Contribution of Nucleus Accumbens to Reward-Seeking Behaviors: Role of Dopamine-Mediated ERK Signaling

by

Merissa Lynn Remus

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This dissertation was presented

by

Merissa Lynn Remus

It was defended on

January 19, 2015

and approved by

Nathan Urban PhD, Interim Provost and Head of Department, Department of Biological Sciences, Carnegie Mellon University

Susan Sesack PhD, Professor, Department of Neuroscience, University of Pittsburgh

Alison Barth PhD, Professor, Department of Biological Sciences, Carnegie Mellon University

Dissertation Advisor: Edda Thiels PhD, Associate Professor, Department of Neurobiology, University of Pittsburgh

To my students at BASIS Peoria

Contribution of Nucleus Accumbens to Reward-Seeking Behaviors:

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Exposure to environmental cues that have been associated with rewards tend to increase behaviors that serve to procure the reward. The excitatory modulation that so-called conditioned cues exert over reward seeking depends on the nucleus accumbens (NAc). A critical molecular step within the NAc in this behavioral phenomenon is activation of extracellular signal-regulated kinase (ERK) in the NAc. The first set of experiments served to investigate the subregional pattern of ERK activation within the NAc during cue-potentiation of reward seeking and the contribution of different elements in the behavioral situation to the distribution of ERK signaling in the NAc of rats. We found that the occurrence of reward-seeking behavior did not affect ERK activation in either the core or the shell of the NAc. In contrast, presentation of the conditioned cue caused a significant increase in ERK activation in both subregions of the NAc. Different from the pattern evoked by the cue, presentation of the reward itself had no effect on ERK activation in the core but caused a pronounced increase in ERK activation in the shell. Taken together, these results demonstrate that ERK signaling in the NAc during cue-evoked reward seeking involves both the core and the shell and is driven by the conditioned cue irrespective of whether or not the situation permits engagement in reward-seeking behavior. Furthermore, the results show that the subregional distribution of ERK signaling in the NAc evoked by rewards differs from that evoked by cues that predict them. The second set of experiments served to determine the contribution of NAc dopamine type 1 (D1) versus type 2 (D2) receptors to cue-evoked NAc ERK activation, and to identify the phenotype of NAc cell in which cue-evoked ERK activation occurs. We found

that NAc D1 receptor antagonism markedly blunted the cue-evoked increase in ERK signaling in both the core and the shell, whereas NAc D2 receptor antagonism had no effect on ERK signaling. In addition, we found that every cell in the NAc of rats exhibiting cue-potentiated reward seeking that stained immunopositive for active ERK also stained for prodynorphin, a neuropeptide that in the NAc is expressed selectively by D1 receptor-containing medium spiny neurons. These results demonstrate that conditioned cue-evoked ERK activation is mediated by D1 receptors and takes place in D1 receptor expressing NAc projection cells. Taken together with findings that D1 receptor antagonism abrogates the ability of conditioned cues to potentiate reward seeking, the results point to a molecular mechanism through which dopamine mediates enhanced reward seeking in the presence of reward-predictive cues. Furthermore, the results suggest that inputs to and projection targets of D1 receptor-expressing NAc output cells are candidate elements of the neural circuit underlying cue-potentiation of reward seeking. Collectively, the findings may lead to the development of targeted interventions for the treatment of conditions characterized by maladaptive reward seeking, such as drug addiction and obesity.

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ABBREVIATIONS

ac, anterior commissure

AC, adenylyl cyclase

AMYG, amygdala

AMPA, alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

ANOVA, analysis of variance

APV, (2R)-amino-5-phosphonovaleric acid

BLA, basolateral amygdala

cAMP, cyclic adenosine monophosphate

CeA, central nucleus of the amygdala

ChAT, choline acetyltransferase

CS, conditioned stimulus

CSs, conditioned stimuli

D1, dopamine receptor type 1

D2, dopamine receptor type 2

DA, dopamine

DARPP-32, dopamine- and cAMP –regulated phosphoprotein of 32 kDA

DAT, dopamine transporter

DYN, dynorphin

ENK, enkephalin

ERK, extracellular signal-regulated kinase

fMRI, functional magnetic resonance imaging

FSCV, fast-scan cyclic voltammetry

GABA, gamma aminobutyric acid

IHC, immunohistochemistry

IR, immunoreactivity

MD, mediodorsal thalamus

MEK, mitogen-activated protein kinase kinase

mPFC, medial prefrontal cortex

MSNs, medium spiny-sized neurons

mvVP, medioventral ventral pallidum

NAc, nucleus accumbens

NMDAR, N-methyl-D-aspartate receptor

pERK, phosphorylated ERK (activated ERK)

PIT, Pavlovian-instrumental transfer

PKA, protein kinase A

PDYN, prodynorphin

PENK, proenkephalin

pDMS, posterior medial dorsal striatum

PV, parvalbumin

RAC, D2 receptor antagonist raclopride

REWARD, reward group

SCH23390, D1 receptor antagonist

SNr, substantia nigra pars reticulata

SP, substance P

TO, tone-only group

TOY, tone-only yoked group

US, unconditioned stimulus

VP, ventral pallidum

VTA, ventral tegmental area

1.0 GENERAL INTRODUCTION

A broad challenge that all organisms encounter is how to select and respond efficiently and effectively to stimuli in their environment in order to accomplish important biological functions such as the acquisition of nutrients and sexual partners. One way in which a neutral stimulus gains the ability to facilitate behavior is through its association with rewards. Indeed, it is well established that neutral stimuli that have been associated with rewards, and thereby have acquired predictive information regarding those rewards, are both able to guide and to increase the frequency of an organism's future behavioral pursuits. Cues that have been associated with rewards acquire the capacity to evoke conditioned motivational states of "wanting" and "craving" that invigorate behavior to obtain the reward (Berridge & Robinson, 1998; Berridge, 2012). It is widely assumed that this adaptive feature of associative learning evolved as a mechanism to improve fitness and survival of species (Everitt *et al.*, 2003; Domjan, 2005); however, in addition to providing a beneficial role in guiding organisms to obtain resources and carry out biological functions, conditioned cues also have the capacity to trigger harmful behaviors such as binge eating, drug addiction, and other compulsive reward-seeking behaviors.

