# An investigation into the role of mesoaccumbal $GABA_A$ receptor α2 subunit in mediating cocaine-facilitated conditioned behaviours using the RNA interference system

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DPhil in Psychology

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July 2018

I hereby declare that this thesis has not been and will not be submitted in whole or in part to another University for the reward of any other degree.

Signature .....

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α2 subunit-containing $GABA_A$ receptors (α2-$GABA_A$Rs) are abundantly expressed in the nucleus accumbens (NAc), a region thought to be important in mediating cocaine's reinforcing properties. This thesis develops viral-based RNAi tools in efforts to investigate the functional role of mesoaccumbal α2-$GABA_A$Rs in mediating cocaine's ability to facilitate conditioned behaviours (i.e. behavioural sensitisation and conditioned reinforcement).

RNA interference (RNAi)-mediated knockdown of the α2 subunit expression in the NAc core neither affected appetitive Pavlovian learning nor instrumental learning maintained by the conditioned reinforcer, but blocked cocaine facilitation of conditioned reinforcement. This behavioural phenotype was also observed upon α2 knockdown specifically in NAc core dopamine D2 receptor (D2R)-containing neurons, whereas α2 knockdown in mesoaccumbal D1R-containing neurons reduced the level of discriminated approach during Pavlovian learning. Further, α2 knockdown in the NAc core or shell did not block cocaine-induced sensitisation as previously observed in the constitutive knockouts (Dixon et al., 2010), but the latter increased acute locomotor responses to cocaine.

Data presented within this thesis indicate that GABAergic signalling via α2-$GABA_A$Rs within the NAc is involved in some of the motivation-enhancing properties of cocaine, most likely via interactions with the dopaminergic system.

# Acknowledgments

First, I would like to express my sincere gratitude to my wonderful supervisors Sarah King and Dai Stephens for the constant support and guidance during these past four years. I have been extremely fortunate to have Sarah as my primary supervisor, who believes in my abilities and has given me wonderful opportunities to be part of the lab. It has also truly been a privilege to be supervised by Dai, who has given me countless expert advice on my behavioural work and continues to inspire me to do better. I've thoroughly enjoyed our brainstorming sessions! Special mention goes to my assessor, Eisuke Koya for providing such valuable advice and helpful feedback to my work. I am beyond grateful to have such an amazing supervisory team!

I am so grateful to all my colleagues who have helped me along the way. I am particularly indebted to Jonny Robertson, for patiently teaching me new techniques, sitting with me during long surgery sessions, and helping me along the way with the animal work. I would also like to thank Claire Dixon for the helpful advice and creating the Med-PC programmes and macros for my experiments, as well as for being a great mentor in the lab.

I would like to express my gratitude to Majid Hafezparast for providing plasmids and cells used in this thesis, and to members of the Hafezparast Lab, Fabio Simoes, Greig Joilin, and Muruj Barri for helping me with my molecular/cell culture work. I would also like to thank members of the Koya Lab, Ella (the most hard-working, meticulous research technician I know!), Joe, and Meike, for their help along the way. Completing this work would have been all the more difficult if it were not for the support and friendship provided by these guys. Also, thanks to the University of Sussex Ancillary Unit team (Steph, Steve, Danielle, and Mike) for looking after my mice.

I would like to thank my mum and dad for their endless support and taking interests in my research. You have always shown me the value of having good work ethics and encouraged me to be the best I can be. Huge thanks to my brother, Martin, and my sister-in-law, Olivia, for reminding me to relax when I work too much!

I would like to dedicate this thesis to my husband, Juraj Izold. Without your constant support, I wouldn't have come this far. You have always been there for me in every step of my academic journey and are always happy to discuss my research. Thank you for everything you have done for me, I will forever be grateful and I hope I have made you proud.

Lastly, I would like to thank University of Sussex for providing financial support, which allowed me to undertake this research and gave me the opportunity to attend a conference and a summer school.

# Communications arising from this thesis

Sindarto, M. S., Dixon, C. I., King, S. L., & Stephens, D. N. (2017). An investigation into the role of α2-containing $GABA_A$ receptors in conditioned reinforcement and its facilitation by cocaine. *EPBS Biennial Meeting 2017.*

Sindarto, M. S., Dixon, C. I., King, S. L., & Stephens, D. N. (2016). An investigation into the role of α2-containing $GABA_A$ receptors in conditioned reinforcement and its facilitation by cocaine. *FENS Summer School, 2016.*

## Chapter 1. General Introduction

## Chapter 5. Effects of GABAAR α2 subunit knockdown in the nucleus accumbens D1 or D2 receptor-containing neurons on cocaine-facilitated conditioned reinforcement



## Chapter 6. General Discussion

# List of abbreviations

| | | |
|---|---|---|
| α2-GABAAR | = | α2-containing GABAA receptor |
| α4-GABAAR | = | α4-containing GABAA receptor |
| AAV | = | Adeno-associated virus |
| aca | = | anterior commissure |
| BAC | = | Bacterial Artificial Chromosome |
| BZ | = | Benzodiazepine |
| BLA | = | Basolateral Amygdala |
| CeA | = | Central Nucleus of the Amygdala |
| Cl- | = | Chloride |
| CNS | = | Central Nervous System |
| CR | = | Conditioned Reinforcer |
| Cre | = | Cre Recombinase |
| CRf | = | Conditioned Reinforcement |
| CS | = | Conditioned Stimulus |
| DIO | = | Double-floxed Inverted Orientation |
| DO | = | Double-floxed Orientation |
| DS | = | Dorsal Striatum |
| EGFP | = | Enhanced Green Fluorescent Protein |
| EPSP | = | Excitatory Postsynaptic Potential |
| FSI | = | Fast-spiking Interneurons |
| GABA | = | γ-aminobutyric acid |
| $GABA_AR$ | = | $GABA_A$ receptor |
| GFP | = | Green Fluorescent Protein |
| HC | = | Hippocampus |
| HEK293 cells | = | Human Embryonic Kidney 293 cells |
| ICC | = | Immunocytochemistry |
| IHC | = | Immunohistochemistry |
| IPSP | = | Inhibitory Postsynaptic Potential |
| mIPSC | = | miniature Inhibitory Postsynaptic Current |
| MSN | = | Medium Spiny Neuron |

| | | |
|---|---|---|
| NAc | = | Nucleus Accumbens |
| NCR | = | Non-conditioned reinforcer |
| PFC | = | Prefrontal Cortex |
| PIT | = | Pavlovian to Instrumental Transfer |
| qRT-PCR | = | Quantitative Reverse Transcriptase Polymerase Chain Reaction |
| RNAi | = | RNA interference |
| shα2 | = | α2-targeting short hairpin RNA |
| shScr | = | scrambled short hairpin RNA |
| siRNA | = | small interfering RNA |
| SNP | = | Single Nucleotide Polymorphism |
| TM | = | Transmembrane |
| VI | = | Variable Interval |
| VP | = | Ventral Pallidum |
| VTA | = | Ventral Tegmental Area |
| WT | = | Wildtype |

# Chapter 1
# Introduction

## 1.1. Overview

Drug addiction is now recognised as a neuropsychiatric disorder and its consequences on both physical and mental health continue to place a heavy burden on the public health system. According to the World Drug Report (2017), the market for cocaine continues to expand in recent years, reflected by increases in coca bush cultivation and seizures, raising further concerns pertaining to growing cocaine use worldwide. In the UK, deaths involving cocaine in 2012-2016 have been reported to reach an all-time high (Office for National Statistics, 2016). Whilst various interventions have been put in place to manage and restore social and economic impacts associated with drug misuse, there remains a lack of effective and accessible treatments to tackle drug misuse problems. Further research is therefore needed to better understand the biological and behavioural aetiology of drug addiction, in the continuing effort to provide effective treatments.

## 1.2. Genetic basis of addiction: Focusing on *GABRA2* polymorphism

Human studies of addictive disorders converge on the relevance of genetic heritability in the development of drug addiction (Kendler, Karkowski, Neale, & Prescott, 2000; Kendler, Karkowski, & Prescott, 1999; Verhulst, Neale, & Kendler, 2014). Computations of the weighted mean heritability for addictions from large twin studies further revealed that while heritability is lowest for hallucinogenic drugs (0.39), it is highest for cocaine (0.72) (Goldman, Oroszi, & Ducci, 2005). Among a plethora of genes linked to cocaine addiction to date, polymorphic variations of *GABRA2*, encoding the α2 subunit of the Gamma-Aminobutyric acid A receptors ($GABA_ARs$), have recently been associated with cocaine addiction, particularly in individuals with a history of childhood trauma (Dixon et al., 2010; Enoch et al., 2010). There also exists a large body evidence linking *GABRA2* variation to individual susceptibility to heroin (Enoch et al., 2010), alcohol (Edenberg et al., 2004; Li et al., 2014; also reviewed in Stephens, King, Lambert, Belelli, & Duka, 2017), as well as polydrug (Agrawal et al., 2006; Matthews, Hoffman, Zezza, Stiffler, & Hill, 2007) abuse.

Maladaptive actions that characterise the addiction phenotype often stem from unbalanced neural interactions within circuits implicated in goal-directed behaviours (Volkow, Wang, Tomasi, & Baler, 2013). GABA is the major inhibitory neurotransmitter in the mammalian central nervous system (CNS), and therefore plays an integral role in regulating the dynamics of neural activity and excitability. It exerts its inhibitory and in

minor cases, excitatory, role by acting via ionotropic $GABA_A$ receptors ($GABA_ARs$) (Bracci & Panzeri, 2006; Farrant & Nusser, 2005; Obata, Oide, & Tanaka, 1978). The α2 subunit-harbouring $GABA_ARs$ (α2-$GABA_ARs$), in particular, are densely expressed in a region by which cocaine and other drugs of abuse exert their reinforcing properties, i.e. the nucleus accumbens (NAc), as well as in other loci implicated in motivational processes and incentive learning, such as the prefrontal cortex, amygdala, and the hippocampus (Cardinal, Parkinson, Hall, & Everitt, 2002; Hörtnagl et al., 2013; Pirker, Schwarzer, Wieselthaler, Sieghart, & Sperk, 2000; Tracy, Jarrard, & Davidson, 2001).

In this thesis, we sought to explore the role of α2-$GABA_ARs$, specifically in the NAc, in mediating cocaine-induced dopamine-dependent behaviours. This introduction will begin by describing the NAc architecture and circuitry, $GABA_AR$ properties, and the role of the GABAergic system in mediating cocaine-induced responses. Finally, this chapter will also introduce RNA interference (RNAi) as a strategy to study the functional importance of α2-$GABA_ARs$ in a site- and/or pathway-specific manner.

## 1.3. The Nucleus Accumbens (NAc)

Goal-directed action largely depends on our ability to accurately integrate and assess the value of the anticipated incentives with the effort-related costs (Salamone, Correa, Farrar, & Mingote, 2007; Salamone & Correa, 2012). One of the most investigated systems, implicated in the expression of motivated actions, is the basal ganglia (BG) circuitry, which comprise a set of subcortical structures, forming looped circuits with the

thalamus and cortex (Hollerman, Tremblay, & Schultz, 2000; Lanciego, Luquin, & Obeso, 2012).

The striatum serves as the main input nucleus to the basal ganglia. Anatomically, the striatal region can be partitioned into dorsal and ventral territories, and it is the ventral portion of the striatum (i.e. the nucleus accumbens, NAc), which forms the prime focus of this thesis. The NAc receives highly converging inputs from cortical and subcortical structures and projects to motor areas to govern behavioural output (Voorn, Vanderschuren, Groenewegen, Robbins, & Pennartz, 2004). The net output projections from the NAc are also influenced or fine-tuned by the intra-accumbal microcircuitry, consisting of collateral projections between neighbouring NAc neurons, as well as regulation by local GABAergic and cholinergic interneurons (Tepper, Wilson, & Koós, 2008; discussed further below).

### 1.3.1. The NAc architecture

The NAc is a part of the ventral striatum (VS), originally coined by Heimer (Heimer, Switzer, & Van Hoesen, 1982). The NAc can be further segregated into two subterritories on the basis of cellular morphology, projection patterns, neurochemistry, and biological functions, i.e. NAc shell and core (Heimer, Zahm, Churchill, Kalivas, & Wohltmann, 1991; Voorn et al., 2004). Based on anatomical landmarks, the core region lies directly beneath and continuous with the dorsal striatum (DS) and surrounds the anterior commissure (aca), whereas the shell subdivision surrounds the core

medially, laterally, and ventrally. Given that the core-shell division can be most easily distinguished in a more caudal part of the NAc, the rostral part of the NAc is now referred to as the rostral pole (Zahm & Brog, 1992).

The GABA-releasing medium spiny neurons (MSNs) make up the majority (~95%) of the striatal neuronal cell population (Matamales et al., 2009; Ouimet, Langley-Gullion, & Greengard, 1998) and the remaining ~5% of striatal neurons comprise several classes of interneurons, i.e. parvalbumin (PV)-expressing fast-spiking GABAergic interneurons (FSIs), neuropeptide Y (NPY)/ nitric oxide synthase (NOS)/ somatostatin (SOM)-expressing GABAergic interneurons, calretinin (CR)-expressing GABAergic interneurons, and cholinergic interneurons (CINs) (Tepper & Bolam, 2004).

### 1.3.2. Cell populations

#### 1.3.2.1. Medium Spiny Neurons

The GABAergic MSNs exhibit a characteristic hyperpolarised resting membrane potential (RMP) of approximately ~-80 mV. This is known as the 'down state', due to a strong inward rectification mediated by high amounts of inwardly rectifying Kir2 K+ channels (Gertler, Chan, & Surmeier, 2008; Mermelstein, Song, Tkatch, Yan, & Surmeier, 1998; Wilson & Kawaguchi, 1996; Wilson, 1993). A barrage of highly convergent glutamatergic inputs, impinging onto the dendritic spines of MSNs, depolarise the neurons, thereby overwhelming and promoting the closure of the dendritic Kir2 K+ channels and inactivation of the neighbouring Kv4 (A-type) K+ channels. These events lead to increased input impedance of the MSN dendrites,

yielding an 'up' state as the somatic membrane potential approaches the spike threshold. It is at this state that MSNs fire (Day, Wokosin, Plotkin, Tian, & Surmeier, 2008; Surmeier, Carrillo-Reid, & Bargas, 2011; Wilson, 1993).

Based on their neuropeptide expression, electrophysiological properties and projection patterns, the striatal MSNs can be further classified into two main categories. Namely, the D1-MSNs are enriched with Dopamine D1-receptors (D1Rs), substance P and dynorphin, whereas the D2-MSNs selectively express Dopamine D2-receptors (D2Rs) and enkephalin (Gerfen et al., 1990; Surmeier, Song, & Yan, 1996). Also note at least 6% of MSNs in the mouse striatum co-express D1Rs and D2Rs (Perreault, Hasbi, O'Dowd, & George, 2011).

Morphologically, the dendrites of the D1- and D2-MSNs contain a similarly high number of spines, though the latter were found to possess more, resulting in a greater number of synaptic contacts onto these neurons (Gertler et al., 2008). Electrophysiological evidence further suggests that the dendrites of the D2-MSNs are more excitable than those of the D1 counterparts (Day et al., 2008). Nevertheless, unlike projections originating from the DS, recent evidence suggests that the canonical 'striatal-like' segregated projection pattern does not apply to efferents from the mouse NAc (further discussed in Section 1.3.3.4 below) (Kupchik et al., 2015; Smith, Bevan, Shink, & Bolam, 1998).

#### 1.3.2.2. GABAergic interneurons

Approximately 3-4% of striatal neurons are aspiny GABAergic interneurons (Tepper, Tecuapetla, Koós, & Ibáñez-Sandoval, 2010). They were first characterised by [$^3$H] GABA uptake combined with Golgi staining and were found to accumulate [$^3$H] GABA to a significantly greater extent than the MSNs (Bolam, Clarke, Smith, & Somogyi, 1983). Later findings further identified three types of GABAergic interneurons on the basis of their calcium-binding proteins. These neurons either express PV, CR, or NPY-NOS-SOM, representing 0.7%, 0.5%, or 0.6% of the total striatal neuron population in the rat, respectively (Rymar, Sasseville, Luk, & Sadikot, 2004).

##### 1.3.2.2.1. Fast-spiking interneurons (FSIs)

The PV-immunoreactive (PV+) interneurons exhibit a fast-spiking electrophysiological profile and are, thus, commonly referred to as fast-spiking interneurons (FSIs). This subpopulation of striatal interneurons was first documented in the Gerfen, Baimbridge, & Miller (1985) study. PV+ FSIs can further be classified into two subtypes based on the firing characteristics – i.e. exhibiting continuous firing (maintained by continuous current injection) vs. "stuttering" response. The latter denotes a brief series of action potentials separated by silent periods of variable interval, which have been detected specifically in the rodent NAc (Stefano Taverna, Canciani, & Pennartz, 2007), though such a phenotype was found to be less frequent in mice (Freiman, Anton, Monyer, Urbanski, & Szabo, 2006).

Nevertheless, it remains tentative whether these aforementioned firing properties reflect different states of the same FSIs or are indicative of distinct FSI subpopulations.

Neurochemically, FSIs express high amounts of the main synthetic enzyme for GABA, i.e. glutamate decarboxylase (GAD), specifically the GAD67 isoform (i.e. GABA-synthesising enzyme) (Kita, Kosaka, & Heizmann, 1990; Lenz, Perney, Qin, Robbins, & Chesselet, 1994). FSIs make $GABA_A$ receptor-mediated synapses primarily onto the somatic membrane of both D1- and D2-MSN subtypes (Bennett & Bolam, 1994; Kita et al., 1990), and have been demonstrated to make connections with other FSIs, but not with other classes of interneurons (Gittis, Nelson, Thwin, Palop, & Kreitzer, 2010). Specifically, paired recordings showed that FSI-MSN synapses exhibit effective temporal summation, low failure rates (<1%), and, importantly, spiking of the FSIs is potent enough to delay or completely block MSN firing (Koós & Tepper, 1999). The occurrence of such powerful inhibition has been linked to an increase in cortical activity, indicating descending excitatory afferents from the cortex to the PV+ FSIs (i.e. feedforward inhibition) (Mallet, Le Moine, Charpier, & Gonon, 2005).

#### 1.3.2.2.2. Low-threshold spiking interneurons

The NPY-NOS-SOM+ interneurons exhibit a low-threshold calcium spike (LTS), high input resistance (>600 MΩ), and a more depolarised RMP (~-56 mV). Based on these characteristics, this class of interneurons was initially termed PLTS interneurons (PLTSIs) due to the **LTS** and **p**ersistent

depolarising plateau potentials (Kawaguchi, 1993). Unlike FSIs, PLTSIs form synaptic contacts on distal regions of the MSN dendrites and spines, avoiding the soma, as well as on cholinergic interneurons (Kubota & Kawaguchi, 2000). Intriguingly, the GABAergic inhibitory post-synaptic currents (IPSCs) evoked by LTSI and consequently, the synaptic responses in target MSNs are relatively weak. These findings cast doubts as to whether the main neuroactive substance released by PLTSIs is GABA, considering that GAD and GABA expression levels are relatively low in PLTSIs (Gittis et al., 2010; Tepper et al., 2010).

More recently, the original premise that the NPY-NOS-SOM+ neurons represent a single subpopulation of interneurons has been disputed, as immunolabelling studies identified NOS-SOM+ interneurons that did not harbour NPY (Figueredo-Cardenas, Morello, Sancesario, Bernardi, & Reiner, 1996) and stereological cell counting experiments revealed differences in the numbers of SOM+ and NPY+ neurons (Rymar et al., 2004). For example, a class of NPY+ neurogliaform interneurons (NPY-NGF) has recently been identified and intriguingly, the NPY-NGF interneurons were found to mediate powerful inhibition to MSNs (Du et al., 2017). These neurons exhibit similar electrophysiological characteristics to those of the MSNs, i.e. hyperpolarised membrane potential, low input resistance, and marked inward rectification (Wilson, 1993). This subclass of interneurons forms extremely high probability of synaptic contacts with MSNs with no failures observed. They are thought to mediate feedforward

inhibition of MSNs, but with slower kinetics relative to that mediated by FSIs (Ibáñez-Sandoval et al., 2011).

#### 1.3.2.2.3. Calretinin-expressing interneurons

Out of the three classical types of striatal GABAergic interneurons, the least is known about the CR+ subtype. Though these neurons represent ~0.5% of striatal neurons in based on stereological cell counts of immunoreactive neurons in the rat striatum (Rymar et al., 2004), the activity of these neurons has never been recorded and they have not been intracellularly labelled, in part due to the lack of transgenic mice whereby CR is tagged with a fluorescent protein (e.g. EGFP-CR+) to enable selective investigation of this neuronal subtype (Tepper et al., 2010). As a result, current knowledge of these neurons remains in its infancy and is limited to their morphological structure based on immunostaining experiments, which revealed at least three morphologically different subtypes of CR+ interneurons based on their somatic size (Prensa, Giménez-Amaya, & Parent, 1998; Rymar et al., 2004; Schlösser, Klausa, Prime, & Ten Bruggencate, 1999).

### 1.3.2.3. Cholinergic interneurons

The cholinergic interneurons (CINs) only make up ~1% of striatal neurons, but these neurons ramify extensively and provide the main source of striatal acetylcholine (ACh) (Bolam, Wainer, & Smith, 1984; Contant, Umbriaco, Garcia, Watkins, & Descarries, 1996). The CINs are distinguishable from other striatal cells as they have large soma (>15 μm)

and display a unique electrophysiological profile, such as a more depolarised RMP (~-60 mV), high input resistance (~ 200 MΩ), and exhibit a tonic firing rate of 3-10 Hz, therefore classed as tonically active neurons (TANs) (Calabresi et al., 1997; Lee, Dixon, Freeman, & Richardson, 1998; Wilson, Chang, & Kitai, 1990). Immunohistochemical analyses further indicated that the striatum contains the highest density of cholinergic markers, which include ACh, choline acetyltransferase (ChAT), as well as acetylcholinesterase (AChE), yielding a hypothesis that cholinergic signalling may play a crucial role in regulating striatal activity and its output (Lim, Kang, & McGehee, 2014). The mesostriatal cholinergic system has been previously implicated in cocaine self-administration, reinforcement learning, and sensitization. In turn, chronic cocaine exposure has also been shown to induce changes in the cholinergic system (reviewed extensively in Williams & Adinoff, 2008).

### 1.3.3. Afferents to and efferents from the NAc

The NAc receives dense excitatory afferents from cortical and subcortical structures (Brog, Salyapongse, Deutch, & Zahm, 1993; O'Donnell & Grace, 1995; Powell & Leman, 1976) and form output projections to ventral mesencephalon and the ventral pallidum (VP) (Floresco, Todd, & Grace, 2001; Heimer et al., 1991; Kupchik et al., 2015; Xia et al., 2011), which subsequently innervate the thalamic regions and ultimately, the cortical regions. Collectively, these projections form the cortico-striato-pallidal-thalamo-cortical loop (Parent & Hazrati, 1995), which has long been

implicated in behavioural responses to rewards (Satoshi Ikemoto, Yang, & Tan, 2015).

#### 1.3.3.1. Excitatory afferents

The primary afferent regions providing glutamatergic inputs to the NAc are the distinct, albeit interconnected, subregions within the prefrontal cortex (PFC) (see Heidbreder & Groenewegen, 2003 for review), the basolateral nucleus of the amygdala (BLA) (Howland, Taepavarapruk, & Phillips, 2002), the thalamus (Wright & Groenewegen, 1995) and ventral hippocampus (vHC) (Blaha, Yang, Floresco, Barr, & Phillips, 1997; Totterdell & Smith, 1989), which also form reciprocal excitatory connections with one another (Jackson & Moghaddam, 2001; O'Donnell & Grace, 1995) (Figure 1.1).

Importantly, there are differences between the origin of afferents innervating the core versus the shell subcompartments. Namely, the NAc shell is densely innervated by the ventral regions of the infralimbic, prelimbic, insular cortices, whereas the core primarily receives afferents from the dorsal agranular insular, anterior cingulate, and dorsal areas of the prelimbic cortices (Brog et al., 1993; Groenewegen, Wright, Beijer, & Voorn, 1999). Nevertheless, stimulation of the PFC, which would typically be powerful enough to trigger accumbal MSN firing, failed to do so in fornix-transfected rats. These data further suggest that the hippocampal afferents to the NAc, travelling in the fornix, are required to permit PFC inputs to elicit neuronal firing in the NAc by switching the neurons to a more

depolarised state ("up" state) (O'Donnell & Grace, 1995). Note, however, that recordings were performed from neurons in the medial NAc (medial core and shell). A recent study by Britt et al. (2012) further showed that glutamatergic inputs from vHC stably depolarised NAc neurons particularly those located in the medial NAc shell due to greater NMDA-mediated inward currents exhibited by the vHC-NAc synapses, thus providing a potential mechanism by which hippocampal inputs have a unique ability to depolarise MSNs.

The topographical organisation of inputs from the BLA, on the other hand, is more complex. It generates an elaborate 'rostral to core' to 'caudal to shell' topography that also varies according to the patch-matrix divisions of the NAc (Wright, Beijer, & Groenewegen, 1996). These amygdalar inputs play a key role in monitoring changes in the affective salience of the stimuli (Shiflett & Balleine, 2010), as well as in instrumental learning solely maintained by a conditioned reinforcer (Burns, Robbins, & Everitt, 1993; also reviewed in Everitt et al., 1999). Finally, the NAc also receives excitatory thalamic afferents, with inputs to the core arising from intermediodorsal and those to the medial shell originating from paraventricular nuclei of the thalamus (Berendse & Groenewegen, 1990). Glutamatergic afferents from the thalamus have been demonstrated to modulate dopaminergic tone in the NAc, which are thought to be involved in mediating behavioural processes relating to arousal (Parsons, Li, & Kirouac, 2007). Further, dopaminergic drugs, e.g. cocaine, have been shown to induce alterations in the thalamic-accumbal projections (Neumann et al., 2016).

These excitatory inputs typically synapse onto the dendritic shafts and spine heads of MSNs (Ligorio, Descarries, & Warren, 2009; Pinto, Jankowski, & Sesack, 2003). They also synapse onto the dendrites of local interneurons (Meredith & Wouterlood, 1990).

Taken together, temporally converging cortical inputs can effectively depolarise MSNs first by promoting a depolarised 'up' state, therefore increasing the likelihood of action potential generation (O'Donnell & Grace, 1995). Intriguingly, adequate and converging glutamatergic inputs in the striatal MSNs have the characteristic ability to generate long-lasting dendritic plateau potentials. As a result, fewer excitatory inputs (tens) are required to transition from the hyperpolarised RMP to this 'up' state (~20-30 mV state transition) and ultimately, to generate action potentials (Oikonomou, Singh, Sterjanaj, & Antic, 2014; Plotkin, Day, & Surmeier, 2011). Given the highly converging information each NAc neuron receives from various afferent sources, it is argued that the intra-NAc microcircuitry could impose an elaborate gating mechanism allowing information provided by certain subsystem(s) to preferentially affect behavioural outputs (discussed below).

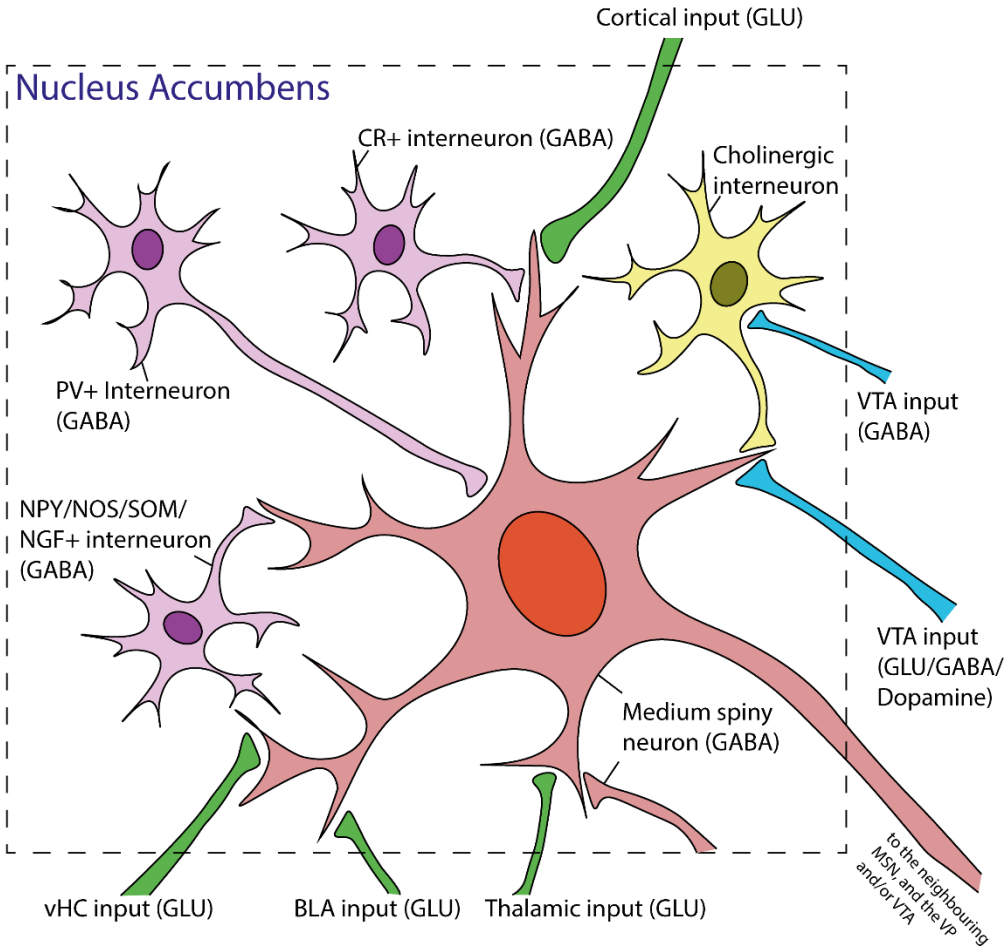



***Figure 1.1.*** A schematic representation of afferents into the accumbal medium spiny neurons. The NAc receives excitatory or glutamatergic inputs from the ventral hippocampus (vHC), basolateral nucleus of the amygdala (BLA), the prefrontal cortex, and the thalamus, whereas GABArgic inputs are provided by neighbouring MSNs (lateral inhibition) and the GABA-expressing interneurons. The majority of cholinergic inputs into the accumbal spiny neurons are derived from the local cholinergic interneurons, whereas dopaminergic inputs into the NAc primarily originate from the VTA dopamine neurons (adapted from Brown et al., 2012; Russo & Nestler, 2013; Tritsch et al., 2012).

### 1.3.3.2. Modulatory/inhibitory afferents

Dopaminergic innervation of the NAc, primarily derived from the ventral tegmental area (VTA) has, by far, received the most attention given its involvement in mediating reinforcement and motivational processes (Saunders, Richard, Margolis, & Janak, 2017; Figure 1.1). Dopamine release within the NAc can be further dissociated based on the modes of firing, i.e. phasic and tonic signalling. The spike-dependent release of dopamine triggered by primary or conditioned reward is known as the phasic dopamine response. The magnitude of phasic dopamine signals has been shown to encode the expected availability or the size of reward (Schultz, 1998). Dopamine also accumulates in the extracellular space, usually evoked via sustained firing of dopamine neurons or glutamate-induced presynaptic stimulation of dopamine terminals at a steady-state concentration that is too low to activate post-synaptic dopamine receptors but is sufficient to stimulate autoreceptors to counteract phasic dopamine response. This phenomenon has been termed the tonic dopamine response (Floresco, West, Ash, Moore, & Grace, 2003; Grace, 1991; Grace, 2000).

Importantly, dopamine binding to the G protein-coupled dopamine D1 or D2 receptors (D1Rs or D2Rs), primarily residing on the MSNs, exert excitatory or inhibitory effects on the MSNs via the modulation of voltage-dependent ion channels and ionotropic receptors on the dendrites (Bertran-Gonzalez et al., 2008; West & Grace, 2002). It is commonly accepted that dopamine's action at D1Rs activates the $G_{s/olf}$ family of G-proteins, leading

to cyclic AMP (cAMP) production by adenyl cyclase, PKA activation and the subsequent phosphorylation of PKA substrates, e.g. DARPP-32 (dopamine- and cAMP-regulated phosphoprotein of $M_r$ 32 kDa) and a transcription factor, CREB (Greengard, Allen, & Nairn, 1999; Surmeier, Ding, Day, Wang, & Shen, 2007), whereas D2R activation inhibits adenylyl cyclase activity via $G_{i/o}$ proteins. This results in the downregulation of cAMP production, leading to reduced activation of PKA (Beaulieu & Gainetdinov, 2011). The D2Rs are expressed both postsynaptically on the dopamine target neurons, as well as presynaptically on dopamine neurons (D2 autoreceptors). The latter regulate dopamine transmission by indirectly affecting expression and activity of the tyrosine hydroxylase (Lindgren et al., 2001). Phasic bursts of dopamine primarily increase average D1R occupancy, whilst having minimal effects on that of D2R (Dreyer, Herrik, Berg, & Hounsgaard, 2010). In contrast, slower tonic dopamine release predominantly activates the high affinity dopamine D2Rs (Floresco et al., 2003; Goto & Grace, 2005)

Intriguingly, recent findings documented in the Tritsch et al. (2012; 2014) studies provided evidence for the co-release of GABA, alongside glutamate and dopamine from the VTA dopaminergic neurons, indicating an alternative mechanism by which dopaminergic neurons inhibit striatal output. The lack of GAD expression in these neurons further suggested that GABA co-released by VTA dopamine neurons is not synthesised *de novo.* Instead, they rely on GABA reuptake from the extracellular space, given the high expression of membrane GABA transporters (mGAT1 and mGAT4) in

these neurons. The NAc also receives GABAergic inputs from the VTA GABA neurons (primarily innervating the accumbal CINs) (Brown et al., 2012; Taylor et al., 2014; Van Bockstaele & Pickel, 1995), as well as reciprocal connections from the VP (Stefanik, Kupchik, Brown, & Kalivas, 2013). GABAergic afferents from the forebrain areas have also been detected, though current understanding of their functional importance remains rudimentary (Brog et al., 1993; Lee, Vogt, Rubenstein, & Sohal, 2014). Notably, the striatal MSNs also receive GABAergic afferents from neighbouring MSNs and the local interneurons, thus forming a highly intricate microcircuits, consisting of the feedforward and lateral inhibitory systems (further discussed below) (Tepper et al., 2008).

Moreover, the NAc receives the majority of its cholinergic inputs from the local CINs (Rymar et al., 2004; Figure 1.1). Optogenetic activation of the accumbal CINs has been demonstrated to increase the frequency of $GABA_AR$-mediated post-synaptic currents (Witten et al., 2010) and evoke DA release in the NAc via nictonic acetylcholine (ACh) receptor (nAChR)-mediated increase in the excitability of dopamine terminals (Cachope et al., 2012), whereas optogenetic inhibition of these neurons was found to increase MSN firing rate (Witten et al., 2010). Behaviourally, activity of the CINs has been linked to goal-directed actions, reward learning, and motivation (Aitta-aho et al., 2017; Ostlund, LeBlanc, Kosheleff, Wassum, & Maidment, 2014; Witten et al., 2010). Further, the NAc also receives noradrenergic inputs primarily from the nucleus of the solitary tract (NTS) and, to a lesser extent, from the locus coeruleus (LC) (Delfs, Zhu, Druhan, &

Aston-Jones, 1998). Activation of noradrenergic α receptor subtypes in the NAc has been implicated in enhancement of memory for arousing events (Kerfoot & Williams, 2011).

#### 1.3.3.3. The NAc microcircuitry: Focusing on the feedforward and lateral inhibition

Though most of the neurons in the striatum are GABAergic, most of the synapses (~80%) consist of asymmetric glutamatergic synapses originating from cortical and thalamic regions (see Wilson, 2007 for review). The local striatal GABAergic circuitry, however, plays a central role in modulating striatal output by preventing overexcitation of neurons, as blockade of $GABA_A$Rs was found to markedly augment spontaneous firing *in vivo* (Nisenbaum & Berger, 1992).

The fast inhibitory transmission in the striatum is mediated through the action of GABA at the ionotropic $GABA_A$ receptors, located on the dendritic and perisomatic membranes of the MSNs (Straub et al., 2016). The two primary sources of fast inhibition in the striatum are the *feedforward* and *lateral* inhibition from GABAergic interneurons and axon collaterals of MSNs respectively (depicted in Figure 1.1).

##### 1.3.3.3.1. Feedforward inhibition by striatal interneurons

The FSIs receive strong excitatory inputs from the cortex, that somewhat differs from those innervating the MSNs. Cortical stimuli that are insufficient to evoke excitatory responses in the MSNs have been shown

to elicit strong responses (i.e. large scale immediate early gene expression) in the FSIs (Parthasarathy & Graybiel, 1997). The primary target of these neurons are the proximal dendrites and the somatic membrane of the MSNs, both of which are electrotonically favoured locations. In mature rat brain slices, FSI-MSN GABAergic transmission was found to evoke a large inhibitory post synaptic potential (IPSP) with extremely low failure rates, mediated exclusively by $GABA_AR$s, strong enough to delay or block neuronal firing (Koós & Tepper, 1999; 2002).

Similarly, another subclass of GABAergic interneurons with characteristics somewhat similar to PLTSIs, have been shown to exert relatively powerful inhibitory effect on the MSN spike timing and are capable of blocking an action potential generation (Koós & Tepper, 1999). More recently, another subpopulation of striatal GABAergic interneurons, i.e. NPY-NGF neurons, was characterised. The size of synaptic currents evoked by these neurons, as well as the reliable nature of the synapse led to a premise that these neurons are powerful mediators of feedforward inhibition to the MSNs. However, note that the identification of these cells was made based on observations in the DS. It is yet to be investigated whether these neurons are also involved in the mesoaccumbal feedforward circuitry. Akin to the FSIs, these subtypes of GABAergic interneurons also respond to cortical stimulation (Ibáñez-Sandoval et al., 2011; Tepper et al., 2010).

Nevertheless, despite compelling evidence for the high efficacy of FSI-mediated perisomatic inhibition (Tepper, Koós, & Wilson, 2004), a study by

Du et al. (2017) observed little effect of GABAergic inputs from FSIs on dendritic plateau coupled with high frequency of excitatory inputs suggesting that likely role of FSIs was to alter the somatic membrane potential rather than switching off the plateau potential altogether, preventing an action potential generation. Instead, powerful inhibition of dendritic plateau potential was observed to be achieved via GABAergic IPSC with slower kinetics, perhaps mediated by the NPY-NGF interneurons, whilst more subtle inhibitory control appeared to originate from connectivity with neighbouring MSNs (discussed below) and PLTSIs.

Lastly, the MSN dendritic inhibition can take place in the form of *shunting inhibition*, partly mediated by the opening of extrasynaptic $GABA_A$Rs, which not only generate persistent hyperpolarising current, but also decrease the membrane resistance (Lee & Maguire, 2014). In summary, research to date has reported at least three types of GABAergic interneurons which inhibit MSNs in a feedforward manner through dendritic and/or the perisomatic targeting of the MSNs. However, precisely how each of these GABAergic neurons sculpt MSN activity in awake, behaving animals remains mysterious.

#### 1.3.3.3.2. Lateral inhibition by MSN axon collaterals

In addition to extrastriatal output projections, intracellular and immunocytochemical labelling studies indicated that the MSNs form recurrent collaterals with neighbouring MSNs, i.e. lateral inhibition (Bolam et al., 1983; Tunstall, Oorschot, Kean, & Wickens, 2002). These connections are unidirectional as no reciprocal connections have been observed (Tunstall et al., 2002). Most of these axons synapse onto the distal dendrites or the spine shaft of the target MSN, with a very small percentage of axosomatic connections (Bolam et al., 1983). This form of inhibition was found to be "weak or non-existent" (Jaeger, Kita, & Wilson, 1994) and exhibited high failure rate (>38%), indicated by the low inhibitory postsynaptic currents and potentials (IPSCs and IPSPs) (Tunstall et al., 2002).

Notably, MSN-MSN synapses are not randomly distributed. While D2-MSNs innervate both D1- and D2-MSNs, D1-MSNs almost exclusively form connections with other D1-MSNs (Dobbs et al., 2016; Taverna, Ilijic, & Surmeier, 2008). The unitary IPSC amplitudes in D2- to D2-MSN pairs were larger compared to those of D1- to D1-MSN pairs, presumably due to more $GABA_A$Rs residing on the D2-MSNs (Taverna et al., 2008). Though the functional significance of lateral inhibition is considered to be minimal due to the distal synaptic locations and the high failure rate to induce IPSP/IPSC, recent optogenetic evidence presented in the Dobbs et al. (2016) study suggests that synchronous activation of D2-MSNs in the NAc core produced a large IPSP in the majority of neighbouring putative D1-MSNs,

which was sufficient to inhibit neuronal excitability and action potential generation, thereby restricting output projections.

#### 1.3.3.4. Efferents from the NAc

Mounting evidence derived from tracer studies reveals heterogeneity in the efferent projection targets of the NAc core and shell subregions. Namely, the NAc core predominantly innervates the dorsolateral portion of the VP, entopeduncular nucleus, and the substantia nigra pars reticulata (SNr), whereas NAc shell primarily projects to ventromedial VP, lateral hypothalamus (almost exclusively innervated by the accumbal D1-MSNs), substantia innominata, VTA, the amygdala, parabrachial nucleus, the substantia nigra pars compacta (SNc), periaqueductal gray, as well as pedunculopontine tegmental nucleus (PPTg) (Heimer et al., 1991; Scofield et al., 2016; Usuda, Tanaka, & Chiba, 1998; Williams, Crossman, & Slater, 1977). The rostral pole of the NAc appears to share output projections with the core and shell, with the medial part giving rise to shell-like projections and the lateral part resembling the core-like innervations (Zahm & Heimer, 1993). It is noteworthy that projections originating from the core and shell form different limbic loops, potentially subserving distinct motivational processes (Scofield et al., 2016).

The striatal D1- and D2-MSNs have been canonically distinguished based on their distinct projection profiles (Gerfen & Surmeier, 2011). Thus, manipulations of the accumbal D1-MSNs are thought to exclusively affect the striatomesencephalic pathway, whereas D2-MSN regulation is relevant

to the striatopallidal circuitry (MacAskill, Little, Cassel, & Carter, 2012; Yawata, Yamaguchi, Danjo, Hikida, & Nakanishi, 2012), providing a mechanistic explanation for the opposing roles of D1- and D2-MSNs in mediating reward-related behaviours (for example, see Lobo et al., 2010). Nevertheless, an increasing amount of evidence indicates that such a segregated projection pattern does not apply to accumbal efferents, specifically from the NAc core. Though the ventral mesencephalon is exclusively innervated by D1-MSNs (Bocklisch et al., 2013; Kupchik et al., 2015; Watabe-Uchida, Zhu, Ogawa, Vamanrao, & Uchida, 2012), NAc-VP projections were found to contain a mixture of D1- and D2-MSN axons (Kupchik et al., 2015; Lu, Ghasemzadeh, & Kalivas, 1998; Smith, Lobo, Spencer, & Kalivas, 2013; Zhou, Furuta, & Kaneko, 2003), warranting reconsideration of the current understanding pertaining to D1-D2 projection selectivity from the NAc (see Figure 1.2).

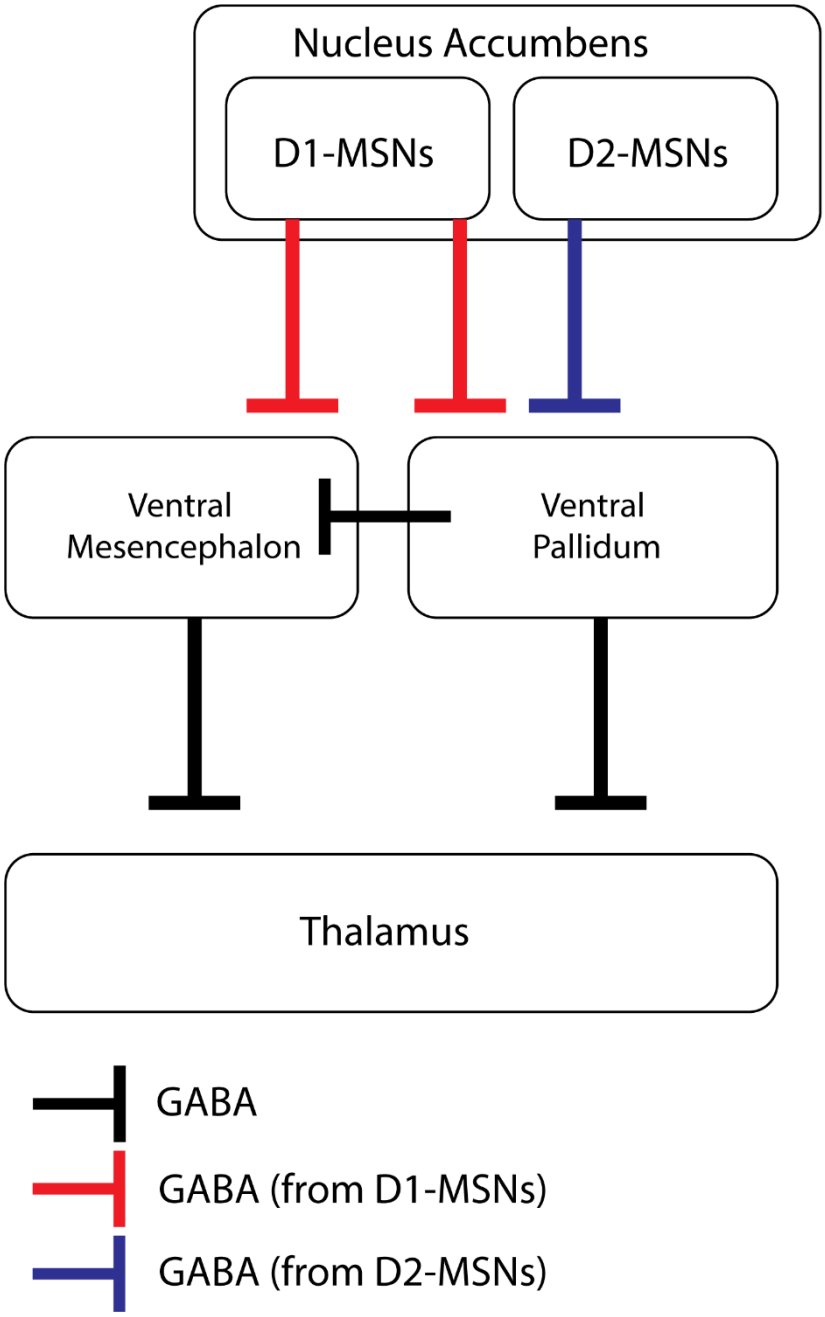



***Figure 1.2.*** Current view on the efferents from the accumbal D1- and D2-expressing MSNs (adapted from Kupchik & Kalivas, 2017)

### 1.3.4. Summary

The NAc is part of the main input structure of the basal ganglia, receiving glutamatergic inputs from various cortical and subcortical structures. These excitatory afferents are thought to be critically involved in

driving behavioural responses to salient events, whilst dopaminergic and GABAergic inputs serve to fine tune these glutamatergic inputs. The NAc, then relays this information to the basal ganglia output structures, which ultimately govern motor actions. Nevertheless, note that the NAc core and shell subdivisions exhibit distinct, but overlapping, patterns of connectivity (Zahm, 1999) and thus, are believed to play distinct roles in motivational and emotional processes (see Parkinson, Olmstead, Burns, Robbins, & Everitt, 1999; Shiflett & Balleine, 2010 for examples).

## 1.4. $GABA_A$ Receptors

### 1.4.1. Receptor structure and subunit composition

The $GABA_A$ receptors ($GABA_ARs$) are considered to be the major inhibitory neurotransmitter receptors in the mammalian brain and are targets for various clinically important drugs (Farrant & Nusser, 2005a; Sieghart, 1995; Erwin Sigel & Steinmann, 2012). They belong to the family of Cys-loop ligand-gated ion channels, which members also include the nAChRs, strychnine-sensitive glycine receptors, and 5-hydroxytryptamine type-3 receptors ($5\text{-}HT_3Rs$) (Betz, 1990). The binding of GABA to $GABA_ARs$ triggers the opening of the ion channels, thereby increasing membrane permeability to chloride and to a lesser extent, bicarbonate ions, mostly leading to a hyperpolarising post-synaptic response in adult neurons (Kaila, Pasternack, Saarikoski, & Voipio, 1989). This phenomenon is critical for the regulation of neural activity by a diverse set of GABA-releasing neurons and deficits in the functional expression of $GABA_ARs$ have been reported to

underpin the pathogenesis of various neurological disorders, including substance abuse (Edenberg et al., 2004; Enoch et al., 2010; Hines, Davies, Moss, & Maguire, 2012; Lieberman, Kranzler, Joshi, Shin, & Covault, 2015; Tan, Rudolph, & Lüscher, 2011).

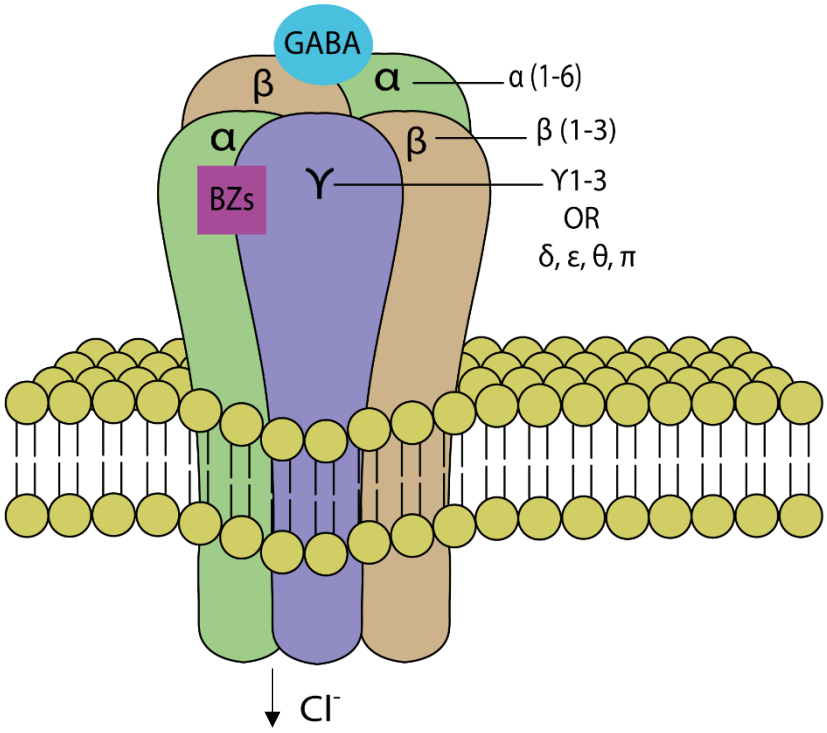
Structurally, the $GABA_AR$s are heteropentamers that are composed of five homologous subunits (Figure 1.3A), sharing a common topological organisation, i.e. a large extracellular N-terminal domain and four transmembrane domains (i.e. TM1-4). TM2 lines the ion channel and a large intracellular domain is located between TM3 and TM4, which is the prime target for post-translational modifications that can affect receptor function or aid in receptor localisation and/or membrane trafficking (Jacob, Moss, & Jurd, 2008; Figure 1.3B). Mature $GABA_AR$ subunits are approximately 450 amino acid residues in length and to date, 19 $GABA_AR$ subunits categorised into eight distinct classes, i.e. α(1–6), β(1–3), γ(1–3), δ, ε, θ, π and ρ(1–3), have been cloned and sequenced in the mammalian CNS (Sigel & Steinmann, 2012). The subunit diversity is further increased by the existence of alternatively spliced variants (Daniel & Ohman, 2009). Examples include the short and long forms of the γ2 subunit, i.e. γ2S and γ2L respectively, which differ in an eight amino acid stretch within the intracellular domain of γ2L variant (Kofuji, Wang, Moss, Huganir, & Burt, 1991). Though the multiplicity of subunits theoretically permits the formation of a plethora of $GABA_AR$ subtypes, only a few subtypes have been shown to exist, suggesting a highly selective oligomerisation (see Figure 1.3A). The most likely subunit stoichiometry of $GABA_AR$ pentamers in the

CNS comprises two α, two ß, and one γ subunit, with α1, ß2 and γ2 subunits-containing $GABA_AR$s being generally accepted as the most abundant subtype, followed by α2ß3γ2 and α3ß3γ2 subtypes (Connolly, Krishek, McDonald, Smart, & Moss, 1996; Klausberger, Fuchs, Mayer, Ehya, & Sieghart, 2000; Knight, Stephenson, Tallman, & Ramabahdran, 2000; Tretter, Ehya, Fuchs, & Sieghart, 1997). There are also less abundant isoforms where γ2 is replaced by γ1, γ3, δ, ε, or π subunits, or θ subunit replacing the ß subunit, in certain regions and/or cell types (Sieghart & Sperk, 2002).

Importantly, the $GABA_AR$ subunit composition dictates the biophysical properties of the receptor (i.e. binding and kinetics of the ion channels) and thus, the magnitude of response following ligand binding. Each subunit has a principal (+) and the complementary (-) side. GABA binds to $GABA_AR$ within the accessible intersubunit pockets at the two ß(+)α(–) interfaces, whereas the benzodiazepine (BZ) allosteric modulator of $GABA_AR$s binding site is located at the α(+)γ(–) interface (Ernst, Brauchart, Boresch, & Sieghart, 2003). The δ-containing receptors exhibit the highest affinities for GABA, whereas synaptic α3ß3γ2 receptor isoform displays the lowest affinity for GABA (Böhme, Rabe, & Lüddens, 2004). However, replacing the γ2 subunit with a δ dramatically decreases channel conductance independent of the α subunit type, indicating that δ subunit-containing receptors exhibit a relatively low efficacy despite the high affinity for GABA (for example, see Zheleznova, Sedelnikova, & Weiss, 2008). Moreover, amino acid residues that line the BZ binding site play a

pivotal role in determining binding and/or efficacy of drugs acting at this site. Namely, the BZ-sensitive $GABA_AR$ subtypes (α1, α2, α3 and α5) harbour a histidine residue at a conserved location, whereas the BZ-insensitive ones carry an arginine residue at the corresponding position. Replacing the histidine with an arginine, as does eliminating or substituting the γ2 subunit, was found to render the BZ-sensitive receptors insensitive to BZ without altering sensitivity to GABA (Günther et al., 1995; Rudolph et al., 1999; Sieghart & Sperk, 2002). Intrasubunit pockets also contain residues, important for binding and/or efficacy of modulatory drugs, including volatile anaesthetics (Nishikawa, Jenkins, Paraskevakis, & Harrison, 2002).

(A)




(B)

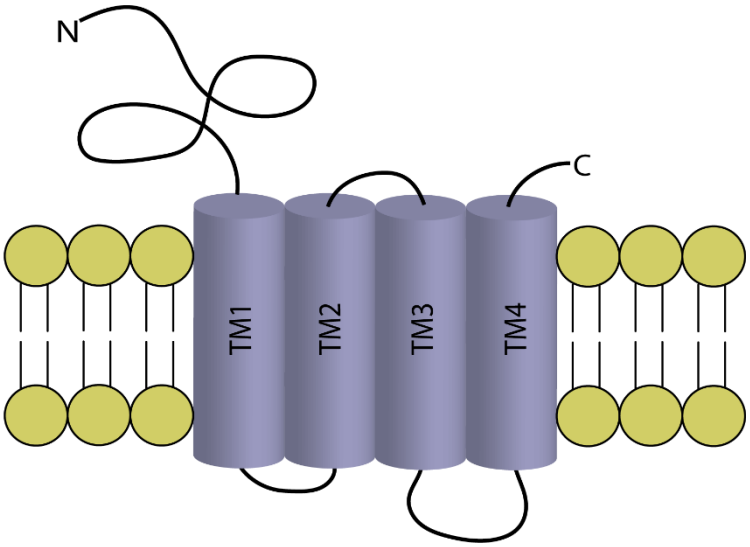


***Figure 1.3.*** **(A)** The $GABA_A$ receptor ($GABA_AR$) is a chloride-permeable heteropentameric channel, composed of five subunits from seven subunit subcategories (α,β,γ,δ,ε,θ,π). For the majority of $GABA_ARs$ in the brain, a typical subunit stoichiometry of 2α:2β:1γ is commonly observed. **(B)** The receptor subunits consist of four transmembrane domains (TM1-4). The N terminus serves as GABA binding site. TM2 lines the pore of the channel, while the intracellular domain between TM3 and TM4 serves as a site for various protein interactions and post-translational modifications, which can, in turn, regulate receptor activity (adapted from Jacob et al., 2008).

## 1.4.2. Cellular and Subcellular localisation of $GABA_AR$s

### 1.4.2.1. Cellular localisation

The heterogenous subunit composition dictates not only the biophysical and pharmacological properties of the various $GABA_AR$ subtypes, but also their cellular/subcellular localisation. Immunohistochemical and *in situ* hybridisation studies have led to a conclusion that whilst some $GABA_AR$ isoforms are broadly expressed throughout the CNS (e.g. α1 subunit-containing $GABA_AR$s), others show a more confined expression (e.g. α6-containing $GABA_AR$s) (Fritschy & Mohler, 1995; Laurie & Wisden, 1992; Sieghart & Sperk, 2002). Also note that outside the CNS, $GABA_AR$s are expressed in the peripheral and enteric nervous system (PNS & ENS) (Akinci & Schofield, 1999; Magnaghi et al., 2006).

Intriguingly, the expression of $GABA_AR$ α1 and α2 subunits is developmentally regulated (Fritschy, Paysan, Enna, & Mohler, 1994). Namely, immunofluorescent analyses detected a developmental switch in the rat α1 and α2 subunit expression, whereby an increase in the α1 and a decrease in the α2 subunit expression were observed. At birth, the onset of α1 became evident in most regions during the first postnatal week and reached the formation of adult subunit expression pattern by post-natal day (PND) 20, except within the striatum and the olfactory bulb granule cell layer whereby its expression was found to remain low at every age examined. Despite the overall decrease in the α2 subunit expression,

immunoreactivity for this subunit remains high in a few regions, including the striatum, hippocampus, olfactory bulb, and superficial cortical layers (Fritschy et al., 1994). These immunohistochemical data are further corroborated by pharmacological data on BZ I ($\alpha 1\beta\gamma$) and II ($\alpha 2/3/5\beta\gamma$) receptors (Candy & Martin, 1979; Lippa, Beer, Sano, Vogel, & Meyerson, 1981).

