in the DMS. However, after extinction and relapse testing, vulnerable animals exhibit reduced miR-212 levels (19). Notably, in the DS we only found changes in miR-212 expression in the DMS – supporting the hypotheses suggest that dysregulated function in action-outcome control contributes to a premature shift in the expression of habit-like responding. Taken together, these data suggest that miR-212 expression is increased during cocaine consumption to counteract drug-induced neuroadaptations but, over time, this protective mechanism is exhausted in addiction-vulnerable individuals (19).

To our knowledge, previous studies have not investigated miR-212 expression within the NAC. Cocaine tended to increase miR-212 in the NACsh of cocaine rats compared to saline controls with this increase sustained in addiction vulnerable compared to resilient rats. These data are interesting with regards to the protective role miR-212 plays in the DMS and the different temporal profile of expression that appears to occur in the VS.

4.6.5 Expression of miR-137 in the striatum is associated with relapse vulnerability

Similar to miR-212, we saw a distinct expression profile in miR-137 in the striatal subregions across the addiction cycle. Variants in the *MIR137* gene locus are associated with increased schizophrenia risk (46). Because of the co-morbidity between addiction and schizophrenia (26) we were interested in examining the potential role for miR-137 in addiction vulnerability. Further, miR-137 has recently been ascribed a role in the regulation of presynaptic plasticity. Siegert et al., 2015 showed that miR-137 over-expression significantly impaired the induction of LTP in the hippocampus (24). Disrupted hippocampal-dependent contextual fear learning was also observed in mice over-expressing miR-137, providing a behavioural surrogate for impaired LTP (24). Moreover, miR-137 regulates gene expression in the presynapse including synatotagmin-1 (*Syt1),* N-ethylmaleimide sensitive fusion protein *(Nsf) and* complexin-1 *(Cplx1),* proteins important for vesicle trafficking and neurotransmitter release (24). We observed significant changes in the expression of miR-137 in the NAC and DS at different stages of the addiction cycle. Cocaine use decreased miR-137 expression in the NACc compared to saline, whereas miR-137 levels were increased in the NACsh of addiction-relapse vulnerable animals after reinstatement. Further, miR-137 was decreased in the DMS of cocaine animals. These findings are interesting in light of evidence that cocaine substantially enhanced presynaptic drive to medium spiny neurons in the DMS (3). In contrast to the reduction in miR-137 seen at early addiction stages, miR-137 is increased in the DLS after relapse. Interestingly, it has been suggested that addiction is facilitated in part by a

shift in control from the goal-directed DMS to the habit-like DLS. Our results support a role for miR-137 in this shift, and may reflect critical neuroadaptations that promote addiction and relapse.

4.6.6 Expression of miR-101b in the NAC is associated with relapse vulnerability through interactions with Drd1

We have shown that cocaine use decreased miR-101b expression in the NACc. Previous studies have found that miR-101b regulates *mTOR* expression (47), and we have demonstrated that miR-101b has the capacity to regulate *Drd1* in vitro. Both *mTOR* and *Drd1* have been implicated in the regulation of addiction-relevant behaviour and drug use and their expression is modified by drug use. The NACc is considered important for encoding the conditioned reinforcing properties of stimuli associated with drug use (7, 48). Importantly, NACc dopamine release has been shown to be important in the acquisition of conditioned stimuli, and lesions or *Drd1* antagonists have been shown to disrupt memory consolidation for conditioned stimuli (49). The decrease in miR-101b observed during cocaine drug use could lead to a subsequent increase in *Drd1* expression, allowing the memory of these cocaine associated stimuli to be acquired.

In contrast, after relapse, vulnerable animals displayed increased expression of miR-101b compared to resilient rats in both the NACc and NACsh. These data occur in parallel to reduced expression of *Drd1* and *mTOR* (18). The increase in miR-101b may contribute to the reduction in *Drd1* expression. Reductions in *Drd1* expression are observed in the striatum of rodents and primates with significant cocaine experience (50). These changes

reflect a pattern of molecular changes consistent with chronically elevated dopamine release in the NACsh during cocaine taking and withdrawal. Furthermore, as the NACsh is engaged during context reinstatement and plays a primary role encoding the rewarding properties of cocaine, these changes may reflect neuroadaptations relevant to ongoing drug craving and relapse, including the integration of the emotional valance of drug-associated stimuli (51, 52). It will be important for further studies to better understand how these changes relate to a shift in control from ventral to dorsal striatal control of behaviour.

4.6.7 Conclusion

In summary, we used a novel BMA model to identify animals that exhibit susceptibility to addiction during an early phase of the addiction cycle. Interestingly, with respect to the NAC, cocaine-induced changes in miRNA appear to be largely localised to the core, where as addiction vulnerability is associated with changes in the shell. miRNA that are differentially expressed across the course of the addiction cycle include miR-101b, which targets *Drd1*, miR-212 and miR-137. We suggest that these miRNA may represent key compensatory mechanisms that render some individuals unable to counteract drug- changes in synaptic structure and function. By understanding the mechanisms by which these miRNA influence synaptic plasticity it may be possible to identify therapeutic targets to aid in recovery from drug addiction.

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4.8 Supplementary Methods

4.8.1 Animals

Male Sprague-Dawley rats (Animal Resources Centre, WA, AUS) weighing 200-250g upon arrival were housed two per cage on a reverse 12-hour light/dark cycle (lights off 0700h) with *ad libitum* access to food and water. All procedures were carried out in strict accordance with protocols approved by the University of Newcastle Animal Care and Ethics Committee, New South Wales Animal Research Act and Regulations and the Australian Code of Practice for the care and use of animals for scientific purposes.

4.8.2 Intravenous Catheter Surgery

Rats were anaesthetized with isofluorane (1-3% with a flow rate of 2L/min) and a Silastic catheter was surgically implanted into the right jugular vein using aseptic techniques as described previously (18). Prior to surgery rats received a 0.3ml intramuscular injection of a broad-spectrum antibiotic (112.5mg/ml benzathine penicillin, 150mg/ml procaine penicillin; Norbrook Laboratories, UK) and a 0.2ml subcutaneous injection of a 50mg/ml solution of Caprofen (Norbrook Laboratories, UK). Following surgery catheters were flushed with 0.3ml of a 50mg/ml solution of Cephazolin (Mayne Pharma, Australia). Rats were allowed to recover for 7 days prior to commencement of cocaine self-administration training. Catheters were flushed daily with 0.2ml of 50U heparanized saline to ensure catheter patency.

4.8.3 Drugs

Cocaine hydrochloride (Johnson Matthey, Edinburgh, UK) was dissolved in sterile physiological saline (2.5mg/mL) (18).

4.8.4 Behavioural Testing Equipment

Behavioural procedures were conducted in standard operant conditioning chambers (Med Associates, VT, USA). Chambers were equipped with two retractable levers (6 cm above the floor), a white cue light above each lever, two speakers to deliver auditory stimuli, and a white house light at the top of the chamber wall opposite the levers. Auditory stimuli were produced by a white noise generator connected to a speaker that produced a 70dB white noise adjacent to the house light (Med Associates, VT, USA), and a tone source located in the top left corner (Sonalert with volume control, Med Associates, VT, USA), that was connected to a speaker that produced an intermittent tone (0.5 Hz at 70dB). A syringe pump (5 rpm motor, Med Associates, VT, USA) located on the outside of the cubicle delivered the IV cocaine. Behavioural testing equipment was controlled by a Windows-based PC, using MED-PC IV software (Med Associates, VT, USA).

4.8.5 Bayesian Model Averaging

The set of candidate predictor variables included the following: from the FR1, FR3 and FR5 training schedules – latency to first lever press in each drug available period, number of infusions earned across each session, mean interval between successive infusions, number of responses in the 20 second time out period that followed each infusion, the number of ‘bursts’ (5 infusions

received in a 5 minute window), number of responses in the last 9 minutes of a session, and the number of responses in the NDA period; and from the PR sessions the number of responses in the 20 second time out period that followed each infusion, and the break point. These predictor variables were extracted separately from each session within each schedule (i.e., FR1, FR3, FR5, PR) and then averaged across sessions within a schedule to obtain a single value of the predictor for each schedule. Mean values were log-transformed for normality over rats where required. Of the 45 rats, 3 were excluded from BMA analysis due to missing data in the FR3 sessions.