While dopamine (DA) and dopaminergic pathways have been identified as a key modulator of conditioned motivation (Wise, 2004; Berridge, 2007), the specific molecular pathways underlying conditioned motivation remain elusive. Knowledge of both the specific

neural populations that process environmental stimuli and the mechanisms by which these environmental stimuli are processed is critical for understanding the fundamental biology of how cues that signal reward availability are used to guide behavior.

The overall aim of this dissertation is to elucidate neurobiological mechanisms that underlie the ability of reward-predictive cues to influence behavior to pursue rewards. To that end, this work utilizes a combination of behavioral, pharmacological, and immunohistochemical (IHC) techniques to determine the contributions of nucleus accumbens (NAc) DA to the meditation of extracellular signal regulated kinase (ERK), a molecular substrate in the NAc that plays a role in the ability of reward-predictive cues to potentiate reward-seeking behavior.

In this introductory chapter, I will first cover the cellular and systems level mechanisms within the mesolimbic DA system with emphasis on the NAc. Second, I will discuss the processes that govern learning and reward-seeking behaviors. Third, these ideas will be integrated to further examine the theories and evidence that link NAc dopamine and reward-motivated behaviors. Finally, a summary of the aims and results is provided.

1.1 DOPAMINE

DA (3-hydroxytyramine) is a modulatory neurotransmitter belonging to the catecholamine group of neurotransmitters, which have been characterized structurally by their constitution of a single amine group, a central catechol (a benzene ring containing two adjacent hydroxyl groups), and a side chain of ethylamine or one of its derivative (Vallone *et al.*, 2000). Unlike traditional neurotransmitters, DA serves to modulate the gain of other

neurotransmitters. DA release has significant effects on many aspects of cognition and behavior and therefore has been implicated in a multitude of cognitive processes including—but not limited to—learning, facilitation of movement, attention, motivation, decision making and reward encoding. In addition to these functions, compromised DA transmission has shown to be associated with a variety of neurological conditions, including schizophrenia, Huntington's disease, Parkinson's disease, Attention deficit hyperactivity disorder (ADHD), depression, and addiction, among others.

1.1.1 The mesolimbic dopamine system

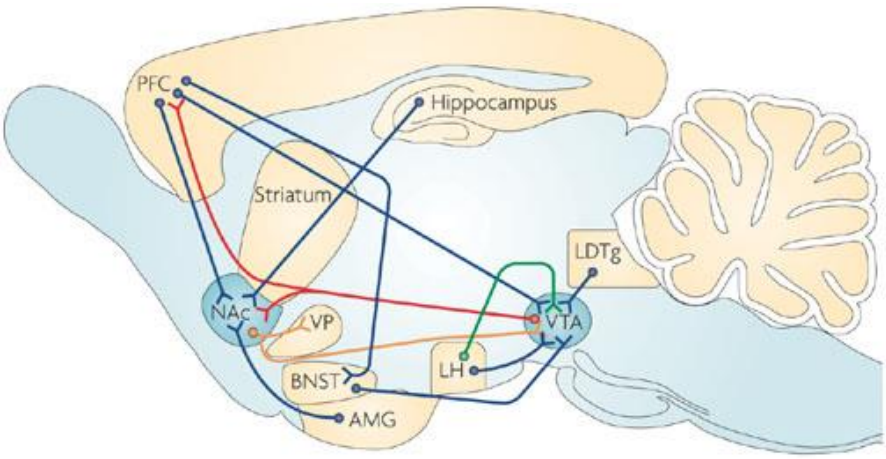

Figure 1: Simplified schematic of the circuitry comprising the mesolimbic dopamine system in the rat brain highlighting the major inputs to the NAc and VTA: glutamatergic projections, blue; dopaminergic projections, red; GABAergic projections, orange; orexinergic projections, green). Adapted from Kauer and Malenka (2007).

The mesolimbic dopamine projection consisting of medium aspiny DA neurons arises from a portion of the ventral tegmental area (VTA) that is located ventrally to the red nucleus in the midbrain (Fallon & Moore, 1978). These DA terminals project to a number of targets including the NAc, the prefrontal cortex (PFC), the amygdala, the hippocampus, the ventral

pallidum (VP), and the olfactory tubercle (Fallon & Moore, 1978; Ikemoto, 2007); however, the projection from the VTA to the NAc is the densest of the foregoing projections (Fields *et al.*, 2007). While it is widely accepted that the dopaminergic innervation of the NAc contributes to several reward-motivated behaviors, the precise functions of the mesolimbic DA system remain unclear. By virtue of the complex nature of the inputs to and projections from mesolimbic DA neurons, it is not surprising that there are several hypotheses for mesolimbic DA's role in motivated behaviors. Increased mesolimbic DA has been implicated in many processes including learning and memory (Kelley, 2004), reward prediction (Schultz *et al.*, 1997; Colombo, 2014), incentive salience (Berridge, 2007), and general motivation and effort (Horvitz, 2000; Salamone *et al.*, 2003) among others. When considering the motivational functions of DA, it should be recognized that motivational processes are often dissociable as smaller yet interacting parts (Phillips, 1984). Thus, DA is likely to perform several functions by utilizing the activation of several receptor subtypes and several signaling cascades to motivate behavior.

1.1.2 Dopamine receptor signaling and pharmacology

A major intricacy in the understanding of the many roles of DA in the processing of reward-motivated behaviors arises from the framework that DA exerts its actions by binding to five types of DA membrane receptors, which belong to the family of seven transmembrane (7TM) G- protein-coupled receptors (GPCRs; Gingrich & Caron, 1993). The binding of DA to its receptors promotes second messengers and drives the activation or repression of specific signaling pathways. In fact, binding to one receptor subtype can propagate multiple

molecular events which in turn may result in various and potentially complex behavioral outcomes.