### 1.4.2.2. Subcellular localisation

#### 1.4.2.2.1. Postsynaptic $GABA_ARs$

Upon release in the synaptic cleft, GABA rapidly diffuses away from its release site, and the fast increase in IPSC followed by a rapid decay further indicate that there is a high density of $GABA_ARs$ located near the transmitter release sites, i.e. the postsynaptic sites. These rapid and transient GABAergic events are termed *phasic* inhibition. A key feature of the phasic mode of receptor activation is the short duration of high (millimolar) concentrations of vesicular GABA to which the receptors are exposed, given that the rate at which GABA binds to the postsynaptic receptors is relatively slow compared to its clearance rate from the release site (Brickley, Cull-Candy, & Farrant, 1999; Farrant & Nusser, 2005; Mozrzymas, 2004). In most mature neurons, the activation of postsynaptic $GABA_ARs$ yields IPSPs, thus temporarily moving the membrane potential away from the spike threshold required for the generation of an action potential (Connors, Malenka, & Silva, 1988). An essential role of phasic inhibition, primarily through the action of GABAergic interneurons at

$GABA_ARs$, is to prevent overexcitation of neurons, which could lead to the development of pathological states within the circuitry, and more importantly, to regulate input integration and thus, synchronise cellular activities (Somogyi & Klausberger, 2005; Whittington & Traub, 2003)

The α(1-3)ß2/3γ2 subunit-containing $GABA_ARs$ are the predominant receptor subtypes that are enriched at the postsynaptic regions, thus mediating phasic inhibition (Figure 1.4). However, these subunits can also be found at the extrasynaptic sites, due to the dynamic mobility and rapid shift of γ2-containing receptors between postsynaptic and extrasynaptic compartments (i.e. receptor trafficking) (Jacob et al., 2005; Thomas, Mortensen, Hosie, & Smart, 2005). The γ2 subunit, in particular, plays a key role in facilitating the clustering of postsynaptic GABAARs through interactions with the GABAAR-associated protein, gephyrin (Jacob et al., 2005). Namely, global deletion of γ2 in mice reduced gephyrin expression and disrupted the clustering of α1, α2, and ß2/3 subunits, leading to the widely held notion that γ2 is indispensable for synaptic enrichment of $GABA_ARs$ (Schweizer et al., 2003). However, more recent data have demonstrated synaptic clustering in the absence of γ2, indicating an alternative mechanism for synaptic localisation (Kerti-Szigeti, Nusser, & Eyre, 2014). Further corroborating this view is the evidence for extrasynaptic clustering of α5ß3γ2 $GABA_ARs$ (Brünig, Scotti, Sidler, & Fritschy, 2002) and deletion of the α5 subunit eliminated tonic conductance in cultured hippocampal neurons (Caraiscos et al., 2004), though there also

exists evidence that this receptor subtype is also found at dendritic synapses of hippocampal pyramidal cells (Serwanski et al., 2006).

#### 1.4.2.2.2. Extra/perisynaptic $GABA_ARs$

The α4 and α6 subunits primarily co-assemble with δ subunits in the adult brain and these δ-containing receptor complexes are almost exclusively localised remotely from the synapses, i.e. at the extrasynaptic dendritic and somatic membranes of the cell (Maguire et al., 2014; Nusser, Sieghart, & Somogyi, 1998; Wei, Zhang, Peng, Houser, & Mody, 2003; refer to Figure 1.4). Low concentrations of GABA escaping from the synaptic cleft (within the submicromolar range) can activate these $GABA_ARs$, leading to repetitive activation of these receptors and, thus, *tonic* inhibition (Farrant & Nusser, 2005b). Common features of extrasynaptic $GABA_ARs$ include high affinity for GABA and little or no receptor desensitisation (Adkins et al., 2001; Yeung et al., 2003). Tonic activation of $GABA_ARs$ triggers a persistent increase in the cell's input conductance, thus, reducing the magnitude of EPSP, narrowing the spatial and temporal window for synaptic integration and, ultimately, affecting cell excitability (Farrant & Nusser, 2005).

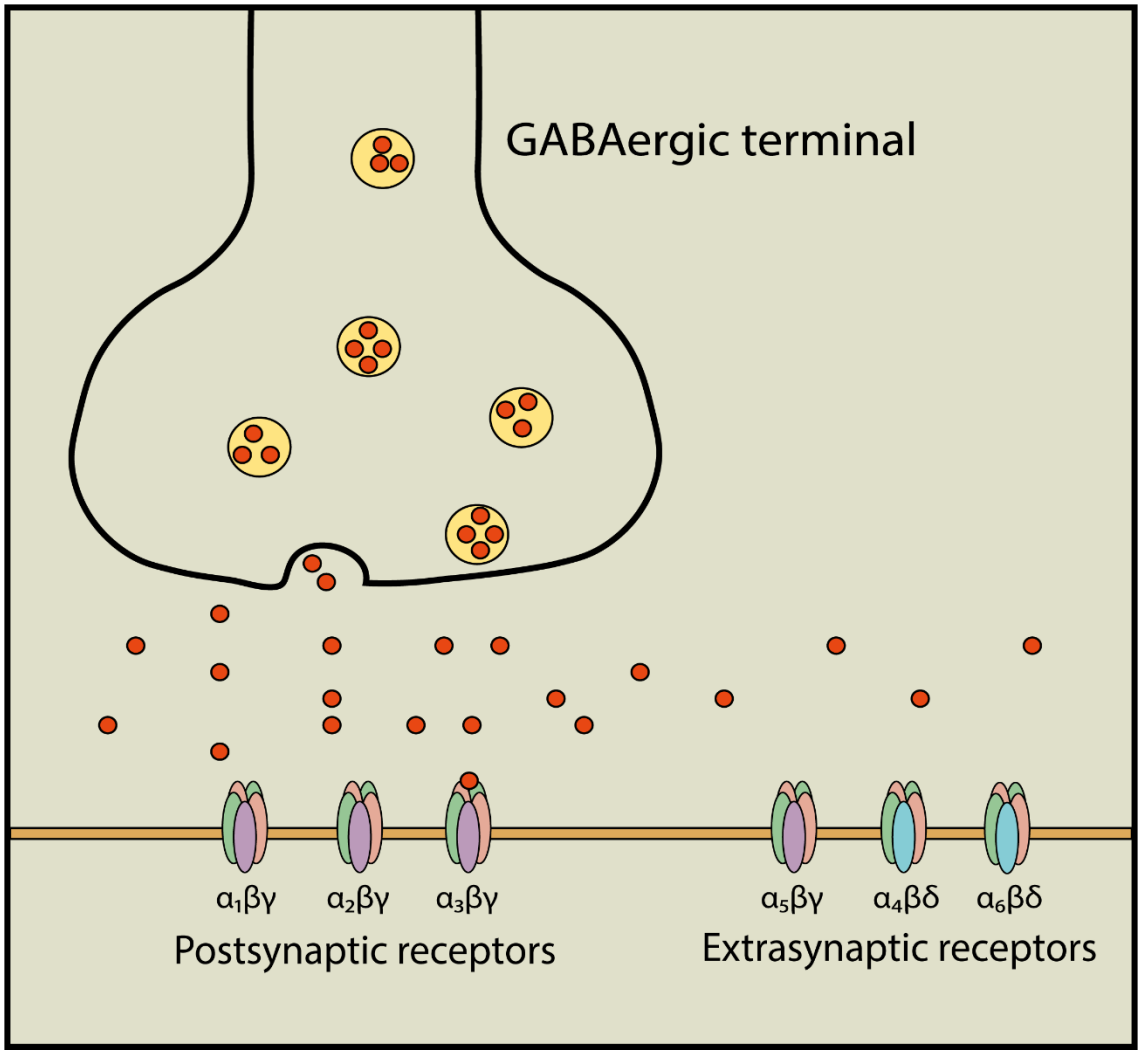



***Figure 1.4.*** The $GABA_AR$ subunit stoichiometry determines its subcellular localisation. Receptors harbouring α(1–3) subunits with ß and γ subunits are primarily localised at the postsynaptic sites, whereas the α5ßγ are extrasynaptically localised. The α(4,6)ßδ-harbouring, benzodiazepine-insensitive receptors can be found at extrasynaptic compartments (adapted from Jacob et al., 2008).

### 1.4.3. Post-translational modification of $GABA_AR$s

Many endogenous processes are known to modulate $GABA_AR$ expression and function post-translationally. These include *palmitoylation, phosphorylation* and *ubiquitination*. Following the assembly in the endoplasmic reticulum (ER), mature $GABA_ARs$ are trafficked to the Golgi apparatus prior to transport and insertion to the plasma membrane. This process requires a number of proteins performing various forms of post-translational modifications, some of which are discussed below (extensively reviewed in Vithlani, Terunuma, & Moss, 2011).

Firstly, the intracellular loop of the $\gamma_2$ subunit is subject to *palmitoylation*, i.e. the covalent attachment of the saturated fatty acid palmitate to a specific protein, by the thioacyltransferase Golgi-specific DHHC zinc finger domain protein (GODZ). This process plays a role in the accumulation of γ2 subunit-containing $GABA_AR$ targeting at postsynaptic sites and its inhibitory function (Fang et al., 2006; Keller et al., 2004). In support of this notion, RNAi-induced knockdown of GODZ produced a marked downregulation in miniature IPSC (mIPSC), due to a decrease in postsynaptic $GABA_ARs$ (Fang et al., 2006).

Secondly, the $GABA_AR$ subunits are subject to *phosphorylation* by several kinases, including cAMP-dependent protein kinase (PKA), calcium/phospholipid-dependent protein kinase (PKC), calcium/calmodulin-dependent kinase II (CaMKII), protein kinase B (PKB) (reviewed in Vithlani et al., 2011). The phosphorylation of $GABA_ARs$ has been implicated in

processes relating to channel kinetics, receptor sensitivity to allosteric modulators, and interactions between protein, to name a few (Vithlani & Moss, 2009). For instance, *in vitro* studies demonstrated lower GABA-activated currents upon cAMP-dependent protein kinase (PKA)-induced phosphorylation of ß1, but an opposite effect was observed upon ß3 phosphorylation (McDonald et al., 1998).

Finally, the $GABA_A$Rs may also be targets for modification by ubiquitin– i.e. covalent attachment of the 76-residue ubiquitin monomer to lysine residues of a given protein, a process known as *ubiquitination* (Vithlani et al., 2011). Whilst the reversible monoubiquitination is more involved in triggering endocytosis, polyubiquitination is required for transport of proteins from the ER back to the cytosol for degradation by proteases (Saliba, Michels, Jacob, Pangalos, & Moss, 2007). Polyubiquitination of $GABA_A$R ß3 subunits, for example, markedly downregulated the number of assembled $GABA_A$Rs and therefore, the amplitude and frequency of mIPSC (Saliba et al., 2007).

### 1.4.4. Pharmacological modulation of $GABA_A$Rs by endogenous and exogenous ligands

The $GABA_A$R has a rich pharmacology as it not only contains bindings sites for its primary ligand (GABA), but also for endogenous and exogenous allosteric modulators. Prime examples of the endogenous modulators include the neurosteroids, synthesised *de novo* in neuronal and glial cells or produced by the metabolism of precursors from the peripheral

steroidogenic organs (Callachan et al., 1987; Lambert, Peters, & Cottrell, 1987; Lambert, Belelli, Hill-Venning, & Peters, 1995; Lambert, Belelli, Peden, Vardy, & Peters, 2003; Tanaka & Sokabe, 2012), whereas synthetic neurosteroids (Lambert et al., 1987), typical and atypical benzodiazepines (BZs) (Rudolph et al., 1999; Schofield et al., 1987; Watanabe, Shibuya, Khatami, & Salafsky, 1986), as well as general anaestehetics, including barbiturates (Schumacher & McEwen, 1989), etomidate (Belelli, Lambert, Peters, Wafford, & Whiting, 1997), and propofol (Belelli & Lambert, 2005) are exogenous modulators.

Akin to their physiological properties, the pharmacological properties of $GABA_AR$s are also determined by variations in the receptor subunit composition. For instance, neuronal $GABA_AR$ subunit composition appears to play a role in determining heterogeneity in neurosteroid sensitivity. The binding sites for neurosteroids within the $GABA_AR$s are positioned within the transmembrane domains of α and ß subunits (Hosie, Wilkins, & Smart, 2007). Previous evidence has shown that GABA-evoked responses via α1ß1Y2 and α3ß1Y2 were enhanced by a low dose of the pregnane steroid, 3α,5α-THPROG, whereas much higher doses of 3α,5α-THPROG were required to induce similar facilitatory influence on GABAergic responses via α2-, α4-, α5-, α6-harbouring receptors (Belelli, Casula, Ling, & Lambert, 2002). Despite the low steroid sensitivity of α4ß1Y2 $GABA_AR$s, the δ-containing α4-$GABA_AR$s were found to be highly steroid sensitive (Belelli, 2002).

One of the most well-researched examples of a $GABA_AR$ modulator is a group of BZ drugs (and their derivatives). BZ binds to a specific regulatory site located at the interface of adjacent α and γ subunits and increases the opening frequency of GABA-gated chloride channels by inducing a conformational change to the receptor structure, thus increasing the mean opening time of the ion channel and, ultimately, the chloride conductance (Pritchett et al., 1989; Sigel & Buhr, 1997).

As outlined in Section 1.4.1, changing the histidine residue at a conserved position within the α subunit affects sensitivity to the typical BZ (Rudolph et al., 1999), as does substituting γ2 with a δ, ε or π subunit (Barnard et al., 1998) or point mutation at the γ2 subunit (M130L) (Buhr & Sigel, 1997). Intriguingly, more recent data from mutagenesis experiments in *Xenopus* oocytes reported a non-canonical BZ-$GABA_AR$ interaction, whereby diazepam was found to bind to β2+/ γ2- site of the $GABA_AR$. The functional implication of this interaction remains to be investigated (Wongsamitkul et al., 2017).

Finally, the enhancement of tonic currents has been observed following the administration of the δ subunit-specific positive allosteric modulator, DS2 (4-chloro-N-[2-(2-thienyl)imidazo[1,2-a]pyridine-3-yl benzamide) (Jensen et al., 2013)or the GABA analog, THIP (4,5,6,7-tetrahydroisoxazolo[5,4-c]pyridin-3-ol) (Drasbek & Jensen, 2006; Maguire et al., 2014). The latter is a full or 'super' agonist at the extrasynaptic α4β3δ receptors (exceeding the maximum response to GABA), but it also serves as

a partial agonist at the synaptic α4ß3γ2 receptors (Mortensen, Ebert, Wafford, & Smart, 2010).

Lastly, a few examples of $GABA_AR$ inhibitors include picrotoxin, bicuculline, and gabazine. Picrotoxin and bicuculline are known to be negative allosteric modulators as both of these agents block GABA-activated chloride influx at an alternative (allosteric) site, which does not overlap with the GABA-binding site. Gabazine, however, acts as a true competitive antagonist of $GABA_ARs$ (reviewed in Olsen, 2018; Stephens et al., 2017). In addition, certain atypical BZs negatively modulate some $GABA_AR$ subtypes in an allosteric fashion, whilst acting as positive allosteric modulators when bound to α4- and α6-containing receptors (Stephens et al., 2017; Walker & Semyanov, 2008).

### 1.4.5. Summary

The heteropentameric $GABA_ARs$ are considered to be the major inhibitory neurotransmitter receptors in the brain. As discussed above, the subunit composition confers not only the cellular and subcellular localisation of these receptors, but also their physiological and pharmacological properties. In specific, postsynaptic $GABA_ARs$ (most commonly harbouring α1, α2, and α3) mediate phasic inhibition, whereas those located extrasynaptically, i.e. α4-, α5-, and α6-containing receptors, are involved in mediating tonic inhibition (Farrant & Nusser, 2005; Sigel & Steinmann, 2012).

## 1.5. Psychostimulants and the GABAergic system

Though the neurobiological mechanisms underpinning psychostimulant high and its acute effects have been relatively well-characterised, a critical question remains as to how the brain adapts to repeated drug exposure, rendering individuals increasingly susceptible to addiction. Whilst the complete answers do not yet exist, research to date has shed light on a variety of short- and intermediate-term changes within the brain, some of which may give rise to further effects that persist for longer periods and may be irreversible (Nestler, 2005).

### 1.5.1. Neurobiological effects of cocaine: Initial and intermediate-term effects

Central to mediating reinforcing properties of psychostimulant drugs is their ability to elevate dopaminergic activity within the NAc (Abi-Dargham, Kegeles, Martinez, Innis, & Laruelle, 2003; Volkow, Fowler, & Wang, 1999). Psychostimulant drugs evoke dopamine buildup in the extracellular space within the NAc by increasing dopamine release (e.g. amphetamine-type stimulants and cocaine), thus delaying dopamine reuptake from the synaptic cleft and prolonging dopamine action (Fleckenstein, Volz, Riddle, Gibb, & Hanson, 2007) and/or by acting as a competitive inhibitor of the dopamine transporter (e.g. amphetamine, cocaine, and methylphenidate) (Fleckenstein et al., 2007; Kuczenski & Segal, 1997; Venton et al., 2006). In this thesis, I particularly focus on the effects of acute and repeated cocaine exposure.

Though cocaine also inhibits the reuptake of other neuromodulators, i.e. serotonin and norepinephrine, its effect on dopaminergic system has long been thought to be most important (Nestler, 2005; Uhl, Hall, & Sora, 2002). An increase in dopamine transients in the NAc triggers a sophisticated mechanism of feedback inhibition whereby dopamine D2 autoreceptors on the dopamine cell bodies within the midbrain, as well as on the terminals within the NAc are activated to inhibit continuous firing and ultimately, dopamine release. Despite this elaborate feedback regulatory system, the increase in extracellular dopamine detected following cocaine exposure raises a further speculation that there exists an alternative mechanism by which cocaine overcomes this (Ford, 2014; Venton et al., 2006).

Several reports point towards the possibility that cocaine may also influence the release of dopamine (Lee, Balu, Davidson, & Ellinwood, 2001; Shore, 1976; Stamford, Kruk, & Millar, 1989). Early work indicated that 80% of dopamine is unavailable for release (i.e. stored in the reserve pool) (Javoy & Glowinski, 1971; Korf, Grasdijk, & Westerink, 1976) and the segregation of vesicles into a reserve pool away from the site of exocytosis requires synapsins, interacting with the synaptic vesicles (Greengard, Valtorta, Czernik, & Benfenati, 1993). More recent data however demonstrated that cocaine (Venton et al., 2006) can, in fact, regulate dopamine release via the mobilisation of the reserve pool of dopaminergic vesicles. In specific, cocaine's ability to enhance dopamine release evoked by electrical stimulation was markedly attenuated in mice devoid of three

synapsin genes, encoding Synapsin I, II, and III (i.e. triple knockouts; TKO), suggesting that this process is synapsin-dependent, though the mechanism by which cocaine interacts with synapsins remains elusive. Cocaine-induced enhancement in dopamine release was particularly evident when the contribution of releasable pool was compromised by inhibiting its synthesis, further indicating that cocaine acts on a pool of dopamine that is not readily releasable and this process is independent of the rate of dopamine synthesis (Venton et al., 2006).

Alternatively, previous studies have also implicated the cholinergic system in cocaine-enhanced dopamine transients, given previous observations of cocaine-evoked increases in the firing rate of CINs (Witten et al., 2010) and that antagonism at acetylcholine receptors and optogenetic inhibition of CINs consistently reduced dopamine levels and blocked cocaine conditioning in a CPP paradigm respectively (Witten et al., 2010; Yorgason, Zeppenfeld, & Williams, 2017; also refer to Williams & Adinoff, 2008 for a thorough review), collectively implicating the significance of CIN activity in influencing cocaine-induced effects. Nevertheless, increasing CIN activity did not reliably increase dopamine transients and there exists evidence for the absence of cocaine-induced effects on CIN activity (Yorgason et al., 2017).

The memory of intense pleasure associated with cocaine use is encoded in the "memory centres" of the brain (i.e. the amygdala and the hippocampus) (Volkow, Fowler, & Wang, 2003). Additionally, the activity of frontal cortex serves as 'cognitive control', regulating the activity of the

limbic regions implicated in drug abuse (Goldstein & Volkow, 2011) and serving to suppress drug-taking urges in non-addicted individuals (Nestler, 2005; Volkow et al., 2003). Dysfunction of the prefrontal cortex is commonly observed in addicted individuals, providing a biological basis for the low tendency of addicted individuals to prevail over drug-taking urges, leading to uncontrollable drug use (Goldstein & Volkow, 2011). Though some of the cocaine's addictive properties are attributable to its ability to elevate extracellular dopamine concentration within the NAc (Everitt & Robbins, 2013; Kalivas & Stewart, 1991; Kiyatkin, 1994), it should be noted that cocaine also has other pharmacological actions in the central and peripheral nervous system. To name a few, it acts as a local anaesthetic and blocks voltage-gated sodium, calcium, and calcium-activated potassium channels (Crumb & Clarkson, 1990; Premkumar, 2005; Ruetsch, Böni, & Borgeat, 2001).

Repeated exposure to cocaine yields a host of neuroadaptations, particularly within the NAc. Alterations in gene expression are particularly intriguing as they tend to be long-lasting and are likely to contribute to the transition to addiction (Nestler, 2005). One of the well-studied examples is the long-lasting effects of cocaine on ΔFosB expression. Cocaine increases the production of ΔFosB and once created, it lasts for 6-8 weeks. Cocaine abuse would therefore trigger the accumulation of ΔFosB, which has been linked to increased drug sensitivity, self-administration and a greater motivational drive for cocaine in rodents (McClung et al., 2004; Nestler, 2001). Conversely, blocking the activity of ΔFosB produced the opposite

effects, i.e. blunted responses to the drug effects (Nestler, 2001). Longer persistence of cocaine use has been reported long-term alterations to the physical structure of neurons particularly within the NAc and thus, connectivity between neurons (Nestler, 2001; Shen et al., 2009). The drug-induced morphological plasticity, partly triggered by changes in gene expression, may have lifelong persistence and have been linked to the expression of addiction related behaviours (Li, Acerbo, & Robinson, 2004; Russo et al., 2010).

### 1.5.2. Cocaine reinforcement

Dopamine release is stimulated by encountering novel, biologically salient stimuli, such as rewards. However, such responses dissipate once a reward becomes fully predicted. Instead, dopamine neurons respond to the presentation of the reward-predicting stimuli, thereby allowing them to gain motivational significance (i.e. Reward Prediction Error (RPE) theory, (Schultz, Dayan, & Montague, 1997). Nonetheless, note that cocaine-induced increases in dopamine transients remain unabated with every encounter, due to its action at DATs (Nestler, 2005). It is, thus, not surprising that cocaine, in particular, has been demonstrated to generate stronger conditioned reinforcers than food reward (Tunstall & Kearns, 2016).

Intriguingly, growing evidence shows that cocaine is a weaker reinforcer compared to food when animals were given a choice (Cantin et al., 2010; Lenoir, Serre, Cantin, & Ahmed, 2007; Tunstall & Kearns, 2016). Similarly, behavioural economic studies also revealed that when cocaine

users were presented with a choice between cocaine and a small non-drug reward (small token or food), they consistently chose the latter (Foltin & Fischman, 1994; Higgins, Roll, & Bickel, 1996). A more recent study further demonstrated that cocaine addicted individuals assigned the highest subjective valence to drug rewards when asked to recall the 'under drug influence' or cue-related situations (otherwise, food > drug) (Goldstein et al., 2010). These findings collectively suggest that the primary reinforcing strength of cocaine alone is not sufficient to explain its ability to cause addiction. Corroborating this argument is the incentive salience theory of addiction proposed by Robinson & Berridge (1993), which posits that it is the motivating power of the drug-associated stimuli, impelled by the physiological states and learned values, that elicits an intense sense of "wanting", without necessarily evoking a sense of liking for that drug. This is thought to contribute to the manifestation of cue-driven drug-seeking behaviours and the associated intrusive thoughts (i.e. craving), rendering individuals susceptible to developing compulsive drug-taking behaviours and relapse in abstaining addicts. To date, there is mounting evidence from pre-clinical (Grimm, Hope, Wise, & Shaham, 2001; Lee, Milton, & Everitt, 2006) and clinical (Childress et al., 1999; Garavan et al., 2000) studies supporting the notion that cocaine-associated stimuli are potent triggers of craving and relapse. Imaging studies further revealed that these are paralleled by activation in the limbic regions (i.e. the amygdala and anterior cingulate) (Childress et al., 1999), dorsolateral prefrontal cortex (Maas et

al., 1998), as well as increased dopaminergic activity in the dorsal striatum (Volkow et al., 2006).

Finally, cocaine also has the ability to energise conditioned behaviours, potentially by enhancing the incentive value of the reward-conditioned stimulus (Chu & Kelley, 1992; Dixon et al., 2010; Robbins, 1975). There exists compelling evidence that this cocaine effect is dependent upon its ability to increase mesoaccumbal dopamine transients (Parkinson et al., 1999; Taylor & Robbins, 1986). Such a facilitatory effect of cocaine will be discussed further in this thesis.

### 1.5.3. Cocaine-induced regulation of $GABA_AR$ expression: Evidence for Dopamine x GABA interaction

Cocaine indirectly regulates GABAergic activity through alternate neurotransmitter systems, i.e. dopaminergic, serotonergic, and noradrenergic systems (e.g. see Bocklisch et al., 2013; Li & Kirby, 2016). Previous research has reported changes in $GABA_AR$ expression in response to cocaine treatments (Chen et al., 2007; Purgianto, Loweth, Miao, Milovanovic, & Wolf, 2016). In turn, altering GABAergic activity via manipulations (i.e. whole-brain deletion) of the $GABA_AR$ subunit expression also altered behavioural responses to cocaine (Dixon et al., 2010; Macpherson et al., 2016; Maguire et al., 2014) and other drugs of abuse (Dixon, Walker, King, & Stephens, 2012; Duka et al., 2015).

The GABAergic MSNs constitute approximately 95% of the neurons in the NAc, the prime locus of cocaine action, with a large proportion of the

remainder consisting primarily of GABAergic interneurons (Kawaguchi, 1993; Tepper & Bolam, 2004). In the NAc, specifically, the $GABA_AR$ α4, α2, and α1 subunits are expressed on the MSNs and/or interneurons (Boyes & Bolam, 2007; Dixon et al., 2010; Maguire et al., 2014; Schwarzer et al., 2001; Mitchell et al., 2018). Changes in the expression of $GABA_AR$ α2 subunit (Chen et al., 2007; Purgianto et al., 2016) have been reported following cocaine exposure and during withdrawal. It is, thus, important to consider how other transmitter systems interact with GABA to shape MSN activity, which ultimately mediates the expression of reward-related behaviours (Dixon, Halbout, King, & Stephens, 2014).

Evidence for dopamine × GABA interaction was first derived from receptor binding studies investigating the effect cocaine on BZ pharmacology and receptor expression, and vice versa. BZ acts primarily at $GABA_ARs$ (Sigel & Buhr, 1997), though other BZ binding sites have been identified, which are beyond the scope of this thesis (Le Fur et al., 1983). Using [$^3$H]Ro 15-1788 to label BZ receptors, early work demonstrated that chronic cocaine treatments induced a significant increase in BZ receptor numbers within the caudate nucleus and the cerebellum, but was decreased in the frontal cortex following chronic cocaine (Goeders, 1990;1991). In keeping, chronic cocaine also enhanced BZ, i.e. [$^3$H]flunitrazepam, receptor binding in the hippocampal and striatal regions of the rat brain, which persisted for at least 21 days following the cessation of cocaine treatment (Lipton, Olsen, & Ellison, 1995). In chronically cocaine-treated animals, the binding of [$^{35}$S]t-butylbicyclo phosphorothionate ([$^{35}$S]TBPS), a high affinity

ligand for the picrotoxin site of the $GABA_ARs$, was also significantly increased in the frontal cortex and hippocampus during withdrawal, accompanied by an increase in cortical and hippocampal α1 and ß3 mRNA expression (Suzuki et al., 2000). The authors further postulated that the increase in GABAergic activity particularly in the cortex might contribute to the hypofunction of dopamine system in the cortical area observed during cocaine withdrawal (Suzuki et al., 2000). Importantly, cocaine-induced enhancement of BZ binding was markedly attenuated upon dopamine depletion using neurotoxin 6-hydroxydopamine (6-OHDA), suggesting that cocaine effect on BZ receptor expression and binding is largely mediated by dopamine (Goeders 1990;1991).

### 1.5.4. Alpha4-containing $GABA_ARs$ and cocaine-induced responses

In the striatum of an adult mouse brain, tonic inhibitory current is primarily mediated by the α4ßδ-harbouring $GABA_ARs$ (Heiman et al., 2008). This is further supported by mounting immunohistochemical and electrophysiological evidence for the high expression level of these receptors in the accumbal MSNs and interneurons (Hörtnagl et al., 2013; Maguire et al., 2014; Pirker, Schwarzer, Wieselthaler, et al., 2000).

Previous work in our laboratory has demonstrated that mice devoid of the Gabra4 gene did not differ from the wild-type counterparts in the expression of cocaine-induced conditioned place preference (CPP) or cocaine-facilitated conditioned reinforcement (CRf). However, α4 knockout specific

to the D1R-expressing neurons facilitated CPP, whereas α4 deletion specifically in the D2R-expressing neurons abolished cocaine facilitation of CPP (Maguire et al., 2014). Further, enhanced activation of α4-$GABA_A$Rs with THIP blocked cocaine enhancement of CPP (Maguire et al., 2014) and CRf (Macpherson et al., 2016). Given that these cocaine-induced behavioural phenotypes are dependent upon cocaine-induced increases in mesoaccumbal dopamine (Cervo & Samanin, 1995; Hnasko, Sotak, & Palmiter, 2007; Taylor & Robbins, 1986), it is likely that the cell type-specific deletion of α4 modulated the action of cocaine by disrupting the functional interactions between the dopaminergic and GABAergic subsystems.

Intriguingly, past research has also yielded evidence for dopamine × GABA interaction at the intracellular level. Namely, agonism at, or acute activation of dopamine D1 receptors augmented GABAergic tonic conductance, thus, dampening the excitatory effect of dopamine acting at D1Rs, whereas the blockade of G-protein coupling via the intracellular GDP-ßs prevented the enhanced tonic current (Maguire et al., 2014). By contrast, only prolonged, but not acute, activation of D2Rs modestly reduced tonic current, thus collectively providing a short-term homeostatic balance in neuronal activity within the striatum by preventing overexcitation of the neurons (Maguire et al., 2014).

### 1.5.5. Summary

Studies to date have provided convincing evidence that whilst the primary reinforcing properties of cocaine may play an important role in the initial episodes of drug use, these properties cannot fully account for the transition from repeated use to addiction. Instead, it is widely believed that the ability of addictive drugs to heighten Pavlovian and/or instrumental responsiveness to drug-associated cues may lead to aberrant drug use, despite the adverse consequences (Belin, Jonkman, Dickinson, Robbins, & Everitt, 2009; Everitt & Robbins, 2005). Others have also reported cocaine-induced potentiation of instrumental responding for cues associated with other reinforcers (e.g. food) (Dixon et al., 2010; Chu & Kelley, 1992).

Biologically, this aberrant motivational process is thought to be mediated by the drug's ability to increase mesoaccumbal dopamine concentration (Everitt & Robbins, 2005; Taylor & Robbins, 1986). There, however, exists evidence that manipulations of GABAergic activity via the α2- or α4-containing $GABA_A$Rs alter cocaine's ability to potentiate conditioned behaviours (Dixon et al., 2010; Maguire et al., 2014; Macpherson et al., 2016), perhaps by modulating dopamine × glutamate interactions, particularly in the NAc, that are deemed important in the manifestation of cocaine-facilitated motivated actions (Burns, Everitt, Kelley, & Robbins, 1994).

## 1.6. The role of α2βγ $GABA_A$ receptors in cocaine addiction: What do we know so far?

A strong link between $GABA_AR$ α2 subunit-encoding gene, *GABRA2* (or Gabra2 in the mouse), polymorphism and alcohol use disorder has been well-documented in the literature through the use of various study designs, including genome-wide association studies (Bierut et al., 2010), genetic linkage studies (Edenberg et al., 2004; Enoch, Schwartz, Albaugh, Virkkunen, & Goldman, 2006; Soyka et al., 2008), and haploytpe analyses (Covault, Gelernter, Hesselbrock, Nellissery, & Kranzler, 2004). More recently, emerging evidence has further implicated *GABRA2* polymorphic variations in cocaine addiction vulnerability in some populations (Dixon et al., 2010; Enoch et al., 2010). To date, the molecular mechanism by which *GABRA2* variation influences the risk for cocaine addiction is unknown.

Among the SNPs characterised in the Dixon et al. (2010) study, one risk SNP linked to cocaine addiction, rs279871, is in 100% linkage disequilibrium with rs279858 (linked to alcohol dependence) (Stephens et al., 2017). In a more recent study by Lieberman et al. (2015), lower α2 mRNA expression was detected in neural cell cultures derived from alcohol dependence-associated risk (rs279858*C) allele carrier. The rs279858-containing *GABRA2* haplotype block harbours a functional polymorphism that also has a regulatory effect on the expression of all $GABA_AR$ subunit genes on chr4p12 (i.e. *GABRB1, GABRA2, GABRA4, GABRG1*) (Lieberman et al., 2015). It is, thus, tempting to hypothesise that the level of α2

expression in cocaine addiction-associated risk (rs279871*G) allele carriers may also be lower than those carrying the protective haplotype (rs894269*T, rs2119767*T, rs929128*G) (Dixon et al., 2010; Duka et al., 2015).

Another *GABRA2* SNP, rs11503014, has also been linked to cocaine addiction vulnerability in individuals with a history of childhood trauma (Enoch et al., 2010). Given the SNP position, within a DNA sequence that is similar to exon splicing enhancers: srp55, srp40, sf2, and sc35, it is conceivable that polymorphic variations may affect α2 expression via alternative splicing (Enoch et al., 2010). Indeed, analyses of *GABRA2* mRNA from the human brain revealed at least four different isoforms of the α2 subunit (Tian, Chen, Cross, & Edenberg, 2005).

Interestingly, whole-brain deletion of Gabra2 in the mouse failed to disrupt cocaine self-administration or reinstatement of cocaine seeking when compared to the wild-type (WT) counterparts, suggesting that α2-$GABA_AR$-mediated activity does not play a key role in mediating cocaine reward (Dixon et al., 2014). Instead, the α2 knockout mice appeared to be insensitive to cocaine, as well as methylphenidate, enhancement of conditioned behaviours, i.e. instrumental responding for CRf and locomotor sensitisation (Dixon et al., 2010; Duka et al., 2015). Intriguingly, whilst deletion of Gabra2 blocked cocaine-induced locomotor sensitisation (Dixon et al., 2010), selective activation of mutant α2(H101R)-$GABA_ARs$ (refer to Section 1.4.1) with Ro15-4513 induced sensitising effects (Morris et al., 2008).

Mesoaccumbal dopamine is implicated in these forms of conditioned behaviours (Chu & Kelley, 1992; Heidbreder, Thompson, & Shippenberg, 1996; McCreary & Marsden, 1993; Taylor & Robbins, 1986; Wolterink et al., 1993)). Supporting this notion, α2(H101R) mice sensitised to Ro 15-4513 displayed an enhanced stimulant response to cocaine (10 mg/kg), but Ro15-4513 failed to enhance conditioned activity in cocaine-sensitised α2(H101R) mutants. Thus, cocaine's ability to enhance conditioned activity (i.e. cross-sensitisation) in α2(H101R) mutants is most likely to depend upon its ability to increase accumbal dopamine level (Morris et al., 2008). Moreover, the Morris et al. (2008) study further demonstrated the importance of α2-containing receptors in mediating BZ (midazolam) potentiation of cocaine-induced hyperactivity.

Histological examinations, profiling the α2 expression patterns throughout the brain, reveal that this subunit is abundant in regions implicated in reinforcement learning and motivational processes, including the hippocampus, cortex, amygdala and striatal subcompartments (Burns et al., 1993; Cardinal et al., 2002; Dixon et al., 2010; Hörtnagl et al., 2013; Pirker, Schwarzer, Wieselthaler, et al., 2000; Tracy et al., 2001). A thorough understanding of GABAergic signalling via the α2-containing receptors may thus provide valuable insights into the development of aberrant behaviours linked to addiction.

## 1.7. Behavioural strategies for investigating cocaine-induced reinforcement

As delineated above, cocaine exerts reinforcing effects, some of which rely on its action on mesolimbic dopamine neurons. Some of these cocaine-induced effects can be modelled using the locomotor sensitisation and conditioned reinforcement paradigms, which are the main focus of this thesis (Vanderschuren & Kalivas, 2000; Parkinson, Olmstead, Burns, Robbins, & Everitt, 1999). Intriguingly, previous data from our laboratory showed that α2 knockout mice failed to display cocaine-induced locomotor sensitisation and cocaine facilitation of CRf, leading to a further speculation that these cocaine-induced responses may share a similar mechanism involving the α2-$GABA_AR$-mediated system, particularly in the NAc.

### 1.7.1. Locomotor sensitisation to cocaine

Repeated exposure to cocaine elicits a progressive increase in a subset of responses to the drug, a phenomenon termed *sensitisation*. Though note that while some effects of the drug sensitise, tolerance develops to other effects of the drugs, and both sensitisation and tolerance often co-exist (Stewart & Badiani, 1993). Drug-induced sensitisation is commonly assayed by monitoring increases in motor activity following non-contingent, intermittent drug administration and this form of sensitisation has been shown to persist for at least a year (Kalivas & Stewart, 1991; Paulson, Camp, & Robinson, 1991). However, sensitisation has also been reported to occur in a conditioned place preference (CPP) paradigm, whereby increases

in the amount of time spent in a cocaine-paired chamber were observed (Lett, 1989). Thus, in addition to the motor stimulant effects, the positive unconditioned properties of the drug appear to gradually strengthen over time.

It has been argued that many aspects of behavioural sensitisation seem to reflect Pavlovian associations between the unconditioned stimulant properties of the drug and the context where drug effect is experienced, such that the context itself can enhance activity in the absence of the drug (Le Merrer & Stephens, 2006; Pert, Post, & Weiss, 1990). Corroborating this account is the evidence that a robust sensitisation induced by psychostimulants is highly context-specific and can be blocked by a context switch (Mattson et al., 2008; Vezina, Giovino, Wise, & Stewart, 1989; Wang & Hsiao, 2003). There also exists evidence that context-dependent sensitisation can be extinguished following repeated pairings of saline and the test environment (Hinson & Poulos, 1981) and that locomotor sensitisation to a non-motor-stimulant reward, i.e. food, has been observed (Le Merrer & Stephens, 2006). However, given that under some conditions, drug-induced sensitisation can still occur in a context-independent manner (Partridge & Schenk, 1999), it has been argued that drug-context learning may aid, but is not necessary or sufficient for, the development of psychostimulant-induced sensitisation.

The development of drug-induced sensitisation can be examined in two temporal domains, i.e. initiation and expression, which are anatomically distinct. *Initiation* denotes acute neural events involved in the induction of

sensitisation, whereas *expression* is defined by the long-term alterations of these initial neural events. Only changes that persist for at least two weeks after withdrawal from a drug can be deemed significant in the expression of sensitisation (Kalivas & Stewart, 1991). The initiation and expression of sensitisation are linked to activities within the VTA and NAc respectively, as early work demonstrated that repeated microinjections of amphetamine into the NAc merely induced hyperactivity, but no sensitising effects, whereas injections of amphetamine in the VTA produced the latter (Kalivas & Weber, 1988). More recent evidence further demonstrated that both initiation and expression of cocaine-induced locomotor sensitisation involved accumbal activity (reviewed in Vanderschuren & Kalivas, 2000). Nonetheless, it should be noted that even during the so-called initiation phase, neural plasticity is already taking place and expressing itself in the form of increases in locomotor activity. Thus, for clarity, this thesis addressed the *initiation* phase as the 'early stage' or the 'development' of sensitisation.

Much of the early work on neuroadaptations underlying sensitisation primarily focused on the mesocorticolimbic dopamine transmission (Kalivas & Duffy, 1993; Parsons & Justice, 1993; Burger & Martin-Iverson, 1994; Sorg, Davidson, Kalivas, & Prasad, 1997; Williams & Steketee, 2005). More recent evidence however further suggests the importance of excitatory afferents to the VTA and the NAc. Electrical stimulation of excitatory projections to the VTA was found to induce sensitisation (Schenk & Snow, 1994) and drug-induced sensitisation was blocked by glutamate receptor

antagonist micro-injected into the VTA (Kalivas & Alesdatter, 1993). Similarly, inhibiting or lesioning excitatory transmission in the NAc blocked the long-term expression of sensitisation (Karler, Calder, & Brent Bedingfield, 1994; Pierce, Reeder, Hicks, Morgan, & Kalivas, 1998; Vanderschuren & Kalivas, 2000). Other studies have also implicated the hippocampus, laterodorsal tegmentum, amygdala, and the paraventricular nucleus of the thalamus (PVN) in the development of sensitisation (Degoulet, Rouillon, Rostain, David, & Abraini, 2008; Yong Li et al., 1999; Nelson, Wetter, Milovanovic, & Wolf, 2007; Wolf, Dahlin, Hu, Xue, & White, 1995), most likely by influencing the dopamine system (discussed in Steketee & Kalivas, 2011).

### 1.7.2. Cocaine-facilitated conditioned reinforcement

In addition to enhancing locomotor activity, cocaine and other psychostimulant agents also have the capacity to enhance the expression of a learned instrumental response maintained by conditioned reinforcement (CRf) (Beninger, 1983; Cador, Taylor, & Robbins, 1991; Chu & Kelley, 1992; Dixon et al., 2010; Robbins, 1978). CRf is a phenomenon whereby a discrete arbitrary stimulus (e.g. tone or light stimulus) acquires the capacity to reinforce instrumental actions in its own right (i.e. conditioned reinforcer, CR) upon Pavlovian association with a primary natural reinforcer (unconditioned reinforcer, UR) (Kelleher & Gollub, 1962). The key neural circuitry subserving CRf primarily involves BLA-NAc interactions as lesion to this region impaired the ability of the CR to support the acquisition of a

new instrumental response (Burns, Robbins, & Everitt, 1993; Cador, Robbins, & Everitt, 1989).

Whilst Pavlovian stimulus-reward association provides knowledge of reward predictability, it is not motivation in itself (Berridge, 2012; Fernando, Urcelay, Mar, Dickinson, & Robbins, 2013). Despite the remembered knowledge of stimulus-reward association, the motivating power of a CR is generated anew with every encounter and thus, can vary in its ability to evoke desire. For example, the same food-associated stimulus that elevates the motivation to seek food rewards, or drug-related stimuli that could trigger relapse in recovering addicts, might have been successfully resisted on previous encounters. There are therefore contributing factors that transform this knowledge to motivation and ultimately, the expression of goal-directed actions (Berridge, 2012). Of these, fluctuations in neurobiological states, including changes in mesocorticolimbic dopamine, are considered to be one of the critical modulators of motivated behaviours (extensively reviewed in Salamone & Correa, 2012). It is thus not surprising that drugs, acting to modulate the dopaminergic system, can alter the expression of CR-governed motivated behaviours (discussed below).

The notion that psychostimulant drugs have the capacity to increase the efficacy of rewarding stimuli was first proposed by Stein (as cited in Robbins, 1975) and as an extension of this hypothesis, Hill (as cited in Robbins, 1975) further observed selective enhancement of responding for the CR by pipradrol (10 mg/kg), while, in the absence of the CR, pipradrol

reduced responses, indicating a true interaction between drug effect and CRf. To date, there is compelling evidence that systemic or intra-accumbal administration of psychostimulant drugs, including pipradrol, amphetamine, methylphenidate, potentiates responding CRf, though evidence pertaining to cocaine effect was less consistent in the rat literature (Beninger, Hanson, & Phillips, 1981; Cador et al., 1991; Chu & Kelley, 1992; Robbins, 1975, 1978; Robbins, Watson, Gaskin, & Ennis, 1983). Studies with mice however have consistently observed cocaine's (10 mg/kg) potentiating effects on instrumental responding for CRf (Dixon et al., 2010; Macpherson et al., 2016).

Biologically, a host of pharmacological studies to date, primarily with rats, provide empirical support for the notion that the mesoaccumbal dopamine action particularly at D1R and D2R subtypes is critical for psychostimulant potentiation of CRf (Cador et al., 1991; Chu & Kelley, 1992; Ranaldi & Beninger, 1995; Taylor & Robbins, 1984; Wolterink et al., 1993). For instance, microinfusion of D1R or D2R agonists (SKF 38393 or quinpirole respectively) in the NAc has been shown to facilitate responding for CRf, but blockade of either receptor subtype only impaired *d*-amphetamine's CRf-potentiating effect without retarding the reinforcing efficacy of the CR (Wolterink et al., 1993). Nevertheless, there also exists evidence that co-activation of D1Rs and D2Rs is required for psychostimulant enhancement of CRf as agonism at D1Rs (CY 208-243) or D2Rs (quinpirole) alone failed to potentiate responding for CRf (Chu & Kelley, 1992). In fact, under some conditions, D1R agonist (including SKF

38393) impaired preferential responding for CRf (Beninger & Rolfe, 1995; Beninger & Ranaldi, 1992; Collins & France, 2015; Collins & Woods, 2009; Ranaldi & Beninger, 1995). Methodological variations, including the routes of the drug administration (i.e. systemic vs. localised treatments), are thought to underlie the discrepancy in the literature (Beninger & Rolfe, 1995). The significance of the dopaminergic system was further emphasised by findings that intra-accumbal dopamine, but not noradrenaline infusion produced increases in responding on the CR-associated lever (Cador et al., 1991). Likewise, 6-hydroxydopamine-induced dopamine, but not noradrenaline, depletion in the ventral, but not dorsal striatum abolished the CRf-enhancing properties of intra-NAc *d*-amphetamine micro-infusion (Taylor & Robbins, 1986). Taken together, there is compelling evidence to date that cocaine-induced increases in mesoaccumbal dopamine is involved in its ability to facilitate the expression of conditioned behaviours, i.e. conditioned reinforcement and behavioural sensitisation.

## 1.8. RNA interference: A method to study the lack-of-function phenotype

Over the past decades, the RNA interference (RNAi) technology has revolutionised the way we study genome. It reveals an array of conserved pathways in which small (20-30 nucleotide) double-stranded, non-coding RNA (dsRNA) molecules associate with the enzymatic machinery of RNAi to orchestrate post-transcriptional control over gene expression through sequence-specific targeting of the target messenger RNAs (mRNAs)

(Elbashir, 2001; Fire et al., 1998). To date, RNAi-induced gene silencing remains to be a powerful analytical modality to study gene function in various organisms ranging from unicellular parasites to mammalian cells (Hannon, 2002).

### 1.8.1. A brief history of RNAi discovery

Prior to the discovery of RNAi by Andrew Fire and Craig Mello (Fire et al., 1998), pioneering observations of post-transcriptional gene silencing were initially reported in plants, but several years later, this phenomenon was also reported in most, if not all, eukaryotes. The first known report of this phenomenon was detailed in the Napoli, Lemieux, and Jorgensen (1990) study, whereby the overexpression of chalcone synthase (CHS) gene with the aim to generate violet petunia flowers instead resulted in white petunia flowers. Transcript analyses further revealed that the transgenic flowers had 50-fold lower CHS mRNAs than the wildtype counterparts. The researchers argued that the introduction of exogenous transgene suppressed the endogenous CHS level, which was termed "co-suppression". Years later, a number of studies reported a similar phenomenon occurring in the nematode worm, *Caenorhabditis elegans.* For example, endogenous small regulatory RNA, also known as microRNA (miRNA), *lin-4* was found to negatively regulate the expression of LIN-14 protein via antisense RNA-RNA interactions with the 3' untranslated region (UTR) of the *lin-14* transcript (Lee, Feinbaum, & Ambros, 1993). However, a more recent attempt to silence *par-1* gene in *C. elegans* by introducing antisense RNA in the experimental condition, with the sense RNA as a control condition,

yielded an unexpected result. That is, the introduction of sense RNA, which could not hybridise with the endogenous target mRNA, induced a silencing effect (Guo & Kemphues, 1995).

In 1998, the work by Fire and colleagues provided an explanation for the Guo and Kemphues's (1995) findings. They postulated that the gene silencing trigger was in the form of double-stranded RNA (dsRNA), instead of single-stranded RNA (ssRNA). Injection of antisense or sense RNA targeting the *unc-22* gene in *C. elegans* produced minimal interference activity, requiring a high dose of RNA molecules to induce observable effects. In contrast, injection with a mixture of sense and antisense RNA produced potent *unc-22* silencing activity and further electrophoretic analyses revealed that the injected material was mainly double stranded. It is thus less likely that the highly efficient silencing activity by sense and antisense RNA was triggered by alternative strand interactions.

Since the discovery of RNAi, several important findings have emerged, including: (1) the RNAi is a well conserved mechanism (Bellés, 2010; Davis et al., 2010; also see studies above), (2) miRNA-induced silencing mechanism appears to be widespread. Within the human genome, for example, approximately ~2000 miRNA molecules have been identified, each serving to orchestrate the expression of proteins involved in various vital processes, including cell growth, proliferation, differentiation, as well as those involved in disease pathologies (Calin et al., 2004; Chan, Krichevsky, & Kosik, 2005; Choi et al., 2011; also reviewed in Ha, 2011). Contrary to miRNA, short interfering RNAs (siRNAs) are synthetic effectors

of the RNAi pathway, though some may arise due to viral infections and are fully complementary to their targets. The effective siRNA duplexes are ~21-23 basepairs (bp) in length, containing the mRNA sequence of the target gene (sense strand) and its complement (active antisense strand) have been extensively used in biomedical research to allow selective suppression of the target gene expression (Elbashir et al., 2001). Throughout this thesis, the term RNAi will be predominantly used to describe siRNA-based gene silencing.

### 1.8.2. RNAi in eukaryotes

In many eukaryotes, RNAi serves primarily as a defence mechanism against mobile genetic components, e.g. viruses and transposable elements, though the role of RNAi as a natural antiviral response in mammalian cells, remains unclear (Cullen, 2006; Yang Li, Lu, Han, Fan, & Ding, 2013; Schütz & Sarnow, 2006; Zambon, Vakharia, & Wu, 2006). The key microprocessor proteins involved in eukaryotic RNAi are Dicer, an endoribonuclease with a helicase domain and a dimer of RNAse III domains, a dsRNA-binding protein (dsRBP) such as TAR RNA-binding protein (TRBP), and an Argonaute family protein, which is involved in the recognition of the guide (or antisense) siRNA strand, target mRNA cleavage, and recruitment of other proteins involved in RNAi. It is the existence of multiple paralogues of this machinery that creates the diversity of RNAi systems among all major eukaryotic lineages (extensively reviewed in Hutvagner & Simard, 2008; Wilson & Doudna, 2013).

In principle, RNAi-induced gene silencing involves four major steps, (1) mature small-interfering RNA (siRNA) biogenesis; (2) recruitment of siRNA antisense or guide strand to the protein complex to form the RNA-induced silencing complex (RISC); (3) target mRNA recognition and cleavage; and (4) target mRNA degradation and ultimately, the inhibition of protein synthesis. The siRNA duplexes can either be generated from cleavage of long dsRNA precursor (viral RNA) by the RNase III domain of Dicer, or exogenously synthesised and introduced into the cells in its mature siRNA form (Brummelkamp, Bernards, & Agami, 2002; Ding & Lu, 2011; Sui et al., 2002; Xia, Mao, Paulson, & Davidson, 2002).

One of the important advances in the RNAi field was the discovery of short-hairpin RNA, as a substitute for the use of synthetic siRNAs. The main advantage of shRNA over siRNA is that it permits more stable silencing of gene expression. It is synthesised in the nucleus upon delivery via various methods of transfection or viral-mediated delivery, transported and processed into the cytoplasm via the miRNA machinery to yield mature siRNA molecules prior to RISC loading (depicted in Figure 1.5). As its name suggests, the primary transcript forms a hairpin-like structure with stem and loop components. In the nucleus, it is transcribed by RNA Polymerase II or III, and further processed by a complex containing RNaseIII enzyme, Drosha, and a dsRNA-binding protein, DiGeorge syndrome critical region gene 8 (DGCR8), which collectively measure the hairpin and excise the stem-loop structure to yield mature shRNAs with a 2 nucleotide (nt) 3' overhang prior to being transported to the cytoplasm by Exportin-5. In the

cytoplasm, shRNAs are further processed by Dicer and TRBP where loop processing takes place to yield mature double-stranded siRNA. The guide strand of mature siRNA is anchored into a specific binding pocket of the Argonaute protein by Dicer and further aided by TRBP, whilst the passenger strand is rapidly degraded. Collectively, the complex containing the siRNA guide strand and the three proteins constituting the basic RNAi machinery, i.e. Argonaute, Dicer, and TRBP, form a transient RISC, allowing the guide strand to identify the target mRNA through perfect or near-perfect complementarity, followed by target mRNA cleavage by the endonucleolytically active Argonaute protein and its degradation (Wilson & Doudna, 2013; Yi, Doehle, Qin, Macara, & Cullen, 2005).

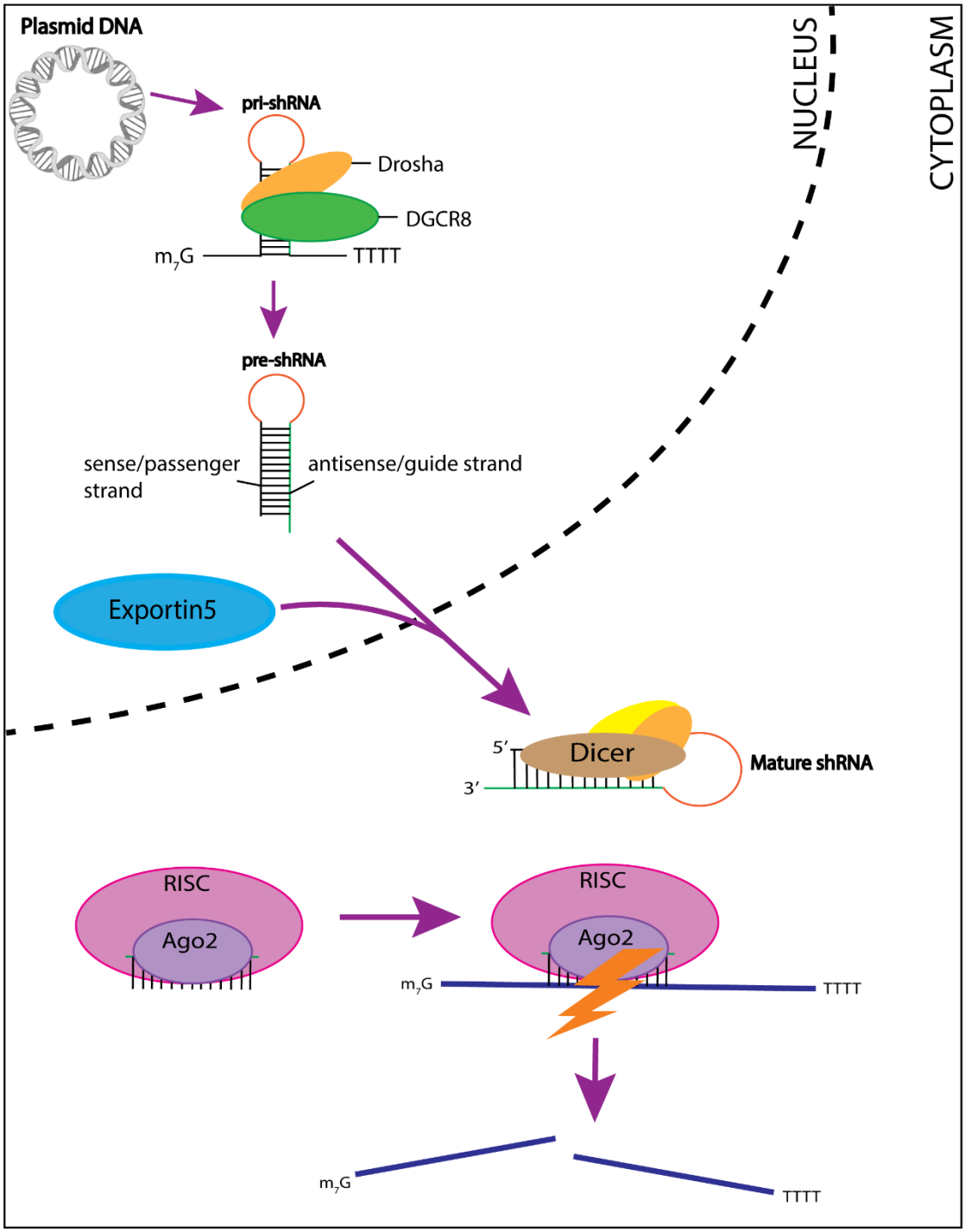



***Figure 1.5.*** A schematic representation of RNAi interference with the short-hairpin RNA (adapted from de Fougerolles, Vornlocher, Maraganore, & Lieberman, 2007; Torrecilla, Rodríguez-Gascón, Solinís, & del Pozo-Rodríguez, 2014).

### 1.8.3. siRNA vs. shRNA

Despite extensive similarities in the mechanistic actions of shRNA- and siRNA-based silencing, notable differences have been documented, pertaining to requirements for sequence composition, duration of knockdown and off-target effect profiles (Rao, Vorhies, Senzer, & Nemunaitis, 2009; refer to Table 1.1 for comparison).

Synthetic siRNA can be introduced into the cytoplasm, ready for RISC assembly for RNAi function. However, this process has been shown to be 10-fold less efficient than shRNA, which assimilates into the miRNA pathway. However, it has been shown that increasing the length of siRNA with 2nt 3' overhangs on the antisense strand improves efficacy (Kim et al., 2005; Sano et al., 2008). Further, whilst tracing experiments revealed high degradation and turnover rate of synthetic siRNA (Järve et al., 2007), shRNA can be continuously synthesised due to its nuclear localisation, thus permitting prolonged knockdown effect (Rao et al., 2009).

It was originally assumed that the siRNA and shRNA would share nearly identical properties given that siRNA duplexes are final products of the hairpin-based RNA intermediates. Therefore, sequences that were proven optimal with siRNA have often been used for shRNA construction (Taxman et al., 2006). Nonetheless, it has been demonstrated that functional shRNA products possessed distinctive key features for efficient target silencing. For instance, strong preferences for G/C at position 11 and A/U at positions 9 and 12 were observed in functional shRNA constructs but

these preferences were less pronounced or even reversed in functional siRNAs. The observed differences in nucleotide preferences were believed to be shRNA-specific features that may not be the priorities in advanced siRNA design algorithms (Li, Lin, Khvorova, Fesik, & Shen, 2007).