We used BMA over linear regression models to test how well the set of 23 variables predicted the relapse score, which involved comparison of the relative fit of $2^{23} \approx 8.3$ million models. We used methods described in the Bayesian Model Averaging library ('BMS' package) in the R programming environment (39, 40). We assumed a uniform prior over model size and birth/death Markov chain Monte Carlo (MCMC) sampling. We excluded the first 100,000 samples as burn in and sampled 200,000 iterations from the stable posterior distribution. MCMC convergence was examined by comparing the frequency that the MCMC sampler drew from the best 2000 models with the marginal likelihood of the best 2000 models. The correlation between the two measures was r =0.994, indicating good convergence. Our primary outcome measure was the strength of the linear relationship between observed and predicted relapse scores.

4.8.6 Bioinformatics Analysis of miRNA Interactions

The computational algorithms miRanda (http://www.microrna.org/) and TargetScan (http://www.targetscan.org/) were used to identify miRNA regulators of synaptic plasticity genes previously found to be altered (18).

4.8.7 Luciferase Reporter Assay

To validate miR-101b regulation of *Drd1*, the miRNA recognition element (MRE) from 3' UTR of *Drd1* was cloned into pMIR-REPORT Luciferase miRNA Reporter Vector (Ambion, Mulgrave, Australia) according to manufacturer's instructions. Reporter gene transfections and assays were performed essentially as described (41). Briefly, HEK-293 cells were co-transfected with 4ng of reporter construct, 20ng of pRL-TK renilla luciferase construct and 100nM antisense inhibitor. The Dual-Luciferase Reporter Assay System (Promega, USA) was used to measure luciferase activity on a BioTek Synergy 2 plate reader. The ability of each AS inhibitor to bind to the miRNA and thus prevent repression of reporter-MRE was determined using the ratio of firefly luciferase activity to renilla luciferase activity (transfection control). Data were normalised to negative controls.

4.8.8 Tissue Dissection

qPCR was performed on dissections of NACsh, NACc, DMS and DLS. NACsh and NACc tissue for qPCR and were macrodissected from coronal sections made on a Cryostat (Leica Biosystems CM1900, Melbourne, Australia) using 0.8mm diameter tissue punches (bregma levels 2.76 to 0.96). DMS and DLS tissue for qPCR and were macrodissected from coronal

sections using 0.8mm and 1mm diameter tissue punches respectively (bregma levels 2.28 to 0.00).

4.8.9 RNA Extraction

Total RNA was extracted using QIAGEN miRNeasy Mini Kit (QIAGEN, Venlo, Netherlands) according to the manufacturer's instructions. Concentrations of RNA were determined using a Nanophotometer (Implen, Munich, Germany).

4.8.10 Reverse Transcription and qPCR

miRNA reverse transcription was performed on 150ng of RNA treated with DNase-1 (Invitrogen, Mulgrave, Australia). Reactions were performed using Superscript III with miRNA-specific primers in a pooled reverse transcription mix according to manufacturer's instructions. qPCR reactions were performed essentially as described (18). Cycle threshold (C_t) for miRNAs were compared to the housekeeper β-Actin (ΔC_t). $\Delta\Delta C_t$ method was used to compare expression between addiction resilient and vulnerable cohorts.

4.8.11 Statistical Analyses

Shapiro-Wilk test for normality was used to assess distribution. Unpaired two-tailed t-tests were performed for behavioural and qPCR data. Due to the prediction that miR-101b would negatively regulate *Drd1*, one-tailed t-tests were used for luciferase assay data. Mann-Whitney U tests conducted were required for data without normal distribution. An alpha value of 0.05 was adopted for all tests.

4.9 Supplementary Results

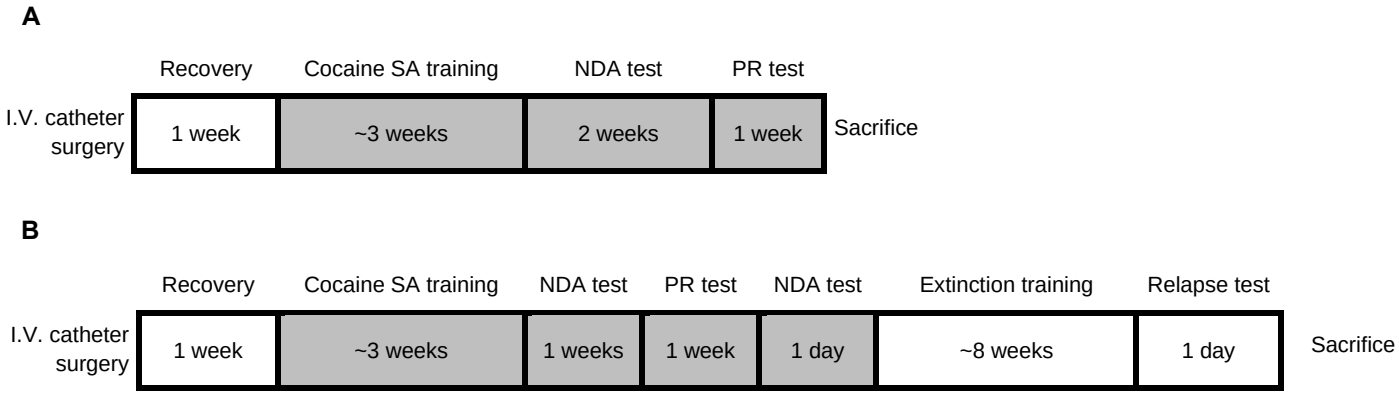

Figure S1

Figure S1: *Experimental timeline for phenotyping of addiction vulnerable and resilient groups.* To phenotype animals into addiction vulnerable or resilient groups, animals were first implanted with an i.v. catheter and trained to self-administer cocaine (0.25 mg/0.1ml i.v.) on an FR1 schedule of responding which progressed to an FR3 and finally an FR5 schedule of responding. Animals were then tested for inability to refrain from drug seeking during periods of signalled drug non-availability (NDA). To assess changes during the drug-taking phase of the addiction cycle (A) rats were tested for NDA responding for 10 sessions, followed by 5 consecutive sessions of progressive ratio (PR) testing. Rats were sacrificed twenty-four hours following final PR session. To assess changes following relapse (B) rats were tested for NDA responding for 5 days. Following this animals were tested for motivation to consume drug using a progressive ratio test, followed by a final FR5 cocaine session. Drug-seeking behaviour was then extinguished by exposing animals to the operant chamber. Drug was absent and lever pressing did not result in a drug reward. Once responding returned to baseline levels, animals were re-exposed to cues associated with drug availability in a reinstatement test and sacrificed 24 hours later. For detailed description of behavioural training and phenotyping see Brown et al. 2010. For both groups, animals that scored in the top third of the distribution for each behavioural test were deemed to be addiction vulnerable, while those in the bottom third of the distribution were identified as addiction resilient.