Five types of DA receptors (D1, D2, D3, D4, and D5) have been isolated, characterized, and divided into two major subfamilies: the D1-like and D2-like classes of DA receptors. The D1-like subfamily is comprised of D1-receptors and D5-receptors (originally identified as D1B) (Tiberi *et al.*, 1991), while the D2-like family includes D2-, D3-, and D4-receptors (Andersen *et al.*, 1990). Although the signaling pathways activated by DA receptors are numerous, this two-family classification system is based largely on biochemical evidence showing that the activation of DA type D1 receptors and DA type D2 receptors have opposing effects on adenylyl cyclase (AC) activity and the production of the second-messenger cyclic adenosine monophosphate (cAMP). Activation of D1 receptors was shown to be linked to G_s, or stimulatory proteins and therefore positively coupled to both AC activity and cAMP production. To the contrary, activation of D2 receptors is linked to $G_{i/o}$, or inhibitory proteins and results in inhibition of AC and suppression of cAMP production (Spano *et al.*, 1978; Kebabian & Calne, 1979). In addition to modulating cAMP activity, co-activation of D1 and D2 receptors activates the signaling of phospholipase C (PLC), which leads to increased intracellular calcium signaling (Lee *et al.*, 2004). Activation of D2 receptors also activates signaling pathways other than inhibition of AC. D2 receptor activation also modulates glycogen synthase kinase (GSK)(Bonci & Hopf, 2005). However, the molecular determinants that regulate these alternate signaling pathways are not fully understood.

DA neurons display two main patterns of firing activity that have been shown to modulate the level of extracellular DA: tonic and phasic firing (Grace, 1991; Grace *et al.*, 2007). It has been posited that low frequency, irregular “tonic” firing of DA neurons results

in a tonically low level of DA release, whereas transients bursts of firing from DA neurons results in a large, but transient increase in DA (Grace *et al.*, 2007). Release of DA can either facilitate or suppress the activity of NAc cells. DA modulates the excitability of NAc cells via its binding onto D1 and D2 receptors (Surmeier *et al.*, 2007; Dreyer *et al.*, 2010). DA receptors are not restricted to the synaptic cleft and are predominantly located extrasynaptically (Sesack *et al.*, 1994; Hersch *et al.*, 1995; Yung *et al.*, 1995; Beaulieu & Gainetdinov, 2011). Termination of DA signaling within the NAc is primarily achieved by the reuptake of DA by the DA transporter (DAT), which is also extrasynaptic (Leviel, 2011). The DA receptor subtypes have differing affinity for DA (Watson *et al.*, 2006; Beaulieu & Gainetdinov, 2011; Covey *et al.*, 2014). Burst firing of DA neurons, such as that evoked by a reward-predictive cue, produces a large transient release of DA that primarily activates postsynaptic D1 receptors (Richfield *et al.*, 1989). On the other hand, D2 receptors have high affinity for DA in order to respond to tonic DA levels and inhibit postsynaptic activity. Interestingly, the phasic and tonic firing patterns of DA neurons are thought to mediate different aspects of reward-motivated behaviors: phasic burst firing has been shown to promote cue-reward associations (Tsai *et al.*, 2009; Zweifel *et al.*, 2009), whereas tonic DA firing has been shown to promote behavioral flexibility (Meck & Benson, 2002).

1.2 NUCLEUS ACCUMBENS

As will be detailed below, the NAc receives cortical-limbic input from the amygdala, the hippocampus, and the PFC. Based on the convergence of these glutamatergic inputs and the dopaminergic input from the VTA and the observation that locomotion evoked by

infusions of DA in the NAc could be blocked by the inhibition of NAc output neurons, Mogenson and colleagues proposed the NAc as a "limbic motor interface" (Mogenson *et al.*, 1980). Since then the NAc has been repeatedly demonstrated as the site in which information about reward, learning, and motivational states is used to guide behavior (for a review, see Day & Carelli, 2007). Information about rewards is relayed to the NAc via its glutamatergic inputs from the amygdala, the hippocampus, and the PFC. Each of these inputs processes distinct types of information. The NAc integrates information about reward with information regarding an organism's motivational state to modulate behavior. DA transmission in the NAc has been shown to be an important modulator of NAc neurons and their cortical-limbic inputs (Taepavarapruk *et al.*, 2000; Floresco *et al.*, 2001b; a; Jones *et al.*, 2010). Thus it has been posited that DA transmission in the NAc may bias the responses of NAc cells to specific inputs that in turn may facilitate behavioral responding to obtain reward (Floresco, 2015).

1.2.1 Medium spiny neurons

Medium spiny neurons (MSNs), are striatal projection neurons that make up approximately 90-95% of the cells in the NAc similar to the composition of the dorsal striatum (Meredith, 1999). They are medium in size, ranging from 12-18 μm in diameter, with prominent dendritic spines. MSNs exert their inhibitory function via release of their primary neurotransmitter, gamma aminobutyric acid (GABA). In addition to DA and GABA receptors, MSNs express both alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and N-methyl-D-aspartate (NMDA) glutamate receptors (Tallaksen-Greene *et al.*, 1992; Meredith, 1999), which allow cortical-limbic inputs to activate MSNs (O'Donnell & Grace, 1995; Goto & O'Donnell, 2002; Goto & Grace, 2005; Grace *et al.*, 2007).

Accordingly, DA input from the VTA and glutamatergic input from cortical-limbic afferents congregate onto common dendrites and spines of MSNs (Totterdell & Smith, 1989; Sesack & Pickel, 1990; Smith & Bolam, 1990).

MSNs also contain a variety of neuropeptides, which may act as neuromodulators on MSNs or their targets (Meredith, 1999). For instance, dynorphin peptides are expressed in and secreted from axons of a subpopulation of MSNs that contain D1 receptors (Gerfen & Young, 1988). Secreted dynorphin peptides are preferential agonists of the κ-opioid receptors that are located on presynaptic DA afferents (James *et al.*, 1982) and presynaptic excitatory synapses (Svingos *et al.*, 1999). Activation of κ-opioid receptors was shown to attenuate DA release in the NAc (Maisonneuve *et al.*, 1994). As will be discussed in greater detail below, there is a strong correlation between the types of neuropeptides that a MSN expresses and the location to which its axons project.

