The Role of Ventral Tegmental Area and Nucleus Accumbens in the Kamin Blocking Effect

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The Role of Ventral Tegmental Area and Nucleus Accumbens in the Kamin Blocking Effect

by

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Declaration of Original and Sole Authorship

I, Mayank Aggarwal, declare that this thesis entitled *The Role of Ventral Tegmental Area and Nucleus Accumbens in the Kamin Blocking Effect* and the data presented in it are my own work.

I confirm that:

- This work was done solely while a candidate for the research degree at the Okinawa Institute of Science and Technology Graduate University, Japan.
- No part of this work has previously been submitted for a degree at this or any other university.
- References to the work of others have been clearly acknowledged. Quotations from the work of others have been clearly indicated, and attributed to them.
- In cases where others have contributed to part of this work, such contribution has been clearly acknowledged and distinguished from my own work.
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Abstract

The overall aim of the research described in this thesis is to identify neural substrates underlying the Kamin blocking effect. This phenomenon is crucial for understanding of the neural mechanisms of associative learning. Kamin blocking refers to the finding that conditioned responding to a cue is attenuated when it is paired with a reinforcer in the presence of another cue which has previously been conditioned using that reinforcer. The blocking effect suggests that associative learning is driven by prediction errors, and is not based purely on temporal contiguity between events. In the context of appetitive classical conditioning, recent evidence suggests that the ventral tegmental area and the nucleus accumbens play a role in computing reward prediction error. The current study shows that blocking inhibition in the ventral tegmental area or inactivating the nucleus accumbens neurons during compound cue conditioning attenuates Kamin blocking. Inactivating the nucleus accumbens during single cue conditioning also attenuates Kamin blocking. Taken together, these findings suggest that inhibition in the ventral tegmental area, inhibitory output from the nucleus accumbens, and learning in the nucleus accumbens play crucial roles in the Kamin blocking effect. Previous studies show that dopamine transients track the theoretical reward prediction error during appetitive classical conditioning, and the reduction in the dopamine response evoked by the reward when it is expected has been suggested to play a role in the Kamin blocking effect. In support of this hypothesis, the current study also found that goal tracking rats, in which expected rewards have previously been shown to evoke a robust dopamine response, did not express the Kamin blocking effect. Conversely, sign trackers, in which expected rewards evoke a diminished dopamine response, expressed the blocking effect. These findings are discussed in relation to psychological theory of learning and the possible underlying neural mechanisms.

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Second, I thank Prof. Bernard Balleine, whose feedback on my thesis proposal, as part of the thesis proposal examination, was crucial in narrowing the focus my project on a few goals which were realistically achievable within the time frame of my PhD. His suggestions on reading material regarding associative learning theories and experimental implementation of classical conditioning was very helpful in improving my conceptual knowledge in this area.

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wasted labor. Sho was very helpful and prompt in clarifying any questions I had about behavioral protocols, animal handling and surgery and running behavioral experiments. Sho's guidance in matters related to animal experiments was crucial in improving my experimental technique and contributed a lot towards the successful completion of my experiments.

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List of Abbreviations

ANOVA – Analysis of Variance

CNO – Clozapine-N-Oxide

CR – Conditioned Response

CS – Conditioned Stimulus

CSF – Cerebrospinal Fluid

DAB – 3,3′-Diaminobenzidine

DREADD – Designer Receptors Exclusively Activated by Designer Drugs

GABA – Gamma-aminobutyric Acid

GIRK – G-protein Inward Rectifying Potassium

GT – Goal Tracker

i.p. – Intraperitoneal

ITI – Inter Trial Interval

NAc – Nucleus Accumbens

NMDA – N-methly-D-aspartate

SEM – Standard Error of the Mean

SNc – Substantia Nigra Pars Compacta

ST – Sign Tracker

TH – Tyrosine Hydroxylase

US – Unconditioned Stimulus

VTA – Ventral Tegmental Area

Table of Contents

List of Figures

1. Introduction and Statement of the Problem

1.1 Introduction

The neural mechanism for learning is a central topic in neuroscience. Classical conditioning, one of the major forms of learning, is the learning of associations between environmental cues and the reinforcers that they foreshadow. Extensive experimental laboratory investigation of learning in the early part of last century led to psychological theories of classical conditioning which assumed that the temporal contiguity between the cue and the reinforcer was sufficient for conditioning to occur. However, the discovery of the Kamin blocking effect (Kamin, 1968; Kamin, 1969a; Kamin, 1969b), called into question this assumption. Kamin demonstrated that conditioned responding to a cue that is paired with a temporally contiguous reinforcer is attenuated if, during the pairing, the cue is presented simultaneously with another cue that has previously been conditioned using the same reinforcer. Kamin explained this phenomenon by proposing that the reinforcer has to be surprising or unexpected for conditioning to occur. This finding shifted the theoretical framework for classical conditioning from theories that required only temporal contiguity to those that relied on prediction error. Models of associative learning that rely on prediction errors have since been elaborated into sophisticated computational theories.

Advances in psychology have been complemented by neurobiological analysis of behavioral effects of localized lesions and pharmacological manipulations of the brain and the correlation of neural activity with behavior, which have suggested possible neural substrates for behavioral learning. Notable among these is the identification of transient increases in the firing of midbrain dopamine neurons, which track the theoretical reward prediction error

signal (Mirenowicz & Schultz, 1994; Schultz *et al.*, 1997; Hollerman & Schultz, 1998) and are correlated with the occurrence of classical conditioning (Waelti *et al.*, 2001), and the identification of glutamate receptor mediated synaptic plasticity, such as that mediated by N-methly-D-aspartate (NMDA) receptors, and its modulation by dopamine (Calabresi *et al.*, 2000; Gurden *et al.*, 2000; Reynolds *et al.*, 2001; Tye *et al.*, 2008; Koralek *et al.*, 2012), as possible neural substrates for learning. However, much remains to be discovered about the neural substrates and mechanisms underlying associative learning and controlling the firing of dopamine neurons in the context of learning. In particular, psychology has shown that learning is an active process. Learning is not the imprinting of every fragment of experience onto memory, but rather a selection process. Thus, a current topic of research is to discover the neural mechanisms that not only permit learning to occur, but also determine what is learnt.

The work in this thesis revisits the Kamin blocking effect from a new biological perspective. As mentioned earlier, this phenomenon showed that associative learning is not based purely on temporal contiguity, but also depends on the predictability of the reinforcer. This finding had a strong influence on theories of associative learning. However, the neural substrates underlying the Kamin blocking effect have not yet been fully elucidated.

1.2 Statement of the problem

The overall aim of the research reported in this thesis is to identify some important neural substrates of the Kamin blocking effect. Contemporary theories of learning suggest that regions involved in the computation of prediction errors may play a key role in the expression of the Kamin blocking effect (Rescorla & Wagner, 1972; Mackintosh, 1975; Pearce & Bouton, 2001). This theoretical framework assumes that learning of new associations cannot

occur when there is no, or diminished, prediction error. To develop this theory further, there is a need to investigate the causal role, in the expression of the Kamin blocking effect, of structures that compute prediction errors.

A review of the literature suggests that the ventral tegmental area (VTA) dopamine neurons, and the control of input to these neurons by the nucleus accumbens (NAc), plays a key role in the computation and expression of reward prediction error. The Kamin blocking effect, at least in the context of appetitive reinforcers (referred to as rewards in this thesis), may invoke mechanisms that rely on these reward prediction error computations. To investigate these mechanisms, the research reported in this thesis tests the role of the ventral tegmental area and the nucleus accumbens in the expression of the Kamin blocking effect.

1.3 Hypotheses

The overall research hypothesis is that the suppression of a dopamine reward prediction error signal when a reward is fully predicted underlies the Kamin blocking effect. There are three specific experimental hypotheses:

I. Inhibition of the dopamine neurons in the ventral tegmental area at the expected time of reward delivery is necessary for reducing the reward evoked dopamine reward prediction error signal when the reward is expected. Thus, inhibitory input to the ventral tegmental area is necessary during compound cue conditioning for the expression of the Kamin blocking effect.

II. Output of the nucleus accumbens region of the striatum controls the activity of the ventral tegmental area dopamine neurons in the context of reward related learning and contributes to the dopamine reward prediction error signal. Thus, output from

the nucleus accumbens is necessary during compound cue conditioning for the expression of the Kamin blocking effect.

III. The output of the nucleus accumbens acquires control over the activity of the ventral tegmental area dopamine neurons through neuronal learning during the single cue conditioning phase. Thus, learning in the nucleus accumbens during the single cue conditioning phase is necessary for the expression of the Kamin blocking effect.

Based on these hypotheses, the aim of the research described in this thesis was to answer three specific questions:

i) Does blocking inhibition in the ventral tegmental area during the compound cue conditioning phase of the Kamin blocking procedure disrupt the Kamin blocking effect?

ii) During compound cue conditioning, does inactivating the nucleus accumbens disrupt the Kamin blocking effect?

iii) During single cue conditioning, does inactivating the nucleus accumbens disrupt the Kamin blocking effect?

These questions are addressed by measuring the effect of anatomically selective manipulations on behavioral measures of the Kamin blocking effect.

1.4 Outline of the thesis

This thesis consists of five chapters.

Chapter one (this chapter) provides an overall statement of the problem, hypotheses, and experimental questions.

Chapter two is a review of the literature concerning the Kamin blocking effect as a behavioral phenomenon, the possible role of dopamine in the expression of the blocking effect, and the likely roles of the ventral tegmental area and the nucleus accumbens as the neural substrates underlying the Kamin blocking effect.

Chapter three reports findings of three experimental studies. In the first, inhibition onto the ventral tegmental area neurons was blocked during the compound cue conditioning phase by bilaterally infusing bicuculline into the ventral tegmental area. In the second, the firing of the neurons in the nucleus accumbens was inhibited during the compound cue conditioning phase, thus disrupting the output from this region. In the third, during the single cue conditioning phase, the firing of the neurons in the nucleus accumbens was inhibited, thus disrupting learning in this region during this phase. The effects of these three manipulations on the Kamin blocking effect are reported.

In chapter four, data from the studies mentioned in chapter three is reanalyzed, grouping animals on the basis of the nature of their response to the conditioned cue during the single cue conditioning phase i.e., goal trackers and sign trackers. Following the Flagel et al., (2011) finding that the reduction in the reward evoked dopamine response when the reward becomes expected occurs only in sign trackers and not in goal trackers, the overall research hypothesis of this thesis (see section 1.3) predicts that only sign trackers will express the Kamin blocking effect.

Chapter five summarizes the results reported in this thesis and discusses their implications within the theoretical framework of associative learning, and for future attempts at demonstrating the Kamin blocking effect. The various associative structures acquired during

classical conditioning, their possible neural pathways, their influence on conditioned responding and subsequent learning, and the importance of experimental protocols designed to distinguish between behaviors attributable to different associative structures are discussed. Chapter 5 also extends on a previously proposed mechanism (Aggarwal *et al.*, 2012) for acquiring timed inhibition of the dopamine neurons during classical conditioning. Lastly, the overall significance of the results reported in this thesis and future directions to build on the current results are discussed.

2. Review of Literature

This chapter introduces selected concepts of associative learning relevant to this thesis, and provides the background to my hypotheses. The chapter starts with the classical conditioning phenomena that contribute to associative learning theory. Section 2.2 discusses the role of prediction error in the Kamin blocking effect, which substantiates the idea that only unexpected events drive associative learning. Section 2.3 discusses the role of the dopamine reward prediction error signal in Kamin blocking of appetitive classical conditioning. Driven by the hypothesis – that a reduction in the reward evoked dopamine signal when the reward is expected is important for Kamin blocking to occur – sections 2.4-2.7 identify neural substrates to be tested for their possible role in the Kamin blocking effect. Lastly, section 2.8 summarizes the hypotheses whose tests are reported on in chapter 3.

2.1 Classical Conditioning

Classical conditioning refers to the learning of an association between two temporally correlated events. When a cue precedes the delivery of a reinforcer, and this pairing is repeated, an association is formed between the cue and the reinforcer. The cue is said to have become conditioned and is referred to as the conditioned stimulus (CS). The reinforcer is called the unconditioned stimulus (US). The presentation of the cue, once conditioned, evokes a behavioral response as a result of the conditioning procedure. This response is called the conditioned response (CR) and is used as the measure for the extent of classical conditioning. Pavlov (1927) was the first to describe such conditioning. He trained a dog using a buzzer CS paired with a food US, an appetitive reinforcer. He observed that after

repeated pairings of the buzzer and the food, the buzzer began to elicit a salivation response (CR) from the dog.

In the early part of the 20th century, it was thought that temporal contiguity between the cue and reinforcer was necessary and sufficient for classical conditioning to occur. However, a number of classical conditioning phenomena, discovered in the latter half of the 20th century, suggested that temporal contiguity was not sufficient for classical conditioning.

Rescorla (1968) argued that contingency between the CS and the US plays an important role in determining the strength of the CR. Rescorla (1968) found that conditioning depends on the probability of US given the CS and probability of the US in the absence of the CS. US presentation in the absence of the CS interfered with conditioning. When the probability of occurrence of US given CS was held constant, conditioning changed from excellent to negligible simply by increasing the rate of occurrence of US in the absence of CS (Rescorla, 1988). These experiments showed that classical conditioning involves more than a simple CS-US contiguity. Rather, the CS-US contingency is an important factor in determining the extent of conditioning.

Lubow (1973b; a) found that a cue repeatedly presented in the absence of a US is difficult to condition when it is subsequently used in a classical conditioning procedure. This phenomenon, called latent inhibition, suggested that temporal contiguity is not sufficient for classical conditioning, and that the associability of the cue plays a role in determining the extent of conditioning. It further showed that the associability of a cue is not constant, and changes based on past experience with the cue.

The discovery of the Kamin blocking effect (Kamin, 1968; Kamin, 1969a; Kamin, 1969b) provided a major conceptual advance in the understanding of the factors necessary for

associative learning, suggesting that the US needs to deviate from expectations for learning to occur. The blocking effect is central to this thesis and is discussed in the next section.

2.2 The Kamin Blocking Effect

Kamin (1968; 1969a; 1969b) described an attenuation in conditioned responding to a CS paired with a US if, during the pairing, the CS is presented simultaneously with another CS which has previously been associated with the same US. This is known as the Kamin blocking effect, or transreinforcer blocking (Ganesan & Pearce, 1988b; a).

In the Kamin blocking experiment (blocking group – Fig. 2.1), a first cue, A, undergoes conditioning using a reinforcer as the US. Once the conditioning is complete, a second cue, B, is simultaneously presented with cue A and this compound A + B is conditioned using the same reinforcer. Finally, responding to the presentation of cue B alone is tested in the absence of the reinforcer (in extinction). Blocking is said to have occurred if, during the extinction test, the blocking group's conditioned response to cue B is significantly lesser than that of animals undergoing control procedures. One commonly used control procedure is for animals to undergo compound cue conditioning without prior conditioning of cue A (control group – Fig. 2.1). A significant difference between the two groups (Fig. 2.1) in their conditioned response to cue B during the extinction test suggests that prior conditioning of cue A is responsible for the blocking group's (Fig. 2.1) diminished response to cue B.

The Kamin blocking effect can be explained by prediction error theories of learning. In these theories, learning is based on the difference between what is predicted and what actually happens, that is, the prediction error (Rescorla & Wagner, 1972; Pearce & Bouton, 2001; Bouton, 2007). In this formulation, associative learning between the cue and the reinforcer occurs only when reinforcement exceeds or falls short of expectation. No associative learning

	Blocking Group	Control Group
Phase 1:	Cue A → Reinforcer	Cue Z → Reinforcer
Phase 2:	Cue A + B → Reinforcer	Cue A + B → Reinforcer
Phase 3:	Cue B → No Reinforcer	Cue B → No Reinforcer

Figure 2.1: **Schematic representation of the Kamin blocking procedure**, which consists of three phases. Phase 1 is a single cue conditioning phase, phase 2 is a compound cue conditioning phase, and phase 3 is an extinction test. In the blocking group, conditioning of cue A occurs in phase 1, prior to conditioning of the compound cue A + B. In the control group, cue Z is conditioned in phase 1 (to keep the number of presentations of the reinforcer the same in the two groups during this phase), prior to conditioning of the compound cue A + B. Blocking occurs if, in phase 3, cue B presentations evoke a stronger response in the blocking group than in the control group.

occurs if the reinforcement does not deviate from expectations. This formulation, when applied to the Kamin blocking experimental design (Fig. 2.1), automatically results in the Kamin blocking effect, and is explained as follows:

According to prediction error based learning models, when cue A is followed by a reinforcer (blocking group in Fig 2.1) in the early stages of conditioning, the reinforcer is completely unexpected and therefore leads to the formation of an association between cue A and the reinforcer. As classical conditioning progresses, the cue A – reinforcer association is gradually strengthened. The cue A – reinforcer association reaches an asymptote once cue A fully predicts the reinforcer. After learning, when the compound cue A + B is conditioned using the same reinforcer (blocking group, Phase 2 in Fig 2.1), the reinforcer is accurately predicted by cue A and can therefore not support conditioning of cue B. Thus, cue B does not

get conditioned during the compound cue conditioning phase. According to prediction error based learning theories, it is the accurate prediction of the reinforcer by cue A that produces the Kamin blocking effect. The extent of blocking of conditioning of cue B depends on how well cue A predicts the reinforcer – the smaller the prediction error in the compound cue phase, the stronger is the blocking effect. It is important to note here that the Kamin blocking effect can also be explained by theories in which temporal contiguity between a cue and a reinforcer is sufficient for associative learning to occur. Such theories rely on cue competition during performance in phase 3 to explain blocking, for example, the extended comparator hypothesis (Stout & Miller, 2007).

The discovery of the Kamin blocking effect had a huge impact on the theoretical framework for understanding classical conditioning. It implied that the reinforcer had to be surprising for conditioning to occur, thus shifting the focus from temporal contiguity to prediction error.

A further development in this theoretical framework came from a different direction, namely the neurobiology of reinforcement. This is elaborated in the next section, but briefly, Schultz and colleagues (Mirenowicz & Schultz, 1994; Schultz *et al.*, 1997; Hollerman & Schultz, 1998; Waelti *et al.*, 2001) found that neuronal responses of the dopamine neurons in the midbrain conformed to the rules of prediction error theories of learning. In particular, the dopamine neurons fired in a manner consistent with prediction error signaling when rewards, such as food or water, were used as the US in classical conditioning procedures. Further, Waelti et al., (2001), using the Kamin blocking paradigm, found a correlation between learning and dopamine response evoked by the reward. Specifically, behavioral learning occurred only when the reward evoked a robust increase in dopamine neuron firing rate. Conversely, behavioral learning did not occur when the reward did not evoke a robust dopamine response. These findings suggested a possible neural substrate for the reward prediction error signal necessary for appetitive classical conditioning. Given the significance

of these findings for Kamin blocking, the next section provides a brief background to the firing modes of dopamine neurons, and then reviews the role of the dopamine reward prediction error signal in the expression of the Kamin blocking effect.

2.3 Dopamine, Reward Prediction Error and the Kamin Blocking Effect

2.3.1. Midbrain dopaminergic pathways

Midbrain dopamine neurons form three distinct cell groups: the ventral tegmental area, the substantia nigra compacta, and the retrorubral area (Anden *et al.*, 1964; Dahlstroem & Fuxe, 1964; Felten & Sladek, 1983; Williams & Goldman-Rakic, 1998). The efferent projections of the midbrain dopamine neurons form a number of important dopaminergic pathways (Fallon & Moore, 1976; Bjorklund & Lindvall, 1978; Fallon *et al.*, 1978; Fallon & Moore, 1978a; b; Lindvall & Bjorklund, 1983). Of particular relevance to this thesis are the mesocorticolimbic and nigrostriatal pathways (Fig. 2.2).

The mesocorticolimbic pathway is divided into the mesolimbic pathway, consisting of mainly the projections from the ventral tegmental area to the ventral striatum (Voorn *et al.*, 1986; Jimenez-Castellanos & Graybiel, 1987), and the mesocortical pathway, which refers to the projections from the ventral tegmental area to the prefrontal cortex (Thierry *et al.*, 1973; Berger *et al.*, 1974; Bentivoglio & Morelli, 2005; Bjorklund & Dunnett, 2007). The nigrostriatal pathways consists of projections from the substantia nigra pars compacta to the dorsal striatum (Gerfen, 1984; Gerfen *et al.*, 1987; Jimenez-Castellanos & Graybiel, 1987).

The mesocorticolimbic and nigrostriatal pathways play a role in motivating behavior and learning, regulating emotions, forming reward associations, controlling voluntary movement, and may also play a role in signaling the saliency of environmental stimuli, amongst others.

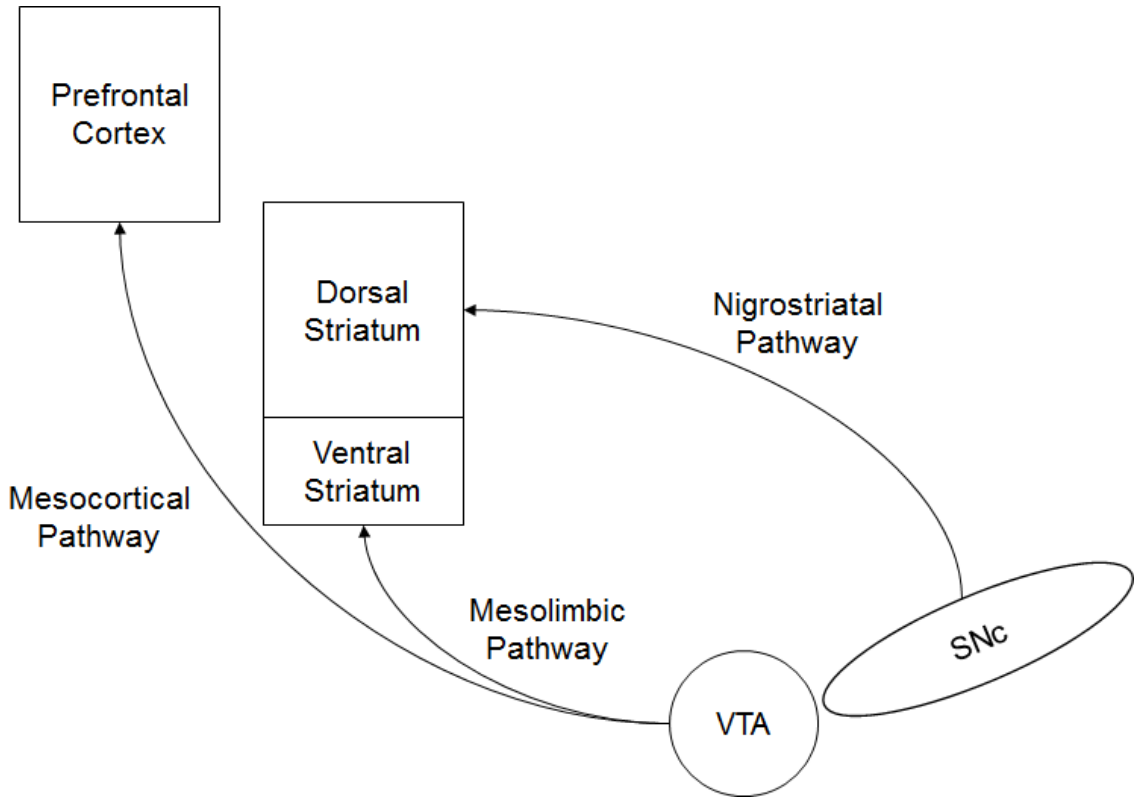

Figure 2.2: **Schematic representation of the midbrain dopaminergic pathways.** The mesocortical pathway consists of projections from the VTA to the prefrontal cortex. The mesolimbic pathway consists of projections from the VTA to the ventral striatum. The nigrostriatal pathway consists of projection from the substantia nigra pars compacts (SNc) to the dorsal striatum.

2.3.2 Physiology of the midbrain dopamine neurons

To appreciate the mechanism underlying the dopamine prediction error signal, it is first important to understand how inputs to the dopamine neurons modulate their firing rate. Dopamine neurons in the midbrain exhibit two different modes of firing. Usually, dopamine neurons fire in a single spiking mode at a low, irregular rate (Grace & Bunney, 1983; 1984b; Grace & Onn, 1989; Lacey *et al.*, 1989; Paladini & Tepper, 1999; Wilson & Callaway, 2000; Grillner & Mercuri, 2002; Hyland *et al.*, 2002). Input activity can sometimes excite these single spiking dopamine neurons into a burst firing mode, consisting of multiple consecutive spikes (Grace & Bunney, 1984a; Hyland *et al.*, 2002; Lodge & Grace, 2006).

The induction of burst firing depends on both the excitatory and inhibitory inputs to the dopamine neurons. Floresco et al., (2003) found that increasing excitatory input to the ventral tegmental area by activation of excitatory afferents or via glutamate agonists induced burst firing in only those dopamine neurons which were already firing in the single spike mode. In neurons that were inactive, increase in excitatory input had little or no effect, presumably due to GABA-mediated hyperpolarization (Lodge & Grace, 2006; Tepper & Lee, 2007). Conversely, decreasing the inhibitory input by decreasing the tonic GABAergic transmission to the ventral tegmental area resulted in an increase in the number of spontaneously active dopamine neurons. Decreasing the inhibitory input, however, did not induce burst firing without concurrent activation of the excitatory inputs to the dopamine neurons (Floresco *et al.*, 2003). Further, induction of burst firing using NMDA agonists was abolished by bath application of $GABA_A$ agonists (Paladini *et al.*, 1999; Tepper & Lee, 2007). This effect could not be reversed by depolarizing current injection, which counters the GABA induced hyperpolarization (Tepper & Lee, 2007), emphasizing the importance of direct inhibitory synaptic inputs to the dopamine neurons (Aggarwal *et al.*, 2012). These findings suggest that the induction of burst firing mode in the dopamine neurons requires a coordinated increase in excitatory input and a decrease in inhibitory input (Floresco *et al.*, 2003; Lodge & Grace, 2006; Tepper & Lee, 2007; Joshua *et al.*, 2009; Aggarwal *et al.*, 2012).

A large population of dopamine neurons enter a burst firing mode when unexpected rewards are encountered (Schultz, 1986; Schultz *et al.*, 1997; Hyland *et al.*, 2002; Joshua *et al.*, 2009). The induction of such synchronous burst firing in a population of dopamine neurons suggests that rewards induce an increase in excitatory input and decrease in inhibitory input to occur in a coordinated manner across a very large number of dopamine neurons.

2.3.3 The dopamine reward prediction error signal

Unexpected rewards, such as food presented to a hungry rat without warning, induce a dopamine response in the midbrain dopamine neurons that consists of a transient increase in the firing rate (Schultz *et al.*, 1997) and a transient increase in dopamine release in the striatum (Day *et al.*, 2007). When a cue is classically conditioned using a reward as the US, the cue gradually comes to evoke a dopamine response. At the same time the dopamine response to the reward, when preceded by the cue, gradually declines (Pan *et al.*, 2005; Day *et al.*, 2007) (Fig. 2.3). This may correspond to the development of an expectation of the reward. Thus, according to formal theory, dopamine signals the error in reward prediction (Montague *et al.*, 1996; Schultz *et al.*, 1997; Waelti *et al.*, 2001).