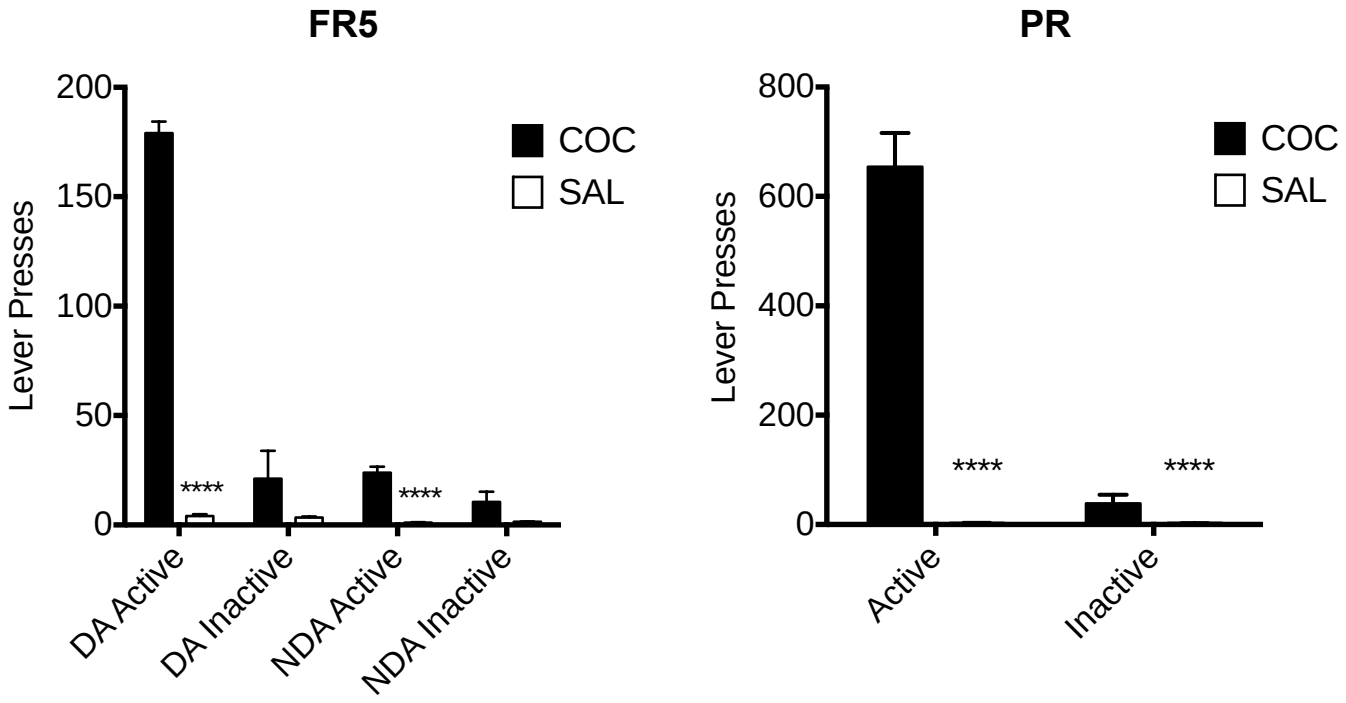

Figure 2

Figure S2: *Addiction-like behaviour in cocaine versus saline rats.* We assessed the lever pressing of cocaine (n=16) versus saline yoked (n=7) rats to confirm cocaine was eliciting drug seeking behaviour. Cocaine significantly increased active lever responding in the FR5 sessions (A) in both drug available (DA) and non-drug available (NDA) periods. Cocaine also increased active lever responding in PR sessions (B). ****p<0.0001. Data presented +/- SEM.

Supplementary Table S3

Rats	Predicted Reinstatement Score
44	72.09796104
5	63.47772139
86	59.97417645
15	57.63939954
21	56.21890503
112	55.34410846
34	53.13629643
103	50.65379484
20	50.13753082
12	48.43907316
96	46.01642
74	45.98304445
82	45.97622067
67	44.77076821
93	44.44708743
92	43.77574175
105	43.54935504
55	43.09766651
2	41.68065588
114	41.18846721
102	40.77107349
68	39.80657684
70	39.67643357
16	39.17181338
31	37.94429036
69	37.23766541
18	37.07895048
106	36.93943617
104	36.22182192
24	36.0974956
77	35.78811158
13	35.71827701

Rats	Predicted Reinstatement Score
30	35.62877823
49	35.48173679
29	34.68838093
37	33.51467118
71	33.45101601
95	30.79638843
22	30.48891209
23	30.13108138
11	29.96488904
110	29.95713267
113	29.79482842
47	28.72938804
19	27.77105274
81	26.54786524
108	25.61617488
9	25.17770039
76	24.47802632
39	23.90444059
4	23.37580385
107	22.48407714
28	22.45193191
8	21.54610291
72	21.47933818
33	20.80808538
91	19.88396543
32	15.98595473
10	15.17155176
65	11.47159114
6	10.39395168
14	10.38798437
80	0

Table S3: *Predicted reinstatement scores generated from BMA model*. Early behavioural data from rats (n=63) was analysed using our BMA model to generate predicted reinstatement score to be used to phenotype animals as addiction vulnerable or relapse.

CHAPTER FIVE: Distinct miRNA expression in dorsal striatal subregions is associated with risk for addiction in rats.

Quinn, Rikki K., Brown, Amanda L., Goldie, Belinda J., Levi, Emily M., Dickson, Phillip W., Smith, Doug W., Cairns, Murray J., Dayas, Christopher V.

Translational Psychiatry 2015, 5, e503

Author contributions to this manuscript

Rikki K Quinn	Designed and performed research, analyzed data and wrote the manuscript	
Amanda L Brown	Designed and performed research, analyzed data	
Belinda J Goldie	Performed research and analyzed data	
Emily M Levi	Performed research	

Phillip W Dickson	Wrote the manuscript	
Doug W Smith	Wrote the manuscript	
Murray J Cairns	Designed the research and wrote the manuscript	
Christopher V Dayas	Designed the research and wrote the manuscript	

July 2016

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OPEN

Citation: Transl Psychiatry (2015) **5**, e503; doi:10.1038/tp.2014.144

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ORIGINAL ARTICLE

Distinct miRNA expression in dorsal striatal subregions is associated with risk for addiction in rats

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Recently, we published data using an animal model that allowed us to characterize animals into two groups, addiction vulnerable and addiction resilient, where we identified that addiction/relapse vulnerability was associated with deficits in synaptic plasticity-associated gene expression in the dorsal striatum (DS). Notable was the strong reduction in expression for activity-regulated cytoskeleton-associated protein (*Arc*) considered a master regulator of synaptic plasticity. In the present study, we confirmed that *Arc* messenger RNA was significantly decreased in the DS, but importantly, we identified that this reduction was restricted to the dorsomedial (DMS) and not dorsolateral striatum (DLS). There is recent evidence of microRNA (miRNA)-associated posttranscriptional suppression of *Arc* and animal models of addiction have identified a key role for miRNA in the regulation of addiction-relevant genes. In further support of this link, we identified several differentially expressed miRNA with the potential to influence addiction-relevant plasticity genes, including *Arc*. A key study recently reported that miR-212 expression is protective against compulsive cocaine-seeking. Supporting this hypothesis, we found that miR-212 expression was significantly reduced in the DMS but not DLS of addiction-vulnerable animals. Together, our data provide strong evidence that miRNA promote ongoing plasticity deficits in the DS of addiction-vulnerable animals.

Translational Psychiatry (2015) **5,** e503; doi:10.1038/tp.2014.144; published online 3 February 2015

INTRODUCTION

A key feature of addiction is the loss of behavioral control over drug taking.[1] A prominent hypothesis that has been proposed to explain this phenomenon is devolution of control from brain areas involved in goal-directed decision making to those involved in habitual behaviors.[1] One of the brain regions strongly linked with the development and expression of habits is the dorsal striatum (DS).[2–5] However, the cellular and molecular adaptations that occur within the DS have received far less attention than those that occur in the nucleus accumbens (NAc) of the ventral striatum. Accordingly, we recently investigated gene expression profiles for key synaptic plasticity molecules in the DS of animals trained to self-administer cocaine and screened for expression of compulsive drug-seeking traits, including reinstatement—a rodent analog of human relapse.[6] This model allowed us to characterize animals into two groups at opposite ends of the addition vulnerability spectrum.[6,7] Using this approach, we observed decreased expression of synaptic plasticity-associated genes in the DS,[6] including the activity-regulated cytoskeleton-associated protein (*Arc*). These changes are consistent with reports that chronic cocaine-taking leads to a loss of plasticity at excitatory synapses in the striatum, albeit in the NAc,[8–10] and the role for *Arc* as a master regulator of synaptic plasticity.[11]

Importantly, while the DS is a key region involved in the formation of habits, this role appears to be restricted to the dorsolateral division (DLS).[4,12] In fact, a clear functional segregation exists between the DLS and dorsomedial striatum (DMS), which is implicated in goal-directed decision making.[5,13] This functional heterogeneity has significant implications for understanding how decision-making processes become disrupted in neuropsychiatric conditions. However, few studies have assessed how addiction-relevant changes in gene expression in the DS might be sustained. Such information may be relevant for explaining the behavioral switch that appears to drive compulsive drug seeking in addiction.[1,14–17]