1.2.2 Interneurons

Local circuit neurons, or interneurons, comprise the remaining 5-10% of NAc neurons (Heimer *et al.*, 1991). There are at least four types of interneurons in the NAc: cholinergic, parvalbumin (PV), and calretinin (CR) interneurons, and a group of interneurons that express both somatostatin (SS) and neuropeptide Y (NPY) (Meredith, 1999). It is known that these four groups provide input to MSNs, and that PV and cholinergic cells receive input from both local and cortical neurons (Meredith *et al.*, 1993). The PV, CR, and SS subtypes are all GABAergic interneurons. The largest of these local neuron types are the cholinergic interneurons, whose cell bodies are 20-50 μM in diameter and reliably stain positive for choline acetyltransferase (ChAT; Kawaguchi *et al.*, 1995; Hussain *et al.*, 1996). ChAT

interneurons express D2 receptors and low amounts of D5 receptors (D1-class; Le Moine *et al.*, 1991; Meredith, 1999). The population of PV-containing cells are fast-spiking inhibitory cells that make up only 3-5 % of neurons in the striatum (Kawaguchi *et al.*, 1995). PV interneurons express D5 receptors (Centonze *et al.*, 2003). The SS containing neurons represent only 1-2% of the striatal population and express D5 receptors (Kawaguchi *et al.*, 1995; Centonze *et al.*, 2003).

1.2.3 Connectivity of the Core and Shell Subregions

The NAc is a heterogeneous structure (Graybiel & Ragsdale, 1978), comprised of two major subregions, the core and the shell, which have been distinguished by their anatomical projections, neurochemistry, and functional specialization (Zahm & Brog, 1992; Zahm, 1999; 2000). The core, which is located directly beneath the dorsal striatum, encircles the anterior commissure (ac), and the shell extends medially, ventrally and laterally around the core. Although these subregions are in close proximity to each other, they have noteworthy differences in their afferent and efferent projections. For example, the NA core receives input from the prelimbic region and lateral orbitofrontal cortex, whereas the NA shell receives input from the infralimibic cortex and the medial lateral orbitofrontal (Berendse *et al.*, 1992). Both core and shell receive input from distinct regions of the hippocampus: the dorsal subiculum projects to the core, whereas the ventral subiculum projects to the shell (Heimer *et al.*, 1991). Subregions of the amygdala project differently within the core and the shell (Wright *et al.*, 1996). Furthermore, core and shell are dissociable by their pallidal targets. The NAc core preferentially projects to the caudolateral VP, which then projects to the subthalamic nucleus and substania nigra, whereas the NAc shell preferentially projects to the

rostromedial VP, which then projects to the VTA and mediodorsal thalamus (Heimer *et al.*, 1991; Zahm & Brog, 1992; Zahm & Heimer, 1993). Uniquely, the shell projects to limbic regions including the lateral hypothalamus and brainstem autonomic centers. Further, the shell is the only region within the striatum that directly projects to regions outside of the basal ganglia. The output from the medial shell is topographically organized; whereas the dorsal medial shell sends projections to the lateral hypothalamus and lateral pre-optic area, the ventral medial shell sends projections to adjacent areas of the same structures but also to the parabrachial nucleus and the periacqueductal grey (Usuda *et al.,* 1998). Beyond the core and the shell, it has been posited that distinct neuronal ensembles with distinct input-output connectivity exist within the NAc (Pennartz *et al.*, 1994; Kelley, 1999; Cruz *et al.*, 2013), which raises the possibility that ensembles of NAc neurons which enable CSs to potentiate reward seeking receive functionally distinct innervation containing the information necessary to guide and invigorate reward-seeking behavior.

1.2.4 Direct and Indirect Pathways

MSNs in the dorsal striatum have generally been classified into two distinct populations based on their DA receptor and neuropeptide content, as well as their axonal projections. One population of MSNs, known as the direct pathway, monosynaptically projects to both the substantia nigra pars compacta and the internal segment of the globus pallidus, and expresses D1 receptors and the neuropeptides Substance P (SP) and dynorphin (DYN; Le Moine *et al.*, 1991; Le Moine & Bloch, 1995; Lu *et al.*, 1997; Lu *et al.*, 1998). The second population of MSNs, known as the indirect pathway, projects to the external segment of the globus pallidus and expresses D2 receptors and enkephalin (ENKs; Le Moine *et al.*,

1990; Le Moine & Bloch, 1995; Lu *et al.*, 1998). Control over behavior is facilitated by the MSNs in the direct and indirect pathways. Direct pathway MSNs serve to inhibit neurons in the basal ganglia output structures. Since basal ganglia output neurons also inhibit behavior (Sakamoto & Hikosaka, 1989; Mink, 1996), it has been proposed that firing of the direct pathways MSNs results in the facilitation of behavior, whereas firing indirect pathway MSNs increases firing of the neurons in the basal ganglia output structures resulting in the suppression of competing behaviors (Alexander & Crutcher, 1990; Nicola, 2007).

There is emerging evidence that MSNs in the NAc also project through the direct and indirect pathways. The available evidence for this dorsal striatal-like organization is stronger for the NAc core than the NAc shell (Brog *et al.*, 1993; Nicola *et al.*, 2000; Nicola, 2007). The NAc core parallels the traditional basal ganglia circuitry of the dorsal striatum, sending outputs through the VP, subthalamic nucleus, and substantia nigra pars reticulate (SNr), and these outputs in turn project via the thalamus to premotor cortical areas. Direct pathway neurons in the NAc core predominantly target the SNr followed then by the mediodorsal thalamus (Montaron *et al.*, 1996), whereas a sparse projection of NAc core neurons targets the dorsal lateral VP followed then by the mediodorsal thalamus (Zahm *et al.*, 1996; O'Donnell *et al.*, 1997). Utilizing this direct loop of the basal ganglia circuitry, cortical activation of the NAc MSNs leads to disinhibition of motor behavior to facilitate reward seeking. The indirect pathway NAc neurons of the NAc core target the dorsolateral VP and subthalamic nucleus before reaching the SNr. Activation of MSNs in the indirect pathway is thought to inhibit motor plans that are counterproductive for behavior to obtain reward. On the other hand, MSNs in the NAc shell are not easily parsed into the direct and indirect pathways because MSNs of the NAc shell target limbic regions in addition to basal ganglia structures (Zahm & Heimer, 1990; Heimer *et al.*, 1991; Zahm & Brog, 1992).