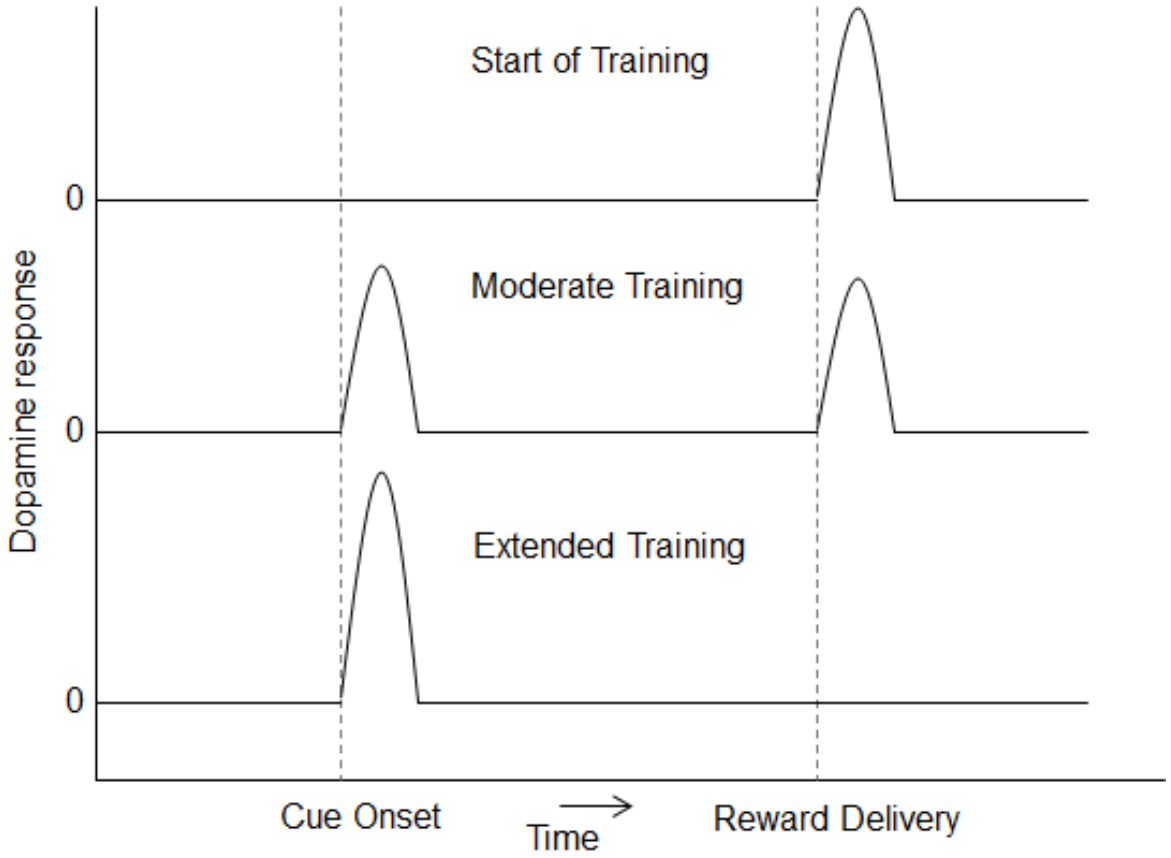

Figure 2.3: **Schematic representation of the dopamine reward prediction error signal**, showing the gradual development of a dopamine response to the cue being a conditioned and a gradual decline in the dopamine response evoked by the reward, as classical conditioning progresses.

Recent research suggests that this dopamine reward prediction error signal plays a key role in the expression of the Kamin blocking effect (Waelti *et al.*, 2001; Steinberg *et al.*, 2013; Sharpe *et al.*, 2017). Steinberg et al. (2013) showed that the reduction in the reward evoked dopamine response when the reward is expected (compared to when it is unexpected) is crucial to the Kamin blocking effect.

Steinberg et al. (2013) conditioned the first cue, A, using a liquid sucrose reward in two groups of animals (Fig 2.4). During the compound cue conditioning phase (Phase 2 in Fig 2.4), only group 1 received optogenetic activation of the ventral tegmental area dopamine neurons at the same time the sucrose reward was delivered (Sucrose + Stim), thereby artificially increasing their firing rate at the time of delivery of the expected reward. The final result of this manipulation was that the expected reward evoked a much higher dopamine response in group 1 (optogenetic activation group) than in group 2 (control group). Next, when the conditioned response to presentation of the second cue, B, alone was tested, group 1 responded more strongly to cue B than group 2. Thus, artificially increasing the dopamine response at the time of the expected reward during the compound cue conditioning phase attenuated the Kamin blocking effect.

It is important to note here that Steinberg et al. (2013) used optogenetic excitation of the ventral tegmental area dopamine neurons, which leads to dopamine release in excess of that evoked by unexpected natural rewards. This excess dopamine may interfere with the Kamin blocking effect in ways other than those restricted to the disruption of the dopamine reward prediction error signal To test the importance of the dopamine reward prediction error signal in Kamin blocking, there is a need to determine whether expected reward evoked dopamine release during the compound cue conditioning phase (phase 2), but not excess dopamine release, attenuates the Kamin blocking effect. This test requires manipulations of the brain

A

	Group 1 (optogenetic activation)	Group 2 (control)
Phase 1:	Cue A → Sucrose	Cue A → Sucrose
Phase 2:	Cue A + B → Sucrose + Stim	Cue A + B → Sucrose
Phase 3:	Cue B → No Reward	Cue B → No Reward

B

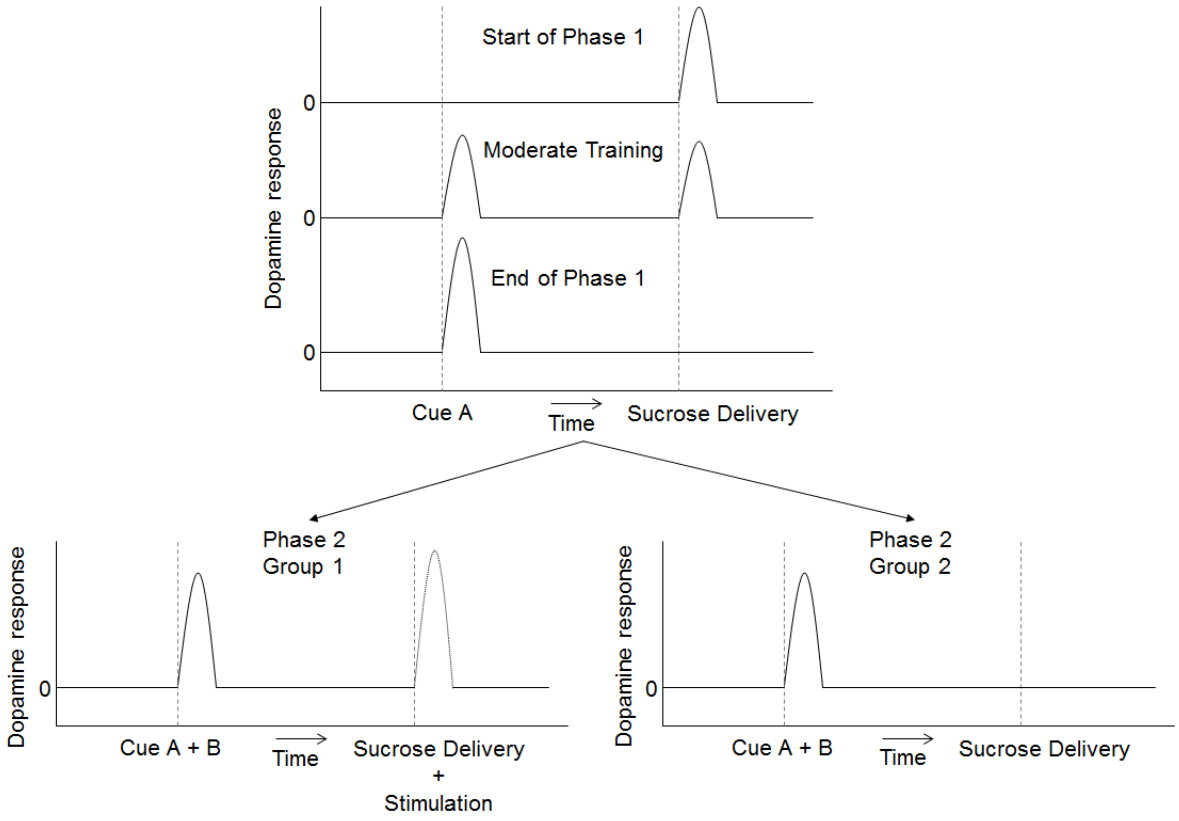

Figure 2.4: **The Kamin blocking procedure used by Steinberg et al., (2013).** (A) A schematic of the Kamin blocking procedure. The role in the Kamin blocking effect, of the reduction in the reward evoked dopamine response when the reward is expected, is exemplified in Phase 3 where cue B presentations evoke a stronger behavioral response in group 1 than in group 2. (B) A schematic representation of the expected difference in dopamine response during phase 2 between groups 1 and 2. In group 1, the presence of optogenetic stimulation induces a dopamine response at the time of sucrose delivery during phase 2. In group 2, sucrose delivery does not induce any dopamine response during phase 2.

that block the reduction in the dopamine reward prediction error signal evoked by rewards when they are expected.

Evidence reviewed in the following sections suggests neural substrates that may play a crucial role in the reduction of the dopamine response evoked by rewards when they are expected. This leads to the experimental part of this thesis, in which the effects of manipulating these neural substrates on the Kamin blocking effect are studied.

2.4 Reduction in the reward evoked dopamine response when the reward is expected

As explained in section 2.3, unexpected rewards induce a transient net excitation of the midbrain dopamine neurons, producing a transient increase in their firing rate. This dopamine response evoked by the reward is diminished when the reward is expected. The decline in the dopamine response elicited by the reward, when preceded by the cue, occurs gradually over the course of classical conditioning. After extended conditioning of the cue-reward association, the reward is accurately predicted by the conditioned cue, and the dopamine response evoked by reward delivery eventually vanishes (Schultz *et al.*, 1993; Schultz *et al.*, 1997). However, unexpected presentation of the reward without the preceding cue evokes a strong dopamine response similar to the one seen at the start of conditioning (Ljungberg *et al.*, 1991; Mirenowicz & Schultz, 1994). Thus, even though the dopamine response declines when the reward is expected, this decline does not remove the potential for the reward to activate the dopamine neurons when it is not preceded by the conditioned cue.

The forgoing findings suggest that the presentation of the conditioned cue initiates a neural process that prevents the following reward from inducing a net excitation of the dopamine neurons. The conditioned cue can reduce reward evoked increase in dopamine neuron firing rate by two mechanisms. Either, the cue could reduce the reward induced excitatory input

onto the dopamine neurons. Alternatively, the cue could increase the inhibitory input onto the dopamine neurons. These mechanisms are not mutually exclusive and may act together to counteract the excitatory drive provided by the reward.

Existing evidence supports the hypothesis that synaptic inhibition of the dopamine neurons occurs at the time of reward delivery. Owesson-White *et al.* (2008) used direct electrical stimulation of the ventral tegmental area (and therefore the dopamine neurons) as the reward in a classical conditioning paradigm. The intensity of this electrical stimulation reward is controlled by the experimenter and is held constant. Thus, the electrical stimulation reward always provides the same reward induced excitatory drive to the dopamine neurons on every reward delivery. Even under these conditions the predicted electrical stimulation reward evokes a diminished dopamine response. This suggests that an increase in direct inhibitory input onto the dopamine neurons at the time of the predicted reward counteracts the excitation produced by the electrical stimulation. However, there remains the possibility that the reduction in dopamine response evoked by the electrical stimulation reward, as the number of trials increased, occurred because of fatigue of a restricted releasable pool of dopamine (Nicolaysen *et al.*, 1988; Montague *et al.*, 2004; Owesson-White *et al.*, 2008), or some other reduction in the excitatory effect of the electrical stimulation.

Further supporting the hypothesis that the conditioned cue increases the synaptic inhibition onto the dopamine neurons to counteract the reward induced excitatory drive, Cohen et al. (2012) found that gamma aminobutyric acid (GABA) containing neurons in the ventral tegmental area undergo a sustained increase in their firing rate on presentation of the conditioned cue. They are not modulated by actual reward. Activation of these GABAergic interneurons inhibits ventral tegmental area dopamine neurons and reduces their firing rate (Tan *et al.*, 2012). Conversely, inactivation of GABAergic interneurons in the ventral tegmental area disinhibits the dopamine neurons (Bocklisch *et al.*, 2013) and increases the

phasic response of dopamine neurons to rewards (Eshel *et al.*, 2015). Tian et al, (2016) further showed that GABAergic neurons in the ventral tegmental area, the ventral pallidum, the rostromedial tegmental nucleus and the ventral striatum all show similar responses to reward expectation, and all provide inhibitory input to the ventral tegmental area dopamine neurons (Fig. 2.5). Thus, these four regions provide reward expectation modulated inhibitory input to the dopamine neurons, and most likely play a role in reducing the reward evoked dopamine response when rewards are expected.

Lastly, inhibitory inputs constitute 65-70% of all the synaptic inputs received by the dopamine neurons (Tepper & Lee, 2007). Thus, GABAergic control seems to dominate the input to the dopamine neurons and most likely plays a major role in controlling the firing rate of these neurons.

The evidence reviewed above suggests that increase in the inhibitory input to the dopamine neurons plays a role in reducing the reward evoked dopamine response when rewards are expected. However, the foregoing argument does not preclude the possible role of reduction in the reward induced excitatory input to the dopamine neurons, and both mechanisms may act together to counteract the excitatory drive provided by the reward.

This section reviewed evidence that suggests that synaptic inhibition at the dopamine neuron plays a role in the reduction in dopamine response to rewards when they are expected. Furthermore, firing the ventral tegmental area dopamine neurons during the delivery of the expected reward during compound cue conditioning abolished the Kamin blocking effect (Steinberg *et al.*, 2013). These considerations led to the hypothesis that inhibitory inputs onto the ventral tegmental area neurons during compound cue conditioning are necessary for the expression of the Kamin blocking effect (hypothesis 1). Chapter 3 reports a test of this hypothesis.

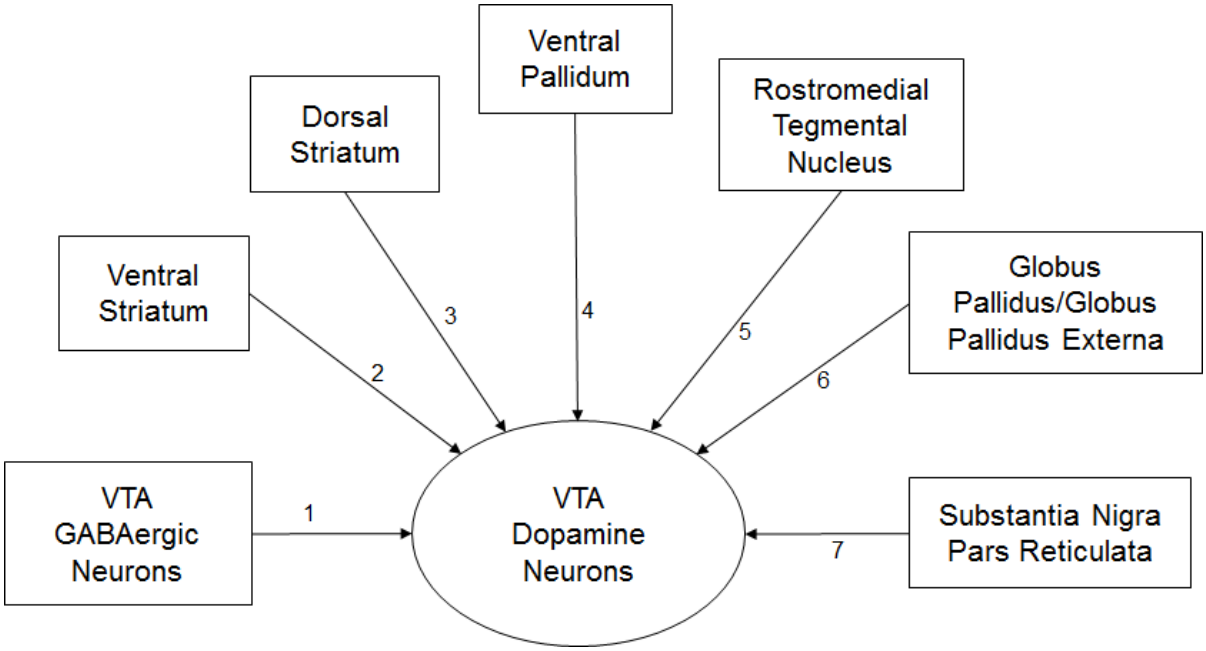

Figure 2.5: **Major sources of inhibitory afferents to the VTA dopamine neurons.** The supporting evidence for each numbered inhibitory input is cited as follows: 1. Tan et al. (2012), Bocklisch et al. (2013), Eshel et al. (2015), Tian et al. (2016), Edwards et al. (2017); 2. Heimer et al. (1991), Groenewegen et al. (1999), Geisler and Zahm (2005), Watabe-Uchida et al. (2012), Tian et al. (2016); 3. Watabe-Uchida et al. (2012), Tian et al. (2016); 4. Groenewegen et al. (1993), Geisler and Zahm (2005), Watabe-Uchida et al. (2012), Hjelmstad et al. (2013), Tian et al. (2016); 5. Jhou et al. (2009), Kaufling et al. (2010), Barrot et al. (2012), Tian et al. (2016); 6. DeVito and Anderson (1982), Watabe-Uchida et al. (2012); 7. Deniau et al. (1982), Geisler and Zahm (2005), Watabe-Uchida et al. (2012)

2.5 Bridging the delay between the cue and the reward

In the previous section, it was argued that the process that results in reduction of the dopamine response evoked by an expected reward is a cue initiated process. The conditioned cue and the following reward are usually separated by several seconds. Thus, the process triggered by the conditioned cue needs to remain active during the delay between the cue onset and reward delivery. Adding complexity to the requirements of this cue triggered process is the finding that presenting a conditioned cue but omitting the following expected

reward results in a decrease in the firing rate of the dopamine neurons to below the baseline firing rate. This depression in dopamine neuron activity occurs exactly at the time at which the reward is expected to occur (Ljungberg *et al.*, 1991; Schultz *et al.*, 1993; Schultz *et al.*, 1997; Hollerman & Schultz, 1998; Waelti *et al.*, 2001; Pan *et al.*, 2008; Cohen *et al.*, 2012) and results in a corresponding dip in the extracellular dopamine concentration in the striatum (Hart *et al.*, 2014). This finding adds a very specific timing requirement to the cue initiated process such that its effect on the modulation of dopaminergic activity is specific to the expected time of occurrence of the reward.

In section 2.4, it was argued that a process initiated by the presentation of a conditioned cue directly inhibits the dopamine neurons, and plays a role in the reduction of dopamine response evoked by the reward when the reward is expected. In section 2.5 it was argued that the effect of the cue initiated process on the modulation of dopaminergic activity is specific to the expected time of reward delivery. Taken together, these arguments suggest that the net increase in inhibitory input onto the dopamine neurons (which reduces the reward evoked dopamine response when the reward becomes expected) is timed to occur exactly at the time at which the reward is expected. This process requires a timing mechanism to bridge the delay between the cue and the expected reward, and this hypothetical timing mechanism is triggered by the presentation of the conditioned cue.

The rest of this section is concerned with two related questions. First, what are the neural substrates underlying the timing mechanism that bridges the delay between the conditioned cue and the expected reward? Second, how do the neural substrates underlying the timing mechanism control the firing of the dopamine neurons?

The striatal neural network has the capacity to generate sequential neural activity unique to an input (Carrillo-Reid *et al.*, 2008; Ponzi & Wickens, 2010), suggesting that it can act as a

timer (Meck *et al.*, 2008) for predicting reward after the cue input (Aggarwal *et al.*, 2012). Many alternative brain regions are also capable of such timing processes (Grillner *et al.*, 2005; Itskov *et al.*, 2011). However, it has been suggested that the striatal contribution to the modulation of these timing process plays an important role in reward related learning (Matell *et al.*, 2003; Matell & Meck, 2004). The striatum also provides direct and indirect inhibitory input to the midbrain dopamine neurons and has been proposed to regulate the firing of dopamine neurons (Gerfen *et al.*, 1987; Brown *et al.*, 1999; Lisman & Grace, 2005; Grace *et al.*, 2007; Hong & Hikosaka, 2008; Bromberg-Martin *et al.*, 2010a; Bromberg-Martin *et al.*, 2010b). Thus, the striatum is a strong candidate neural substrate for the timing mechanism that bridges the delay between the conditioned cue and the expected reward and for coordinating the timed inhibitory input to the dopamine neurons.

The striatum has previously been hypothesized to provide the timed inhibitory input to the dopamine neurons (Brown *et al.*, 1999; Aggarwal *et al.*, 2012), although the numerous sources of input to the dopamine neurons (Watabe-Uchida *et al.*, 2012; Tian *et al.*, 2016) and their effects on dopamine release in the striatum (Floresco *et al.*, 2003; Grace *et al.*, 2007; Stopper *et al.*, 2014) leaves room for many other possibilities. It is also possible that multiple regions act in concert to time the cue triggered process and provide the necessary modulation of dopamine neuron activity to reduce the dopamine response evoked by the reward when it is expected. However, the modulation of striatal neuronal activity by reward expectation (Tian *et al.*, 2016), and the extensive control of the striatal output over the inhibitory input to the dopamine neurons (see section 2.7), suggests that the striatum plays a role in the cue triggered delayed inhibition of the dopamine neurons at the expected time of reward delivery.

In section 2.3.3, it was argued that the reduction in the dopamine response evoked by the reward when it is expected is crucial for the expression of the Kamin blocking effect. In section 2.5, it was further proposed that the striatum is likely to play a role in modulating

dopaminergic activity at the expected time of reward delivery, given its timing capabilities, as well as its direct and indirect inhibitory inputs to the dopamine neurons. The experiments reported in chapter 3 will therefore investigate whether the striatum plays a role in the expression of the Kamin blocking effect. In particular, the experiments will test whether inactivating the striatum, thus disrupting the output from this region, during the compound cue conditioning phase disrupts the Kamin blocking effect (hypothesis 2). For reasons discussed in section 2.7, the experiments will focus on the role of the nucleus accumbens region of the striatum.

2.6 Neuronal learning of the timed inhibition of the dopamine neurons

Section 2.5 discussed the suitability of the striatal neural network to act as the timer for predicting the reward and timing the postulated net inhibition of the dopamine neurons such that it occurs at the expected time of the reward delivery. The resultant reduction in the dopamine response evoked by the reward, when preceded by the cue being conditioned, occurs gradually as classical conditioning progresses. The gradual nature of this reduction suggests a reciprocal gradual increase in the inhibition of the dopamine neurons at the time of reward delivery as learning progresses over the course of classical conditioning. Section 2.6 considers the role of neuronal learning in the striatal neural network in gradually increasing the strength of the timed inhibition of the dopamine neurons as classical conditioning progresses.

Synaptic plasticity is an influential model for the neural basis of learning. Synaptic plasticity in the cortical (Calabresi *et al.*, 2000; Reynolds & Wickens, 2000; Kerr & Wickens, 2001; Reynolds *et al.*, 2001), hippocampal (Floresco *et al.*, 2001b) and amygdala (Floresco *et al.*, 2001a; b) inputs to the striatum is modulated, both *in vitro* and *in vivo*, by dopamine

transients, such as those produced by rewards. In particular, activity in the afferents to the striatal medium spiny neurons (the output neurons of the striatum) followed by activation of the striatal neurons without a concurrent transient increase in dopamine efflux in the striatum resulted in long-term depression of the synaptic inputs (Reynolds & Wickens, 2000). The presence of a concurrent dopamine efflux or the application of a D1 receptor agonist resulted in long-term potentiation of these synapses (Reynolds & Wickens, 2000; Kerr & Wickens, 2001; Reynolds *et al.*, 2001). This synaptic potentiation was blocked by D1 receptor antagonists (Kerr & Wickens, 2001).

Reynolds et al., (2001) demonstrated dopamine D1 receptor mediated strengthening of cortico-striatal synapses using electrical stimulation of dopamine neurons. This stimulation was shown to act as a reward in a behavioral paradigm. In these experiments, pressing of a lever triggered electrical stimulation of the dopamine neurons, and animals gradually learnt to press the lever to obtain dopamine neuron stimulation. Reynolds et al. (2001) found that the magnitude of dopamine dependent strengthening of cortico-striatal synapses was negatively correlated with the time taken to learn the lever pressing behavior. They thus postulated that dopamine dependent strengthening of the cortico-striatal synapses may be a neural mechanism for learning of rewarded behavioral responses. Further, many studies have demonstrated the necessity of striatal dopamine in the expression of classical conditioning (Parkinson *et al.*, 2002; Parker *et al.*, 2010; Parker *et al.*, 2011; Darvas *et al.*, 2014). Taken together, this evidence suggests that dopamine mediated synaptic plasticity occurs in the striatum during reward-related learning and may serve as a neural substrate for reward mediated learning.

Given the importance of dopamine mediated learning in the striatum, Aggarwal et al., (2012) and Brown et al., (1999) hypothesized that strengthening of the cue evoked activity in the

striatum by the reward evoked dopamine release may enable learning of the timed inhibition of the dopamine neurons during classical conditioning.

Therefore, it is important to investigate the role of learning in the striatum in the expression of the Kamin blocking effect. The experiments reported in chapter 3 investigate whether inactivating the striatum during the single cue conditioning phase disrupts the Kamin blocking effect (hypothesis 3). For reasons discussed in section 2.7, the experiments focus on the role of the nucleus accumbens region of the striatum.

2.7 The nucleus accumbens subdivision of the striatum – behavioral and anatomical considerations

Sections 2.4-2.6 discussed the role of a gradually learnt net inhibition of dopamine neurons, occurring exactly at the expected time of reward delivery, in the reduction of the dopamine response evoked by reward when it is expected. Section 2.7 reviews behavioral and anatomical findings that make the nucleus accumbens a likely substrate for the learning and mediation of this timed inhibition.

Darvas et al., (2014) found that mice with only 5% of normal dopamine in the striatum were severely impaired in the acquisition of a conditioned response during classical conditioning. This impairment was ameliorated by pharmacological restoration of dopamine synthesis with L-dopa. They further found that virus-mediated restoration of dopamine synthesis in the ventral striatum, and not in the dorsal striatum, restored the ability of acquire a conditioned response in completely dopamine-deficient mice. These findings suggest that the action of dopamine in the ventral striatum is necessary for the learning of a conditioned response during classical conditioning. Similar impairments in the acquisition as well as performance of conditioned behavior were observed when the dopaminergic afferents to the nucleus

accumbens were lesioned (Parkinson *et al.*, 2002) or dopamine receptors in the nucleus accumbens were blocked (Di Ciano *et al.*, 2001). Taken together, these findings suggest that dopamine mediated learning in the nucleus accumbens is important for classical conditioning.

From the anatomical point of view, the ventral tegmental area dopamine neurons, whose activation at the time of the expected reward abolishes the Kamin blocking effect (Steinberg *et al.*, 2013), predominantly project to the ventral striatum, and are the major source of dopaminergic input to the ventral striatum (Brog *et al.*, 1993; Groenewegen *et al.*, 1999). Conversely, the nucleus accumbens region of the ventral striatum sends inhibitory projections to the dopamine neurons in the ventral tegmental area (Groenewegen *et al.*, 1999; Watabe-Uchida *et al.*, 2012). The nucleus accumbens also projects to other areas that in turn project to the dopamine neurons in the ventral tegmental area (Aggarwal *et al.*, 2012), such as the ventral pallidum (Heimer *et al.*, 1991; Watabe-Uchida *et al.*, 2012), the GABAergic interneurons in the ventral tegmental area (Xia *et al.*, 2011; Edwards *et al.*, 2017), and the lateral hypothalamus (Groenewegen *et al.*, 1999; Watabe-Uchida *et al.*, 2012). Thus, the output of the nucleus accumbens is capable of directly and indirectly modulating the firing of the dopamine neurons in the ventral tegmental area (Fig. 2.6).

Two points in the foregoing argument are, first, that dopamine mediated learning in the nucleus accumbens is important in the context of classical conditioning. Second, the accumbens output has the capability to modulate the firing of the dopamine neurons in the ventral tegmental area. Taken together, these two findings make it likely that dopamine mediated plasticity in the nucleus accumbens plays a role in the learning of the timed net inhibition of the dopamine neurons at the expected time of reward delivery. This timed net inhibition, as discussed in earlier sections, is postulated to be important for the reduction in dopamine response to the reward when the reward is expected.