One level of molecular control responsible for sustaining addiction-relevant reductions in synaptic plasticity gene expression are microRNA (miRNA), short, noncoding RNA molecules that posttranscriptionally regulate messenger RNA (mRNA).[18] Several miRNA have been implicated in promoting addiction-relevant behaviors. For example, altered expression of miR-181a, let-7a and miR-124 are implicated in the regulation of cocaine-induced conditioned place preference.[19] In a key study, Kenny and colleagues showed that the expression of miR-212 was significantly increased in the DS of rats that self-administer cocaine over an extended period of drug access.[16] Importantly, overexpression of miR-212 significantly reduced cocaine-taking, whereas its knockdown had the opposite effect.[16] These findings suggest that the expression of miR-212 is increased following protracted drug taking and possibly acts as a homeostatic control to protect against further cocaine-induced plasticity.[16] Importantly, in these previous studies, manipulation of miRNA expression was not restricted to DS subregions. Furthermore, our model allows us to behaviorally separate addiction-vulnerable from resilient animals, despite consuming similar levels of cocaine. On the basis of the evidence above, we predict that miR-212 expression should be decreased in the DMS in vulnerable versus resilient animals owing to the importance of this region in the regulation of goal-directed behavior. Critically, the animals used in this study have been denied access to drug for a period of up to 8 weeks. As such, any

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Received 18 November 2014; accepted 26 November 2014

changes we observed likely reflect long-term neuroadaptions that may contribute to an increased propensity to relapse.

Accordingly, the primary purpose of the present study was to assess the possible contribution of miRNA to the altered expression of *Arc* detected in our previous study in the DMS and DLS subregions of the DS.[6] We also assessed changes in miRNA identified using pathway analysis as having an involvement in long-term depression (LTD) and potentiation (LTP) signaling pathways.[20,21] Finally, we addressed the hypothesis that addiction/relapse-vulnerable animals display deficits in miR-212 expression in the DMS.

MATERIALS AND METHODS

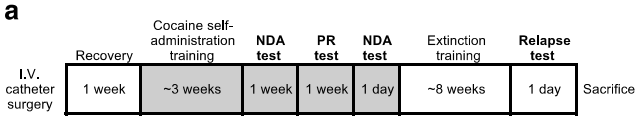
Tissue samples

Tissue samples ($n = 6$ per group) were obtained from animals previously phenotyped as addiction/relapse-vulnerable or resilient as described in detail in Brown *et al*.[6] Briefly, the animals were trained to self-administer cocaine (0.25 mg per 0.1 ml intravenously) for 3 h per day for ~5 weeks, during which time they were tested for three addiction-relevant behaviors: inability to refrain from drug seeking during a period of non-drug availability, motivation to consume drug using repeated progressive ratio tests and cue-induced reinstatement of drug-seeking (Figure 1). The animals were killed 24 h after reinstatement testing. Animals scoring in the top 40% of the distribution for reinstatement as well as the top 30% of the distribution for the remaining behaviors were phenotyped as addiction vulnerable, whereas those in the bottom of the distribution were phenotyped as addiction resilient.

Tissue dissection

The current study was performed on fresh dissections (opposite hemisphere) of DMS and DLS of rats previously phenotyped as vulnerable versus resilient.[6] Tissue for quantitative PCR and western blot analyses were macrodissected from 100 µm or 400 µm coronal sections, respectively, made on a Cryostat (Leica Biosystems CM1900, North Ryde, NSW, Australia) using 0.8–1 mm^2 diameter tissue punches (bregma levels 2.52 to 0.96).

a



b

Behavior	Vulnerable (mean ±SEM)	Resilient (mean ±SEM)	*P* value
NDA responding	150.1 ±47.88	8.1 ±1.26	0.008
PR breakpoint	222.9 ±18.63	46.9 ±7.76	<0.001
Reinstatement	122.7 ±9.29	35 ±8.65	<0.001

Figure 1. Experimental timeline for phenotyping of addiction vulnerable and resilient groups. (**a**) To phenotype animals into addiction vulnerable or resilient groups, animals were first implanted with an intravenous (i.v.) catheter and trained to self-administer cocaine (0.25 mg per 0.1 ml intravenously) on an FR1 schedule of responding which progressed to an FR3 and finally an FR5 schedule of responding. Animals were then tested for inability to refrain from drug seeking during periods of signalled drug non-availability (NDA) for 5 days. Following this, animals were tested for motivation to consume drug using a progressive ratio test, followed by a final FR5 cocaine session. Drug-seeking behavior was then extinguished by exposing animals to the operant chamber. Drug was absent and lever pressing did not result in a drug reward. Once responding returned to baseline levels, animals were re-exposed to cues associated with drug availability in a reinstatement test and killed 24 h later. Animals that scored in the top third of the distribution for each behavioral test were deemed to be addiction vulnerable, whereas those in the bottom third of the distribution were identified as addiction resilient. For detailed description of behavioral training and phenotyping, see Brown *et al*. (2010). (**b**) Animals phenotyped as addiction vulnerable showed significantly higher NDA responding, PR breakpoint and reinstatement scores that animals phenotyped as addiction resilient.

RNA extraction

Total RNA was extracted using QIAGEN miRNeasy Mini Kit (QIAGEN, Venlo, Netherlands) according to the manufacturer's instructions. Concentrations of RNA were determined using a Nanophotometer (Implen, Munich, Germany).

Bioinformatics analysis of miRNA interactions

The computational algorithms miRanda (http://www.microrna.org/) and TargetScan (http://www.targetscan.org/) were used to identify miRNA regulators of previously identified dysregulated synaptic plasticity genes. To identify candidate miRNA involved in LTD and LTP, we used Ingenuity Pathway Analysis (Ingenuity Systems, Redwood City, CA, USA).[22]

Reverse transcription and quantitative PCR

For mRNA expression analysis, 150–450 ng total mRNA was reverse transcribed using Superscript III reverse transcriptase and $oligo_{(dT)}$ primers according to manufacturer's instructions. miRNA reverse transcription was performed on 150 ng of RNA treated with DNase-1 (Invitrogen, Mulgrave, VIC, Australia). Reactions were performed using Superscript II with miRNA-specific primers in a pooled reverse transcription mix as previously described.[23,24]

Quantitative PCR reactions were performed essentially as described.[6] mRNA expression was analyzed with respect to the geometric mean of GAPDH (glyceraldehyde 3-phosphate dehydrogenase) and 18S. Relative miRNA expression was compared with the housekeeper β-actin (ΔC_t). $\Delta\Delta C_t$ method was used to compare expression between addiction resilient and vulnerable cohorts.

Protein extraction

Following macrodissection, tissue was stored at −80 °C until required. 100 µl of homogenizing buffer (50 mM Tris/HCl pH 7.5, 1 mM EGTA, 1 × complete protease inhibitor cocktail tablet, 1 mM DTT, 80 µM ammonium molybate, 1 mM sodium pyrophosphate, 5 mM β-glycerophosphate, 1 mM sodium orthovandate, 2 µM microcystin, final concentration) was added and tissue sonicated for 3 × 10 s pulses at 4 °C using a microsonicator (UP50H, Hielscher Ultrasonics GmbH, Teltow, Germany). 10% SDS was added to a final concentration of 2.5% and the samples were boiled for 5 min, then centrifuged at 15 000 r.p.m. for 10 min at 25 °C. Supernatants were collected and the protein concentration determined using Pierce BCA assay (Thermo Fisher Scientific, Scoresby, VIC, Australia) according to the manufacturer's instructions. The samples were stored at −80 °C until required.