Anatomical studies have indicated both the DA receptor and neuropeptide content of NAc projection neurons are considerably segregated. As described above for the dorsal striatum, MSNs of the NAc are also generally segregated into two populations. One population of MSNs expresses D1 receptors, DYN, and substance P whereas a second population expresses D2 receptors and ENK peptides (Lu *et al.*, 1998; Sonomura *et al.*, 2007). MSNs that target the VTA and predominantly arise from the shell consist predominantly of neurons that express D1 receptors (Brog *et al.*, 1993). For neurons projecting to the VP, a greater percentage of neurons in the shell (considered to be direct pathway neurons) are D1R-expressing; however, neurons in the core (considered to be indirect pathway neurons) express both D1 and D2 receptors (Lu *et al.*, 1998; Zhou *et al.*, 2003). The direct pathway core neurons that project to the SNr are predominantly D1R-expressing neurons (Berendse *et al.*, 1992; Le Moine & Bloch, 1995). The DA receptor content of the unique projections originating from the medial shell, as described above, have yet to be investigated. Although D1 receptors are ubiquitously distributed throughout both NAc subregions, D2 receptors were shown to be more prevalent in the NAc core than the NAc shell (Bardo & Hammer, 1991; Lu *et al.*, 1998).

The aforementioned classification system has depicted the D1 receptors and D2 receptors as discrete populations of MSNs. However, evidence has emerged which suggests that this two-family classification may be an oversimplification in the NAc. Analysis using bacterial artificial chromosome (BAC) mice has revealed that D1 and D2 receptors are co-expressed in 6% of core MSNs and in 17% shell MSNs (Bertran-Gonzalez *et al.*, 2008). Another study reported that as much as 25% of MSNs co-express both receptor subtypes (Hasbi *et al.*, 2010). On the basis of these findings, it is reasonable to speculate the D1 and D2 receptors interact in a third yet smaller population NAc MSNs. Moreover, the coincident

activation of both D1 and D2 receptors has been demonstrated to be critical for certain biochemical and behavioral effects such as cocaine sensitization (Capper-Loup *et al.*, 2002); however this synergy between D1 and D2 receptor signaling alone does not imply that the two receptor subtypes are localized to the same cell. Interestingly, both D1 and D2 receptor mechanisms have been implicated in cue-potentiation of reward-seeking (Lex & Hauber, 2008); however it is not known whether D1 and D2 receptors engage a common or different signaling pathway during CS modulation of reward-seeking behavior.

1.3 BEHAVIORAL CONDITIONING

1.3.1 Appetitive Pavlovian Conditioning

When an initially neutral stimulus, such as an auditory tone, is repeatedly presented with a motivationally relevant stimulus, such as a food or drug reward, the neutral stimulus becomes a conditioned stimulus (CS) through the learning process of Pavlovian conditioning (or classical conditioning) (Dickinson, 1980). Initially, the individual shows little or no response to the presentation of the CS, but exhibits a measurable innate response to the presentation of the reward (the unconditioned stimulus; US), such as salivation. Through repeated CS-US pairings, the tone becomes a CS, which is capable of eliciting the same response as the US. Thus, the individual learns to respond to the previously neutral stimulus with the same response elicited by the US. This type of learning is sensitive to a number of variables including, the frequency of CS-US pairings, the temporal delay between the CS and US, and the valence and saliency of stimuli employed (for a review see, Domjan, 2005).

Ivan Pavlov (1927), a Russian physiologist, was the first to describe this phenomenon and proposed that a conditioned "reflex" (or conditioned response, CR) developed because an association formed between the representation of the CS and the US. According to Pavlov, the presentation of the US activates an area of the brain responsible for processing the US which leads to the activation of a brain center responsible for generating the unconditioned response. When the CS was presented, it activates a specific area of brain, but when the CS was presented just before the US, both brain areas were activated at the same time creating a pathway between the activated brain areas. Pavlov's theory, known as stimulus-substitution theory, proposed that the CS elicits the CR as a result of the CS' ability to activate the region of the brain which is responsive to the US.

Pavlovian conditioning was further described to occur in two forms: consummatory and preparatory conditioning (Konorski, 1967). Consummatory conditioning occurs when the behavior exhibited by conditioned response reflects sensory properties of the US. An example of a consummatory response is Pavlov's original experiment where dogs salivate in the presence of the CS, which has previous been paired with food. Instead of reflecting specific sensory properties of the US, preparatory conditioning reflects the nature of the US (Konorski, 1967; Dickinson & Balleine, 2002). That is, the dogs salivate in the presence of the CS in a similar manner that the dogs will salivate in the presence of the US. Examples of preparatory responses include fluctuations in heart rate and conditioned approach behavior. Conditioned approach is when a CS predictive of reward elicits approach to the source of the reward. "Sign-tracking" and "goal-tracking" are also Pavlovian responses elicited by CSs. "Sign-tracking" refers to when the subject approaches the cue or lever predictive of reward upon CS presentation; whereas, "goal-tracking" describes when the subject is directed toward the site of reward delivery upon CS presentation (Flagel *et al.*, 2008; Flagel *et al.*, 2009).

1.3.1.1 Neurobiology of Appetitive Pavlovian Conditioning

The amygdala has been implicated extensively in the acquisition of appetitive Pavlovian conditioning. Excitotoxic lesions of the central nucleus of the amygdala (CeA) were shown to impair the acquisition of the appetitive CS-generated CR, whereas responding to the US was unaffected (Gallagher *et al.*, 1990; Hatfield *et al.*, 1996; Parkinson *et al.*, 2000a). On the other hand, lesions of the BLA do not preclude acquisition of the CR (Hatfield *et al.*, 1996; Parkinson *et al.*, 2000a). Importantly, following BLA-lesions rats were unable to adjust their responding following the devaluation of the reward suggesting that the BLA is necessary for the CS to retrieve the affective value of the reward (Everitt *et al.*, 2003). As a result of the foregoing studies, a widely accepted hypothesis is that BLA is the neural site of appetitive stimulus-outcome representations, whilst the CeA provides an output pathway by which CSs gain access to appropriate responses (Everitt *et al.*, 2003).