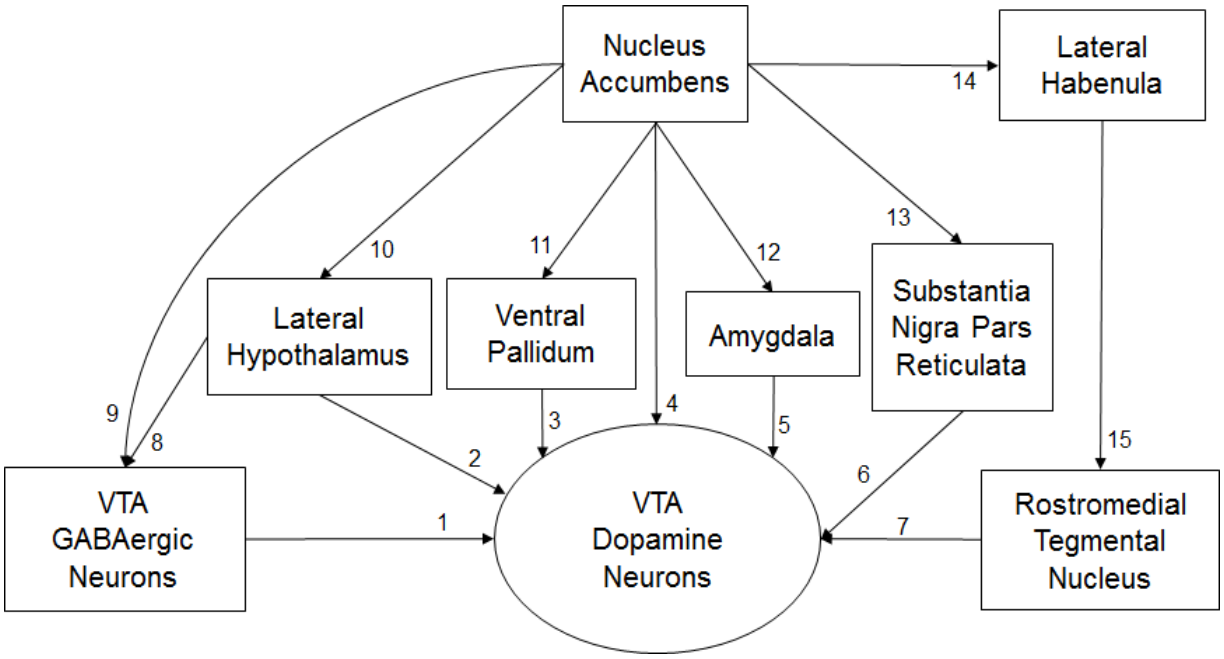

Figure 2.6: **Nucleus accumbens is capable of directly and indirectly modulating the firing of VTA dopamine neurons.** Schematic representation of some of the neural pathways through which the nucleus accumbens can influence the activity of VTA dopamine neurons. The supporting evidence for each numbered connection is cited as follows: 1. Tan et al. (2012), Bocklisch et al. (2013), Eshel et al. (2015), Tian et al. (2016), Edwards et al. (2017); 2. Watabe-Uchida et al. (2012), Tian et al. (2016); 3. Groenewegen et al. (1993), Geisler and Zahm (2005), Watabe-Uchida et al. (2012), Hjelmstad et al. (2013), Tian et al. (2016); 4. Watabe-Uchida et al. (2012), Tian et al. (2016); 5. Geisler and Zahm (2005), Zahm et al. (2011), Watabe-Uchida et al. (2012); 6. Deniau et al. (1982), Geisler and Zahm (2005), Watabe-Uchida et al. (2012); 7. Jhou et al. (2009), Goncalves et al. (2012), Barrot et al. (2012); 8. Nieh et al. (2016), Tyree et al. (2017); 9. Xia et al. (2011); 10. Mogenson et al. (1983), Kirouac and Ganguly (1995); 11. Heimer et al. (1991), Usuda et al. (1998); 12. Ito et al. (1974), Russchen et al. (1985), Salgado and Kaplitt (2015); 13. Heimer et al. (1991), Groenewegen et al. (1999); 14. Nauta et al. (1978), Salgado and Kaplitt (2015); 15. Jhou et al. (2009), Goncalves et al. (2012), Barrot et al. (2012).

2.8 Summary of the hypotheses

The foregoing review developed the hypothesis that inhibitory control of the dopamine neurons in the ventral tegmental area at the time of the expected reward plays a role in the

reduction in dopamine response to the reward when it is expected, and is therefore necessary for the expression of the Kamin blocking effect. The review further identified the nucleus accumbens output as a likely orchestrator of the timed inhibitory control of the dopamine neurons. This inhibitory control needs to be acquired during the process of classical conditioning. Dopamine mediated synaptic plasticity in the nucleus accumbens was identified as a strong candidate as the underlying process. Therefore, the aim of the thesis research is to answer three specific questions (as stated in section 1.3):

i) Does blocking inhibition in the ventral tegmental area during the compound cue conditioning phase of the Kamin blocking paradigm disrupt the Kamin blocking effect?

ii) During compound cue conditioning, does inactivating the nucleus accumbens, thus disrupting the output from this region during this phase, disrupt the Kamin blocking effect?

iii) During single cue conditioning, does inactivating the nucleus accumbens, thus disrupting learning in this region during this phase, disrupt the Kamin blocking effect?

These questions are addressed in the experiments reported in chapter 3.

2. The Role of Ventral Tegmental Area Inhibitory Inputs and the Nucleus Accumbens in the Kamin Blocking Effect

3.1 Introduction

As outlined in the previous two chapters, this thesis is concerned with answering three specific questions:

i) Does blocking inhibition in the ventral tegmental area during the compound cue conditioning phase (phase 2) of the Kamin blocking paradigm (Fig. 2.1) disrupt the Kamin blocking effect?

ii) Does inactivating the nucleus accumbens, thus disrupting the output from this region, during the compound cue conditioning phase disrupt the Kamin blocking effect?

iii) Does inactivating the nucleus accumbens during the single sue conditioning phase (phase 1), thus disrupting learning in this region, disrupt the Kamin blocking effect?

Towards answering the first question, bicuculline, a $GABA_A$ receptor antagonist, was injected bilaterally into the ventral tegmental area before each compound cue conditioning session. This blocks $GABA_A$ receptor mediated inhibition on all neurons in the ventral tegmental area, including the dopamine neurons.

Towards answering the second and third questions, a designer receptor (see section 3.2.8 for details) was expressed, bilaterally, on the neurons in the nucleus accumbens. The activation of this designer receptor hyperpolarizes the neuron expressing the receptor, thus attenuating

neural activity. This receptor, once expressed in the nucleus accumbens, was activated during either phase 2 (question ii) or phase 1 (question iii) of the Kamin blocking paradigm.

The use of a designer receptor over direct microinjections of chemicals that inhibit neuronal firing has many advantages. First, the designer receptor can be activated by systemic injections of the activating drug and does not require the chronic implants necessary for microinjections into the brain. Second, systemic i.p. injections are less stressful for the animals as compared to microinjections. Third, the spread in the expression of the designer receptor, and thus the volume of the brain affected, can be verified during histology. In the case of microinjections using guide cannulae, it is generally not possible to verify the volume of spread of the microinjected drug - only the final placement of the guide cannula can be verified during histology.

3.2 Methods

All experimental procedures involving animals were approved by the Committee for Care and Use of Animals at Okinawa Institute of Science and Technology.

3.2.1 Subjects

Male Long-Evans rats (Charles River Laboratories) aged 7-8 weeks on arrival were provided food and water ad libitum and placed on a 12h light/dark schedule until 5 days before behavioral experiments. They were then food restricted to approximately 85% of their average body weight before the start of habituation, and maintained at this weight throughout the behavioral experiments.

3.2.2 Surgical Procedures

Rats were aged between 9-11 weeks at the time of surgery. Rats used in experiment 1 received bilateral chronic implants of guide cannulae (0.46 mm O.D., Plastics One) targeted 1mm above the VTA (DV 7.0, AP 5.4-6.0, ML 1.0). These coordinates targeted the posteromedial and posterolateral VTA because they have a high density of dopamine neurons and project to the nucleus accumbens (Ikemoto, 2007; Breton *et al.*, 2019). Further Rats used in experiment 2 and 3 received bilateral microinjections of AAV2-hSyn-hM4Di(Gi)-mCherry (Addgene) or saline into the NAc (DV 7.0, AP 2.0, ML 1.0). Such a medial placement in the NAc was selected because posteromedial and posterolateral VTA dopaminergic neurons project to the medial NAc (Ikemoto, 2007; Breton *et al.*, 2019) and the medial NAc exerts direct inhibitory control over VTA dopaminergic neurons (Yang *et al.*, 2018).

3.2.3 Behavioral Apparatus

Operant chambers equipped with two levers, a food magazine, a house light, a pure tone generator (4.5 KHz) and a white noise generator (Med Associates) were used for all behavioral experiments. The two levers and the food magazine were located on the same wall of the operant chamber, with one lever on either side of the food magazine. The two sound generators were placed on the wall opposite to the food magazine.

3.2.4 Free exploration of the operant chamber

To familiarize rats to the experimental chambers, rats were allowed to freely explore the behavioral chambers after recover from surgery. This recovery period was 10 days in experiment 1. In experiments 2 and 3, a minimum of 21 days were allowed for recovery to allow for robust viral expression. Rats were familiarized with experimental chamber by placing them in the chamber for 30 minutes on two consecutive days. Prior reports suggest

that such exposure is sufficient for familiarization to the experimental chamber (Bronstein *et al.*, 1974).

3.2.5 Magazine Training

After free exploration, all rats had three sessions of magazine training over three days. Each session consisted of 25 deliveries of food reward (one sucrose pellet, 45mg) into the food magazine. The food deliveries were separated by a 60-80s variable interval (uniform random distribution).

3.2.6 The Kamin Blocking Procedure

Rats that collected all the 25 food pellets on the last day of magazine training progressed to the Kamin blocking procedure (Fig. 3.1), which consisted of three phases – single cue conditioning phase, followed by compound cue conditioning phase, and finally an extinction test.

Phase 1 consisted of 12 single cue conditioning sessions over 12 days. Cues consisted of extension of either the left of right lever for 5 seconds. One lever (L1, Fig. 3.1) was paired with food reward which was delivered to the food tray immediately after lever retraction. The other lever (L2, Fig. 3.1) was not rewarded. Each session consisted of 50 presentations of each lever cue. The inter-cue

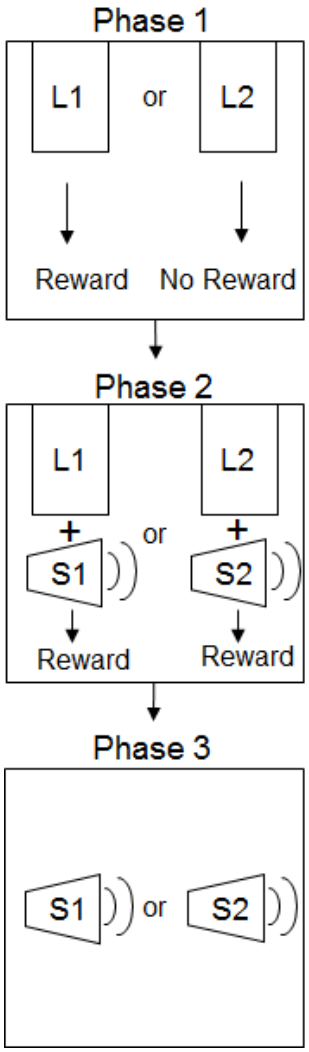

Figure 3.1: **A schematic of the Kamin blocking experimental design.** Phase 1 is a single cue conditioning phase, in which only one of the cues (L1 or L2) is followed by reward. Phase 2 is a compound cue conditioning phase, in which compound cues (L1+S1 or L2+S2) are presented, and both compound cues are followed by reward. Phase 3 is an extinction test in which only the cues added in phase 2 (S1 and S2) are presented and no rewards are given.

interval ranged from 15-75s. Left and right levers were counterbalanced. The percentage of trials with a lever press response to the lever cues was used as the measure of conditioned responding (Davey *et al.*, 1981; Day *et al.*, 2007).

Phase 2 of the procedure consisted of 6 compound cue conditioning sessions over 6 days. In each session two compound cues were presented 25 times each. The presentation of each compound cue was separated by a 35-75s variable interval. One of the compound cues consisted of one of the lever cues used in phase 1 plus a pure tone (4.5 KHz, 5s). The other compound cue consisted of the other lever cue plus a white noise (5s). Food reward immediately followed the termination of each compound cue and was the same as that used in phase 1.

Phase 3 of the Kamin blocking procedure consisted of a single extinction session in which the development of a conditioned response (due to learning in phase 2) to separated presentations of the two sound cues was tested. This session consisted of 25 presentations of each of the 5s sound cues. The presentation of each sound cue was separated by a 35-75s variable interval. No lever cues were presented and no rewards were delivered during this session. The total duration of nose poking into the food magazine during the 5s presentation of the sound cues was used as the measure for conditioned responding. Nose poke duration during the 5s period immediately preceding each cue was used as the baseline response for that cue.

Note that the intervals between cue presentations were different in phase 1 than in phases 2 and 3 because the inter-cue intervals were adjusted across phases to keep the session duration the same across phases.

3.2.7 Allocating animals to experimental groups

Each experiment involved a control group and an experimental group. In experiments 1 and 2, the experimental manipulation occurred in phase 2. Therefore, rats were divided into control and experimental groups by matching performance on the lever press measure in the two groups. This was done by separating rats at the end of phase 1 into two groups – ones that showed a robust lever press conditioned response to the paired lever, and ones that did not show a lever pressing conditioned response to the paired lever. Then, equal numbers of rats from each of these two groups were randomly assigned to the control and experimental groups. Therefore, both the control and experimental groups contained an equal number of rats that showed the lever press conditioned response and an equal number of rats that did not show the lever press conditioned response. In experiment two, this control group was further divided into two groups (as two groups of controls were needed), putting half the animals that lever pressed in one of the control groups, and the other half in the other control group. In experiment 3, the experimental manipulation took place in phase 1. Therefore, no performance matching could be done and rats were randomly assigned to the experimental group or one of the two control groups after the magazine training sessions ended.

3.2.8 Experimental manipulations

3.2.8.1 Bicuculline microinjections into the ventral tegmental area - Experiment 1

Bicuculline was used to block the inhibitory inputs to the VTA neurons. Bicuculline solution was prepared by dissolving bicuculline methiodide (Tocris) in saline. Either 200nl of 1mM bicuculline solution or 200nl of saline solution was bilaterally injected into the VTA 10 minutes before the start of each of the six compound cue conditioning sessions (Celada *et al.*, 1999). For the purpose of microinjections, an injection cannula protruding 1mm further than the guide cannula was inserted into the guide cannula and attached to a 10µl syringe

(Hamilton). The syringe was placed in a KDS 101 syringe pump (KDS Scientific) and the solution was infused into the VTA over 2.5 min. The injection cannula was left inside the brain for 2 minutes after infusion to allow diffusion to occur and was then manually retracted.

3.2.8.2 Viral microinjections into the nucleus accumbens during surgery – experiments 2 and 3

To express the hM4Di receptor on accumbens neurons in experiments 2 and 3, AAV2-hSyn-hM4Di(G_i)-mCherry (Addgene) was injected bilaterally into the NAc, 300nl per hemisphere. Activation of the hM4Di receptor inhibits neural activity via two mechanisms. One is via the activation of G-protein inward rectifying potassium (GIRK) channels, hyperpolarizing the neuron and attenuating neuronal activity (Armbruster *et al.*, 2007; Zhu & Roth, 2014; Vardy *et al.*, 2015). Another is via inhibition of axonal release of neurotransmitters (Stachniak *et al.*, 2014; Vardy *et al.*, 2015; Roth, 2016). The hM4Di receptor was activated by subthreshold doses of clozapine (Hand *et al.*, 1987; Melis *et al.*, 1999; Gomez *et al.*, 2017).

The hM4Di designer receptor was initially designed to be activated by the inert ligand clozapine-N-oxide (CNO) (Armbruster *et al.*, 2007). However, recently it has been shown that CNO gets reverse metabolized into clozapine (Gomez *et al.*, 2017; Manvich *et al.*, 2018), which is a psychoactive drug (Hand *et al.*, 1987; Melis *et al.*, 1999; Schwieler & Erhardt, 2003). CNO doses necessary to activate the hM4Di designer receptor result in cerebrospinal fluid (CSF) clozapine concentrations high enough to activate several of its natural target receptors, as well as the hM4Di designer receptors (Raper *et al.*, 2017). Gomez et al., (2017) also found that extremely low doses of clozapine, at which clozapine does not activate its natural target receptors in the brain (Hand *et al.*, 1987; Melis *et al.*, 1999), are sufficient to activate the hM4Di designer receptor. Given the activation of the h4MDi receptor by converted clozapine when CNO is injected systemically, the high concentrations of clozapine

in the CSF after CNO injections, and the subthreshold nature of the clozapine doses necessary to activate the hM4Di receptor, Gomez et al., (2017) concluded that DREADDs should be activated using subthreshold doses of clozapine rather than by CNO. Thus, in experiments 2 and 3, subthreshold doses of clozapine were used to activate the hM4Di designer receptors.

3.2.8.3 Clozapine intraperitoneal injections - Experiments 2 and 3

Either clozapine solution (0.1mg/kg) or 0.45ml vehicle was injected intraperitoneally 25-35mins before the start of each conditioning session during experiments 2 and 3 as indicated in figures 3.6A and 3.8A. For preparing the clozapine solution, clozapine (Sigma-Aldrich) was dissolved in DMSO (Tocris) and then diluted in saline to form a 1mg/ml clozapine solution (0.5%DMSO). The vehicle control solution was 0.5% DMSO in saline.

3.2.9 Histology

Histological protocols were as previously described (Aoki *et al.*, 2015; Liu *et al.*, 2017; Aoki *et al.*, 2018). Brains were removed for histological verification of injection sites and viral expression after induction of anesthesia (sodium pentobarbital or isoflurane) and perfusion with 4% paraformaldehyde (PFA, 100 mM in sodium phosphate buffer, pH 7.4). The extracted brains were post-fixed in PFA at 4^{o}C for at least 3 days. Subsequently, the brains were gelatin embedded and then cut in 80µm coronal sections for verification of injection sites in the VTA. Coronal sections of 60µm thickness were cut for the verification of viral expression in the NAc. Sections were cut using a vibratome (VT1000S, Leica).

The injection sites in the VTA were determined by immuno-histochemical staining for tyrosine hydroxylase (TH). The extent of viral expression was determined by immuno-histochemical staining for mCherry. A metal enhanced 3,3’-dia-minobenzidine-tetrahydrochloride method (DAB) was used to visualize all immuno-histochemical staining.

All sections were first treated with 3% H_2O_2 (Wako) for 30 min and then blocked with 5% goat serum and 0.2% Triton X-100 (Bio-Rad) in PBS for one hour. To visualize injection sites in the VTA, the 80µm sections were then incubated in an anti-TH primary antibody (1:1000, rabbit anti-TH, Enzo Life Sciences). To visualize the expression of hM4Di on the neurons in the NAc, the 60µm sections were incubated in an anti-mCherry primary antibody (1:500, rabbit anti-mCherry, Abcam). Primary antibody incubation was carried out in 2% goat serum and 0.2% Triton-X 100 in PBS for 48h at 4ºC. This was followed by incubation in a biotinylated secondary antibody (1:500, Biotin conjugated Goat anti-rabbit IgG, Thermo Fisher). Secondary antibody incubation was carried out in 2% goat serum and 0.2% Triton-X 100 in PBS for 3h at 25ºC. Then all sections were incubated with streptavidin-conjugated horseradish peroxidase (Standard ABC Peroxidase Kit, Vector Laboratories) in 0.2% Tween-20 and PBS for one hour at 25°C and then visualized using DAB (Thermo Fisher). DAB-stained sections were dry mounted on frosted glass slides (Matsunami Glass) and then counterstained with 0.02% thionin (Alfa Aesar) for 2 minutes, run through an ethanol dehydration series and cover-slipped with Entellan mounting medium (Merck).

3.2.10 Inclusion criteria

Two inclusion criteria were applied. One was behavioral and the other was anatomical. The behavioral inclusion criteria were applied first. The anatomical inclusion criterion was applied on those rats that passed the behavioral inclusion criteria.

Behavioral inclusion criteria: Only those rats that developed a conditioned response to the paired lever during phase 1 were included for data analysis. Further, only those rats that did not develop a conditioned response to the unpaired lever were included. Three quantitative criteria were set to assess the development of the conditioned response to the levers. For inclusion on the basis of behavior during phase 1, the rat had to pass any one of the three

criteria for its response to the paired lever, and fail all three criteria for its response to the unpaired lever. The percentage of trials with a lever press response was used as the measure for responding to the levers. The following are the three quantitative criteria:

1.1) Above 40% response on the paired lever on one of the last three sessions.

1.2) Above 30% response on the paired lever on two of the last six sessions

1.3) Above 20% response on the paired lever on three of the last six sessions

Anatomical inclusion criterion: The rats that passed the behavioral inclusion criteria then had to pass the anatomical inclusion criterion. For experiment 1, the injection sites in both the hemispheres had to be in the dopamine neuron containing region of the ventral tegmental area for inclusion. Injection sites in the ventral tegmental area were verified using anti-tyrosine hydroxylase immunostaining. For experiments 2 and 3, hM4Di expression had to be restricted to the extent of the nucleus accumbens for inclusion. Expression of hM4Di was verified by immunostaining for mCherry.

In experiment 1, a total of 41 rats were used. Out of the 41, only 20 rats met the inclusion criteria. Two rats died before completing phase 2 and the brain of one rat was not perfused properly due to technical problems during perfusion. One of the rats took 2-3 hours to complete compound cue conditioning sessions on 3 out of 6 occasions. These four rats were excluded from data analysis. Ten rats did not meet the behavioral inclusion criteria and were thus excluded from data analysis (Fig. 3.2A, B). Seven rats did not meet the anatomical inclusion criteria for experiment 1 and were thus excluded from data analysis.

In experiment 2, a total of 44 rats were used. Out of the 44, only 19 rats met the inclusion criteria. Eighteen rats did not meet the behavioral inclusion criteria and were thus excluded from data analysis (Fig. 3.2C, D). Seven rats did not meet the anatomical inclusion criteria for experiment 2 and were thus excluded from data analysis.

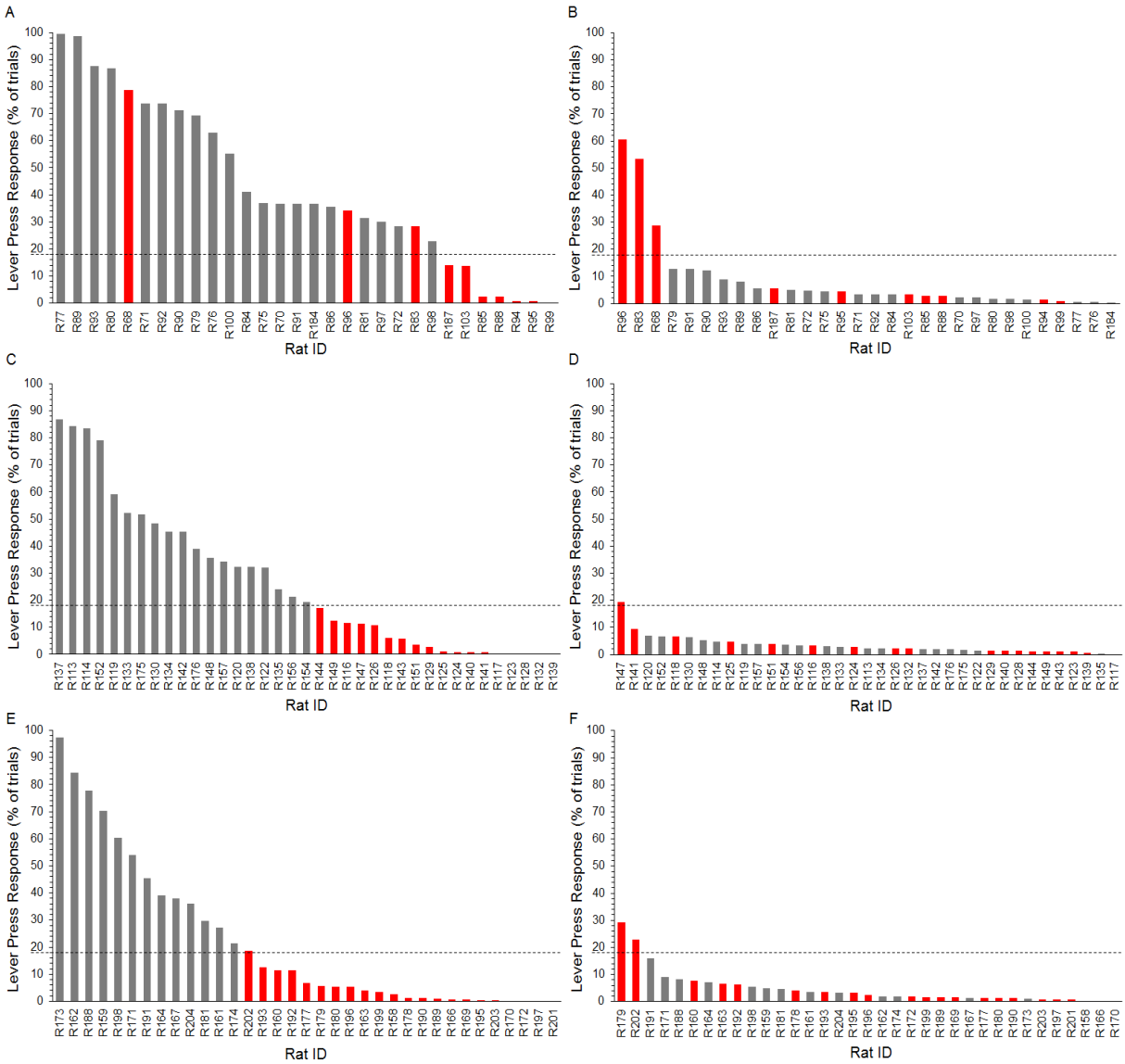

Figure 3.2: **Lever press responses averaged across sessions 7-12.** Animals that were included in the data analysis for experiments 1 (A, B), 2 (C, D) and 3 (E, F) (grey bars) and animals that were excluded on the basis of behavior (red bars) are shown. Rats common to experiments 2 and 3 are shown only in the experiment 2 data (C, D). Lever press responses to L1 (A, C, E) were above the dotted line and lever press responses to L2 (B, D, F) were below the dotted line in all animals that passed the behavioral inclusion criteria.

In experiment 3, a total of 54 rats were used. Out of the 54, only 18 rats met the inclusion criteria. One rat died before completing phase 2 and the brain of one rat was not perfused

properly due to problems during perfusion. These two rats were excluded from the data analysis. Another 28 rats (19 from the control groups and 9 from the experimental group) did not meet the behavioral inclusion criteria and were thus excluded from data analysis (Fig. 3.2E, F). Six rats did not meet the anatomical inclusion criteria for experiment 3 and were thus excluded from data analysis.

Note: Experiments 2 and 3 were run concurrently, and 12 rats were common to experiments 2 and 3, constituting the control group for hM4Di expression. Out of these 12 rats, only 5 rats met the inclusion criteria.

3.2.11 Statistical analysis

Experiment 1 had 9 control and 11 experimental rats. Experiments 2 and 3 had two control groups each. The two control groups in each of experiments 2 and 3 did not show a difference in performance in phase 3 of the Kamin blocking procedure, and were thus combined for the purpose of statistical testing. This meant that each experiment had only one control group and one experimental group during statistical testing. Experiment 2 had 12 control and 7 experimental rats. Experiment 3 had 10 control and 8 experimental rats. The statistical analysis justifying the amalgamation of the two control groups in experiments 2 and 3 is as follows.

In experiment 2, there was no significant difference between the two control groups in their lever press responses to L1 ($F=0.956$; $p=0.351$) or L2 ($F=0.003$; $p=0.961$) during phase 1. There was a significant difference between L1 and L2 directed lever press response in both the control groups ($F=36.103$, $p<0.001$; $F=31.055$, $p<0.001$) during phase 1. In addition, there was no significant difference between the two control groups in their nose poke responses to S1 and S2, and in their nose poking during the 5s immediately preceding S1 and S2 during phase 3 (closest $F=1.139$; $p=0.311$). There was no significant difference between the two

control groups in their normalized responses to S1 (F=1.119; p=0.315) and S2 (F=1.593; p=0.236) during phase 3. These analyses showed that the two control groups in experiment 2 did not differ. Thus, they were combined to form one control group.

In experiment 3, there was no significant difference between the two control groups in their lever press responses to L1 (F=0.124; p=0.734) or L2 (F=1.795; p=0.217) during phase 1. There was a significant difference between L1 and L2 directed lever press response in both the control groups (F=47.410, p<0.001; F=33.852, p<0.001) during phase 1. In addition, there was no significant difference between the two control groups in their responses to S1 and S2, and to the baselines of S1 and S2 during phase 3 (closest F=1.793; p=0.217). There was no significant difference between the two control groups in their normalized responses to S1 (F=0.574; p=0.470) and S2 (F=2.638; p=0.143) during phase 3. These analyses showed that the two control groups in experiment 3 did not differ. Thus, they were combined to form one control group.

To verify the development of a conditioned response to the paired lever cue during phase 1, a three way mixed ANOVA (Greenhouse-Geisser correction) was conducted on the lever press response measure, with session and cue as within subject factors, and group as the between subject factor. This was followed by an analysis of simple main effects to compare the difference in responding to the two lever cues within each group (L1 vs L2 in both the control and experimental groups), and to compare the differences between the two groups in their response to each of the lever cues (control vs experimental for both L1 and L2). Bonferroni adjustment was used to control for multiple comparisons. Statistical tests were considered significant if the probability of finding a false positive (type I error) was below 0.05 (alpha < 0.05).

To verify conditioned responding to the sound cues in phase 3 (extinction test) of the Kamin blocking procedure, the average duration of nose poking during each of the sound cues (averaged over all 25 trials) was compared to the average baseline response preceding that cue. A two way mixed ANOVA was conducted on the average nose poke duration measure, with cue as the within subject factor and group as the between subject factor. This was followed by an analysis of simple main effects (Bonferroni) to compare the difference in responding to the sound cues with their respective baselines within each group (S1 vs baseline_S1 and S2 vs baseline_S2 in both the control and experimental groups).