Western blot

Western blotting was performed essentially as previously described.[25] 15µg of protein sample was mixed with sample buffer (1% SDS, 10% glycerol, 0.5% DTT, 0.1% bromphenol blue, final concentration) and subjected to SDS–polyacrylamide gel electrophoresis before being transferred to nitrocellulose (Hybond ECL, GE Healthcare, Rydalmere, NSW, Australia). Nitrocellulose membranes were stained with Ponceau S (0.5% Ponceau in 1% acetic acid) to assess the efficacy of the transfer. The membranes were then washed in TBST (Tris-buffered saline with Tween) (150 mM sodium chloride, 10 mM Tris, 0.075% Tween-20, pH 7.5) and blocked in 5% skimmed milk powder in TBST for 1 h at 25 °C. The membranes were washed in TBST and incubated with anti-Arc (1:2000 Synaptic Systems #156002), overnight at 4 °C. The membranes were washed in TBST and incubated with horse-radish peroxidase-linked anti-IgG secondary specific antibodies for 1 h at 25 °C. The membranes were visualized on Fujifilm Las-3000 imaging system (Fuji, Stamford, CT, USA) using Luminata Forte Western HRP substrate (Millipore, Billerica, MA, USA). The density of bands was measured using a MultiGauge V3.0 (Fuji). *Arc* protein levels were normalized to β-actin. All the results are expressed as a fold change relative to the addiction/relapse resilient group.

Luciferase reporter assay

To validate miR-431 regulation of *Arc*, the miRNA-recognition element from 3′-UTR of *Arc* was cloned into pMIR-REPORT Luciferase miRNA Reporter

Vector (Ambion, Mulgrave, VIC, Australia) according to manufacturer's instructions. Reporter gene transfections and assays were performed essentially as described.[26,27] Briefly, HEK-293 cells were co-transfected with 4 ng of reporter construct, 20 ng of pRL-TK renilla luciferase construct and 100 nM chemically modified antisense (AS) inhibitor. The Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA) was used to measure luciferase activity on a BioTek Synergy 2 plate reader. The ability of each AS inhibitor to bind to the miRNA and thus prevent repression of reporter miRNA-recognition element was determined using the ratio of firefly luciferase activity to Renilla luciferase activity (transfection control). The data were normalized to negative controls.

Statistical analyses

Two-tailed independent sample *t*-tests were used to analyze miRNA and luciferase assay data. The Mann–Whitney nonparametric *U*-test was conducted for data that violated the assumptions. An alpha value of 0.05 was adopted for all the tests. Statistics were conducted using IBM SPSS v19 (IBM, Armonk, NY, USA).

RESULTS

Behavioral phenotyping of addiction vulnerable versus resilient

Behavioral data from the animals used in this study have been published previously.[6] Briefly, animals were trained to self-administer cocaine and tested for three addiction-relevant behaviors: non-drug availability responding, progressive ratio breakpoint and cue-induced reinstatement of drug-seeking (Figure 1). Animals that scored in the top or bottom 40% of the distribution for reinstatement, and the top or bottom 30% for the two remaining behaviors were phenotyped as addiction/relapse-vulnerable or resilient, respectively.[6]

Analysis of Arc mRNA and protein in the DMS and DLS in addiction-vulnerable versus resilient animals

We have previously shown that the synaptic plasticity related gene *Arc* is significantly downregulated in the DS of addiction-vulnerable versus resilient rats.[6] To extend these findings, we examined the expression of *Arc* within the DMS and DLS. We found that *Arc* mRNA was significantly decreased in the DMS of animals phenotyped as addiction vulnerable versus resilient ($t_9 = 3.845$, $P = 0.004$, Figure 2), with no significant change detected in the DLS ($P > 0.05$). Interestingly, *Arc* protein was significantly decreased in both the DMS ($t_{10} = 3.295$, $P = 0.008$) and DLS ($t_{10} = 2.88$, $P = 0.01$).

Identification of candidate miRNA targeting Arc and synaptic plasticity-associated genes

To identify miRNA with the potential to regulate *Arc*[21,28] we used the miRNA-target prediction algorithms miRanda and TargetScan. This approach identified miR-431 and miR-221 as potential regulators of *Arc* mRNA.

In our previous work, we identified a general pattern of downregulated gene expression consistent with deficits in the ability to evoke synaptic plasticity. Further, dysregulated striatal LTP and LTD is thought to be a hallmark of addiction in experimental models.[8,29] Therefore, we used Ingenuity Pathway Analysis to identify candidate miRNA involved in regulation of genes in the LTP and LTD signaling pathways. Using this approach, we identified several miRNA, including miR-181a, miR-212, miR-132, miR-101b, miR-222, miR-342-5p, miR-382, miR-495, miR-7a, miR-708 and miR-99a, which are putative regulators of genes within the LTP and LTD pathways (Figure 3).

Analysis of Arc-relevant miRNA expression in the DMS and DLS of addiction-vulnerable versus resilient animals

After predicting a potential relationship between *Arc* transcript and the expression of miR-431 and miR-221, we investigated their expression in both the DLS and DMS subregion dissections. Interestingly, miR-431 expression was significantly increased in both the DMS ($t_{10} = 2.168$, $P = 0.05$) and DLS ($t_{10} = 2.71$, $P = 0.02$) of addiction-vulnerable compared with resilient rats (Figure 4). No changes in miR-221 expression were observed in the DMS ($P = 0.07$) or DLS ($P > 0.05$) between the addiction vulnerability groups.

To validate a potential functional interaction between miR-431 and *Arc,* we used a luciferase reporter assay. Relative luciferase activity from the construct containing the *Arc* miRNA-recognition element was increased by 17% when transfected with AS-431 compared with AS control ($t_{14} = 3.539$, $P = 0.003$, Figure 5). These data suggest that miR-431 has the capacity to regulate its cognate recognition elements in *Arc*.

Analysis of LTD- and LTP-associated miRNA expression in addiction-vulnerable versus resilient animals

Ingenuity Pathway Analysis was used to identify candidate miRNA within LTD and LTP pathways. We then used the quantitative PCR to analyze the expression of selected miRNAs (Figure 4) in the DMS and DLS of addiction-vulnerable versus resilient animals. miR-101b expression was significantly increased in the DMS (Mann–Whitney *U*-test = 6.00, $P = 0.05$) and DLS (Mann–Whitney *U*-test = 2.00, $P = 0.01$) of addiction-vulnerable animals. miR-181a was

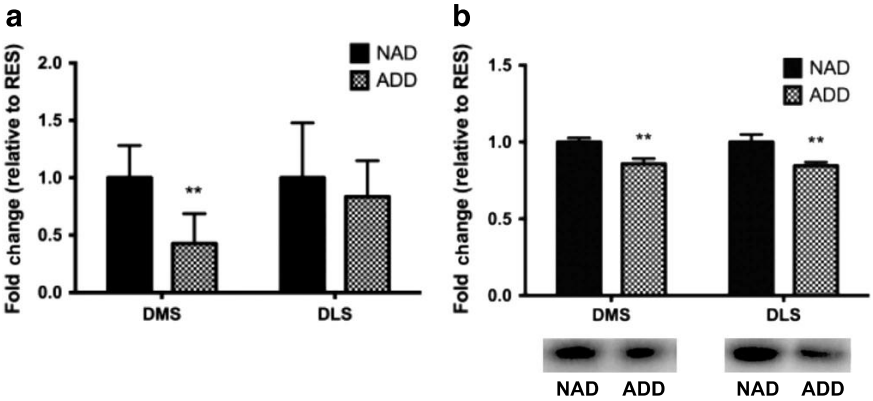


Figure 2. Changes in *Arc* expression in the DS subregions of addiction vulnerable animals. (**a**) Animals phenotyped as addiction vulnerable displayed altered Arc mRNA expression in the DMS but not DLS compared with addiction resilient controls. (**b**) *Arc* protein was significantly decreased in both the DMS and DLS subregions of addiction vulnerable versus resilient animals. $^*P < 0.05$; $^{**}P < 0.01$. n=6 per group. DLS, dorsolateral striatum; DMS, dorsomedial striatum; DS, dorsal striatum; mRNA, messenger RNA.