### 1.8.4. RNAi activity in neuronal cells

#### 1.8.4.1. si/shRNA triggers

Long dsRNA serves as a potent effector of RNAi in various organisms. However, it has been found to induce a robust immune response, i.e. the interferon (IFN) response, in mammalian cells as the first defence mechanism to limit viral replication. This subsequently leads to a global inhibition of protein synthesis and ultimately apoptotic effects, thus rendering long dsRNA inefficient as a potential activator of the RNAi pathway in mammalian systems (Clemens, 1997). More recent findings documented in the Elbashir et al. (2001) and Caplen et al. (2001) studies, however, showed that shorter RNA duplexes, i.e. 21-25 mer in length are potent and specific effectors of the RNAi machinery, bypassing the dsRNA detectors of the innate immune response.

Mounting evidence to date indicates that RNAi appears to be operative in neurons. For example, the introduction of exogenously-synthesised siRNAs directed against MAP2 and YB-1 transcripts into cultured rat primary hippocampal and cortical neurons via cationic lipid-based method caused efficient suppression of gene expression (Krichevsky & Kosik, 2002). Similarly, the success of viral-based mediated shRNA-based

RNAi has been reported in mouse and rat neuronal or non-neuronal cells within the CNS, targeting various genes in distinct regions (e.g. Heitz et al., 2014; Hommel, Sears, Georgescu, Simmons, & DiLeone, 2003; Sapru et al., 2006).

Table 1.1

| Criterion | siRNA | shRNA |
|---|---|---|
| **Synthesis** (Kim et al., 2005; Sano et al., 2008) | Exogenously synthesised | Nuclear expression |
| **Delivery method** (Foged, 2012; Vorhies & Nemunaitis, 2009; Moore et al., 2010) | Synthetic polymer-/Lipid-based delivery to the cytoplasm | Viral- (*in vivo*) or non-viral delivery (*in vitro*) methods to the nucleus |
| **Duration of knockdown** (Jarve et al., 2007; Rao et al., 2009) | Short-term (99% degraded after 48 hours) | Long-term |
| **Sequence-specific off-target effects** (Rao et al., 2009a) | Higher than shRNA | Lower than siRNA |
| **Activation of immune/inflammatory responses** (Singh et al., 2011) | Higher than shRNA | Lower than siRNA |

***Table 1.1.*** Differences between siRNA and shRNA.

### 1.8.4.2. Off-target effects

Despite the reported high potency and specificity of RNAi-induced gene knockdown, there are a number of impediments to the use of RNAi for phenotypic analysis of gene function. Previous studies have identified multiple mechanisms through which the delivery of silencing triggers can cause effects other than the intended mRNA depletion, termed *off-target effects*. Unintended transcripts with <100% base-pair interactions with the siRNA can be targeted for RNAi-mediated knockdown and this is thought to occur due to high availability of cytoplasmic siRNA (Alemán, Doench, & Sharp, 2007).

First, off-target phenotypes are commonly observed due to partial complementarity between the 5' region of siRNA and the 3' UTR hexamers of the off-target transcripts, reminiscent of micro RNA (miRNA)- mRNA interaction. Given that siRNA and miRNA share the same RNAi machinery, the off-target gene suppression could potentially result from siRNA functioning as miRNA on unintended targets (Jackson & Linsley, 2010; Siolas et al., 2005). Several proposed ways to mitigate off-target effects include the introduction of a short overhang on the guide strand, the introduction of single/double base mismatches, as well as chemical modification of the passenger strand or the guide strand to prevent its participation in silencing or to reduce the entry of siRNA constructs into the endogenous miRNA-based silencing pathway respectively (Bramsen et al., 2010; Jacksonet al., 2006; 2006a).

Under certain conditions, the delivery of synthetic siRNA or shRNA, despite its short length, has been reported to trigger perturbations within the cellular environment, independent of the interaction of RNAi construct and a transcript – activating a host of immune mediated responses and subsequently, a cascade of mRNA decay pathways, ultimately resulting in aptoptosis (Bridge, Pebernard, Ducraux, Nicoulaz, & Iggo, 2003). Alternately, cellular toxicities have also been specifically associated with nucleotide composition of the siRNA. Sequence analyses documented in the Fedorov et al. (2006) study revealed that a significant proportion of toxic siRNAs contain motifs enriched with AU-rich sequences. AU-rich-elements that exist in the 3' untranslated region (UTR) of transcripts have been implicated in transcript stability, and toxic siRNAs reported in this study were found to contain AU-rich pentamers, potentially targeting and affecting the stability of off-target transcripts.

The current view posits that shRNA produces less downregulation of off-target transcripts than the corresponding siRNA, dosed to achieve similar levels of suppressive effect. Such difference has been postulated to occur due to mechanistic variations by which siRNA and shRNA enter the RNAi pathways (Rao, Senzer, Cleary, & Nemunaitis, 2009). This further indicates that off-target effects seen with shRNA may not be observed with siRNA with identical targeting sequences and vice versa. For instance, liver-directed viral-based RNAi induced dose-dependent, albeit not sequence-specific, lethality in the mouse. It was later found to be due to the overexpression of shRNA oversaturating the endogenous miRNA system,

particularly the Exportin-5-mediated export to the cytoplasm and the downstream Argonaute-2 activity, resulting in toxic effects (Grimm et al., 2006). The shRNA-mediated off-target dysregulation of endogenous miRNA pathway has also been detected in neuronal cells. Namely, both the active shRNA construct and the scrambled control yielded neuronal migration defect by disrupting endogenous *let7* miRNA levels (Baek et al., 2014) in the mouse brain and degenerative changes in cell morphology due to shRNA-induced saturation of miRNA pathway have also been reported in the rat brain (van Gestel et al., 2014), masking true effects of specific gene expression knockdown. The discrepancies between shRNA- and siRNA-induced off-target effects should therefore be taken into account when designing and optimising RNAi assays.

Finally, specific delivery vehicles of exogenous RNAi constructs may also contribute to the manifestation of these perturbations. For instance, adenoviral (AdV) vectors have been found to evoke sustained and strong expression of chemokines and cytokines in transduced human cells, i.e. HeLa cells (Zaiss et al., 2002). Marked inflammatory responses have also been detected in mouse neurons following injection of replication-competent or incompetent AdVs (Byrnes, Wood, & Charlton, 1996; Ohmoto et al., 1999), thus indicating that the pharmacodynamics of small RNA delivery system are not only dependent on the structure and chemistry of the constructs but also upon the biomaterials used for delivery (Kanasty, Whitehead, Vegas, & Anderson, 2012). Adequately addressing these concerns is essential for minimising the introduction of confounding

artefacts and therefore maximising the potential of RNAi-based applications.

### 1.8.4.3. Targeted delivery

A suitable delivery method of dsRNA intermediates into mouse neurons largely depends on the experimental setup. In cultured neurons, siRNA can be directly introduced into single neurons via lipid-based approaches, such as lipofection (Kao, Krichevsky, Kosik, & Tsai, 2004), cationic lipid-based transfection (Krichevsky & Kosik, 2002), and lipid nanoparticle delivery vehicles (Rungta et al., 2013), whereas plasmid-based shRNA can be readily introduced into the nucleus of post-mitotic neurons by nucleofection or using viral vectors (for example, see Katsu-Jiménez et al., 2016; Zeitelhofer et al., 2007).

For *in vivo* applications, the use of viral-based delivery vehicles via intracranial injection remains a widely popular and the most proficient method for delivery of RNAi effectors. Virus-mediated delivery is usually based on delivery of shRNA, though note that a few non-viral-based approaches, e.g. systemic injection of targeted exosomes or recombinant protein fusion have recently been developed to facilitate siRNA delivery to the mouse brain (Alvarez-Erviti et al., 2011; Haroon et al., 2016). In terms of viral-mediated delivery, the type of viral vector used is primarily governed by specific research requirements, i.e. the duration of knockdown, cell type, and the safety profile of the viral vector. For instance, adeno-associated viruses (AAVs) and Lentiviruses (LVs) are favoured if long-term

gene knockdown is required. These vectors are also considered relatively safe to use and pseudotyping the vectors with different envelope (LV) or capsid (AAV) structures improves tissue tropism (Burger et al., 2004; Couto & High, 2010; Cronin, Zhang, & Reiser, 2005; Hommel et al., 2003; Stewart et al., 2003).

### 1.8.5. Summary

Since its discovery, RNAi continues to be considered as an important tool for functional genomics (Agrawal et al., 2003) and is a promising therapeutic strategy to suppress the expression of the disease-relate genes (Chen & Xie, 2012). Two of the most common RNAi effectors are the exogenously synthesised siRNA and its precursor, plasmid-based shRNA (Rao et al., 2009). Despite its applicability across various cell lines and the advantages associated with RNAi over the traditional deleterious mutation strategy (i.e. more spatial and temporal control over gene expression and the lack of genetic compensation) (Rossi et al., 2015), there are caveats associated with RNAi, such as the off-target effects (Jackson & Linsley, 2010).

## 1.9. Viral-mediated DNA delivery: A focus on recombinant adeno-associated viral vectors

### 1.9.1. Adeno-associated virus (AAV) and its natural diversity

The adeno-associated viruses (AAVs) are helper-dependent members of the family Parvoviridae, which require helper viruses, such as adenoviruses (AdVs) and herpes simplex viruses (HSVs), or DNA damaging

agents for a productive infection. These helper functions elicit changes in the cellular environment, thereby aiding AAV replication and gene expression. In the absence of helper viruses, AAV lies dormant within the cell but can be rescued by subsequent infection of the helper virus. Despite its prevalence in humans, AAV has not been associated with any human disease and is thus widely considered as a non-pathogenic virus (Berns & Giraud, 1996; Weitzman & Linden, 2011).

There are currently more than 100 known serotypes of AAV, discovered fortuitously as contaminants from AdV preparations from various species (12 human serotypes and more than 100 serotypes from nonhuman primates). In specific, AAV serotypes 2,3,5 and 6 were first discovered from human cells and the rest were isolated from the nonhuman primate origins (Daya & Berns, 2008; Weitzman & Linden, 2011). Though the general organisation of the AAV genome is conserved across different serotypes, the capsid structure of the numerous serotypes reveals distinct surface topologies determining the tissue tropisms. Namely, there are serotype-specific variations pertaining to antigenicity, receptor interactions and intracellular pathways (Aschauer, Kreuz, Rumpel, Tsao, & Cerniauskas, 2013; Zincarelli, Soltys, Rengo, & Rabinowitz, 2008).

### 1.9.2. AAV2 structure and biology of infection

Among all of the known AAV serotypes, AAV type 2 (AAV2) is the most extensively studied serotype to date and therefore commonly serves as a prototype for the AAV family. The AAV2 genome consists of a single-

stranded DNA (ssDNA) molecule of approximately 4.7kb in length and at both ends of the genome exist the inverted terminal repeats (ITRs). These ITR regions form a T-shaped hairpin structure and contain *cis*-elements essential for viral replication and packaging. Within the wild-type AAV2 genome are the *rep* and *cap* genes encoding proteins required for viral replication (i.e. Rep40, Rep52, Rep 68 and Rep78) and structural proteins that produce the capsid (i.e. VP1, VP2 and VP3) respectively (Srivastava, Lusby, & Berns, 1983; Wistuba, Weger, Kern, & Kleinschmidt, 1995). In order to produce recombinant AAVs, the *rep* and *cap* genes from the AAV genome can be replaced by a transgene (4.7-5kb) corresponding to the size of wild-type AAV genome and provided *in trans* during transfection for viral assembly (Zolotukhin, 2005).

The AAV ssDNA is encapsidated within the non-enveloped icosahedral capsid architecture of approximately 20nm in diameter. The AAV virion consists of VP1, VP2, and VP3 in 1:1:10 ratio. While VP1 and VP3 are crucial for viral infectivity, i.e. implicated in virus escape from endosomes and receptor usage respectively, VP2 is not crucial for viral assembly or infection (Girod et al., 2002; Rabinowitz, Xiao, & Samulski, 1999).

Successful AAV infection requires the following key steps: (1) capsid-receptor interaction, (2) endocytic uptake, (3) release from endosomal compartments, (3) entry into the nucleus, (4) capsid uncoating and DNA release, (5) second strand synthesis and finally, (6) viral DNA transcription (Bartlett, Wilcher, & Samulski, 2000). The heparan sulfate proteoglycan

(HSPG) was first discovered to be the preferred docking partner of AAV2 and the binding region was then mapped to amino acids 585-588 of the viral capsid protein (Opie et al., 2003). Within the CNS, AAV2 preferentially infect the neurons due to higher HSPG availability on the surface of neurons in comparison with non-neuronal cells, e.g. glia (Hsueh et al., 1998). To corroborate this argument, AAV2 delivery in the brain yielded a largely neuronal transduction profile and direct injection of soluble heparin into the brain has been shown to improve transduction efficiency of intracranially injected AAV2 (Bartlett, Samulski, & McCown, 1998; Nguyen, Sanchez-Pernaute, Cunningham, & Bankiewicz, 2001). In addition to mediating neurotropic bias of AAV2, binding to the heparan sulfate is interestingly associated with the limit of CNS volume effectively targeted by AAV vectors (Murlidharan, Samulski, & Asokan, 2014).

Following cell surface attachment and internalisation into the cell, via clathrin-coated vesicles, AAV enters the nucleus of the host cell and reveals its genomic content though the trafficking pathway of AAV particles from the cell surface to the nucleus is not fully understood. In the CNS, paravascular cerebrospinal fluid (CSF) transport and axonal transport are the two main systems thought to regulate spread of AAV vector within the interstitial space and the intracellular environment correspondingly. Intact AAV2 virions are known to exclusively undergo anterograde axonal transport where they enter the somatic region of the host neuron, travel along the axon and are subsequently released at the projection site. These

released virions are then free to transduce neighbouring cells within the region (Aschauer et al., 2013; Murlidharan et al., 2014).

### 1.9.3. Manufacturing recombinant AAV (rAAV) vectors: The helper-free system

Early methods for rAAV production require auxiliary viruses, e.g. adenoviruses, to provide helper functions described above. However, a pivotal advancement in the AAV manufacturing field was made through the identification and cloning of AdV regions important for AAV replication and encapsidation, thus eliminating the use of helper viruses and decreasing the risk of contamination during AAV preparation (Matsushita et al., 2004; Samulski & Shenk, 1988). The helper-free system utilises the HEK293 cell line, transiently transfected with: (i) recombinant vector genome plasmid, (ii) rep/cap plasmid and (iii) AdV helper plasmid, allowing viral assembly to occur inside the host cells (Xiao, Li, & Samulski, 1998).

### 1.9.4. Advantages and disadvantages of using rAAV vectors: Technical considerations

To date, AAV continues to establish its position as one of the most popular gene delivery systems in dividing and non-dividing cells for numerous reasons. A major advantage of rAAV vectors, and in fact, retrovirus-derived vectors (e.g. LV vectors), is the long-term expression of the transgene following *in vivo* gene delivery. While LV vector system aids long-term expression of the gene of interest by integrating its genome within

the host genome, the rAAV genome primarily forms a stable concatemer episomally, lowering the risk of insertional mutagenesis (McCarty, Young, & Samulski, 2004; Schnepp, Clark, Klemanski, Pacak, & Johnson, 2003; Singer & Verma, 2008). Although rAAV is small in size reflecting its restricted packaging capacity, this may provide an advantage over larger vectors, such as LVs, with regards to viral spread (see Parr-Brownlie et al., 2015for review). Thirdly, the non-pathogenic and replication defective nature of AAVs ensure the safety of this vector system, reflected by lowered biosafety level requirements (Flotte & Carter, 1995; Nayak & Herzog, 2010).

Nevertheless, there are several impediments associated with AAV vector use. For many years, the use of rAAV was limited by the inefficient methods of virus production, but this issue has been resolved by a number of research groups (discussed above in Section 1.9.3), thus currently leaving slow onset of transgene expression and restricted packaging capacity in a single vector approach (~4.7kb) as primary obstacles of rAAV use. In experimental settings where a fast onset of transgene expression is required, the use of self-complementary AAV (scAAV) expression construct, where two halves of the single-stranded AAV (ssAAV) genome can form intramolecular double strands, may be more desirable given that the formation of dsDNA in the ssAAV expression construct is considered to be a rate-limiting step for AAV-mediated transgene expression. While this approach significantly shortens the onset of transgene expression, it reduces the effective genome size to approximately 2.3kb (Ferrari, Samulski, Shenk, & Samulski, 1996; Fisher et al., 1996; Raj, Davidoff, & Nathwani, 2011).

Furthermore, findings from transduction experiments revealed that AAV vectors carrying larger genomes (>5.3kb) transduced cells much less efficiently than those carrying WT size genomes. This was postulated to be due to preferential degradation of viral particles encapsidating larger genomes as the addition of proteasome inhibitor rectified this issue. To improve AAV packaging capacity, researchers have also harnessed the ability of rAAV genome to link together in strings or doublets (Flotte, 2000). A few examples of developed techniques include overlapping two independent vectors by homologous recombination, the *trans*-splicing approach where exons can be reconstituted through splicing, the hybrid dual vector system (Duan, Yue, Engelhardt, Boss, & Kerem, 2001; Ghosh et al., 2008; Ghosh & Duan, 2007).

### 1.9.5. Summary

To date, the AAV remain to be one of the most popular vectors of choice for DNA/gene delivery *in vivo,* due to its efficiency both in dividing and non-dividing cells (Aschauer et al., 2013; Xiao et al., 1998). Upon insertion into the target cells, the AAV genomes form stable concatemers episomally, thus minimising the risk of mutagenesis within the host genome (Singer & Verma, 2008). Nonetheless, it is noteworthy that different AAV serotypes exhibit distinct tissue tropism and specifically, though all serotypes have the general ability to transduce all major cell types in the brain (i.e. neurons, astrocytes, microglia, and oligodendrocytes), transgene

expression was found to vary for specific cell type and serotype combinations (Aschauer et al., 2013).

## 1.10. Aims and structure of the thesis

### 1.10.1. Chapter 2

Chapter 2 describes the experimental procedures used for this thesis. This includes AAV design and production, *in vitro* and *in vivo* testing of AAV functionality, as well as the behavioural experiments (sensitisation and conditioned reinforcement).

### 1.10.2. Chapter 3

Chapter 3 details the design and production of viral vectors, harbouring α2-targeting (or non-targeting) shRNA, as tools to study the function of α2-$GABA_A$Rs in the region, as well as in a specific cell type, of interest. Given the novelty of the silencing construct used in this thesis, its knockdown potency was first characterised *in vitro* prior to viral assembly and subsequently, *in vivo* testing. Specifically, quantitative real-time PCR (qRT-PCR) and immunocyto/histochemistry were performed to analyse the silencing potency of the RNAi effectors, both at the mRNA and protein levels. These viral tools were then used to characterise the role of mesoaccumbal α2-$GABA_A$Rs in mediating cocaine-facilitated conditioned behaviours, detailed in the following chapters.

### 1.10.3. Chapter 4

Previous research in our laboratory demonstrated that the whole-brain deletion of Gabra2 blocked cocaine-potentiated conditioned behaviours (i.e. locomotor sensitisation and conditioned reinforcement). It was further hypothesised that such phenotypes might have been linked to the loss of Gabra2 particularly in the nucleus accumbens (NAc) (Dixon et al., 2010). This chapter therefore seeks to investigate the effects of α2 knockdown in the NAc core and shell (using the RNAi strategy) on cocaine-induced locomotor sensitisation and cocaine facilitation of CRf. In the sensitisation experiment, mice were given repeated, intermittent cocaine (10mg/kg) over 10 daily sessions. Conditioned activity (with a sham injection) was measured seven days following the final day of sensitisation. The CRf experiment was also carried out to probe whether silencing α2 in the NAc core/shell would affect the potentiating effect of cocaine (0, 3, 10 and 30 mg/kg cocaine, Latin-square design) on selective nosepoking for CRf.

### 1.10.4. Chapter 5

Approximately 95% of neurons within the NAc are GABAergic medium spiny neurons (MSNs), which could be subdivided into two main populations, i.e dopamine D1R- and D2R-expressing MSNs (Matamales et al., 2009; Ouimet et al., 1998). This chapter therefore seeks to further probe the roles of α2-$GABA_AR$-mediated signalling in D1R- and D2R-expressing neurons within the NAc core in mediating cocaine-facilitated conditioned reinforcement using the Cre-dependent RNAi strategy.

# Chapter 2

# Materials and Methods

## 2.1. Molecular cloning/subcloning procedures

### 2.1.1. Methods for DNA preparation for cloning

#### 2.1.1.1. Annealing of single-stranded oligonucleotides

Single-stranded oligonucleotides (ssDNA), comprising the top and bottom strands of a DNA fragment, were synthesised commercially by Eurofins Genomics (final concentration = 100μM). Each pair of ssDNA was annealed by mixing forward and reverse oligonucleotides (100nmoles/ml final concentration for each of the oligonucleotides) in 1× annealing buffer (10 mM Tris, pH 7.5 - 8.0, 50 mM NaCl, 1 mM EDTA), with a total volume of 50μl. Each reaction was set up in a 0.2ml thin-walled PCR tube (Alpha Laboratories, LW2340). The reaction was incubated at 95˚C for two minutes in a thermal cycler (Bio-Rad T100™) and cooled to 25˚C for 3 hours before further use. The dsDNA was then phosphorylated to aid ligation into the recipient plasmid (see Section 2.1.3). Note that all oligonucleotides used in this thesis were synthesised by Eurofins Genomics.

#### 2.1.1.2. Polymerase chain reaction (PCR)-based isolation of DNA fragment

To introduce new restriction sites to dsDNA fragments for subcloning purposes, PCR-based isolation of DNA fragment was performed. The dsDNA was isolated from a plasmid by amplifying the sequence of interest using primers containing new restriction sites. Note that all primers used in this thesis were synthesised by Eurofins Genomics.

Phusion® High-Fidelity DNA Polymerase (New England Biolabs (NEB), M0530) was used for all PCR procedures unless stated otherwise. PCR reactions were set up in 0.2ml thin-walled PCR tubes in a total volume of 50µl. A typical reaction contained 10ng of plasmid DNA, 0.5µM of Forward and Reverse primers, 200µM dNTPs, 1× Phusion GC buffer, 3% DMSO. The DNA fragment was isolated from the plasmid and amplified by PCR with an initial denaturation step at 98°C for 30 seconds, followed by 35 PCR cycles [98°C for 10 seconds, 45-72°C (depending on the melting temperature of the primers) for 30 seconds, and 72°C for 15-30 seconds per kb], with a final extension of 72°C for 10 minutes using a thermal cycler. The PCR products were electrophoresed on a 1% agarose gel, purified using the QIAquick gel extraction kit (Qiagen, 28704) (see Section 2.1.2), and phosphorylated as non-phosphorylated primers were used (refer to Section 2.1.3 below)

### 2.1.1.3. Plasmid digestion with restriction enzymes

To isolate a DNA fragment from a plasmid by restriction digests, 3µg plasmid DNA was used. A typical restriction digest reaction (50µl total volume), set up in a 0.2ml PCR tube, contained 3µg DNA, 10 units of each restriction enzyme (for a double digest reaction, refer to specific experiments below), and 1× NEB buffer. The restriction digest reaction was then incubated at the recommended temperature (refer to the manufacturer's protocol of the restriction enzyme used for incubation temperature and duration). Next, digested DNA fragments were electrophoresed on a 0.5%

agarose gel and purified using the QIAquick gel extraction kit as described in Section 2.1.2. Also note that the ends of blunt-ended, recipient plasmids were dephosphorylated to prevent self-ligation, prior to gel electrophoresis and DNA purification steps (see Section 2.1.3).

### 2.1.2. Agarose gel electrophoresis and DNA purification

PCR products or linearised DNA were visualised and purified by agarose gel electrophoresis, followed by gel extraction. PCR products were electrophoresed on a 1% agarose gel (Fisher Bioreagents, BP160-500), whereas restriction digest reactions were electrophoresed on a 0.5% agarose gel. Agarose was dissolved in 1× TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA, pH 8.0) by heating the agarose mixture in the microwave until boiling. The solution was then cooled to approximately 55˚C before adding Ethidium bromide (0.5 μg/ml final concentration from a 10mg/ml stock solution, Sigma-Aldrich, E1510). For estimation of fragment size, a 100bp (NEB, N0467S) or 1kb ladder (NEB, N0468S) was used. Gel electropheoresis was carried out in a horizontal tank containing 1x TAE buffer and run at 100V for approximately 30 minutes or until the DNA fragments were fully resolved. Gels were imaged using a UV transilluminator.

Next, DNA bands were excised from the gel as 200mg agarose slices using a scalpel blade and purified using the QIAquick gel extraction kit according to the manufacturer's instructions. DNA was eluted from the

QIAquick spin column with 30µl of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5).

### 2.1.3. Phosphorylation and dephosphorylation of DNA fragments

Phosphorylation and/or dephosphorylation of DNA ends were performed to aid ligation. DNA duplexes (up to 300 pmol of 5' termini) were phosphorylated by T4 Polynucleotide Kinase (PNK; NEB, M0201), in a 50µl reaction (see manufacturer's protocol). To prevent religation of plasmid DNA, dephosphorylation of linearised DNA (1pmol of DNA ends) was performed using Calf Intestine Phosphatase (1 unit; NEB, M0290) prior to gel electrophoresis and DNA purification (see manufacturer's instructions).

### 2.1.4. Ligation

DNA fragments were ligated at a 1:3 vector:insert molar ratio (i.e. 0.02pmol vector and 0.06pmol insert) in a 20µl reaction using T4 DNA Ligase (400 units) (NEB, M0202S) according to the manufacturer's instruction. The ligation reaction was incubated overnight (~18 hours) at 16˚C in a thermal cycler. After incubation, ligase was heat inactivated at 65˚C for 10 minutes and the ligation mixture was chilled on ice for an hour prior to bacterial chemotransformation.

### 2.1.5. Bacterial chemotransformation

Chemically-competent One Shot™ Stbl3™ *E. coli* (Invitrogen, C737303) were used to propagate recombinant plasmids. Cell aliquots were gently thawed on ice before use. Next, 5µl of the ligation mix, or dd$H_2O$

(negative control), was added to a 50µl vial of cells, followed by incubation on ice for 30 minutes. Heat-shock transformation was performed according to the manufacturer's protocol. Next, 100µl of the cell mixture was streaked onto LB (Luria Broth) agar plates containing the appropriate antibiotics, i.e ampicillin or kanamycin (100µg/ml), and incubated overnight at 37˚C.

For blue/white colony screening, 100 µl of 20 mg/ml X-gal stock (Roche, 03117073001) and 100 µl of 10 mM Isopropyl-ß-D-thiogalactoside (IPTG; Roche, 010724815001) were spread on to each pre-made LB agar plate (containing 100 µg/ml ampicillin).

### 2.1.6. Colony PCR

This step was performed to identify the presence (or absence) of insert DNA in the recipient vector, as well as to determine insert size and orientation. For this, the Megamix Blue PCR mastermix (Microzone, 2MMB) with the experiment-specific primer sets were used. Each individual colony was isolated with a sterile toothpick and dipped into the reaction tube (PCR tube) containing the mastermix and primers. The toothpick was removed from the tube and streaked onto an agar plate containing the appropriate antibiotics (with X-Gal and IPTG for blue/white screening). The plate was then incubated overnight at 37˚C.

A typical PCR condition for colony PCR consists of an initial denaturation step of 95˚C for 15 minutes, followed by 35 PCR cycles (95˚C for 30 seconds, 55˚C for 60 seconds, and 72˚C for 45 seconds), with a final

extension at 72˚C for 10 minutes. PCR products were then electrophoresed on a 1% agarose gel, followed by gel imaging (see Section 2.1.2).

### 2.1.7. Miniprep isolation of recombinant plasmids

The positive clones (i.e. individual colonies) were selected and grown for 16 hours in 5ml LB broth containing the appropriate antibiotics (100 µg/ml ampicillin or kanamycin) at 37˚C in a shaker-incubator (250rpm). Plasmids were then isolated from 3 ml of the liquid cultures using the QIAprep Spin Miniprep Kit (Qiagen, 27104) according to the manufaturer's instructions. Using a sterile loop, some of the liquid cultures were streaked on agar plates containing the appropriate antibiotics and grown overnight at 37˚C. The concentration and purity of DNA extracted were measured using Nanodrop 2000 Spectrophotometer (Thermo Scientific, ND2000) (refer to Section 2.1.9 for sample preparation for DNA sequencing).

### 2.1.8. Maxiprep isolation of recombinant plasmids

The positive clones (i.e. individual colonies) were selected and grown for 8 hours in 5ml LB broth containing the appropriate antibiotics at 37˚C in a shaker-incubator (250rpm). Next, 250µl of the liquid culture was added to 100ml of the LB broth containing the appropriate antibiotics for high copy plasmids (or 500ml for low copy plasmids), followed by a 16-hour incubation at 37˚C in a shaker-incubator (set to 250rpm). Some of the overnight bacterial cultures were stored in 15% glycerol at -80˚C (i.e. 500µl liquid culture + 500µl 30% glycerol (v/v), mixed by vortexing).

Plasmids were isolated from the ~100ml (or ~500ml) liquid cultures using the PureLink® HiPure Plasmid Filter Maxiprep Kit (Invitrogen, K210017) according to the manufacturer's protocol. The DNA pellet was resuspended in 200µl TE buffer (or in 100µl of TE buffer for the low copy plasmids).

### 2.1.9. Sample preparation for DNA sequencing

Sequencing of plasmid DNA was carried out by Eurofins Genomics (TubeSeq Service). DNA samples (80 ng/µl sample concentration) and the sequencing primers were prepared according to their sample submission guideline.

## 2.2. Mammalian cell culture

### 2.2.1. Maintenance of adherent HEK293 cells

All cell culture work was performed in a laminar flow hood under aseptic conditions. Human embryonic kidney (HEK)293 cells were used for *in vitro* experiments and rAAV production in this thesis. This cell line was provided by Dr. Majid Hafezparast as laboratory stock (University of Sussex). HEK293 cells were maintained in growth medium of Dulbecco's Modified Eagle Medium (DMEM; Gibco, 11960044), supplemented with 6mM L-Glutamine (Gibco, 25030081), 10% Fetal Bovine Serum (Gibco, 26140079), and Penicillin/ Streptomycin (final concentration of 100U/ml/ 100µg/ml; Gibco, 15140122). These cells were grown in Corning T175 (175cm$^2$) cell culture flasks (Sigma-Aldrich, CLS431080) at 37˚C with 5% $CO_2$, and passaged when cells reached 80-90% confluency.

Prior to cell passage, growth medium, 1× Dulbecco's Phosphate Buffered Saline (DPBS; Gibco, 14190250) and Trypsin were pre-warmed at 37˚C. Firstly, growth medium was removed from the flask and cells were washed once with sterile DPBS and 9ml of Trypsin-EDTA (0.25%; Gibco, 25200056) was added to the cell monolayer, followed by 5-minute incubation at 37˚C to promote cell detachment from the flask. Trypsin was deactivated by adding the same amount of 10% Fetal Bovine Serum-containing growth medium to the flask. The single cell suspension was centrifuged at 300*g* at room temperature (~25˚C) for 5 minutes followed by removal of the supernatant and cell pellet resuspension in 10ml of the growth medium. Cells were counted using a glass haemocytometer (refer to Section 2.2.3 for cell counting). Once counted, cells split into new T175 flasks at a ratio of 1:10 for maintenance or seeded into 10-cm dishes or multi-well plates for subsequent experiments. Cells were then returned to the incubator (37˚C, 5% $CO_2$).

### 2.2.2. Freezing and recovery of HEK293 cells

For long-term storage, cells were kept in the liquid nitrogen (<150˚C). Prior to storage, 80% confluent cells with a low-passage number (i.e. split less than 3×) were trypsinised and centrifuged to form a pellet as described in Section 2.2.1. Cells were resuspended in growth medium and counted (see Section 2.2.3). Each cell suspension was formed with 1 million cells in 1ml of growth medium containing 10% dimethyl sulfoxide (DMSO; Sigma-Aldrich, M81802) to protect cells from damage at low temperature conditions. The

cell suspensions were kept in a Nalgene™ cryovials (Thermo Scientific, 5000-1012), stored overnight in -80˚C freezer in a Nalgene™ Mr. Frosty cryo freezing container (Thermo Scientific, 5100-0001) prior to storage in liquid nitrogen.

To retrieve HEK293 cells that were previously stored in liquid nitrogen, cryovials containing HEK293 cells were heated to 37˚C until cells were 80% thawed. In the cell culture hood, cells were transferred from the cryovial to 10ml of pre-warmed growth medium. The cell suspension was then centrifuged at 300 G at room temperature (~25˚C) for 5 minutes. The supernatant was removed and the cell pellet was resuspended in fresh growth medium before being transferred to a T175 flask. The appropriate volume of growth medium was then added to the flask to allow cell growth and the flask was kept in the 37˚C incubator (with 5% $CO_2$).

### 2.2.3. Viable cell counting with trypan blue

Cells were counted using the trypan blue staining procedure with a glass haemocytometer. Following resuspension of cells with the growth medium, 500µl of cell suspension was transferred into a 1.5ml Eppendorf tube (Eppendorf, Cat no. 0030120086). From this, 100µl cells were transferred to a new 1.5ml tube containing 400µl of 0.4% Trypan Blue (0.32% final concentration; Sigma-Aldrich, T8154). Next, 100µl of the cell-Trypan Blue mix was applied to the haemocytometer by gently filling both chambers underneath the coverslip. Using a light microscope (at10x magnification), count the number of unstained, live cells in one set of 16

squares (Figure 2.1 highlighted in blue). The number of live cells in the other three sets of corner squares were also counted.

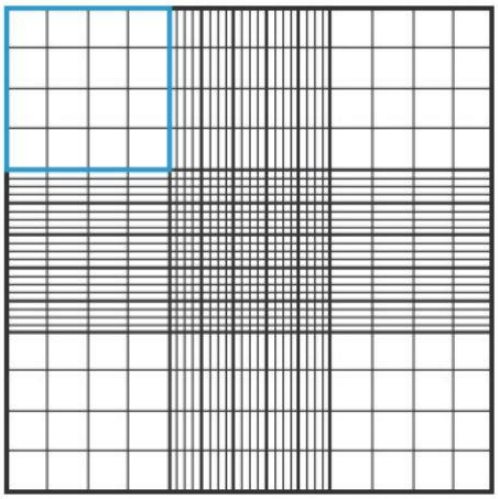


***Figure 2.1.*** Haemocytometer grids for cell counting (image retrieved from Abcam; https://www.abcam.com/protocols/counting-cells-using-a-haemocytometer)

To calculate the number of viable cells per ml, cell counts from the four sets of 16 squares were averaged and multiplied by $10^4$. The value was then multiplied by 5 to account for the 1 in 5 dilution from the addition of Trypan Blue.

### 2.2.4. Transfection of HEK293 with Lipofectamine 3000

Transfection experiments for *in vitro* assays of pAAV functionality were performed using Lipofectamine 3000 (Invitrogen, L3000015) in 24-well plates (Sigma-Aldrich, CLS3527) on round glass coverslips (13mm diameter; Fisher Scientific, 12392128) according to the manufacturer's instructions. HEK293 cells were seeded the day before so that they were 80% confluent

on the day of transfection (approximately $2\times10^5$ cells/well). Also note that for each well, 1µl of Lipofectamine 3000 was used and cells were incubated for 48 hours at 37˚C prior to imaging and/or immunocytochemical analyses. Imaging of live cells transfected with fluorescent constructs were performed using the epifluorescent Zeiss Axiovert 200 motorized inverted microscope.

### 2.2.5. Immunocytochemical staining

Forty-eight hours post-transfection, cells were washed once with 1ml of DPBS (per well), then fixed with 4% paraformaldehyde (PFA; Sigma-Aldrich, 158127) for 10 minutes. Cells were washed 3× with 1ml of DPBS post-fixation.

Fixed cells were permeabilised in 0.2% Triton X-100 (Sigma-Aldrich, T8787) in sterile PBS for 2 minutes with gentle agitation before blocking with 5% Bovine Serum Albumin (BSA; Sigma-Aldrich, A9418) in PBS (i.e. BSA-PBS) for 30 minutes. Cells were then incubated with the primary antibody (diluted in 1% BSA-PBS) at 4˚C overnight (~16 hours).

On the following day, cells were washed with PBS-TS (PBS, 0.1% Tween 20 (Sigma-Aldrich, P2287), 0.02% Sodium Dodecyl Sulfate (SDS; Fisher BioReagents, BP166-100)) for 5 minutes (1ml per well) with gentle agitation to remove unbound primary antibodies. Cells were incubated with the secondary antibody in 1% BSA-PBS for 1 hour at room temperature with gentle agitation. Note that this and the subsequent steps were performed in the dark given the light-sensitive fluorophores. Following incubation with the secondary antibody, cells were washed 3× in PBS-TS for 5 minutes each.

The coverslips were then mounted on to 76 × 26mm glass microscopic slides (Fisher Scientific, 13192131) with Fluoroshield™ (Sigma-Aldrich, F6182). The edges of the coverslips were sealed with nail varnish and slides were stored at 4˚C before imaging on the microscope. Slides were imaged using the Olympus BX53 epifluorescent microscope (at 10x magnification). Images were captured using a QI click camera (Qimaging) attached to the microscope, viewed using the iVision software (version 4.0.15, Biovision Technologies), and analysed in Fiji.

## 2.3. RNAi vector design and preparation

### 2.3.1. Design of the shRNA constructs

Two shRNA sequences, containing Gabra2-targeting siRNA constructs (i.e. shα2(A) and shα2(B)) with a non-targeting scrambled control sequence were designed in the present research. The shRNA construct used in this thesis contained 24-nucleotide (nt) siRNA stem sequences, with a 6-nt loop structure (5'-CTTCCTGTC-3') (refer to Table 2.1 for siRNA sequences). The siRNAs used in the present research were designed according to the Reynolds' design algorithm (Reynolds et al., 2004) using RNAi explorer (www.genelink.com/sirna/siRNAorder.asp). BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch) analyses on these sequences were then performed to minimise off-target effects (i.e. targeting non-target endogenous genes and/or those within the pAAV construct).

To facilitate cloning into the pAAV-EGFP-shRNA vector (kindly donated by Ralph DiLeone, Yale University), XbaI and SapI sites were added to the shRNA construct (see Figure 2.2). For ligation into the Cre-dependent pAAV vector, (blunt-end ligation; Figure 2.5), a"T' base was added at the 5' end of the hairpin sequence to aid transcription by the mU6 promoter (Ma et al., 2014).

Table 2.1

| Sequence | Sense (5'-3') | Antisense (5'-3') |
|---|---|---|
| shα2(A) | GGA GAC AGT ATT ACT GAA GTC TTC | GAA GAC TTC AGT AAT ACT GTC TCC |
| shα2(B) | GGA TGA TGG AAC ATT GCT ATA TAC | GTA TAT AGC AAT GTT CCA TCA TCC |
| shScr | GGA TGC TAG AAC ATC CCT ATA TGC | GCA TAT AGG GAT GTT CTA GCA TCC |

*Table 2.1.* Sense and antisense siRNA sequences of Gabra2-targeting and scrambled shRNA constructs.

## 2.3.2. Construction of pAAV-EGFP-shRNA

### 2.3.2.1. Vector and insert DNA preparation

The shRNA constructs (Gabra2-targeting constructs and the non-targeting control) used in this thesis are listed in Table 2.1 and the pAAV-EGFP-shRNA vector was kindly donated by Ralph DiLeone (Yale University).

Firstly, shRNA oligonucleotides (synthesised by Eurofins Genomics) were annealed and subsequently phosphorylated as described in Sections 2.1.1.1 and 2.1.3. The recipient plasmid was then digested with XbaI (NEB,

R0145S) and SapI (NEB, R0569S) enzymes at 37˚C for 1 hour (see Figure 2.2 for the cloning site; also refer to Section 2.1.1.3) and dephosphorylated with CIP (see Section 2.1.3). The digested plasmid was then isolated and purified by agarose gel electrophoresis on a 0.5% agarose, followed by gel extraction (refer to Section 2.1.2).

#### 2.3.2.2. Ligation and transformation

The vector and the shRNA (shα2(A), shα2(B), or shScr) insert were ligated according to the protocol described in Section 2.1.4 and the ligated products were transformed into Stbl3 cells as outlined in Section 2.1.5.

#### 2.3.2.3. Miniprep of pAAV-EGFP-shRNA

Five individual colonies from each shRNA-carrying vector were picked and plasmid DNA was extracted from these colonies using the miniprep kit (see Section 2.1.7).

#### 2.3.2.4. Diagnostic restriction digest and sequence verification

Diagnostic restriction digest with SapI or XbaI was then performed to identify the positive clones (refer to Section 2.3.2.1 for digestion condition). A successful ligation of the shRNA was marked by the loss of SapI, but not XbaI, recognition site (refer to Chapter 3 for results). DNA sequence integrity was further determined through DNA sequencing

<u>shRNA sequencing primers (synthesised by Eurofins Genomics):</u>

F (5'-3'): CAC AGA CTT GTG GGA GAA GC

R (5'-3'): CCC CTG AAC CTG AAA CAT AAA)

### 2.3.2.5. Maxiprep of pAAV-EGFP-shRNA

Plasmids from positive clones were then further amplified and extracted from bacterial cells using the maxiprep kit (see Section 2.1.8).

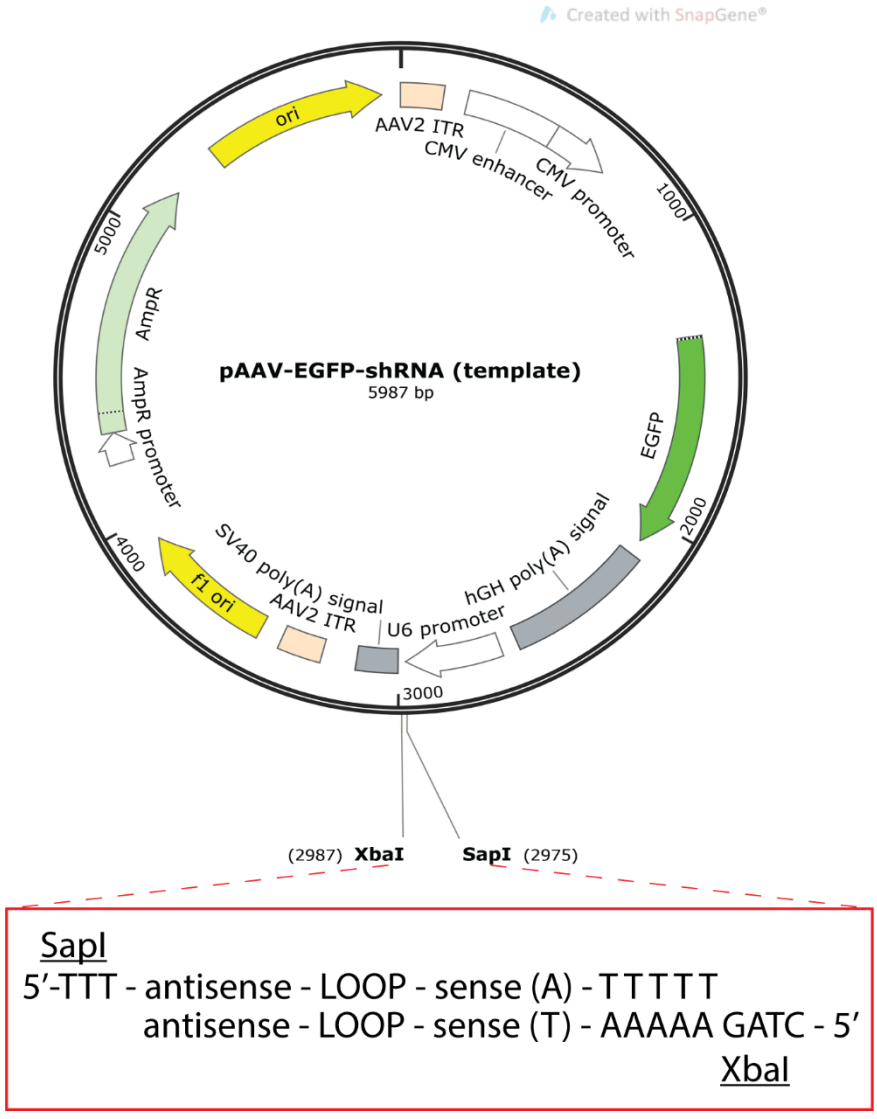



*Figure 2.2.* shRNA cloning site within pAAV-EGFP-shRNA.

## 2.3.3. Construction of pAAV-EGFP_mCherry(DO_DIO)-shRNA

This Cre-dependent RNAi vector was constructed using elements from two plasmids, i.e. pAAV-Ef1a-DIO-mCherry-WPRE-pA (kindly gifted by Bernardo Sabatini, Addgene plasmid #37083) and pSico (a gift from Tyler Jacks, Addgene plasmid #11578).

### 2.3.3.1. Subcloning EGFP (pSico) to pBluescriptII SK+

Firstly, the EGFP construct was isolated from pSico and AscI sites were introduced at both ends of the gene through PCR-based DNA isolation (refer to Section 2.1.1.2) to facilitate ligation to pAAV-Ef1a-DIO-mCherry-WPRE-pA.

For this purpose, 1ng of plasmid DNA template was used, and the PCR condition consisted of an initial denaturation step at 98˚C for 30 seconds, 35 cycles (98˚C for 10 seconds, 68˚C for 30 seconds, and 72˚C for 30 seconds), with a final extension at 72˚C for 10 minutes.

Primers, with protective sequences (in red), used to isolate EGFP from pSico (in black) and introduce AscI sites (in purple):

F: TAA TTG GCG CGC CTT AGT GAA CCG TCA GAT CCG

R: TTA TTA GGC GCG CCT TAT GCG GCC GCT ACT TGT

The PCR product was then electrophoresed on a 1% agarose gel and purified (Section 2.1.2), phosphorylated (Section 2.1.3), and stored temporarily at 4˚C.

Next, pBluescript II SK+, provided by Dr. Majid Hafezparast as laboratory stock (University of Sussex), was digested with EcoRV-HF® (NEB, R3195) at 37˚C for 1 hour and dephoshorylated to prevent religation of the blunt-ended, digested plasmid (refer to Sections 2.1.2 and 2.1.3). The linearised plasmid was then run on a 0.5% agarose gel and further purified using the gel extraction kit as described in Section 2.1.2.

The linearised vector and the EGFP insert were ligated as described in Section 2.1.4, followed by bacterial transformation for propagation of recombinant plasmids (refer to Section 2.1.5). Transformation with pBluescript required blue/white colony screening (see Section 2.1.5 for details) due to the presence of lacZ within the vector. Successful ligation of the insert into the multiple cloning site (MCS) of pBluescript disrupts the lacZ sequence, thus leading to a loss of functional ß-galactosidase (colonies should appear white). If lacZ remains intact and ß-galactosidase is produced, hydrolysis of X-Gal to form 5-bromo-4-chloro-indoxyl occurs and its dimerization produced an insoluble blue pigment, hence the blue colonies (Juers, Matthews, & Huber, 2012).

Plasmid from white colonies were subsequently extracted from the bacterial cells using the miniprep kit (refer to Section 2.1.7) and sequence integrity was checked through DNA sequencing using (Section 2.1.9) using pBluescript KS (5'- CGA GGT CGA CGG TAT CG -3') and SK (5'- TCT AGA ACT AGT GGA TC -3') primers.

#### 2.3.3.2. Subcloning EGFP into pAAV-Ef1a-DIO-mCherry-WPRE-pA to produce pAAV-EGFP_mCherry(DO_DIO)

The EGFP construct (flanked by AscI sites) was isolated from pBluescript by restriction digest with AscI (NEB, R0558S) enzyme (incubated at 37˚C for 1 hour). The pAAV-Ef1a-DIO-mCherry-WPRE-pA was also digested with AscI (Section 2.1.1.3) to permit EGFP insertion into the plasmid, then dephosphorylated (Section 2.1.3) to prevent religation.

Linearised DNA was, then, electrophoresed on a 1% agarose gel and extracted from the gel (Section 2.1.2). The EGFP construct was phosphorylated by T4 PNK as previously described in Section 2.1.3. The linearised mCherry vector and EGFP insert were ligated as described in Section 2.1.4, followed by bacterial transformation (refer to Section 2.1.5).

Next, colony PCR (Section 2.1.6) was performed to identify colonies harbouring the recombinant plasmids, as well as to determine size (approximately 1kb) and orientation of EGFP (see Figure 2.3), with the following primers (5'-3' orientation):

F: CAT TAT ACG AAG TTA TGG CGC G

R: TAT GCG GCC GCT ACT TGT AC

A positive clone was selected based on the colony PCR outcome and the plasmid DNA was extracted by miniprep for sequencing (Section 2.1.7) and by maxiprep for subsequent steps (refer to Section 2.1.8). The EGFP

sequence was checked by DNA sequencing (Eurofins Genomics), with the following sequencing primers (5'-3' orientation):

F: GGT AGC TGG ATT GTA GCT GC

R: TAA TGC AGA AGA AGA GGA TGG G

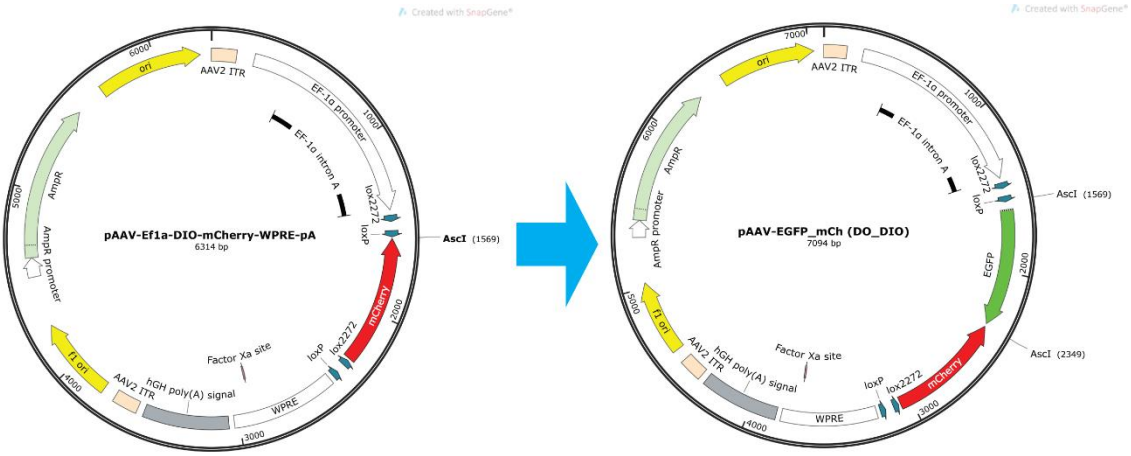



***Figure 2.3.*** Insertion of the EGFP (with AscI sites) construct into pAAV-Ef1a-DIO-mCherry-WPRE-pA.

### 2.3.3.3. Substituting CMV-EGFP in pSico with a STOP cassette

In pSico, the presence of CMV+EGFP served to prevent shRNA transcription in the absence of Cre and enable visualisation of Cre-negative, transfected cells. For our Cre-dependent RNAi vector, we substituted the region spanning CMV promoter and EGFP within pSico with a 82-bp STOP cassette (synthesised by Eurofins Genomics). The single-stranded DNA molecules were annealed as described in Section 2.1.1.1, followed phosphorylation of DNA ends (Section 2.1.3).

STOP cassette

Top strand (5'-3'): AAT TCA ACT TGT TTA TTG CAG CTT ATA ATG GTT ACA AAT AAA GCA ATA GCA TCA CAA ATT TCA CAA ATA AAG CAT TTT TTG C

Bottom strand (5'-3'): GGC CGC AAA AAA TGC TTT ATT TGT GAA ATT TGT GAT GCT ATT GCT TTA TTT GTA ACC ATT ATA AGC TGC AAT AAA CAA GTT G

Next, pSico was digested with EcoRI (NEB, R0101) and NotI-HF® (NEB, R3189) at 37˚C for 1 hour to remove CMV+EGFP (refer to Section 2.1.1.3), dephosphorylated (Section 2.1.3), electrophoresed on a 0.5% agarose gel and purified using the gel extraction kit (Section 2.1.2).

The STOP cassette and pSico vector were ligated as described in Section 2.1.4 and the ligated product was transformed into Stbl3 *E.coli* cells (Section 2.1.5).

Individual colonies were miniprepped to extract plasmid DNA and the cloning of STOP cassette into pSico was checked through DNA sequencing (refer to Section 2.1.9 for sample preparation).

Sequencing primers to check the presence of the STOP cassette and its sequence integrity (5'-3'):

F: AGT TTG GTT AGT ACC GGG CC

R: TCG TGA AGC GAG CTT ATC GAT A

### 2.3.3.4. Construction of the pAAV-EGFP_mCherry(DO_DIO)-shRNA template plasmid

Next, a region spanning the mU6 promoter, the STOP cassette, and the shRNA cloning site within pSico (insert DNA) was amplified by PCR (refer to Section 2.1.1.2) with the following primers (5'-3'):

F: AGT TTG GTT AGT ACC GGG CC

R: TCG TGA AGC GAG CTT ATC GAT A

The PCR cycle condition for this consisted of an initial denaturation step at 98˚C for 30 seconds, 35 PCR cycles (98˚C for 10 seconds, 55˚C for 30 seconds, and 72˚C for 30 seconds), with a final extension at 72˚C for 10 minutes. PCR products were purified by agarose gel electrophoresis on a 1% gel, followed by gel extraction (Section 2.1.2), and phosphorylated by T4 PNK (Section 2.1.3).

The pAAV-EGFP_mCherry(DO_DIO) vector was then digested with EcoRV-HF® (37˚C incubation for 1 hour), dephosphorylated (Section 2.1.3), then purified by agarose gel electrophoresis on a 1% gel, followed by gel extraction (Section 2.1.2).

The linearised vector and the insert, containing mU6, STOP cassette, and the shRNA cloning site, were ligated and chemically transformed into Stbl3 cells as previously described (Sections 2.1.4 and 2.1.5). Colony PCR (1832bp product size; Section 2.1.6) was then performed to determine

positive clones (i.e. recombinant plasmids with the insert positioned in the right orientation) with the following primers (5'-3'):

F: CGC ACC ATC TTC TTC AAG GAC GAC

R: TCG TGA AGC GAG CTT ATC GAT A

Recombinant plasmids (Figure 2.4) were extracted from the bacterial cells using the miniprep kit for DNA sequencing and using the maxiprep kit for the subsequent steps.

<u>Primers used for sequence confirmation (5'-3'):</u>

F: AGT TTG GTT AGT ACC GGG CC

R: TCG TGA AGC GAG CTT ATC GAT A

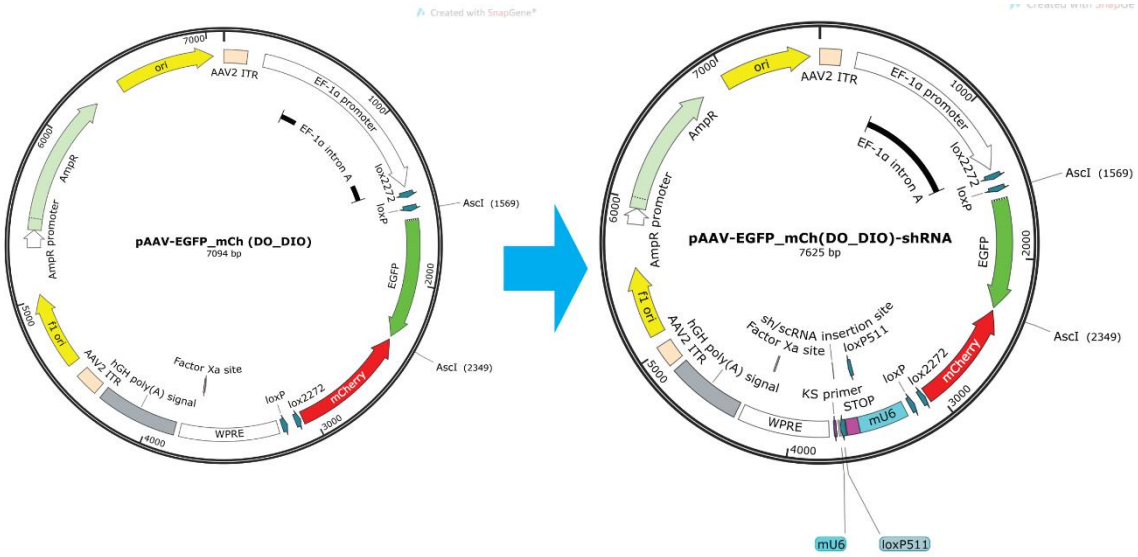



***Figure 2.4.*** Construction of pAAV-EGFP_mCherry(DO_DIO)-shRNA template vector. A region spanning mU6 promoter, STOP cassette, and the shRNA cloning site from the modified pSico (refer to Section 2.3.3.3) was subcloned into pAAV-EGFP-mCherry(DO_DIO).

### 2.3.3.5. Construction of pAAV- EGFP_mCherry (DO_DIO)-shRNA (final plasmid)

The design of the shRNA (shα2(A) and shScr) used for the Cre-dependent pAAV is described in Section 2.3.1. The single-stranded shRNA oligonucleotides were annealed and phosphorylated as previously outlined in Sections 2.1.1.1 and 2.1.3.

The pAAV-EGFP_mCherry(DO_DIO)-shRNA template plasmid was digested with HpaI (NEB, R0105) at 37˚C for 1 hour (refer to Figure 2.5) and dephoshphorylated to prevent religation (Sections 2.1.1.3 and 2.1.3). The linearised plasmid was purified by agarose gel electrophoresis on a 0.5% agarose, followed by gel extraction (see Section 2.1.2).