Then, the response to each cue was normalized to each cue's baseline response according to equation 1, in order to compare inter-cue and inter-subject responding.

$$\text{Relative Response Index} = \sqrt{\left[\frac{\sum_i^n \frac{\text{Cue}_i}{\text{Cue}_i + \text{Baseline}_i}}{n}\right].\left[\frac{\sum_i^n \text{Cue}_i}{\sum_i^n \text{Cue}_i + \sum_i^n \text{Baseline}_i}\right]} \qquad (1)$$

i → Trial number; n → Total number of trials

The relative response index is the square root of the product of two different normalized measures of the nose poke response. The first measure is the relative nose poke response index, and is the nose poke duration during the cue divided by the sum of the nose poke duration during the cue and the 5s preceding the cue for each trial, averaged across trials. This sets a maximum on the contribution that performance on any one trial can have on the overall relative nose poke response index measure at 1/n. Thus, the first measure reflects the consistency of nose poke performance across trials, and ensures that no single trial can skew this measure drastically. However, because the relative nose poke response index sets a maximum on single trial contribution, it cannot capture the vigor of the nose poke response. For example, if on a particular trial the baseline response is zero, then it does not differentiate

between a 100ms nose poke response and a 5s nose poke response during the cue, and both these response durations during the cue contribute 1/n to the first measure. The second measure is the relative response vigor index, and is the sum of the nose poke duration during all the cue presentations during a session divided by the sum of nose poke duration during all the cue presentations and the 5s periods preceding the cue presentations. This measure, also called the elevation ratio (Blaisdell *et al.*, 2009), captures the vigor of the nose poke response but also allows single trial performance to skew the measure. For example, if the rat does not nose poke during the 5s period preceding the cue on any of the cue presentations, and only nose pokes during cue presentation on one of the trials, then this measure will give a read out of 1, which is the maximum this measure can be. In this study, the two measures were combined to form the relative response index to take into account both response vigor and the consistency of response across trials, and to dilute the effects of single trial performance on the final measure.

A two way mixed ANOVA was conducted on the relative response index (see equation 1), with cue as the within subject factor and group as the between subject factor. This was followed by an analysis of simple main effects (Bonferroni adjustment) to compare the difference in responding to the two sound cues within each group (S1 vs S2 in both the control and experimental groups) and the difference between the groups in their response to each cue (control vs experimental for both S1 and S2).

To analyze responding to the compound cues during phase 2 of the Kamin blocking paradigm, two 3-way mixed ANOVAs were conducted, one on the lever press measure, and the other on the relative response index. Cue and session were the within subject factors and group was the between subject factor. This was followed by an analysis of simple main effects (Bonferroni adjustment) to compare differences in responding to the two compound

cues within each group, and the difference between the groups in their response to each cue (control vs experimental for both L1+S1 and L2+S2).

For all statistical testing, alpha < 0.05 was used to determine significance. Bonferroni adjustment was used to control for multiple comparisons during the analysis of simple main effects.

3.3 Results

3.3.1 Blocking $GABA_A$ receptors in the ventral tegmental area attenuates the Kamin blocking effect

To test the role of inhibition of VTA neurons in Kamin blocking, injections of the $GABA_A$ receptor antagonist, bicuculline, were made into the VTA bilaterally (Fig. 3.3) before each compound cue conditioning session. A total of 11 bicuculline-injected and 9 saline-injected rats met the criteria for inclusion (see Methods). During phase 1 of the Kamin blocking paradigm (Fig 3.4A), both the experimental and control rats gradually developed a lever pressing conditioned response to the presentation of the lever cue paired with the food reward (Fig. 3.4B). Neither group developed a conditioned response to the presentation of the unpaired lever (Fig. 3.4B). A three way mixed ANOVA showed a significant main effect of lever cue ($F=162.102$; $p<0.001$; Greenhouse-Geisser) and a significant interaction between cue and session ($F=10.691$; $p<0.001$; Greenhouse-Geisser). No other main effect or interaction was significant (interaction between cue and group came closest with $F=3.982$; $p=0.061$). A simple main effects comparison using the Bonferroni adjustment showed a significant effect of the lever cue in both the control ($F=52.395$; $p<0.001$) and the experimental groups ($F=120.500$; $p<0.001$). There was no significant difference between the two groups in their responses to the paired lever ($F=1.823$; $p=0.194$) or the unpaired lever

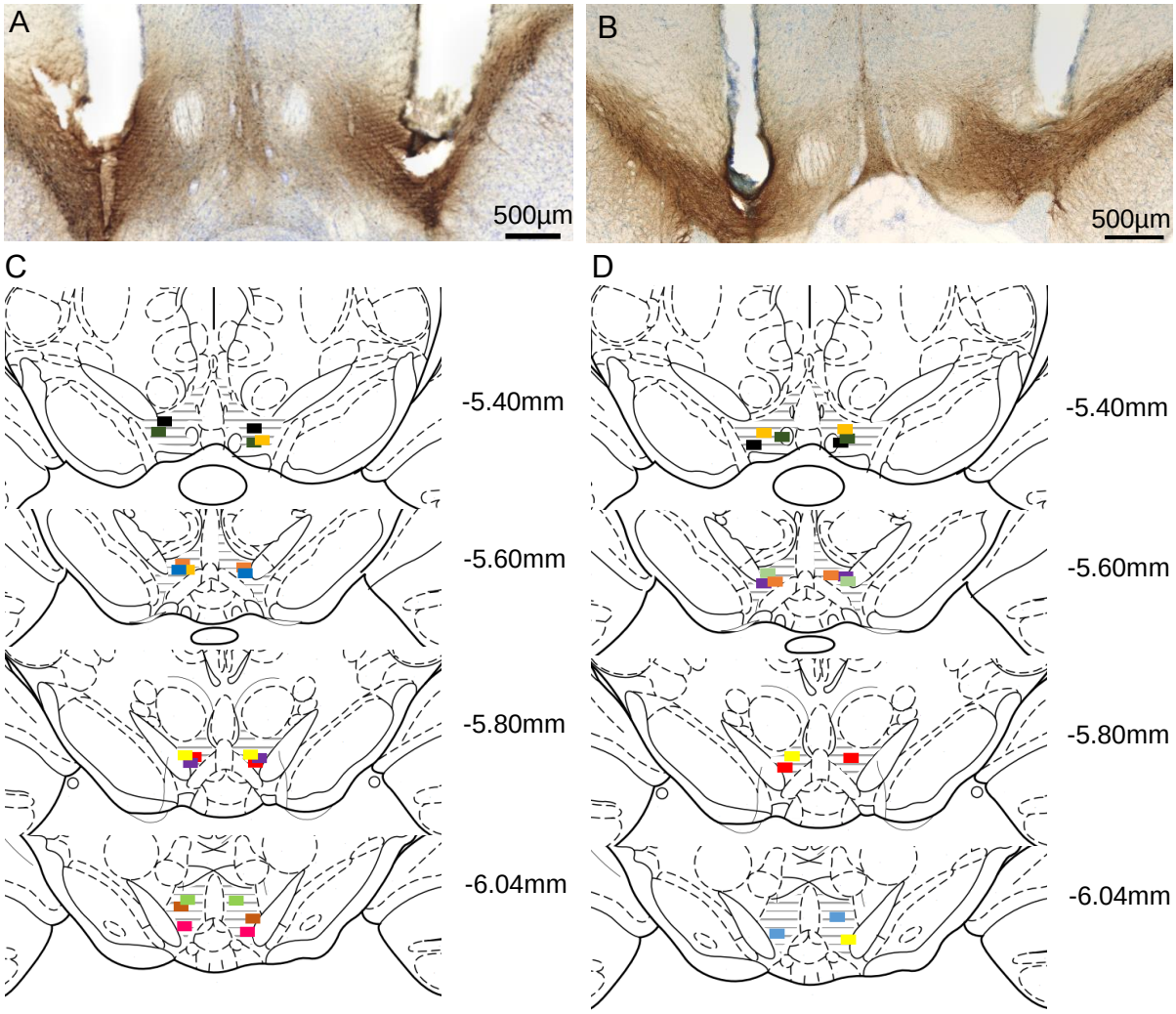

Figure 3.3: **Histological verification of injection sites in the VTA.** (A-B) Representative examples of anti-TH immunostaining in the experimental (A) and control (B) groups, verifying the position of the injection cannulae. (C-D) Histologically verified injection sites (rectangles) of the 11 experimental (C) and 9 control (D) rats. Injection site for each rat is shown in a different color. The numbers indicate AP distance of the corresponding section from Bregma (Adapted from Paxinos and Watson (1998)). Shaded regions indicate the VTA.

(F=0.867; p=0.364). These results show that both control and experimental groups acquired discriminative conditioned responding to the lever paired with food reward, and there was no significant difference between the groups.

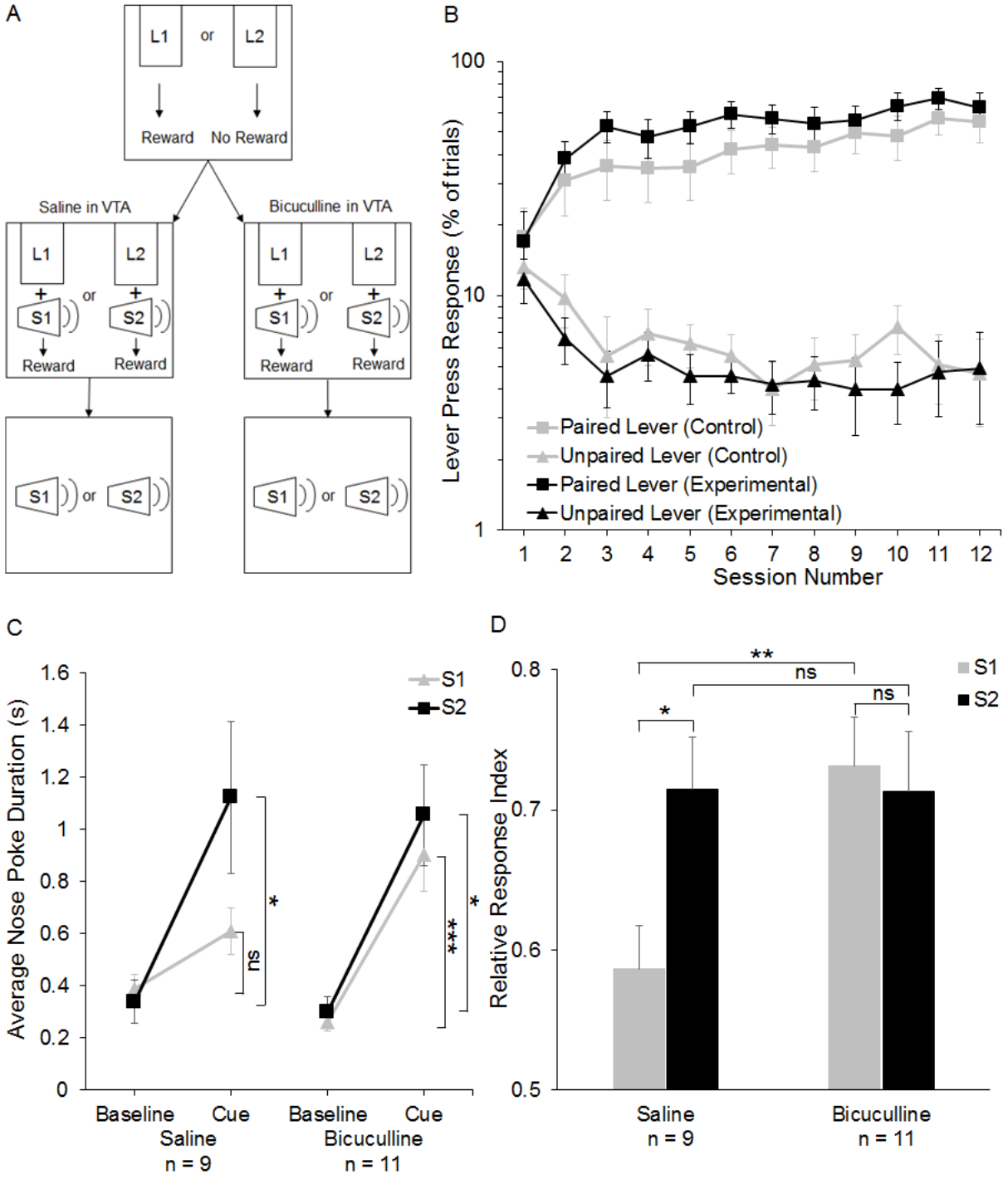

Figure 3.4: **Blocking inhibitory inputs to the VTA in phase 2 attenuates the Kamin blocking effect.** (A) Schematic of the experimental design (see methods). Phase 1 is a single cue conditioning phase, in which only one of the cues (L1 or L2) is followed by reward. Phase 2 is a compound cue conditioning phase, in which compound cues (L1+S1 or L2+S2) are presented, and both compound cues are followed by reward. Phase 3 is an extinction test in which only the cues added in phase 2 (S1 and S2) are presented and no rewards are given. After phase 1, rats were divided into control and experimental groups.

Figure 3.4 contd…

Before each session of phase 2, the control group received bilateral microinjections of saline and the experimental group received microinjections of bicuculline into the VTA. (B) Lever press responses of the control and experimental groups during phase 1. (C) Nose poke responses to the sound cues in phase 3. Baseline response is measured during the 5s immediately preceding the respective sound cue. Cue response is measured during the cue, which lasts for 5s. (D) Responses to the sound cues normalized (equation 1) to their respective baseline. Data are presented as mean ± SEM.

To test whether prior conditioning of the paired lever cue blocks the acquisition of a conditioned response to added cues, both the experimental and the control group then underwent compound cue conditioning (phase 2). In this phase, a sound cue (S1 or S2) was added to each of the lever cues (Fig. 3.4A), and both the compound cues (L1 + S1 and L2 + S2) were followed by food reward. Before each session of compound cue conditioning, the experimental group was injected with bicuculline, and control with vehicle, bilaterally into the VTA. Then, their response to the separate presentations of the sound cues alone was tested in extinction (phase 3). During the extinction test, the controls increased their nose poke response from baseline only during S2 ($t=3.294$; $p=0.024$; two way mixed ANOVA – simple main effects – Bonferroni), which is the sound cue that was given as a compound along with the unpaired lever in phase 2 (Fig. 3.4C). The controls did not increase their nose poke response during S1 ($t=1.457$; $p=0.971$) (Fig. 3.4C). Thus, the controls showed conditioned responding to S2 but not to S1, showing they expressed Kamin blocking. In contrast, the experimental group responded to both S1 ($t=4.728$; $p=0.001$) and S2 ($t=3.5$; $p=0.015$) (Fig. 3.4C). However, even though the experimental group responded to both S1 and S2 during the extinction test, this is not sufficient to conclude that Kamin blocking has not occurred, because there could be a significant difference between their responses to S1 and S2.

To compare conditioned responding to S1 and S2, the responses to the sound cues were normalized (equation 1) to their respective baselines (Fig. 3.4D). In agreement with the foregoing analysis of raw nose poke data, the controls responded significantly more to S2 than to S1 ($F=5.045$; $p=0.037$; two way mixed ANOVA – simple main effects - Bonferroni). This confirms, as above, that the controls expressed the Kamin blocking effect. However, the experimental group did not show a significant difference in their response to S1 and S2 ($F=0.125$; $p=0.728$) during phase 3 (Fig. 3.4D). This means that the experimental group did

not express the Kamin blocking. The experimental group also responded significantly more to S1 than the control group ($F=8.326$; $p=0.010$). There was no difference in the responses of the two groups to S2 ($F=0.001$; $p=0.979$). This means that blocking the $GABA_A$ receptors in VTA during phase 2 had the specific effect of increasing the conditioned responding to S1 in phase 3. These results show that blocking $GABA_A$ receptor mediated inhibition in the VTA during compound cue conditioning attenuates the Kamin blocking effect.

It is important to note here that the pattern of responding to S1 and S2 in the control and the experimental groups observed in phase 3 could occur either due to differences in nose poke response during S1 and S2 or due to differences in nose poking during the baseline period of S1 and S2. The baseline period is the 5s immediately preceding the presentation of a sound cue. Nose poke duration in the 5s immediately preceding S1 was not significantly different from nose poking during the 5s immediately preceding S2 in the control ($t=0.754$; $p=1.000$) and experimental groups ($t=0.645$; $p=1.000$). There was also no significant difference between the two groups in their nose poke duration in the 5s before S1 ($t=2.000$; $p=0.062$) or S2 ($t=0.368$; $p=0.716$). These results show that the pattern of conditioned responding to S1 and S2 observed in phase 3 in the two groups is not due to differences in baseline nose poking.

3.3.2 Inactivating the nucleus accumbens during the compound cue conditioning phase attenuates the Kamin blocking effect

One of the major sources of inhibitory input to the VTA is the nucleus accumbens.

To test the role of the nucleus accumbens in Kamin blocking, an inhibitory designer receptor exclusively activated by designer drugs (G_i DREADD) was used. This approach had the advantage of enabling the anatomical extent of the drug action to be determined. To express

the DREADD, viral AAV2-hSyn-hM4Di(Gi)-mCherry was bilaterally injected into the NAc during surgery, three weeks before behavioral testing. Figure 3.5 shows a representative example of the expression of the virus, verified by immunostaining, and summarizes the extent of viral expression in each animal. The virus was expressed in both the shell and core regions of the NAc.

To inactivate the neurons expressing the DREADD during compound cue conditioning (phase 2), i.p. injections of clozapine were administered to rats in the experimental group before each session in phase 2. Two controls were used. One control group was injected with saline without virus in the nucleus accumbens, and given i.p. clozapine injections during phase 2. Another control group was injected with the virus in the nucleus accumbens, and given vehicle i.p. injections during phase 2. A total of 12 control and 7 experimental rats met criteria for inclusion (see methods). Figure 3.6A shows the experimental design. For data analysis, the two control groups were combined.

Both the experimental and the combined control group gradually developed a lever pressing conditioned response to the presentation of the lever paired with the food reward (Fig. 3.6B). Neither group developed a conditioned response to the presentation of the unpaired lever during phase 1 (Fig. 3.6B). A three way mixed ANOVA using Greenhouse-Geisser adjustment showed a significant effect of lever cue ($F=115.140$; $p<0.001$) and a significant interaction between cue and session ($F=9.364$; $p<0.001$). No other main effect or interaction was significant (interaction between cue and group came closest with $F=0.435$; $p=0.518$). A simple main effects comparison using the Bonferroni adjustment showed a significant effect of the lever cue in both the control ($F=88.029$; $p<0.001$) and the experimental groups ($F=40.147$; $p<0.001$). There was no significant difference between the two groups in their responses to the paired lever ($F=0.053$; $p=0.821$) or the unpaired lever ($F=0.312$; $p=0.584$).

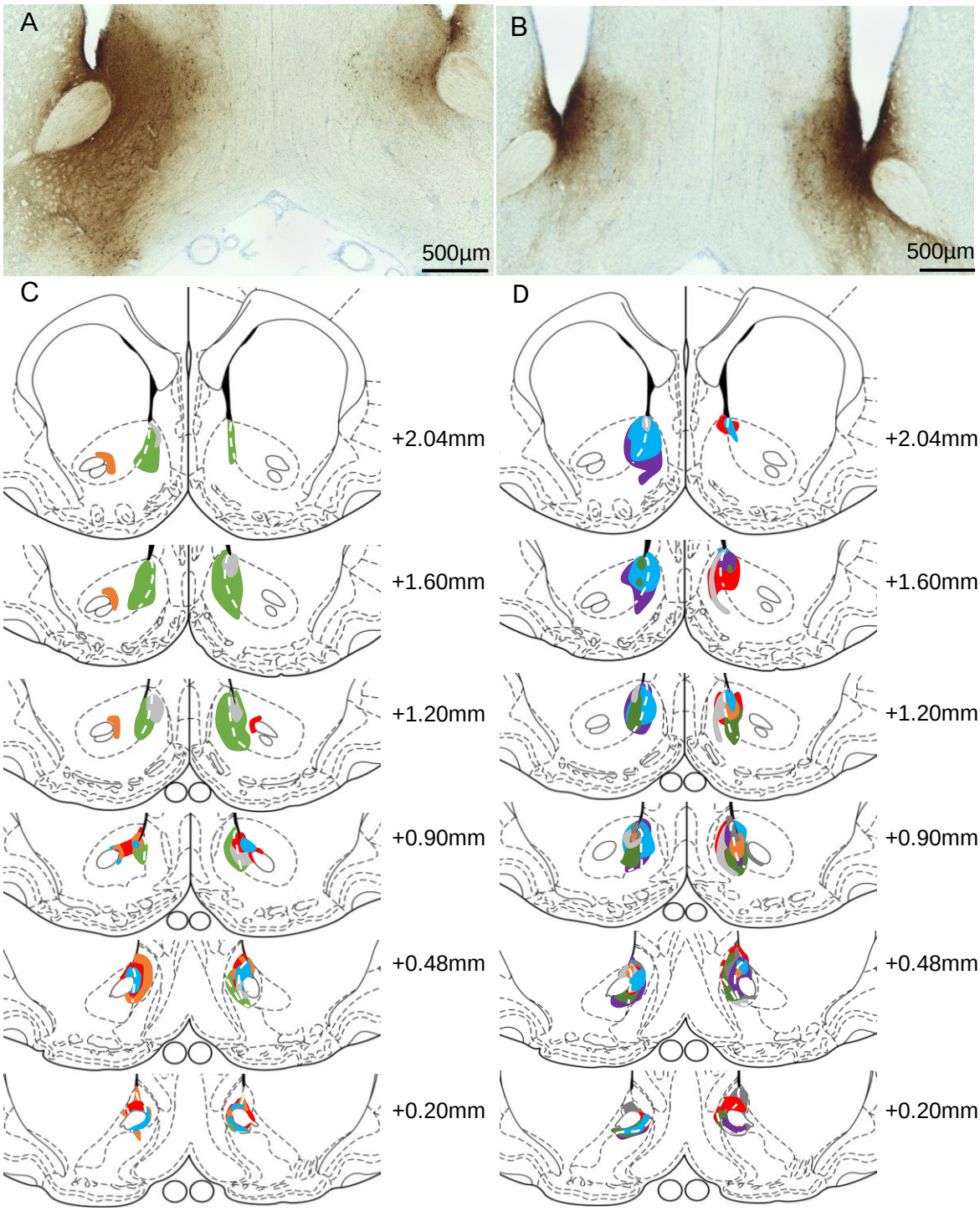

Figure 3.5: **Histological verification of viral expression in the NAc.** (A) Representative examples of anti-mCherry immunostaining in the control (A) and experimental (B) groups, verifying the expression of the injected virus. (C-D) Histologically verified viral expression in 5 control (C) and 7 experimental (D) rats. Viral expression for each rat is shown in a different color. The numbers indicate AP distance of the corresponding section from Bregma (Adapted from Paxinos and Watson (1998)).

These results show that both experimental and control rats acquired discriminative conditioned responding to the lever paired with food reward, and there was no significant difference between the two groups.

To test the role of the output of the nucleus accumbens in Kamin blocking, the experimental group was then intraperitoneally injected with clozapine, and the two control groups with vehicle and clozapine respectively, before each compound cue conditioning session (phase 2). Then, their response to the presentation of the sound cues alone was tested in extinction (phase 3). During the extinction test, the controls significantly increased their nose poke response above the baseline response only during S2 ($t=5.517$; $p<0.001$; two way mixed ANOVA – simple main effects – Bonferroni), which is the sound cue that was given as a compound along with the unpaired lever in phase 2 (Fig. 3.6C). The controls did not increase their nose poke response during S1 ($t=1.382$; $p=1.000$) (Fig. 3.6C). Thus, the controls showed conditioned responding to S2 but not to S1, showing that they expressed the Kamin blocking effect. The experimental group increased their nose poke response during both S1 ($t=4.017$; $p=0.005$) and S2 ($t=3.005$; $p=0.047$) (Fig. 3.6C), suggesting that the Kamin blocking effect was attenuated in this group.

To directly compare conditioned responding to S1 and S2, the responses to the sound cues were normalized (equation 1) to their respective baselines (Fig. 3.6D). In agreement with the foregoing analysis of raw nose poke data, the controls responded significantly more to S2 than to S1 (Fig. 3.6D). A two way mixed ANOVA showed a significant main effect of cue ($F=6.35$; $p=0.022$) and a significant interaction between cue and group ($F=5.019$; $p=0.039$). A simple main effects comparison using the Bonferroni adjustment showed that the controls responded significantly more to S2 than to S1 ($F=15.376$; $p=0.001$). This confirms that the controls expressed the Kamin blocking effect. In contrast, the experimental group did not

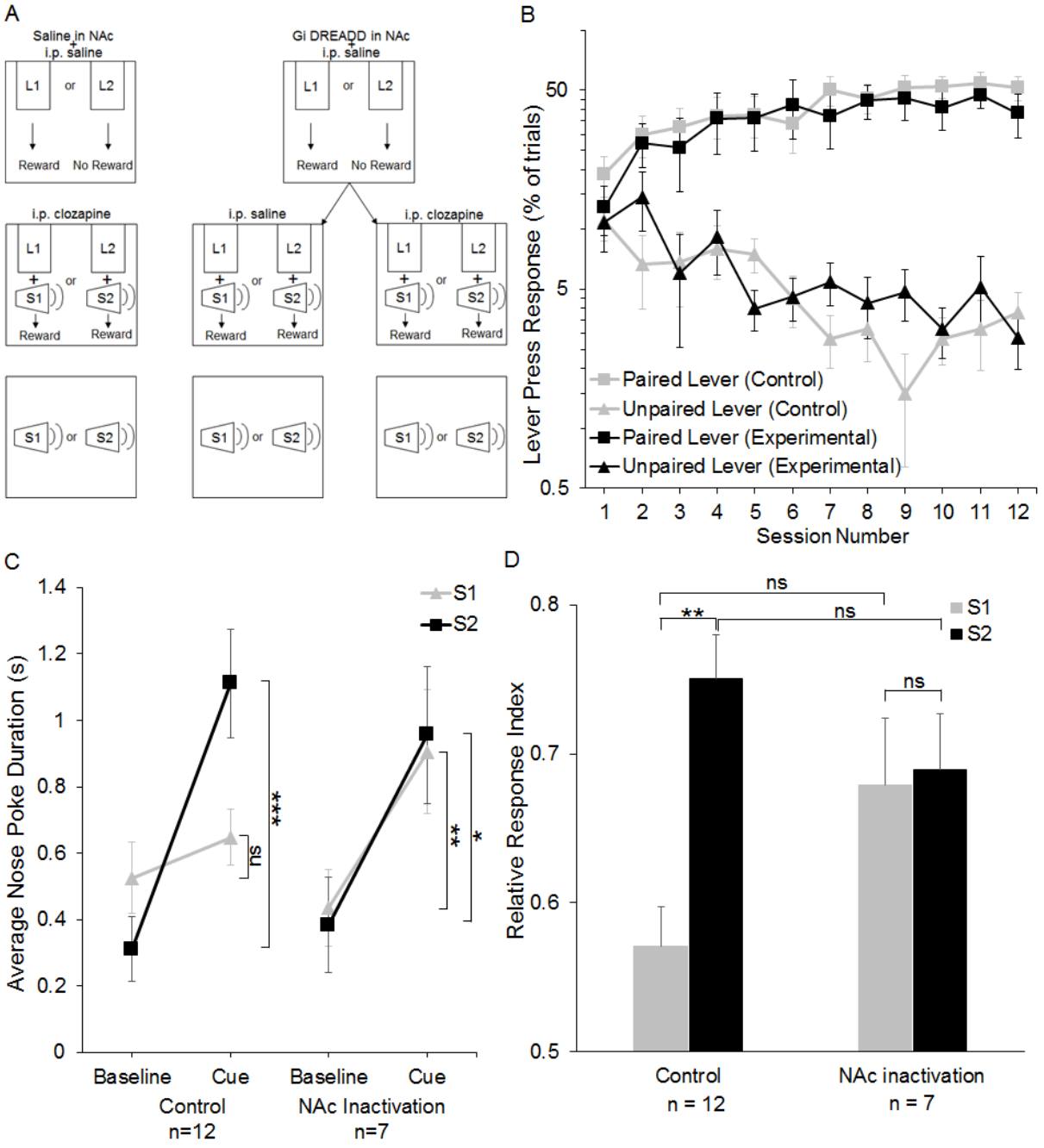

Figure 3.6: **Inactivating NAc during phase 2 attenuates the Kamin blocking effect.** (A) Schematic of the experimental design (See also legend of Fig. 3.4 and methods). (B) Lever press responses of the control and experimental groups during phase 1. (C) Nose poke responses to the sound cues in phase 3. Baseline response is measured during the 5s immediately preceding the respective sound cue. Cue response is measured during the cue, which lasts for 5s. (D) Responses to the sound cues normalized (equation 1) to their respective baseline. Data are presented as mean ± SEM.

show a significant difference in their response to S1 and S2 (F=0.031; p=0.862) during phase 3 (Fig. 3.6D). This means that the experimental group did not express Kamin blocking. There was no significant difference in the responses of the two groups to S1 (F=4.394; p= 0.051) and S2 (F=1.419; p=0.250) (Fig. 3.6D). These results show that inactivating the NAc neurons, and thereby blocking the output from this region, during the compound cue conditioning phase of the Kamin blocking paradigm attenuates the Kamin blocking effect.