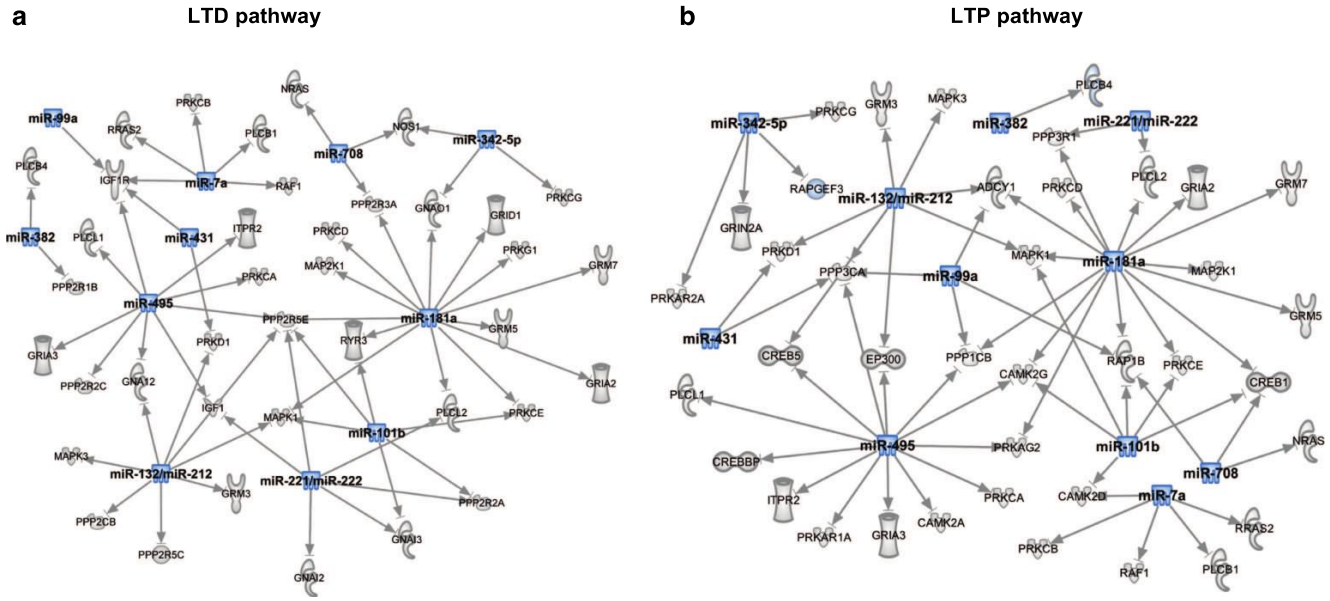


Figure 3. Predicted interactions between miRNA and mRNA targets within addiction relevant signaling pathways. IPA was used to identify miRNA involved in regulation of genes within synaptic plasticity-associated signaling pathways. Putative interactions are shown between miRNA and mRNA targets within the synaptic LTD (**a**) and synaptic LTP (**b**) pathways. IPA, ingenuity pathway analysis; LTD, long-term depression; LTP, long-term potentiation; mRNA, messenger RNA; miRNA, microRNA.

increased in the DLS ($t_{10} = 2.735$, $P = 0.02$) but not DMS of addiction-vulnerable animals. Interestingly, this miRNA has previously been shown to be altered in a number of brain regions, including the NAc, following cocaine exposure.[19,30] miR-708 was significantly increased in the DLS (Mann–Whitney *U*-test = 5.00, $P = 0.03$) but not DMS ($P > 0.05$) of addiction-vulnerable animals.

The expression of miR-222, miR-342-5p, miR-382, miR-495, miR-99a and miR-7a was not altered between phenotyped groups ($P > 0.05$) in either the DMS or DLS.

We also predicted that addiction-vulnerable animals would display reduced expression for miR-212 expression in the DMS compared with resilient animals, consistent with the protective role miR-212 has been shown to have in controlling cocaine consumption.[16,17] Consistent with this hypothesis, addiction-vulnerable animals displayed significantly reduced DMS miR-212 expression ($t_{10} = 2.876$, $P = 0.01$) but no significant changes were observed in the DLS ($P = 0.07$). Of note, the expression of the closely related miR-132 was significantly increased in the DLS ($t_{10} = 2.208$, $P = 0.05$), but not the DMS.

DISCUSSION

In this study, we examined the role of miRNA in the regulation of specific synaptic plasticity genes and signaling pathways associated with addiction/relapse vulnerability. We identified several miRNA in the DMS and DLS of addiction-vulnerable animals with the potential to regulate genes within LTP and LTD pathways including *Arc,* a 'master' regulator of plasticity. We also identified a pattern of miR-212 expression consistent with the hypothesis that loss of function of this miRNA in the DMS leads to compulsive drug-seeking and relapse risk.

miRNA control of Arc expression and relevance to addiction

In previous work, we observed that *Arc* expression was reduced in the DS of addiction-vulnerable animals,[6] however, when we investigated subregion-specific transcript changes here, this effect was restricted to the DMS. Importantly, we also observed a significant decrease in *Arc* protein in the DMS. Surprisingly, DLS *Arc* protein was also decreased. The discrepancy between *Arc* mRNA and protein in the DLS may indicate temporal differences in *Arc* recruitment, translational control and transcript degradation or stability between DS subregions.[11] The decrease in *Arc* mRNA detected was at baseline (that is, 24 h after reinstatement testing), and is likely to have persisted for many weeks after cocaine-taking had ceased. We hypothesized that the long-lasting decrease in *Arc* observed in our previous work would be associated with upregulated expression of miRNA that can bind to 3-prime end of this gene. Using bioinformatics, we identified miR-431 as a potential candidate for regulation of *Arc*. The expression of this miRNA was increased in the DMS and DLS of addiction-vulnerable animals. Further, we demonstrated using luciferase assays that miR-431 does regulate *Arc* expression *in vitro*. Thus, we predict that the dysregulation of *Arc* synthesis may have resulted in ongoing deficits in striatal plasticity and act as a molecular mediator of brain addiction processes.

Interestingly, other studies have also identified addiction-relevant changes in *Arc* expression. However, in contrast to the data presented here, Hearing *et al.*[31] found that *Arc* mRNA was increased in both the DMS and DLS of animals re-exposed to a cocaine-paired environment. These differences may be due to the different time point that brains were harvested or the use of forced abstinence model versus extinction of drug-seeking used in our model. In our study, we collected brains 24 h after reinstatement testing, whereas Hearing *et al.*[31] harvested tissue immediately after testing. Another possible factor is that our phenotyped groups did not differ in the levels of cocaine consumed, thereby controlling for the direct action of cocaine on *Arc* expression. Thus, the increase in *Arc* reported in previous studies could be due to pharmacological effects of cocaine. Interestingly, a subsequent study by the same group demonstrated that inhibition of *Arc* in the DLS did not alter drug-seeking during a context test. However, although the response of control animals decreased during subsequent extinction tests, inhibition of *Arc* in the DLS prevented this decrease in responding.[32] Despite these differences, both data sets implicate *Arc* recruitment and dysregulated signaling in the addiction process.