The vector and shRNA insert was ligated and chemically transformed into Stbl3 cells as previously outlined in Sections 2.1.4 and 2.1.5. Colony PCR was then performed to identify recombinant plasmids carrying the shRNA insert in the orientation with the following primers (5'-3'):

F (shRNA-specific primer): AGC CTT GTT TGA AGA CTT CA (for shα2) or AGC CTT GTT TAT ATA GGG AT (for shScr)

R: TCG TGA AGC GAG CTT ATC GAT A

Recombinant plasmids were extracted from the bacterial cells using the miniprep kit for DNA sequencing and using the maxiprep kit for subsequent experiments and rAAV production. The following primer pair was used to check sequence integrity of the ligated shRNA (5'-3'):

F: AGT TTG GTT AGT ACC GGG CC

R: TCG TGA AGC GAG CTT ATC GAT A

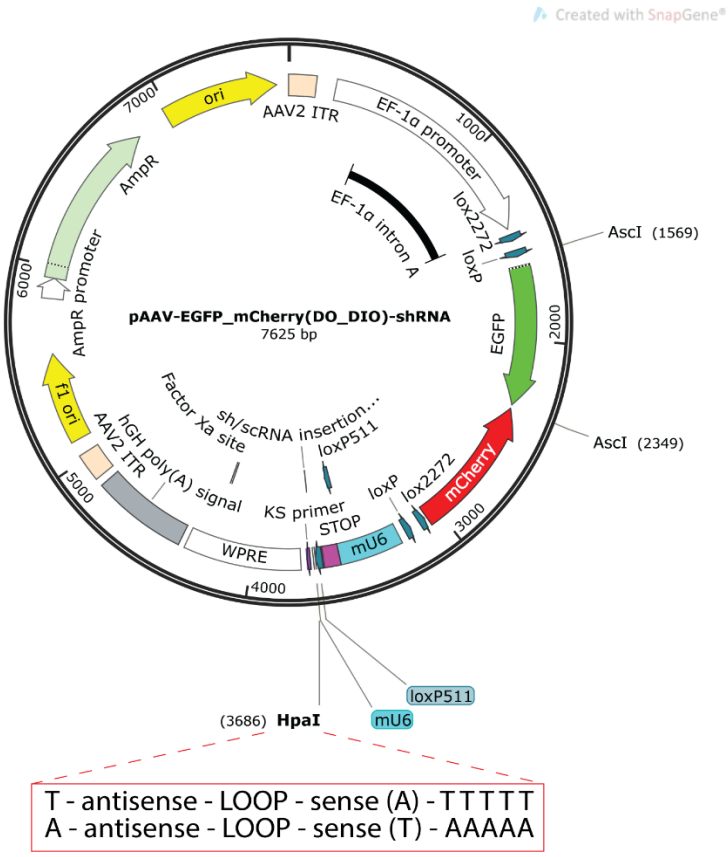



***Figure 2.5.*** shRNA cloning site within pAAV-EGFP_mCherry(DO_DIO)-shRNA

## 2.4. *In vitro* assays of pAAV functionality and shRNA potency

### 2.4.1. Assaying the knockdown potency of Gabra2-targeting shRNA constructs

Prior to the start of the experiment, the amount of pCMV-Gabra2 (Origene, MR225561) was amplified through bacterial chemotransformation

(grown in kanamycin-containing media) and the plasmid was extracted from the cells using the maxiprep kit as described in Sections 2.1.5 and 2.1.8.

Figure 2.6 illustrates the co-transfection conditions. All experimental conditions were set up in triplicates. Each well of cells was co-transfected with mouse Gabra2 cDNA-containing plasmid DNA and pAAV-EGFP-shRNA containing either shα2(A), shα2(B), or the shScr control at a 1:3 ratio (125ng Gabra2 cDNA + 375ng shRNA-containing plasmid), or sterile dd$H_2O$ (negative control) using Lipofectamine 3000 as described in Section 2.2.4. Next, cells were fixed in 4% PFA and immunofluorescent analyses of EGFP expression to probe for transfection efficiency and mouse α2 expression to probe the knockdown efficacy of the shα2 constructs relative to the control condition (co-transfection with Gabra2 cDNA and shScr) were performed as described in Section 2.2.5. For this purpose, cells were stained with the Chicken Anti-GFP (1:2000) and Rabbit Anti-α2 (1:500) primary antibodies. Secondary labelling with Donkey Anti-Chicken CF™ 488 (1:500) and Goat Anti-Rabbit Alexa Fluor 568 (1:500) was performed (refer to Table 2.2 for details). Quantitative assays of fluorescence intensity are described in Section 2.12.1

| | | | | | |
|---|---|---|---|---|---|
| pCMV-Gabra2 (125ng) + pAAV-EGFP-shα2(A) (375ng) | pCMV-Gabra2 (125ng) + pAAV-EGFP-shα2(A) (375ng) | pCMV-Gabra2 (125ng) + pAAV-EGFP-shα2(A) (375ng) | | | |
| pCMV-Gabra2 (125ng) + pAAV-EGFP-shα2(B) (375ng) | pCMV-Gabra2 (125ng) + pAAV-EGFP-shα2(B) (375ng) | pCMV-Gabra2 (125ng) + pAAV-EGFP-shα2(B) (375ng) | | | |
| pCMV-Gabra2 (125ng) + pAAV-EGFP-shScr (375ng) | pCMV-Gabra2 (125ng) + pAAV-EGFP-shScr (375ng) | pCMV-Gabra2 (125ng) + pAAV-EGFP-shScr (375ng) | | | |
| Negative control | Negative control | Negative control | | | |

***Figure 2.6***. Co-transfection conditions. HEK293 cells were transfected with pCMV-mGabra2-Myc and pAAV-EGFP-shRNA in a 24-well plate setup.

### 2.4.2. Assaying the efficacy of the Cre-dependent pAAV-EGFP_mCherry(DO_DIO)-shRNA

In this experiment, we assessed the functionality of the Cre-dependent switch of the fluorescent markers (i.e. EGFP and mCherry) and the functionality of the silencing (i.e. shα2(A)) construct. Note that prior to the start of the experiment, the amount of pAAV-Ef1a-Cre (kindly gifted by Karl Deisseroth, Addgene #55636) was amplified through bacterial chemotransformation (grown in ampicillin-containing media) and the

plasmid was extracted from the cells using the maxiprep kit as described in Sections 2.1.5 and 2.1.8.

HEK293 cells were seeded in a 24-well plate (on glass coverslips) and co-transfected with pAAV-EGFP-mCherry(DO_DIO)-shRNA, harbouring shScr or shα2 (214ng), pCMV-gabra2 (71ng), and pAAV-EF1α-iCre (214ng) at a 3:1:3 ratio using Lipofectamine 3000 (refer to Section 2.2.4). All experimental conditions were set up in triplicates (refer to Figure 2.7 for the experimental setup).

| Cre+ mCherry + EGFP staining experiment | | | α2 + mCherry staining experiment | | |
|---|---|---|---|---|---|
| pCMV-Gabra2 (71ng) + Cre-dependent pAAV- shα2 (214ng) + pAAV-Ef1a-Cre (214ng) | pCMV-Gabra2 (71ng) + Cre-dependent pAAV- shα2 (214ng) + pAAV-Ef1a-Cre (214ng) | pCMV-Gabra2 (71ng) + Cre-dependent pAAV- shα2 (214ng) + pAAV-Ef1a-Cre (214ng) | pCMV-Gabra2 (71ng) + Cre-dependent pAAV- shα2 (214ng) + pAAV-Ef1a-Cre (214ng) | pCMV-Gabra2 (71ng) + Cre-dependent pAAV- shα2 (214ng) + pAAV-Ef1a-Cre (214ng) | pCMV-Gabra2 (71ng) + Cre-dependent pAAV- shα2 (214ng) + pAAV-Ef1a-Cre (214ng) |
| pCMV-Gabra2 (71ng) + Cre-dependent pAAV- shScr (214ng) + pAAV-Ef1a-Cre (214ng) | pCMV-Gabra2 (71ng) + Cre-dependent pAAV- shScr (214ng) + pAAV-Ef1a-Cre (214ng) | pCMV-Gabra2 (71ng) + Cre-dependent pAAV- shScr (214ng) + pAAV-Ef1a-Cre (214ng) | pCMV-Gabra2 (71ng) + Cre-dependent pAAV- shScr (214ng) + pAAV-Ef1a-Cre (214ng) | pCMV-Gabra2 (71ng) + Cre-dependent pAAV- shScr (214ng) + pAAV-Ef1a-Cre (214ng) | pCMV-Gabra2 (71ng) + Cre-dependent pAAV- shScr (214ng) + pAAV-Ef1a-Cre (214ng) |
| Negative control | Negative control | Negative control | | | |
| | | | | | |

*Figure 2.7.* Co-transfection conditions (for two separate staining experiments) to assess the functionality of the RNAi Cre-dependent pAAV.

Two separate staining experiments were performed. Firstly, to assess whether the fluorescent switch was mediated by Cre recombinase, cells were stained with the Rabbit Anti-GFP (1:1000), Chicken Anti-mCherry (1:1000), and Mouse Anti-Cre (1:600) primary antibodies. Secondary labelling was then performed with Goat Anti-Rabbit Alexa Fluor 488 (1:500), Goat Anti-Chicken Alexa Fluor 568 (1:500), and Goat Anti-Mouse Alexa Fluor 647 (1:500) (Section 2.2.5)

Secondly, to assess the Cre-dependent shα2-induced Gabra2 knockdown, immunocytochemical staining was performed, whereby cells were stained Chicken Anti-mCherry (1:1000) and Rabbit Anti-α2 antibodies (1:500), followed by secondary labelling with Goat Anti-Chicken Alexa Fluor 568 (1:500) and Goat Anti-Rabbit Alexa Fluor 647 (1:500) (refer to Section 2.2.5). Quantitative measurements of α2 knockdown are described in Section 2.12.1.

Table 2.2

| Primary Antibodies | | | |
|---|---|---|---|
| **Antibody** | **Dilution** | | **Source** |
| | **ICC** | **IHC** | |
| Chicken Anti-GFP | 1:1000 | 1:10,000 | Abcam (RRID:AB_300798) |
| Rabbit Anti-α2 | 1:500 | 1:1000 | Synaptic Systems (RRID:AB_2108839) |
| Rabbit Anti-GFP | 1:1000 | 1:10,000 | Abcam (RRID:AB_303395) |
| Chicken Anti-mCherry | 1:1000 | 1:6000 | Abcam (RRID:AB_2722769) |
| Mouse Anti-Cre | 1:600 | 1:2000 | Millipore (RRID:AB_2085748) |

| Secondary Antibodies | | | |
|---|---|---|---|
| **Antibody** | **Dilution** | | **Source** |
| | **ICC** | **IHC** | |
| Donkey Anti-Chicken CF 488 | 1:500 | | Sigma-Aldrich (RRID:AB_2631230) |
| Goat Anti-Rabbit AF 568 | | | ThermoFisher (RRID:AB_143157) |
| Goat Anti-Mouse AF 647 | | | Abcam (ab150119) |
| Goat Anti-Rabbit AF 488 | | | Abcam (RRID:AB_2630356) |
| Goat Anti-Chicken AF 568 | | | Abcam (ab175477) |
| Goat Anti-Rabbit AF 647 | | | Abcam (RRID:AB_2714032) |

***Table 2.2.*** A list of the primary and secondary antibodies used for immunocytochemical and immunohistochemical analyses in this thesis.

## 2.5. Recombinant AAV (rAAV) production

### 2.5.1. Plasmid preparation

The pAAV-RC, containing the viral *rep* and *cap* genes, and pHelper, carrying the adenovirus E2A, E4, and VA RNA genes, from the AAV Helper-Free System (Stratagene, 240071) were provided by Dr. Ralph DiLeone (Yale University). These plasmids were amplified by bacterial chemotransformation and the DNA was extracted using the maxiprep kit (Sections 2.1.5 and 2.1.8). The concentration of each of these plasmids and the pAAV expression vectors (i.e. pAAV-EGFP-shRNA and pAAV-EGFP_mCherry(DO_DIO)-shRNA) was adjusted to 1μg/μl in TE buffer (pH 7.5) prior to rAAV production.

### 2.5.2. Preparing the HEK293 cells

Forty-eight hours before transfection, HEK293 cells were seeded into 30× 10cm dishes so that they were confluent on the day of transfection (refer to Section 2.2 for recovery and maintenance of HEK293 cells). Cells with a low passage number (<5) were used for transfection.

### 2.5.3. Transfecting HEK293 cells

For rAAV production, calcium phosphate transfection method was used. For each dish, 10μg (10μl) of each plasmid (pHelper, pAAV-RC, and pAAV expression vector) was added to a 15ml conical tube. Next, 1ml of 0.3M $CaCl_2$ (Sigma-Aldrich, 449709) was added to the DNA mixture and mixed gently. Then, 1 ml of 2× HEPES-buffered saline [280 mM NaCl

(Sigma-Aldrich, S3014), 1.5mM $Na_2HPO_4$ (Sigma-Aldrich, 255793), 50mM HEPES (Sigma-Aldrich, H3375), pH adjusted to 7.10 with NaOH] was added to a second 15ml conical tube. The 1.03ml of DNA-$CaCl_2$ mixture was then added to the 2× HBS solution dropwise and the solution was mixed by inverting the tube twice. The solution was then immediately applied to the dish of HEK293 cells in a dropwise manner while swirling the dish gently. The dish was returned to the 37˚C incubator (5% $CO_2$) for 6 hours. Note that the precipitate (small grains about the size of bacteria) should be visible approximately 1 hour after transfection. Six hours post-transfection, the medium was replaced with 10ml of fresh growth medium. The dishes were then incubated for 72 hours before harvesting the rAAVs.

### 2.5.4. Harvesting rAAVs

Seventy-two hours post-transfection, the colour of the growth medium turned yellow and a large amount of cell death was observed. Growth medium and cells were collected from the dishes by scraping the cells off the dish using cell scrapers (Thermo Scientific, 179693) and transferred into a 50ml conical tube. Four dishes were harvested at a time into the same 50ml tube for each rAAV and the cells were pelleted by centrifugation at 250*g* for 5 minutes at 20˚C. These steps were repeated until cells from all 30 dishes were pelleted.

Next, the pellet was resuspended in 8ml of lysis buffer (refer to Khan, Hirata, & Russell, 2011 for recipe) and subjected to two rounds of freeze-

thaw cycles in dry ice/ethanol bath and in a 37˚C water bath. The pellet was then stored at -80˚C until virus purification.

### 2.5.5. rAAV purification

The rAAVs in the present research was purified using the Iodixanol gradient purification method as described in the Khan et al. (2011) protocol.

### 2.5.6. Titering purified rAAV stocks

The rAAV stocks used in this thesis were purified according to the McClure et al. (2011) protocol. However, following cell fixation and prior to cell counting, transduced HEK293 cells were stained with the Rabbit Anti-GFP primary antibody (1:1000), followed by secondary labelling with Goat Anti-Rabbit Alexa Fluor 488 (1:500) (refer to Table 2.2 for further details on antibody dilutions).

## 2.6. Animals

Dopamine receptor D1- or D2-specific Cre recombinase hemizygous mice (strain name; D1-Cre = Tg(Drd1-cre)EY217Gsat; D2-Cre = Tg(Drd2-cre)ER44Gsat), supplied by Mutant Mouse Regional Resource Centers (MMRRC), were maintained at the University of Sussex by breeding D1-Cre or D2-Cre mice with wild-type (WT) C57BL/6 obtained from Charles River Laboratories. Some of the experimental WT mice used in this thesis were obtained from Charles River Laboratories (refer to specific experiments for more details on the number of animals used).

All mice were 7-8 weeks old at the start of experiments. They were housed under a 12-hour light/dark cycle (lights on at 7:00 A.M.), at the maintained humidity level of 50±5% and temperature of 21±2˚C. During Conditioned Reinforement (CRf) experiments (refer to Chapters 4 and 5), mice were food-restricted to 85%-90% *ad libitum* body weight one week before and throughout the behavioural experiment. All experiments were carried out in accordance with the United Kingdom (Home Office) Animal Act 1986.

## 2.7. Genotyping

### 2.7.1. DNA extraction

Mouse ear punches were collected and digested in a 20µl solution containing Proteinase K (1mg/ml final concentration, Roche, RPROTK-RO), 20mM Tris HCl (Sigma-Aldrich, 857645), and 10mM EDTA (Sigma-Aldrich, EDS) lysis buffer. Each solution was overlayed with two drops of mineral oil (Sigma-Aldrich, M5904), prior to incubation at 55˚C for 2 hours and at 95˚C for 15 minutes in a thermocycler. Extracted samples were then diluted in 100µl RNase-free $H_2O$ (Invitrogen, AM9932).

### 2.7.2. PCR

A pair of Cre primers were used to confirm the presence (or absence) of Cre (102 bp product) in D1- and D2-Cre mouse lines.

PCR primers used for Cre detection (5'-3'):

F: GCG GTC TGG CAG TAA AAC TAT C

R: GTG AAA CAG CAT TGC TGT CAC TT

PCR was performed using the Megamix Blue PCR mastermix (Microzone, 2MMB) according to the manufacturer's protocol (with 0.5µl of extracted DNA in each reaction). Solutions were overlayed with two drops of mineral oil, then incubated at 95˚C for 5 minutes (initial denaturation step), followed by 35 PCR cycles (95˚C for 30 seconds, 55˚C for 30 seconds and 72˚C for 1 minute), and incubation at 72˚C for 10 minutes (final extension step).

### 2.7.3. Agarose gel electrophoresis and DNA visualisation

PCR products were electrophoresed on a 1.5% agarose gel as described previously (Section 2.1.2) alongside a 100bp ladder. Gels were imaged using the UV transilluminator.

## 2.8. Stereotaxic viral injection

One day before surgery (and for 3 days post-surgery or until no pain symptoms present), Metacam (Meloxicam, Boehringer Ingelheim) was administered orally in wet mash.

Prior to the surgical procedure, mice were anaesthetised with a gaseous mix at 1L/min flow rate (70% $O_2$, 50% $N_2O$), containing isoflurane (Abbott Laboratories). Anaesthesia was induced using 3% isoflurane for around 3 minutes and maintained at 1-1.5% to keep the animal at a surgical plane. Once anaesthetised, mice were mounted onto the stereotax (Kopf Instruments) and stereotaxically infused with 1µl (0.5µl per side) of either saline or rAAV vectors expressing shScr or shα2, bilaterally into the NAc

core (coordinates AP 1.18; L+/-1.00; DV -4.20), shell (coordinates AP 1.18; L+/-0.50; DV -4.50), or into the dorsal striatum (coordinates AP+1.18, L+/-1.00, DV -3.30). Viral infusion was carried out using 33-gauge steel infusers (Cooper's needleworks) connected to 5µl Hamilton Gastight syringes via polyethylene tubing (Harvard Apparatus) at a rate of 0.2µl per minute for five minutes, and infusers were left to settle for additional five minutes. Infusers were gently removed and the scalp incision was closed with a non-absorbable, sterile polypropelene surgical suture (Ethicon, W8871T).

## 2.9. *In vivo* assays of α2 knockdown with the rAAV vectors

### 2.9.1. Knockdown of α2 expression with rAAV-EGFP-shα2

#### 2.9.1.1. Stereotaxic viral injection

Two groups of mice were used in this experiment, bilaterally injected with rAAV-EGFP-shScr (n = 7) or rAAV-EGFP-shα2(A) (referred to as shα2; n = 8) into the NAc core (coordinates: AP +1.18, L ±1, DV +4.20 (Paxinos and Franklin, 2001). The procedure for stereotaxic viral injection is described in Section 2.8.

#### 2.9.1.2. Immunohistochemical analysis

Four weeks post-surgery, four animals from each group were deeply anaesthetised with 200mg/kg sodium pentobarbital and transcardially perfused with ice-cold 1× PBS for 5 minutes, then with 4% PFA (Sigma-Aldrich, P6148) in PBS for 20 minutes. Brains were removed, then further

fixated in 4% PFA overnight and cryoprotected in 30% in sucrose-PBS solution. Next, brains were frozen in crushed dry ice.

Coronal sections (30µm in thickness) were prepared using a Leica CM1900 cryostat. Sections, containing the striatum, were free-floated in 1× TBS (50 mM Tris-Cl (Sigma-Aldrich, 93352), pH 7.6; 150 mM NaCl) were rinsed twice in TBS for 1 minute each with gentle agitation, blocked in 5% normal goat serum (Vector Laboratories, S-1000) in 0.2% Triton X-100 TBS (TBS-Tx). The sections were then stained overnight at 4˚C in the Chicken Anti-GFP (1:10000) and Rabbit Anti-α2 (1:1000) antibodies in TBS-Tx. Sections were rinsed three times (five minutes each) with TBS to remove excess primary antibodies prior to secondary labelling with Donkey Anti-Chicken 488 (1:500) and Goat Anti-Rabbit 568 (1:500) for 2 hours. This and the following steps were performed in the dark given the light sensitive nature of the fluorophores. Sections were then mounted onto Superfrost slides (Cole-Parmer, WZ-48512-00) with Fluoroshield. Images were captured using the Leica TCS SP8 confocal system attached to a DMI 6000 AFC Inverted Motorised Research Microscope at 20x magnification (zoom factor 1), and analysed in Fiji.

#### 2.9.1.3. Quantitative reverse transcriptase PCR (qRT-PCR) mRNA analysis

##### 2.9.1.3.1. Preparation of tissue samples and phase separation

Four weeks post-surgery, brains of three mice injected with shScr-containing rAAVs and those of four mice injected with rAAV-EGFP-shα2

were dissected and tissue samples from the NAc were collected using a 1.5mm biopsy punch (Kai Medical Inc., BP15F).

Accumbal punches were homogenised in 600µl of Trizol (Invitrogen, 15596026) and 200µl of RNAse-free $H_2O$ (Invitrogen, AM9932). Chloroform (160µl; Sigma-Aldrich, 528730) was then added to the homogenised sample and phase separated in a peqGOLD PhaseTrap A phase-lock tube (PEQLAB) by centrifugation for 15 minutes.

#### 2.9.1.3.2. RNA precipitation

The nucleic acid-containing aqueous layer was decanted into a new 1.5ml Eppendorf tube, then mixed with isopropanol (Fisher BioReagents, 10284250), 50µl of sodium acetate (Invitrogen, AM9740) and 5µl of glycoblue (Invitrogen, AM9516). Each sample was then incubated at -80˚C overnight and thawed at room temperature before centrifugation (12,000*g*) at 4°C for 20 minutes until a blue RNA pellet was formed. The supernatant was discarded and the RNA pellet was washed with 1ml of 75% EtOH (Fisher BioReagents, 10041814) for 5 minutes at 4°C. The wash was removed and the pellet was left to air dry for approximately 30 minutes before being resuspended in 87.5µl of RNase-free $H_2O$.

#### 2.9.1.3.3. RNA cleanup

RNA cleanup was performed using the RNeasy MinElute Cleanup Kit (Qiagen, 74204) according to the manufacturer's instructions. The amount

and purity of RNA were measured using the Nanodrop 2000 Spectrophotometer.

#### 2.9.1.3.4. cDNA synthesis

The iScript cDNA Synthesis kit (Bio-Rad, 1708891) was used for cDNA synthesis with 100ng of each RNA sample according to the manufacturer's protocol. The amount and purity of cDNA were measured using Nanodrop 2000 Spectrophotometer.

#### 2.9.1.3.5. qPCR

The qPCR assays were performed using the QuantiTect SYBR Green PCR kit (Qiagen, 204145) according to the manufacturer's instructions. Sample concentrations were determined based on the serial dilution concentration curves and each reaction was set up in triplicate. 1µl of each sample was used as the template cDNA (or 1µl of RNase-free $H_2O$ as the no template control), with 0.3µM of each of the forward and reverse primers (GAPDH (housekeeping gene), GFP, and $GABA_AR$ α2 subunit primer pairs; see Table 2.3). The total reaction volume of each reaction was 20µl. The qPCR assay was performed on the Mx4000 multiplex quantitative PCR sampler (Stratagene). Quantification of mRNA expression levels is described below in Section 2.12.2. Note that the α2 primer pairs were positioned upstream of the RNAi-mediated excision site for a more accurate measure of gene expression knockdown (Holmes, Williams, Chapman, & Cross, 2010).

Table 2.3

| Primer | Forward (5'-3') | Reverse (5'-3') |
|---|---|---|
| GAPDH | TGCCCCCATGTTTGTGATG | TGTGGTCATGAGCCCTTCC |
| $GABA_AR$ α2 subunit (Gabra2) | TGGCTGAACAGAGAATCGGT | TCCCAAGCCCATCCTCTTTT |
| EGFP | CGCACCATCTTCTTCAAGGACGAC | GCGGATCTTGAAGTTCACCTTGATGCC |
| mCherry | GAACGGCCACGAGTTCGAGA | CTTGGAGCCGTACATGAACTG |

***Table 2.3.*** Primer sequences used for qRT-PCR assays of RNAi-mediated α2 knockdown.

## 2.9.2. Knockdown of α2 expression with rAAV-EGFP_mCherry(DO_DIO)-shα2

### 2.9.2.1. Confirmation of mouse genotype by *in situ* hybridisation (RNAscope)

The presence of Cre in either D1R- or D2R-harbouring neurons in D1- (n = 3) or D2-Cre (n = 3) mice respectively was assessed by fluorescent *in situ* hybridisation, i.e. RNAscope Fluorescent Multiplex reagent kit (Advanced Cell Diagnostics, 320850). The probes used for this assay, designed by Advanced Cell Diagnostics, targeted Cre (GenBank accession number KC845567.1; Cat no. 312281-C2) and Drd1a (GenBank accession

number NM_010076.3; Cat no. 406491-C3) or Drd2 (GenBank accession number NM_010077.2; Cat no. 406501) transcripts.

For this assay, mice were sacrificed by cervical dislocation and the brains were removed and rapidly frozen in isopentane (Sigma-Aldrich, 270342) at -50˚C. Next 10µm striatal slices were prepared using a Leica CM1900 cryostat and RNAscope *in situ* hybridisation was performed as previously described (Rubio et al., 2015). The probes were incubated with the brain sections at 40˚C for 2 hours. The sections from each genotype (i.e. D1- or D2-Cre mice) were hybridised with probes against Cre recombinase, and Drd1a or Drd2 transcripts to visualise the presence (or absence) of Cre in a specific cell population. Next, they were incubated with the three-step preamplifier an amplifier probes prior to incubation with the fluorescently-labelled probes (Atto 550 and Atto 647). Finally, the slides containing brain sections were coverslipped with VECTASHIELD HardSet Antifade Mounting Medium with DAPI (Vector Laboratories, H-1500) and imaging was performed on the Olympus BX53 microscope (at 20x magnification). Images were captured using the QI click camera (Qimaging) attached to the microscope, viewed using the iVision software (version 4.0.15, Biovision Technologies), and analysed in Fiji.

#### 2.9.2.2. Immunohistochemical analysis of the Cre-mediated fluorescent switch

D2-Cre (n = 3) and WT (n = 3) male mice were injected with rAAV-EGFP_mCherry(DO_DIO)-shα2 into the NAc core as previously described in

Section 2.8. Four weeks post-surgery, animals were perfused, brains were removed, cryopreserved and cut into 30μm sections using a cryostat. Sections were then subjected to immunohistochemical analyses as described in Section 2.9.1.2. Sections were stained with Rabbit Anti-GFP (1:10,000), Chicken Anti-mCherry (1:6000), and Mouse Anti-Cre (1: 2000) primary antibodies, followed by secondary labelling with Goat Anti-Rabbit 488 (1:500), Goat Anti-Chicken 568 (1:500), and Goat Anti-Mouse 647 (1:500).

#### 2.9.2.3. qRT-PCR analysis

Five D2-Cre mice, injected with Cre-dependent rAAVs harbouring shScr (n = 3) or shα2 (n = 2) were used. The qPCR assay was performed as described in Section 2.9.1.3 with GAPDH (housekeeping gene), mCherry, and $GABA_AR$ α2 subunit primer pairs (refer to Table 2.3).

## 2.10. Conditioned Reinforcement

### 2.10.1. Animals

A total of 97 mice were used for the conditioned reinforcement (CRf) experiments in this thesis (refer to Table 2.4 for more details). Thirty-nine of these mice were obtained from Charles River Laboratories, while the rest were bred and maintained at the University of Sussex. All mice were 7-8 weeks old at the start of the experiment (refer to Section 2.6 for maintenance conditions).

Table 2.4

| Genotype | Number | | Breeding |
|---|---|---|---|
| | Male | Female | |
| Wild-type | 39 | 0 | Charles River Laboratories |
| Wild-type | 11 | 9 | University of Sussex |
| D1-Cre | 11 | 8 | University of Sussex |
| D2-Cre | 10 | 9 | University of Sussex |

*Table 2.4.* The number and genotype of animals used in the conditioned reinforcement experiment (refer to Chapter 4, Figure 4.2 for details on viral manipulations).

### 2.10.2. Apparatus

Eight operant chambers (Med Associates Inc.), enclosed in sound-attenuating boxes, were used to assess conditioned reinforcement. Each animal was placed in a single unit, equipped with a food magazine delivering sweetened pellets (5TUL; Test Diets, 1811142), two nose-poke modules, a tone generator above the food magazine and two LED stimulus lights located opposite the nose-poke modules, and a ventilation fan. Head entries into the food magazine were detected using an infrared photodetector. The number of head entries and/or nose-poke inputs in both training and testing phases was recorded using the Med-PC IV software.

### 2.10.3. Drugs

Cocaine Hydrochloride (Macfarlan Smith, UK) was dissolved in 0.9% saline solution to a reach a final concentration of 0, 3, or 10 mg/kg and injected into the peritoneal cavity at a volume of 10 ml/kg.

### 2.10.4. Stereotaxic viral injection

Animals were bilaterally injected with the rAAV-EGFP-shRNA (Chapter 4) or with the rAAV-EGFP_mCherry(DO_DIO)-shRNA (Chapter 5) into the NAc core. The procedure for stereotaxic viral injection surgery and coordinates were detailed in Section 2.8.

### 2.10.5. Pavlovian Conditioning

Three weeks post-surgery, all animals received at least one week of acclimatisation to food restriction to maintain 85-90% of free-feeding body weight, which continued throughout the experiment. Mice then underwent 10 daily 60-minute Pavlovian training sessions (with no house light illumination) in the operant chambers. In each session, two stimuli were presented; 16 presentations of 10-second flashing lights and 16 presentations of 10-second tone. One (CS+) always served to predict food delivery, i.e. stimulus commenced five seconds prior to food delivery and continued for five seconds after, whereas presentations of the other (CS-) yielded no outcome (counterbalanced across animals). The order of stimulus presentation was randomly mixed and each stimulus presentation was separated by variable inter-trial intervals (i.e. 80-120s between trials). The

percentage of magazine entries during the presentations of CS+ or CS- was then calculated to provide a measure of Pavlovian conditioning. To ensure stable levels of performance, we established a learning criterion (≥80% magazine entries during CS+ presentations over the 60-minute session).

### 2.10.6. Conditioned Reinforcement

Following the completion of Pavlovian training, two nosepoke modules were introduced in each operant chamber. Mice were trained to nosepoke into the 'active' module to yield CS+ presentation. Nosepoking into the 'inactive' module led to CS- presentation (counterbalanced across animals). Rates of nosepoke responses for CS+ and CS- were measured following i.p. cocaine, ie. 0, 3, and 10mg/kg, in a Latin square arrangement.

### 2.10.7. Immunohistochemical analyses of rAAV placement

#### 2.10.7.1. α2 knockdown in wild-type mice with rAAV-EGFP-shα2

Immunohistochemical analyses were performed as described in Section 2.9.1.2

#### 2.10.7.2. α2 knockdown in D1-or D2-Cre mice with rAAV-EGFP_mCherry(DO_DIO)-shα2

Immunohistochemical analyses were performed as described in Section 2.9.1.2. Sections were incubated with Rabbit Anti-GFP (1:10,000) and Chicken Anti-mCherry (1:6000) primary antibodies, followed by secondary labelling with Goat Anti-Rabbit Alexa Fluor 488 (1:500) and Goat Anti-Chicken Alexa Fluor 568 (1:500).

## 2.11. Cocaine-induced locomotor sensitisation

### 2.11.1. Animals

A total of 34 wild-type C57BL/6 mice, bred at Sussex University, were used in the sensitisation experiments (males = 16, females = 18) (refer to Figure 4.2 in Chapter 4 for more details). All animals were 7-8 weeks of age at the start of the experiment (refer to Section 2.6 for details on housing conditions).

### 2.11.2. Apparatus

Locomotor activity was measured in 16 annular black Perspex runways, with a diameter of 24cm and annulate width of 6.5cm. These were placed in a clouded Perspex sheet on an elevated frame. A digital camera, positioned underneath the sheet, was used to capture the silhouettes of the boxes' edges and the mice within them. The MatLab (MathWorks, UK) video analysis programme and Microsoft Excel macro were used to transform video data into a quantitative measure of distance travelled (in meters).

### 2.11.3. Drugs

Cocaine Hydrochloride (Macfarlan Smith, UK) was dissolved in 0.9% saline solution to a reach a final concentration of 10 mg/kg and injected into the peritoneal cavity at a volume of 10 ml/kg.

### 2.11.4. Stereotaxic viral injection

Mice in the sensitisation experiments were bilaterally injected with shα2- or shScr-containing rAAV-EGFP-shRNA vectors into the NAc core or shell (refer to specific experiments in Chapter 4). The stereotaxic viral injection surgery is detailed in Section 2.8.

### 2.11.5. Locomotor sensitisation to cocaine

Prior to the sensitisation experiment, all mice underwent two habituation sessions on two consecutive days. On the first day, mice were habituated to the runway for 60 minutes before being returned to the homecages and on the second day, they were habituated to the equipment for 30 minutes, then received sham i.p. injections followed by a 60-minute habituation session. Subsequently, all mice received repeated treatment of 10 mg/kg cocaine for 10 consecutive daily sessions. Their locomotor activity was recorded for 60 minutes in each session.

### 2.11.6. Conditioned activity

Seven days following the completion of the sensitisation experiment described above, all mice received 10 ml/kg saline injections and their activity within the runway was recorded for 60 minutes.

### 2.11.7. Immunohistochemical analyses of rAAV placement

Immunohistochemical analyses were performed as described in Section 2.9.1.2

## 2.12. Statistical analyses

### 2.12.1. Immunocytochemistry/immunohistochemistry

All fluorescent images from the immunocytochemical and immunohistochemical experiments were analysed using Fiji. The potency of the shα2 constructs designed in the present research was quantified by measuring differences in the average fluorescence intensity (integrated density value; IDV) of α2, normalised to the corresponding IDV of GFP or mCherry (for the Cre-dependent rAAVs) to control for variations in transfection/transduction efficiency in the shα2 condition(s) relative to that in the shScr group. Data are presented as the mean fold change ± SEM.

All statistical analyses described here and in subsequent sections were performed using SPSS Statistics 24 (IBM), and data were plotted using GraphPad Prism (GraphPad Software). First, a one-way analysis of variance (ANOVA) was performed to analyse differences in α2 expression upon transfection of HEK293 cells with pAAV-EGFP-shRNA constructs harbouring either shScr, shα2 (A), or shα2 (B) with group as the independent variable and the normalised α2 IDV as the dependent variable. Independent-sample t-tests were also carried out to compare the IDV of α2 expression (normalised to that of GFP) in accumbal neurons of mice transduced with rAAV-EGFP-shα2 and those transduced with rAAV-EGFP-shScr, as well as to compare the IDV of α2 expression (normalised to that of mCherry) in HEK293 cells transfected with shScr- or shα2-harbouring Cre-dependent pAAVs.

### 2.12.2. qRT-PCR

Data on the RNA expression level were collected using the Mx4000 data analysis software (Stratagene, CA, USA), then exported to an Excel worksheet. Averaged α2 reaction triplicates were normalised against GFP (or mCherry for the Cre-dependent rAAVs) to provide a measure of the delta CT. Normalising the CT value of α2 against that of GFP or mCherry would account for variations in transduction efficiency of the virus. The delta CT value of the accumbal samples obtained from mice injected with the shα2-containing rAAVs was then normalised against those of mice injected with the shScr-containing rAAVs to give a measure of the delta delta CT. Lastly, a mathematical model was used to calculate the fold change based on the delta-delta CT value (Pfaffl, 2001).Independent t-tests were then performed to analyse differences in α2 mRNA levels in the NAc of mice injected with the shα2-containing rAAVs relative to those injected with shScr-containing rAAVs, with group as an independent variable and delta CT as the dependent variable.

### 2.12.3. *In situ* hybridisation (RNAscope)

Colocalisation of the Cre signal with that of Drd1a or Drd2 visualised using the *in situ* hybridisation technique was quantified by first defining the region of interest (i.e. cell nuclei via DAPI staining), then, measuring bivariate Pearson's correlation cefficient of fluorescence intensity (i.e. IDV) of Cre and that of Drd1a or Drd2, given that the expression of Cre is

controlled by Drd1a or Drd2 promoters in D1- or D2-Cre mice respectively (Gong et al., 2007).

### 2.12.4. Conditioned Reinforcement

#### 2.12.4.1. Pavlovian Conditioning

Pavlovian conditioning data presented in Chapters 4 and 5 were assessed using three-way mixed ANOVAs with groups as the between-subjects factor, conditioned stimulus (CS; CS+ and CS-) and session as the within-subjects factors, and magazine entries as the dependent variable. The latency to approach food magazine following CS+ onset was also analysed using two-way mixed ANOVAs, with group as the between-subjects factor and session (1 vs. 10) as the within-subjects factor with the latency (in seconds) as the dependent variable.

Importantly, note that for all ANOVAs described here and throughout this thesis, assumptions of normality (Kolmogorov-Smirnov's test) and homogeneity of variance (Levene's test) were checked. If violated, log-transformed data were used. The assumption of sphericity was also checked and violation of this assumption led to the use of Greenhouse-Geisser's corrected value.

#### 2.12.4.2. Conditioned Reinforcement

The baseline rates of nosepoke responses for the conditioned reinforcer (CR) and the non-conditioned reinforcer (NCR) presentations were analysed using two-way mixed ANOVAs, with stimulus-associated

nosepoke modules (i.e. nosepokes) as the within-subject factor, group as the between subject factor and the nosepoke responses for the CR vs. NCR as the dependent variable.

The pattern of CR-maintained responding over the 60 minute session, broken down in 10 minute time bins, was further analysed using two-way mixed ANOVAs with time as the within-subjects factor and group as the between-subjects factor, with the number of nosepoke responses in each 10 minute timebin as the dependent variable.

#### 2.12.4.3. Cocaine effects on Conditioned Reinforcement

To assess cocaine effects on responding for CRf, three-way mixed ANOVAs were performed with group as the between-subjects factor, and cocaine dose and stimulus-associated nosepoke modules (i.e. nosepokes) as the within-subject factors. To aid interpretation of the three-way interaction, post-hoc two-way repeated measures ANOVAs (with Bonferroni correction for multiple comparisons), with nosepokes and cocaine dose as the within-subject factors were performed, with the number of nosepoke responses as the dependent variable.

### 2.12.5. Cocaine-induced Sensitisation

#### 2.12.5.1. Locomotor response to acute cocaine

Locomotor response to cocaine was measured by statistical comparison of the average distance travelled post sham injection prior to the sensitisation procedure and that following the first cocaine injection (i.e.

sensitisation day 1). Two-way mixed ANOVAs were performed with group as the between-subjects factor and cocaine dose as the within-subjects factor, with the distance travelled in meters as the dependent variable.

### 2.12.5.2. Sensitisation

Locomotor sensitisation to cocaine in mice injected with rAAV-EGFP-shα2 or rAAV-EGFP-shScr, in the NAc core or shell, was analysed using two-way mixed ANOVAs with group as the between-subjects factor and session (either Session 1 to Session 10, or Sessions 1 and 10) as the within-subjects factor. The distance travelled in meters was the dependent variable.

### 2.12.5.3. Conditioned activity

Conditioned activity following repeated cocaine exposure was analysed using two-way mixed ANOVAs with group as the between-subjects factor and sham injection (pre- vs. post-sensitisation) as the within-subjects factor, and meters travelled in the locomotor runway as the dependent variable.

# Chapter 3

# Design and construction of mouse Gabra2-targeting RNAi vectors

## 3.1. Introduction

Polymorphic variation of the $GABA_A$ receptor α2 subunit-encoding gene, *GABRA2* (or Gabra2 in the mouse), is a well-established genetic marker of alcohol dependence in various populations (Edenberg, Dick, Xuei, & Tian, 2004; Enoch, 2008; Matthews, Hoffman, Zezza, Stiffler, & Hill, 2007; Philibert et al., 2009) and is more recently linked to cocaine addiction (Dixon et al., 2010; Enoch et al., 2010). The intronic *GABRA2* single-nucleotide polymorphism (SNP) rs11503014, located within the 5' untranslated region (UTR) of the alternative *GABRA2* transcript and thought to be implicated in exon splicing, has been associated with cocaine addiction particularly in African-American individuals with a history of childhood adversity (Enoch et al., 2010). Haplotype analyses of subjects of Caucasian origin (with a mix of African and Native American ancestry) further indicated that the susceptibility to develop cocaine addiction is linked to the SNP rs894269, located in the promoter block approximately 9kb away from the transcription start site, alongside other markers (rs279871, rs279845, and rs279836) within the *GABRA2* transcript. A protective haplotype, i.e. rs894269 (T), rs2119767 (T), rs929128 (G) positioned within the promoter block, has also been identified. Individuals carrying this haplotype are approximately 30% less likely to develop cocaine addiction, relative to those carrying alternative ones (Dixon et al., 2010).

If rs11503014 (or other risk SNPs in the vicinity) is in fact implicated in exon splicing, it may influence *GABRA2* expression, thus providing a potential molecular mechanism by which *GABRA2* contributes to the development of cocaine addiction. Some support for this interpretation is derived from a recent study using the induced pluripotent stem cell (iPSC) model, demonstrating that neural cultures derived from *GABRA2* rs279858*C risk allele carriers, previously linked to alcohol dependence, displayed significantly lower *GABRA2* mRNA expression (Lieberman et al., 2015).

Using mice devoid of the $GABA_AR$ α2 subunit, we have previously established that Gabra2 is not critical for the primary rewarding effects of cocaine, but is paramount for its "energising" effects on motivated behaviours. Namely, whole-brain deletion of Gabra2 blocked cocaine-induced locomotor sensitisation and cocaine-potentiated CRf (Dixon, Halbout, King, & Stephens, 2014; Dixon et al., 2010). Some of these findings have recently been extended to another psychostimulant drug, i.e. methylphenidate (Duka et al., 2015).

Central to mediating these behavioural effects is the psychostimulants' ability to elevate extracellular dopamine concentration in the NAc (Kalivas & Duffy, 1993; Parkinson, Olmstead, Burns, Robbins, & Everitt, 1999; Williams & Steketee, 2005; Wolterink et al., 1993), where $GABA_AR$ α2 subunit is the predominant $GABA_AR$ (Chen et al., 2007; Dixon et al., 2010; Hörtnagl et al., 2013; Pirker, Schwarzer, Wieselthaler, Sieghart, & Sperk, 2000). It is thus conceivable that the phenotypic disparities

observed between Gabra2 null mutants and the wild-type (WT) counterparts might have been attributed to the loss of α2, specifically in the NAc. To address this hypothesis, this study sought to develop viral-based RNAi tools, which continue to be routinely used to study gene function in discrete brain regions (Hommel et al., 2003; Salahpour, Medvedev, Beaulieu, Gainetdinov, & Caron, 2007). These molecular tools would not only aid in providing further anatomical specificity to our previous knockout data presented in the Dixon et al. (2010) study, but would also be valuable for advancing our understanding of the functional significance of α2-$GABA_A$Rs in the brain.

In concept, the fundamental question that gene knockout- and RNAi-based studies seek to answer are somewhat aligned, i.e. what is the phenotype when gene X is silenced? It is however important to note that RNAi downregulates, rather than completely ablates, gene expression to varying degrees depending on the efficacy of the silencing construct, i.e. si/shRNA, as well as on the proportion of transduced cells, thus yielding hypomorphic phenotypes that do not always mirror the loss-of-function phenotypes induced by genetic mutations. Thus, in many cases, its application serves to complement rather than fully substitute gene targeting approaches, e.g. gene knockout, for investigating gene function (Boettcher & McManus, 2015; Salahpour et al., 2007).

As detailed in Chapter 1, the RNAi technology reduces gene expression through base-pair interactions between the siRNA and the target mRNA, thus inhibiting protein synthesis with an unprecedented degree of specificity (Elbashir, 2001; Fire et al., 1998). The high efficacy and success

rate of RNAi-induced gene expression silencing has been documented in the literature using various cell types, including neurons (Heitz et al., 2014; Hommel et al., 2003; Krichevsky & Kosik, 2002). However, a proper selection of functional siRNA remains to be one of the most challenging aspects of RNAi-based gene silencing and although there is no guarantee of a successful knockdown until empirically proven, numerous groups have developed siRNA design algorithms in the continuing effort to maximise the potentials of RNAi-based gene expression knockdown (for examples, see Huesken et al., 2005; Reynolds et al., 2004; Ui-Tei et al., 2004; Vert, Foveau, Lajaunie, & Vandenbrouck, 2006).

Several lines of research to date have employed the Cre-regulated RNAi to exert greater temporal and spatial control over gene expression. Namely, transcription of shRNA is enabled by the presence of Cre recombinase (Cre), thus gene expression knockdown only occurs in Cre-expressing cells (i.e. CRE-ON system) (Fritsch et al., 2004; Kasim, Miyagishi, & Taira, 2004; Ventura et al., 2004). In brief, Cre/Lox recombination involves Cre-mediated excision of specific DNA sequences flanked by a *direct repeat* of loxP sites (Sauer & Henderson, 1988). Alternatively, if the DNA sequence of interest is flanked by two pairs of *inverted* lox sites (e.g. loxP and lox511 pairs), a one way, Cre-dependent genetic inversion (FLEx switch) occurs. Both lox sites are recognised by Cre, however, an efficient recombination occurs only between pairs of identical lox sites. For instance, lox511 can only recombine with another lox511, albeit not with loxP (Schnütgen et al., 2003). To achieve the genetic switch,

Cre-mediated recombination may first induce the inversion mediated by either loxP or lox511 sites, producing a direct repeat of either loxP or lox511 pairs. This is then followed by Cre-mediated excision of DNA sequence located between the two loxP/lox511 sites, leading to an irreversible switch (Figure 3.1).

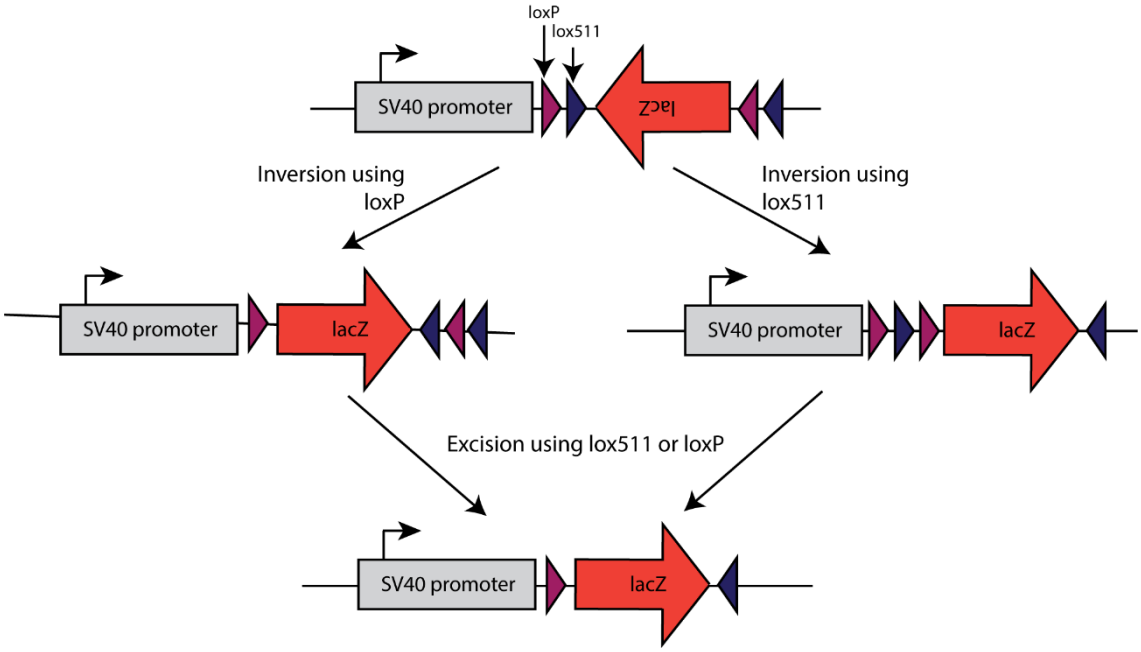



***Figure 3.1.*** The FLEx switch system (adapted from Schnütgen et al., 2003)

The α2-containing receptors are preferentially expressed on the striatal MSNs, i.e. D1- and D2-MSNs, thought to play somewhat distinct roles in motor control and motivated behaviours (Durieux, Schiffmann, & de Kerchove d'Exaerde, 2012; Natsubori et al., 2017; Smith, Lobo, Spencer, & Kalivas, 2013; Soares-Cunha et al., 2016). It is also therefore of interest to investigate cell-type-specific roles of mesoaccumbal α2 and one plausible method to address this is by utilising Cre-regulated RNAi tools in either D1- or D2-Cre transgenic mice. However, there currently exists no suitable Cre-

dependent RNAi vector that also permits the visualisation of Cre activity, where shRNA activation occurs. This study thus further aimed to construct a novel Cre-dependent RNAi vector, which harbours: (i) two fluorescent markers (EGFP and mCherry) to enable the visualisation of Cre-positive and Cre-negative neurons using the FLEx switch system (A. Saunders, Johnson, & Sabatini, 2012; Schnütgen et al., 2003), and (ii) an α2-targeting shRNA construct (shα2) or its non-targeting scrambled control (shScr), placed under the control of the lox site-containing mutant mouse U6 promoter (Ventura et al., 2004).

Finally, all shRNA vectors generated in the present research were packaged into recombinant AAV2 (rAAV2) vectors for targeted delivery into the region of interest (mainly the striatal subcompartments). This study specifically harnessed the restricted transduction efficiency or viral spread (Aschauer et al., 2013) and the preferential neuronal tropism of rAAV2 (Hsueh et al., 1998) for highly specific targeting of neurons in NAc subcompartments, i.e. core and shell.

## 3.2. Experimental design

In this chapter, we constructed and tested the efficacy of RNAi vectors targeting the mouse Gabra2 transcripts (with the non-targeting scrambled control). A brief summary of the experimental timeline, comprising the design, construction, and assays of the Gabra2-targeting RNAi vectors, is depicted in Figure 3.2.


***Figure 3.2.*** An experimental workflow of the rAAV-shRNA design and production.

## 3.3. Results

### 3.3.1. shRNA design

Figure 3.3A demonstrates the stem-loop structure of the shRNA used in the present research (Hommel et al., 2003). The stem portion comprises a sense strand, comprising a 24-nt region of the α2 transcript or a scrambled control sequence and the corresponding antisense strand. A one-base mismatch was introduced in the former, positioned 5' of the PolyT stretch, to aid antisense strand incorporation into the RISC (Hommel et al., 2003). The 10-nucleotide loop consists of bases with low complementarity to avoid loop collapse and non-canonical cleavages (Gu et al., 2012; Li et al., 2007). Two shα2 constructs (i.e. shα2(A) and shα2(B)) were designed and tested in the present research. The stem portion of the shRNAs comprises 24-nt α2-

targeting siRNA sequences (i.e. siα2(A) and siα2(B)), which were designed in compliance with the Reynolds' siRNA design algorithm (Reynolds et al., 2004). The siα2(A) and siα2(B) scored 6 and 7 points based on the algorithm respectively.

Table 3.1

| Sequence | Sense (5'-3') | Antisense (5'-3') |
|---|---|---|
| shα2(A) | GGA GAC AGT ATT ACT GAA GTC TTC | GAA GAC TTC AGT AAT ACT GTC TCC |
| shα2(B) | GGA TGA TGG AAC ATT GCT ATA TAC | GTA TAT AGC AAT GTT CCA TCA TCC |
| shScr | GGA TGC TAG AAC ATC CCT ATA TGC | GCA TAT AGG GAT GTT CTA GCA TCC |

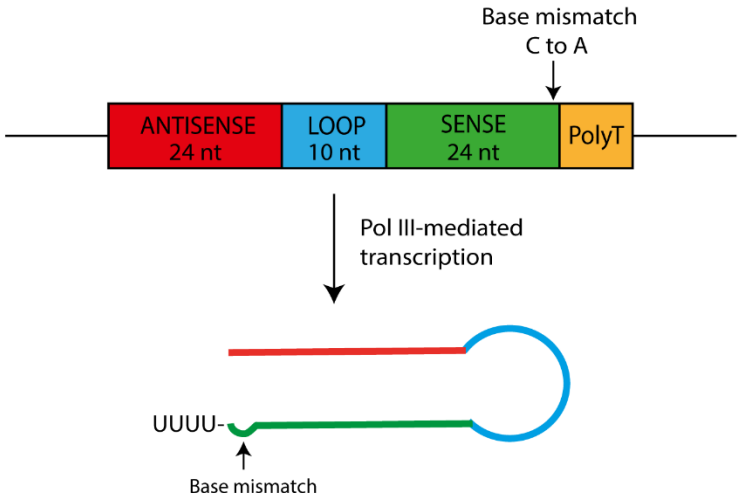
***Table 3.1.*** Sense and antisense siRNA sequences of Gabra2-targeting and scrambled shRNA constructs.

A scrambled hairpin construct (shScr) harbours a non-targeting siRNA sequence. A BLAST analysis against the mouse genome revealed that scr is unlikely to target α2 transcripts, either by siRNA-mRNA interaction or by a miRNA-like seed pairings (Carthew & Sontheimer, 2009; Ellwanger, Büttner, Mewes, & Stümpflen, 2011). The list of siRNA sequences used in the present research is vsummarised in Table 3.1.

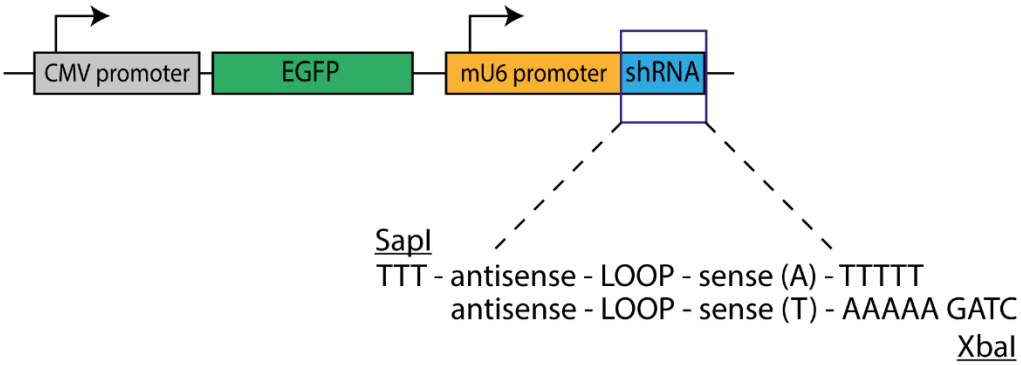
### 3.3.2. Construction of the pAAV-EGFP-shRNA vector

Each shRNA construct was ligated into the shRNA expression vector (i.e. pAAV-EGFP), kindly donated by Ralph DiLeone (Yale University; also used in Hommel et al., 2003). To facilitate ligation into the vector, SapI and XbaI recognition sites were added to the ends of the shRNA oligonucleotides, as illustrated in Figure 3.3B. The loss of SapI site represents successful ligation of the shRNA construct into the pAAV-EGFP vector. Sequence integrity was then confirmed by DNA sequencing (Eurofins Genomics) (Figure 3.3C).

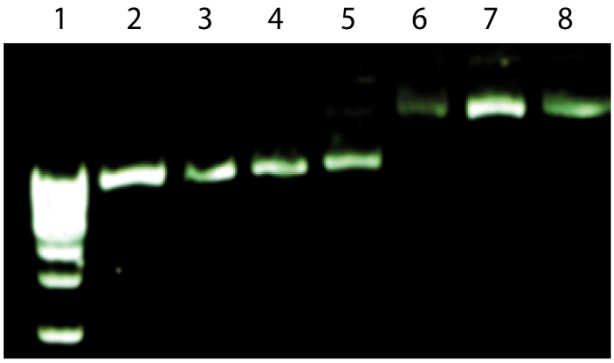
(A)




(B)




(C)




Lane 1 - 1kb ladder
Lane 2 - pAAV-EGFP (digested with XbaI)
Lane 3 - pAAV-EGFP (digested with SapI)
Lanes 4&5 - pAAV-EGFP-shRNA (digested with XbaI)
Lane 6 - pAAV-EGFP (uncut)
Lane 7 - pAAV-EGFP-shRNA (uncut)
Lane 8 - pAAV-EGFP-shRNA (digested with SapI)

***Figure 3.3.*** Construction of pAAV-EGFP-shRNA vector **(A)** The shRNA stem-loop structure. **(B)** SapI and XbaI recognition sites were added to aid shRNA insertion into the pAAV-EGFP vector and **(C)** successful cloning of the construct was initially verified by restriction analysis (i.e. loss of SapI recognition site).

## 3.3.3. RNAi-mediated silencing of Gabra2 expression

### 3.3.3.1. *In vitro* assays

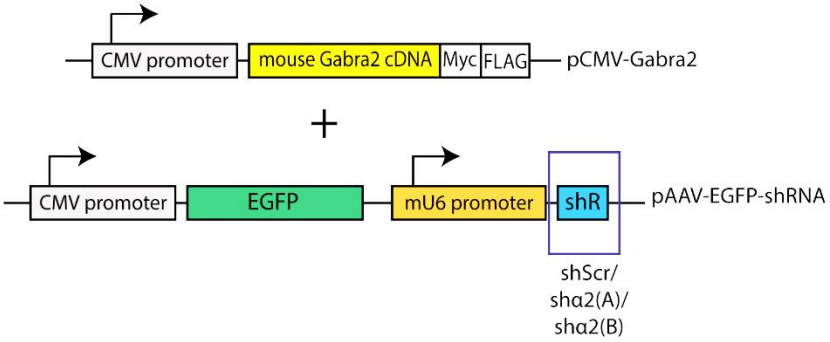
An *in vitro* assay of the shRNA silencing efficacy was performed prior to recombinant virus production. For this, human embryonic kidney 293 (HEK293) cells were co-transfected with pCMV-Gabra2 (a plasmid that harbours the mouse Gabra2 cDNA under the control of the CMV promoter) and pAAV-EGFP-shRNA vector carrying either shα2(A), shα2(B) or shScr, at a 1:3 ratio respectively (Figure 3.4A). Note that each co-transfection condition was set up in triplicate. Forty-eight hours post-transfection, cells were fixed and immunocytochemical (ICC) analyses of EGFP and α2 expression were performed to assess the silencing efficacy of the shα2 constructs.

The extent of α2 knockdown was quantified by measuring the integrated density value (IDV; intensity or mean gray value × area) of α2 (normalised to the corresponding IDV of GFP to control for variations in transfection efficiency) in shα2(A), shα2(B) conditions relative to that in the shScr counterpart. Given that scr should not modulate the level of α2 expression, it was therefore presumed that the normalised α2 IDV in the shScr condition was indicative of pCMV-Gabra2 transfection efficiency, thus yielding a fold change of 1. Figure 3.4B depicts the mean fold change ± SEM of normalised α2 IDV (n = 3) in shScr, shα2(A) and shα2(B) conditions.