The NAc is critical for both the acquisition and performance of appetitive Pavlovian conditioning. Bilateral lesions of the NAc core, but not the shell, were shown to disrupt the acquisition of appetitive Pavlovian behaviors (Parkinson *et al.*, 1999; Parkinson *et al.*, 2000b; Cardinal *et al.*, 2002b). NAc dopamine depletions were shown to impair both the acquisition and performance of appetitive Pavlovian conditioning, but the impairment on acquisition was more pronounced than effects on performance (Parkinson *et al.*, 2002). Likewise, NAc core infusions of the D1/D2 receptor antagonist flupenthixol decreased approach rate during both acquisition and performance (Di Ciano *et al.*, 2001). Moreover, it was shown that inactivation of D1 but not D2 receptors in the NAc impaired acquisition (Dalley *et al.*, 2005). Further, DA enhancement in the NAc increased appetitive Pavlovian approach (Pecina *et al.*, 2003). Interestingly, a recent study revealed that intra-NAc core infusions of flupenthixol prevented the expression of sign-tracking (attraction to the CS), but not of conditioned

approach (Saunders & Robinson, 2012), which supports the hypothesis NAc DA is responsible for the stated of conditioned motivation exhibited by sign-trackers. A disconnection experiment between the NAc core and the anterior cingulate cortex revealed that afferents from the anterior cingulate to the NAc core were critical for the acquisition of autoshaping (Parkinson *et al.*, 2000b). Whereas NAc core infusions of a NMDA receptor antagonist disrupted acquisition, infusions of an AMPA/kainate receptor disrupted performance (Di Ciano *et al.*, 2001). Taken together, these findings support the hypothesis that DA release in the NAc is necessary to modulate the synaptic input from cortical and limbic inputs in order to support both the acquisition and performance of appetitive Pavlovian behaviors.

1.3.2 Instrumental Conditioning

Through the process of instrumental (or operant) conditioning, the subject learns to associate actions or responses with motivationally relevant outcomes. Thus responses on the operant will increase or decrease depending on whether the outcome is reinforcing or not (Thorndike, 1933; Skinner, 1938). One fundamental difference between Pavlovian and instrumental conditioning is that in instrumental conditioning, delivery of the reward is contingent on emission of the operant whereas no specific response is required for the reward delivery in Pavlovian conditioning. Thus, in instrumental conditioning but not in Pavlovian conditioning the subject exerts control over the frequency of reward delivery.

1.3.2.1 Neurobiology of Instrumental Conditioning

A number of cortical, limbic, and striatal regions have been implicated in instrumental conditioning. The medial prefrontal cortex (mPFC) is required for acquisition, but not the performance of instrumental responding (Ostlund & Balleine, 2005). Likewise, the coincident activation of NMDA and D1 receptors in the NAc mPFC is required for the acquisition of instrumental conditioning (Baldwin *et al.*, 2002). The dorsomedial striatum (pDMS) is critical for both the acquisition and the performance of instrumental conditioning (Yin *et al.*, 2005b; Corbit & Janak, 2010; Shiflett *et al.*, 2010). Acquisition of instrumental conditioning was shown to be dependent on activation of NMDA receptors in the pDMS (Yin *et al.*, 2005a) and the NAc (Kelley *et al.*, 1997). Likewise, the coincident activation of NMDA and D1 receptors in the NAc core is also required for learning (Smith-Roe & Kelley, 2000). Interestingly, the activation of ERK in the pDMS is required for both acquisition and performance (Shiflett *et al.*, 2010). A disconnection experiment further revealed that a BLA → pDMS projection is important for learning, but not the performance of instrumental conditioning (Corbit *et al.*, 2013). These studies implicate that the NAc is critical for acquisition of instrumental conditioning whereas the pDMS has been implicated in both the acquisition and the performance of instrumental behavior.

1.3.3 Conditioned "Incentive" Motivation

An important outcome of associative learning is the acquisition of motivational responses (Rescorla & Solomon, 1967; LeDoux, 2000). CSs that predict rewards acquire "incentive salience" which is said to reflect the motivational value of the reward (Berridge, 2004). Consequently, CSs motivate reward-seeking behavior by eliciting a state of

conditioned motivation or desire. It has been posited that phasic DA release in the NAc may serve as a mechanism for incentive motivation during both the acquisition (Berridge & Robinson, 2003; Aragona *et al.*, 2009) and maintenance of Pavlovian associations (Stuber *et al.*, 2005). The effects of incentive salience are assessed using the Pavlovian-instrumental transfer paradigm as discussed below.

1.4 PAVLOVIAN INSTRUMENTAL TRANSFER

This ability of the CS to motivate reward-seeking behavior can be demonstrated and assessed effectively using a behavioral paradigm called Pavlovian-instrumental transfer (PIT; Estes, 1948; Lovibond, 1983). The PIT paradigm occurs in three stages: Pavlovian conditioning, instrumental conditioning, and the PIT transfer test. In independent sessions, subjects are trained on Pavlovian conditioning and instrumental conditioning. During the PIT test, the presence of a CS that has been previously paired with a reward has the ability to potentiate behavior of an independently acquired motor response, such as lever pressing, for the same reward. In contrast to other behavioral paradigms, the PIT test is the first time that the subject experiences the presentation of the CS in the presence of reward-seeking opportunity. A clear demonstration of the PIT effect is a pronounced increase in the rate of lever pressing specifically during the presentation of the CS compared with periods during which the CS is not present.