Baseline nose poking during the 5s immediately preceding S1 was significantly different from nose poking before S2 in the control group (t=3.627; p=0.012). There was also a significant difference in responding during S1 compared to during S2 in the control group (t=3.171; p=0.033). Thus, both the difference in baseline nose poking before S1 and S2 and the difference in nose poke response during S1 and S2 contributed to the significant difference in conditioned responding to S1 and S2 observed in the control group. However, these results also show that there was a significant difference in responding to S1 and S2 in the control group, which cannot be explained by the difference in baseline nose poking.

In the experimental group, there was no significant difference in baseline nose poking during the 5s immediately preceding S1 and S2 (t=0.662; p=1.000) and no significant difference in responding during S1 and S2 (t=0.262; p=1.000). There was also no significant difference between the two groups in their baseline response before S1 (t=0.517; p=0.612) or S2 (t=0.412; p=0.686). These results show that the pattern of conditioned responding to S1 and S2 observed in phase 3 in the experimental group is not due to differences in baseline nose poking.

3.3.3. Inactivating the nucleus accumbens during the single cue conditioning phase (phase 1) attenuates the Kamin blocking effect

The motivation for the choice of neural substrates tested in the previous two experiments was their postulated role in the reduction in dopamine response evoked by the reward when the reward is expected. This reduction in the dopamine response evoked by the reward when preceded by the cue being conditioned occurs gradually over the course of classical conditioning. Thus, the control of inhibition in the VTA by the NAc needs to be acquired through gradual neuronal learning during the single cue conditioning phase. Given the postulated role of dopamine mediated synaptic plasticity in the NAc in reward related learning, experiment three aims to test the role of neuronal learning in the NAc during phase 1 in Kamin blocking.

Towards this goal, the G_i DREADD expressing virus AAV2-hSyn-hM4Di(Gi)-mCherry was bilaterally injected into the NAc during surgery. Figure 3.7A shows a representative example of viral expression in one animal, verified by immunostaining for mCherry. Figure 3.7B shows the extent of viral expression in each animal of the experimental group. The control group for viral injections was the same as in experiment 2 (Fig. 3.5C). The virus was expressed in both the shell and core regions of the NAc. To inactivate the neurons expressing the DREADD, the DREADD was activated by i.p. injections of clozapine in the experimental group before every single-cue conditioning session (phase 1; Fig. 3.8A). One control group was injected with saline in the nucleus accumbens and given i.p. clozapine injections before each single-cue conditioning session (Fig. 3.8A). Another control group was injected with the virus in the nucleus accumbens and given vehicle i.p. injections (Fig. 3.8A). A total of 10 control and 8 experimental rats met the criteria for inclusion (see methods). For data analysis, the two control groups were combined.

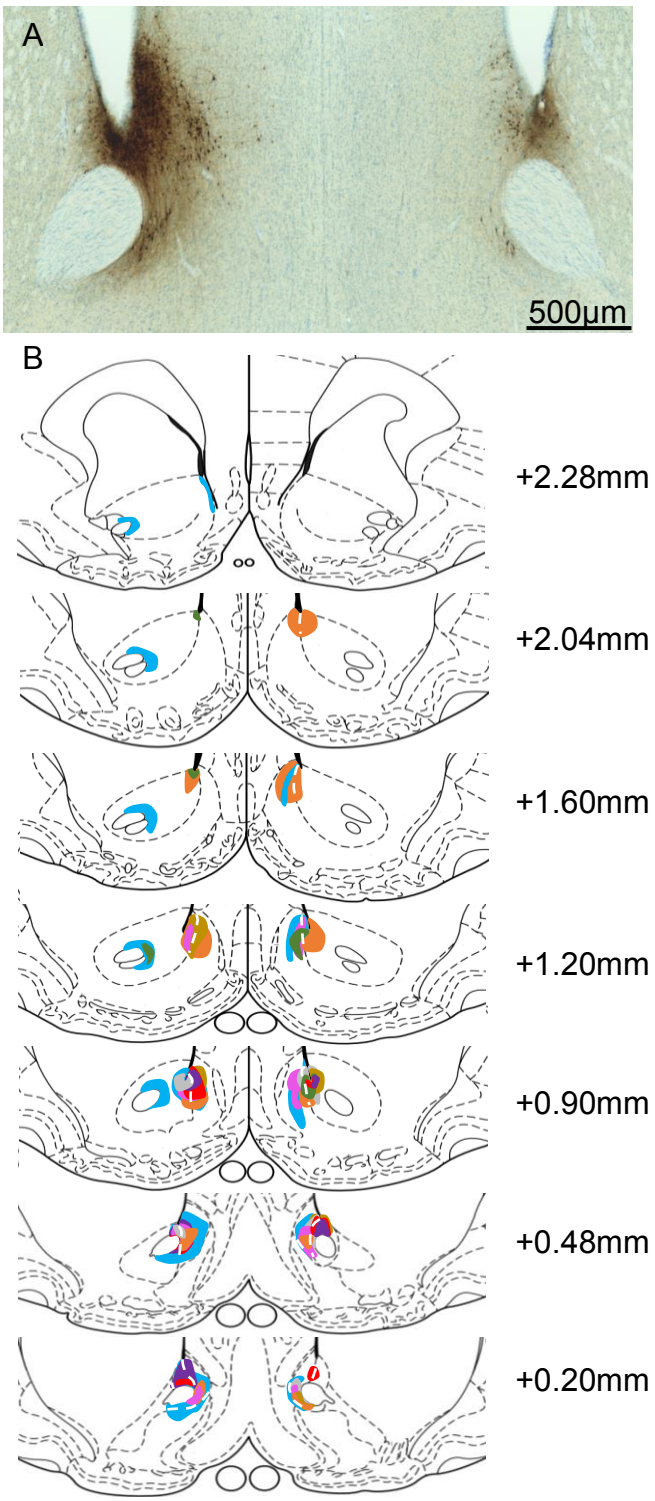

Figure 3.7: **Histological verification of viral expression in the NAc.** (A) Representative example of anti-mCherry immunostaining in one animal, verifying the expression of the injected virus. (B) Viral expression in all 8 animals of the experimental group (control group with viral injections was the same as in experiment 2 – see Fig. 3.5C). Each animal is represented in a different color. Numbers indicate AP distance of the corresponding section from Bregma (Adapted from Paxinos and Watson (1998)).

Both the experimental and the combined control group gradually developed a conditioned response (lever pressing) to the presentation of the lever paired with the food reward (Fig. 3.8B). Neither group developed a conditioned response to the presentation of the unpaired lever during phase 1 (Fig. 3.8B). A three way mixed ANOVA showed a significant effect of lever cue ($F=121.403$; $p<0.001$; Greenhouse-Geisser) and a significant interaction between cue and session ($F=10.859$; $p<0.001$; Greenhouse-Geisser). No other main effect or interaction was significant (main effect of group came closest with $F=1.994$; $p=0.177$). A simple main effects comparison using the Bonferroni adjustment showed a significant effect of the lever cue in both the control ($F=67.035$; $p<0.001$) and the experimental group ($F=55.644$; $p<0.001$). There was no significant difference between the two groups in their responses to the paired lever ($F=0.751$; $p=0.399$) or the unpaired lever ($F=1.530$; $p=0.234$). These results show that both experimental and control rats acquired discriminative conditioned responding to the lever paired with food reward.

Both the experimental and control groups then underwent compound cue conditioning (phase 2; Fig. 3.8A). Subsequently, their response to the presentation of the sound cues alone was tested in extinction (phase 3). During the extinction test, the controls increased their nose poke response from baseline only during S2 ($t=4.685$; $p=0.001$; two way mixed ANOVA – simple main effects – Bonferroni) (Fig. 3.8C), which is the sound cue that was given as a compound along with the unpaired lever in phase 2. The controls did not increase their nose poke response during S1 ($t=2.207$; $p=0.257$) (Fig. 3.8C). Thus, the controls showed conditioned responding to S2 but not to S1, showing that they expressed the Kamin blocking effect. The experimental group responded to both S1 ($t=3.888$; $p=0.008$) and S2 ($t=3.713$; $p=0.011$) (Fig. 3.8C), suggesting that the Kamin blocking effect was attenuated in this group.

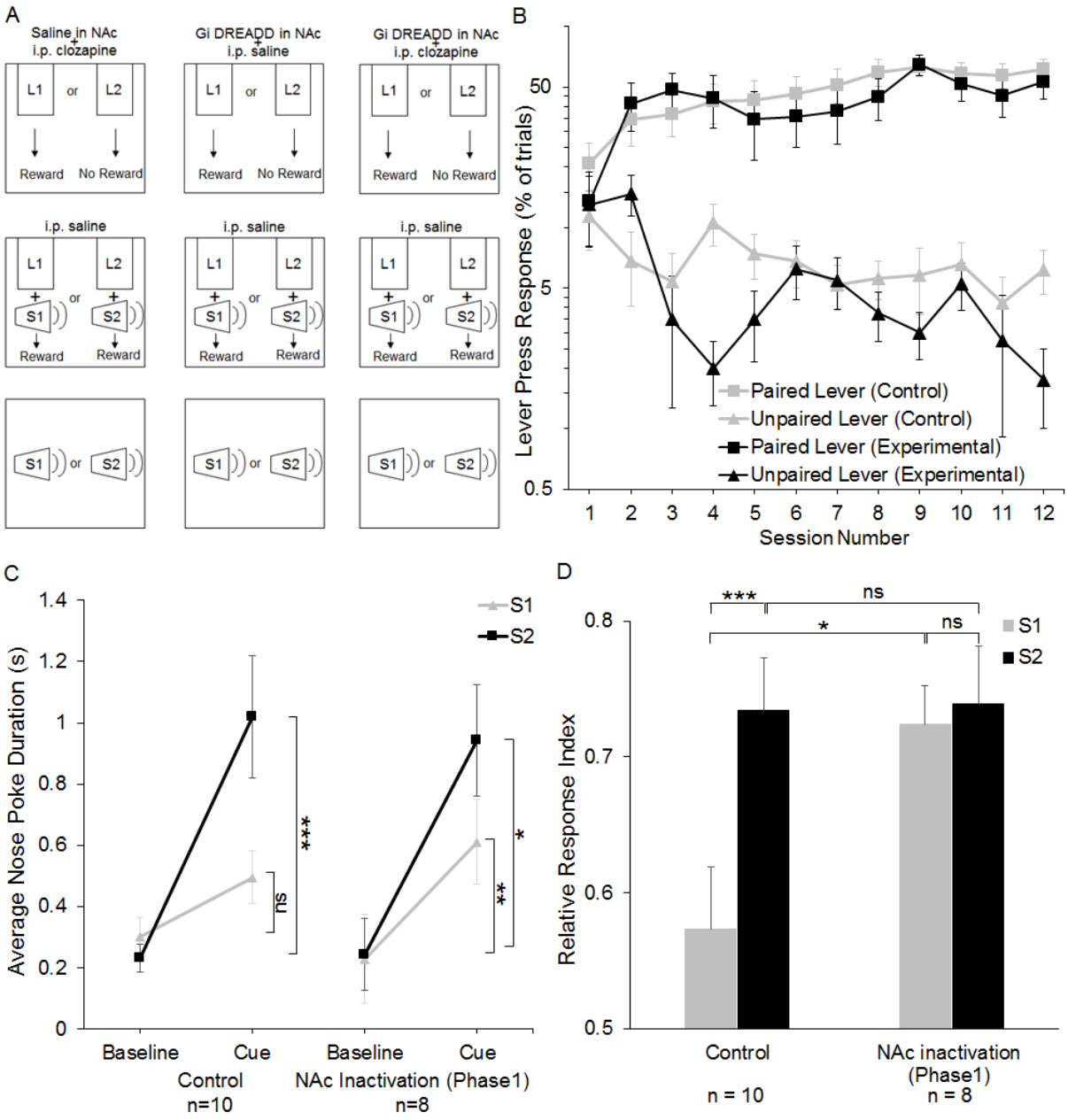

Figure 3.8: **Inactivating NAc during phase 1 attenuates the Kamin blocking effect.** (A) Schematic of the experimental design (See legend of Fig. 3.4. and methods). Animals were divided into two control groups and one experimental group before the start of phase 1. For the purpose of data analysis, the data from the two control groups was combined to form one control group. (B) Lever press responses of the control and experimental groups during phase 1. (C) Nose poke responses to the sound cues in phase 3. Baseline response is measured during the 5s immediately preceding the respective sound cue. Cue response is measured during the cue, which lasts for 5s. (D) Responses to the sound cues normalized (equation 1) to their respective baseline. Data are presented as mean ± SEM.

When the responses to the sound cues were normalized (equation 1) to their respective baselines (Fig. 3.8D), the controls responded more to S2 than to S1 (Fig. 3.8D). A two way mixed ANOVA showed a significant main effect of cue ($F=8.034$; $p=0.012$) and a significant interaction between cue and group ($F=5.446$; $p=0.033$). A simple main effects (Bonferroni adjustment) showed that the controls responded significantly more to S2 than to S1 ($F=15.023$; $p=0.001$) (Fig. 3.8D). This confirms that the controls expressed the Kamin blocking effect. The experimental group did not show a significant difference in their response to S1 and S2 ($F=0.113$; $p=0.741$) during phase 3 (Fig. 3.8D). This means that the experimental group did not express the Kamin blocking effect. The experimental group also responded significantly more to S1 than the control group ($F=6.118$; $p=0.024$) (Fig. 3.8D). There was no significant difference in the responses of the two groups to S2 ($F=0.006$; $p=0.937$). This means that inactivating the NAc during phase 1 had the specific effect of increasing the conditioned responding to S1 in phase 3. These results show that, during the single cue conditioning phase of the Kamin blocking paradigm, inactivating the NAc, and thereby blocking neuronal learning in this region, attenuates the Kamin blocking effect.

Baseline nose poke duration in the 5s immediately preceding S1 was not significantly different from the baseline before S2 in the control ($t=1.108$; $p=1.000$) and experimental groups ($t=0.205$; $p=1.000$). There was also no significant difference between the two groups in their baseline before S1 ($t=0.474$; $p=0.640$) or S2 ($t=0.105$; $p=0.919$). These results show that the pattern of conditioned responding to S1 and S2 observed in phase 3 in the two groups is not due to differences in baseline nose poking.

3.3.4 Experimental manipulations did not affect performance during compound cue conditioning sessions

Experimental manipulations during the compound cue conditioning phase in experiments 1 and 2 could have affected performance during the compound cue conditioning phase. However, two 3-way mixed ANOVAs, followed by simple effects comparison using Bonferroni adjustment showed that there were no significant differences between the control and the experiments groups in their lever press response (Fig. 3.9A, C, E) (comparison between the two groups in their lever press response to L2+S2 during the fifth compound cue session in experiment 3 came closest to significance with $F=1.869$; $p=0.190$) or their nose poke response (Fig. 3.9B, D, F) (comparison between the two groups in their relative response index during the second compound cue session in experiment 1 came closest to significance with $F=4.454$; $p=0.051$) to the two compound cues during any of the 6 compound cue conditioning sessions. These results show that experimental manipulations of disrupting $GABA_A$ receptor mediated inhibition in the VTA or inactivating the NAc during compound cue conditioning sessions, or inactivating the NAc during single cue conditioning sessions, did not affect performance on the lever press and nose poke response measures during the compound cue conditioning sessions.

In addition, both the control and the experimental groups showed a significant difference in their nose poke response to the two compound cues in experiment 1 (Control: $F=9.984$, $p=0.005$; Experimental: $F=16.728$, $p=0.001$), experiment 2 (Control: $F=25.009$, $p<0.001$; Experimental: $F=9.376$, $p=0.007$), and experiment 3 (Control: $F=27.920$, $p<0.001$; Experimental: $F=21.383$, $p<0.001$). Further, the comparison between the nose poke response to the two cues for the two groups was significant for all the compound cue conditioning sessions in all the three experiments except for session 1 of the control group in experiment 1

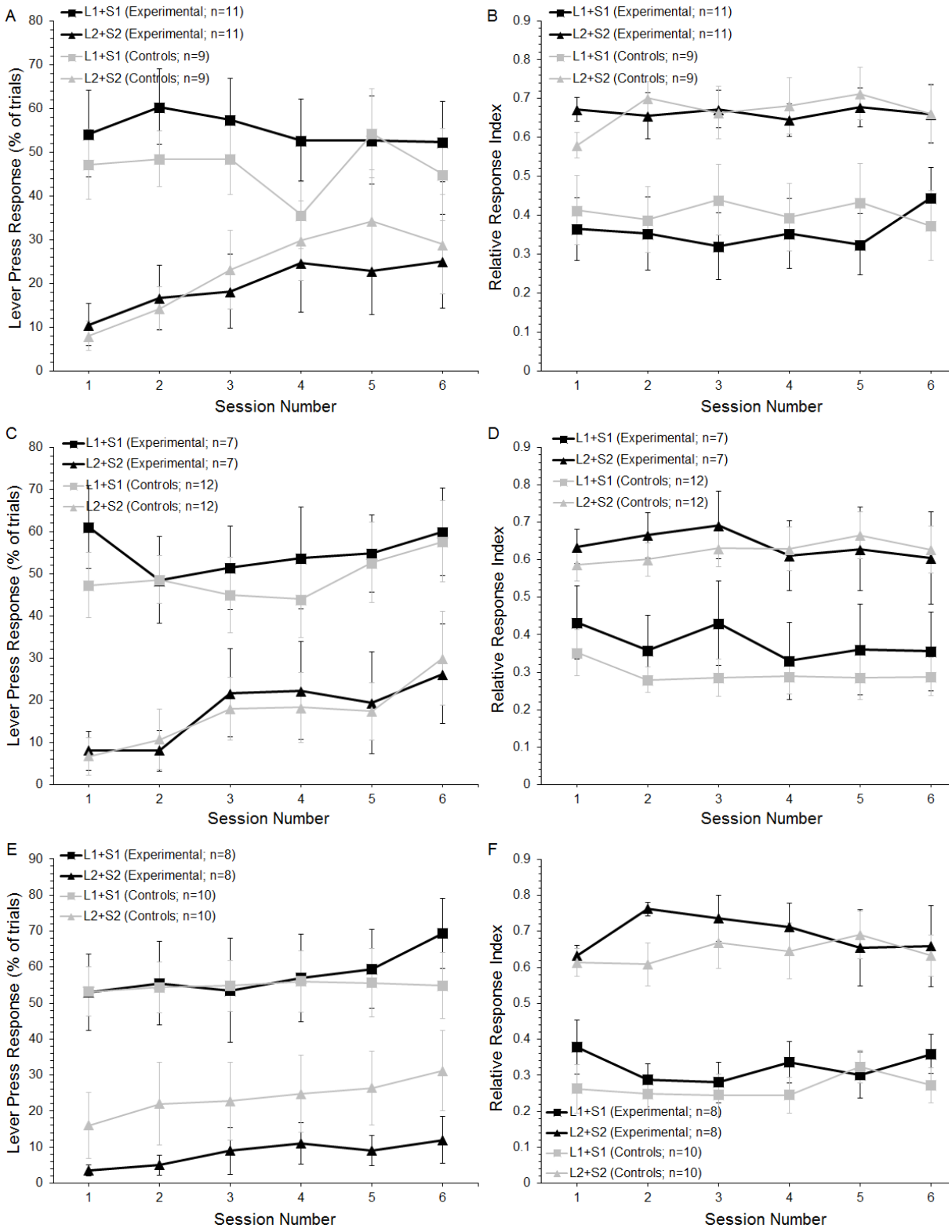

Figure 3.9: **Lever press and nose poke responses during the compound cue conditioning phase.** (A) Lever press responses and (B) nose poke responses to the two compound cues in experiment 1. (C) Lever press responses and (D) nose poke responses to the two compound cues in experiment 2. (E) Lever press responses and (F) nose poke responses to the two compound cues in experiment 3. Data are presented as mean ± SEM.

(F=3.212; p=0.090). These results show that the experimental manipulations in experiments 1-3 did not have an effect on the within subject measure of nose poke responding during the compound cue conditioning phase. This within subject measure of nose poke responding is used as the behavioral measure in phase 3, to test for the expression of the Kamin blocking effect.

3.3.5. The difference in responding to S1 and S2 in the control groups is due to Kamin blocking, and not due to overshadowing or manipulation induced generalization decrement

During the extinction test the rats in the control group increased their nose poke response from baseline only during S2, which is the sound cue that was given as a compound along with the unpaired lever in phase 2. The controls did not increase their nose poke response during S1, which is the sound cue given along with the paired lever in phase 2. Further, a comparison of the normalized responses showed that the controls responded significantly more to S2 than to S1 during the extinction test. These results were interpreted as demonstrating the Kamin blocking effect in the control group. However, the phenomenon of overshadowing can also account for these results, calling into the question the demonstration the Kamin blocking effect in experiments 1-3.

Overshadowing refers to the following phenomenon. Consider pairing a compound cue consisting of two previously neutral cues, A and B, with a reinforcer. After compound cue conditioning, when conditioned responding to separate presentation of the elements, A and B, of the compound cue A + B is assessed, if A elicits a stronger conditioned response than B, then A is said to overshadow B. Overshadowing occurs as a result of differences in the psychological salience of the physical characteristics of the two cues used as elements of a compound cue.

In experiments 1-3, overshadowing of the sound cue S1 by the lever cue L1 during phase 2 also explains the difference in responding to S1 and S2 during the extinction test in the control group. The reasoning is as follows.

L1 overshadows S1 during phase 2 resulting in less conditioning of S1 and therefore less responding to S1 during the extinction test. This could occur without conditioning L1 at all during phase 1. On the other hand, L2 fails to overshadow S2 because L2 is not paired with reward during phase 1 and thus gets ignored by the rats during phase 2. Therefore, conditioning of S2 progresses normally during phase 2 and the rats respond significantly more to S2 than to S1 during the extinction test.

The results of the experimental manipulations in experiments 1 and 2 can also be explained by the phenomenon of overshadowing. Manipulations in experiments 1 and 2 occur during phase 2 and could act by reinstating the prediction error signal or by preventing overshadowing of S1 by L1. In both cases, the differences in responding to S1 and S2 during the extinction test will be attenuated.

However, overshadowing cannot explain the result of experiment 3. In experiment 3, the experimental manipulation occurs in phase 1. The overshadowing explanation would require the experimental manipulation in phase 1 to reduce the ability of L1 to overshadow S1 in phase 2.

Overshadowing occurs as a result of differences in cue characteristic and salience. Prior experience with lever cues in phase 1 cannot affect the ability of the lever cues to overshadow the sound cues on the basis of differences in cue characteristic. However, experience with lever cues in phase 1 can affect the salience of the lever cues in phase 2, thereby modulating their ability to overshadow the sounds cues in phase 2. This is used in the overshadowing explanation of the control's difference in responding to S1 and S2 during the extinction test,

explained on the basis of L2 being ignored in phase 2 due to prior experience with L2 as an unrewarded, and thus not salient, cue during phase 1. It follows that driving up the salience of L1 during phase 1 will increase its ability to overshadow S1. However, the only way inactivating the accumbens in phase 1 can prevent L1 from overshadowing S1 during phase 2 is by reducing the salience of L1.

If inactivating the nucleus accumbens during phase 1 reduces the salience of L1 at the end of phase 1 in the experimental group, then there should be a significant difference between the control and the experimental groups in their response to L1 towards the end of phase 1 in experiment 3. There was no significant difference between the two groups in their response to L1 on sessions 10 ($F=0.404$; $p=0.534$), 11 ($F=1.881$; $p=0.189$), and 12 ($F=1.281$; $p=0.274$) of phase 1. Thus, inactivating the nucleus accumbens during phase 1 did not have a significant effect on the final salience of L1 at the end of phase 1.

Another possibility is that the manipulation in phase 1 resulted in a loss in salience of L1 from phase 1 to phase 2, rather than in phase 1 itself. This could happen if the manipulation in phase 1 induced a physiological state which can be interpreted as a different context. If this were the case, then the context shift between phase 1 and phase 2 would result in generalization decrement and thus a loss in the salience of L1 from phase 1 to phase 2. The resulting reduction in overshadowing of S1 by L1 can explain the increase in response to S1 in the experimental versus the control group. However, generalization decrement can only reduce the ability of L1 to overshadow S1, not abolish it. Thus, it is also expected that the experimental group would have responded more to S2 than to S1 during the extinction test. However, there was no significant difference between responding to S1 and S2 in the experimental group during the extinction test in experiment 3 ($F=0.113$; $p=0.741$).

Further, if the manipulation in experiment 3 caused a context shift resulting in generalization decrement, the generalization decrement should also have happened in experiment 2, where the same manipulation was done in phase 2. The shift in context from phase 2 to phase 3 in the experimental group of experiment 2 is expected to decrease the salience of S1 and S2 from phase 2 to phase 3. Thus, it is expected that the controls would respond more to S2 than the experimental group during the extinction test in experiment 2. However, there was no significant difference in the response to S2 between the two groups during the extinction test in experiment 2 ($F=1.419$; $p=0.250$).

Thus, the experimental results do not support the existence of a generalization decrement caused by manipulation induced context shifts across phases in the experimental group in experiments 2 and 3.

Taken together, the experimental results find no support for the hypothesis that inactivating the nucleus accumbens in phase 1 resulted in a loss in salience of L1. As explained previously, this was the only way that inactivating the accumbens in phase 1 could have prevented L1 from overshadowing S1 during phase 2. Thus, overshadowing cannot explain the result of experiment 3.

Given that the experimental manipulation did not alter the salience of the lever cues, and the manipulation itself was absent in phase 2 and so could not directly affect overshadowing, L1 should have overshadowed S1 in the experimental group in experiment 3. The absence of overshadowing in the experimental group of experiment 3 serves as the overshadowing control, showing that the differences observed between responding to S1 and S2 in the control groups were not due to overshadowing but due to Kamin blocking.

3.4 Discussion

The present study showed that the Kamin blocking effect was attenuated by blocking inhibition in the VTA or by inhibiting neurons in the NAc. These effects were seen if the manipulations were performed during the compound cue conditioning phase. In addition, inhibiting NAc neurons during the single cue conditioning phase also attenuated the Kamin blocking effect. Conceptually, the Kamin blocking effect is important because it led to the idea that classical conditioning occurs only when outcomes deviate from expectations, in other words, when there is a prediction error. The results reported here identify specific neural structures that are involved in Kamin blocking, and thus suggest the involvement of these structures in computing prediction error in the context of appetitive classical conditioning.

Previous work (Mirenowicz & Schultz, 1994; Schultz *et al.*, 1997; Hollerman & Schultz, 1998; Waelti *et al.*, 2001) has shown that midbrain dopamine neurons fire in a manner consistent with prediction error signaling during appetitive classical conditioning. In particular, the dopamine response evoked by rewards is reduced when they are expected. Waelti et al. (2001) suggested that this reduction in the dopamine signal evoked by the reward when it is expected plays a crucial role in the Kamin blocking effect. In support of this idea, Steinberg et al. (2013) showed that overriding this reduction by optogenetic stimulation of the VTA dopamine neurons when the expected reward is delivered attenuates the Kamin blocking effect. The present study found that blocking inhibition in the VTA during compound cue conditioning similarly attenuates the Kamin blocking effect. This shows that naturally occurring inhibition in the VTA is necessary for the Kamin blocking effect, and suggests that inhibitory inputs to the VTA neurons play a role in reducing the dopamine response evoked by the reward when it is expected.

The present study also found that inhibiting the NAc neurons during compound cue conditioning attenuates the Kamin blocking effect. These neurons send direct inhibitory inputs to the VTA dopamine neurons (Watabe-Uchida *et al.*, 2012; Tian *et al.*, 2016) and may be an important source of the inhibition occurring in the VTA at the expected time of reward delivery. In addition, NAc projection neurons also synapse onto the GABA interneurons in the VTA (Xia *et al.*, 2011). Moreover, NAc neurons also project to other brain regions that are in turn afferent to the VTA dopamine neurons (Groenewegen *et al.*, 1999; Hong & Hikosaka, 2008; Bromberg-Martin *et al.*, 2010a; Bromberg-Martin *et al.*, 2010b). Given this connectome, the present results suggest that the NAc directly or indirectly contributes to the inhibition of VTA dopamine neurons at the expected time of reward delivery, and this inhibitory control is necessary for the Kamin blocking effect.