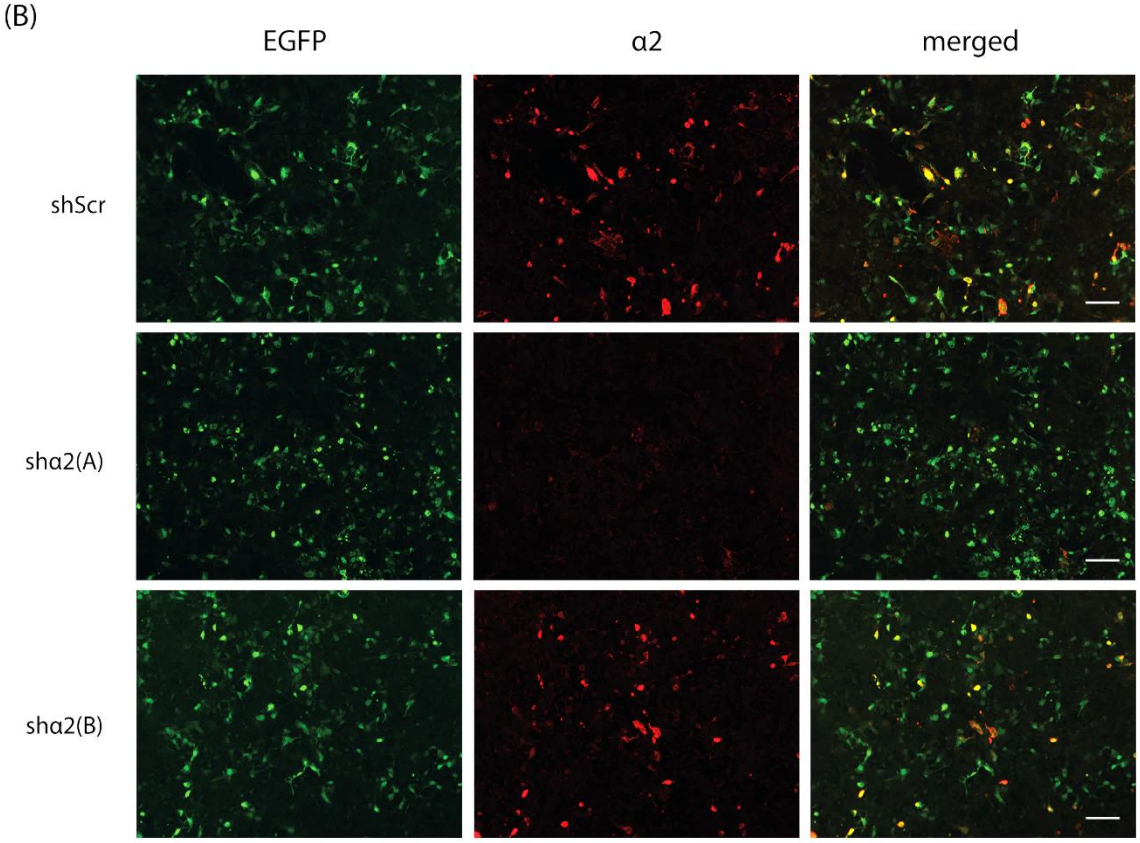
A one-way ANOVA, with Tukey's correction for multiple comparisons, comparing relative α2 expression levels between the experimental

conditions revealed a significant main effect of group ($F(2,8) = 11.41$, $p < .01$). The post-hoc test further indicated that α2 expression in the shα2(A) condition was significantly lower (86.37%) than that in the shScr counterpart ($p < .01$), but the difference in α2 expression between the shScr and shα2(B) conditions was statistically non-significant ($p = .240$). Overall, these data suggest that shα2(A), but not shα2(B), effectively silenced α2 expression *in vitro* and the former was therefore used for subsequent *in vivo* experiments.

(A)


CMV promoter
mouse Gabra2 cDNA
Myc
FLAG
pCMV-Gabra2
+
CMV promoter
EGFP
mU6 promoter
shR
pAAV-EGFP-shRNA
shScr/
shα2(A)/
shα2(B)

(B)


EGFP
α2
merged
shScr
shα2(A)
shα2(B)

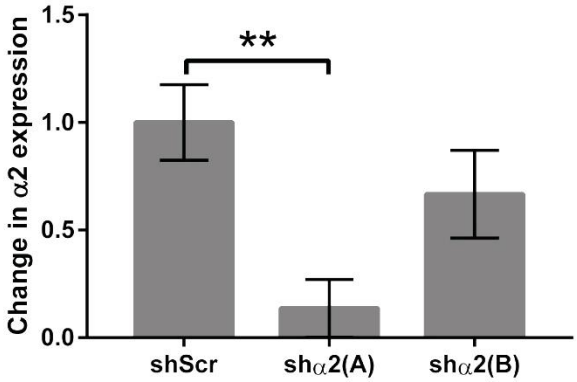

Change in α2 expression
1.5
1.0
0.5
0.0
**
shScr
shα2(A)
shα2(B)

***Figure 3.4.*** *In vitro* analyses of shα2 silencing potency. **(A)** HEK293 cells co-transfected with pCMV-Gabra2 and pAAV-EGFP-shRNA at a 1:3 ratio. **(B)** Cells transfected with shα2(A), but not shα2(B), displayed markedly less α2 staining in the immunocytochemical assay relative to the shScr condition. Scale bar 100μm. ** $p < .01$.

### 3.3.3.2. *In vivo* assays

For *in vivo* assays, shScr and shα2(A) (hereafter referred to as shα2) were individually packaged into AAV2 capsid proteins to generate recombinant AAV2 vectors (i.e. rAAV-shScr and rAAV-shα2) for targeted delivery into the brain, with respective viral titres of $1x10^9$ and $5x10^8$ infectious units/ml. Titres were measured in HEK293 cells using methods previously described in the McClure, Cole, Wulff, Klugmann, & Murray, (2011) study.

The functionality of the rAAVs was tested by injecting these viruses bilaterally into the NAc core (injection volume of 0.5µl per side) (see Figure 3.5A for the injection site). Two groups of mice, receiving either rAAV-shScr or rAAV-shα2 injection into the NAc core, were used in this experiment. Twenty one days post viral infusion, reverse transcription qPCR (RT-qPCR) (n = 3-4 per group) and immunohistochemical (n = 4 per group) analyses were performed to visualise rAAV transduction profile and/or assess the silencing efficacy of shα2 *in vivo.*

First, the RT-qPCR analysis was carried out to assess the extent of RNAi-induced α2 knockdown at the transcriptomic level. Relative expression levels of α2 mRNA (normalised to EGFP to control for transduction efficiency) in shScr and shα2 conditions are summarised in Figure 3.5B. Current data showed a ~57% shα2-induced downregulation in α2 mRNA expression and this difference reached statistical significance, $t(5) = 2.52$, $p < .05$, one-tailed t-test.

By contrast, data from the immunohistochemical (IHC) experiment corroborated the ICC data. Using the same method of quantification as the ICC experiment described above, the α2 expression was significantly lower in the shα2 group than that in the shScr group (70.41% decrease, $t(6) = 8.83$, $p < .001$) (Figure 3.5C).

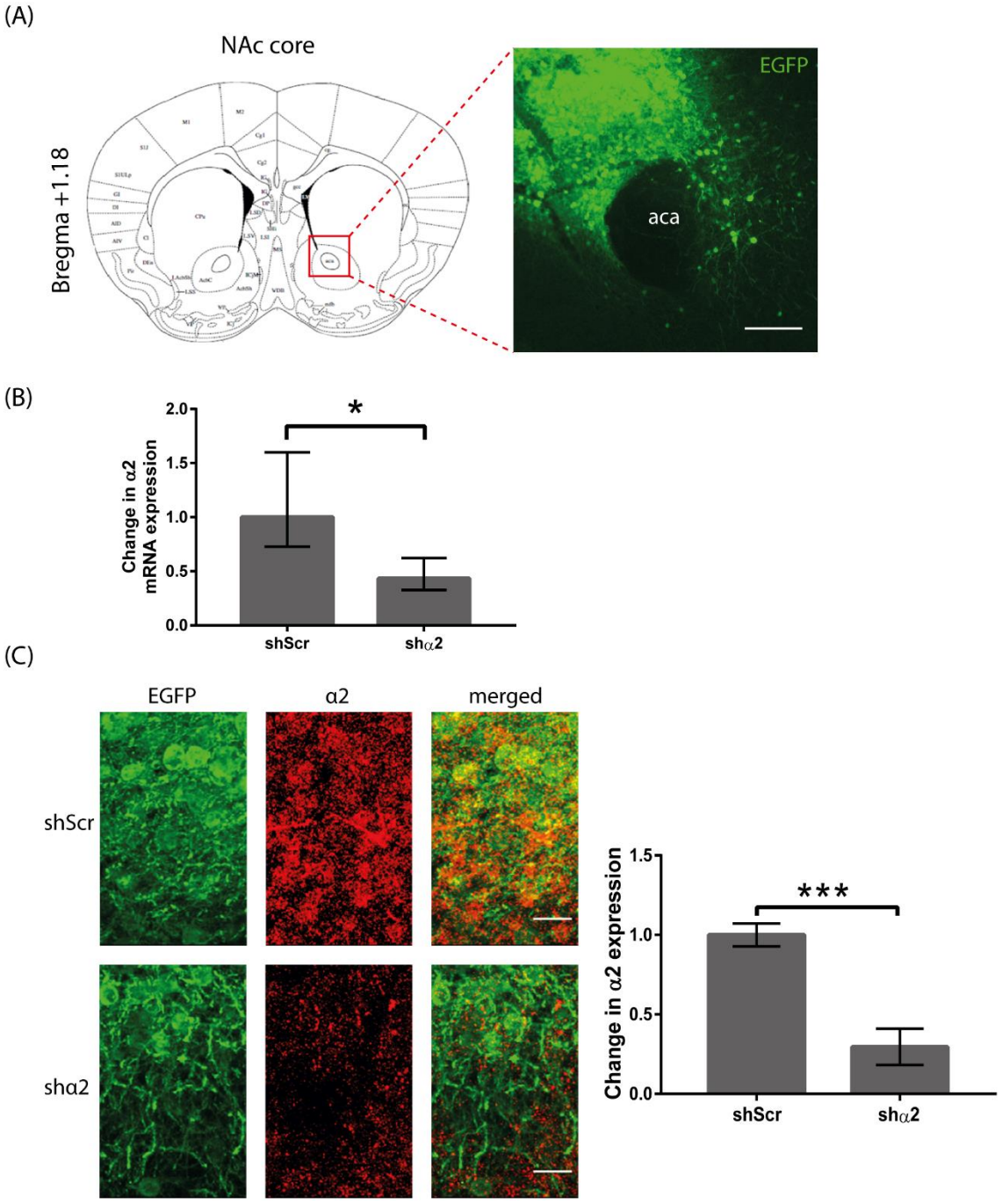
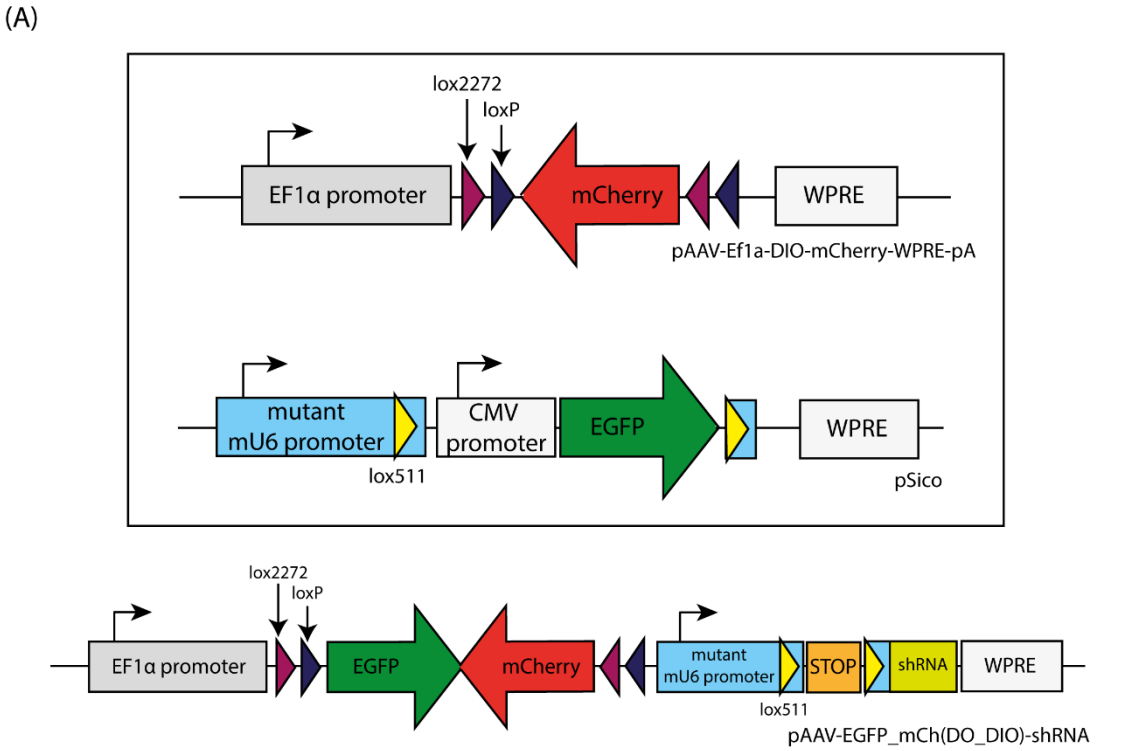



***Figure 3.5.*** **(A)** Histological examination of EGFP expression after virus infusion into the NAc core (area marked with the red box indicates the target injection site; Paxinos & Franklin, 2001) Scalebar 200µm. Analyses of α2 knockdown by **(B)** RT-qPCR and **(C)** double immunofluorescence (i.e. colocalisation of EGFP and α2). Scale bar 20µm *** $p < .001$.

### 3.3.4. Construction of the Cre-dependent pAAV-EGFP_mCh(DO_DIO)-shRNA vector

In efforts to study the role of Gabra2 in D1R- and D2R-expressing neurons selectively within the NAc, a Cre-dependent shRNA-harbouring vector (pAAV-EGFP_mCh(DO_DIO)-shRNA) was constructed from two plasmids, i.e. pSico and pAAV-Ef1a-DIO-mCherry-WPRE-pA, kindly donated by Tyler Jacks (Massachusetts Institute of Technology; Ventura et al., 2004; Addgene plasmid # 11578) and Bernardo Sabatini (Harvard University; Saunders et al., 2012; Addgene plasmid # 37083) respectively (Figure 3.6A).

Figure 3.6B summarises the cloning steps for pAAV-EGFP_mCh(DO_DIO)-shRNA construction in a chronological order. Firstly, PCR-based cloning was performed to insert an EGFP construct into pAAV-Ef1a-DIO-mCherry-WPRE-pA. This would enable the labelling of Cre-negative (EGFP-ON) and Cre-positive (mCherry-ON) cells in a population of transduced neurons. Next, the CMV + EGFP construct within pSico was substituted with the 80-bp STOP cassette containing a Poly(T) tail to prevent shRNA transcription in the absence of Cre recombinase. The shRNA was then inserted at the HpaI site and finally, the region within pSico spanning the mU6 promoter to the shRNA was then subcloned into the EGFP-harbouring pAAV-Ef1a-DIO-mCherry-WPRE-pA at the EcoRV site, yielding a 5092-bp rAAV genome. DNA sequencing was performed after each cloning step to check sequence integrity and the presence of mutations.



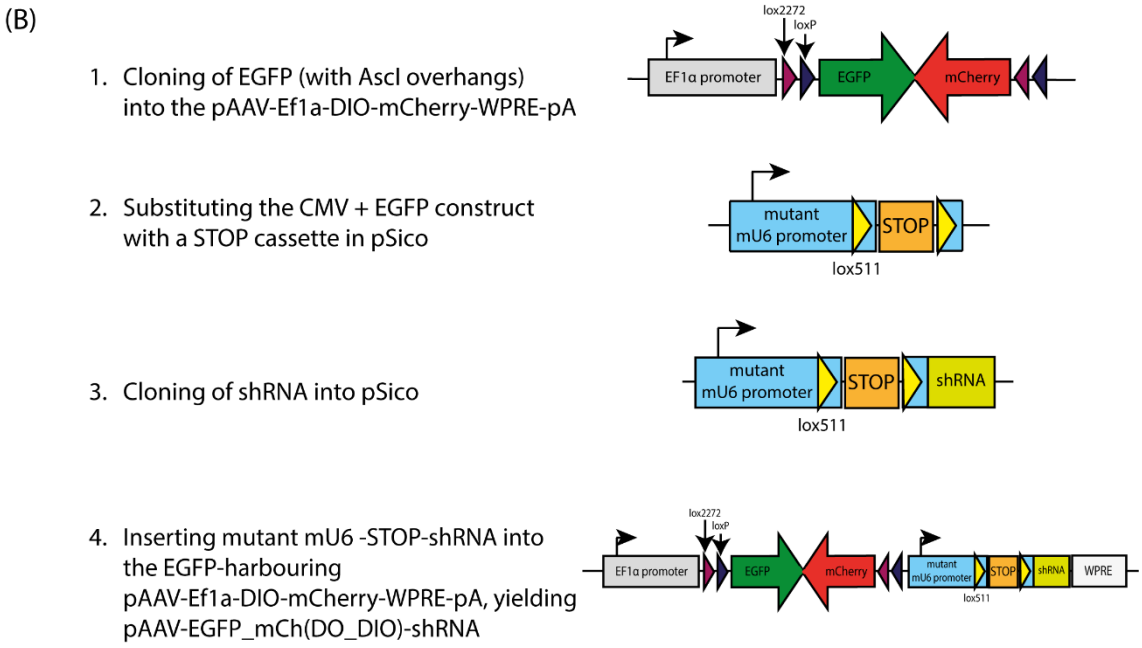




***Figure 3.6.*** **(A)** Design of and **(B)** cloning procedures to generate pAAV-EGFP_mCh(DO_DIO)-shRNA vector.

## 3.3.5. Cre-dependent RNAi-mediated silencing of Gabra2 expression

### 3.3.5.1. *In vitro* assays

An *in vitro* analysis of Cre-driven α2 knockdown was performed by co-transfecting HEK293 cells with pAAV-EGFP-mCherry(DO_DIO)-shRNA, harbouring shScr or shα2, pCMV-gabra2, and pAAV-EF1α-iCre (a gift from Karl Deisseroth, Stanford University; Addgene plasmid # 55636) at a 3:1:3 ratio (see Figure 3.7). Cells were fixed 72 hours post-transfection and subsequently subjected to immunocytochemical analyses. Firstly, to probe whether the molecular switch of the fluorescent markers was successfully induced in the presence of Cre, cells were stained with antibodies against EGFP, mCherry, and Cre (n = 3 per condition). As illustrated in Figure 3.8A, mCherry, but not EGFP, co-localises with Cre, suggesting a Cre-mediated switch of the fluorescent constructs.

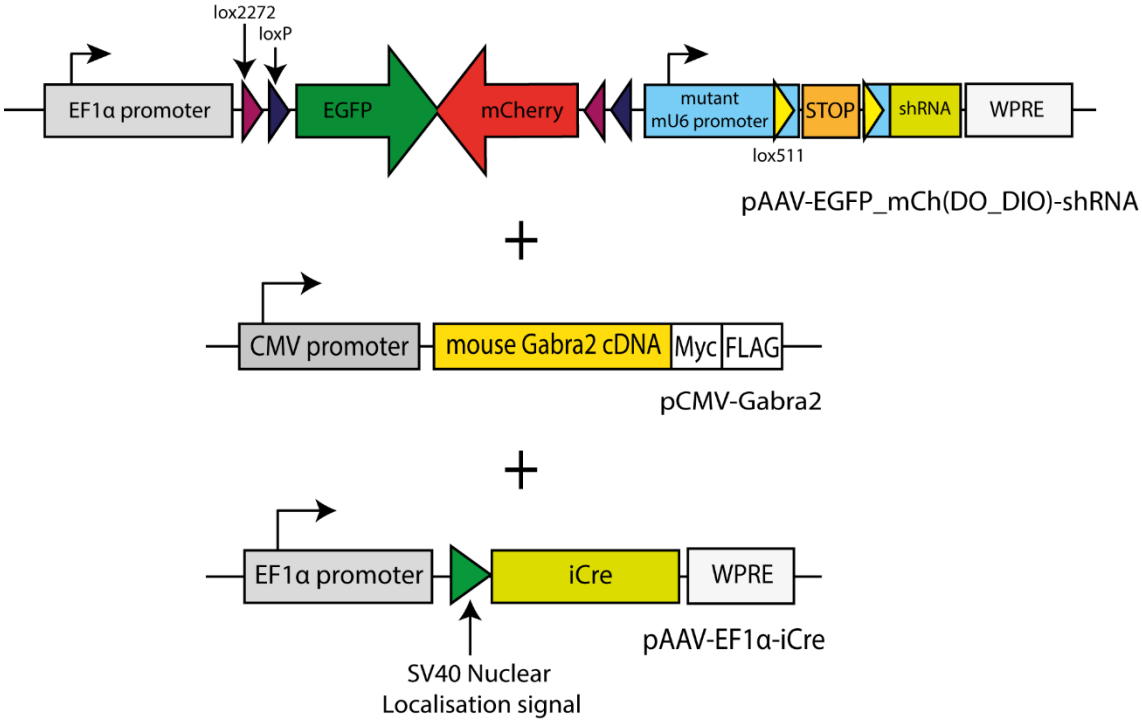
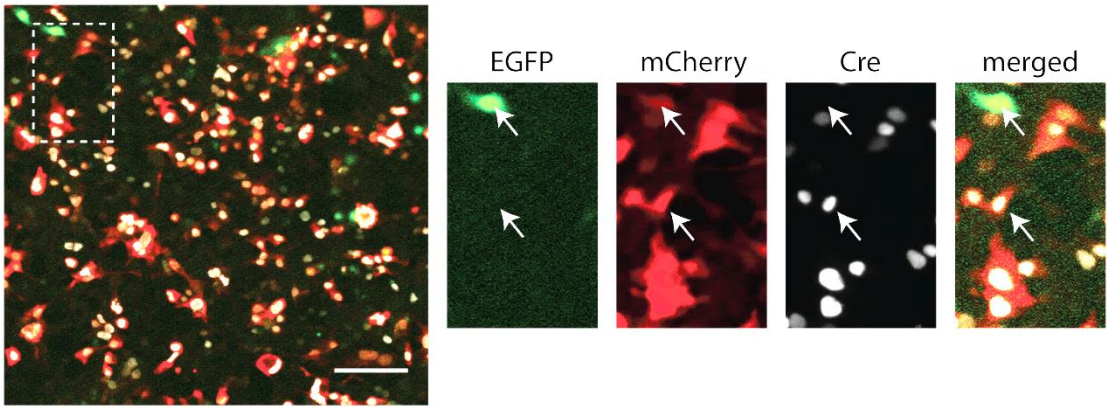


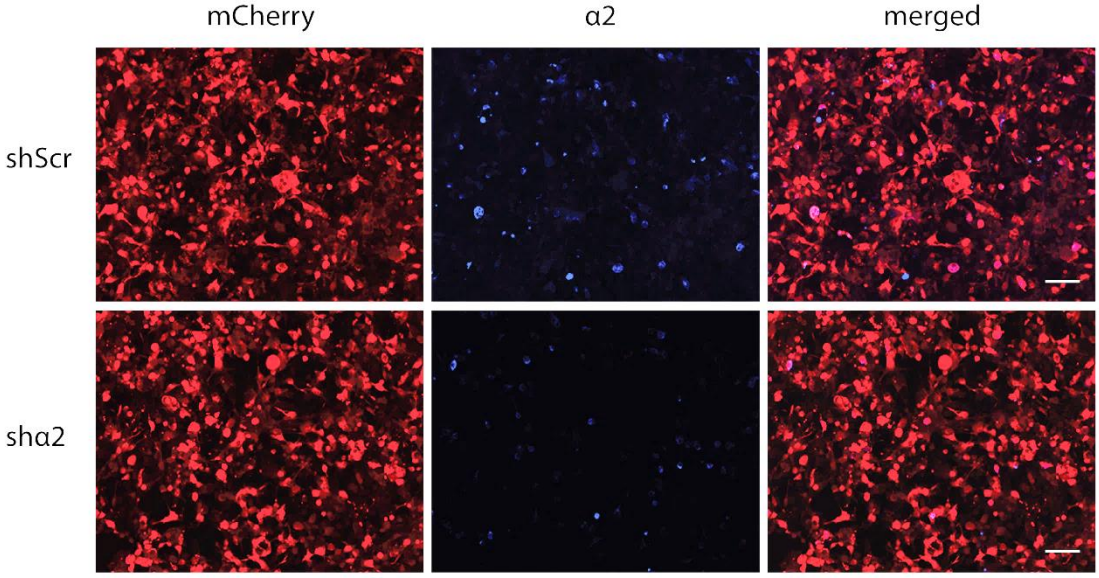

***Figure 3.7.*** The functionality of the Cre-dependent pAAV was tested by co-transfecting HEK293 cells with pAAV-EGFP-mCherry(DO_DIO)-shRNA, carrying either shScr or shα2, pCMV-gabra2, and pAAV-EF1α-iCre at a 3:1:3 ratio.

In the following immunocytochemical experiment, cells were stained with antibodies against mCherry and α2 (n = 3 per condition) to assess the extent of Cre-dependent α2 knockdown. To quantify this, the IDV of α2 was normalised to that of mCherry to control for variations in transfection efficiency of the Cre-expressing cells. Results are illustrated in Figure 3.8B. A t-test comparison between shScr and shα2 manipulations indicated that the α2 expression in the latter was significantly lower (82.03%) relative to that in the shScr condition, $t(4) = 23.06$, $p < .001$.

(A)


EGFP
mCherry
Cre
merged

(B)


mCherry
α2
merged
shScr
shα2

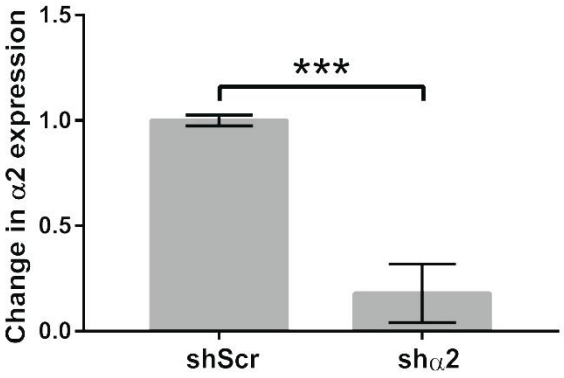
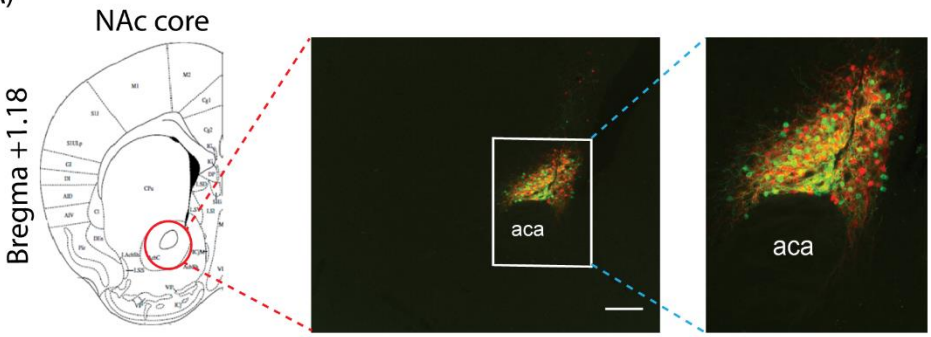

Change in α2 expression
1.5
1.0
0.5
0.0
***
shScr
shα2

***Figure 3.8.*** **(A)** A Cre-mediated switch of fluorescent constructs. The mCherry-, but not EGFP-positive cells co-express Cre recombinase, suggesting a Cre-dependent activation of mCherry. **(B)** Immunocytochemical analysis of Cre-dependent α2 knockdown demonstrated significantly lower α2 mRNA expression in cells treated with shα2 relative to those treated with shScr. Scale bar 100μm *** $p < .001$.

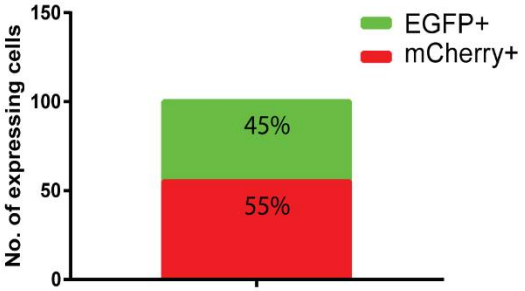
### 3.3.5.2. *In vivo* assays

Next, the Cre-dependent rAAV harbouring shScr or shα2 (titre = 2-3 × $10^8$ infectious units/ml) was injected into the NAc core of D2-Cre and wild-type (WT) mice (n = 3 in each group)to test its functionality in vivo (see Figure 3.9A for targeted injection site). Results from the manual cell count revealed that in the NAc core of a D2-Cre mouse, for example, of 118 cells counted, 53 and 65 cells stained positive for EGFP and mCherry respectively (Figure 3.9B). Figures 3.9C&D depict the representative confocal images of EGFP, mCherry, and Cre expression in the transgenic and WT mice respectively. As anticipated, the mCherry expression was notably absent in the WT brain and thus, its expression only occurred in the presence of Cre.

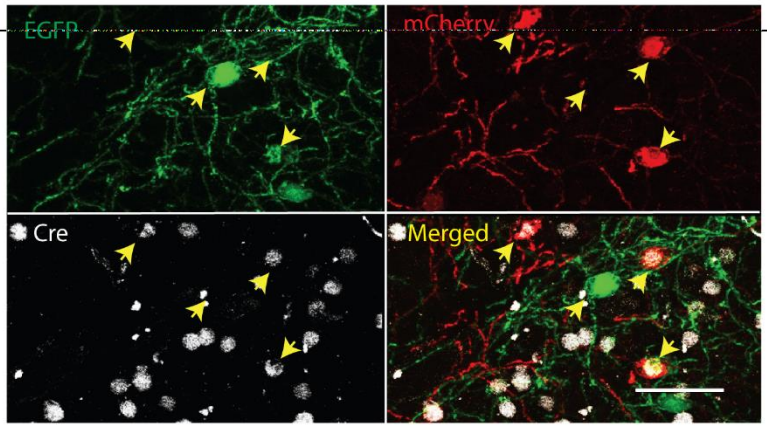
(A)


NAc core
Bregma +1.18
aca
aca

(B)


150
100
50
0
No. of expressing cells
EGFP+
mCherry+
45%
55%

(C)


EGFP
mCherry
Cre
Merged

(D)

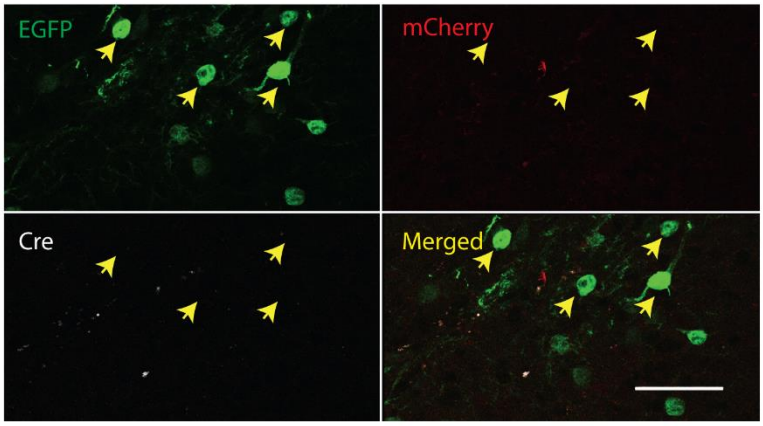

EGFP
mCherry
Cre
Merged

*Figure 3.9.* Immunohistochemical analysis of the Cre-switch dual fluorescent system **(A)** Histological examination of EGFP and mCherry expression after virus infusion into the NAc core of a D2-Cre mouse (area marked with the red circle indicates the target injection site; Paxinos & Franklin, 2001). Scalebar 200μm. **(B)** Neurons expressing mCherry and EGFP are non-overlapping (i.e. no neurons stained positive for both fluorophores were detected). **(C)** The mCherry-, but not EGFP-positive neurons were found to colocalise with Cre in the D1- and D2-Cre mice, but **(D)** mCherry and Cre expressions were notably absent in the NAc core of a wildtype mouse injected with the Cre-dependent rAAV harbouring shα2, collectively suggesting that mCherry expression was dependent on Cre activity. Scale bar 50μm.

The RT-qPCR assays were then performed to assess Cre-dependent knockdown of α2 expression *in vivo*. Two groups were used in these experiments, i.e. D2-Cre mice injected with either shScr- or shα2-harbouring Cre-dependent rAAV into the NAc core. Firstly, the RT-qPCR assay revealed that the α2 mRNA level (normalised to mCherry) in the shα2 group (n = 2) was 35.26% lower relative to that in the shScr (n = 3) condition ($t(3) = 2.74$, $p < .05$, one-tailed *t*-test; Figure 3.10).

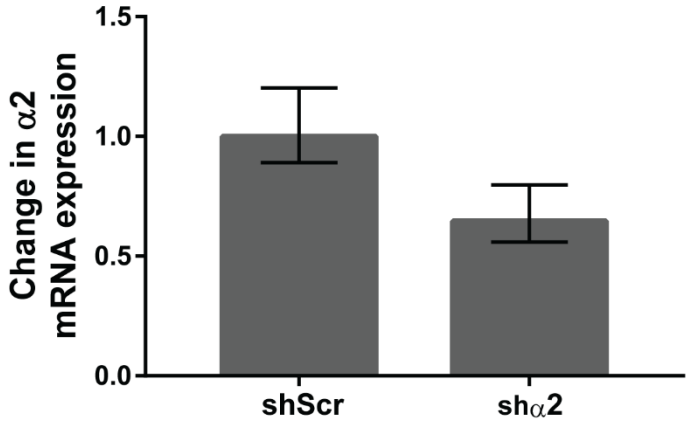



***Figure 3.10.*** Quantification of Cre-dependent α2 mRNA knockdown *in vivo* by RT-qPCR.

## 3.4. Discussion

Since its discovery, RNAi continues to be recognised as a powerful tool for inhibition of gene expression. Here we have demonstrated that a novel α2-targeting silencing construct effectively silenced α2 expression in WT and Cre-expressing cells, further extending RNAi applicability to study $GABA_AR$ function. While there already exists published data utilising α2-targeting siRNA vector in the rat (Liu et al., 2011), to the best of our knowledge, this study is first to report the use of RNAi strategy to target Gabra2 expression in the adult mouse brain. These molecular tools would undoubtedly aid future investigations into the functional roles of α2-$GABA_ARs$ in various regions as well as in specific cell subpopulations through the use of the Cre-dependent RNAi vector developed here.

### 3.4.1. The shα2 design and efficacy

Two shRNA sequences were originally tested for their potency *in vitro*. The 24-nt stem sequences comprised α2-directed siRNAs designed in accordance with the Reynold's algorithm (Reynolds et al., 2004). This algorithm was selected for the present research owing to the high reported potency of siRNAs generated with this algorithm (i.e. all beyond 50% gene expression knockdown, mostly 80% and above), as well as due to the high success rate, i.e. generating 29 out of 30 effective RNAi effectors (Reynolds et al., 2004). In line with these findings, shα2(A) induced >70% knockdown of α2 expression in WT or Cre-expressing cells when tested *in vitro* and *in vivo*, though our analysis of transcript abundance revealed less robust α2

mRNA knockdown *in vivo.* One potential reason for such discrepancy might be the incomplete degradation of target mRNA fragments that persisted following RNAi-mediated cleavage, compromising the RT-qPCR detection of knockdown. This is, however, unlikely given that the primer pair used to detect knockdown in the present research amplified the 5' mRNA fragment, not the 3' fragment that was reported to accumulate following RNAi cleavage possibly due to RNA-protein interactions, thereby acting as template for cDNA synthesis (Holmes et al., 2010). Instead, the differences in ICC/IHC and RT-qPCR findings might have been driven by the technical limitations discussed further below.

By contrast, the shα2(B) reduced α2 expression only by ~33% relative to that in the shScr condition *in vitro*. This is somewhat surprising as siα2(B) exhibited perfect complementarity to the Gabra2 transcript and fulfilled more design criteria in the algorithm compared to the siα2(A), giving rise to a higher overall score. Firstly, although scr was designed by introducing five base mismatches to siα2(B) and previous evidence suggests that some mismatches can be tolerated (Du, Thonberg, Wang, Wahlestedt, & Liang, 2005), it is unlikely that the shScr induced silencing effects on α2 mRNA levels, masking shα2(B)-induced knockdown, as the mismatches were introduced in the seed region of the siα2(B) to preclude not only siRNA-mRNA interaction, but also miRNA-like seed interaction with the α2 transcripts (Carthew & Sontheimer, 2009; Ellwanger et al., 2011; Jackson, Burchard, Schelter, et al., 2006).

One potential reason for the low silencing efficacy of shα2(B) could be its target position within the α2 transcript. In fact, a large variation in the efficacy of siRNAs targeting different sites within the same transcript have been reported (Holen, Amarzguioui, Wiiger, Babaie, & Prydz, 2002), leading to continuously emerging rules for improvement of siRNA design. In addition to the design criteria used in the present study, the target sites for both siRNAs in the present research were within the recommended parameters defined by previous research (i.e. located at least 100 nucleotides away from the AUG codon and not within 50-100 of the termination codon, also avoiding the UTR regions) (Mocellin & Provenzano, 2004). However, there exist additional sequence-specific discriminants that have been reported to improve mRNA susceptibility to RNAi-induced perturbations. These include specific nucleotide preferences, thermodynamic characteristics and other sequence features proposed in other design algorithms (Huesken et al., 2005; Ui-Tei et al., 2004; Vert et al., 2006), as well as local target accessibility or secondary structure (Heale, Soifer, Bowers, & Rossi, 2005; Luo & Chang, 2004; Westerhout, Ooms, Vink, Das, & Berkhout, 2005). These parameters might have, in part, elucidated variations in the silencing efficacy of the α2-targeting shRNAs designed here.

Alternatively, the answer may lie in the inherent variations between shRNA- and siRNA-based silencing. It was originally surmised that shRNA and siRNA were governed by the same mechanism, thus sequences that were effective siRNAs were often placed in the scaffold of shRNA. Recent

research however indicated that functional shRNA and siRNA exhibit similar, but not identical, target preferences. Namely, functional shRNAs display preferences for G/C and A/U at positions 11 and 9 respectively, whereas functional siRNAs strongly favour G/C at position 9 (Li et al., 2007). Intriguingly, the functional shα2(A) and the non-functional shα2(B) harbour A and C bases at position 9 respectively, which might somewhat explain the low efficacy of shα2(B). Nonetheless, given the small shRNA samples used in the Li et al. (2007) study and the lack of existing evidence supporting these data, this interpretation should be treated with caution.

### 3.4.2. Design and functionality of the Cre-dependent RNAi vector

This study further constructed and tested the functionality of a Cre-dependent RNAi vector to improve the spatial resolution of α2 knockdown, i.e. only in a defined cell population (Cre-expressing cells; CRE-ON system) within a specified region (Ventura et al., 2004). It also permits two fluorescent transgenes to be simultaneously expressed in spatially intermingled Cre-expressing and non-expressing cells (Saunders et al., 2012). Note that mCherry was used in the present research, instead of the TdTomato in the Saunders' Cre-switch vector due to the rAAV genome size constraints (Hermonat, Quirk, Bishop, & Han, 1997; Saunders et al., 2012; Srivastava et al., 1983).

Findings from the ICC and IHC analyses consistently demonstrated that the FLEx genetic switch was induced only in the presence of Cre, as co-

localisation between EGFP and Cre was not observed. The ICC data also revealed that the shα2 appeared to evoke potent knockdown of α2 expression in comparison to the shScr control, when placed under the control of a modified form of U6 promoter, which constitutes a TATA-containing bifunctional lox site. Such modification was reported to retain WT promoter activity, given that the critical elements within the promoter, i.e. proximal sequence element (PSE), the TATA box, as well as spacing between PSE and TATA (17bp) and TATA and transcription start site (25bp), were preserved (Paule & White, 2000; Ventura et al., 2004). Preliminary data generated in the present research further confirmed that the disparity in α2 expression between the shScr and shα2 co-transfection conditions was likely to be Cre-dependent as the silencing effect of shα2 was abolished in the absence of Cre, i.e. in WT cells (Appendix A), further demonstrating the functionality of the STOP cassette to prevent shRNA transcription in the absence of Cre and substantiating previous evidence for the lack of effective recombination between the three pairs of distinct lox sites, i.e. loxP, lox2272, and lox511 (Araki, Araki, & Yamamura, 2002; Lee & Saito, 1998; Saunders et al., 2012; Schnütgen et al., 2003).

Nevertheless, comparison of relative mRNA expression by RT-qPCR revealed that shα2 appeared to induce marginal silencing effects (< 50%) on the α2 mRNA level in the NAc core of D2-Cre mice. This is not surprising as only ~50% of the striatal neurons express D2Rs (Gerfen et al., 1990; Gertler, Chan, & Surmeier, 2008; Saunders et al., 2012; also shown by the cell count data in the present research), thus the degree of α2 knockdown with the

Cre-dependent rAAVs should be approximately 50% lower than that achieved by rAAV-EGFP-shα2 (also refer to technical limitations outlined below). Additional characterisations of total and surface α2 expression knockdown *in vivo* also remain to be performed.

### 3.4.3. Conclusions and technical considerations

To conclude, findings from the present research showed that α2 expression did not appear to be resilient to RNAi-induced perturbations. Nonetheless, the varying abilities of shα2(A) and shα2(B) to evoke translational repression may, in part, be explained by our limited understanding of RNA-target recognition, as well as the contribution of various contextual factors. To further improve the specificity of gene expression knockdown, this study also constructed a Cre-dependent RNAi vector, which not only harbours a silencing construct for RNAi application, but also permits visualisation of the full range of transduced cells (i.e. Cre-positive and Cre-negative cells) to enable cell-type-specific analyses when appropriate.

Nevertheless, some of the current findings should be interpreted with caution due to technical limitations and therefore warrant further investigation. Firstly, using the current measures of α2 knockdown in the in-vitro analyses, this study cannot at present eliminate the possibility that differences in α2 expression levels might have been an artefactual result of variations in the transfection efficiency of the Gabra2-harbouring plasmid or the lack of co-localisation of both plasmids within the same host. One

plausible way of rectifying this issue would be to utilise a pCMV-Gabra2 that carries a fluorescent marker, controlled by a separate promoter, to which α2 could be normalised as it would serve as a better indicator of its transfection efficiency. Though current *in vivo* assays of α2 knockdown should provide a more accurate indicator of shα2's silencing potency due to endogenous α2 expression, a few technical limitations should be noted. Firstly, using the SYBR Green RT-qPCR method of assay, the presence of dsDNA contaminants in the samples, target abundance, and inaccurate targeting of the NAc core might result in overestimation of the target and thus, undermine the true suppressive capacity of shα2 (Arvey, Larsson, Sander, Leslie, & Marks, 2010; Smith & Osborn, 2009).

Of equal importance, despite the ubiquitous use of viral vectors for delivery of genetic materials in the CNS, creating high viral titres remains to be a challenge in rAAV production and is often impeded by viral genome sizes (Dong, Fan, & Frizzell, 1996). Lower titres of the Cre-dependent rAAVs in the present research could thus be explained by the size of the viral transgene (slightly under 5.1 kb), which exceeded the small packaging capacity of AAV virions (4.7 kb) (Srivastava et al., 1983). Though there exists evidence that the rAAV particles are able to accommodate 900 extra nucleotides above the aforementioned wild-type packaging capacity, yielding a maximum packaging limit of 5.6 kb (Hermonat et al., 1997), rAAVs have been observed to exhibit reduced titres as the genome size increases (Dong et al., 1996). To circumvent this issue, one could resort to the use of other AAV capsids to generate AAV hybrids in efforts to improve viral spread or

transduction efficiency (Aschauer et al., 2013), or opt for the lentiviral vector system for the delivery of larger constructs (Kumar, Keller, Makalou, & Sutton, 2001).

Finally, there remains a need for future experiments, such as electrophysiological or radioligand binding experiments, to probe whether the extent of α2 knockdown achieved in the present research would be sufficient to alter α2-$GABA_AR$ number. This is fuelled by previous evidence that oligomerisation of $GABA_AR$ subunits is highly inefficient with less than 25% of subunits being assembled into pentameric receptors (Gorrie et al., 1997; also reviewed extensively in Jacob, Moss, & Jurd, 2008).

Over the past years, tremendous progress has been made to harness RNAi pathway as a method to decipher gene functions in various organisms. Recent years have also witnessed growing advances in the delivery method of RNAi effectors tailored for specific experimental or therapeutic purpose, thereby further expanding its utility (Capel et al., 2018; Kannan, 2018; Prakash, Malhotra, & Rengaswamy, 2010). Despite the aforementioned caveats, the superiority of this approach over the conventional reverse genetic approaches continues to make RNAi a method-of-choice in modern biology (Silva, Chang, Hannon, & Rivas, 2004).

# Chapter 4

# Effects of $GABA_AR$ α2 subunit knockdown in the nucleus accumbens on cocaine-facilitated conditioned reinforcement and locomotor sensitisation

## 4.1. Introduction

The original findings by (Olds & Milner, 1954) that rats would work for electrical stimulation to specific brain regions soon propelled the search for circuitry and interconnections between areas that underlie reward processing. Of these, the nucleus accumbens (NAc) has been demonstrated to be a key component of the neural pathway implicated in reinforcement and motivational processes (Carelli, 2002; Di Chiara et al., 2004; Mogenson, Jones, & Yim, 1980; also refer to Salamone, 2006).

To date, the NAc continues to receive much attention in the field of reinforcement-related research given its unique role as a "limbic-motor interface" (Mogenson et al., 1980), integrating and filtering converging inputs from cortical and subcortical structures, including the prefrontal cortex (Christie, James, & Beart, 1985; Groenewegen, Room, Witter, & Lohman, 1982; Sesack & Pickel, 1992) and subcortical structures, including the amygdala (French & Totterdell, 2003; Robinson & Beart, 1988), hippocampus (Floresco et al., 2001; Kelley & Domesick, 1982), thalamus (Berendse & Groenewegen, 1990; Robinson & Beart, 1988), and the VTA (Brown et al., 2012; Fallon & Moore, 1978; Hasue & Shammah-Lagnado, 2002; Van Bockstaele & Pickel, 1995), and subsequently, transmitting this information to downstream motor regions that ultimately govern behaviour (Mogenson et al., 1980). It is also noteworthy that the NAc core and shell subdivisions display functional heterogeneity, due to histochemical and input-output pathway disparities (Heimer, Zahm, Churchill, Kalivas, &

Wohltmann, 1991; Zahm, 1999; also refer to Chapter 1 for afferents and efferents to and from the NAc core and shell).

Approximately 90-95% of neurons in the NAc are GABAergic, yet the role of GABA in mediating cocaine-induced responses remains overlooked. Cocaine evokes an array of behavioural effects, many of which are linked its ability to increase extracellular dopamine level in the NAc and some of these include cocaine-induced locomotor sensitisation (Brenhouse, Montalto, & Stellar, 2006; Filip & Siwanowicz, 2001; Kalivas & Duffy, 1990; Kalivas & Stewart, 1991; Nakagawa et al., 2011), and its ability to enhance conditioned behaviours (Chu & Kelley, 1992; Dixon et al., 2010; Rutsuko Ito, Robbins, & Everitt, 2004; Taylor & Robbins, 1986; Wolterink et al., 1993). Intriguingly, previous work in our laboratory using mice devoid of the $GABA_AR$ α2 subunit-encoding gene (Gabra2) established that whole-brain ablation of Gabra2 blocked cocaine's ability to induce locomotor sensitisation, as well as to invigorate instrumental responding for CRf (Dixon et al., 2010). More recent data investigating the effect of methylphenidate further confirmed the latter (Duka et al., 2015), suggesting that intact GABAergic functioning at α2-$GABA_ARs$ is a prerequisite for these dopamine-dependent conditioned behaviours.

Given that the α2-$GABA_ARs$ are densely expressed in the NAc (Dixon et al., 2010; Hörtnagl et al., 2013; Pirker, Schwarzer, & Wieselthaler, 2000), the experiments reported here sought to address the standing hypothesis that the loss of cocaine specifically in the NAc might have contributed to these phenotypes observed in the α2 knockout ($\alpha2^{-/-}$) mice. We thus

examined the effects of RNAi-induced silencing of α2 expression in the NAc core and shell on cocaine's ability to induce locomotor sensitisation, and in the NAc core on cocaine's ability to potentiate CRf. Note that the CRf data from the NAc shell manipulation were not presented here due to low sample size.

## 4.2. Experimental design

### 4.2.1. Conditioned Reinforcement

This experiment examined whether RNAi-induced silencing of $GABA_AR$ α2 subunit expression in the NAc core affected the acquisition of nosepoking for CRf and its modulation by cocaine. Two groups of mice, injected with the shα2- or shScr-harbouring vectors into the region of interest (i.e. the NAc core), were used (refer to Figure 4.2C for group details). Note that data from NAc shell manipulation were inconclusive and thus, not included in this thesis due to small sample size.

A summary of the experimental procedure is depicted in Figure 4.1. In brief, all animals were trained to associate a stimulus, either flashing lights or tone (counterbalanced across animals), with food reward for 10 consecutive days (Pavlovian conditioning, PC). Next, all animals were subjected to a conditioned reinforcement (CRf) test session, whereby the rates of nosepoking for the food-paired, conditioned reinforcer (CR) and the control, unpaired stimulus (non-conditioned reinforcer, NCR) were measured. The effects of cocaine (0, 3, and 10mg/kg) on CR- and NCR-maintained responding were also measured in the present research.

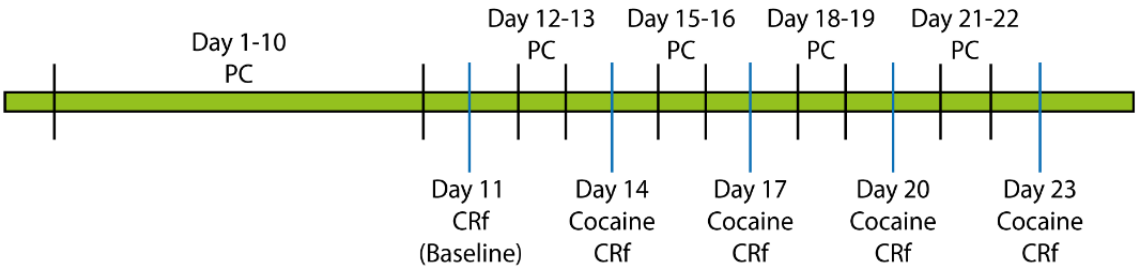



*Figure 4.1.* An overview of the conditioned reinforcement experiment.

### 4.2.2. Cocaine-induced locomotor sensitisation

This experiment examined whether RNAi-induced silencing of $GABA_AR$ α2 subunit expression in the NAc core or shell affected the development of sensitisation to cocaine. Two groups of mice, injected with shα2- or shScr-containing rAAVs into the NAc core or shell, were used (see Figure 4.2C for group details). Sensitisation to cocaine was assessed by measuring locomotor activity following repeated, daily cocaine over the course of 10 days. Conditioned locomotor activity (with a sham injection) was then measured seven days post-sensitisation (refer to Figure 4.7A below).

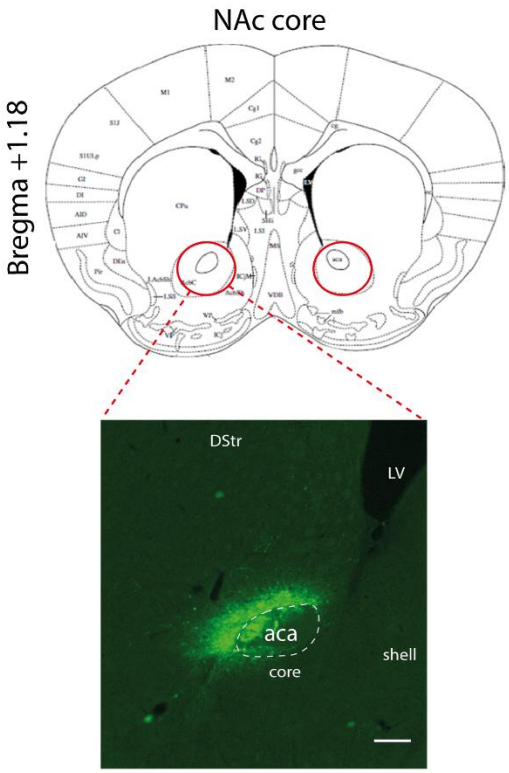
## 4.3. Results

### 4.3.1. Histological assessments

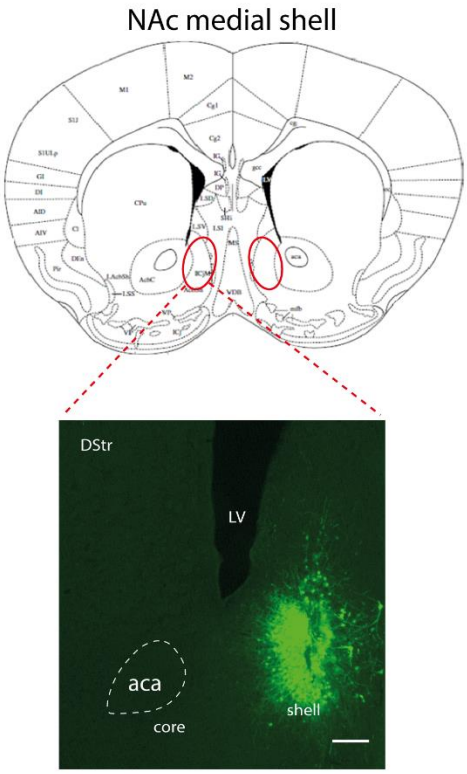
To examine the role of intra-accumbal α2-$GABA_ARs$ in mediating cocaine potentiation of conditioned behaviours, viral vectors carrying shα2 or shScr (rAAV-EGFP-shRNA) were injected in the NAc core or medial shell of adult C57BL/6J WT mice, three weeks prior to the start of the behavioural experiment. Figure 4.2 depicts the targeted injection sites for NAc core (coordinates AP 1.18; L+/-1.00; DV -4.20; Figure 4.2A) and shell (coordinates AP 1.18; L+/-0.50; DV -4.50; Figure 4.2B) (Paxinos & Franklin, 2001). The transduction profile and silencing potency of the rAAV vectors used in this study were previously characterised, as detailed in Chapter 3. Behavioural results from mice without viral infection or those with

inaccurate targeting of intracerebral viral injection into one or the other hemisphere were excluded from the analyses (refer to Figure 4.2C)

(A)




(B)




(C)

| Experiment | Group | No. of mice used in experiment | No. of mice excluded | No. of mice used for analysis |
|---|---|---|---|---|
| Cocaine facilitation of conditioned reinforcement (α2 knockdown in the NAc core) | shScr | 16 | 5 | 11 (all males) |
| | shα2 | 15 | 6 | 9 (all males) |
| Cocaine Sensitisation (α2 knockdown in the NAc core) | shScrCore | 10 | 0 | 10 (5 males and 5 females) |
| | shα2Core | 11 | 1 | 10 (5 males and 5 females) |
| Cocaine Sensitisation (α2 knockdown in the NAc shell) | shScrShell | 6 | 2 | 4 (3 males and 1 female) |
| | shα2Shell | 7 | 2 | 5 (3 males and 2 females) |

***Figure 4.2.*** Histological assessment of bilateral viral infusions into the NAc. Fluorescent immunostaining of EGFP shows rAAV-EGFP-shRNA-infected neurons within the **(A)** NAc core or **(B)** dorsomedial shell subdivision from adult mice, taken approximately 10 weeks after viral injection. Areas marked in red indicate the target injection sites. Scalebar 200µm. **(C)** A detailed summary of the experimental groups in the conditioned reinforcement and sensitisation experiments. Animals without viral infection or with inaccurate viral placement in one hemisphere or the other were excluded from the analyses (refer to Chapter 2 for the immunohistochemical methodology).

### 4.3.2. The effects of α2 knockdown in the NAc core on conditioned reinforcement and its facilitation by cocaine

#### 4.3.2.1. Pavlovian conditioning

The effect of α2 knockdown in the NAc core on the Pavlovian discriminated approach is illustrated in Figure 4.3A, calculated as the percentage of magazine entries during the onset of a stimulus associated with food delivery (conditioned stimulus, CS+) or the unpaired, control stimulus (i.e. CS-). All animals reached the set criterion of 80% magazine entries during the CS+ presentations by session 10. On average, the shScr and shα2 groups reached 82.3 ±3.30% and 86.3 ±2.35% entries during the CS+ period respectively. A three-way mixed ANOVA, comparing the percentage of magazine entries during CS+ and CS- presentations between the experimental groups across 10 daily sessions yielded a significant three-way (CS × session × group) interaction ($F(9,162) = 2.15$, $p < .05$). These data further showed that over time, both experimental groups were more likely to approach the food magazine upon CS+, than CS-, onset (i.e. significant main effect of CS ($F(1,18) = 336.36$, $p < .001$) and session × CS interaction ($F(9,162) = 36.74$, $p < .001$)). However, there were no differences between groups in this particular measure of discriminated approach, either through the main effect of group ($F(1,18) = 0.49$, $p = .491$), the session × group interaction (Greenhouse-Geisser corrected, $F(2.51, 45.23) = 0.56$, $p = .611$, $\varepsilon = .279$), or through the CS × group interaction ($F(1,18) = 0$, $p = .986$).

The expression of stimulus-reward learning was further probed by analysing changes in the latency to approach the food magazine following CS+ onset (CS+ latency) in both experimental groups on sessions 1 vs. 10 (Figure 4.3B). A food pellet was delivered into the magazine 5 seconds into each CS+ presentation. On average, the CS+ latencies for the shScr group was 8.74 ±0.26 seconds (session 1) and 4.84 ±0.37 seconds (session 10), whereas the CS+ latencies for the shα2 group was 7.86 ±0.50 seconds and 4.95 ±0.52 seconds on sessions 1 and 10 respectively. An ANOVA comparing CS+ latency on sessions 1 and 10 between shScr and shα2 groups indicated that the CS+ latency of both groups combined was significantly lower on session 10 (estimated marginal means = 4.90 ±0.31 seconds) relative to session 1 (estimated marginal means = 8.30 ±0.27 seconds) ($F(1,18) = 65.22$, $p < .001$). However, there were no differences between groups, either through session × group interaction ($F(1,18) = 1.33$, $p = .263$) or the main effect of group ($F(1,18) = 0.93$, $p = .347$). Overall, these data suggest that Pavlovian learning with food reward was unaffected by RNAi-induced silencing of α2 in the NAc core.