Two types of PIT have been studied in the laboratory: general and stimulus-specific PIT. To establish general PIT in the laboratory, subjects receive Pavlovian training on one stimulus paired with a single reward, followed by instrumental conditioning on one operant

to obtain reward. Finally, when given the opportunity to perform the operant in the presence and absence of the CS, the participant presses the lever more in the presence of the CS than in the absence of the CS. The enhanced performance of the instrumental response in the presence of the CS is widely assumed to be evidence of a motivating attribute of the US reflected in the CS (Dickinson & Balleine, 2002). A paradigm that distinguishes stimulus-specific PIT and general PIT was introduced by Corbit and Balleine (2005). To establish stimulus-specific PIT in the laboratory, subjects receive Pavlovian conditioning with two different stimuli, such as tone and light, which result in distinct outcomes, such as sugar water and food pellets. Next, the same subjects receive instrumental conditioning on two levers that result in the two distinct appetitive outcomes. During the transfer test, one of the CSs is presented while the subjects are free to respond on both levers. Stimulus-specific PIT is demonstrated by enhanced responding on the lever that produces the particular reward during the presentation of the CS with which the reward was previously paired. It has been proposed that the stimulus-specific PIT effect occurs because the CS and the instrumental response activate a specific sensory aspect, including the visual appearance, feel, and smell of the US (Cardinal *et al.*, 2002a). Thus, stimulus-specific PIT has also been called stimulus-specific PIT or outcome-specific PIT. During both general and stimulus-specific PIT, the presence of the CS leads to heightened reward-seeking behavior; however, as will be discussed further, different neural substrates have been implicated in these similar, but distinct processes (Corbit & Balleine, 2005; 2011). This dissertation addresses the roles of NAc DA and ERK signaling in general PIT.

1.4.1 Neurobiology of Pavlovian-Instrumental Transfer

PIT has been shown to rely upon several brain structures that collectively compose part of the mesolimbic DA system, including the VTA, the NAc, and the amygdala. Of particular importance, the NAc has been shown to play a central role in guiding reward-related behavior (for review, see Day & Carelli, 2007) and as such, the NAc has been identified as a crucial substrate for PIT. Pharmacological lesions of the NAc core abolished the general potentiating effect of CSs over instrumental responding (Hall *et al.*, 2001; de Borchgrave *et al.*, 2002; Corbit & Balleine, 2011), whereas disruptions of stimulus-specific PIT were observed following similar manipulations to the NAc shell (Corbit *et al.*, 2001; Corbit & Balleine, 2011). Importantly, the preceding results indicate that the core mediates the general excitatory influence of the CS on operant responding, whereas the shell mediates the outcome specific influences of the CS on operant responding (Corbit & Balleine, 2011). Consistent with the dichotomous view that motivational aspects of the US are encoded by general PIT, whereas the sensory aspects on the US are encoded by stimulus-specific PIT, the CeA has been identified as a crucial substrate of general PIT (Hall *et al.*, 2001; Holland & Gallagher, 2003), whereas the basolateral amygdala (BLA) has been found to mediate stimulus-specific PIT (Blundell *et al.*, 2001; Corbit & Balleine, 2005). The CeA does not send input directly to the NAc; however, it may affect the NAc indirectly through polysynaptic pathways that connect the CeA to the VTA. For instance, the VTA may be influenced by the pathway from the CeA to the lateral hypothalamus or the pathway from the CeA to the prefrontal cortex. Both of these pathways terminate in the VTA (Zahm, 2006), which in turn projects to the NAc. Indeed, bilateral inactivation of the VTA via local infusions of GABA agonists precluded rats from exhibiting PIT (Murschall & Hauber, 2006).

Importantly, the structures downstream of the NAc required for PIT are not known. Thus, the phenotyping and receptor pharmacology experiments contained in this dissertation lay the foundation for determining which NAc output regions should be investigated in further study of PIT.

1.4.2 The Role of Nucleus Accumbens Dopamine in Pavlovian-instrumental Transfer

A substantial body of electrophysiological, behavioral, and pharmacological evidence supports the emerging hypothesis that DA release within the NAc is required for heightened motivation to pursue reward-seeking in the presence of reward-predictive stimuli during PIT. The presentation of reward-predictive CSs modulates the firing rate of NAc neurons (Roitman *et al.*, 2005; Day *et al.*, 2006; Day *et al.*, 2007; Aragona *et al.*, 2009; Ambroggi *et al.*, 2011). Inactivation of the VTA, which interrupts the major DA input from the VTA and reduces DA release in the NAc (Westerink *et al.*, 1996), was shown to disrupt PIT (Murschall & Hauber, 2006; Corbit & Janak, 2007). Systemic administration of DA antagonists was shown to abolish PIT (Dickinson *et al.*, 2000), and infusion of DA receptor antagonists directly into either the NAc core or shell likewise was shown to impair PIT (Lex & Hauber, 2008; Ostlund & Maidment, 2012). Microinfusions of amphetamine, a DA agonist, into the NA shell selectively potentiated the excitatory effect of CSs on instrumental responding for a sucrose reward (Wyvell & Berridge, 2000). More recently, it was shown that microinfusions of amphetamine throughout the vast majority of both the NAc core and the shell produced amplifications of PIT (Pecina & Berridge, 2013). Micro-dialysis studies have shown that exposure to appetitive CSs elevates NAc DA (Bassareo & Di Chiara, 1999; Ito *et al.*, 2000). Fast-scan cyclic voltammetry (FSCV) studies indicate that DA release occurs transiently

upon exposure to reward-predictive cues (Robinson *et al.*, 2001; Roitman *et al.*, 2004; Flagel *et al.*, 2011). Interestingly, it was recently demonstrated that phasic releases of DA during presentation of a CS predicted the magnitude of the PIT effect (Wassum *et al.*, 2013). Collectively, these studies support the conclusion that increased DA release in the NAc is both necessary and sufficient for CS to increase the magnitude and frequency of reward-seeking behaviors.

1.5 FUNCTIONAL ROLES OF THE NUCLEUS ACCUMBENS CORE AND SHELL

Since the proposal by Mogenson and colleagues (1980) ascribing the NAc with the role of transforming motivational information from limbic areas of brain into appropriate behavioral actions to obtain reward, there have been unremitting efforts to encompass the structural organization of the NAc into a theory of NAc function. Due to the unique neural features defining each of the NAc subregions (detailed above), considerable attention has been devoted to understanding their putative functional roles in reward-related behaviors. The NAc core and shell are both implicated in the processing of various aspects of reward-related stimuli, including natural rewards.