Exactly how loss of *Arc* function might lead to the dysregulation of synaptic plasticity in the DMS and contribute to compulsive

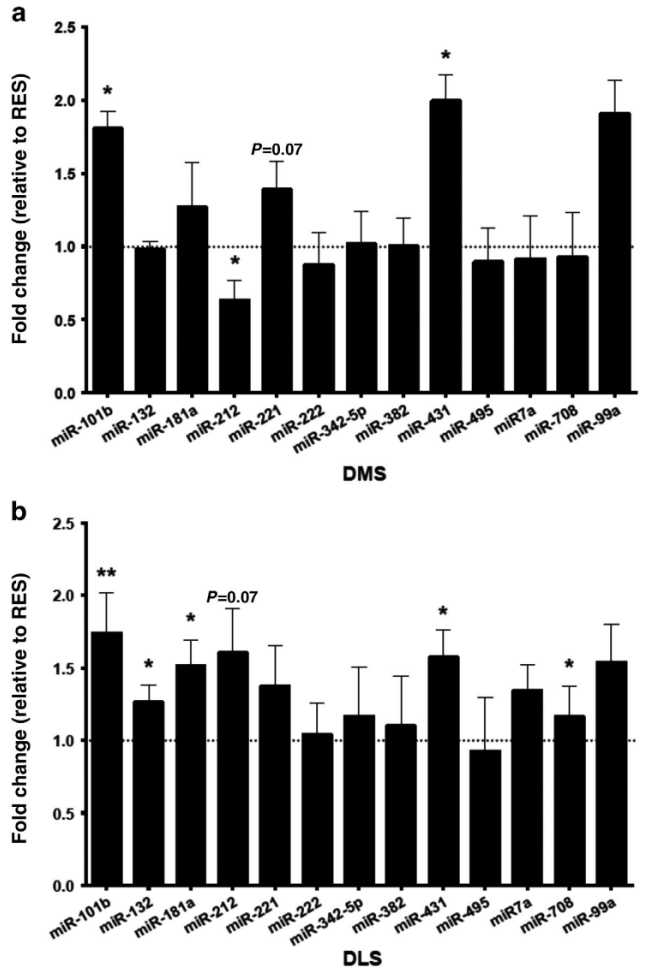


Figure 4. Changes in miRNA expression in addiction vulnerable versus resilient rats. Animals phenotyped as addiction vulnerable displayed altered miRNA expression profiles in both the DMS (**a**) and DLS (**b**) compared with addiction resilient controls. $^{*}P < 0.05$; $^{**}P < 0.01$. n=6 per group. DLS, dorsolateral striatum; DMS, dorsomedial striatum; miRNA, microRNA.

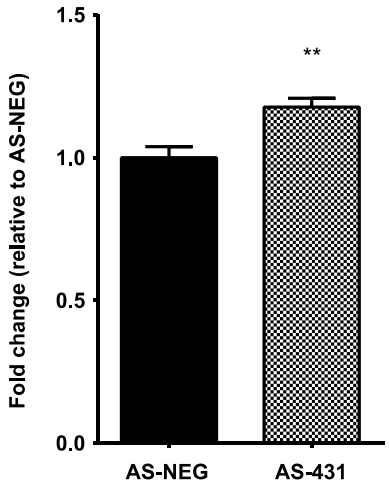


Figure 5. Luciferase reporter gene expression assay. AS-431 increased luciferase activity when co-transfected in HEK-293 cells with Arc MRE luciferase construct, suggesting miR-431 has the ability to regulate Arc expression *in vitro*. $^{*}P < 0.05$; $^{**}P < 0.01$.

drug-seeking is unclear. *Arc* is trafficked to activated synapses, translated into protein[33] and can promote both synaptic strengthening and weakening.[11] *Arc*-induced modulation of LTP is thought to occur through F-actin-mediated enhancement of AMPAR GluA trafficking, postsynaptic density remodeling and localization of translation machinery.[11,34] Modulation of LTP by *Arc* appears to be mediated by the recruitment of dynamin and endophilin 2/3,[34] promoting the internalization of *GluA1* AMPARs. Interestingly *Arc*-dependent LTD has been shown to require activation of Group I mGluRs, and dysregulated signaling through *Grm5* contributes to the expression of addiction-relevant behaviors.[35,36] Knockout of *Arc* expression in the hippocampus has been shown to impair memory formation.[37] Given the role of *Arc* in synaptic activation and memory formation, it is possible that suppressed *Arc* transcription in the DMS may negatively impact goal-directed decision-making process. By default, these changes may result in a predominance of habit-relevant neuroadaptations in the DLS, which manifests as a state of behavioral inflexibility. Given that *Arc* is an immediate early response gene, persistent deficits in *Arc* expression may alter the effects of novelty and impair the formation of new memories that allow the individual to adapt to changes in the value of the drug and contribute to the persistent nature of addiction.

Importantly, vulnerable versus resilient animals did not exhibit differences in days to reach the extinction criterion,[6] suggesting that there were no major deficits in extinction learning. It is possible, however, that dysregulated striatal plasticity might still result in a more subtle failure to learn that cocaine cues no longer predict drug reward. Interestingly, a persistent state of 'anaplasticity' has been reported in the NAc of addiction-vulnerable animals, whereas in resilient cocaine-taking rats this plasticity recovered.[8] These findings are consistent with several other important studies reporting impairments in the ability to evoke LTD and LTP at excitatory synapses in the NAc of animals that have had a significant history of cocaine self-administration.[29,38] Interestingly, a recent study by Corbit *et al*.[39] demonstrated that cocaine exposure led to a more rapid shift in behavioral control from the DMS to DLS. This study found that animals exposed to cocaine that were subsequently trained to self-administer food rewards became insensitive to outcome devaluation more rapidly than saline controls. Further, they showed that cocaine exposure altered glutamatergic transmission only in the DMS with no effect seen in the DLS. These results align with the hypothesis that synaptic plasticity impairments in addiction-vulnerable animals lead to a premature shift in behavioral control from goal-directed to habitual.

Decreased miR-212 expression in the DMS of addiction-vulnerable versus resilient animals

miR-212 is the best characterized miRNA with respect to compulsive drug taking and addiction. Hollander *et al*.[16] showed that lenti-viral-mediated overexpression of miR-212 in the DS decreased cocaine consumption, whereas its knockdown had the opposite effect. Furthermore, they showed that miR-212 regulated compulsive cocaine consumption through a complex homeostatic interaction with *MeCP2* and *BDNF*.[17] Thus, overexpression of miR-212, which suppressed cocaine consumption, led to a decrease in *MeCP2* and *BDNF*.[17] These findings accord well with data demonstrating that increased striatal *BDNF* helps to promote drug-seeking behaviors.[40,41] Consistent with the hypothesis that miR-212 negatively regulates and is protective against cocaine-taking, we found that miR-212 expression was significantly decreased in the DMS of addiction-vulnerable animals but there was no significant change in DLS miR-212 expression.[16] This disparity is interesting given the functionally distinct roles of these subregions. We speculate that the decrease in miR-212 observed in the DMS may lead to a cascade of signaling changes that shifts

the balance of DS control over behavioral responding to the DLS. Together, these data support a role for miR-212 in addiction-relevant neuroplasticity but also identify a subregion or temporal specificity in the actions of miR-212 in the DS.

Expression of LTP- and LTD-associated miRNA in the DMS and DLS of addiction-vulnerable animals

Pathway analysis identified a number of candidate miRNAs with the potential to influence the expression of genes associated with LTP and LTD signaling pathways. For example, miR-101b, which was significantly increased in both the DLS and DMS, is predicted to target *MAPK1, PRKC, PP2a* and genes encoding the guanine nucleotide-binding proteins. miR-181a was significantly increased in the DLS of addiction-vulnerable animals and pathway analyses predict potential interactions with molecules linked with alterations in synaptic plasticity including the Group 1 metabotropic glutamate receptor *Grm5* and calcium impermeable AMPARs (*GluA2*). The expression of miR-431 was increased up to twofold in DS subregions and has been shown to decrease *BRAF* expression *in vitro*.[42] The serine/threonine protein kinase encoded by *BRAF* regulates the MAP kinase/ERKs signaling pathway.[43] miR-181a has previously been linked with cocaine-related addiction behavior. For example, silencing of miR-181a expression increased the rate of extinction of cocaine-induced conditioned place preference, effects that were accompanied by decreased dopamine receptor 3 and *MeCP2* expression in the NAc.[19] Notably, the homeostatic interaction between *MeCP2* and miR-212 is a key regulatory mechanism preventing runaway maladaptive changes in the striatum that can lead to compulsive drug-seeking.

CONCLUSIONS

A major clinical hurdle for addiction is the prolonged propensity for relapse, which can endure for many years. This suggests that underlying changes in brain circuits are also persistent and we have focused our attention on longer-term influences. It is important to reiterate that our measurements of gene and miRNA expression were made up to 8 weeks after drug exposure and extinction, and therefore likely reflect changes that underpin the more persistent aspects of addiction neurobiology. Using this approach, we have identified several addiction-relevant miRNA with the potential to regulate *Arc* and other synaptic plasticity genes, in the DMS and DLS of addiction/relapse-vulnerable animals. Importantly, we found subregion-specific changes in *Arc* expression focused in the DMS. We also provide new data to support the role for miR-212 in the neuroadapatations that promote addiction. Together our study has identified a number of miRNA that may contribute to the neuroadaptations that lead to the persistent risk of relapse associated with cocaine addiction. Our findings provide further support for proposals, which state that cocaine exposure promotes deficits in striatal synaptic plasticity.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGMENTS

These studies were supported by funding from the Australian National Health and Medical Research Council, the Hunter Medical Research Institute and the University of Newcastle through project grants to CVD.