#### 4.3.2.2. Conditioned reinforcement

The effect of α2 knockdown in the NAc core on the baseline rates of instrumental responding (i.e. nosepoking into a port) for the conditioned reinforcer (CR, previously CS+) and for the control stimulus (NCR, previously CS-) is depicted in Figure 4.3C. On average, the shScr group made 96 ±17.24 and 32 ±3.06 CR and NCR nosepokes respectively, whereas

the shα2 counterpart made 110 ±12.74 and 42 ±6.20 CR and NCR nosepokes respectively. Note that statistical analyses displayed below were performed with the log-transformed data to maintain homogeneity of variance.

An ANOVA comparing the rates of nosepoking for CR and NCR in both groups demonstrated a statistically significant main effect of CS ($F(1,18) = 61.58$, $p < .001$), though neither the main effect of group ($F(1,18) = 2.46$, $p = .134$) nor the group × CS interaction ($F(1,18) = 0.02$, $p = .896$) was significant. These data therefore indicate that α2 knockdown in the NAc core had minimal effects on the average *total* rates of responding solely governed by a reward-associated stimulus. The pattern of CR-maintained responding across the 60-minute session, broken down in 10-minute time bins, also indicated no differences between the experimental groups (i.e. non-significant time × group interaction ($F(5,90) = 0.35$, $p = .883$). However, the main effect of time was statistically significant ($F(5,90) = 10.31$, $p < .001$), indicating marked time-dependent changes in CR-maintained responding, with responding for the CR decreasing over time (Figure 4.3D).

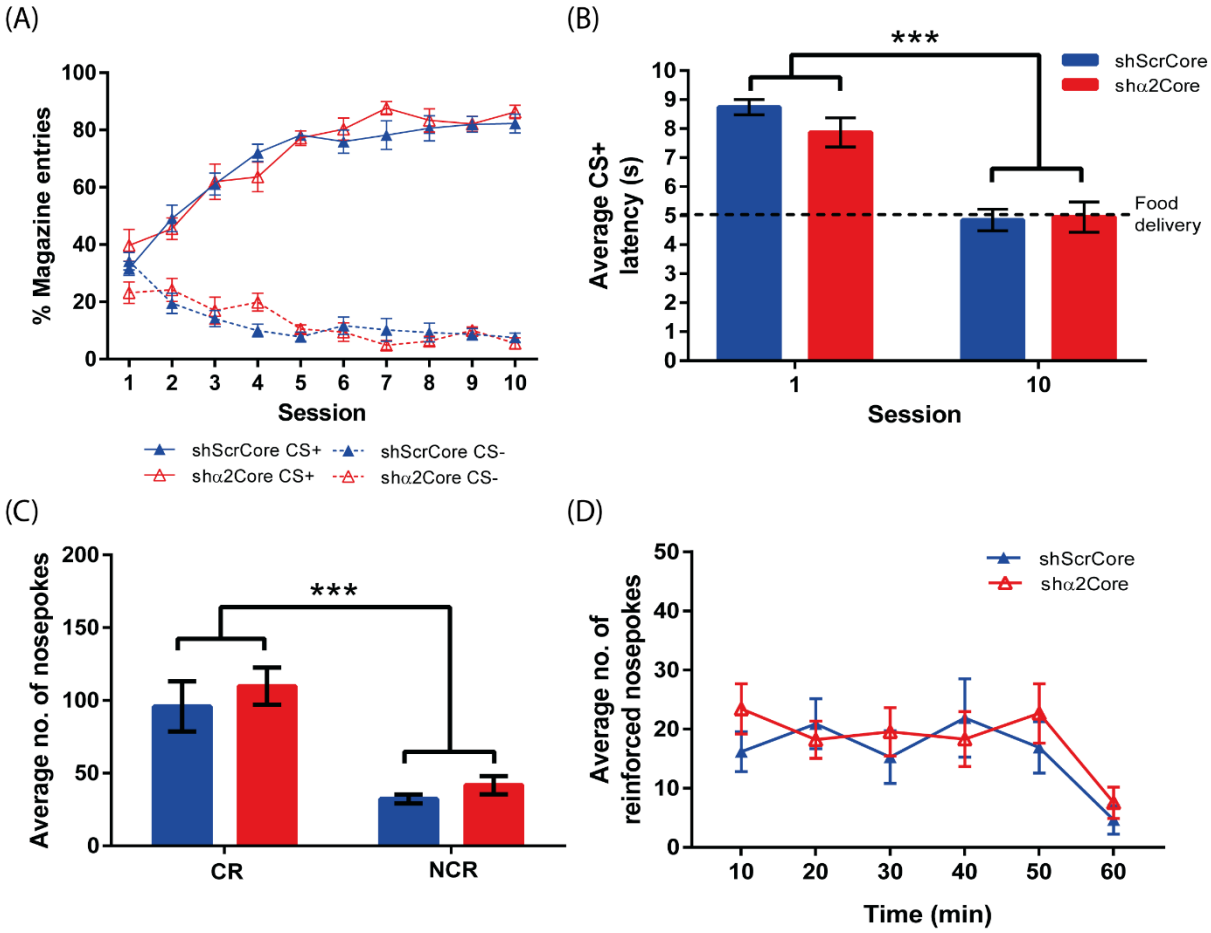



***Figure 4.3.*** Effects of α2 knockdown in the NAc core on conditioned reinforcement. **(A)** Both shScr (n = 11) and shα2 (n = 9) groups of mice learned the Pavlovian stimulus-reward association to a similar degree, as indicated by the level of discriminated approach over the course of 10 daily sessions and **(B)** the amount of time taken (latency) to approach the food magazine upon CS+ onset on sessions 1 and 10. **(C)** The rates of reinforced and non-reinforced nosepoke responses during a conditioned reinforcement test. Both groups made more nosepoke responses into the reinforced, than in the non-reinforced module. Reducing α2 expression in the NAc core affected neither the total nor **(D)** the pattern of the reinforced response rates. Data are presented as mean ± SEM. ***$p < .001$

#### 4.3.2.3. Cocaine enhancement of conditioned reinforcement

The rates of nosepoking for CR and NCR with acute cocaine pre-treatments, administered in a Latin-square arrangement, are summarised in Figure 4.4. Data were log-transformed to maintain homogeneity of variance. A three-way mixed ANOVA comparing CR vs. NCR nosepokes in different groups pre-treated with multiple doses of cocaine revealed statistical significance for the three-way interactions (nosepokes × group × dose) ($F(2,36) = 4.57$, $p < .05$), as well as for all of the two-way interactions, i.e. dose × group ($F(2,36) = 8.15$, $p = .001$), nosepokes × dose ($F(2,36) = 6.73$, $p < .01$) and nosepokes × group ($F(2,36) = 10.88$, $p < .01$) interactions. These collectively suggest that the interaction between cocaine treatments and the rates of nosepoking for CR and NCR was likely to be affected by manipulation of α2 subunit expression in the NAc core. Given the complex nature of the three-way interaction, post-hoc analyses of simple main effects and interactions within each experimental group were performed.

Firstly, shScr group made significantly more CR over NCR nosepokes regardless of the dose of cocaine administered (i.e. significant main effect of nosepokes $F(1,10) = 62.76$, $p < .001$) and the stimulant properties of cocaine were also evident through a significant main effect of dose ($F(2,20) = 33.54$, $p < .001$). Importantly, a significant nosepokes × dose interaction, $F(2,20) = 45.90$, $p = .001$) further suggests that cocaine selectively increased nosepoking for CRf. Repeated measures ANOVAs (with Bonferroni correction for multiple comparisons), conducted to explore cocaine effects on

the rates of nosepoking for the CR and NCR separately, revealed significant main effects of dose on reinforced nosepokes ($F(2,20) = 42.60$, $p < .001$), as well as on non-reinforced nosepokes ($F(2,16) = 6.58$, $p < .01$), indicating dose-dependent increases and decreases in reinforced and non-reinforced nosepokes respectively in the shScr group (see Figure 4.4A).

Similarly, suppressing α2 expression in the NAc core left instrumental responding for CRf intact (i.e. a significant main effect of nosepokes, $F(1,8) = 113.14$, $p < .001$). However, this manipulation completely abolished cocaine's ability to selectively enhance CR-driven responding (i.e. a significant main effect of dose, $F(2,16) = 7.54$, $p < .01$ and a non-significant nosepokes × dose interaction, $F(2,16) = 0.15$, $p = .865$) (Figure 4.4B)

In summary, both experimental groups made more responses on the reinforced than the non-reinforced module, regardless of experimental manipulations (i.e. cocaine pre-treatment and/or RNAi-induced knockdown of α2 expression in the NAc core) introduced in the present research. Nevertheless, whilst responding on the CR-associated module was selectively enhanced by acute cocaine in the shScr group, it was not in the shα2 counterpart.

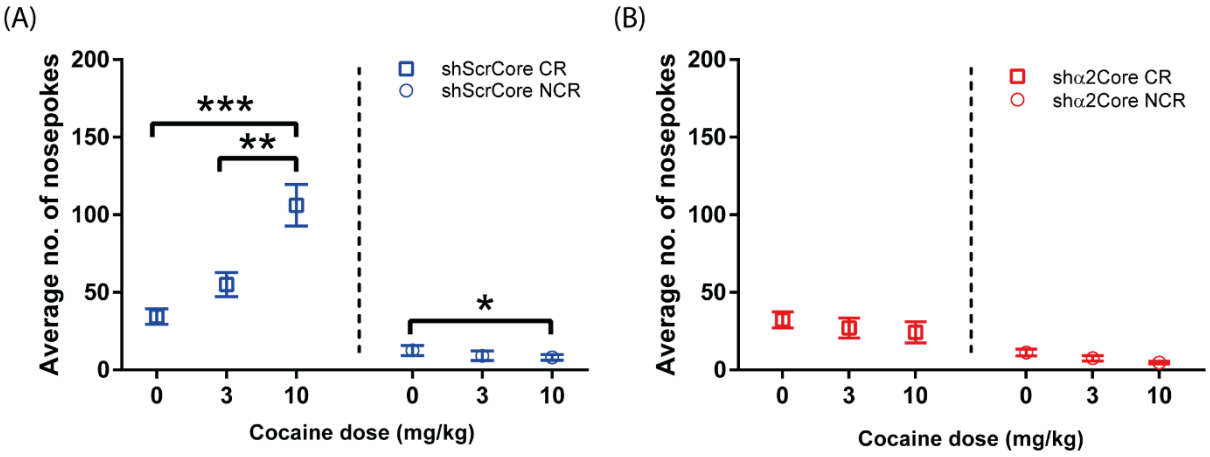



***Figure 4.4.*** Cocaine facilitation of conditioned reinforcement in shScr and shα2 groups. **(A)** Cocaine markedly increased responding for the conditioned reinforcer and decreased responding for the non-conditioned reinforcer in the shScr group, but **(B)** yielded no marked effects in the shα2 counterpart.

### 4.3.3. The effects of α2 knockdown in the NAc core and shell on cocaine-induced locomotor sensitisation

Next, two separate experiments (i.e. BS-CORE and BS-SHELL experiments) were conducted to probe whether α2 expression in the NAc core and/or shell was critical for cocaine-induced locomotor sensitisation (for group details. For each experiment, two experimental groups, i.e. shScrCore and shα2Core in the BS-CORE experiment, and shScrShell and shα2Shell in the BS-SHELL experiment, were used (for group details, see Figure 4.2C). A brief summary of the sensitisation experimental timeline is shown in Figure 4.5A.

Sensitisation to cocaine was assessed by measuring locomotor activity following repeated, daily cocaine over the course of 10 days (Day 3-12) and the conditioned activity to the cocaine-associated context was analysed by comparing locomotor activity following sham injections pre- and post-cocaine sensitisation (i.e. Day 2 vs. Day 19). Acute locomotor effects of cocaine were analysed by comparing locomotor activity post-sham injection (Day 2) and following the first exposure to cocaine (Day 3).

Locomotor activity in each session is presented as the average distance travelled (forward movement) in metres and each session lasted for 60 minutes. Also, note that one animal from the shα2Core group (BS-CORE experiment), as well as four animals, two from shScrShell and two from shα2Shell groups, in the BS-SHELL experiment were excluded from the

experiment due to inaccurate targeting of either side of the NAc core or shell.

#### 4.3.3.1. Acute locomotor responses to cocaine: Effects of α2 knockdown in the NAc core or shell

All groups of mice regardless of α2 manipulation in the NAc displayed increases in locomotor activity to acute cocaine (i.e. significant main effect of dose in BS-CORE and BS-SHELL experiments, $F_{CORE}(1,17) = 19.08$, $p < .001$; $F_{SHELL}(1,7) = 6.96$, $p < .05$; Figures 4.5B&C). In fact, RNAi-induced perturbations of α2 levels in the NAc core or shell did not seem to affect acute responses to cocaine when compared to the respective shScr counterparts, indicated by statistically non-significant dose × group interaction ($F_{CORE}(1,17) = 0.02$, $p = .886$; $F_{SHELL}(1,7) = 2.08$, $p = .192$) and main effect of group ($F_{CORE}(1,17) = 0.08$, $p = .777$; $F_{SHELL}(1,7) = 2.22$, $p = .180$).

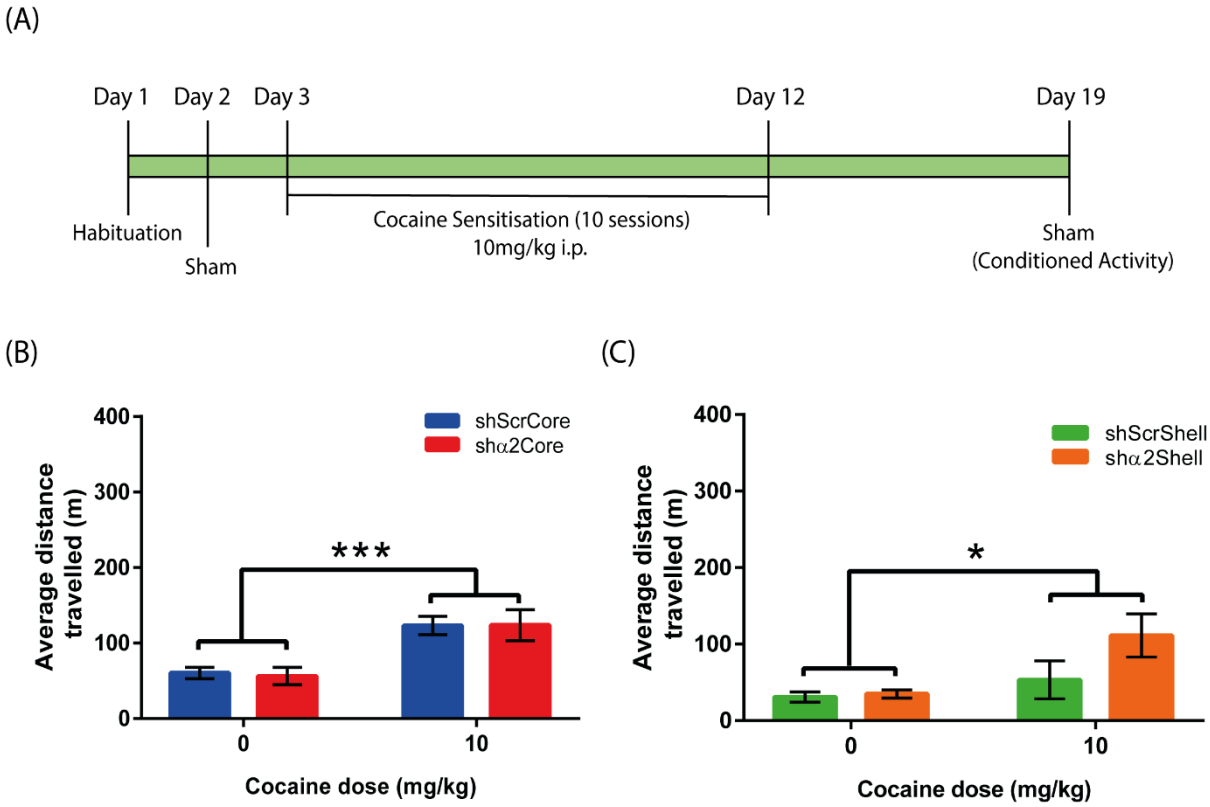



*Figure 4.5.* (A) Timeline of the cocaine sensitisation experiment. (B) Reducing α2 expression in the NAc core or (C) in the shell subdivision failed to alter acute locomotor responses to cocaine. Data are presented as mean ± SEM. *** $p < .001$ * $p < .05$

### 4.3.3.2. Sensitisation to cocaine: Effects of α2 knockdown in the NAc core (BS-CORE)

Figure 4.6A displays the effects of repeated, non-contingent cocaine administration (10 mg/kg i.p.) on locomotor activity in the shScrCore and shα2Core groups. An ANOVA comparing group differences in the locomotor responses to cocaine across 10 days revealed a significant main effect of session (Greenhouse-Geisser corrected, $F(4.95,84.24) = 7.781$, $p < .001$, $\varepsilon = .551$), but no significant main effect of group ($F(1,17) = 0.74$, $p = .402$) or group × session interaction (Greenhous-Geisser corrected, $F(4.95,84.24) = 0.38$, $p = .863$, $\varepsilon = .551$), suggesting that both groups of mice sensitised to cocaine to a similar degree. Locomotor sensitisation to cocaine was further evidenced by a significant elevation in cocaine-induced responses on session 10 relative to those on session 1, through a significant main effect of session with the estimated marginal means (± SEM) of the distance travelled of 120.43 ±11.51 metres and 174.90 ±14.53 metres for sessions 1 and 10 respectively ($F(1,17) = 12.53$, $p < .01$) (Figure 4.6C). Intriguingly, there was no evidence of conditioned activity in either group, as no differences in locomotor activity following sham injections pre- and seven days post-sensitisation were observed (i.e. non-significant main effect of session ($F(1,17) = 0.14$, $p = .716$), group ($F(1,17) = 0.24$, $p = .631$) or session × group interaction ($F(1,17) = 0.69$, $p = .419$)) (Figure 4.6E).

#### 4.3.3.3. Sensitisation to cocaine: Effects of α2 knockdown in the NAc shell (BS-SHELL)

Figure 4.6B illustrates the effects of repeated, non-contingent cocaine administration (10 mg/kg; i.p.) on locomotor activity in the shScrShell and shα2Shell groups. The ANOVA comparison of acute locomotor responses to cocaine (over 10 sessions) between experimental groups yielded a significant main effect of session ($F(9,63) = 15.57$, $p < .001$), but the group × session interaction was statistically non-significant ($F(9,63) = 0.67$, $p = .734$), indicating that cocaine-induced sensitisation was evident in both groups. Importantly, α2 knockdown in the NAc shell also failed to disrupt cocaine-induced sensitisation in contrast to observations in the constitutive knockout mice (Dixon et al., 2010). Instead, manipulating α2 levels specifically in the NAc medial shell appeared to enhance acute responses to cocaine, indicated by the significant main effect of group ($F(1,7) = 10.95$, $p < .05$). This was further confirmed by an ANOVA comparison of locomotor responses to cocaine on sessions 1 and 10, through a non-significant group × session interaction ($F(1,7) = 0.90$, $p = .375$), but significant main effects of group ($F(1,7) = 9.92$, $p < .05$) and session ($F(1,7) = 41.09$, $p < .001$), with estimated marginal means of 82.32 ±19.35 metres and 242.66 ±16.58 metres for sessions of 1 and 10 respectively (Figure 4.6D).

In marked contrast to the BS-CORE data, both groups in the BS-SHELL experiment displayed a significantly higher locomotor activity following sham injections post-, compared to pre-sensitisation, with the

estimated marginal means of 32.81 ±4.19 metres and 102.54 ±10.32 metres pre- and post-sensitisation respectively (i.e. significant main effect of session $F(1,7) = 76.03$, $p < .001$), thereby providing evidence for conditioned activity in the cocaine-associated locomotor chamber (Figure 4.6F). However, no group differences were observed, either via group × session interaction ($F(1,7) = 2.19$, $p = .183$) or via the main effect of group ($F(1,7) = 1.34$, $p = .285$). Overall, the current data show that α2 knockdown in the NAc core or shell did not disrupt cocaine-induced locomotor sensitisation. Silencing α2 expression specifically in the NAc shell, however, appeared to enhance the acute locomotor effects of cocaine without affecting its conditioned behavioural effects.

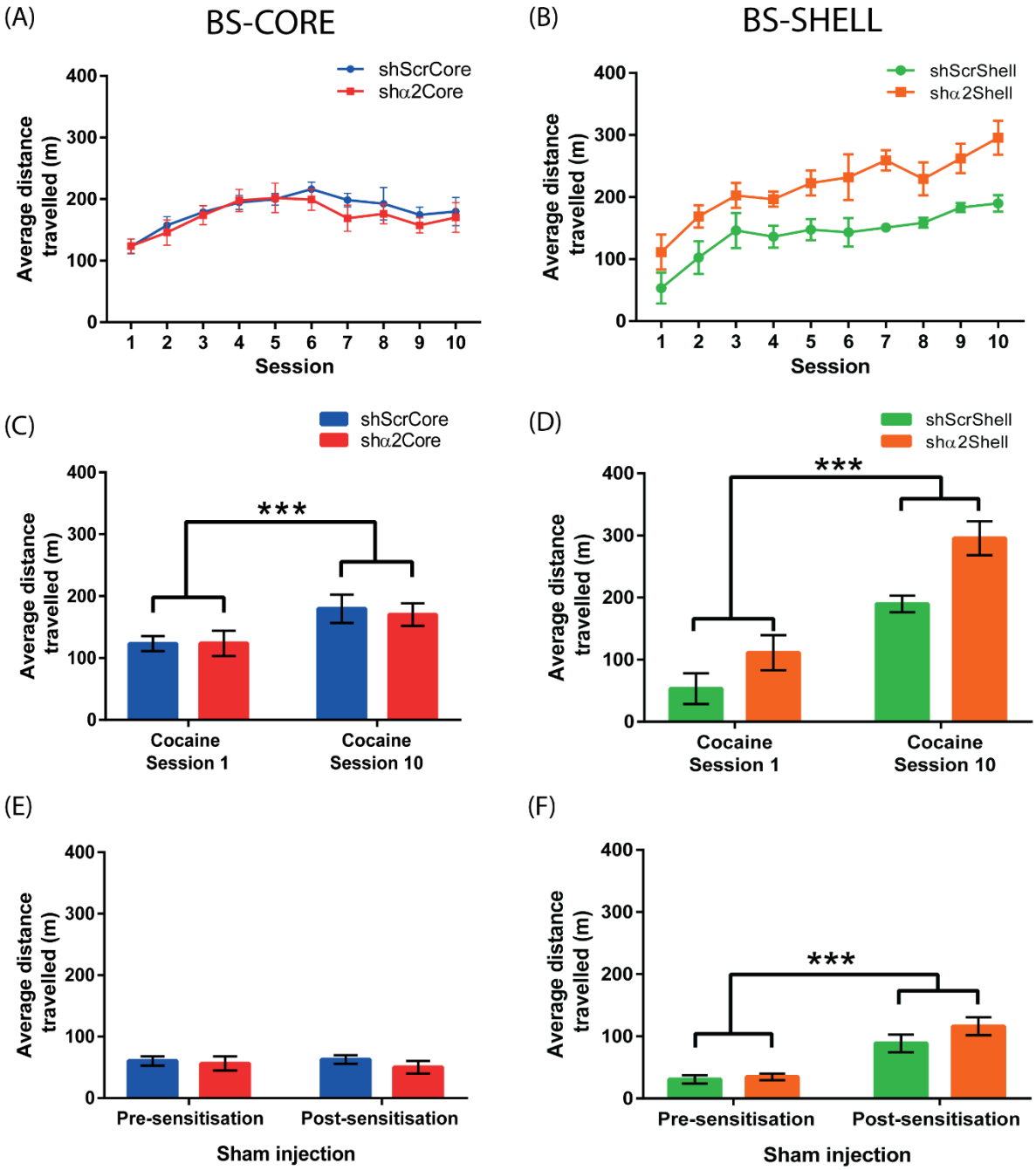

(A)
BS-CORE
shScrCore
shα2Core
Average distance travelled (m)
Session
(B)
BS-SHELL
shScrShell
shα2Shell
Average distance travelled (m)
Session
(C)
shScrCore
shα2Core
***
Average distance travelled (m)
Cocaine Session 1
Cocaine Session 10
(D)
shScrShell
shα2Shell
***
Average distance travelled (m)
Cocaine Session 1
Cocaine Session 10
(E)
Average distance travelled (m)
Pre-sensitisation
Post-sensitisation
Sham injection
(F)
***
Average distance travelled (m)
Pre-sensitisation
Post-sensitisation
Sham injection

***Figure 4.6.*** Effects of repeated intermittent cocaine (10mg/kg) on locomotor activity. **(A&C)** Knockdown of α2 expression in the NAc core or **(B&D)** shell did not abolish cocaine's sensitising effects. The shα2Shell (n = 5) mice displayed higher acute locomotor responses to cocaine compared to the shScrShell (n = 4) group ($p < .05$). **(E)** Conditioned locomotor activity was notably absent in shScrCore (n = 10) and shα2Core (n = 10) groups in the BS-CORE experiment. **(F)** By contrast, cocaine-sensitised shScrShell and shα2Shell groups (BS-SHELL experiment) displayed conditioned activity ($p < .001$), though no between-group differences were observed. Data are presented as mean ± SEM. ***$p < .001$

## 4.4. Discussion

This study offers an important insight into the interaction between dopaminergic and GABAergic systems by demonstrating the functional significance of the $GABA_AR$ α2 subunit in the NAc in the expression of cocaine-induced responses. Namely, RNAi-induced silencing of α2 expression selectively in the NAc core abolished cocaine's ability to invigorate the expression of a learned behaviour (i.e. nosepoking for CRf), though manipulation of α2 in the NAc core or shell failed to affect cocaine-induced locomotor sensitisation. Instead, α2 knockdown specifically in the NAc shell enhanced acute locomotor, but not the conditioned, effects of cocaine. These data thus question the initial hypothesis, also proposed in the Dixon et al. (2010) study, that both cocaine's sensitising and CRf-enhancing properties may share a common circuitry involving mesoaccumbal α2-containing receptors, downstream of the dopaminergic pathway.

Although both of the behavioural models used in this thesis appear to engage an overlapping circuitry involving the mesolimbic dopamine system, there exists evidence for the mechanistic dissociation between psychostimulant-facilitated locomotor sensitisation and CRf. For example, the VTA and NAc (especially for cocaine) are key structures for the development of psychomotor sensitisation (reviewed in Vanderschuren & Kalivas, 2000), whereas psychostimulant enhancement of CRf has been

shown to implicate both the NAc and VP (Fletcher, Korth, Sabijan, & DeSousa, 1998; Figure 4.7)

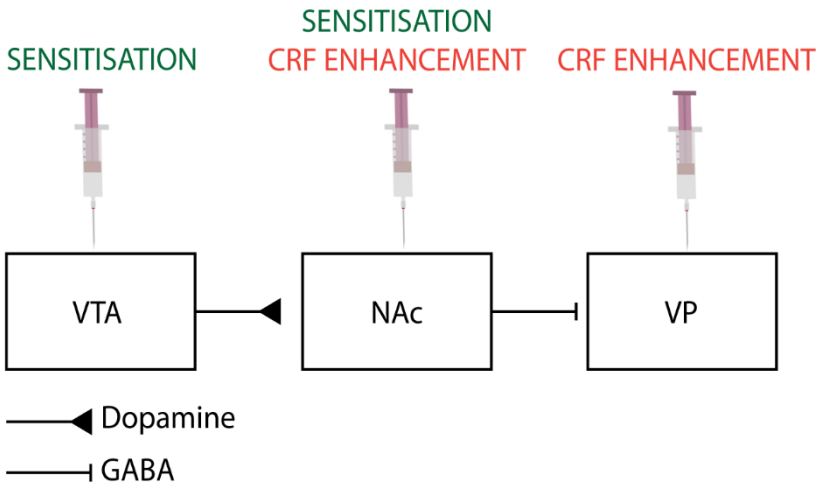



***Figure 4.7.*** Dissociation between psychostimulant-facilitated locomotor sensitisation and CRf revealed by localised drug administration studies (Fletcher et al., 1998; Vanderschuren & Kalivas, 2000).

### 4.4.1. The effects of α2 knockdown in the NAc core and shell on locomotor sensitisation to cocaine

Emerging evidence to date has implicated the role of GABA in the expression of drug-induced locomotor sensitisation. Namely, reducing $GABA_AR$-mediated inhibition via whole-brain ablation of Gabra2 and optogonetic activation of GABA-expressing neurons in the NAc have been shown to block cocaine-induced sensitisation respectively (Dixon et al., 2010; Wang et al., 2014). Similarly, decreasing inhibitory tone in the NAc by selectively silencing of parvalbumin-expressing FSIs blocked early ad later stages of amphetamine-induced locomotor sensitisation (Wang et al., 2017),

collectively pointing to mesoaccumbal GABAergic activity as a critical substrate underpinning psychostimulant-induced sensitisation. This is further substantiated by the Henry & White (1995) study, which detected a sensitised response of NAc neurons to iontophoretically applied GABA after one day of withdrawal in cocaine-sensitised rats. Nevertheless, it is important to note that the mechanism by which deletion of α2-$GABA_A$Rs affects the net neuronal activity and output remains unclear, given the varying roles of phasic inhibitory systems depending on the receptor's subcellular localisation (i.e. somatic vs. dendritic localisation) (extensively reviewed in Koos, Tepper, & Wilson, 2004; Tepper, Tecuapetla, Koós, & Ibáñez-Sandoval, 2010; Tepper, Wilson, & Koós, 2008). Due to the hyperpolarised RMP of striatal MSNs, which is more negative than the $Cl^-$ reversal potential (i.e. ~-60mV), GABA action at $GABA_A$Rs can have excitatory effects on MSN activity. In the MSNs, this is likely to occur when GABAergic post-synaptic potentials (PSP) shortly precede glutamatergic inputs in a time window of 140ms (with maximum effect reached between 50-60ms, limiting the influence of shunting effects) (Bracci & Panzeri, 2006; also discussed further in Chapter 1). Based on evidence available to date, it appears that the blockade of locomotor sensitisation to psychostimulants is associated with altered GABAergic control of the NAc neurons.

Findings from the present research, however, demonstrated that reducing $GABA_A$R α2 subunit expression in the NAc core or shell by RNAi, potentially dampening phasic inhibitory control of neuronal activity (Dixon et al., 2010), did not affect the development of cocaine-induced locomotor

sensitisation. This is in line with the widely-accepted notion that the NAc is primarily implicated in psychostimulant-induced locomotor hyperactivity, but not necessarily in the development of locomotor sensitisation per se (see Kalivas & Weber, 1988; Pijnenburg & Rossum, 1973), though note that this view was primarily built upon observations on dopaminergic activity. Moreover, based on the previous knockout data (Dixon et al., 2010), one could posit that suppressing α2 level in the NAc core or shell specifically might not be sufficient to induce marked effects on sensitisation. This seems unlikely as a study by Engin et al. (2014) demonstrated that targeted α2 knockdown in the whole NAc using the site-specific Cre/Lox recombination technology also failed to produce marked effects on locomotor sensitisation to cocaine.

Virtually all drugs of abuse decrease neuronal firing in the NAc (Bourdelais & Kalivas, 1990; reviewed in Wise, 1998) and such decreases in accumbal activity have been linked to drug-induced locomotor hyperactivity (Henry & White, 1995; Pennartz, Groenewegen, & Lopes da Silva, 1994). Slice electrophysiological data also identified decreased synaptic strength at excitatory synapses in the NAc shell of cocaine-sensitised mice (Thomas, Beurrier, Bonci, & Malenka, 2001). Increasing NAc neuronal activity by means described in previous studies might therefore block drug-induced sensitisation and mere downregulation, instead of complete ablation, of α2 subunit in the NAc might not be sufficient to alter NAc activity to an extent that would adversely influence cocaine-induced effects that underlie the development of locomotor sensitisation. While this remains a plausible

speculation, note that in some cases, effects that are masked due to compensatory changes in the constitutive knockouts could be more easily observed in the knockdowns due to minimal or no compensations (Engin et al., 2014). Without electrophysiological evidence, this study cannot eliminate the possibility that α2 knockdown might, in fact, induce a larger effect on the mIPSC characteristics relative to the knockouts.

Some of the key regions implicated in the development of sensitisation, including the PFC (Yong Li et al., 1999; Wolf et al., 1995) and the dorsal hippocampus (Degoulet et al., 2008) harbour high amounts of the $GABA_AR$ α2 subunit (Hörtnagl et al., 2013). The phenotypic discrepancy observed between the current data and those from the α2 constitutive knockouts (Dixon et al., 2010) might stem from the absence of α2 in region(s) other than the NAc (discussed further in Chapter 6). Developmental factors and the extended period α2 deletion are also among the variables that are likely to contribute to the loss of sensitisation in the α2 knockouts, which are not applicable to gene silencing by RNAi. Overall, findings from this study indicate that α2 expression in the NAc core or shell is not required for cocaine-induced sensitisation.

Conditioning has long been thought to be an important process in aiding the expression of sensitisation. In some cases, this form of learning is critical for the development of sensitisation (Crombag et al., 2001; Dykman, 1976; Le Merrer & Stephens, 2006; Mattson et al., 2007). However, in the BS-CORE experiment, conditioned activity was not observed using the current measure, irrespective of the α2 manipulation, perhaps suggesting

that sensitisation might have occurred in a context-independent manner. It should be noted that given the lack of saline control groups in the sensitisation experiments, the measure of conditioned activity is distinct from those typically presented in previous research. Alternatively, one could argue that the damage resulting from viral injection into the NAc core might have somewhat disrupted context-dependent learning and/or other phenotypes that may influence the expression of conditioned activity, such as reactivity to novel environment. While the former explanation is unlikely given that such procedure induced no effect on other forms of conditioning observed in the present research (see CRf discussion below), the latter remains a possibility given that NAc lesioned-rats displayed significantly higher locomotor activity in a novel environment than the sham controls (Burns, Annett, Kelley, Everitt, & Robbins, 1996). The occurrence of such a behavioural effect might have masked conditioned activity in the present research and thus, elucidated the lack of conditioned locomotor activity in both groups. One way of addressing this would be to further habituate the mice to the locomotor runways prior to the first cocaine exposure. It is also worth noting that dopamine-depleting lesions in the NAc core have previously been found to impair conditioned locomotor response (Sellings & Clarke, 2006).

Notably, α2 knockdown in the NAc shell, but not in the core, potentiated the acute locomotor effects of cocaine, whilst having no effects on cocaine-induced conditioned activity. Complementary to these findings, lesion to the dorsomedial shell of the NAc disrupted neither the baseline

locomotor activity nor locomotor responses to the initial (acute) exposure to cocaine (15mg/kg; i.p.). However, the lesion group exhibited significantly lower locomotor activity compared to the sham controls on the fifth (last) day of repeated cocaine regimen (Todtenkopf, Carreiras, Melloni, & Stellar, 2002). Similar findings implicating the importance of NAc shell in psychostimulant-induced locomotor hyperactivity were also reported in the Parkinson et al. (1999) study. Interestingly, dampening GABAergic activity, via local injection of $GABA_AR$ antagonist picrotoxin in the NAc, also increased locomotor activity, mimicking the effect of intra-accumbal dopamine injection (Wachtel & Anden, 1978; Costall & Naylor, 1975; Pijnenburg & Rossum, 1973). It is thus conceivable that intact GABAergic activity, particularly in the NAc shell, may serve to attenuate psychostimulant-induced locomotor hyperactivity such that in a state of reduced inhibition, heightened locomotor responses to the drug were observed.

Secondly, the lack of group differences in conditioned activity in the BS-SHELL experiment extends previous and current observations that α2-$GABA_ARs$ are not essential for mediating the unconditioned properties of food and psychostimulant rewards that facilitate conditioning, either in the sensitisation, CRf, or in the drug self-administration paradigm (Dixon, Halbout, King, & Stephens, 2014; Dixon et al., 2010; also see the CRf discussion below).

### 4.4.2. The effects of α2 knockdown in the NAc core on conditioned reinforcement and its facilitation by cocaine

Despite having no effect on cocaine-induced sensitisation, α2 depletion specifically in the NAc core abolished cocaine's ability to invigorate instrumental responding maintained by the CR, most likely through GABA × dopamine interactions. Corroborating this view is the findings from pharmacological and lesion studies, collectively yielding a conclusion that the mesolimbic dopamine system does not mediate CRf, but is critical for its potentiation by psychostimulant agents (Taylor & Robbins, 1986; Wolterink et al., 1993).

Results from this study also extend existing evidence on cocaine's ability to energise instrumental responding for CRf (Chu & Kelley, 1992; Dixon et al., 2010; Macpherson et al., 2016). Namely, the shScr group displayed markedly enhanced reinforced nosepoking with cocaine (10 mg/kg) pre-treatment. The magnitude of cocaine effect was, in fact, comparable to that observed in a separate group of mice receiving sham injection into the NAc core to control for both the surgical procedure and activation of the RNAi pathway (see Appendix B).

Firstly, the loss of cocaine facilitation in the shα2 group did not arise from their lack of ability to learn stimulus-reward (Pavlovian) or action-outcome (instrumental) association. Similar findings were also reported with the α2 knockouts, suggesting that GABA action α2-containing receptors is not required for basic reward-learning processes. In keeping, it

has been reported that $GABA_AR$-mediated activity in the NAc, primarily in the shell, appears to subserve an inflexible behavioural component of feeding that is independent of its incentive motivational property via disinhibition of neurons within the lateral hypothalamus (Stratford & Kelley, 1997, 1999), as well as the VP (Stratford & Wirtshafter, 2012) that mediate motor programs specific to ingestion. This mechanistic pathway is thought to bypass inputs that are relevant to food-seeking behaviour, including appetitive conditioning. Further, deficits in the acquisition of CRf responding were observed only upon selective excitotoxic lesion of the BLA, but not in CeA-, NAc core-, or shell-lesioned rats, relative to the respective controls (Cador et al., 1989; Parkinson et al., 1999; Robledo, Robbins, & Everitt, 1996), implicating the significance of BLA integrity in the expression of CR-governed motivated behaviours.

Importantly, providing anatomical specificity to published data with the α2 constitutive knockouts is the finding that intact functioning of α2-$GABA_ARs$ specifically in the NAc core is critical for cocaine facilitation of CRf. Firstly, note that cocaine enhancement of CRf is not a result of cocaine-induced increases in general activity, thus eliminating the argument that the loss of cocaine's facilitatory effect on CRf in the shα2 mice was simply attributed to insensitivity to cocaine's motor stimulant effect. The locomotor data presented above lend further support to this argument. Alternatively, due to the predictive value of the CR (i.e. predicting food delivery), as well as the physiological state of the animals at the time of testing, one could posit that cocaine might have affected other aspects of food-seeking

behaviour in the shα2 group, e.g. increasing conditioned Pavlovian approach or goal-tracking responses (Holden & Peoples, 2010), thus masking its CRf-potentiating effect. This is also unlikely as this study failed to observe cocaine-induced potentiation in the rates of magazine entries during the CRf sessions (Appendix C). Thirdly, a pilot CRf experiment, performed to investigate the effect of α2 knockdown in the DS further questioned the possibility that the phenotypes observed in the shα2 group were artefactual results of viral leakage in the DS (Appendix D).

Instead, the answer may lie in the synergistic interaction between GABA and dopamine in the NAc, namely, how RNAi-induced downregulation of α2 expression affects dopamine action such that cocaine-evoked increases in extracellular dopamine in the NAc core can no longer act as a 'gain-amplifier' controlling the vigour of the goal-directed response. To date, a few mechanisms by which GABAergic and dopaminergic systems interact have been proposed. Firstly, it is now well-established that dopamine favours the activation of D1-MSNs but dampens the excitability of those harbouring D2Rs (Gerfen & Surmeier, 2011; Harsing & Zigmond, 1997). At the intercellular level, reducing α2-$GABA_AR$ expression may interfere with the synaptic communication, i.e. lateral and feedforward inhibition, both of which are deemed critical in the manifestation of cocaine-induced responses, e.g. locomotor hyperactivity and self-administration, to name a few (Dobbs et al., 2016; Yu et al., 2017). Alternatively, a recent study indicated that midbrain dopamine neurons can inhibit striatal MSNs through a non-canonical release of GABA, i.e. via the membrane uptake, not

synthesis of GABA (Tritsch, Jun, Ding, & Sabatini, 2012; Tritsch, Oh, Gu, & Sabatini, 2014). Though the understanding of the functional relevance of GABA co-released with dopamine remains in its infancy, it may be of significance when considering the interaction between drug-induced elevation in extracellular dopamine and CR-induced *firing* of dopamine neurons, co-releasing GABA and dopamine (Schultz, 1998).

At the intracellular level, the action of dopamine at D1Rs and D2Rs activate and inhibit protein kinase A (PKA) through $G_s/G_{olf}$ and $G_{i/o}$ respectively (Stoof & Kebabian, 1984; also refer to Chapter 1 for more details). The $GABA_AR$ ß1 and ß3 subunits are subject to PKA-mediated phosphorylation, whereby phosphorylation of the former reduces, whereas ß3 phosphorylation facilitates GABAergic currents (McDonald et al., 1998). D1R stimulation and/or internal PKA application were reported to facilitate α5- and δ-containing $GABA_AR$-mediated tonic conductance in the D1-MSNs of juvenile and adult mice respectively, whereas D1R- or D2R-stimulation marginally prolonged the mIPSC decay kinetics in juvenile mice (Janssen, Ade, Fu, & Vicini, 2009; Maguire et al., 2014). It is yet to be determined whether dopamine effect on phasic GABA currents is somewhat distinct in adult mice and potentially possesses functional significance that may underlie some of cocaine's reinforcing properties.

Somewhat corroborating the current findings are those presented in the (Parkinson et al., 1999) study, which also implicated the NAc core as a critical structure for psychostimulant enhancement of CRf. The fundamental difference between the current and previous data however lies

in the type of manipulations, affecting neuronal activity in a seemingly antagonistic fashion. Namely, at a more cellular level, both reducing α2 expression, thus presumably reducing inhibitory tone and increasing net activity (though also note the depolarising effects of GABA in striatal neurons discussed above), and the loss of NAc core activity via excitotoxic lesion (Parkinson et al., 1999) completely abolished psychostimulant's ability to enhance responding for CRf. In fact, similar observations have been observed with agonism or antagonism at AMPA and NMDA receptors in the NAc (Burns et al., 1994). Adding to this complexity, lesioning the ventral subiculum of the hippocampus (Burns et al., 1993) or CeA (Robledo et al., 1996), which provides glutamatergic afferents to the NAc and midbrain dopamine neurons respectively also abolished psychostimulant potentiation of CRf. Taken together, intact functioning of glutamatergic, dopaminergic, and GABAergic transmission in the NAc core, as well as the interaction between these subsystems are vital for psychostimulant's CRf-potentiating effects. A CRf experiment investigating the consequence of α2 knockdown in the shell was also carried out but yielded inconclusive results due to small sample sizes.

### 4.4.3. Conclusions and technical considerations

To date, our understanding of the exact mechanism underlying the relationship between GABA action at α2-$GABA_A$Rs and the expression of cocaine-induced dopamine-mediated behaviours remains rudimentary. This study, to the best of our knowledge, is the first to employ viral-based RNAi

in efforts to investigate the functional role of α2-$GABA_A$Rs in mouse models and using this method, the results indicated that wild-type expression level of $GABA_A$R α2 subunit is critical for cocaine's CRf-enhancing properties, albeit not for reward learning or for cocaine's sensitising properties. Given that whole-brain deletion of Gabra2 also abolished methylphenidate potentiation of CRf (Duka et al., 2015), it is plausible to assume that the effects of intra-accumbal α2 knockdown could be extrapolated to other stimulants though this warrants future investigation.

Of final note, there are several technical considerations that merit consideration in addition to those already mentioned above. Firstly, given the lack of evidence pertaining to RNAi-induced reduction in the *total number* of functional α2-containing receptors on the cell surface, caution should be exercised when interpreting these data, which assayed only the effects of manipulation on the total amount of the α2 subunit expression (refer to Chapter 3). This issue could be addressed by performing electrophysiological, ligand-binding, or immunohistochemical assays to provide a more accurate account of receptor function and/or density. Secondly, though one of the strengths of RNAi-induced knockdown is the lack of compensatory change, one should take into account the intrinsic homeostatic mechanism regulating phasic and tonic inhibition, whereby overexpression of extrasynaptic, α5- or α6-$GABA_A$Rs was found to decrease synaptic GABAergic transmission regardless of extrasynaptic receptor activation (Wu et al., 2013). Thus, this study cannot at present eliminate such homeostatic competition, which might contribute to the observed

phenotypes. One plausible way to rectify this limitation would be to characterise both phasic activity and tonic conductance in neurons targeted by the shRNA-harbouring rAAVs. Overall, findings from the present research add to the field by providing a deeper understanding of the role of α2-containing $GABA_A$Rs, specifically in the NAc subregions, in mediating some of cocaine-induced responses.

# Chapter 5

# Effects of $GABA_AR$ α2 subunit knockdown in the nucleus accumbens D1 or D2 receptor-containing neurons on cocaine-facilitated conditioned reinforcement

## 5.1. Introduction

Although the vast majority of neurons within the NAc are GABAergic, dopaminergic projections to the accumbens play an important role in modulating their function. Dopaminergic activity within the NAc is paramount for learning processes related to reinforcement and the exertion of effort-related actions (Di Chiara, 1998; Di Chiara et al., 2004; Nowend, Arizzi, Carlson, & Salamone, 2001; Salamone, 2006; Salamone & Correa, 2012; Saunders, Richard, Margolis, & Janak, 2017). Illustrating the former is the classic reward prediction error (RPE) model, built upon the observations that dopamine neurons fire not only when an animal received a reward but also when a previously neutral stimulus has been learned to predict its delivery (conditioned stimulus, CS). When a reward is fully predicted, the dopamine response to the reward itself disappears, but dopamine neurons continue to exhibit larger responses if more than the predicted reward occurs (Schultz, Dayan, & Montague, 1997; Schultz, 1998).

Although the mesolimbic dopamine system has long been thought as the biological substrate for *reward* or *hedonia*, originally proposed by Roy Wise (Wise, 1978), there is a wealth of evidence in the literature challenging the traditional tenets of the dopamine hypothesis of reward, thus prompting further theoretical development in the field of dopamine research (extensively reviewed in Nutt, Lingford-Hughes, Erritzoe, & Stokes, 2015; Salamone & Correa, 2012). After decades of investigations, it is now widely accepted that the mesolimbic dopamine is, rather, involved in motivational

processes (e.g. see Herberg, Stephens, & Franklin, 1976). Behavioural measures that were originally thought to measure the hedonic function of mesolimbic dopamine, e.g. progressive ratio breakpoints, are now viewed as measures of effort-related decision making (Salamone, 2006; Salamone & Correa, 2012). It is therefore no surprise that lesions of, or any form of interference with the dopaminergic system in the NAc would, to varying degrees, affect the ability of psychostimulant drugs to augment the expression of motivated behaviours (for examples, see Fritz et al., 2011; Parkinson, Olmstead, Burns, Robbins, & Everitt, 1999; Todtenkopf, Carreiras, Melloni, & Stellar, 2002).

Within the NAc, the modulatory action of dopamine is mediated by five dopamine receptors, classified into two major categories, i.e. D1-like and D2-like receptor classes (D1Rs and D2Rs), that are expressed by largely nonoverlapping subpopulations of MSNs (Gerfen et al., 1990; Gerfen & Surmeier, 2011). Note, however, that dopamine D2Rs are also found on cholinergic interneurons, thus often rendering interpretation of phenotypic changes linked to D2R manipulation difficult (Aubry, Schulz, Pagliusi, Schulz, & Kiss, 1993; Maurice et al., 2004). All dopamine receptors are G protein-coupled and regulate intracellular signalling cascades, serving to enhance or dampen neuronal outputs. D1Rs are coupled to $G_{s/olf}$ proteins. The binding of dopamine to D1Rs typically enhances neuronal excitability and facilitates long-term potentiation by stimulating adenylyl cyclase activity, elevating cyclic adenosine monophosphate (cAMP) and subsequently, activating protein kinase A (PKA). PKA has a wide array of

targets, including various transcription factors, ion channels, and glutamate receptors. In contrast, activation of D2Rs, coupled to the $G_{i/o}$ proteins, inhibits adenylyl cyclase, triggers the activation of $K^+$ channels, and the modulation of numerous ion channels, collectively dampening cell excitability and the facilitation of long-term potentiation (Neve, Seamans, & Trantham-Davidson, 2004; Stoof & Kebabian, 1984).

The conditioned reinforcement (CRf) paradigm has long been considered to provide a stringent measure of the reinforcing or motivational properties of Pavlovian conditioned stimuli (Kelleher, 1966). Akin to other psychostimulant drugs, cocaine has a capacity to energise instrumental actions to obtain the conditioned reinforcer (CR) and this cocaine effect is known to dopamine-dependent (Chu & Kelley, 1992; Dixon et al., 2010; Parkinson et al., 1999; Wolterink et al., 1993; refer to Chapter 1, Section 1.7). Though there is evidence that co-activation of D1Rs and D2Rs is required to enhance responding for CRf (Chu & Kelley, 1992), others have shown that selective activation of either receptor subtype is sufficient to increase the rate of responses for CRf (Wolterink et al., 1993). Studies with dopamine receptor antagonists, however, have yielded more consistent results. Namely, intra-NAc infusion of a D1R antagonist (SCH23390) or a D2R antagonist (raclopride, pimozide, or metoclopramide) completely blocked the potentiating effects of psychostimulants (i.e. *d*-amphetamine, cocaine, or pipradrol) on responding for CRf (Chu & Kelley, 1992; Ranaldi & Beninger, 1993; Wolterink et al., 1993). At higher doses, intra-accumbal microinfusion of SCH23390 or raclopride were found to block CRf in the

absence of amphetamine (Wolterink et al., 1993). These data collectively suggest that dopamine signalling via D1Rs and/or D2Rs is a key mechanism underlying the ability of psychostimulant drugs to enhance instrumental responding for CRf.

Nevertheless, it is ultimately the NAc activity, regardless of originating influences, that governs the behavioural output. The effect of dopamine is merely modulatory and it is glutamate that provides an excitatory drive to the NAc neurons. Consistent with this notion, pharmacological manipulations of the glutamatergic activity in the NAc have been reported to block amphetamine-facilitated CRf and amphetamine-induced locomotor hyperactivity (Burns, Everitt, Kelley, & Robbins, 1994). Similarly, alterations of GABAergic activity within the NAc have also been found to block cocaine-facilitated CRf (see Chapter 4), inhibited the expression of cocaine CPP, and attenuated the expression of cocaine-induced sensitisation (Wang et al., 2014). Together, these findings highlight the significance of intact functional interactions between the dopaminergic, glutamatergic, GABAergic subsystems for the manifestation of cocaine-induced behavioural effects.

Traditionally, D1- and D2-MSNs in the ventral striatum are thought have antagonistic influences on behaviours due to distinct projection targets within the basal ganglia (BG). Namely, D1-MSNs project directly to VM whereas D2-MSNs form indirect projections to the VM via the VP (Kreitzer & Malenka, 2008). Using a combination of optogenetics and electrophysiology, recent data presented in the Kupchik et al. (2015) study

revealed that MSN projections, specifically from the NAc core (ventromedial striatum) of mice, do not display exclusive segregation of the direct and indirect pathways. Instead, there is some degree of anatomical overlap in their projection targets as nearly half of the VP neurons are innervated by D1-MSNs, thus calling into question whether the anatomical and behavioural disparities between D1- and D2-MSNs apply to efferents from the NAc in mice.

Findings from progressive ratio tasks to test the level of effort exerted to obtain food reinforcers revealed that optogenetic inhibition of D1-MSNs or D2-MSNs reduced breakpoints, whereas optogenetic activation of mesoaccumbal D1R- or D2R-expressing neurons increased the number of cumulative presses and breakpoints, indicating an absence of accumbal D1-D2 antagonism (Natsubori et al., 2017; Soares-Cunha et al., 2016; Tsutsui-Kimura et al., 2017). In contrast, emerging literature supports the differential roles of D1- and D2-MSNs in regulating psychostimulant-induced motivated actions. For instance, optogenetic activation of D1- and D2-MSNs was found to generate opposing effects on cocaine-induced conditioned place preference, with the former promoting and the latter reducing conditioned preference for the cocaine-paired chamber (Lobo et al., 2010). Consistent with the Lobo et al. (2010) data, chemogenetic inhibition of D1-MSNs blocked cocaine-induced increases in D1-MSN activity and preference for the drug-paired chamber, leading to a premise that it is D1, albeit not D2, signalling that drives the motivation to enter the cocaine-paired chamber (i.e. the expression of CPP) (Calipari et al., 2016). The

differential involvement of D1-/D2-MSNs pertaining to drug-induced reinforcement has also been observed in the sensitisation (Chandra et al., 2013; Song et al., 2014). In contrast to the findings with food reinforcers, these data lend support to the behavioural distinction between D1- and D2-MSN activity pertaining to motivation.

Nearly all striatal neurons are GABAergic (Bolam et al., 1983; Tepper et al., 2010) and there is evidence that dopamine can modulate MSN activity (i.e. GABA release) in the striatum. Namely, D1R agonist enhances, whereas D2R agonist decreases, electrically-stimulated GABA release in the striatum (Harsing & Zigmond, 1997). These differential modulatory effects of dopamine, particularly on the lateral inhibitory network within the NAc, may be an important mechanism by which cocaine facilitates CRf, given that RNAi-induced knockdown of the α2 subunit expression in the NAc core was sufficient to block cocaine's CRf-enhancing properties (refer to Chapter 4). This study, therefore, postulated that the RNAi-induced translational repression of the α2 subunit in the NAc core D1R- or D2R-harbouring neurons, using the newly developed Cre-dependent RNAi vectors (refer to Chapter 3), would abolish or at least, attenuate cocaine facilitation of CRf.

## 5.2. Experimental design

The present research examined the effects of α2 knockdown in D1R- or D2R-expressing neurons within the NAc core (i.e. D1 and D2 experiments respectively). In each experiment, three groups of mice, i.e. D1- or D2-Cre mice injected with Cre-dependent rAAVs carrying either the shScr or shα2, and WT mice injected with the Cre-dependent shα2-containing vectors (see Section 5.3.1 for more details). A brief summary of the experimental procedure is described in Chapter 4 (refer to Chapter 2, Section 2.10 for detailed methods).

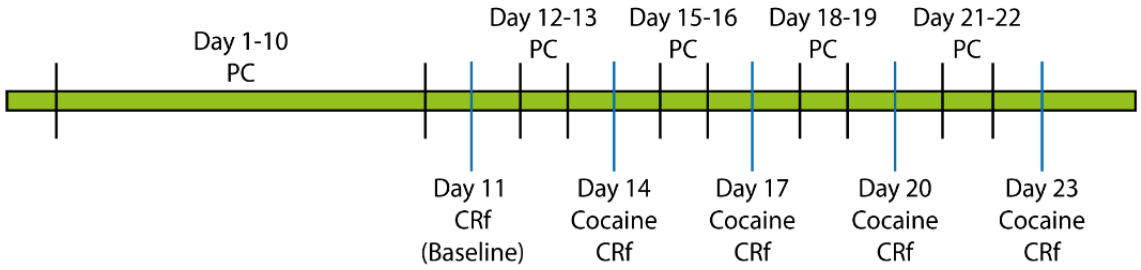



***Figure 5.1.*** An overview of the conditioned reinforcement experiment. In the Pavlovian conditioning session, animals were trained to associate a stimulus, either flashing lights or tone (counterbalanced across animals), with food reward. The position of the reinforced and non-reinforced nosepoke modules during CRf test sessions was also counterbalanced across animals.

## 5.3. Results

### 5.3.1. Histological assessment

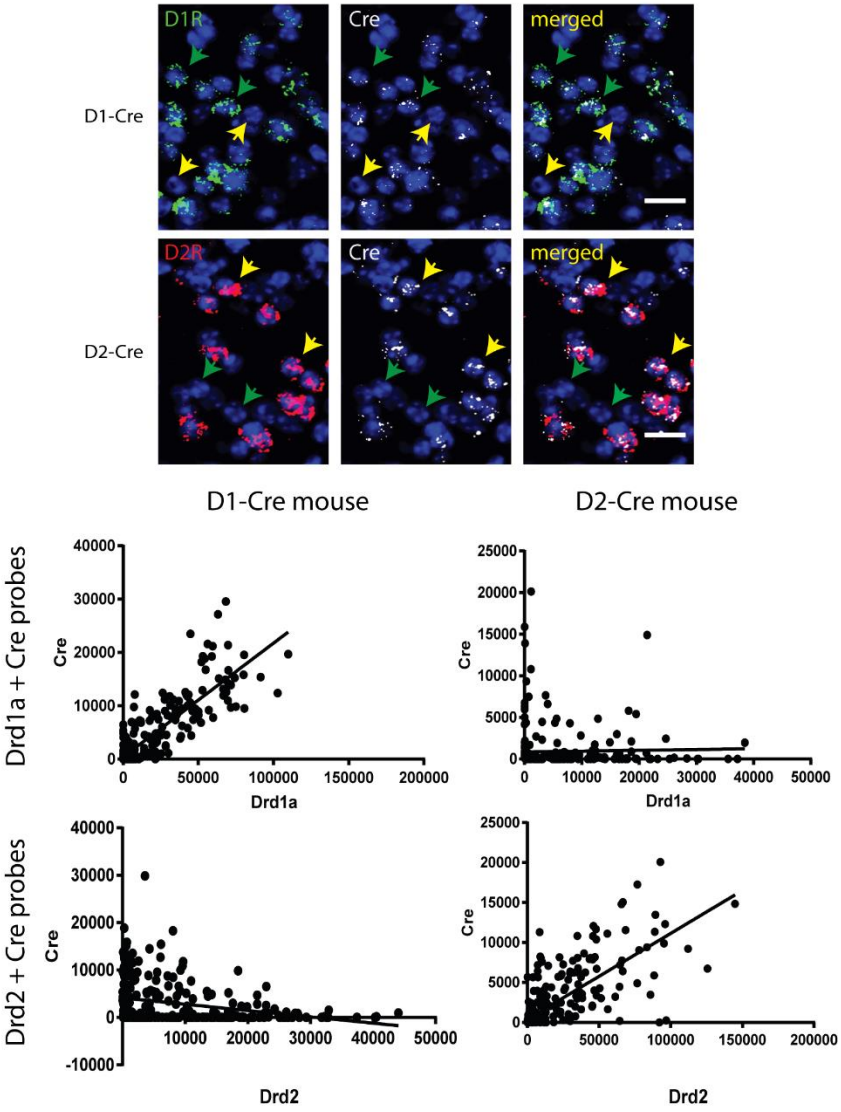
To probe the functional contributions of mesoaccumbal α2-$GABA_A$Rs specifically in D1R- and D2R-expressing neurons in the expression of motivated behaviour (i.e. CRf) and its potentiation by cocaine using the Cre-dependent rAAVs (harbouring shScr or shα2), D1- and D2-Cre transgenic mice were used. RNAscope validation of their genotypes is presented in Figure 5.2A. In the D1 Cre mouse, D1Rs was found to colocalise with Cre, indicated by a strong positive correlation between the integrated density values (IDVs) of Drd1a and Cre signals, $r$ = .869, $p$ < .001 (n = 345 cells) and a weak, but significant negative correlation between the IDVs of Drd2 and Cre signals, $r$ = -.273, $p$ < .001 (n = 278 cells). Similarly, D2Rs colocalise with Cre in the D2 Cre mouse, $r$ = .759, p< .001 (n = 380 cells), and no correlation between the IDVs of Drd1a and Cre signals was observed in the D2-Cre mouse, $r$ = .029, $p$ = 0.630 (n = 280 cells). A separate group of WT mice, injected with shα2-carrying Cre-dependent rAAVs, was also used in each behavioural experiment to control for Cre integration within the genome and to ensure that any observed phenotypic effect of shα2 was dependent on Cre-mediated recombination.