Another result of the present study was that inhibiting the NAc neurons during the single cue conditioning phase attenuated the Kamin blocking effect, even though classical conditioning proceeded normally during this phase. This may come as a surprise because blocking occurs during the subsequent compound cue conditioning phase, at which time there is no experimenter-induced inhibition of the NAc. However, blocking depends on the accuracy, during phase 2, of the reward estimate generated by the cue conditioned in phase 1. Thus, learning about reward estimation during phase 1 directly affects blocking during phase 2. Therefore, the present result suggests that the output of the NAc plays a role in learning about reward estimation during phase 1.

Studies showing that the dopamine response evoked by the reward, when preceded by the cue being conditioned, declines gradually as classical conditioning progresses (Mirenowicz & Schultz, 1994; Schultz *et al.*, 1997; Hollerman & Schultz, 1998; Waelti *et al.*, 2001) suggest potential neurophysiological correlates of reward estimation learning during phase 1. This

gradual decline implies that increased inhibitory input to the dopamine neurons is acquired through neuronal learning during phase 1. Therefore, the new finding, that inactivating the NAc during phase 1 attenuates Kamin blocking, suggests that neuronal learning in the NAc during phase 1 may play a key role in reducing the dopamine response evoked by the reward when it is expected.

Here, it is important to note some caveats to the above mentioned interpretations of the new experimental results reported in this paper. First, bicuculline injections into the VTA blocked $GABA_A$ receptor mediated inhibitory input to all the neurons in this region, and not just the dopamine neurons. Thus, the deficit in the Kamin blocking effect reported here as a result of these injections cannot be attributed to a modification of dopamine signaling without further experiments specific to manipulating only the dopamine neurons in this region. In support of a dopaminergic mechanism, GABA neurons in the VTA inhibit dopamine neurons in this region via $GABA_A$ receptors (Tan *et al.*, 2012). Conversely, inactivation of these neurons disinhibits the dopamine neurons (Bocklisch *et al.*, 2013) and increases the phasic response of dopamine neurons to rewards (Eshel *et al.*, 2015).These GABA neurons increase their firing in response to reward predicting cues (Cohen *et al.*, 2012), making their $GABA_A$ receptor mediated inhibitory input to the dopamine neurons relevant in the context of classical conditioning.

Second, while the present findings point to the involvement of the NAc in regulating the firing of dopamine neurons in response to expected rewards, the anatomical connections of the NAc and its effect on many cue mediated behaviors leaves the room for many other possibilities. The NAc is a key region in mediating the effect of conditioned cues on learning and behavior. Lesions or reversible inactivation of this region, while leaving classical conditioning intact, affect higher order behaviors such as Pavlovian to instrumental transfer

(Corbit *et al.*, 2001; Shiflett & Balleine, 2010; Corbit & Balleine, 2011), outcome devaluation (Corbit *et al.*, 2001; Shiflett & Balleine, 2010), latent inhibition (Weiner *et al.*, 1996; Jongen-Relo *et al.*, 2002; Schiller *et al.*, 2006), and responding maintained by conditioned reinforcers (Di Ciano *et al.*, 2008). The NAc also receives a convergence of inputs from many regions involved in mediating the effects of stimulus reward learning, such as the amygdala and the prefrontal cortex (Brog *et al.*, 1993; Groenewegen *et al.*, 1999) amongst others.

In support of a dopaminergic mechanism underlying the attenuation of the blocking effect by inactivation of the NAc, the output of the NAc is regulated by reward expectation (Tian *et al.*, 2016) and provides both direct and indirect inhibitory input to the VTA dopamine neurons (Groenewegen *et al.*, 1999; Hong & Hikosaka, 2008; Bromberg-Martin *et al.*, 2010a; Bromberg-Martin *et al.*, 2010b). Thus, from an anatomical perspective, the output of the NAc exerts reward expectation regulated control over multiple sources of inhibitory inputs to the VTA dopamine neurons during classical conditioning.

The three experimental results reported here show the importance of the NAc and inhibition in the VTA in the expression of the Kamin blocking effect. The underlying motivation for the choice of neural substrates investigated has been the hypothesis that the reduction in dopamine response evoked by the reward when it is expected is necessary for the expression of the Kamin blocking effect. However, the results cannot be directly extended to imply that the neural substrates investigated here play a role in reducing the dopamine response evoked by rewards when they are expected. Further experiments, directly measuring the effects of the experimental manipulations used in this research on the dopamine response evoked by the expected reward, are needed to verify this hypothesis. Such an investigation will help identify neural substrates that make behaviorally significant contributions to the computation of the dopamine reward prediction error signal.

4. Expression of the Kamin Blocking Effect in Sign Trackers and Goal Trackers

4.1 Introduction

During classical conditioning, when a cue is paired with a reinforcer, the cue being conditioned comes to elicit behavioral responses called conditioned responses. When the conditioning is appetitive, two types of conditioned approach responses occur. Some subjects approach the cue and interact with it. This is called a sign tracking response (Hearst & Jenkins, 1974; Boakes, 1977; Davey & Cleland, 1982). Others approach and interact with the site of expected reward delivery. This is called a goal tracking response (Boakes, 1977; Farwell & Ayres, 1979; Holland, 1979; Davey & Cleland, 1982). This chapter reports the results of an investigation into the expression of the Kamin blocking effect in animals that develop either a sign tracking or a goal tracking conditioned response. The findings have implications for the learning mechanisms involved in classical conditioning, the resulting associative structures, and their roles in producing the conditioned response.

Recent studies indicate that different neural substrates underlie goal tracking and sign tracking behavior. Flagel et al. (2011) found that systemic injections of the dopamine receptor antagonist flupenthixol during classical conditioning impaired both the acquisition and performance of the sign tracking response. Saunders and Robinson (2012) further found that injecting flupenthixol (nonselective dopamine receptor antagonist) specifically into the nucleus accumbens core impaired the acquisition and performance of the sign tracking response. These findings suggest that both the *acquisition* and *performance* of the sign tracking conditioned response depend on dopamine function in the nucleus accumbens core. In contrast, injecting flupenthixol into the nucleus accumbens core did not impair the

acquisition or performance of the goal tracking response (Saunders & Robinson, 2012). The performance of the goal tracking response was impaired under systemic flupenthixol injections (Flagel *et al.*, 2011). These findings suggest that the *acquisition* of the goal tracking response does not depend on dopamine. The *performance* of the goal tracking response does depend on dopamine, but is not dependent on dopamine function in the nucleus accumbens core. The action of dopamine in regions of the brain other than the nucleus accumbens core plays a role in the performance of the goal tracking response. The differences in the neural substrates mediating sign tracking and goal tracking conditioned responses implies that different learning mechanisms and principles may underlie the acquisition of these two types of conditioned responses (Derman *et al.*, 2018).

Flagel et al., (2011) also showed that dopamine transients in animals exhibiting goal tracking conditioned responses do not track the theoretical reward prediction error. They found that the dopamine response evoked by the reward, when preceded by the cue being conditioned, declined only in sign trackers as classical conditioning progressed. In contrast, the reward evoked dopamine response did not decline in animals that developed a goal tracking conditioned response. The absence of this dopamine reward prediction error signal in goal trackers has implications for the Kamin blocking effect.

It was argued in chapter two that the Kamin blocking effect can be explained by models of learning in which learning is based on prediction error. In the case of appetitive classical conditioning, dopamine reward prediction error was suggested as a neural substrate underlying the Kamin blocking effect. Specifically, a reduction in the dopamine response evoked by reward when it is expected was hypothesized to be necessary for the Kamin blocking effect (Waelti *et al.*, 2001; Steinberg *et al.*, 2013; Sharpe *et al.*, 2017). Here, this argument is extended to animals exhibiting goal tracking, which have been found not to show a reduction in the dopamine response evoked by the reward when it is expected. Thus, this

chapter investigates the hypothesis that animals that develop a goal tracking conditioned response do not express the Kamin blocking effect.

Previous investigations into the expression of the Kamin blocking effect in animals that show a sign tracking or goal tracking conditioned response have produced mixed results. Derman et al., (2018) showed that both sign tracking and goal tracking animals express the Kamin blocking effect. However, there is an alternate explanation for their results, if the details of the training procedure they used are taken into account. Derman et al. (2018) used two sound cues in phase 1, one paired with reward (S1→R) and the other not paired with reward (S2→X). In phase 2, they compounded the sound cues by adding simultaneous lever cues (S1+L1 and S2+L2). During their compound cue conditioning sessions, they presented the previously non-reinforced sound cue (S2) 6 times in the absence of reward (S2→X) and presented its compounded form 4 times followed by reward (S2+L2 →R). Similarly, they presented the previously reinforced sound cue 6 times followed by reward (S1→R) and its compounded form 4 times followed by the reward (S1+L1 →R).

The training procedure used by Derman et al. (2018) is expected to lead to the formation of a stronger association between L2 and the reward than a training procedure in which non-reinforced sound cue presentations (S2→X) are not given in phase 2. Therefore, it is suggested here that the use of such a training procedure during compound cue training will lead to the formation of a stronger L2-R association relative to the L1-R association even in the absence of phase 1 (no single cue conditioning phase – no blocking should occur), resulting in a larger response to L2 than to L1 during the post-training extinction test. Derman et al., (2018) indeed found that animals that developed a goal tracking conditioned response showed robust conditioned responding to both the added cues during the extinction test but respond significantly more to L2 than to L1. Derman et al. (2018) argue that this difference in responding reflects the Kamin blocking effect.

The alternative interpretation of Derman et al (2018) suggested here is that the difference in conditioned responding to L2 and L1 observed in the extinction test occurred because of the compound cue training procedure they used. Further, this difference in responding to L1 and L2 would have been observed even if the animals did not undergo phase 1 of their behavioral paradigm, even though no blocking should occur in the absence of phase 1. The fact that the goal tracking animals responded robustly to both the lever cues in their extinction test leaves open the possibility that goal trackers do not express the Kamin blocking effect.

In support of the alternative interpretation presented here, Holland et al., (2014) found evidence that suggests that goal trackers do not show the Kamin blocking effect. They found that goal tracking to auditory cues did not block the acquisition of a sign tracking response to the added lever cues during the compound cue training phase. In contrast, sign tracking to lever cues blocked the acquisition of goal tracking to auditory cues, suggesting that sign trackers do show the Kamin blocking effect. However, there is an alternative interpretation of these findings as well, related to the associability of the different types of cues used. Specifically, Holland et al., (2014) found that the lever cue overshadowed the formation an association between an auditory cue and food reward. Further, the lever cue when added, during the compound cue conditioning phase, to the previously conditioned auditory cue, took conditioned responding away from the auditory cue. These findings suggest that the lever cues were more associable than the auditory cues. Thus further experimental study of the expression of the Kamin blocking effect in animals that exhibit a goal tracking conditioned response is warranted.

Other studies of Kamin blocking using pigeons showed that highly diffuse cues, which lead to the development of goal tracking conditioned responses, interfere with the development of sign tracking conditioned responses to localized cues (Blanchard & Honig, 1976; Leyland & Mackintosh, 1978; Khallad & Moore, 1996). However, diffuse cues do not support the sign

tracking conditioned response because there is no discrete localized cue to direct responding towards, leaving no option but to goal track. Therefore, when diffuse versus discrete cues are used to produce these two types of conditioned responses, there is a problem in the interpretation of the experimental results.

Let us assume that goal tracking and sign tracking form different associative structures and rely on different learning principles. Under these assumptions, it is highly probable that associative structures for each type of learning are acquired simultaneously during classical conditioning. Depending on which associative structure is dominant, either sign tracking or goal tracking conditioned responding is observed. However, diffuse cues do not offer the opportunity to sign track. When cues that do not support a sign tracking conditioned response are used for classical conditioning, it may be expected that even if the associative structure that would otherwise lead to sign tracking is dominant, the conditioned response produced will be goal tracking, simply because that is the only response available. This creates a problem because, under these circumstances, even though the conditioned response is goal tracking, the dominant learning mechanism may be the one that is responsible for the sign tracking conditioned response.

The use of diffuse cues confounds the interpretation of experiments which find similarities between factors controlling goal tracking and sign tracking. For example, Derman et al., (2018) found that the development of a goal tracking conditioned response to diffuse cues blocks the acquisition of a sign tracking conditioned response to localized cues, and vice versa. One interpretation of this finding is that both goal tracking and sign tracking depend on the same reinforcer prediction error mechanism discussed in chapter two. However, if there are indeed two different learning mechanisms in play, goal tracking to a diffuse cue does not necessarily mean that the underlying associative structure dominating learning in these animals is the one acquired through the goal tracking learning mechanism. Thus, even though

the conditioned response is goal tracking, the learning principles being followed may be the same as those underlying the development of a sign tracking conditioned response.

Therefore, during the blocking procedure, both the localized cue and the diffuse cue, even though they elicit different conditioned responses, may engage the same learning principles, resulting in similar blocking effects. However, as proposed above, this experimental result may be due to the confound created by the restrictive nature of the available conditioned responses to the diffuse cue, and thus may not reflect the similarity between the learning mechanisms and associative structures responsible for the acquisition of these two types of conditioned responses in general. It is therefore important to test differences in learning between groups of animals that respond by either goal tracking or sign tracking to the same localized cue. That is, to use cues such that the cue identity is not variable across the goal and sign tracking groups. This chapter reports the results of one such study.

In the experiment reported here, groups of animals that develop either a sign tracking or a goal tracking conditioned response to a lever cue paired with a food pellet reward are investigated for the expression of the Kamin blocking effect. In the previous chapter, lever press was used as the measure of classical conditioning during phase 1 of the Kamin blocking paradigm, and data from animals that did not lever press was not used for data analysis. On further analysis it was observed that a subgroup of the animals that did not develop a lever press conditioned response responded to the paired lever cue by increasing the duration of nose poking into the food magazine, which is a goal tracking response. This naturally occurring group of animals showing a robust goal tracking conditioned response provided an opportunity to test the hypothesis that animals that develop a goal tracking conditioned response do not express the Kamin blocking effect.

4.2 Methods

In experiments 1-3 (chapter 3), the data from only those rats that showed a reliable lever press response in phase 1 was analyzed. Thus, data from only the sign trackers was used for those experiments. However, many of the rats that did not show a lever press response in phase 1 reliably nose poked into the food magazine when the paired lever cue was presented. This is a goal tracking response. This chapter reports on the expression of the Kamin blocking effect in those animals that develop a goal tracking conditioned response to the paired lever cue.

In chapter 2, it was reported that at the time of running the behavioral experiment, both the experimental and control groups of all three experiments had some animals that did not develop a lever pressing conditioned response. These animals were run through the Kamin blocking paradigm but were excluded from the data analysis reported in chapter 3. For the purpose of investigating the expression of the Kamin blocking effect in goal trackers, previously unused data from the animals in the controls groups of experiments 1-3 was utilized.

Among the rats in the control groups of experiments 1-3, 30 rats were excluded from data analysis on the basis of behavioral criteria for development of a lever press conditioned response only to the paired lever cue. Among these 30 rats, those rats that passed the following two behavioral criteria were included into the data analysis as goal trackers.

1) The rats had to satisfy one of the following two conditions assessing conditioned responding to the paired lever.
    a. Relative response index for the paired lever above 0.65 on the last two sessions and above 0.7 on at least one of the last two sessions.

b. Relative response index for the paired lever above 0.7 on the last session and on at least three of the last four sessions.

2) The rats had to satisfy one of the following two conditions to eliminate the development of a conditioned response to the unpaired lever.

a. Relative response index for the unpaired lever below 0.7 on the last four sessions, below 0.65 on at least two of the last four sessions, and below 0.6 on at least one of the last four sessions.

b. Relative response index for the unpaired lever below 0.7 on the last session and below 0.6 on three of the last four sessions.

Out of 30 rats, only 14 rats passed the behavioral criteria for inclusion into the data analysis as rats that developed a goal tracking response to only the lever cue paired with reward (Fig. 4.1A, B). Therefore, there were 14 rats in the goal tracking group.

A total of 26 control rats passed the inclusion criteria for experiments 1-3. These 26 rats developed a sign tracking conditioned response to the paired lever cue. Four of these sign tracking rats, also passed the behavioral criteria for the development of a goal tracking response to the paired lever (Fig. 4.1C, D). Since these 4 rats developed both a robust sign tracking and a robust goal tracking response to the paired lever cue, they were excluded from the sign tracking group. Thus, there were 22 rats in the sign tracking group.

The data from 14 rats that developed only a goal tracking conditioned response to the paired lever cue was analyzed and compared with the 22 rats that developed only a sign tracking conditioned response to the paired lever cue to investigate the expression of the Kamin blocking effect in these two groups of animals.

All experimental procedures are the same as those for the control groups of experiments 1-3, and are described in chapter 3. The statistical testing methods are also the same as those

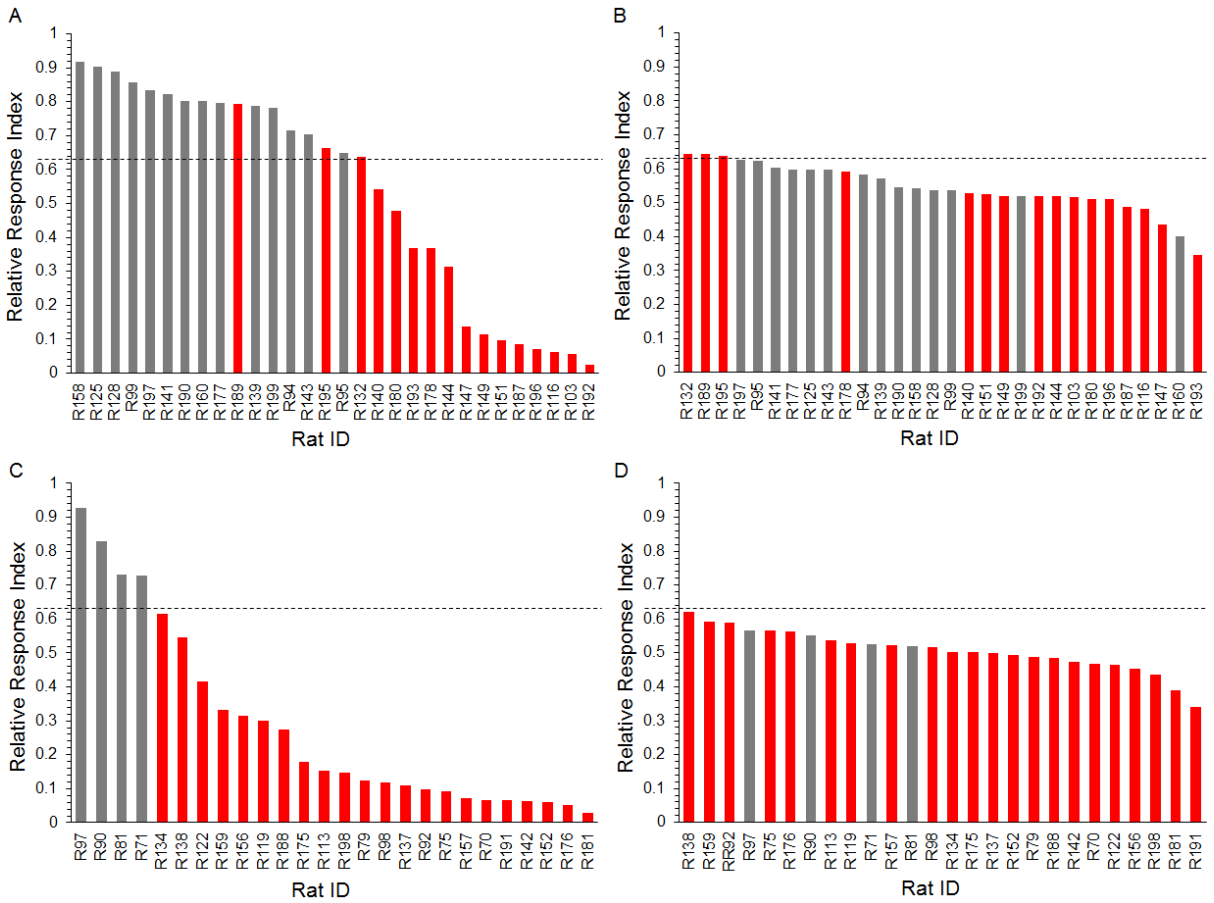

Figure 4.1: **Relative response index averaged across sessions 10-12 of phase 1.** (A, B) Nose poke responses to the paired lever (A) and to the unpaired lever (B) of the 30 rats that were eliminated from the control groups of experiments 1-3 on the basis of behavior. (C, D) Nose poke responses to the paired lever (C) and to the unpaired lever (D) of the 26 control group rats that were included in the data analysis of experiments 1-3. Relative response index to L1 (A, C) was above the dotted line and relative response index to L2 (B, D) was below the dotted line in all animals that passed the behavioral criteria for the development of a discriminatory goal tracking conditioned response to L1 (grey bars). Animals that did not develop a goal tracking response on the basis of behavioral criteria are represented as red bars.

mentioned in chapter 3, except that they were carried out on a different data set where the animals were divided into those that either goal tracked or sign tracked in phase 1 of the Kamin blocking paradigm. In addition, a three way repeated measures ANOVA (Greenhouse-Geisser adjustment), on the relative response index for the goal tracking and

sign tracking group was used to show the development of a nose poking conditioned response during phase 1 in only the goal tracking group.

4.3 Results

Classical conditioning using lever cues paired with a food pellet reward resulted in the development of sign tracking in some animals, and goal tracking in others. Figure 4.2 shows the gradual development of a conditioned response to the lever cue paired with the food reward in both the sign trackers and goal trackers. The sign trackers lever pressed when the lever paired with the food reward was presented and did not lever press when the unpaired lever was presented during phase 1. The goal trackers did not develop a lever pressing conditioned response (Fig 4.2A) but developed a nose poking conditioned response into the food magazine (Fig 4.2B).

Statistical analysis showed that the differences in behavioral patterns apparent in figure 4.2 were statistically significant. A three way mixed ANOVA on the lever press response measure, using the Greenhouse-Geisser adjustment, showed a significant effect of lever cue ($F=51.526$; $p<0.001$), a significant effect of group ($F=110.147$; $p<0.001$), and a significant cue x session ($F=3.449$; $p=0.003$), cue x group ($F=57.825$; $p<0.001$) and cue x session x group ($F=5.516$; $p<0.001$) interaction. A simple main effects comparison using the Bonferroni adjustment showed a significant effect of the lever cue in only the sign trackers ($F=140.477$; $p<0.001$) but not the goal trackers ($F=0.074$; $p=0.787$), showing the development of a lever pressing conditioned response only in the sign trackers. This means that only the sign trackers developed a lever pressing conditioned response in response to the lever cue paired with the reward, while the goal trackers did not develop a lever press response to either of the lever cues.

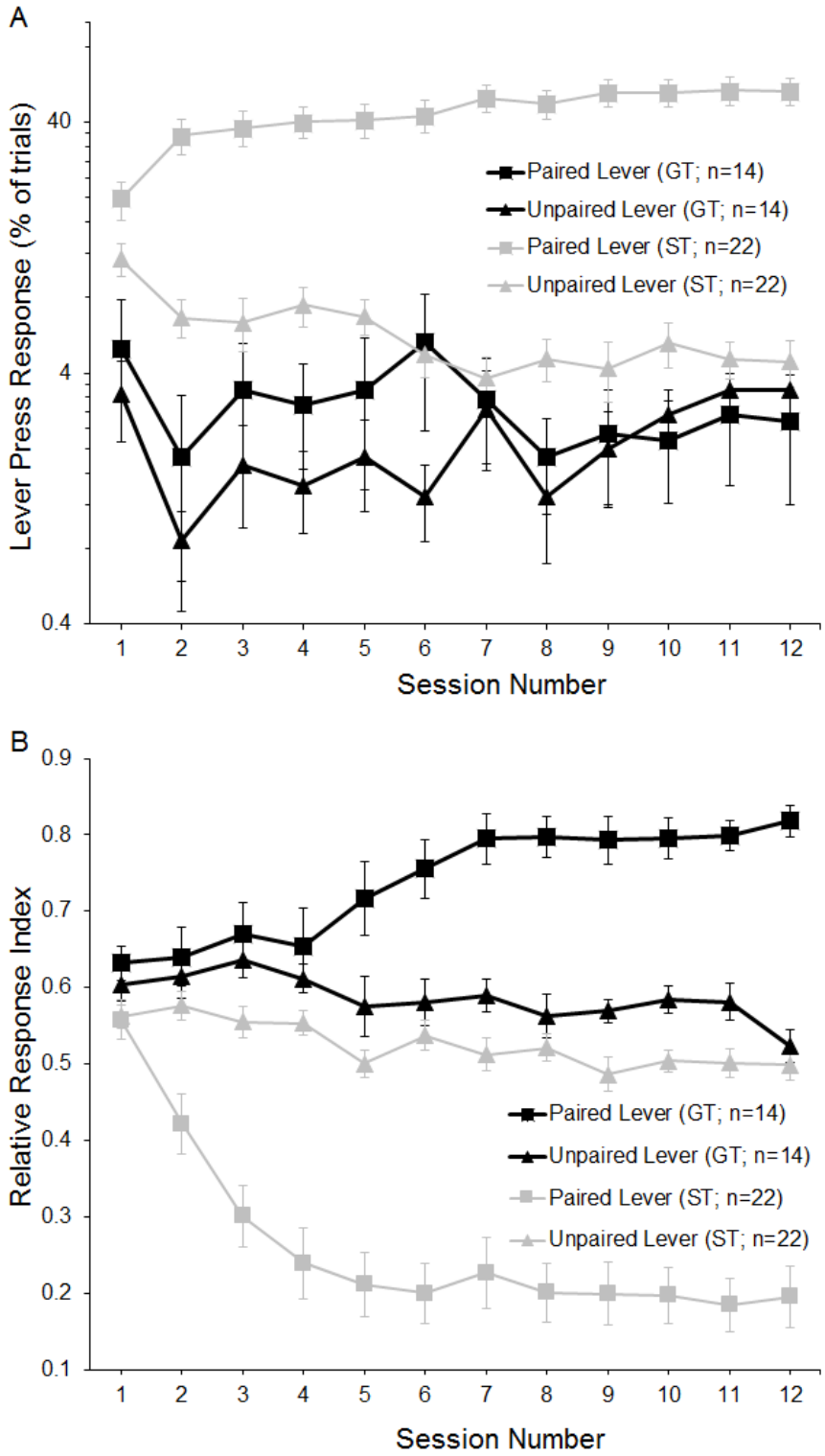

Figure 4.2 **Conditioned responses of the sign tracking and goal tracking groups during phase 1.** (A) Lever press responses of sign trackers and goal trackers during phase 1 of the Kamin Blocking Paradigm. (B) Nose poke responses of sign trackers and goal trackers to lever cues during phase 1. Nose poke responses are normalized to their baselines (response during the 5s immediately preceding the respective lever cue) according to equation 1. Data are presented as mean ± SEM.

Further statistical analysis was necessary to verify the development of a nose poking conditioned response in the goal tracking group. A three way repeated measures ANOVA on the nose poke response measure for the goal trackers and sign trackers, using the Greenhouse-Geissier adjustment, showed a significant main effect of cue ($F=6.608$; $p=0.015$), session ($F=5.317$; $p<0.001$) and group ($F=82.323$; $p<0.001$). In addition, all interactions were also significant – cue x group ($F=92.064$; $p<0.001$), cue x session ($F=3.728$; $p=0.001$), group x session ($F=17.598$; $p<0.001$), and cue x session x group ($F=16.062$; $p<0.001$). A simple main effects comparison (Bonferroni adjustment) showed a significant effect of the lever cue in the goal trackers ($F=20.186$; $p<0.001$). This means that the goal trackers developed a nose poking conditioned response only to the paired lever. There was a significant effect of the lever cue in sign trackers as well ($F=95.144$; $p<0.001$), which occurred because the nose poking response during L1 was suppressed below baseline in this group, as they were interacting with the lever during L1. This means that only the goal trackers developed a nose poking conditioned response in response to the lever cue paired with the reward, while the sign trackers suppressed their nose poke responses to the presentation of L1. There was also a significant effect of session on the nose poke response to L1 in both the goal tracking ($F=3.265$; $p=0.007$) and sign tracking groups ($F=18.911$; $p<0.001$). There was no significant effect of session on nose poke responses to L2 in the sign ($F=1.713$; $p=0.131$) or goal ($F=1.194$; $p=0.342$) tracking groups. This means that, over the course of multiple conditioning sessions, goal trackers developed a nose poke conditioned response and sign trackers developed a suppression in their nose poke response only to that lever which was paired with the food reward.