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CHAPTER SIX: Conclusion

The current thesis identifies deficits in molecular signalling pathways, including mTOR and Arc, that play a key role in synaptic plasticity. These changes appear to be strongly linked with the development and maintenance of an addiction-like phenotype in rats. Interestingly, miRNA with known or predicted binding to regulatory elements on these synaptic plasticity genes displayed temporally specific patterns of expression at different stages of the addiction cycle. Thus, this thesis highlights time dependent expression of molecular adaptations that can contribute to the development of addiction. The identification of these molecular and biochemical changes throughout various stages of the addiction cycle highlight potential targets for the development of effective pharmacotherapuetics to aid in the treatment of addiction.

Critically, we have shown an important role for the mTOR complex 1 (mTORC1) in cocaine taking, withdrawal and drug reinstatement. Withdrawal of cocaine was found to increase markers of mTORC1 activity in the NAC, while inhibition of this signalling pathway using rapamycin attenuated addiction-like behaviour in rats, including reinstatement like behaviour and motivation to consume cocaine. Importantly, systemic administration of rapamycin produced a moderate decrease in body weight in these rats. Notably, intra-NAC or i.c.v rapamycin had minimal impact on bodyweight. The development of small molecules that target mTORC1 in the CNS may provide a strong therapuetic treatment of drug addiction.

Previous work from our group has shown that genes encoding synaptic plasticity genes including for *mTOR* are downregulated in the striatum animals

phenotyped as addiction vulnerable [1]. Until now the underlying mechanisms contributing to these impairments have not been well understood. This thesis identifies miRNA as potential regulators of addiction relevant plasticity genes. Furthermore, we utilised a computational paradigm to identify addiction vulnerable rats early during cocaine taking enabled to better understand the temporal profile of miRNA expression across the addiction cycle.

Chapter 2 of this thesis shows that cocaine increases mTORC1 activity and that inhibition of mTORC1 using rapamycin reduces drug-seeking behaviour. The increase in mTORC1 promoted enhanced translation and phosphorylation of GluA1 AMPAR subunits; actions that contributes to drug-seeking behaviour. Surprisingly though work from our lab indicates that addiction vulnerable animals exhibit suppressed *mTOR* expression following reinstatement of drug seeking [1]. Importantly, work from Chapters 4 and 5 provides a mechanistic link to explain these results. Thus, miR-101b which negatively regulates mTOR gene expression is decreased by cocaine and may explain the increased mTORC activity in response to cocaine (see Figure 1a). However, after withdrawal and reinstatement, miR-101b expression is increased and associated with suppressed mTOR gene expression. A prolonged suppression of mTOR gene expression in vulnerable animals may be a marker of relapse vulnerability and drive ongoing plasticity deficits in addicted individuals. From a therapeutic perspective early administration of rapamycin appears to prevent the initial changes in synaptic plasticity processes that underlie these changes, and as such reduces the expression of addiction like behaviour in rats (see Figure 1b).

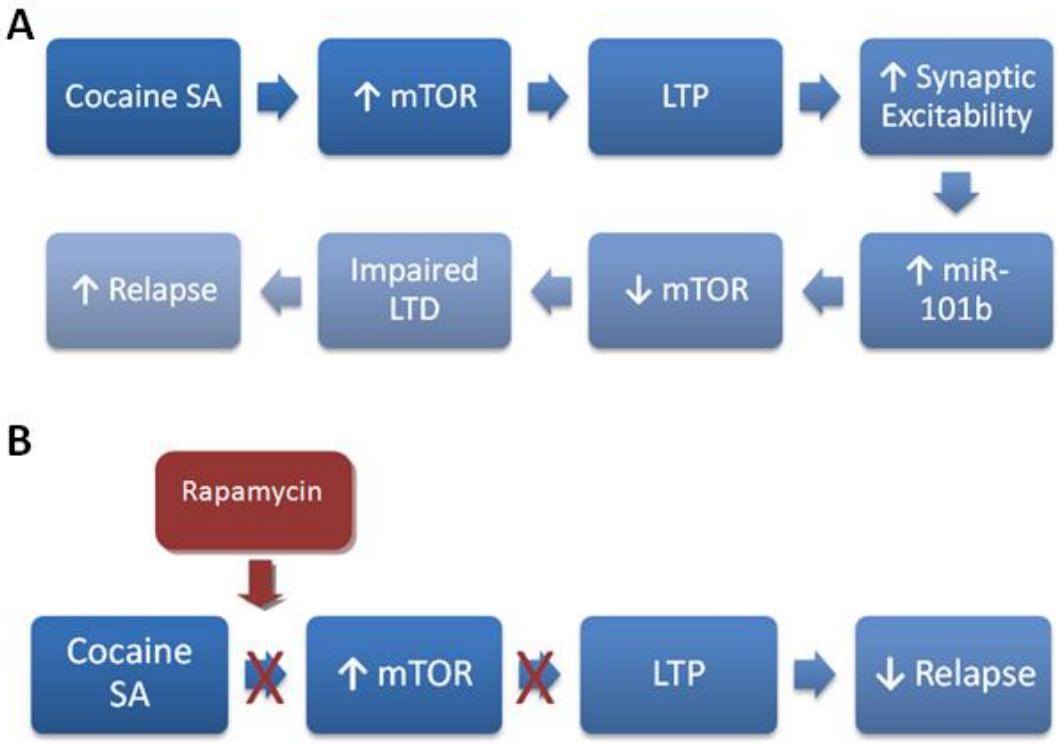

Figure 7: Proposed role of mTOR in addiction. (A) Following self-administration of cocaine, mTOR expression is increased, resulting in an increase in LTP and synaptic exctiability. In an attempt to curb abberant mTOR signalling, miR-101b expression is increased, which in turn decreased mTOR expression. This in turn impairs the ability of neurons to evoke synaptic plasticity in the future, namely LTD, leaving synapses in a state of increased excitability leading to an increase in propensity to relapse into drug seeking. **(B)** Administration of Rapamycin during drug taking prevents the initial increase in mTOR expression, thereby preventing LTP and attenutating the increase in synaptic excitability and the risk of relapse in these individuals.

Another key finding of in this study was the regional and temporal expression profile of miR-212. For example, miR-212 levels were increased in the DMS of vulnerable animals. However, after extinction and relapse testing, the opposite pattern i.e. lower levels of miR-212 were observed compared to resilient animals. This change in miR-212 is particularly interesting given the known role of this miRNA in the regulation of compulsive cocaine seeking. Hollander et al. [2] demonstrated that overexpression of miR-212 reduced drug seeking, while inhibition of this miRNA increased cocaine consumption. Together these data

suggest that during drug taking early stages, miR-212 is increased in an attempt to decrease cocaine consumption, however with continue drug intake the protective actions of miR-212 are exhausted. This leads to a subsequent increase in cocaine consumption and an inability to refrain from drug seeking behaviour. Im et al. [3] have previously demonstrated that MECP2 and miR-212 are locked in negative homeostatic relationship that affects cocaine consumption. As such, targeting this interaction or restoring muR-212 fucntion may provide a potential therapuetic target for treatment of addiction and relapse. Future studies are required to assess the expression changes in MECP2 after cocaine use and in addiction vulnerability groups both during drug taking and follow withdrawal and reinstatement of drug seeking.

In summary, the current thesis demonstrates a critical role for a number of molecular markers of addiction-like behaviour in rats. Future studies will be required to elucidate the downstream signalling pathways, as well as assess the behaviour effects of manipulation of miRNA. Identification of these signalling molecules and downstream targets present potential therapuetic targets for the treatment of relapse vulnerability in addiction.

6.2 References

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