Figure 5.2B illustrates the target viral injection site (i.e. the NAc core; coordinates AP 1.18; L+/-1.00; DV -4.20; Paxinos & Franklin, 2001). When injected into the NAc core of D1- or D2-Cre mice, a mixed population of mCherry- and EGFP-expressing cells was observed, but only EGFP-

expressing cells were detected in the WT brain, indicating the absence of Cre in the latter (refer to Chapter 3 for characterisations of these viruses).

Three groups of mice were used in each experiment, i.e. D1 Cre/shScr (n = 7; males = 4, females = 3), D1 Cre/shα2 (n = 12, males = 7, females = 5) and WT/shα2 (n = 10, males = 5, females = 5) in the D1 experiment, and D2 Cre/shScr (n = 10 males = 5, females = 5), D2 Cre/shα2 (n = 9, males = 5, females = 4) and WT/shα2 (n = 10, males = 6, females = 4) in the D2 experiment. Eight animals from each experiment (i.e. D1 and D2 experiments) were removed from the analysis due to inaccurate placement of the rAAVs (see below for the number of animals in each group used for analysis).

(A)


D1R
Cre
merged
D1-Cre
D2R
Cre
merged
D2-Cre
D1-Cre mouse
D2-Cre mouse
Drd1a + Cre probes
Cre
Drd1a
Drd2 + Cre probes
Drd2

(B)

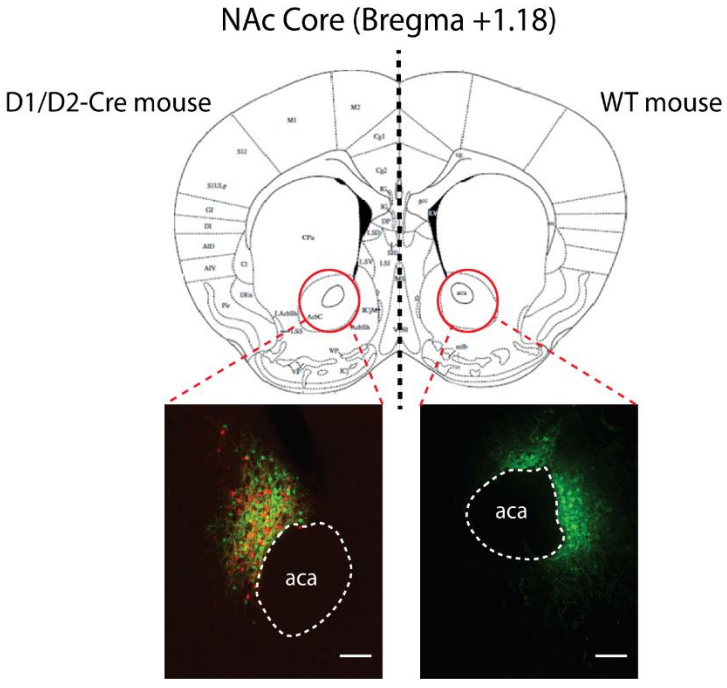

NAc Core (Bregma +1.18)
D1/D2-Cre mouse
WT mouse
aca
aca

***Figure 5.2.*** **(A)** RNAscope verification of Cre expression patterns in the NAc core of D1- and D2-Cre transgenic mice. Positive correlation between Cre and Drd1a or Drd2 signals was observed in D1-Cre or D2-Cre mice respectively **(B)** Histological assessment of bilateral viral infusions into the NAc core. Fluorescent immunostaining of EGFP (Cre-negative cells) and mCherry (Cre-positive cells) in D1- and D2-Cre mice, or of EGFP only in the wildtype mice, shows the Cre-dependent rAAV-infected neurons, taken approximately 10 weeks after viral injection (refer to Chapter 3 for details on the construct design). Areas marked in red indicate the target injection sites. Scalebar 100μm.

## 5.3.2. The effects of α2 knockdown in NAc core D1R- and D2R-expressing neurons in CRf and its facilitation by cocaine

### 5.3.2.1. Pavlovian Conditioning

Figure 5.3 illustrates the effect of α2 knockdown in the NAc core D1R- and D2R-expressing neurons on Pavlovian discriminated approach, displayed as the percentage of magazine entries during CS+ and CS- presentations over the course of 10 daily sessions.

By session 10, D1 Cre/shScr (n = 6, males = 4, females = 2), D1 Cre/shα2 (n = 7, males = 5, females = 2), and WT/shα2 (n = 8, males = 3, females = 5) mice reached 87.82 ±4.61%, 74.50 ±12.13% and 84.23 ±3.08% magazine entries during CS+ presentations respectively. A three-way mixed ANOVA comparing the percentage of magazine entries revealed a non-significant session × CS × group interaction (Greenhouse-Geisser corrected, $F(10.56, 95.08) = 1.13$, $p = .349$, $\varepsilon = .587$). However, significant session × CS interaction (Greenhouse-Geisser corrected, $F(5.28, 95.08) = 32.32$, $p < .001$, $\varepsilon = .587$), as well as significant main effects of session (Greenhouse-Geisser corrected, $F(3.59, 64.59) = 29.57$, $p < .001$, $\varepsilon = .399$) and CS ($F(1,18) = 404.57$, $p < .001$) collectively suggest that all groups of mice regardless of virus manipulation and genotype acquired the stimulus-reward association by displaying higher proportions of magazine entries during CS+ onset. Interestingly, there appears to be significant group differences in Pavlovian learning, through the CS × group interaction, $F(2,18) = 6.40$, $p < .01$) and the main effect of group ($F(2,18) = 3.78$, $p < .05$)). The Bonferroni's post-hoc

test further revealed that the D1 Cre/shα2 mice displayed significantly reduced level of discriminated approach relative to the D1 Cre/shScr counterpart ($p < .05$) (Figure 5.3A).

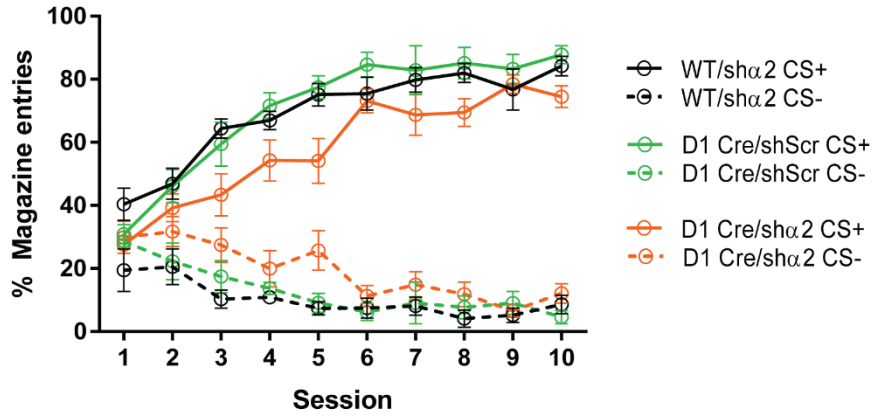
Next, in the D2 experiment, the D2 Cre/shScr (n = 7, males = 4, females = 3), D2 Cre/shα2 (n = 7, males = 5, females = 2), and WT/shα2 (n=7, males = 3, females = 4) groups reached 80.88 ±4.82%, 83.20 ±2.62% and 81.83 ±3.71% magazine entries during CS+ presentations on session 10 respectively. An ANOVA comparison of between-group differences in magazine entries during CS+ vs. CS- presentations over the course of 10 sessions revealed non-significant session × CS × group (Greenhouse-Geisser corrected, $F(8.59, 77.26) = 0.55$, $p = .830$, $\varepsilon = .477$), CS × group ($F(2,18) = 0.04$), and session × group (Greenhouse-Geisser corrected, $F(8.79, 79.19) = 0.70$, $p = .701$, $\varepsilon = .489$) interactions. However, CS × session interaction (Greenhouse-Geisser corrected, $F(4.29, 77.26) = 27.15$, $p < .001$), as well as the main effects of CS ($F(1,18) = 179.93$, $p < .001$) and session (Greenhouse-Geisser corrected, $F(4.40, 79.19) = 42.19$, $p < .001$, $\varepsilon = .489$) were statistically significant. These data indicate that all animals, regardless of genotype or virus manipulation, exhibited Pavlovian learning to a similar degree (Figure 5.3C).

Pavlovian learning was further assessed by measuring changes in the latency to approach the food magazine following CS+ onset (CS+ latency) on sessions 1 and 10. In the D1 experiment, the average CS+ latencies were 8.31 ±0.47, 8.42 ±0.30 and 8.66 ±0.34 seconds on session 1 and 4.19 ±0.22,

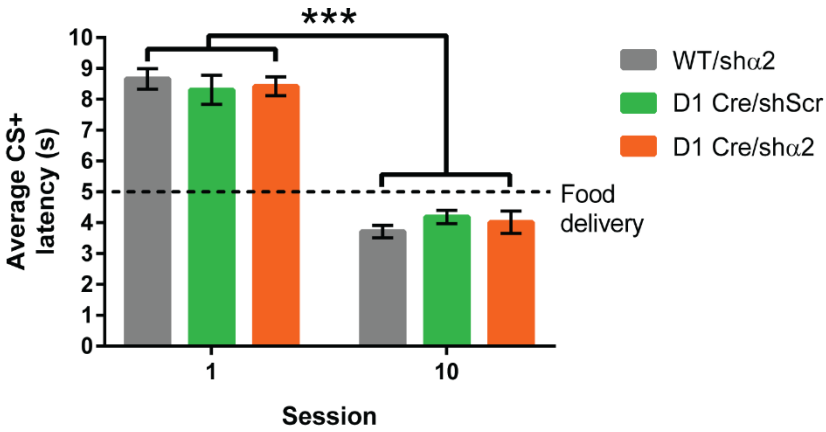
4.02 ±0.36 and 3.71 ±0.20 seconds on session 10 for D1 Cre/shScr, D1 Cre/shα2, and WT/shα2 groups respectively. Though the CS+ latency on session 1 was significantly higher than that on session 10 (i.e. estimated marginal means of 8.46 ±0.21 (session 1) and 3.97 ±0.16 (session 10); significant main effect of session ($F(1,18) = 203.97$, $p < .001$)), differences between groups were statistically non-significant, either via session × group interaction ($F(2,18) = 0.61$, $p = .554$) or the main effect of group ($F(2,18) = 0.03$, $p = .971$) (Figure 5.3B).

The average CS+ latencies in the D2 experiment were 8.69 ±0.27, 8.63 ±0.48 and 7.98 ±0.45 on session 1, and 4.24 ±0.40, 4.07 ±0.74 and 3.84 ±0.43 on session 10 for D2 Cre/shScr, D2 Cre/shα2, and WT/shα2 groups respectively. Akin to the D1 experiment, all groups of mice displayed markedly lower CS+ latency on session 10 ($F(1,18) = 203.97$, $p < .001$), but there were no marked differences in the CS+ latency between groups (i.e. non-significant session × group interaction, $F(2,18) = 0.61$, $p = .554$) (Figure 5.3D). Collectively these findings demonstrated that translational repression of α2 either in the D1- or D2-expressing neurons in the NAc core did not impair appetitive Pavlovian learning, though the former appeared to reduce the level of discriminated approach.

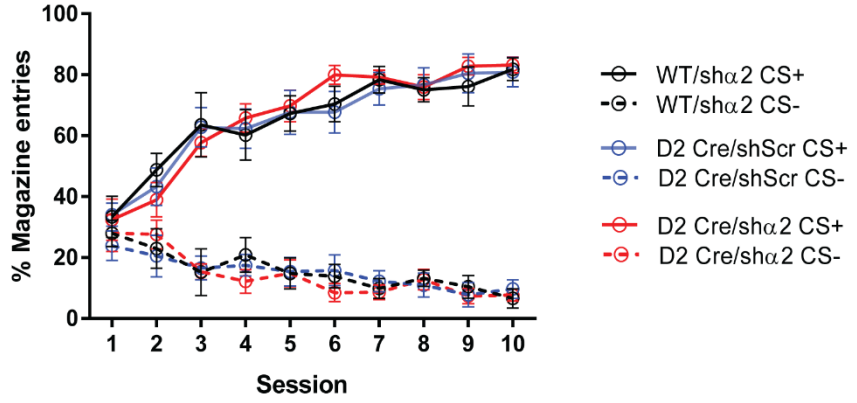
(A)


% Magazine entries
Session
WT/shα2 CS+
WT/shα2 CS-
D1 Cre/shScr CS+
D1 Cre/shScr CS-
D1 Cre/shα2 CS+
D1 Cre/shα2 CS-

(B)


***
Average CS+ latency (s)
Session
Food delivery
WT/shα2
D1 Cre/shScr
D1 Cre/shα2

(C)


% Magazine entries
Session
WT/shα2 CS+
WT/shα2 CS-
D2 Cre/shScr CS+
D2 Cre/shScr CS-
D2 Cre/shα2 CS+
D2 Cre/shα2 CS-

(D)

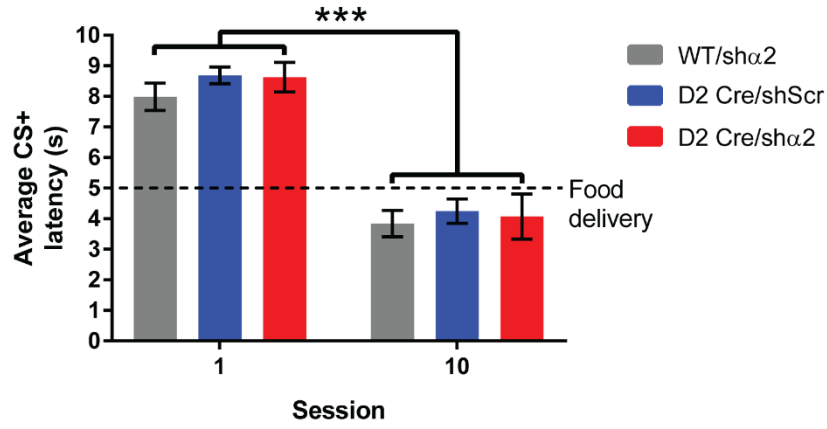

***
Average CS+ latency (s)
Session
Food delivery
WT/shα2
D2 Cre/shScr
D2 Cre/shα2

***Figure 5.3.*** Appetitive Pavlovian learning in the D1 and D2 experiments. **(A)** All groups of mice in the D1 experiment acquired the food-stimulus association, indicated by the levels of discriminated approach over the course of 10 daily conditioning sessions and **(B)** the latency to approach the food magazine during CS+ presentation. The D1 Cre/shα2 mice, however, displayed a reduced level of discriminated approach relative to the shScr counterparts ($p < .05$). **(C)** The α2 knockdown in the D2-expressing NAc core neurons, however, had minimal effects on the Pavlovian discriminated approach and **(D)** CS+ latency. Data are presented as mean ± SEM. ***$p <$ .001

### 5.3.2.2. Conditioned reinforcement

The effects of α2 knockdown in the NAc core D1R- and D2R-expressing neurons on the baseline rates of responding for CRf are summarised in Figure 5.4.

Firstly, in the D1 experiment, the D1 Cre/shScr, D1 Cre/shα2, and WT/shα2 groups made, on average (±SEM), 107.83 ±18.68, 86.29 ±14.01 and 81.63 ±19.50 CR and 41.17 ±7.12, 30.14 ±3.72 and 34.63 ±6.33 NCR nosepokes respectively. As depicted in Figure 5.4A, all groups of mice displayed markedly higher rates of nosepoking for the CR than the NCR ($F(1,18) = 34.56$, $p < .001$). However, manipulations introduced in the present research appeared to have only marginal effects on responding for CRf (i.e. non-significant nosepokes × group interaction, $F(2,18) = 0.35$, $p = .712$). Further analysis of the pattern of reinforced nosepoking rates over the 60-minute session, broken down into six 10-minute timebins, revealed no significant time × group interaction (Greenhouse-Geisser corrected, $F(6.54, 58.83) = 0.95$, $p = .474$, $\varepsilon = .654$) though the main effect of time was significant (Greenhouse-Geisser corrected, $F(3.27, 58.83) = 4.35$, $p < .01$, $\varepsilon = .654$), indicating time-dependent decreases in CR-maintained responding (Figure 5.4B).

Statistical analyses for the D2 experiment were performed with log-transformed data to maintain homogeneity of variance. The D2 Cre/shScr, D2 Cre/shα2, and WT/shα2 groups made 234.86 ±43.82, 194.57 ±39.15 and 130 ±13.15 CR, and 46.71 ±7.15, 47.43 ±9.16 and 27.14 ±3.65 NCR

nosepokes respectively. Akin to the findings from the D1 experiment, all groups of mice displayed preferential responding for the CR over the NCR (i.e. a significant main effect of nosepokes, $F(1,18) = 339.74$, $p < .001$ and though D2 Cre, irrespective of the virus manipulation, appeared to display higher CR-reinforced nosepoking, the nosepokes × group interaction was found to be statistically non-significant ($F(2,18) = 0.63$, $p = .546$) (Figure 5.4C). Analyses of the timebin data further demonstrated the lack of between-group differences in the pattern of reinforced nosepoking (Greenhouse-Geisser corrected, $F(5.20, 46.81) = 1.41$, $p = .241$, $\varepsilon = .520$) (Figure 5.4D). In summary, reducing α2 expression selectively in the NAc core D1R- or D2R-expressing neurons did not markedly affect the acquisition of instrumental responding for CRf.

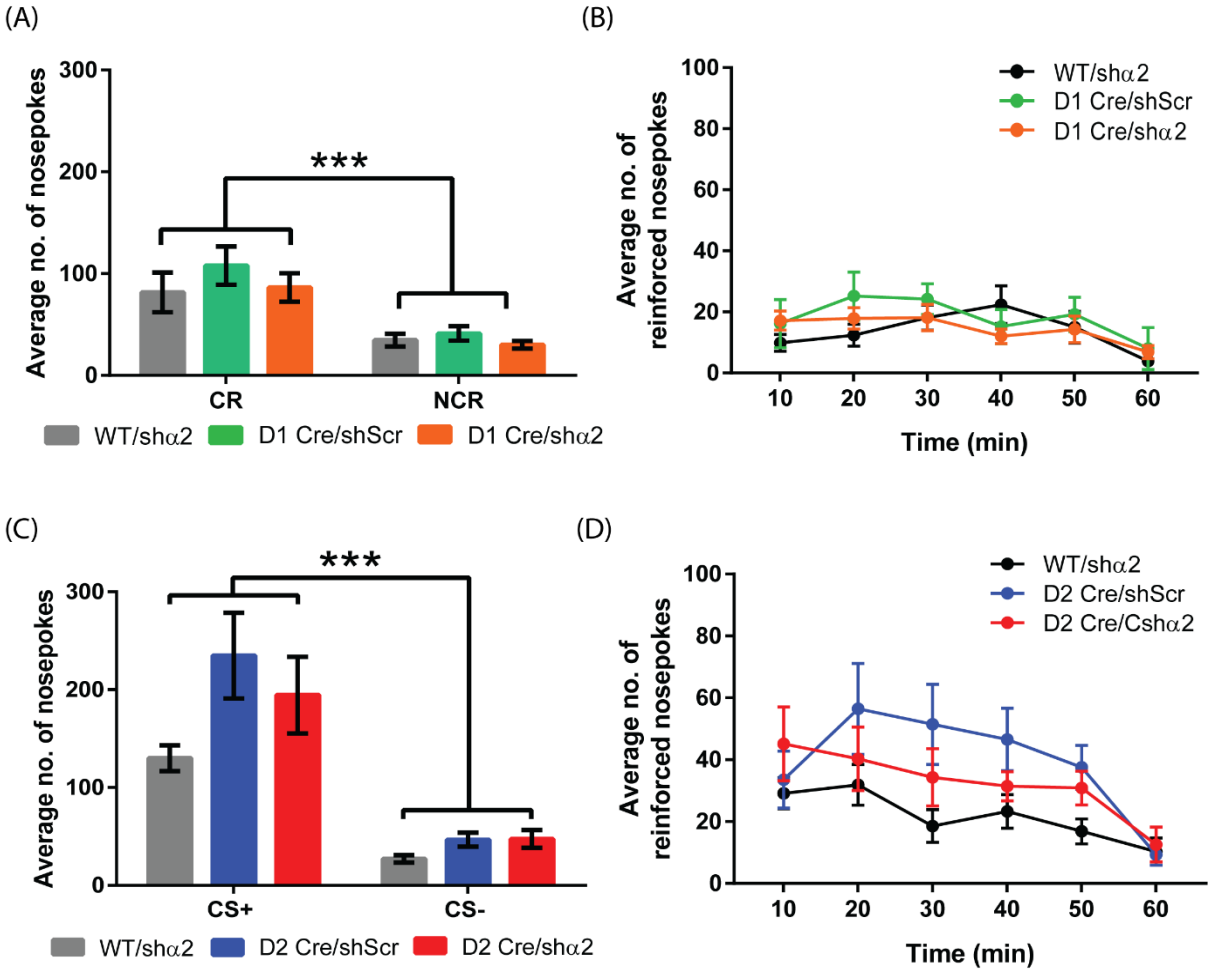



***Figure 5.4.*** The acquisition of an instrumental action (i.e. nosepoking into a port) for conditioned reinforcement in the D1 and D2 experiments. **(A)** All groups of mice in the D1 experiment displayed preferential nosepoking into the CR-associated module. **(B)** Time-dependent decreases in the rates of reinforced nosepoking (60-minute session) were observed in all groups. **(C)** All groups in the D2 experiment also exhibited higher rates of CR-driven nosepoking and **(D)** the rates of reinforced nosepoking appeared to decrease over time. Data are presented as mean ± SEM. ***$p < .001$

### 5.3.2.3. Cocaine enhancement of conditioned reinforcement

Next, this study investigated the effects of α2 expression knockdown in the NAc core on cocaine (0, 3 & 10 mg/kg i.p.) facilitation of CRf, in an attempt to provide pathway specificity to the findings presented in Chapter 4. The rates of nosepoke responses for the CR and NCR with different doses of cocaine administered in a Latin square design are summarised in Figure 5.5.

A three-way mixed ANOVA comparing between-group variations in CR- vs. NCR-maintained nosepoking with different doses of cocaine in the D1 experiment revealed that the nosepokes × dose × group interaction (Greenhouse-Geisser corrected, $F(2.93, 26.33) = 0.81$, $p = .499$, $\varepsilon = .731$), all of the two-way interactions, and main effects of dose and group were statistically non-significant ($p > .05$). However, there was a significant main effect of nosepokes ($F(1,18) = 29.82$, $p < .001$), suggesting that preferential responding for CRf appeared to be intact in all groups, but cocaine's CRf enhancing properties were not evident in this experiment. This was further confirmed by post-hoc two-way ANOVAs of the experimental groups (i.e. significant main effect of CS in each group; $F_{WT/sh\alpha2}(1,7) = 15.67$, $p < .01$; $F_{D1\ Cre/shScr}(1,5) = 7.44$, $p < .05$; $F_{D1\ Cre/sh\alpha2}(1,6) = 8.49$, $p < .05$; Figure 5.5A, 5.5C & 5.5E).

By contrast, the ANOVA comparison in the D2 experiment yielded a significant three-way interaction ($F(4,36) = 5.96$, $p < .01$), as well as significant dose × group ($F(4,36) = 6.91$, $p < .001$), CS × group ($F(2,18) =$

3.83, $p < .05$), and dose × nosepokes ($F(2,36) = 26.95$, $p < .01$) interactions. The main effects of dose and nosepokes were also statistically significant ($F_{dose}(2,36) = 14.62$, $p < .001$; $F_{nosepokes}(1,18) = 112.62$, $p < .001$). Collectively, these data suggest that cocaine differentially altered nosepoke responding for CR and/or NCR presentations in different experimental groups. Post-hoc analyses of simple interactions and main effects within each group were performed to aid data interpretation.

Firstly, all experimental groups exhibited higher rates of responding for CR, than for the NCR, regardless of cocaine pre-treatments (main effects of CS, $F_{WT/sh\alpha2}(1,6) = 241.61$, $p < .001$; $F_{D2\ Cre/shScr}(1,6) = 36.29$, $p < .01$; $F_{D2\ Cre/sh\alpha2}(1,6) = 22.51$, $p < .01$). The stimulant properties of cocaine remained intact in both of the control groups, WT/shα2 and D2 Cre/shScr, indicated by the significant main effect of dose ($F_{WT/sh\alpha2}(2,12) = 13.97$, $p < .01$; $F_{D2\ Cre/shScr}(2,12) = 12.90$, $p < .01$), but were notably absent in the D2 Cre/shα2 group (Greenhouse-Geisser corrected, $F(1.15, 6.92) = 0.52$, $p = .522$, $\varepsilon = .576$). Importantly, cocaine was found to differentially alter CR- and NCR-maintained responding in both of the control groups ($F_{WT/sh\alpha2}(2,12) = 25.52$, $p < .001$; $F_{D2\ Cre/shScr}(2,12) = 12.82$, $p < .01$; Figure 5.5B & 5.5D), but induced no significant effects on responding for CRf in the D2 Cre/shα2 counterpart ($F(2,12) = 0.65$, $p = .539$) (Figure 5.5F).

Repeated measures ANOVAs (with Bonferroni correction for multiple comparisons), performed to probe cocaine effects specifically on the rates of CR- and NCR-maintained nosepoking in the WT/shα2 and D2 Cre/shScr groups, showed that cocaine selectively enhanced reinforced nosepoking in

both groups ($F_{WT/sh\alpha2}(2,12) = 19.25$, $p < .001$; $F_{D2\ Cre/shScr}(2,12) = 13.42$, $p < .01$), whilst having no marked effects on responses for the NCR ($F_{WT/sh\alpha2}(2,12) = 2.02$, $p = .176$; $F_{D2\ Cre/shScr}(2,12) = 2.34$, $p = .139$. Overall, these findings suggest that reducing the total amounts of α2 in D2R-expressing neurons within the NAc core completely blocked cocaine's CRf-potentiating properties.

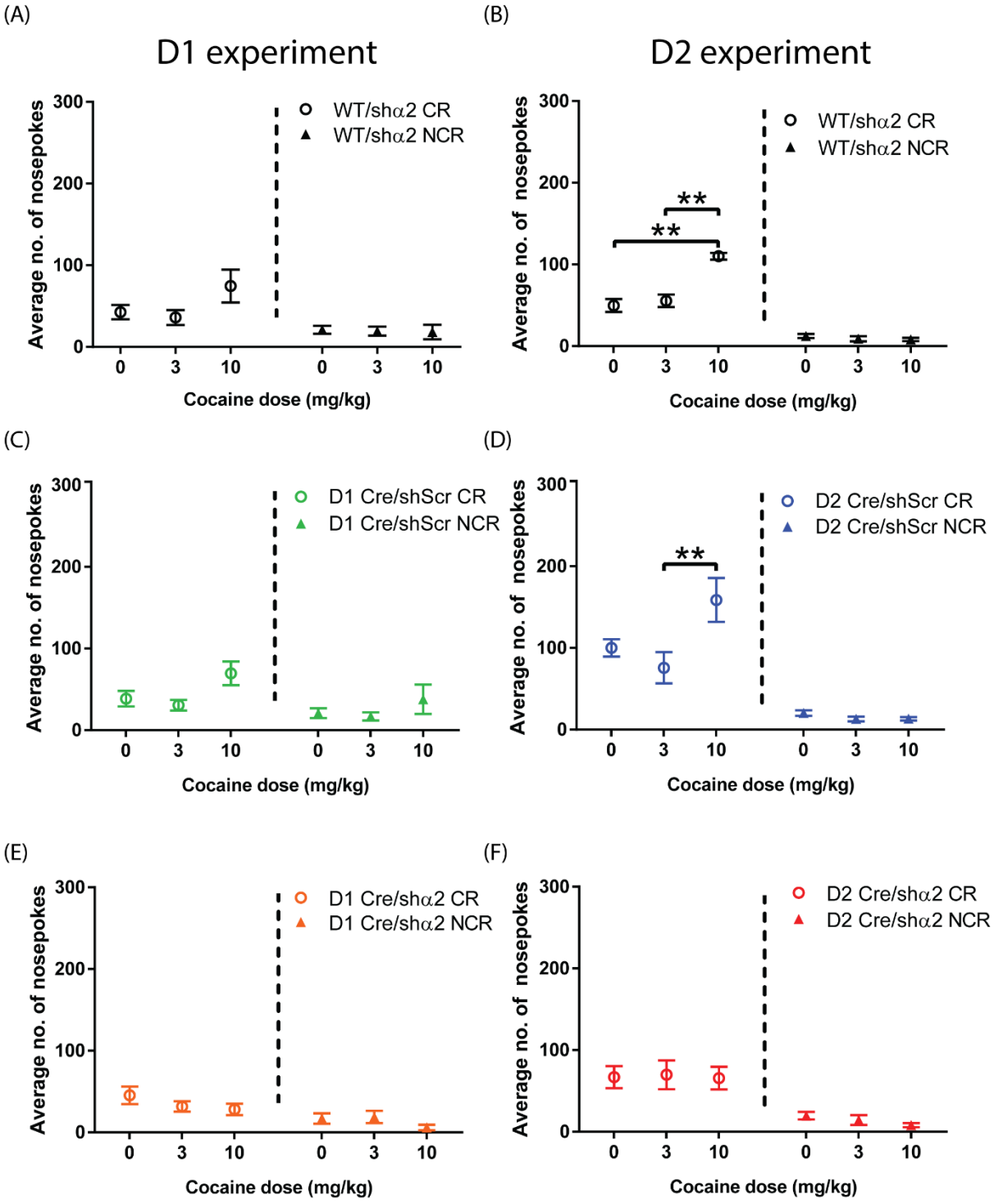

(A)
D1 experiment
WT/shα2 CR
WT/shα2 NCR
Average no. of nosepokes
300
200
100
0
0 3 10 0 3 10
Cocaine dose (mg/kg)
(B)
D2 experiment
WT/shα2 CR
WT/shα2 NCR
**
**
Average no. of nosepokes
Cocaine dose (mg/kg)
(C)
D1 Cre/shScr CR
D1 Cre/shScr NCR
Average no. of nosepokes
Cocaine dose (mg/kg)
(D)
D2 Cre/shScr CR
D2 Cre/shScr NCR
**
Average no. of nosepokes
Cocaine dose (mg/kg)
(E)
D1 Cre/shα2 CR
D1 Cre/shα2 NCR
Average no. of nosepokes
Cocaine dose (mg/kg)
(F)
D2 Cre/shα2 CR
D2 Cre/shα2 NCR
Average no. of nosepokes
Cocaine dose (mg/kg)

***Figure 5.5.*** The effects of cocaine pre-treatments on responding for conditioned reinforcement in the D1 and D2 experiments. In the D1 experiment, cocaine (3 or 10mg/kg i.p.) failed to potentiate nosepoking for conditioned reinforcement in the **(A)** WT/shα2, **(C)** D1 Cre/shScr and **(E)** D1 Cre/shα2 groups. In the D2 experiment, cocaine (10mg/kg) selectively enhanced CR-driven nosepoking in the **(B)** WT/shα2 and **(D)** D2 Cre/shScr groups, but not in the **(F)** D2 Cre/shα2 counterpart. Data are presented as mean ± SEM. $^{**}p < .01$

## 5.4. Discussion

To probe the possibly dissociable functions of α2 in D1R- and D2R-expressing neurons, the Cre-dependent rAAVs, carrying either an α2-targeting silencing construct (shα2) or a non-targeting construct (shScr), were injected into the NAc core of D1 Cre, D2 Cre, and WT mice. The effects of the cell-type-specific α2 knockdown on appetitive Pavlovian behaviour, CRf, and its facilitation by cocaine were subsequently investigated.

Results from the current study showed that reducing α2 expression levels in the NAc core D1R- or D2R-expressing neurons did not impair Pavlovian or instrumental learning, though α2 knockdown in the D1R-expressing cells reduced the level of discriminated approach when compared to the D1 Cre/shScr counterparts, indicating the dissociable roles of α2-$GABA_A$Rs in D1R- or D2R-expressing neurons in appetitive Pavlovian learning. Further, we also demonstrated the functional significance of the $GABA_A$R α2 subunit expression, particularly in the NAc core D2R-expressing neurons, in mediating cocaine facilitation of CRf. Namely, silencing α2 expression in this subpopulation of accumbal neurons completely abolished cocaine's ability to selectively enhance responding for CRf, thus providing pathway specificity to the previous findings described in Chapter 4. Though α2 knockdown in the D1R-expressing neurons also appeared to block cocaine's CRf-enhancing properties, current results from the D1 experiment remain inconclusive, due to the lack of significant

cocaine enhancement in both control groups for potential reasons discussed below.

### 5.4.1. Effects of α2 knockdown in NAc core D1R- and D2R-expressing neurons on associative learning

The mesolimbic dopamine system is involved in the expression of learned behaviours, though the exact nature of its role remains unclear to date. Evidence derived from microdialysis and fast-scan cyclic voltammetry studies has implicated the mesoaccumbal dopaminergic system in appetitive associative learning (Bassareo, Musio, & Di Chiara, 2011; Cheng, de Bruin, & Feenstra, 2003; Flagel et al., 2011; Kiyatkin, 1995; Schultz et al., 1997). This study demonstrated that silencing α2 expression specifically in the D1R- or D2R-expressing neurons in the NAc core of the mouse failed to impair appetitive associative learning, in that over the course of 10 Pavlovian conditioning sessions, all groups of mice in each experiment were progressively more likely to approach the food magazine during the CS+ onset and exhibited shorter latency to approach the magazine upon the CS+ presentation (prior to food delivery). However, α2 knockdown in D1R-containing neurons appeared to reduce the level of discriminated approach. Intriguingly, disruptions in appetitive Pavlovian conditioned responses were also observed upon pharmacological blockade of the D1Rs, suggesting their functional importance in mediating incentive learning (reviewed in Beninger & Miller, 1998). For instance, the D1R antagonist SCH23390 dose-dependently reduced magazine entries during the food-paired stimulus

presentations in a conditioning paradigm (Eyny & Horvitz, 2003) or reduced CS+ consumption in rats trained to associate flavoured saccharin solution with intragastric sucrose infusion (Azzara, Bodnar, Delamater, & Sclafani, 2001). Studies with D2R antagonists, however, yielded mixed results. Namely, D2R antagonist (raclopride) has been shown to have positive effects on learning, i.e. increasing head entries during CS+ presentations compared to the vehicle controls (Eyny & Horvitz, 2003), though, the Azzara et al. (2001) study failed to observe marked effects of raclopride on flavour-intragastric carbohydrate infusion conditioning. Such discrepancy could be explained by mounting evidence supporting the role of D2Rs in motor responding during learning (extensively reviewed in Beninger & Miller, 1998).

Furthermore, the present study showed that RNAi-induced downregulation of α2 expression in either of the neuronal subpopulations failed to disrupt the acquisition of instrumental responding for CRf, as indicated by the total number and the pattern of reinforced response rates. In particular, despite the reduced level of discriminated approach and the failure to meet the Pavlovian learning criterion (i.e. ≥80% magazine entries during CS+ presentations during the 60-minute conditioning session), mice with lower levels of α2 in the mesoaccumbal D1R neurons exhibited similar rates of CS+ - reinforced and non-reinforced nosepoking, relative to those in the control groups, suggesting that the CS+ acquired the capacity to function as reinforcer to a similar extent in all groups of mice in the D1 experiment. Overall, this is not surprising given that lesion of the BLA, but

not of the NAc core or shell, abolished CR-induced control over behaviour, suggesting the importance of BLA integrity in responding for CRf (Burns, Everitt, & Robbins, 1999; Burns, Robbins, & Everitt, 1993; Parkinson, et al., 1999). In keeping, there is evidence that disruptions in Pavlovian associative learning did not necessarily yield deficits in the acquisition of CRf (Olmstead, Robbins, & Everitt, 1998; though also see Beninger & Phillips, 1980).

The NAc is enriched with post-synaptic and extra-synaptic $GABA_A$Rs that mediate phasic and tonic GABAergic activity respectively (Dixon et al., 2010; Farrant & Nusser, 2005; Hörtnagl et al., 2013; Maguire et al., 2014; Pirker, Schwarzer, & Wieselthaler, 2000). Intriguingly, previous research using mice lacking α4-$GABA_A$Rs in D1R-expressing neurons (α4D1KO) found no genotypic differences in appetitive conditioning; in contrast, mice lacking α4-$GABA_A$Rs in D2R-expressing neurons (α4D2KO) exhibited enhanced nosepoking for CRf (Macpherson et al., 2016). However, note that we identified higher CRf-maintained responding in the D2 Cre mice, irrespective of virus manipulation, thus potentially challenging the previous α4D2KO findings, though further investigations remain to be performed. These findings not only extend previous findings pertaining to the role of dopamine signalling via D2Rs in motivation (Natsubori et al., 2017; Soares-Cunha et al., 2016; Tsutsui-Kimura et al., 2017), but together with current data, also show that GABA action at α2- and α4-containing receptors in D1R- or D2R-containing neurons play different roles in distinct aspects of reward learning. Nonetheless, this interpretation should be

treated with caution as the knockout approach lacks regional specificity and the knockout models often display compensatory alterations, which may ultimately affect their phenotypes (Gingrich & Hen, 2000). Overall, current findings suggest that the $GABA_AR$ α2 subunit expression, either in the NAc core D1R- and D2R-expressing neurons, is not a pre-requisite for Pavlovian learning and the acquisition of a novel response maintained by Pavlovian-conditioned stimuli.

### 5.4.2. Effects of α2 knockdown in NAc core D1R- and D2R-expressing neurons on cocaine enhancement of conditioned reinforcement

Importantly, α2 knockdown in the D2R-containing neurons blocked cocaine's ability to further invigorate motivated behaviours directed towards CRf. Though results from the D1 experiment appeared to yield similar findings as those observed in the D2 experiment (also refer to Chapter 6 for discussion on D1/D2 antagonism), cocaine effects in both D1R- control groups failed to reach statistical significance, most likely due to technical reasons discussed further below. Evidence exists that D1-MSN activity is important for the expression of cocaine-induced motivated behaviours. For instance, increased D1-MSN activity was detected immediately prior to entry into a cocaine-paired chamber in a CPP paradigm and the peak amplitude correlated well with the time spent in the drug-paired compartment, suggesting that D1 signalling drives the expression of place preference (Calipari et al., 2016). Consistent with that observation,

chemogenetic inhibition of D1-MSNs abolished preference for the drug-paired chamber (Calipari et al., 2016). For these reasons, follow-up investigations should be carried out to confirm the effects of α2 knockdown in D1R-expressing neurons in the NAc core on cocaine facilitation of CRf.

In line with current data, the CRf-potentiating effects of *d*-amphetamine (Wolterink et al., 1993) and cocaine (Chu & Kelley, 1992) were completely abolished by pharmacological blockade of either D1Rs or D2Rs with SCH23390 or raclopride respectively, suggesting that reducing GABAergic activity via α2-containing receptors primarily in D2R- and possibly, in D1R-harbouring neurons blocked the downstream consequences of dopamine action in the NAc such that it could no longer energise responding for CRf (discussed below). Similarly, intra-NAc infusions of low doses of D2R antagonist were found to impair motivation – i.e. shifting behaviour away from food-reinforced tasks which required a considerable amount of effort and towards low-effort tasks with less reinforcement (Salamone, Correa, Farrar, & Mingote, 2007).

It is, however, noteworthy that motivation, in itself, is a complex process, which involves various behavioural functions underpinned by a vast array of interacting circuits. There are quite distinct facets of motivation, for example, the activational (speed, vigour and persistence) and directional (away from or towards a stimulus) components (Salamone, Yohn, López-Cruz, San Miguel, & Correa, 2016; Salamone & Correa, 2012). This also leads to an important question as to what aspect of motivation is, in fact, mediated by dopamine transmission via D2Rs. Using the random ratio

paradigm to measure effort in instrumental responding, a study by Trifilieff and colleagues (2013) further demonstrated that postsynaptic D2R overexpression in the NAc increased the willingness to work for food reinforcers without affecting consummatory behaviour. Taken together, these data suggest that dopamine transmission via D2Rs enhances motivation by influencing decision making based upon effort expenditure (i.e. the *activational* aspect of motivation, as described by Salamone & Correa, 2012), rather than by altering the representation of the value of the reinforcer per se. For this reason, we hypothesised that RNAi-induced downregulation of α2 expression in D2R-containing neurons blocked cocaine facilitation of CRf by reducing the willingness to work for CR presentations, without necessarily affecting its reinforcing value.

### 5.4.3. GABA × Dopamine: Evidence for D1R-D2R interactions

As discussed in the previous chapter, the interaction between dopamine and GABA in the NAc is crucial for cocaine-facilitated CRf to occur, but the form of interaction between these subsystems that specifically underlies this cocaine effect remains elusive.

Firstly, dopamine can exert modulatory effects on GABA release in the striatum. Pharmacological data presented in the Harsing and Zigmond (1997) study have demonstrated that whilst dopamine action at D1Rs increased GABA overflow, evoked by electrical field stimulation, activation of D2Rs induced an opposite effect. Further, more recent studies demonstrated that the striatal MSNs form complex and highly

asymmetrical connectivity, forming a lateral inhibitory network. Namely, while the D1-MSNs have been shown to almost exclusively innervate neighbouring D1-MSNs, the D2-MSNs project to both subpopulations (Dobbs et al., 2016; Taverna et al., 2008; Tecuapetla, Koos, Tepper, Kabbani, & Yeckel, 2009). Thus, there is a possibility that RNAi-induced silencing of α2 expression in the D2R neurons might have induced a robust effect on cocaine-facilitated CRf via the targeting of D2R-, as well as D1R-expressing neuronal activity within the NAc microcircuitry.

Further, existing data suggest that dopamine × glutamate interaction is implicated in the psychostimulant's CRf-enhancing properties (Burns et al., 1993; 1994). An elegant series of experiments by Levine et al. (1996) demonstrated that mechanistically, the action of dopamine at D1Rs and D2Rs differentially modulates glutamatergic transmission in striatal neurons. Namely, D1R activation enhances NMDA-mediated responses only when the neurons are in a more depolarised, or "up" state, whereas D2R activation may effectively attenuate glutamatergic responses via AMPA receptors during the down state. Though note that tonic D1R or D2R activation was found to increase or decrease striatal neuron excitability respectively, regardless of the membrane potentials (West & Grace, 2002).

To date, it remains unclear how phasic GABAergic action precisely modulates striatal MSN activity in behaving animals (refer to inhibitory and facilitatory accounts of striatal GABAergic function documented in Bracci & Panzeri, 2006; Kiyatkin & Rebec, 1999). Intriguingly, previous research demonstrated that $GABA_AR$ antagonist, bicuculline and picrotoxin,

blocked D2R inhibitory effects on NMDA receptors in the rat PFC (Tseng & O'Donnell, 2004). A similar triadic interaction between the dopaminergic, glutamatergic, and GABAergic systems has also been reported in accumbal MSNs. Recordings from the NAc neurons of adult rats indicated that D2R activation enhanced cortico-accumbal responses to cortical stimulation, but markedly attenuated synaptic responses to cortical stimulation in the presence of $GABA_AR$ antagonist, suggesting D2R recruitment of *depolarising* GABA responses to collectively modulate cortical inputs into the NAc. It was further proposed that D2R activation may have a dual effect within the NAc circuitry, i.e. attenuating corticoaccumbal EPSPs and enhancing feedforward mechanism through the activation of local GABA interneurons, which may exert a depolarising effect onto the hyperpolarised MSNs, but may shunt excitatory responses when neurons are at a more depolarised state (Benoit-Marand & O'Donnell, 2008). Based on these findings, we posit that reducing the α2-$GABA_ARs$ in D2R-expressing neurons might have attenuated D2R-mediated facilitation of excitatory afferents onto the accumbal MSNs, primarily from the cortex (potentially mimicking the effects of D2R antagonism), which serves as a key mechanism underlying psychostimulant enhancement of CRf.

Adding to this complexity, dopaminergic modulation of GABAergic signalling in the NAc has been reported to vary according to the type of the GABAergic connections. Namely, in the NAc shell, quinpirole (a D2R agonist) reliably reduced the amplitude of lateral inhibition (i.e. MSN-MSN

inhibition), but had divergent effects on FSI-MSN connections (Kohnomi, Koshikawa, & Kobayashi, 2012).

### 5.4.4 Conclusions and technical considerations

To conclude, data generated in the present research provide valuable insights into the role of Gabra2 specifically in D1R- and D2R-containing neurons within the NAc core subregion using a newly developed Cre-dependent RNAi vector (refer to Chapter 3 for vector design). Firstly, this research has unravelled the facilitatory role of Gabra2, specifically in D1R-containing mesoaccumbal neurons, in appetitive learning, which was not revealed in the α2 constitutive knockouts (Dixon et al., 2010) or in the region-specific knockdowns (Chapter 4). Importantly, this study further provided cell-type specificity to the findings presented in Chapter 4. Namely, intact GABAergic activity via the α2-$GABA_A$Rs, specifically in the NAc core D2R-expressing neurons, is a pre-requisite for cocaine facilitation of CRf.

There are, nonetheless, several technical limitations that need to be considered when interpreting these data. Firstly, the differences in behavioural phenotypes revealed through the RNAi-induced regulation of α2 in specific cell subpopulations provide strong evidence for the efficacy of the Cre-dependent rAAV tools used in the present research. However, further characterisations, including electrophysiological, immunohistochemical, and/or ligand-binding assays, are warranted to assess RNAi-induced silencing effects on the total amount of subunit expression, as well as on the membrane receptor number. Moreover, the lack of cocaine enhancement of CRf observed across all groups in the D1 experiment was unlikely to be

attributable to the non-specific damage in the NAc core caused by the viral injections, given that targeting the NAc core D2-MSNs with the same amount of shScr-harbouring Cre-dependent rAAVs did not disrupt cocaine's CRf-potentiating effects. Instead, other experiment-specific confounding variables, presumably the physiological state of the animals at the time of testing (i.e. state of hunger) or potential variabilities in the experimental procedures, might have attenuated the magnitude of cocaine effect. A repeat experiment should, thus, be conducted to investigate the effects of α2 knockdown in the NAc core D1R-expressing cells on CRf and its potentiation by cocaine.

Of final note, it is important to highlight that up to 80% of cholinergic interneurons in the NAc also express D2Rs and selective activation of these receptors have been demonstrated to enhance phasic dopamine release in the NAc, thus influencing incentive learning (Alcantara, Chen, Herring, Mendenhall, & Berlanga, 2003; Aubry et al., 1993; Cachope et al., 2012). There is evidence that the α2-containing $GABA_AR$ isoform is preferentially enriched on striatal MSNs, thus leading to a hypothesis that the lack of cocaine enhancement seen in the D2 Cre/shα2 mice was likely to be attributed to reduced levels of α2 in D2-MSNs (Boyes & Bolam, 2007; Schwarzer et al., 2001). However, due to the lack of data in the literature pertaining to α2-$GABA_AR$ cellular and subcellular localisation in the striatum, this study cannot at present eliminate the possibility that this receptor subtype is expressed in the interneurons, including the cholinergic interneurons (CINs), akin to the α4-$GABA_ARs$ (Maguire et al., 2014). To

rectify this issue, the Cre-dependent rAAVs designed in this thesis can be injected into the NAc core of the ChAT-Cre (Bloem et al., 2014) or Adora2a-Cre mice (Durieux et al., 2009) to further probe whether the loss of cocaine facilitation of CRf was specifically attributed to reduced levels of α2 in the NAc core D2-MSNs or the CINs respectively. Overall, these findings not only improve current understanding of the role of the $GABA_AR$ α2 subunit in incentive learning and motivation, but also pave the way for future investigations into the functional roles of the accumbal function in the expression of motivated behaviour.

# Chapter 6

# General Discussion

Data presented in this thesis have demonstrated the role of the mesoaccumbal α2-containing $GABA_A$Rs in mediating cocaine-facilitated conditioned behaviours, i.e. locomotor sensitisation and CRf (see Table 6.1 for the summary of findings). Namely, the RNAi-induced suppression of the α2 subunit expression in the NAc shell was found to affect the locomotor stimulant effects of cocaine without altering its sensitising properties. Additionally, manipulating the level of α2 expression in the NAc core neither affected cocaine-induced hyperlocomotion nor sensitisation, but modulated cocaine's ability to invigorate instrumental responding for CRf. Further discussions of how the present findings fit into the context of ongoing addiction research to date and their wider implications will be presented here.

## 6.1. Summary of findings

### 6.1.1. Developing RNAi tools to target the mouse Gabra2 expression in a site- and/or cell-type-specific manner

Findings presented in Chapter 3 demonstrated the silencing potency of the α2-targeting RNAi effector used for the behavioural experiments in this thesis. Both *in vitro* and *in vivo* immunofluorescent analyses indicated >70% shα2-induced knockdown in the α2 subunit expression, when compared to the amount of the α2 subunit expression in cells transfected or transduced with a non-targeting scrambled control (shScr). A Cre-dependent RNAi (Cre-ON) vector was also developed in this thesis for region- and cell-type-specific silencing of Gabra2 expression. The presence of Cre recombinase in the transfected/transduced cells promoted the mCherry expression and shRNA transcription, whereas transfected/transduced Cre-negative (wild-type) cells expressed EGFP and the shRNA transcription was hindered by the STOP cassette in these cells. Using these tools, we were able to study the functional roles of intra-accumbal GABAergic signalling via α2-$GABA_A$Rs in mediating cocaine-facilitated conditioned behaviours.

### 6.1.2. The role of mesoaccumbal α2-$GABA_A$Rs in cocaine-facilitated conditioned reinforcement

The CRf experiments presented in this thesis (Chapters 4&5) showed that intact GABAergic transmission via α2-$GABA_A$Rs, specifically in the NAc core subdivision, is not required for CRf, but plays a critical role in

cocaine facilitation of CRf. Namely, reducing α2 expression in the NAc core completely abolished cocaine's potentiating effect without disrupting the CR's ability to act as a reinforcer and bias action selection, consistent with previous findings that CRf largely depends on the BLA, rather than the NAc, integrity (Burns et al., 1993; Parkinson et al., 1999). The current findings, together with previous work, further reveal the differential involvement of mesoaccumbal α2- and α4-$GABA_AR$-mediated GABAergic activity in mediating CRf and its modulation by cocaine. Namely, RNAi-mediated silencing of α4 subunit expression in the NAc or whole-brain ablation of the α4 subunit expression increased rates of CRf-maintained responding, but had no marked effects on cocaine-potentiated CRf (Macpherson et al., 2016; also refer to Section 6.4 for further discussion).

Furthermore, reducing the expression level of α2 subunit in the D1R-expressing neurons within the NAc core reduced the level of Pavlovian discriminated approach, whereas RNAi manipulation of α2 expression in the D2R-expressing neurons robustly abolished cocaine's CRf-enhancing properties.

### 6.1.3. The role of mesoaccumbal α2-$GABA_ARs$ in cocaine-induced locomotor hyperactivity and sensitisation

The RNAi-induced knockdown of the α2 expression in the NAc shell, but not in the core, markedly heightened cocaine-induced hyperlocomotion without altering conditioned activity. Intriguingly, this phenotype is not always observed in the constitutive knockouts, potentially due to Gabra2

deletion in other region(s) or compensations, which might nullified the effect (Dixon et al., 2010; Mitchell et al., 2018).

Findings from this thesis are consistent with ample evidence implicating the NAc shell dopamine in locomotor behaviour. To name a few, microinjections of a mixture of D1R- and D2R-agonists in the medial shell of the NAc markedly increased locomotion, whereas pharmacological manipulations in the NAc core induced little or no effects on locomotion (Ikemoto, 2002). Even in a study that reported increases in locomotor activity upon psychostimulant microinfusion into the NAc core, the potency of the drug-induced enhancement in locomotion was reported to be significantly higher when administered in the shell than in the core (Heidbreder & Feldon, 1998).

Nevertheless, neither α2 knockdown in the NAc core nor in the shell induced significant effects on the development of locomotor sensitisation to cocaine or conditioned locomotor activity. Collectively, the current findings revealed a dissociation between cocaine's locomotor-enhancing and positive reinforcing effects that are often considered homologous (Wise & Bozarth, 1987), and that the wild-type expression level of α2, primarily in the NAc shell, appears to play a role in dampening the reactivity towards cocaine's locomotor-enhancing effects.

Table 6.1.

| **Behaviour** | **α2 constitutive knockout** | **α2 knockdown (NAc shell)** | **α2 knockdown (NAc core)** | **α2 knockdown (NAc core/D1-expressing neurons)** | **α2 knockdown (NAc core/D2-expressing neurons)** |
|---|---|---|---|---|---|
| Appetitive Pavlovian learning | Normal (Dixon et al., 2010) | ? | Normal | Reduced discriminated approach | Normal |
| Conditioned Reinforcement | Normal (Dixon et al., 2010) | ? | Normal | Normal | Normal |
| Cocaine-facilitated Conditioned reinforcement | Blocked (Dixon et al., 2010) | ? | Blocked | ? | Blocked |
| Cocaine-potentiated locomotor activity | Normal (Dixon et al., 2010); Enhanced (Mitchell et al., 2018) | Enhanced | Normal | ? | ? |
| Locomotor sensitisation to cocaine | Blocked (Dixon et al. 2010) | Normal | Normal | ? | ? |
| Cocaine-induced conditioned locomotor activity | Normal (Dixon et al., 2010 | Normal | ? | ? | ? |

*Table 6.1.* Summary of the behavioural consequences of whole-brain α2 knockout (Dixon et al., 2010; Mitchell et al., 2018), as well as those of the RNAi-mediated α2 knockdown in the NAc shell and core (including in D1R- and D2R-expressing neurons (data presented in this thesis).

## 6.2. Behavioural relationships between cocaine-induced sensitisation and potentiation of conditioned reinforcement

Repeated, intermittent exposure to cocaine and other psychostimulant drugs can lead to hypersensitivity (sensitisation) to the drug-induced motor-enhancing and incentive-motivational effects (Kalivas & Stewart, 1991; Robinson & Berridge, 1993). According to the sensitisation theory of addiction, repeated drug exposure strengthens the ability of drug-associated stimuli to control behaviour, as the underlying neural system also becomes progressively sensitised, thereby increasing the risk of addiction. Through the CRf paradigm, we have also learned that cocaine, among other psychostimulant drugs, can strengthen stimulus-driven conditioned behaviours. This is of particular importance, especially when considering the phenomenon of polysubstance abuse, whereby the effect of one type of drug may lead to stimulus-driven intensified craving for another drug and therefore, drug-seeking behaviours. To date, there is compelling evidence from animal (Crombag & Shaham, 2002; Grimm, Hope, Wise, & Shaham, 2001; Lu, Grimm, Dempsey, & Shaham, 2004) and human (Childress, McLellan, & O'Brien, 1986; Childress et al., 1999; Drummond, 2000; Foltin & Haney, 2000) studies that drug-associated environmental stimuli are powerful triggers of "craving" (i.e. cue reactivity) both in animal and human studies, thus leading to persistent drug-seeking and drug-taking

behaviours, as well as increasing the propensity of relapse (refer to Perry, Zbukvic, Kim, & Lawrence, 2014 for a comprehensive review).

A drug challenge, following a period of withdrawal, is often reported to induce a more sizeable effect on locomotor activity in psychostimulant-sensitised WT mice than those receiving repeated, intermittent sham injections in a classic behavioural sensitisation experiment (for example, see Cornish & Kalivas, 2001; Jung, Lee, Sim, & Baik, 2013). The differences in the locomotor response to the psychostimulant drug could be, at least partly, explained by its ability to facilitate conditioned activity, which is somewhat reminiscent of the psychostimulant enhancement of CRf. This motivation-enhancing effect of the drug is often difficult to isolate in the traditional sensitisation paradigm, given that the drug itself also serves as the US that promotes conditioning. However, in the study by LeMerrer and Stephens (2006), the effect of acute cocaine exposure on conditioned locomotion was significantly larger in the food-sensitised mice compared to the controls (i.e. cross-sensitisation). These findings suggest that: (1) locomotor sensitisation reflects a conditioned association between the *reinforcing properties* of food and its associated environment, and (2) cocaine can boost the expression of this conditioned behaviour. Further corroborating this notion is the findings documented in the Morris et al. (2008) study, which demonstrated enhanced conditioned activity upon acute cocaine challenge in Ro15-4513-sensitised α2(H101R) mutant mice. However, Ro15-4513 failed to enhance conditioned activity in cocaine-sensitised α2(H101R) mutants. From this line of reasoning, it thus seems plausible that cocaine facilitation of CRf and

locomotor sensitisation reflect an interaction between psychostimulant action and conditioned behaviours, and such a phenomenon may contribute to the development of drug addiction by facilitating cue-driven motivated behaviours.