Both the groups then underwent compound cue conditioning (phase 2). When their response to the presentation of the sound cues alone was tested in extinction (phase 3), the sign trackers increased their nose poke rate from baseline only during the sound that was given as

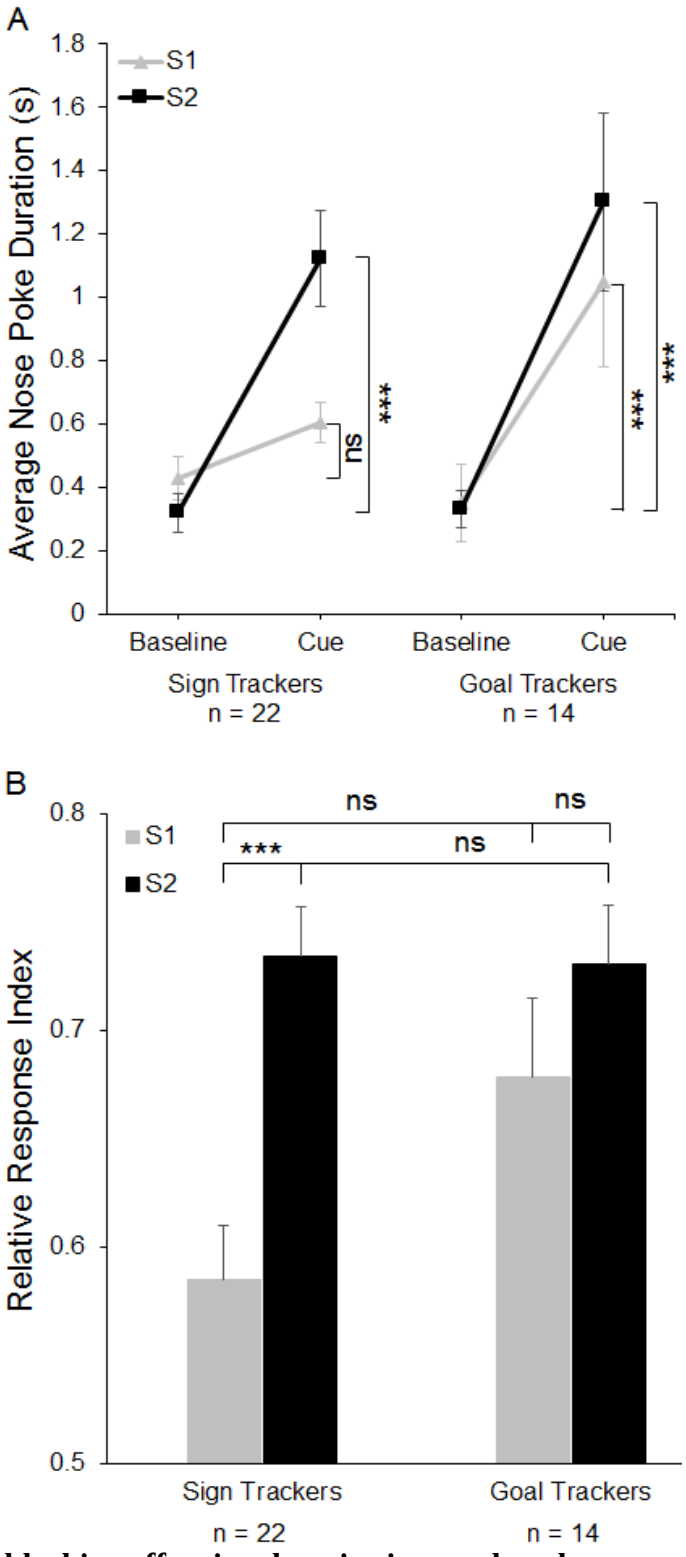

Figure 4.3 **Kamin blocking effect is robust in sign trackers but attenuated in goal trackers.** (A) Nose poke responses for sign and goal trackers when the sound cues are presented in phase 3. Baseline response is measured during the 5s immediately preceding the respective sound cue. Cue response is measured during the cue, which lasts for 5s. (B) Responses to the sound cues normalized (equation 1) to their respective baselines. Data are presented as mean ± SEM.

a compound along with the unpaired lever in phase 2 (S2) ($t=5.463$; $p<0.001$; two way mixed ANOVA – simple main effects – Bonferroni). The sign trackers did not increased their nose poke rate from baseline during the sound cue that was given as a compound along with the paired lever in phase 2 (S1) ($t=1.596$; $p=0.728$) (Fig. 4.3A). These findings show that the sign trackers responded to S2 but not to S1, producing the Kamin blocking effect. The goal trackers responded to both S1 ($t=5.095$; $p<0.001$) and S2 ($t=5.277$; $p<0.001$) (Fig. 4.3A), suggesting that the Kamin blocking effect was attenuated in this group.

In agreement with the foregoing comparison of the raw nose poke scores, when the responses to the sound cues were normalized to their respective baselines (Equation 1 – chapter 3), statistical tests showed an attenuation of the Kamin blocking effect in the goal tracking group. A two way mixed ANOVA showed a significant main effect of cue ($F=14.164$; $p=0.001$). There was no significant effect of group ($F=1.534$; $p=0.224$) or interaction between cue and group ($F=3.306$; $p=0.078$). A simple main effects comparison using the Bonferroni adjustment showed that the sign trackers responded significantly more to S2 than to S1 ($F=20.029$; $p<0.001$) while the goal trackers did not show a difference in their response to S1 and S2 ($F=1.548$; $p=0.222$) during phase 3 (Fig. 4.3B). This means that the sign tracking group showed a robust Kamin blocking effect. The measure of the Kamin blocking effect did not reach significance in the goal tracking group, implying that the effect was attenuated in this group of animals. There was no significant difference in the responses of the two groups to S1 ($F=3.160$; $p=0.084$) and S2 ($F=0.011$; $p=0.917$) (Fig. 4.3B).

These results show that animals that develop a sign tracking conditioned response to the lever cue paired with the food pellet reward during phase 1 express the Kamin blocking effect. In contrast, the blocking effect is attenuated in animals that develop a goal tracking conditioned response to the paired lever cue.

It is important to note here that the pattern of responding to S1 and S2 in the sign and goal tracking groups observed in phase 3 could occur either due to differences in nose poke response during S1 and S2 or due to differences in nose poking during the baseline period of S1 and S2. The baseline period is the 5s immediately preceding the presentation of a sound cue. Nose poking during the 5s immediately preceding S1 was not significantly different from nose poking before S2 in the sign tracking ($t=2.037$; $p=0.297$) and goal tracking groups ($t=0.294$; $p=1.000$). There was also no significant difference between the two groups in their baseline before S1 ($t=0.693$; $p=0.491$) or S2 ($t=0.120$; $p=0.908$). These results show that the pattern of conditioned responding to S1 and S2 observed in phase 3 in the two groups is not due to differences in baseline nose poking.

To check whether the pattern of responding observed in phase 3 was already present in phase 2, a 3-way mixed ANOVA was conducted on the nose poke response to the two compound cues during phase 2 (Fig. 4.4B). A simple main effects comparison using Bonferroni adjustment showed that there was a significant difference in the nose poke response to the two compound cues in the sign trackers ($F=57.898$; $p<0.001$) but not in the goal trackers ($F=2.706$; $p=0.109$). There was also a significant difference between the two groups in their nose poke responses to L1+S1 ($F=143.624$; $p<0.001$), but not in their nose poke responses to L2+S2 ($F=0.070$; $p=0.793$). These differences occurred because nose poke responding during L1+S1 in the sign trackers was suppressed below baseline, just as it was suppressed on L1 presentation during phase 1 in this group. This suppression in nose poke response was not observed in phase 3 in the sign tracking group. Thus, the significant differences observed in phase 2 do not necessarily foreshadow the significant differences observed in phase 3.

The observed suppression in nose poke responding to L1+S1 in the sign tracking group probably occurred due to lever pressing to L1+S1 in this group. In support of this idea, a 3-way mixed ANOVA on lever press responses during phase 2 (Fig. 4.4A), followed by a

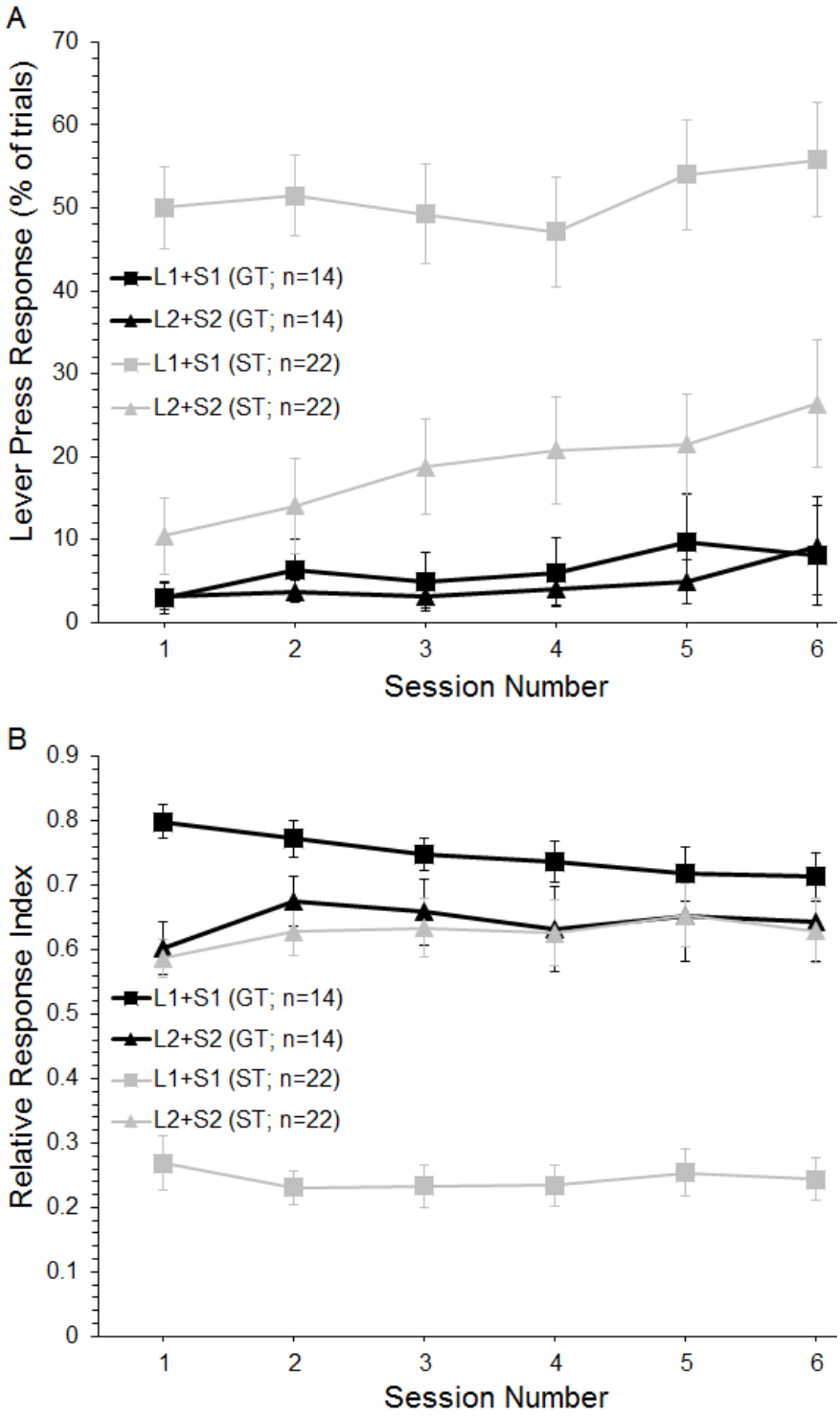

Figure 4.4 **Lever and nose poke responses of the sign tracking and goal tracking groups during phase 2.** (A) Lever press responses of sign trackers and goal trackers to the two compound cues, L1+S1 and L2+S2, during phase 2 of the Kamin Blocking Paradigm. (B) Nose poke responses of sign trackers and goal trackers to the two compound cues lever cues during phase 2. Nose poke responses are normalized to their baselines (response during the 5s immediately preceding the respective lever cue) according to equation 1. Data are presented as mean ± SEM.

simple main effects comparison (Bonferroni adjustment) showed that there was a significant difference in the lever press response to the two compound cues in sign trackers ($F=28.499$; $p<0.001$), but not in goal trackers ($F=0.045$; $p=0.834$). There was also a significant difference between the two groups in their lever press response to L1+S1 ($F=38.563$; $p<0.001$), but not in their lever press response to L2+S2 ($F=3.821$; $p=0.059$). These results suggest that only L1+S1 elicited a lever press conditioned response in the sign trackers. None of the compound cues elicited a lever press conditioned response in the goal trackers.

In addition, figure 4.4A shows an increasing trend in the lever press response to L2+S2 in the sign tracking group across the sessions of phase 2, suggesting that the sign trackers may have been gradually developing a lever press response to L2+S2 during phase 2. However, the effect of session on L2+S2 in the sign tracking group did not reach significance ($F=2.435$; $p=0.057$). There was also no effect of session on the lever press response to L1+S1 in the sign tracking group ($F=1.030$; $p=0.418$) or on the lever press response of the goal tracking group to L1+S1 ($F=0.280$; $p=0.920$) and L2+S2 ($F=0.177$; $p=0.969$).

4.4 Discussion

In this chapter, the expression of the Kamin blocking effect is examined in subjects that develop either a goal tracking or sign tracking conditioned response to the same localized cue. Previously unanalyzed data from experiments 1-3 (chapter 3) was used to investigate the expression of the Kamin blocking effect in animals that develop either a goal tracking or a sign tracking conditioned response to a lever cue paired with a food pellet reward. It was found that, among animals that respond by either sign tracking or goal tracking to the same kind of cue, those that sign track show the Kamin blocking effect while this effect is

attenuated in the goal tracking group. This finding supports the hypothesis that goal trackers do not express the Kamin blocking effect.

Flagel et al. (2011) found that in animals that goal tracking animals, expected reward evoke a robust dopamine response. It was found here that the Kamin blocking effect is attenuated in goal trackers. Conversely, sign tracking animals, in which expected rewards evoke a diminished dopamine response (Flagel *et al.*, 2011), expressed the blocking effect. These findings support the hypothesis that the reduction in dopamine response evoked by the reward when it is expected is necessary for the Kamin blocking effect (Steinberg *et al.*, 2013; Sharpe *et al.*, 2017).

In the introduction of this chapter it was argued, based on the different anatomical substrates of goal tracking and sign tracking conditioned responses, that different learning mechanisms may underlie the development of these two conditioned responses. The experimental finding of a difference in the expression of the Kamin blocking effect between animals that either goal track or sign track to the paired lever cue further suggests that different learning mechanisms underlie the development of these two conditioned responses. A corollary is that only the learning mechanism underlying the sign tracking response supports the Kamin blocking effect, while the learning mechanism underlying the goal tracking response does not support the Kamin blocking effect.

As argued in the introduction of this chapter, two different associative structures may underlie sign and goal tracking conditioned responding. Both these associative structures may be acquired simultaneously during classical conditioning, and the nature of the final conditioned response may be decided by which associative structure is dominant. This means that even if the expressed conditioned response is goal tracking, some learning in the sign tracking system is also expected. If we further follow the argument that only the sign tracking

learning mechanism supports the Kamin blocking effect, it would be expected during phase 2 that, within each animal, the sign tracking system acquires an association between the added cue and the reward depending on the extent of learning in the sign tracking system during phase 1. On the other hand, during phase 2, the goal tracking system will acquire an association between the added cue and the reward irrespective of the amount of learning in this system during phase 1.

Following the foregoing argument, in the goal tracking group, those animals that respond via the goal tracking system in phase 3 will not express the Kamin blocking effect. However, this is only valid if, once the system being used for conditioned behavior is determined by neural processes, the conditioned responding via the chosen system is completely independent of influences from the other system. In the case that the sign tracking system influences conditioned responding via the goal tracking system, the following argument will apply.

Some animals in the goal tracking group may respond via the sign tracking system in phase 3. Learning about the added cue in the sign tracking system in phase 2, and thus responding via this system to the added cue when presented alone in phase 3, depends on the amount of learning about the first cue in the sign tracking system during phase 1. Learning in the sign tracking system during phase 1 in the goal tracking group, though limited, is expected to vary between subjects. Thus, it is expected that goal trackers responding via the sign tracking system in phase 3 will show different degrees of attenuation of the Kamin blocking effect. Further, as mentioned previously, if the sign tracking system influences conditioned responding via the goal tracking system, goal trackers responding in phase 3 via the goal tracking system will also show different degrees of attenuation of the Kamin blocking effect. Therefore, it may also be expected that some of the goal tracking animals may actually express the Kamin blocking effect. In agreement with this idea, some of the animals in the goal tracking group in this experiment showed the Kamin blocking effect (Appendix 1).

Similarly, some animals in the sign tracking group may respond via the goal tracking system during phase 3. The goal tracking system would have acquired an association between the added cue and the reward because the goal tracking system does not support blocking. Thus, animals in the sign tracking group responding via the goal tracking system in phase 3 are expected to show an attenuation of the Kamin blocking effect. In agreement, some of the animals in the sign tracking group did not express the Kamin blocking effect (Appendix 1).

The findings reported in this chapter are discussed further in the general discussion (chapter 5).

5. General Discussion

The main findings reported in this thesis are that blocking inhibition in the ventral tegmental area or inactivating the nucleus accumbens neurons during compound cue conditioning attenuates Kamin blocking. Inactivating the nucleus accumbens during single cue conditioning also attenuates Kamin blocking. Taken together, these findings suggest that inhibition in the ventral tegmental area, inhibitory output from the nucleus accumbens, and learning in the nucleus accumbens play crucial roles in the Kamin blocking effect. In addition, the current study also found that only sign tracking animals express Kamin blocking. The blocking effect is absent in goal tracking animals.

The new findings reported in this thesis provide insight into the biological and psychological nature of the associative structures acquired during classical conditioning. In this chapter, the implications of the foregoing findings for theory of associative learning, and the underlying neural mechanisms are discussed. In section 5.1, the observation that Kamin blocking is absent in goal trackers is discussed with respect to the prediction error explanation of the blocking effect. Section 5.2 discusses another explanation for the deficit in blocking in goal trackers which is not discussed in section 5.1. Section 5.3 discusses the implications of the finding that goal trackers do not express the Kamin blocking effect on future attempts at demonstrating the blocking effect. In section 5.4, the possible roles of the core and shell subdivisions of the nucleus accumbens in the Kamin blocking effect are discussed. Section 5.5 extends a previously proposed (Aggarwal *et al.*, 2012) neural mechanism for reducing the reward evoked dopamine response when rewards are expected. Section 5.6 discusses the overall significance of the findings reported in this thesis. Lastly, section 5.7 discusses future research directions to build on the findings and ideas reported in this thesis.

5.1 Goal trackers and the prediction error learning explanation of the Kamin blocking effect

The discovery of the Kamin blocking effect suggested that learning occurs only when outcomes deviate from expectations, that is, when there is an error in prediction. The prediction error explanation of the Kamin blocking effect has implications at two levels. One is at the neurophysiological level, in which midbrain dopamine neuron activity is assumed to represent reward prediction error. The other is at the level of behavioral theory. At this level, prediction error is abstract (not embodied) and is computed by cognitive processes. The present result that goal trackers do not express the Kamin blocking effect is consistent with the dopamine reward prediction error explanation of the blocking effect. However, this finding poses problems for the prediction error based theoretical explanation of the Kamin blocking effect for the following reason.

During classical conditioning, the cue being conditioned can acquire both incentive and predictive properties. Incentive properties are demonstrated by eliciting approach and interaction with the cue, invigorating ongoing instrumental actions (Pavlovian-instrumental transfer) and acting as a reinforcer (secondary reinforcer). Predictive properties are demonstrated by eliciting approach and interaction with the reward location, and eliciting responses specific to the reward, such as licking or gnawing. Evidence suggests that there is a difference in which of these properties is acquired by the conditioned cue in goal and sign trackers.

Conditioned cues develop incentive properties in sign trackers, but not in goal trackers. Conditioned cues elicit approach and interaction with the cue in sign trackers but not in goal trackers. Instead, in goal trackers, conditioned cues elicit approach and interaction with the expected location of reward delivery, such as the food tray. Further, conditioned cues act as a

secondary reinforcers only in sign trackers but not in goal trackers (Robinson & Flagel, 2009). These findings suggest that conditioned cues develop incentive properties only in sign trackers, and not in goal trackers.

On the other hand, conditioned cues develop predictive properties in both sign trackers and goal trackers. This is evidenced by the finding that conditioned cues elicit reward specific responses, such as licking or gnawing, in both goal trackers and sign trackers (Davey & Cleland, 1982; Flagel *et al.*, 2011; Derman *et al.*, 2018).

The foregoing arguments suggest that in sign trackers conditioned cues develop both incentive and predictive properties. In goal trackers conditioned cues develop only predictive properties (Fig 5.1). However, although the conditioned cue develops predictive properties in goal trackers, the present study found that goal trackers do not express the Kamin blocking effect. This finding seems to contradict the prediction error based theoretical explanation of the blocking effect. In order to unravel this contradiction, one needs to examine what needs to be predicted in order to block conditioning of the added cue, and thus cause Kamin blocking.

Conditioned Cue	Sign Trackers	Goal Trackers
Incentive Properties	Yes	No
Predictive Properties	Yes	Yes

Figure 5.1: **Differences in the development of incentive and predictive properties of the conditioned cue in sign and goal trackers during classical conditioning.**

Evidence indicates that blocking depends on the conditioned cue providing an accurate estimate of the magnitude of the reward induced arousal of a general affective state. Blocking does not depend on the accuracy of prediction of the specific sensory and perceptual features

of the reward. Blocking procedures typically use the same reward during phase 1 and phase 2 of the Kamin blocking procedure. However, blocking occurs even when the rewards used in phase 1 and phase 2 are different flavored food pellets (Burke *et al.*, 2007), or relevant to different deprivation states (hunger, thirst, etc), for example food and water (Ganesan & Pearce, 1988a). Thus, the degree of blocking does not depend on the prediction of the sensory specific features of the reinforcer. Instead, these findings suggest that rewards activate an affective state general to all rewards, and the activation of this general affective state by the prior conditioned cue is responsible for the Kamin blocking effect (Dickinson & Dearing, 1979; Ganesan & Pearce, 1988a; Dickinson & Balleine, 2002; Balleine, 2005). In addition, blocking can be abolished by simply increasing the magnitude of the reinforcer from phase 1 to phase 2, for example, giving one food pellet as the reward in phase 1 and three food pellets as the reward in phase 2 (Holland, 1984). Thus, the magnitude of reward induced arousal of an affective state general to all rewards needs to be accurately predicted for Kamin blocking to occur.

The preceding argument suggests one way to reconcile the prediction error based theoretical explanation of the blocking effect and the finding that goal trackers do not express Kamin blocking despite the conditioned cue developing predictive properties in goal trackers. In goal trackers the conditioned cue acquires predictive properties relating to the sensory specific properties of the reward, thus eliciting a conditioned response consisting of an approach towards the reward location, and reward specific responses such as licking or gnawing. However, the conditioned cue fails to provide an accurate estimate of the magnitude of reward induced arousal of the general appetitive affective state in goal trackers. Thus, the goal trackers do not express the Kamin blocking effect. This explanation can be further elaborated, in terms of the underlying associative structures acquired as a result of learning during classical conditioning.

According to the Konorskian model of the associative structures underlying Pavlovian incentive learning (Fig. 5.2), when a cue is conditioned, it forms an association not only with the general affective state the reinforcer activates (appetitive or aversive), but also with the sensory and perceptual features of the reinforcer representation (Dickinson & Dearing, 1979; Dickinson & Balleine, 2002; Balleine, 2005). The presentation of the conditioned cue can then activate affective processes via either its direct link with the affective state or via the activation of the reinforcer representation, the latter being gated by the presence of the relevant motivational state (hunger, thirst, etc). It is possible that while the goal trackers form an association between the conditioned cue and the sensory and perceptual features of the reward, they form a weak or non-existent association between the conditioned cue and the general affective state activated by the reward. In case of such an associative structure, the conditioned cue would give rise to an underestimate of the affective arousal induced by the reward. This underestimate would result in an increase in prediction error when the expected

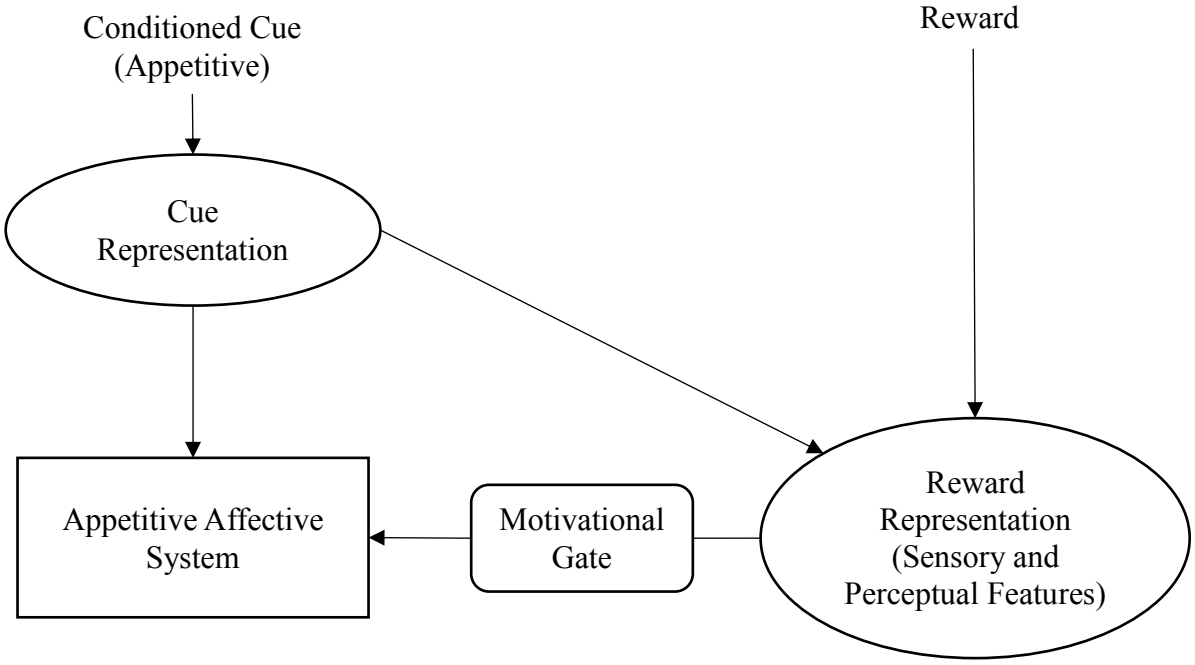

Figure 5.2: **Simplified version of the Konorskian model** showing the two different pathways through which a conditioned cue, conditioned using an appetitive classical conditioning procedure, can activate the appetitive affective system.

reward is delivered, leading to conditioning of the added cue in phase 2 in goal trackers. An explanation for the weakness, in goal trackers, of the acquired association between the conditioned cue and the general affective state activated by the reinforcer is elaborated in the following arguments.

In chapter four, it was argued that goal tracking and sign tracking behavior have different neural substrates, suggesting that different learning systems are responsible for the acquisition of the sign tracking and goal tracking behaviors. Lesaint et al., (2014) described a model framework that uses two parallel learning systems to account for the development of sign tracking and goal tracking behaviors in different individuals undergoing the same experimental paradigm. Their framework also results in the conditioned cue giving rise to an underestimate of the reward value in goal trackers. Here, it is argued that their framework also offers an explanation for the weakness, in goal trackers, of the association between the conditioned cue and the general affective state activated by the reward.

Lesaint et al., (2014) argued that sign trackers focus their attention on the lever (when the lever extends) as well as the food magazine (once the lever retracts) prior to reward consumption and thus assign value to both the lever and the magazine. The goal trackers focus their attention only on the magazine prior to reward consumption and thus assign value only to the magazine. The prediction about the value of the reward is based on the current value of the cue in focus when the reward is delivered i.e. the lever for sign trackers and the magazine for goal trackers. They further argue that unrewarded interactions with the magazine during the inter trial interval (ITI) drive down the value of the magazine during the ITI. The lever is not present during the ITI, leaving the value associated with the lever unchanged during the ITI. Thus, in goal trackers, which use the magazine value to estimate the value of the upcoming reward, the asymptote of reward magnitude prediction is reached when the increment in magazine value, due to the reward prediction error generated when the

expected reward is received, exactly cancels the decrement in magazine value during the ITI. This results in a permanent underestimate of the reward magnitude in goal trackers. Here, it is argued that these unrewarded interactions with the magazine during the ITI also weaken the strength of the associations between the magazine and i) the general affective state activated by the reward, and ii) the sensory and perceptual features of the reward representation.

The model proposed by Lesaint et al., (2014) offers a simple explanation for the observation that goal trackers do not express the Kamin blocking effect. Their model also makes several, behaviorally testable predictions (Lesaint *et al.*, 2015). Some of these are, in goal trackers, i) decreasing the ITI should increase the asymptotic reward magnitude prediction and thus reduce the asymptotic reward prediction error at the time of delivery of the expected reward; ii) increasing the ITI should lead to a larger reduction in magazine value during the ITI, resulting in a smaller asymptotic prediction of reward value and thus an increase in the asymptotic reward prediction error at reward delivery; and iii) removing the food magazine during the ITI should increase asymptotic reward prediction to the level of the value of the reward being used for the conditioning procedure, and thus abolish the reward prediction error when the predicted reward is delivered.