Neurobiologically, cocaine-induced increases in mesoaccumbal dopamine concentrations are heavily implicated in mediating both of these behavioural phenomena. The functional importance of dopamine in mediating psychostimulant enhancement of CRf has been revealed through lesion and pharmacological studies, extensively discussed in Chapters 4&5 (Burns, Everitt, Kelley, & Robbins, 1994; Parkinson et al., 1999; Pierce & Kalivas, 1997; Wolterink et al., 1993). Further, a systemic cocaine challenge, following repeated cocaine exposure, was paralleled by a "sensitised response" of the mesoaccumbal dopamine (Cadoni, Solinas, & Di Chiara, 2000; Chen, Marmur, Paredes, Pulles, & Gardner, 1996; Parsons & Justice, 1993) and similarly, cocaine microinfusion into the NAc was found to induce a sensitised behavioural response in amphetamine-sensitised rats (Cador, Bjijou, & Stinus, 1995).

There are, however, mechanistic differences between psychostimulant-induced sensitisation and psychostimulant-potentiated CRf. In the CRf literature, dopamine signalling via D2Rs is deemed critical for psychostimulant-potentiated CRf. Both systemic and intra-NAc raclopride treatment markedly attenuated stimulant-potentiated reinforced responding (Chu & Kelley, 1992; Wolterink et al., 1993). However, data from the sensitisation experiments yielded somewhat contradictory results.

Systemic raclopride markedly attenuated the expression of methylphenidate-induced enhancement in locomotor activity following 11 days of methylphenidate exposure (Claussen, Witte, & Dafny, 2015). Similar effects were observed with systemic haloperidol in cocaine pre-exposed rats (Mattingly, Rowlett, Ellison, & Rase, 1996). In contrast, intra-NAc core raclopride infusion, RNAi-mediated D2R knockdown, or D2R-expressing cell inactivation failed to induce marked effects on locomotor sensitisation to cocaine (15mg/kg; i.p.) (Hikida, Kimura, Wada, Funabiki, & Nakanishi, 2010; Jung et al., 2013; Sim et al., 2013). Thus, dopamine signalling via D2Rs in the NAc appears to be critical for psychostimulant enhancement of CRf, but not for that of conditioned locomotion.

It should also be noted that, despite the lack of evidence for conditioned activity in both groups in the BS-CORE experiment (potentially due to methodological factors discussed in Chapter 4), it is unlikely that α2 knockdown specifically in the NAc core would have affected associative learning, given the marginal effects of α2 manipulations in the NAc core on Pavlovian appetitive learning. However, this remains to be investigated further due to the nature of the stimuli, i.e. contextual vs. discrete, which are known to be processed differently (Crombag, Badiani, Maren, & Robinson, 2000; Holland & Bouton, 1999; Selden, Everitt, Jarrard, & Robbins, 1991).

## 6.3. Conditioned reinforcement and its facilitation by cocaine: Role of the mesoaccumbal dopamine

Cocaine's ability to elevate dopamine concentration in the NAc underlies its ability to facilitate CRf (Chu & Kelley, 1992; Taylor & Robbins, 1986), but a question remains as to whether the CR-induced activation of dopamine neurons plays a prominent role in the acquisition and expression of CRf-maintained instrumental actions, given that dopamine neurons are activated upon the presentation of the CS, not the primary reinforcer (Schultz, Dayan, & Montague, 1997). This phenomenon has also received a considerable amount of empirical support from other microdialysis (Ostlund et al., 2014), voltammetry (Cacciapaglia, Saddoris, Wightman, & Carelli, 2012), and electrophysiological studies (Kosobud, Harris, & Chapin, 1994).

Firstly, it is crucial to highlight the distinction between the performance during the first CRf session (for baseline measurements) and during subsequent CRf tests with drug pre-treatments. Namely, the first session, during which animals were exposed to the nosepoke modules for the first time, without drug exposure, primarily assessed the acquisition of an instrumental action (i.e. instrumental learning) maintained by the CR, whereas subsequent CRf sessions assayed the expression (or performance) of *learned* actions solely motivated by the CR. Thus, it is likely that in the current study, cocaine facilitated the expression of CR-driven learned actions, rather than instrumental learning per se.

There currently exists compelling evidence implicating the involvement of the accumbal dopaminergic system in mediating some aspects of motivation (i.e. motor function or instrumental behaviour). To illustrate this, a study by Baldo, and colleagues (2002) demonstrated that pharmacological blockade of D1Rs or D2Rs in the NAc core or shell failed to suppress food intake, but impaired motor activity. In keeping, Adamantidis and colleagues (2011) reported that optogenetic stimulation of VTA dopamine neurons did not affect food intake per se, but enhanced food-reinforced lever pressing, collectively suggesting that mesoaccumbal dopamine is not critical for the primary motivation to eat, but is involved in energising the *activational* aspect of motivation (Salamone & Correa, 2012). Dopamine is also heavily implicated in Pavlovian approach behaviours and Pavlovian to Instrumental Transfer (PIT), providing a mechanism by which CS exerts control over behaviour (Lex & Hauber, 2010; Parkinson et al., 2002; Wyvell & Berridge, 2000).

However, excitotoxic lesion of the NAc core or shell (Parkinson et al., 1999) or dopamine depletion in the NAc (J. Taylor & Robbins, 1986) merely abolished *d*-amphetamine potentiation of CRf, without affecting the acquisition and expression of preferential responding for the CRf. Instead, it is lesion to the BLA that reduced the CR's control over behaviour (Burns et al., 1993). Moreover, dopamine depletion in the NAc failed to disrupt sensitivity to reinforcer devaluation, thus, mesoaccumbal dopamine does not appear to be essential for encoding action-outcome association that is critical during instrumental learning (Lex & Hauber, 2010; Yin, Ostlund, &

Balleine, 2008). Also note that although intra-NAc infusion of high doses of dopamine antagonists (i.e. raclopride or SCH23390) has been reported to abolish preferential responding for the CR over the NCR, the authors believed that this might be a result of the overall decrease in locomotor activity (Wolterink et al., 1993). Based on existing data, it is conceivable that the mesoaccumbal dopamine may contribute towards, but is not critical for CRf-governed responding, most likely by affecting the motor output. This also suggests that CR-governed responding is a multifaceted phenomenon, which engages a complex network of activity involving the accumbal dopamine (Burns et al., 1994, 1993; Wolterink et al., 1993).

So, how does psychostimulant-induced increase in dopamine levels enhances the rates of responding for CRf? The answer might lie in the interactions between the different time scales of dopamine transmission evoked by the CR presentation (brief pulses of dopamine) and cocaine (longer-term increases in extracellular dopamine) in the NAc (Hernandez & Hoebel, 1988; Schultz et al., 1997; Venton et al., 2006). Whilst phasic dopamine has been linked to its 'teaching signal' function, tonic dopamine release has been suggested to energise goal-directed actions (Hamid et al., 2015). It is, perhaps, the prolonged response of target neurons to the high levels of extracellular dopamine, resulting from bursts of dopamine release evoked by the CR and cocaine-induced blockade in dopamine reuptake that enhanced instrumental responding selectively for CRf.

Of equal importance, it also remains to be determined whether the CR-induced brief increases in dopamine are sustained throughout the CRf

sessions, especially when the animals have learned that the CR presentation did not predict food reward delivery. We posit that the time-dependent decreases in the rates of reinforced nosepoking observed in the present research might have reflected the lack of dopamine response associated with the CR towards the end of each CRf session.

## 6.4. Conditioned reinforcement and its facilitation by cocaine: Distinct roles of GABAergic signalling via α2- and α4-$GABA_A$Rs

Phenotypic differences resulting from RNAi-induced α2 and α4 knockdown in the NAc in the CRf experiments are likely to be linked to distinct forms of GABAergic signalling mediated by the α2- and α4-harbouring receptors. Whilst the former is likely to participate in synaptic (phasic) GABAergic activity, α4 is largely involved in extrasynaptic (tonic) GABAergic transmission mediated by GABA spillover, affecting α4βδ receptors that are highly abundant in the NAc MSNs of adult mice (Dixon et al., 2010; Farrant & Nusser, 2005; Maguire et al., 2014). These findings further add to growing evidence pertaining to the mechanistic dissociation between baseline responding for CRf and its potentiation by cocaine (e.g. Burns et al., 1993; Parkinson et al., 1999).

At rest, the MSNs exhibit a highly negative membrane potential (-85 to 90 mV) (Jiang & North, 1991; Wilson & Kawaguchi, 1996) that is below the reversal potential for $Cl^-$ ions (-60 mV). This means that the opening of

$GABA_ARs$ would trigger outward movement of the Cl- ions and thus, depolarise the neuron (Mercuri, Calabresi, Stefani, Stratta, & Bernardi, 1991). As a result, the effect of GABA may be excitatory if temporally coupled with a glutamatergic signal (Bracci & Panzeri, 2006). Phasic GABAergic transmission may be best suited to serve such an excitatory effect, whereas sustained opening of the extrasynaptic $GABA_ARs$ yields a shunting inhibitory effect, narrowing the magnitude of excitatory signals (Farrant & Nusser, 2005; Stephens et al., 2017). Differences in the outcome of α2 and α4 manipulations in CRf, presented in this thesis and in the Macpherson et al. (2016) study, might reflect the distinct physiological roles of GABAergic activity via α2- and α4-$GABA_ARs$ in influencing the net MSN output.

As previously discussed in Chapters 4 and 5, there exists a multitude of complex ways by which GABA interacts with dopamine in the NAc that may underlie cocaine-facilitated responding for CRf. Of particular interest, in the accumbal MSNs of adult rats, D2R activation with quinpirole increased the amplitude and frequency of evoked synaptic responses from the cortex. However, these effects were abolished upon treatment with $GABA_AR$ antagonist, picrotoxin, thus revealing the D2R's "inhibitory" action, suggesting a triadic interaction between cortical glutamatergic input, dopamine action at D2Rs, and GABAergic signalling via the $GABA_ARs$. This was further corroborated by the finding that quinpirole increased glutamate-independent depolarising events that were AMPAR antagonist-resistant and $GABA_AR$ antagonist-sensitive (Benoit-Marand &

O'Donnell, 2008). Given the findings presented in Chapter 5, RNAi-induced downregulation of the mesoaccumbal α2-$GABA_A$Rs in the NAc core D2R-expressing neurons might have attenuated the "depolarising/excitatory" GABAergic responses, recruited by the D2Rs to modulate glutamatergic inputs.

With the assumption that the presentation of a CR alone can evoke bursts of dopamine, one may posit that GABA, co-released with dopamine, by the dopaminergic neurons innervating the NAc (Tritsch, Jun, Ding, & Sabatini, 2012; Tritsch, Oh, Gu, & Sabatini, 2014) might serve to control goal-directed actions, perhaps primarily through its action at α4-containing receptors. Reducing the number of α4-containing receptors selectively in the NAc would reduce this and/or other (i.e. lateral inhibition and/or feed-forward inhibition) forms of GABAergic control, leading to an overall increase in neuronal excitability within the NAc (Maguire et al., 2014) and allowing excitatory inputs to exert more powerful influence upon behaviours (i.e. increased CRf responding) (Burns et al., 1993). Intriguingly, α4 deletion in D2R-, but not D1R-expressing neurons, also mimicked the phenotype of α4 constitutive knockout or intra-accumbal α4 knockdown mice (Macpherson et al., 2016). Though further experiments to probe the role of α4-$GABA_A$Rs in D1- or D2-expressing NAc MSNs is yet to be determined, current data together with findings from this thesis (refer to Chapter 5), suggest that intact D2 pathway from the NAc appears to be critical for the expression of CRf (Chu & Kelley, 1992; Macpherson et al., 2016; Wolterink et al., 1993).

The loss of cocaine facilitation of CRf was also observed upon intra-accumbal activation of δ-containing $GABA_AR$s with THIP (4,5,6,7-tetrahydroisoxazolo[5,4-c]pyridin-3-ol). The THIP-induced effect, however, was notably absent in the Gabra4 knockout mice, suggesting that the increase in the α4βδ $GABA_AR$-mediated tonic conductance was responsible for the loss of cocaine-facilitated CRf (Macpherson et al., 2016). However, note that electrophysiological recordings from the accumbal MSN also revealed that the administration of THIP reduced mIPSC and sIPSC frequency (Maguire et al., 2014), thus raising an important question as to whether the loss of cocaine-facilitated CRf with THIP is directly attributable to α4βδ $GABA_AR$ agonism, or to the secondary effects (i.e changes in phasic GABAergic activity).

## 6.5. α2-$GABA_AR$s and cocaine-facilitated CRf: Implicating the accumbal-pallidal connectivity

The long-standing view of CRf posits that following Pavlovian associations, the CR can serve as an incentive to allow motivated behaviours in its own right (Mackintosh, 1974). Alternatively, others posit that stimuli predictive of primary reinforcers merely function as a signal that serve to guide, rather than strengthen, behaviour to obtain the primary reinforcers (Davison & Baum, 2006). According to this "signpost" account of CRf, a response would occur not because the learned stimulus strengthens the response in a reinforcement-like manner, but because its presentation is useful for accessing the primary reinforcer (Shahan, 2010), thus providing a

plausible explanation for the time-dependent decline in the rates of responding during CRf tests, documented in Chapters 4 and 5. It would, nonetheless, be remiss not to consider the possibility that once learned, the CR may acquire both the reinforcing and predictive properties, collectively facilitating the instrumental learning and subsequently, the expression of the learned actions.

It is a popular belief that psychostimulant-induced increases in extracellular dopamine amplify the incentive salience of the CR, rendering it a more potent "motivational magnet" (Berridge, 2012). However, owing to the fact that the mesoaccumbal dopamine could serve several independent functions, it is plausible that the predictive power of the CR (Schultz, 1998) and/or the willingness to work for a reinforcer (Salamone, Correa, Farrar, & Mingote, 2007; Salamone, 1998) may also be susceptible to psychostimulant manipulations, thus contributing to the overall increase in CR-maintained responding.

The mesoaccumbal dopamine system is the neurobiological substrate underpinning cocaine's ability to enhance responding for CRf (Taylor & Robbins, 1984; Taylor & Robbins, 1986). This thesis further showed that intact GABAergic transmission via the α2-containing receptors in the D2R-expressing neurons of the NAc core, is a prerequisite for this cocaine effect to occur. Recent anatomical evidence demonstrated that D2-MSNs in the NAc core of the mouse innervate the VP, but not the VTA (Kupchik et al., 2015), highlighting the importance of accumbal-pallidal connectivity in mediating cocaine-facilitated CRf.

As a major output region for limbic signals, the VP has been directly implicated in reinforcement learning and motivation associated with drug and food reinforcers in operant and place preference conditioning studies (Hiroi & White, 1993; McAlonan, Robbins, & Everitt, 1993; Robledo & Koob, 1993). For instance, decreasing inhibitory transmission to the VP, achieved by selective upregulation of D2Rs or chemogenetic inhibition of D2-MSNs in the NAc core, was found to enhance the willingness to work for food (Gallo et al., 2018; Trifilieff et al., 2013) and augmented cocaine-seeking behaviour in mice (Heinsbroek et al., 2017). Consistently, optogenetic activation of NAc core D2-MSNs, thus facilitating their outputs to the VP, reduced conditioned preference for a cocaine-paired chamber (Lobo et al., 2010).

The VP also receives dopaminergic innervation from VTA neurons (Klitenick, Deutch, Churchill, & Kalivas, 1992; Stout et al., 2016). Intriguingly, bilateral microinfusions of *d*-amphetamine in the NAc or VP enhanced CR-maintained responding, whereas picrotoxin microinfusions in the VP abolished preferential responding for CRf, implicating NAc-VP GABAergic transmission in psychostimulant enhancement of CRf (Fletcher, Korth, Sabijan, & DeSousa, 1998). The exact contributions of psychostimulant-elevated dopamine levels in the VP and NAc in boosting the rates of CRf-maintained responding remain elusive. However, we posit that the RNAi-induced knockdown of the mesoaccumbal $GABA_AR$ α2 subunit expression, particularly in D2R-harbouring MSNs, might have interfered not only with dopamine action at the intra-accumbal level, but the resulting alterations in the net MSN output could also have modulated

dopamine action in the VP, leading to a complete loss of cocaine-facilitated CRf observed in the present research. Given that the D1-MSNs also provide afferents to the VP neurons (Kupchik et al., 2015), it is likely that α2 knockdown in D1R-expressing NAc neurons would alter cocaine-facilitated CRf. Moreover, the effects of α2 knockdown in the NAc shell on cocaine enhancement of CRf are yet to be explored.

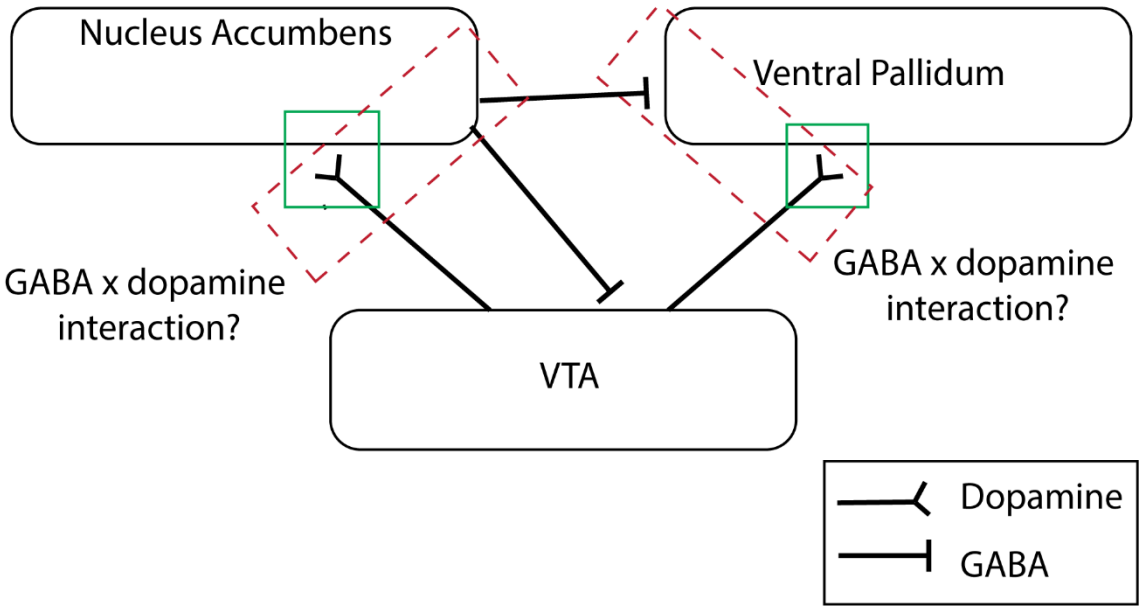



***Figure 6.1.*** The involvement in VTA-accumbal-pallidal and VTA-pallidal connectivity in cocaine-facilitated CRf. Dopamine projections to the NAc (Taylor & Robbins, 1986) and to the VP (Fletcher et al. 1998) have been implicated in cocaine's ability to potentiate CRf. The loss of cocaine-facilitated CRf in the mesoaccumbal α2 knockdown mice might be due to alterations at the accumbal and therefore, perhaps, the pallidal levels.

## 6.6. α2-GABA$_A$Rs and cocaine-facilitated CRf: Challenging D1/D2 antagonism

Mounting evidence to date suggests that D1- and D2-MSN activation in the NAc exerts opposing influences on cocaine-induced responses (refer to Chapter 5 for examples). Though further investigations are yet to be carried out, findings from the present research somewhat suggest a synergistic relationship between these two populations, particularly in mediating cocaine-facilitated CRf. Namely, reducing GABAergic signalling via α2-GABA$_A$Rs in the NAc core, or specifically in the D1- or D2-MSN subpopulations within this region appeared to be sufficient to block cocaine CRf.

Intriguingly, the Chu and Kelley (1992) study concluded that the action of dopamine at both D1R and D2Rs are crucial for psychostimulant enhancement of CRf. It is commonly assumed that dopamine action at D1Rs and D2Rs are largely excitatory and inhibitory respectively (though also see Benoit-Marand & O'Donnell, 2008 for D2R activation and GABA in the NAc). Here, however, we showed that reducing inhibitory control on (potentially enhancing the activity of) both D1R- and D2R-expressing neurons blocked cocaine-facilitated CRf. So, how do our data fit in the current literature? The answer may lie in the complex interactions between dopamine and GABA (Table 6.2). Of particular interest, Benoit-Marand and O'Donnell (2008) demonstrated that D2R activation enhanced cortico-accumbal synapses by recruiting GABA component in the adult rat brain,

revealed by picrotoxin treatment. It is currently not clear whether the D2R recruits both phasic and tonic GABAergic inhibitory systems to achieve such facilitatory effects on cortico-accumbal synapses, given that picrotoxin serves as an antagonist on both synaptic and extrasynaptic receptors (Farrant & Nusser, 2005). However, we speculate that the accumbal D2Rs may interact with the phasic, instead of the tonic, GABAergic system given that its facilitatory effect on MSN activity has been revealed in previous research (Bracci & Panzeri, 2006). Further, recent data from our laboratory showed that agonism at D2Rs had minimal effects on tonic GABAergic currents. For these reasons, we hypothesised that the decrease in $GABA_AR$ number as a result of α2 knockdown might have altered the modulatory action of dopamine on glutamatergic afferents into the NAc neurons, leading to the loss of cocaine-potentiated CRf. Moreover, α2 manipulations specifically in D2R-expressing neurons would similarly affect the net output of the NAc core, leading to the loss of cocaine's CRf enhancing effects.

Table 6.2.

| Genotype | D1R-expressing neuron | D2-expressing neuron |
|---|---|---|
| Wildtype | ↑ excitability | ↑ excitability |
| Mesoaccumbal α2 knockdown | ↑ excitability | ↓ excitability |
| Mesoaccumbal α4 knockdown | ↑ excitability | ↑ excitability |

***Table 6.2.*** Speculated modulatory effects of dopamine on D1R- or D2R-expressing neuron excitability in the NAc core of wildtype and α2 knockdown mice (Benoit-Marand & O'Donnell, 2008).

## 6.7. α2-GABA$_A$Rs and cocaine-induced locomotor sensitisation: Mechanism beyond the NAc?

Dopaminergic neuron activity within the VTA has long been thought to be the neurobiological correlate of the development of sensitisation (Kalivas & Stewart, 1991). The VTA is enriched with the α1-containing GABA$_A$Rs, but the α2 subunit is sparsely expressed in this region (Hörtnagl et al., 2013; Schwarzer et al., 2001). This thesis, therefore, initially hypothesised that the loss of Gabra2 specifically in the NAc could have altered signalling within the VTA, leading to a loss of sensitisation to cocaine observed in the α2 constitutive knockouts (Dixon et al., 2010; Xia et al., 2011). In support of this notion, altering the inhibitory control of the NAc neurons via optogenetic activation of parvalbumin-expressing GABAergic interneurons in the NAc blocked locomotor sensitisation to amphetamine (Wang et al., 2017).

Mounting behavioural and neurobiological evidence to date corroborates the view that psychostimulant-induced sensitisation is not a unitary phenomenon (Wise & Leeb, 1993) and mechanistically, sensitisation has been shown to engage a complex network of subsystems involving the NAc, VTA, amygdala, mPFC, laterodorsal tegmentum, and the subthalamic PVN (Beyer & Steketee, 2002; Kalivas & Alesdatter, 1993; Kalivas & Stewart, 1991; Nelson, Wetter, Milovanovic, & Wolf, 2007; Ujike, Onoue, Akiyama, Hamamura, & Otsuki, 1989; Wolf, Dahlin, Hu, Xue, & White, 1995; Young & Deutch, 1998). As the α2 subunit is densely expressed in

some of these regions (Hörtnagl et al., 2013; Schwarzer et al., 2001), it is plausible that the blockade of sensitisation in the constitutive knockouts might have resulted from the loss of Gabra2 expression in multiple regions, perhaps including the NAc. In keeping, previous research has identified alterations in the $GABA_AR$ expression and function across different regions following repeated methamphetamine exposure. For instance, a radio-ligand binding assay reported decreases in [$^3$H]flunitrazepam binding in the motor and cingulate cortices, caudate putamen, and the NAc in methamphetamine-sensitised mice. These changes were reversed by the 5-HT3 receptor antagonist, MDL72222, which attenuated locomotor sensitisation to methamphetamine (Yoo, Lee, Kim, Lee, & Jang, 2010).

## 6.8. Considerations

### 6.8.1. The RNAi strategy to study α2-$GABA_AR$ function

At present, there are limited methods to study the functional roles of α2-$GABA_ARs$ selectively. Existing pharmacological tools, such as benzodiazepines and their derivatives, lack receptor specificity (Atack et al., 2006; Lüddens & Wisden, 1991; Tan et al., 2011). In this thesis, we have exploited advances in RNAi technology to enable investigations into the functional contributions of the α2-$GABA_ARs$ in a site- and cell-type-specific manner. There are, however several issues that merit consideration.

Firstly, the off-target effects, either of a sequence specific or a non-sequence specific nature, comprise one of the major impediments of sh/siRNA-induced gene expression silencing. In the present research, the

occurrence of non-sequence specific off-target effects, i.e. the activation of the antiviral immune response pathway (Reynolds et al., 2006), was unlikely given the short length of the double-stranded RNAi effectors (Elbashir, 2001). Additionally, though off-target silencing of other genes, either by siRNA- or miRNA-like interaction might have occurred, we postulate that the effect was likely to be minimal, in part due to the high knockdown potency of shα2, as indicated by the immunocytochemical and immunohistochemical analyses presented in Chapter 3.

Furthermore, current data demonstrated that α2 subunit expression did not appear to be resilient to our RNAi manipulations. However, it remains unclear whether the α2 expression knockdown achieved using this approach would sufficiently reduce the level of α2-containing receptor surface expression, as *in vitro* findings documented in the Gorrie et al. (1997) study showed that only a small proportion (<25%) of the $GABA_AR$ subunits underwent oligomerisation to form functional receptors. On the other hand, published data from the Liu et al. (2011) study found that 80% knockdown of Gabra2 expression in the rat significantly reduced radioligand ([$^3$H]flunitrazepam) binding in the CeA. This was thought to be solely triggered by the downregulation of the α2-containing receptors, as no changes in the expression level of the $GABA_AR$ α1 subunit, which is also highly expressed in the CeA, were observed. Though future investigations (i.e. electrophysiological or ligand-binding assays) are warranted, based on existing data, we argued that the extent of α2 knockdown achieved by the shα2 (>70%) used in the present research was likely to downregulate the

receptor number, thus leading to the aforementioned phenotypic differences observed in the present experiments.

Alternatively, the inducible, site-specific gene knockout using the Cre-lox strategy is also a suitable method for targeting specific $GABA_AR$ subunits as it permits site-specific gene knockout and this strategy has been utilised in previous research to study the functional roles of the mesoaccumbal Gabra2 in the mouse (Engin et al., 2014). Recent years have also witnessed growing interests in the use of CRISPR interference (CRISPRi) and Transcription Activator-Like Effector (TALE)-based transcriptional repression techniques as reverse genetic strategies (Boettcher & McManus, 2015). In a direct comparison with the RNAi, CRISPRi appeared to yield more consistent, potent knockdown (>75%) and greater loss-of-function phenotypes (Gilbert et al., 2013, 2014).

Finally, one of the renowned strengths of post-transcriptional gene expression silencing with RNAi is the lack of compensations, commonly associated with gene targeting approaches (Rossi et al., 2015). However, it has been reported that phasic and tonic inhibitions, via synaptic and extrasynaptic $GABA_ARs$ respectively, appear to regulate each other to maintain homeostasis. Namely, overexpression of α6ß3δ receptors in cultured pyramidal neurons produced a substantial reduction in mIPSC frequency and such homeostatic plasticity occurred independent of the receptor activation. Nevertheless, the overexpression of α2ß3γ2 receptors failed to alter THIP-sensitive currents when tested *in vitro* (Wu et al., 2013). Further electrophysiological characterisations of both tonic conductance and

mIPSC characteristics upon RNAi-induced α2 knockdown should, thus, be performed in continuing efforts to understand the functional interactions between these inhibitory systems that may work synergistically in mediating the expression of dopamine-dependent motivated behaviours.

### 6.8.2. Behavioural paradigms

It is also of importance to consider the caveats of the behavioural paradigms used in this thesis. Incentive motivation theory posits that repeated drug exposure can lead to greater salience of some of the drug-induced effects, leading to a sensitised response (Robinson & Berridge, 1993). Many drugs of abuse share a similarity in that some of their effects sensitise, e.g. locomotor effects (Vanderschuren & Kalivas, 2000). It has been postulated that they may share a common mechanism by which they evoke locomotor sensitisation, given that cross-sensitisation between drugs has been reported, despite drug-specific differences in the mechanisms of action (Bonate, Swann, & Silverman, 1997; Cadoni et al., 2000; Cunningham, Finn, & Kelley, 1997). Though this phenomenon has been reported in a plethora of pre-clinical studies to date, the occurrence of sensitisation does not appear to extend to the human case and therefore, sensitisation as a mechanism of drug abuse is considered of somewhat limited explanatory value. In the animal literature, locomotor sensitisation, in specific, is paralleled by a sensitised dopamine neuron reactivity (Vezina, Lorrain, Arnold, Austin, & Suto, 2002). Challenging the notion of sensitisation in humans is the imaging data documented in the Volkow et

al. (1997) study, which identified blunted dopamine responses to a methylphenidate challenge in the striatum of cocaine addicts, though others have modelled sensitisation (i.e. increased dopamine release) in healthy individuals treated with amphetamine (Boileau et al., 2006). The lack of changes in dopamine response upon the methylphenidate challenge in the Volkow et al. (1997) study could have been due to the fact that measurements were taken in the dorsal striatum, rather than in the NAc. Nevertheless, psychostimulant-induced sensitising effects have also been difficult to demonstrate in humans (refer to Leyton & Vezina, 2013 for review of sensitisation studies in humans). The apparent discrepancy between the animal and human literature is likely to be driven by drug administration regimens, withdrawal periods, surrounding cues (contextual and discrete cues), as well as contingent vs. non-contingent drug exposure (Caprioli, Celentano, Paolone, & Badiani, 2007; Leyton, 2007).

Of final note, though paradigms that require noncontingent (experimenter-administered) drug administration have yielded a wealth of evidence on how acute or repeated drug exposure alters neural function, note that the neuroplasticity occurring in response to contingent drug administration may differ (for example, see Miguéns et al., 2008).

## 6.9. Future work

### 6.9.1. Further investigations of the role of α2-GABA$_A$Rs in locomotor sensitisation to cocaine

Findings from Chapter 4 have demonstrated that Gabra2 expression in the NAc core or shell is not critical for the development of cocaine-induced locomotor sensitisation. As discussed above, though it is a possibility the loss of the GABA$_A$R α2 subunit in multiple regions might have accounted for the blockade of the development of cocaine-induced sensitisation in the α2 constitutive knockouts, a recent optogenetic study has shown that optogenetic activation of NAc GABAergic neurons was sufficient to abolish the expression of locomotor sensitisation during a cocaine challenge (Wang et al., 2014). This investigation could therefore be extended by examining the effects of α2 knockdown in the NAc core or shell on the long-term expression of sensitisation.

### 6.9.2. Further investigations of the role of α2-GABA$_A$Rs in cocaine-facilitated CRf

The NAc core and shell subdivisions have been shown to be involved in mediating different aspects of psychostimulant-facilitated CRf. Namely, the NAc core was found to be a critical site for the interaction between the CR's control over behaviour and its potentiation by *d*-amphetamine, whereas the shell subdivision primarily mediates the stimulant effects of *d*-amphetamine (Parkinson et al., 1999). Based on current findings, this study hypothesised

that the lack of mesoaccumbal Gabra2 expression might have specifically disrupted cocaine-induced enhancement in the *willingness to work* for the CR, as the loss of the cocaine facilitation of CRf was observed upon RNAi-induced silencing of α2 expression in D2R-harbouring neurons. There is strong evidence in the literature implicating D2R-mediated dopamine transmission in tasks requiring high effort (Gallo et al., 2018; Soares-Cunha et al., 2016; Trifilieff et al., 2013). At the neurobiological level, we posit that reducing the level of α2 expression in the NAc core might have somewhat dampened the consequences of cocaine-induced D2R-, and possibly D1R-, mediated dopamine signalling, thus leading to enhanced inhibitory transmission to the pallidum and the loss of its CRf-potentiating effects.

Mechanistically, as depicted in Figure 6.2, we further speculated that reducing the number of α2-harbouring receptors in the NAc would enhance MSN outputs to the VP, which in turn, might result in attenuated inhibitory afferents to the VTA dopamine and non-dopamine neurons. Note that in the rat, inhibition from the VP was more prominent in the non-dopamine neurons than in the dopamine counterparts (Hjelmstad, Xia, Margolis, & Fields, 2013). Given that the primary inhibitory control of dopamine neurons originate from the local GABAergic interneurons (Tan et al., 2012), we posit that the increase in GABAergic input from the interneurons may lead to a net decrease in the activity of dopamine neurons, leading to the loss of cocaine-induced effects

Alternately, according to the RPE model, the presentation of a CR should trigger dopamine neuron activation (Schultz et al., 1997). Cocaine-

induced blockade of dopamine transporters might prolong the availability of high dopamine level in the extracellular space triggered by CR presentation, leading to increased CR-maintained responding in the wildtype mice. However, it remains a possibility that in the α2 knockout or knockdown mice, CRs no longer evoked bursts of dopamine during the CRf tests, hence the absence of cocaine's enhancing effect.

Taken together, psychostimulant enhancement of CRf appears to involve dopamine action at the NAc and VP (Fletcher et al., 1998) though detailed mechanisms underlying this behavioural response remains to be fully elucidated. To probe whether cocaine-facilitated CRf requires the functional interaction between the dopaminergic action and GABAergic signalling via the α2-containing receptors strictly at the accumbal level, or at the level of NAc-VP synapses, future experiments should also address the effects of mesoaccumbal α2 knockdown on intra-NAc or intra-VP cocaine-enhanced CRf.

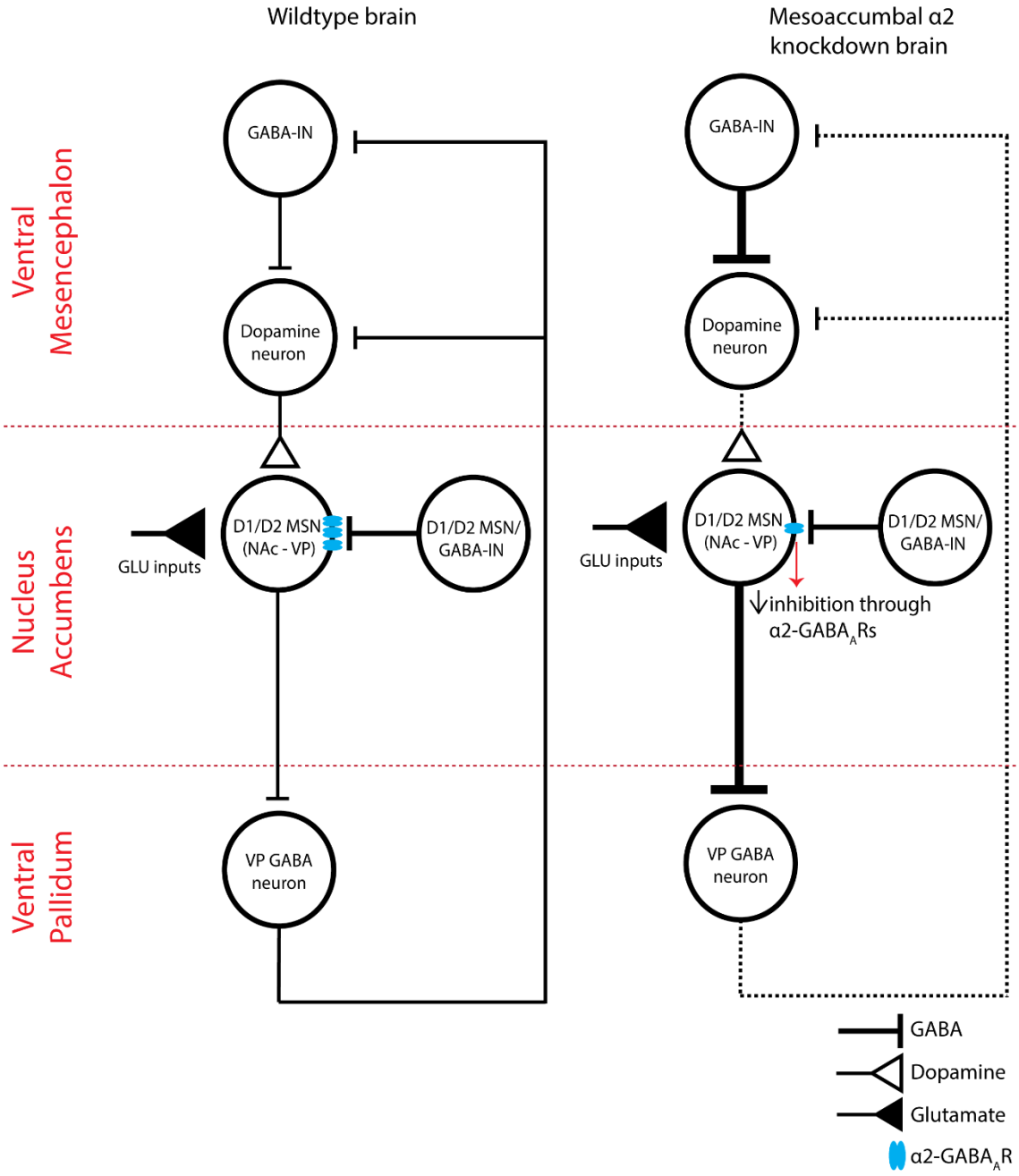

Wildtype brain
Mesoaccumbal α2 knockdown brain
Ventral Mesencephalon
Nucleus Accumbens
Ventral Pallidum
GABA-IN
Dopamine neuron
D1/D2 MSN (NAc - VP)
D1/D2 MSN/ GABA-IN
GLU inputs
VP GABA neuron
inhibition through α2-GABA$_A$Rs
GABA
Dopamine
Glutamate
α2-GABA$_A$R

***Figure 6.2.*** Speculated influence of mesoaccumbal $GABA_AR$ α2 subunit knockdown in the D1- or D2-MSNs on the NAc-VP-VM-NAc circuitry. Reduced inhibitory control by GABA through α2-$GABA_ARs$ may enhance MSN output to the VP, resulting in dampened inhibitory input to dopamine and non-dopamine neurons in the VM (Hjelmstad, Xia, Margolis, & Fields, 2013). Such alterations are likely to elucidate the loss of cocaine-facilitated CRf in the α2 knockouts (Dixon et al., 2010) or in the mesoaccumbal α2 knockdowns.

Whilst the current research has provided robust evidence for importance of α2-$GABA_A$Rs in the NAc core in mediating cocaine enhancement of CRf, the contribution of α2 in the NAc shell remains elusive, warranting future investigations. We postulate that α2 knockdown in the NAc shell, would also affect cocaine facilitation of CRf as dopamine depletion in the NAc shell has also been demonstrated to abolish *d*-amphetamine potentiation of CRf (Parkinson et al., 1999). We further posit that the heightened cocaine-induced hyperlocomotion resulting from α2 knockdown in the NAc shell (refer to Chapter 4: BS-SHELL experiment) might enhance responding on both the CR- and NCR-associated modules (Parkinson et al., 1999).

Furthermore, considering that α4-$GABA_A$R activation with THIP, leading to an overall reduction in mIPSC frequency, also blocked cocaine-facilitated CRf (Macpherson et al., 2016; Maguire et al., 2014), further investigations should be carried out to probe whether the loss of cocaine-potentiated CRf upon RNAi-induced downregulation of α2 expression was attributable to the downregulation of the α2-containing receptors or simply resulted from a general decrease in phasic GABAergic activity in the NAc, mediated by the α2- and potentially, α1-containing receptors (Hörtnagl et al., 2013; Mitchell et al., 2018). Electrophysiological recordings from the NAc core neurons of the Gabra2 knockout mice indicated no genotypic differences in the mIPSC frequency, but revealed a ~33% decrease in the mIPSC amplitude, indicating the presence of other synaptic $GABA_A$R isoform(s) (Dixon et al., 2010). Future studies could probe the effects of

RNAi-induced mesoaccumbal Gabra1 expression knockdown on cocaine's CRf-potentiating properties.

It remains elusive whether the α1- and α2-$GABA_A$Rs display subcellular colocalisation within the accumbal neurons (see Figure 6.3), thus gating the same excitatory inputs. Some argue that the α1-$GABA_A$Rs are preferentially enriched in the GABAergic interneurons, whereas the α2-harbouring receptors are densely expressed in the MSNs (Boyes & Bolam, 2007; Schwarzer et al., 2001). However, more recent *in vivo* data suggest that both the α1- and α2-containing $GABA_A$Rs are present on the MSN dendrites (Gross et al., 2011) and others have identified mixed α1/α2 postsynaptic clusters in the developing MSNs (Arama et al., 2015). Based on the anatomical and THIP data described above, it is tempting to speculate that knocking down Gabra1 expression, thus reducing overall phasic GABAergic activity, would also block cocaine-facilitated CRf. However, note that α1- and α2-$GABA_A$Rs have distinct kinetic properties, i.e. α1-$GABA_A$Rs exhibit fast deactivation and desensitisation (Freund & Buzsáki, 1996), whereas α2-$GABA_A$Rs are often characterised by fast activation, slow deactivation, and higher GABA affinity (Lavoie, Tingey, Harrison, Pritchett, & Twyman, 1997; Levitan et al., 1988).

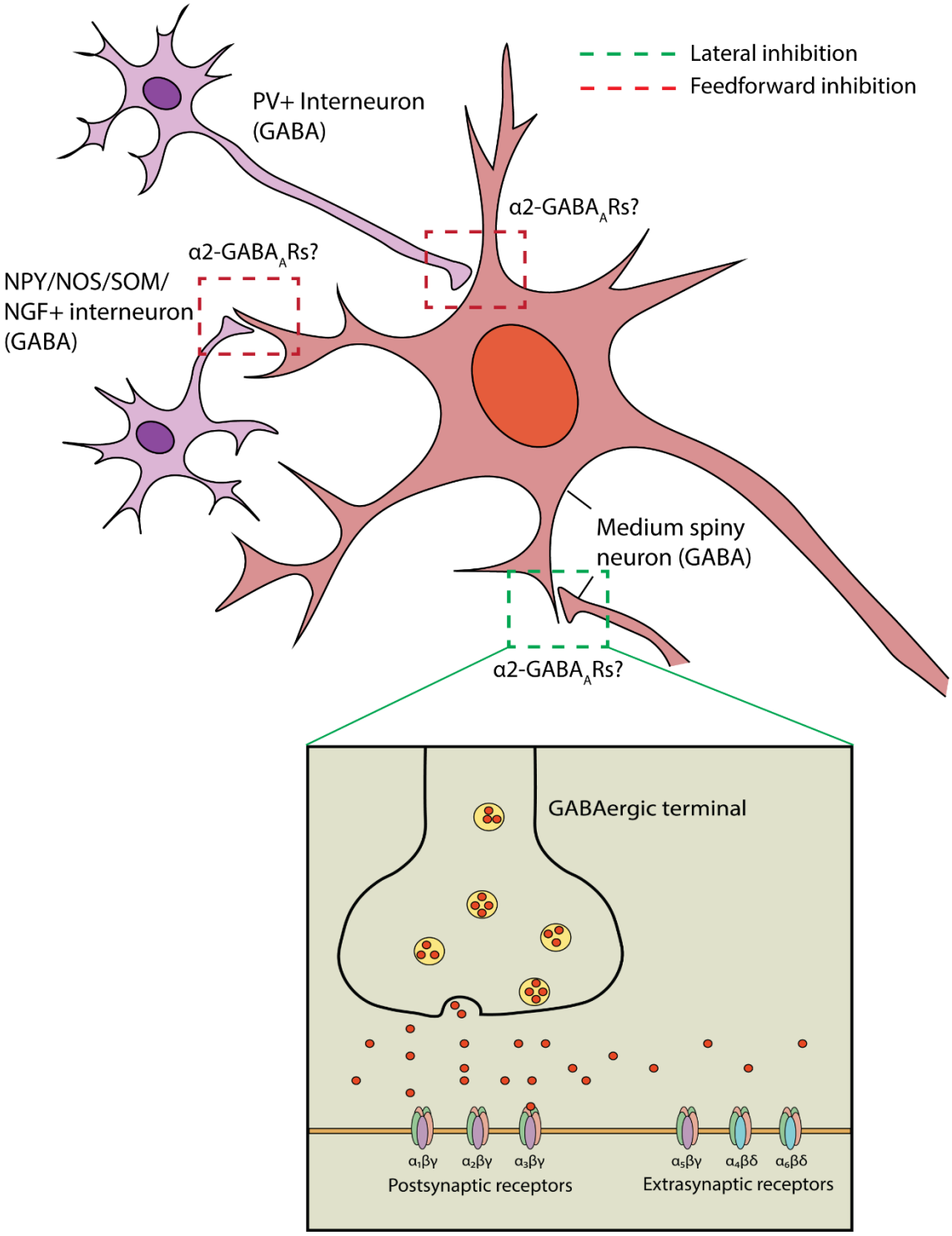



***Figure 6.3.*** Subcellular localisation of α2-GABA$_A$Rs within the NAc. It remains to be determined whether α2-GABA$_A$Rs are localised on the dendritic and/or the somatic membrane of accumbal medium spiny neurons, which are involved in distinct forms of inhibition (i.e. lateral vs. feedforward

inhibition; also refer to Chapter 1 Section 1.3.3.3) (adapted from Stephens et al., 2017).

The findings from Chapter 5 have yielded further insights into the cell-type-specific roles of the intra-accumbal α2 subunit, particularly in Pavlovian learning and cocaine-facilitated responding for CRf. However, given the inconclusive results, it remains unknown whether intact GABAergic signalling via the α2-containing receptors in the NAc core D1R-expressing neurons are also crucial for dopamine's ability to boost CRf. A repeat experiment should therefore be carried out to investigate this further. Additionally, to extend the D1/D2 pathway-specific investigations, future research could utilise optogenetic approaches to further establish causal relationships between the accumbal MSN activity and cocaine enhancement of CRf.

Finally, akin to CRf, PIT also measures how reward-associated stimuli can alter motivation and ultimately, instrumental actions. Lesion studies have implicated activities within the CeA and NAc core, specifically in outcome-specific PIT (Corbit & Balleine, 2005; 2011). Intriguingly, psychostimulant drugs can potentiate the expression of PIT through augmented phasic dopamine signalling (Ostlund et al., 2014; Saddoris, Stamatakis, & Carelli, 2011). Future research could therefore probe whether the mesoaccumbal α2-containing receptors also play crucial roles in the expression of PIT and its potentiation by psychostimulant agents.

## 6.10. Conclusions

To conclude, studies to date have yielded converging evidence for high genetic influences on dopamine-dependent behavioural responses to cocaine (Dixon et al., 2010; Duka et al., 2015; Mitchell et al., 2018). Using the RNAi technology in mouse models, findings presented in this thesis have demonstrated that reducing the expression level of the $GABA_AR$ α2 subunit, specifically in D2R-harbouring neurons of the NAc core, abolished cocaine's ability to boost the expression of a learned instrumental action. Further, though manipulations of α2 expression in the NAc core or shell did not induce marked effects on the development of cocaine-induced locomotor sensitisation, the latter markedly amplified cocaine-induced locomotor hyperactivity. Collectively, this thesis has coalesced current data into a working hypothesis that the mesoaccumbal α2 expression is critical for cocaine's CRf-enhancing properties, but not for its sensitisation-inducing effects. It remains to be determined whether the present findings with cocaine can be extrapolated to other psychostimulant drugs.

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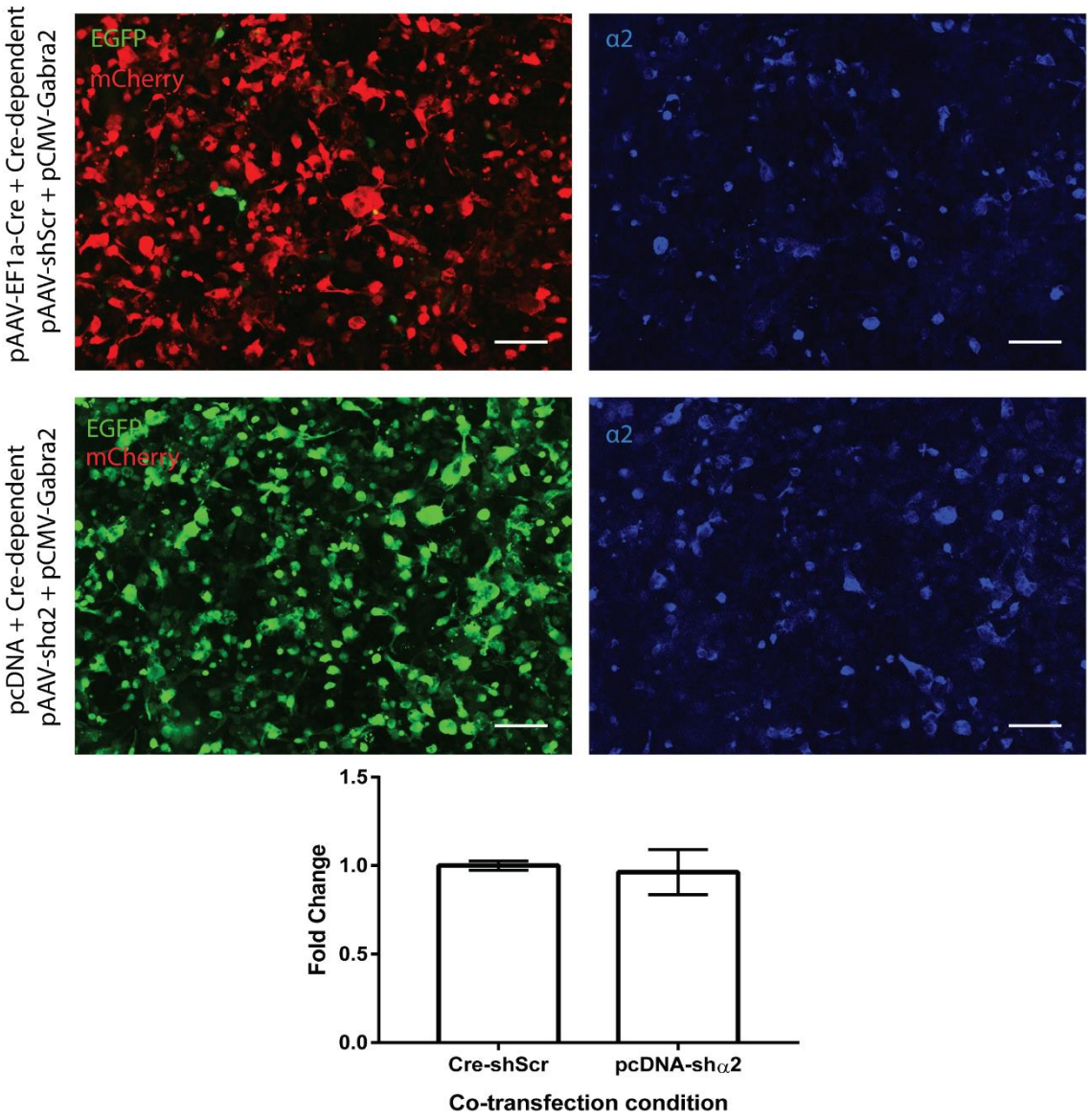
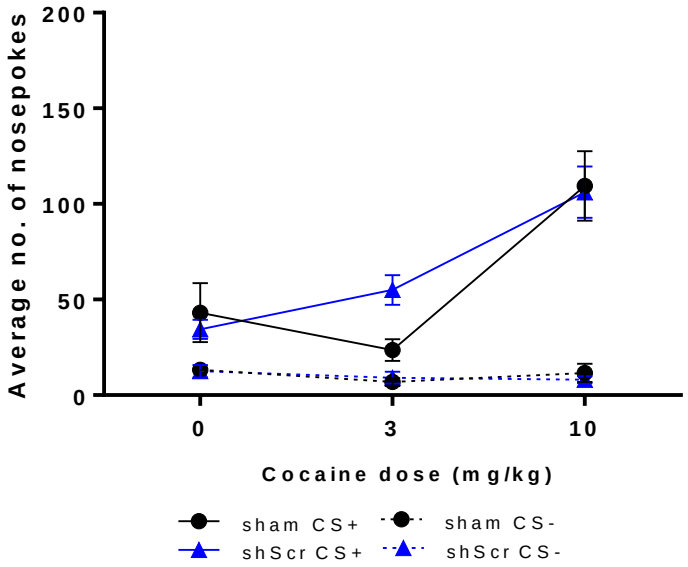
# Appendix A - An *in vitro* assay of the Cre-dependent pAAV-shα2




The amount of α2 expression (signal), normalised to either mCherry or EGFP did not vary significantly between cells co-transfected with Cre- and Cre-dependent shScr-harbouring plasmids and those transfected with pcDNA (No Cre control) and Cre-dependent pAAV-shα2 (experiments were set up in triplicates; scalebar 100µm), $t(4) = 0.29$, $p = .784$. Thus, the α2 knockdown by pAAV-EGFP_mCherry(DO_DIO)-shα2, presented in Chapter 3, occurred only in the presence of Cre.

# Appendix B - Investigating the effects of shScr injections into the NAc core on cocaine-facilitated conditioned reinforcement

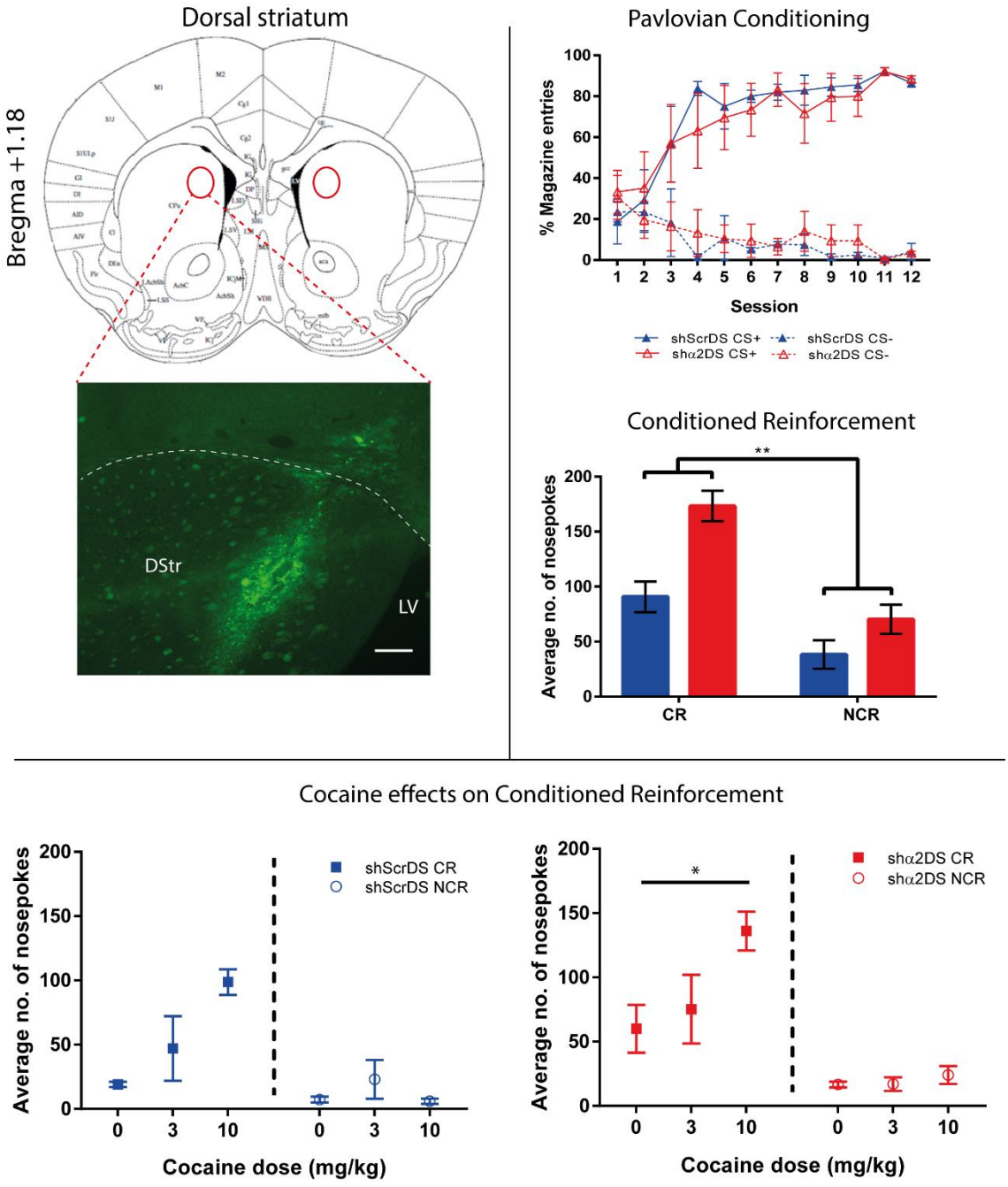



Here, we showed that rAAV-EGFP-shScr injections into the NAc core (n = 11) did not affect baseline responding of conditioned reinforcement and cocaine-facilitated conditioned reinforcement through potential off-target effects when compared to a group of mice receiving sham injections (saline) into the NAc core (n = 14) (i.e. a non-significant three-way interaction between groups, dose, and nosepokes; $F(2,46) = 1.14$, $p = .328$).

# Appendix C - Investigating the effects of cocaine on magazine entries during conditioned reinforcement sessions


Different doses of cocaine failed to affect magazine entries during CRf sessions in both groups, indicated by statistically non-significant group x dose interaction ($F(2,36) = 2.96$, $p = .065$) and main effect of dose ($F(2,36) = 3.16$, $p = .055$).

# Appendix D - Investigating the effects of α2 knockdown in the dorsal striatum on cocaine-facilitated conditioned reinforcement




An additional pilot experiment was conducted to examine whether the loss of cocaine's CRf-enhancing effect seen in the shα2 group above could have been an artefact of viral spread and therefore, α2 knockdown in the dorsal striatum (DS). The target injection sites within the DS (coordinates

AP+1.18, L+/- 1.00, DV -3.30) are shown in Figure 4.5 (Paxinos & Franklin, 2001). Two groups of mice, shScrDS (n = 4, all males) and shα2DS (n = 4, all males) were initially used in this experiment. Suppressing the expression of α2 in the DS did not affect Pavlovian learning with food rewards (i.e. a non-significant three-way (CS × session × group) interaction ($F(11,44) = 0.53$, $p = .875$) and conditioned reinforcement (i.e. a non-significant nosepokes × group interaction ($F(1,4) = 6.13$, $p = .069$)). Further, α2 knockdown in the DS failed to abolish cocaine enhancement of CRf ($F_{CR}(2,4) = 14.81$, $p < .05$; $F_{NCR}(2,4) = 2.00$, $p = .251$).