The predictions made by Lesaint and colleagues (Lesaint *et al.*, 2014; Lesaint *et al.*, 2015) can be extended to make predictions about the expression of the Kamin blocking effect. First, decreasing the ITI should increase the incidence of the blocking effect in goal trackers, and vice-versa. Second, removing the magazine during ITI should result in the expression of the Kamin blocking effect in goal trackers. A third prediction can be made from the theoretical basis for Lesaint and colleagues modelling studies. They argued that unrewarded interactions with the magazine during the ITI drive down its value and thus lead to a permanent underestimate of the reward value. This argument can be extended to argue that unrewarded interactions during the presence of the lever cue also drive its value down. It follows that

increasing the duration of the lever cue should result in a larger decrement in the value of the lever through unrewarded interactions, and thus result in a larger permanent underestimate of the reward value. Such an increase in the dopamine reward prediction error signal evoked by expected rewards has been observed when longer duration cues are used for classical conditioning procedures (Fiorillo *et al.*, 2008). The foregoing argument leads to the prediction that increasing the duration of the lever cue should decrease the incidence of Kamin blocking in sign trackers. Experiments designed to test these predictions are needed to shine further light on the neural mechanisms underlying associative learning.

The hypothesis developed here posits that goal trackers show a deficit in blocking because of what is learnt, or not learnt, during the single cue conditioning phase. From the associative learning theory point of view, it is argued that blocking is absent in goal trackers because they fail to form a robust association between the cue and the general affective properties of the reinforcer, and thus fail to accurately predict the magnitude of reinforcer induced affective arousal. From the neurophysiological substrates of the prediction error signal point of view, it is argued that the reduction in the dopamine response evoked by a reward when it is preceded by the conditioned cue is critical for the expression of blocking, and goal trackers do not show blocking because expected rewards elicit a robust dopamine response in these animals.

To address how causal any criteria are in generating blocking, it is necessary to turn goal trackers into sign trackers to see if they still don’t show blocking and so directly test the hypothesis being developed here. Following either the associative learning theory or the neurophysiological dopamine reward prediction error signal explanation of the absence of blocking in goal trackers, instantaneously converting goal trackers into sign trackers should not lead to blocking in the goal trackers because instantaneous conversion does not change what has been learnt. On the other hand, after the emergence of the goal tracking response,

gradually converting goal trackers into sign trackers by manipulating learning should lead to blocking in this group of converted sign trackers.

Gradual conversion of goal to sign tracking response can be done by manipulating experimental parameters to bias responses towards sign tracking for subsequent conditioning sessions until the goal tracking response changes to a sign tracking response. For example, increasing the inter-trial interval has been shown to increase the instance of sign tracking behavior (Lee *et al.*, 2018).

It might be possible to obtain instantaneous conversion from goal to sign tracking using the following manipulations. It has been suggested that the dopamine response elicited by conditioned cues in sign tracking animals is motivating and endows the conditioned cue with incentive salience, making it attractive and causing the animals to approach and interact with the cue (Flagel *et al.*, 2011). The conditioned cue does not elicit a dopamine response in goal trackers (Flagel *et al.*, 2011). Thus, one way to instantaneously convert goal tackers into sign trackers might be to stimulate the VTA dopamine neurons at the time of presentation of the conditioned cue. The ventral pallidum is also more active on cue presentation in sign trackers than in goal trackers (Ahrens *et al.*, 2016; Ahrens *et al.*, 2018), and inhibiting the ventral pallidum increases goal tracking behavior (Chang *et al.*, 2015). Thus exciting the ventral pallidum at the time of presentation of the conditioned cue may also change goal trackers into sign trackers.

5.2 An alternative explanation for the deficit in blocking in goal trackers

In learning based accounts of Kamin blocking, the sum total of the prediction made by all the cues present before reinforcer delivery is used to calculate the prediction error at the time of reinforcer delivery. This common prediction error term is used to update the associative

strength of each of the reinforcer preceding cues. A critical feature of the learning based explanation of blocking is the use of this common error term.

Another explanation for the deficit in blocking in goal trackers is as follows. Sign trackers use a common error term to update reinforcer predictions, thus showing blocking. On the other hand, goal trackers do not rely on prediction errors for updating associative strengths, and that temporal contiguity is sufficient for conditioning in goal trackers. Thus the goal trackers fail to show blocking.

This alternative explanation can be tested by observing the effect of contingency on the goal tracking conditioned response to localized cues. The prediction would be, that after the development of a goal tracking response to a localized cue, contingency degradation by increasing the probability that the reinforcer occurs in the absence of the cue without changing the probability of reinforcer occurrence in the presence of the cue, will have no effect on the conditioned response.

5.3 Goal trackers do not express the Kamin blocking effect – implications for future attempts at demonstrating the Kamin blocking effect

Recently, Maes et al. (2016) reported several failures to replicate the Kamin blocking effect. The authors suggested that the conditions under which the Kamin blocking effect applies still need to be unraveled. The implications of the present finding – that Kamin blocking is attenuated in goal trackers – are as follows:

First, experiments involving the demonstration of the Kamin blocking effect should only use those subjects that develop a sign tracking response during phase 1. Subjects that develop a goal tracking response in phase 1 should not be used for Kamin blocking procedures because the Kamin blocking effect is expected to be attenuated in these subjects.

Second, following from the foregoing condition, it is essential to use procedures in which sign tracking and goal tracking responses can be distinguished during phase 1, when attempting to demonstrate the Kamin blocking effect. This necessitates the use of only localized cues during phase 1 (see section 4.1). Further, to study Kamin blocking in the context of aversive conditioning, it is important to ascertain which conditioned responses acquired during aversive classical conditioning correspond to the sign and goal tracking responses seen in appetitive classical conditioning.

Lastly, it is important to note that in the experiment reported in this thesis, not all goal trackers showed an attenuation in Kamin blocking, and not all sign trackers expressed the Kamin blocking effect (see appendix 1). This within group variation in the expression of the Kamin blocking effect is expected, as explained in section 4.4, and may at times interfere with attempts to demonstrate blocking in sign tracking subjects, resulting in the measure for the blocking effect not reaching significance.

The finding that the Kamin blocking effect is attenuated in goal trackers provides insight into some of the conditions necessary for blocking to occur, and thus informs future demonstrations of the blocking effect.

5.4 The role of the subdivisions of the nucleus accumbens in the expression of the Kamin blocking effect

The new results reported in this thesis suggest that learning in the nucleus accumbens in phase 1 and the output of the nucleus accumbens in phase 2 are both necessary for the expression of the Kamin blocking effect. The nucleus accumbens consists of the core and shell subregions, and the experimental manipulations used in the experiments described in this thesis affect both the core and the shell. However, the core and shell subregions play

distinct roles in reward related behavior and have different anatomical connections. Thus it is important to consider how the differences between the core and shell subregions of the nucleus accumbens relate to the expression of the Kamin blocking effect.

Anatomically, the NAc shell primarily projects to the VTA while the NAc core projects primarily to the substantia nigra (Groenewegen *et al.*, 1999). This puts the NAc shell output in a unique position to directly modulate the firing of the dopamine neurons in the VTA.

Behaviorally, studies of Pavlovian to instrumental transfer, which measure the effects of presenting classically conditioned cues on ongoing instrumental responding, suggest differences between the core and shell sub regions of the accumbens in mediating the effects of conditioned cues on behavior. Corbit and Balleine (2011) found that lesions of the NAc core abolished general Pavlovian to instrumental transfer while those of the shell abolished outcome specific Pavlovian to instrumental transfer. Further, disrupting the communication between the basolateral amygdala and the nucleus accumbens shell abolished specific Pavlovian to instrumental transfer, while disrupting the connections between the central nucleus of the amygdala and the nucleus accumbens core abolished general Pavlovian to instrumental transfer (Corbit & Balleine, 2005; 2011). These results taken together suggest that the general arousing effects of Pavlovian cues are mediated by the central nucleus of the amygdala – nucleus accumbens core pathway, whereas the basolateral amygdala – nucleus accumbens shell pathway mediates the arousing effects of the specific outcome representations activated by the Pavlovian cues.

In relation to the Konorskian model described earlier (Fig. 5.2), the foregoing arguments suggest that the nucleus accumbens core pathway may directly link the conditioned cue and the affective state associated with the reward. On the other hand, the nucleus accumbens shell pathway may underlie the motivationally gated cue induced activation of the affective state

via an activation of the reward representation. In this formulation, one would expect both the nucleus accumbens core and the shell to play a role in the expression of the Kamin blocking effect, since the total affective arousal depends on both these structures. In support of the view that Kamin blocking depends on both the outcome specific as well as general affective representations triggered by the conditioned cue, Burke et al. (2007) found that conditioned reinforcement can be mediated by the activation of either of these representations.

Given these anatomical, behavioral and theoretical considerations, further experiments should test if the core and shell play different roles in controlling the inhibitory input to the VTA dopamine neurons and in computing the prediction error during classical conditioning, and in the expression of the Kamin blocking effect.

5.5 A mechanism for acquiring the timed inhibition of the dopamine neurons

In chapter 2, it was argued that a net increase in inhibitory input to the dopamine neurons at the time when reward delivery is expected counteracts the excitation produced by the reward, and reduces the dopamine response evoked by the reward when it is expected. The dopamine response evoked by the reward, when preceded by the cue being conditioned, declines gradually as classical conditioning progresses, suggesting that the strength of the timed inhibition of the dopamine neurons increases gradually over the course of classical conditioning. This inhibitory input is time-locked to the occurrence of the reward from the very beginning of the learning process (Pan *et al.*, 2005; 2008), implying that the acquisition of the timing does not involve gradual temporal shift.

Aggarwal et al., (2012) (appendix 2) proposed a learning mechanism by which such an increase in timed inhibitory input to the dopamine neurons may be acquired gradually over the course of classical conditioning. This mechanism is extended in the following arguments.

Experimental findings and theoretical models suggest that the presentation of a cue generates sequential neural activity unique to the cue, both in the striatum (Matell *et al.*, 2003; Carrillo-Reid *et al.*, 2008; Jin *et al.*, 2009; Ponzi & Wickens, 2010; Bakhurin *et al.*, 2017) and in the cortex (Matell *et al.*, 2003; Jin *et al.*, 2009; Kim *et al.*, 2013; Bakhurin *et al.*, 2017). Modelling studies suggest that such sequential activity in the cortex and the striatum results in sequential input-output activity in cortico-striatal synapses unique to the cue (Ponzi & Wickens, 2012; Ponzi & Wickens, 2013). This cue driven sequential cortico-striatal activity has been suggested to depend on the strength of the cue driven cortical inputs to the striatum (Ponzi & Wickens, 2012). Here, it is suggested that the strengthening of cue driven cortical inputs to the striatum by reward induced dopamine transients increases the robustness of the cue generated sequential input-output activity in the cortico-striatal synapses. The following arguments detail how these two processes — the dopamine mediated strengthening of the cue driven cortical inputs to the striatum, and the cue generated sequential input-output activity in the cortico-striatal synapses unique to the cue — together produce the gradually increasing timed inhibition of dopamine neurons at the expected time of reward delivery.

The current extension of the mechanism proposed by Aggarwal et al., (2012) — to account for the timed inhibition of dopamine neurons – makes three assumptions. First, the synaptic strength of the cortical inputs to the striatum is modified according to the three factor rule (Wickens & Kotter, 1995; Reynolds & Wickens, 2002; Izhikevich, 2007; Yagishita *et al.*, 2014). The three factor rule predicts that conjunction of pre and post synaptic activity will strengthen the synapse only in the presence of the phasic dopamine signal. In the absence of this phasic dopamine signal, the same activity pattern results in a depression of synaptic strength. Thus, dopamine transients evoked by a reward result in incremental strengthening of only those inputs to the striatum which are active in conjunction with post synaptic activity at the time of reward delivery.

The second assumption of the proposed mechanism is that at the very beginning of the first classical conditioning session, at least some of the cortico-striatal input-output units directly activated by cue presentation are also active at the time of reward delivery.

The third assumption is that the robustness of the cue driven sequential input-output activity in the cortico-striatal synapses is dependent on the strength of the cue driven cortical inputs to the striatum (Ponzi & Wickens, 2012). Further, increasing robustness of cue generated sequential activity will lead to better conservation of cue generated sequential cortico-striatal activity across separate instances of cue presentation. Improvement of inter-trial conservation means that more of the cortico-striatal input-output units active at time t after cue presentation will be the same on every instance of cue presentation. However, external perturbations, such as inputs unrelated to the cue, will adversely affect inter-trial conservation of cue generated sequential input-output activity. The effects of random perturbations on inter-trial conservation of cue generated input-output activity in cortico-striatal synapses, and on learning of timed inhibition of dopamine neurons, will be discussed later in this section.

The following arguments, using the three foregoing assumptions, describe a mechanism by which the gradually increasing timed inhibition of dopamine neurons at the expected time of reward delivery can be acquired over the course of classical conditioning.

When a cue precedes reward delivery by a fixed duration, cue driven cortical inputs to the striatum active at the time of reward delivery (assumption 2) get incrementally strengthened by the reward induced phasic dopamine transient according to the three factor rule (assumption 1). Over several cue-reward pairings, the incremental strengthening of cue driven cortical inputs to the striatum add up, and result in an increase in the inter-trial conservation of the cue generated sequential input-output activity in the cortico-striatal synapses (assumption 3). As this process gradually strengthens the cue driven cortical inputs

to the striatum, the trial to trial changes or adjustments in the sequential activity generated by the cue become smaller and smaller, until the sequential activity becomes robust and conserved across trials.

In parallel, with the increase in robustness and inter-trial conservation of the cue generated sequential input-output activity in the cortico-striatal synapses, increasingly the same cortico-striatal input-output units are active at time t after cue presentation and therefore at the time of reward delivery (which always occur after a fixed time delay after cue presentation). Thus, increasingly, the same striatal input-output units are active on multiple instances of reward delivery, and the incremental strengthening (by the reward induced dopamine transient) of these inputs gradually adds up. This gradual strengthening of the cue generated input-output activity in the striatum present at the time of reward delivery increases the striatal throughput at the expected time of reward delivery. The increased striatal throughput either directly or indirectly results in the inhibition of the dopamine neurons at the expected time of reward delivery. During this neuronal learning process, the striatal throughput of the now robust cue generated activity pattern is maximally increased specifically at the time of reward occurrence, thus automatically timing the inhibition of the dopamine neurons to the expected time of reward delivery. An asymptote in the strengthening of the synapses is automatically reached when the timed inhibition onto the dopamine neurons completely abolishes the dopamine transients evoked by the reward.

To summarize, the foregoing mechanism proposes that each input-output unit in the striatum active at the time of reward delivery is strengthened by the reward induced phasic dopamine transient in accordance with the three factor rule (defined earlier in this section). This process first leads to the strengthening of the cortical inputs to the striatum activated by the cue, which results in increasing the robustness and inter-trial conservation of the cue generated sequential input-output activity in the cortico-striatal synapses. The increase in inter-trial

conservation of cue generated sequential activity, increases the number of same input-output units active at the time of reward delivery across trials. Thus, increasingly, the same input-output units are strengthened by the reward induced dopamine response. The co-occurrence of these input-output units is always maximum at the time of reward delivery — which always occurs a fixed delay after cue presentation — due to the inter-trial conservation of the cue generated sequential input-output activity in the striatum, and therefore the striatal throughput in increased specifically at the (expected) time of reward delivery. An asymptote in the increase of the timed inhibition of dopamine neurons is automatically reached when the timed inhibition onto the dopamine neurons completely abolishes the dopamine transients evoked by the reward.

The foregoing mechanism accounts for the gradual increase in the strength of inhibition of dopamine neurons as classical conditioning progresses and also accounts for the occurrence of the specific timing of this inhibition of dopamine neurons to the expected time of reward delivery. However, this mechanism relies heavily on the development of robust cue generated sequential input-output activity in the cortico-striatal synapses and its inter-trial conservation. Both the development of robust sequential activity, and its inter-trial conservation, are adversely affected by random inputs unrelated to the cue.

The cue generated activity is overlaid on an ongoing dynamic of input-output activity in the striatum. This ongoing dynamic in the striatum is expected to result in random inputs to the striatum unrelated to the cue which will interfere with the cue generated input-output activity in the striatum. The effect of this interference on the cue generated input-output activity in the striatum is expected to add up as more time elapses after cue presentation. Thus, the probability that a given number of input-output units in the striatum active at time t after cue presentation are a product of cue driven activity decays as time elapses from cue presentation.

The effect of this decay will be more prominent at the beginning of classical conditioning, since at that time the cue driven inputs to the striatum would have yet to undergo strengthening and would thus be relatively weak. This implies that the probability that a given number of cortico-striatal input-output units directly activated by cue presentation are also active at time t after cue presentation will be smaller at time points further away from the time of cue presentation (for larger t).

Following from the foregoing argument, the probability that a given number of cortico-striatal input-output units directly activated by cue presentation are also active at time of reward delivery (assumption 2) will be smaller as longer fixed delays between cue onset and reward delivery are used for classical conditioning. Thus, at longer fixed delays, fewer number of cue driven cortical inputs to the striatum will get incrementally strengthened on every trial, leading to slower per trial addition of the incremental strengthening of the cue driven cortical inputs to the striatum. This will lead to a slower increase in the robustness and inter-trial conservation of the cue generated sequential input-output activity in the cortico-striatal synapses (assumption 3). Therefore, at longer fixed delays between cue onset and reward delivery, the per trial rate of increase － of the number of same input-output units active at the time of reward delivery across trials － will be slower. This will result in slower strengthening of the cue generated input-output activity present in the striatum at the time of reward delivery, and will reduce the per trial rate of increase of the timed inhibition of the dopamine neurons.

The foregoing argument suggests that conditioning using longer time intervals between cue onset and reward delivery will result smaller increment, per trial, of the reduction of the dopamine response evoked by the reward when it is expected. Thus, the current extension of the mechanism proposed by Aggarwal et al., (2012) predicts that more cue-reward pairing

trials will be needed to extinguish the dopamine response evoked by the expected reward when longer time intervals between cue onset and reward delivery are used for conditioning. A corollary of this prediction is that, when the number of cue-reward pairings are held constant, the dopamine response evoked by the reward when preceded by the cue being conditioned will be greater when conditioned using longer time intervals between cue onset and reward delivery.

In support of the current extension of the mechanism proposed by Aggarwal et al., (2012) for learning of the timed inhibition of dopamine neurons, Fiorillo et al. (2008) found that expected rewards evoke a larger dopamine response after a given number of cue-reward pairings when longer fixed time intervals between cue onset and reward delivery are used for conditioning. Fiorillo et al. (2008) conditioned 4 visual cues to the same reward such that the reward was delivered after a particular fixed delay from the onset of the cue. This delay was varied from 1-16s depending on the conditioned cue, that is, one cue was conditioned using a one second fixed delay, another cue was conditioned using a two second fixed delay, and so on. Each cue received an equal number of pairings with the reward.

After more than 600 pairings of each cue with the reward, Fiorillo et al. (2008) found that the dopamine response evoked by the reward was much higher when preceded by the cue conditioned using longer fixed delays between cue onset and reward delivery than when preceded by cues conditioned using shorter fixed delays. Thus, when the number of cue-reward pairings are held constant, the reduction in dopamine response evoked by the expected reward is inversely related to the duration of the cue-reward time interval used for conditioning. This finding supports the current extension of the mechanism proposed by Aggarwal et al., (2012) for neuronal learning of the timed inhibition of dopamine neurons at the expected time of reward delivery, although other explanations and mechanisms cannot be excluded without direct evidence.

A second prediction can be made from the current extension of the proposed mechanism (Aggarwal *et al.*, 2012) and is elaborated as follows. As mentioned earlier, the foregoing mechanism relies on the development and inter-trial conservation of robust cue generated sequential input-output activity in the cortico-striatal synapses, which depends on the strengthening of those cortical inputs to the striatum which are activated by cue presentation. The strengthening of cue driven cortical inputs to the striatum occurs at the time of reward delivery according to the three factor rule, such that those cortico-striatal input-output units active at the time of the reward induced phasic dopamine signal are incrementally strengthened. However, there is a probability that the same input-output unit active at the time of reward delivery as a result of cue presentation may be active by chance outside of the cue-reward pairing protocol. Such chance activation of the input-output unit in the absence of the reward evoked phasic dopamine signal will result in weakening of that input-output unit according to the three factor rule.

Previously in this section it was argued that classical conditioning procedures using longer time intervals between cue onset and reward delivery decreases probability that a given number of cortico-striatal input-output units directly activated by cue presentation are also active at time of reward delivery. Taken together with the foregoing argument, it is expected that at sufficiently long cue-reward time intervals the incremental strengthening of the reduced number of cue related input-output units active at the time of reward delivery on any given trial will be approximately cancelled by the weakening of cue related input-output units active by chance in the absence of the reward induced phasic dopamine signal. When this happens, an asymptote will be reached in the robustness of the sequential cortico-striatal input-output activity generated by the cue being conditioned, and this asymptotic robustness will be lesser than that required to fully and consistently cancel the dopamine response evoked by the reward.

Following from the foregoing argument, the current extension of the mechanism proposed by Aggarwal et al., (2012) also makes the following predictions if very long time intervals between cue onset and reward delivery are used for classical conditioning. First, the asymptotic timed inhibition of dopamine neurons at the expected time of reward delivery acquired through the mechanism described in this section will fail to fully cancel the reward induced excitatory drive onto the dopamine neurons, and thus the expected reward will always elicit a significant dopamine response. Second, the decrease in the asymptotic robustness of the cue generated sequential activity in the striatum may lead to larger trial to trial variation in the timed inhibition of dopamine neurons, thus increasing the variance of the dopamine response evoked by the expected reward. A strict numerical quantification of the cue-reward time intervals necessary to test these predictions requires mathematical formulation of the ideas proposed here which is beyond the scope of this thesis.

5.6 Significance

The results reported in this thesis show that the nucleus accumbens and the inhibitory inputs to the ventral tegmental area are involved in the expression of the Kamin blocking effect. Blocking depends on the accuracy, during the compound conditioning phase (phase 2), of the reward estimate generated by the cue conditioned in the single cue conditioning phase (phase 1). Therefore, the nucleus accumbens and the inhibitory inputs to the ventral tegmental area may play an important role in the reward estimate generated by the conditioned cue.

A neurophysiological correlate of the reward estimate, in the context of appetitive classical conditioning, is the dopamine reward prediction error signal. A reduction in the dopamine reward prediction error signal evoked by rewards when they are expected has previously been suggested to play a role in the Kamin blocking effect. In support of this hypothesis, chapter 4

reports that goal tracking animals, in which expected rewards have previously been shown to evoke a robust dopamine response, do not express the Kamin blocking effect. Conversely, animals in which expected rewards evoke a diminished dopamine response express the Kamin blocking effect.

The finding that goal trackers do not express the Kamin blocking effect offers insight into how the associative structures acquired during classical conditioning interact to produce the conditioned response, and how they affect subsequent learning and the expression of the Kamin blocking effect. These insights are discussed in detail in sections 4.4 and 5.1. Briefly, in the context of appetitive conditioning, it is suggested here that goal tracking and sign tracking conditioned responses emerge depending on which of two associations is acquired during classical conditioning. One is the association between the cue representation and the reward representation. The other is the association between the cue representation and appetitive affective system. Which of these associations is dominant controls behavioral responding. Further, in animals that exhibit only a goal tracking response, acquisition of a direct association between the cue representation and the appetitive affective system activated by the reward during the single cue conditioning phase is weak at best. The weakness of the cue representation – appetitive affective system association results in an underestimate of the reward induced activation of the appetitive affective system in goal trackers, and this inaccurate estimate results in the attenuation of the Kamin blocking effect.

5.7 Future directions

Future research can extend the results reported in this thesis to provide greater insight into the neural mechanisms of associative learning and resulting behavioral adaptation. Experimental testing of the predictions made by the theories put forth in this thesis will increase

understanding of classical conditioning phenomena. Further, the development of experimental procedures aimed to identify different types of conditioned responses will help better investigate classical conditioning phenomena, and the underlying neural mechanisms.

First, it is argued that the inhibitory inputs to the VTA, the output of the nucleus accumbens, and learning in the nucleus accumbens, are crucial to the expression of the Kamin blocking effect because they contribute to reducing the dopamine response evoked by rewards when they are expected. These hypotheses need to be tested by measuring the effects of the manipulations conducted in the experiments reported here, on the expected reward evoked increase in activity of the dopamine neurons in the VTA.

Second, this thesis argues that two different learning mechanisms and associative structures underlie goal and sign tracking behaviors. As mentioned in section 5.1, Lesaint et al., (2014) proposed a model based on two learning mechanisms to account for the variance in the degree of goal and sign tracking behavior observed in rodents during classical conditioning. They made several experimentally testable predictions (Lesaint *et al.*, 2015), which were extended in section 5.1 to make predictions about the expression of the Kamin blocking effect. Experiments designed to test these predictions will provide insight into the neural mechanisms underlying learning.

Third, as argued in section 5.3, the shell and core regions play separate roles in reward related behavior, and may play distinguishable roles in the expression of the Kamin blocking effect. Further experiments specifically manipulating either the core or the shell are needed to test the roles of these sub regions of the nucleus accumbens in the Kamin blocking effect, and in reducing the dopamine response evoked by rewards when they are expected.

Fourth, based on the Konorskian model of the associative structures underlying Pavlovian incentive learning (Fig. 5.2), in section 5.1 it was argued that goal trackers form a weaker

association between the cue representation and the general affective state activated by the reward than sign trackers. In section 5.3 is was argued that the nucleus accumbens core may play a role in the direct link between the conditioned cue and the affective state associated with the reward. It was also argued that the nucleus accumbens shell plays a role in the motivationally gated cue induced activation of the affective state associated with the reward via an activation of the reward representation. Taken together, these arguments of section 5.1 and 5.3 lead to the following prediction. After classical conditioning, blocking the nucleus accumbens core output on probe trials in sign tracking animals is expected to increase the incidence of goal tracking responses on the probe trials in these animals. Similarly, blocking the nucleus accumbens shell output on probe trials in the goal trackers might increase the incidence of sign tracking responses on the probe trials in these animals. Experimental tests of these predictions will provide further insight into the neural pathways through which the different associative structures acquired during classical conditioning affect conditioned responding, and into the role of these different associative structures in producing various conditioned responses.

Fifth, in section 5.4 a dopamine dependent learning mechanism is proposed for acquiring the timed inhibition of the dopamine neurons at the expected time of reward delivery. According to this mechanism, the cue generated cortico-striatal sequential activity becomes more robust and conserved across trials as classical conditioning progresses. The gradual development of this sequential activity needs to be experimentally tested. Further, according to the proposed mechanism, the production of the timed inhibition depends on the sequential cortico-striatal activity generated by cue presentation. Thus, the introduction of large amplitude perturbations in this sequential activity at any time point between cue onset and reward delivery on probe trials, possibly via optogenetic inhibition of excitation of the striatal neurons, might sufficiently perturb the sequential activity and cause an increase in the

dopamine response evoked by the expected reward. Mathematical formulation of the mechanism proposed here will help formalize predictions which can be experimentally tested.

Lastly, as mentioned in section 5.5, it is important for appetitive classical conditioning procedures to use localized cues to allow for the identification of goal and sign tracking conditioned responses. Further, there is a need to identify correlates of sign and goal tracking behavior in the context of aversive classical conditioning. The development and standardization of procedures to evaluate the development of goal and sign tracking conditioned responses will provide a better experimental model to study the different associative structures formed during associative learning from the points of view of neural mechanisms of acquisition, neural pathways through which they influence behavior, and their effects on behavior.

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Appendix I

To answer whether any of the 14 goal tracking animals reported in chapter four seemed to express the Kamin blocking effect, the relative response index scores were compared for individual rats. In the goal tracking group, while 10 of the 14 rats showed equivalent responding to S1 and S2, 4 of the 14 rats showed more responding to S2 than S1. This suggests that while most animals in the goal tracking group showed an attenuation in the Kamin blocking effect, four of these rats may have expressed the effect. In the sign tracking group, 15 of the 17 rats showed a greater response to S2 than to S1, and 2 rats showed almost equivalent responding to both S1 and S2. This suggests that while most of the animals in the sign tracking group expressed the Kamin blocking effect, two rats may not have expressed the blocking effect. The finding that a few rats show differences in the expression of the Kamin blocking effect as compared to the majority in the group they belong to (sign tracking or goal tracking group) suggest that the two learning mechanisms learn in parallel during classical conditioning.