Several studies have implicated the NAc core in the control of reward- and drug-seeking behaviors by discrete cues, whereas the NAc shell is in control of such behaviors by contextual information. Blockade of D1Rs, excitotoxic lesions, and transient inactivation of the NAc core precludes reinstatement of food and drug seeking by discrete cues (Fuchs *et al.*, 2004; Ito *et al.*, 2004; Bossert *et al.*, 2007; Floresco *et al.*, 2008; Chaudhri *et al.*, 2010). On

the other hand, similar manipulations in the shell leave cue-reinstatement of drug seeking intact, but impair contextual-induced reinstatement. This role for the NAc shell is consistent with its unique excitatory input from the ventral subiculum, which has been shown to be important for relaying spatial and contextual information during reward-seeking behaviors (Riedel *et al.*, 1997; Ito *et al.*, 2008).

The NAc core has been ascribed the preferential role of encoding cue-reward associations. For example, CSs predictive of either sucrose or drug reward were shown to alter the firing responses preferentially in the NAc core (Day *et al.*, 2006; Aragona *et al.*, 2009; Ambroggi *et al.*, 2011; Cacciapaglia *et al.*, 2011). Early in conditioning, CSs predictive of cocaine had little effect on phasic DA, as measured using FSCV; however, following one training session of Pavlovian conditioning, CSs predictive of cocaine evoked phasic DA release in the NAc core that correlated with approach behavior (Aragona *et al.*, 2009). This result indicates CS-evoked DA release in the NAc core is contingent upon acquisition of Pavlovian conditioning. Likewise, sign-trackers, which are attracted to the CS itself and are posited to exhibit conditioned incentive motivation, were shown to exhibit phasic DA release in the NAc core upon exposure to a CS (Flagel *et al.*, 2011). In these aforementioned studies, it is important to note that neurons in the NAc core alter firing more reliably compared to neurons in the NAc shell upon exposure to CSs, but that neural responses in the NAc shell are also altered upon exposure to CSs and rewarding stimuli themselves. Neurons of the NAc in the core and the shell have been shown to modulate their firing rates in response to numerous other stimuli including reward delivery (Carelli & Deadwyler, 1994; Carelli, 2002; Day *et al.*, 2006), the anticipation of operant rewards (Carelli & Deadwyler, 1994; Carelli, 2002; Ambroggi *et al.*, 2011), and unexpected reward (Schultz *et al.*, 1997; Day *et al.*, 2007). Thus, the specific involvement of the NAc core versus shell in CS processing is not yet clear.

The NAc shell has been demonstrated to be more influential than the core in both the consumption and the palpability of rewards. Rats rapidly learn to self-administer psychostimulants such as cocaine, amphetamine, or DA receptor agonists into the medial accumbens shell, but not the core (Ikemoto *et al.*, 2005), and a number of drugs were shown to preferentially increase DA in the NAc shell (Pontieri *et al.*, 1995; Heidbreder & Feldon, 1998; Lecca *et al.*, 2004; Giorgi *et al.*, 2005; Aragona *et al.*, 2008; Aragona *et al.*, 2009). Natural rewards themselves were shown to preferentially increase DA in the NAc shell, but not the core (Bassareo & Di Chiara, 1999). Moreover, intra-NAc blockade of AMPA receptors in the rostral medial shell produces intense eating (Maldonado-Irizarry *et al.*, 1995). Likewise, intra-NAc μ-opioid agonists have also been shown to increase food intake, an effect that was more pronounced when μ-opioid agonists were infused in the NAc shell verses the core, whereas infusion of μ-opioid antagonists attenuated consumption (Kelley *et al.*, 1996; Pecina & Berridge, 2000). In addition, opioid agonists were shown to alter hedonic or palatability reactions to both rewarding and aversive stimuli in a discrete area within the medial NAc shell (Pecina & Berridge, 2005). The finding that processing of palatability reactions are localized to an isolated region within the NAc is indicative that certain aspects of reward-related stimuli may be processed by distinct neurotransmitter systems in functionally segregated regions or characteristically distinct ensembles of neurons in the NAc. With this idea in mind, this dissertation addresses the region, the DA receptor subtype, and the cell type specificity of the ensembles of neurons recruited during CS-potentiation of reward-seeking.

In some incidences both core and shell have been proclaimed to support commensurate functionalities. Intra-NAc infusions of DA or amphetamine into both the core and the shell induced heightened locomotion (Swanson *et al.*, 1997; Ikemoto, 2002; Ikemoto

& Witkin, 2003). In an elegant mapping study, Zhang and Kelley (2000) reported that μ-opioid stimulation in both the NAc core and shell enhanced the consumption of palatable foods. Likewise, PIT was enhanced by local infusions of amphetamine or a μ-opioid receptor agonist comparably throughout NAc core and shell (Pecina & Berridge, 2013). Widespread effects in the NAc could be reflective of similar neurochemistry or receptor expression as well as analogous input. Furthermore, anatomical connections between the core and shell have been established (van Dongen *et al.,* 2005; van Dongen *et al.,* 2008). Although the NAc subregions process information from different inputs, the interconnections between the core and the shell combined with evidence of similar processing of reward stimuli suggest that the core and the shell subsist as interacting networks rather than autonomous entities.

1.6 FUNCTIONAL ROLES OF NUCLEUS ACCUMBENS D1 AND D2 RECEPTORS

Several investigations have shown that intra-NAc infusions of D1 and D2 antagonists produce comparable effects on behavior (Baldo *et al.*, 2002; Lex & Hauber, 2008; Liao, 2008; Nicola, 2010; Shin *et al.,* 2010; Reisi *et al.*, 2014; Steinberg *et al.,* 2014). Both D1 and D2 receptor antagonists were shown to diminish cue-evoked excitation, but not cue-evoked inhibition of NAc cells during a discriminative stimulus task for sucrose. Importantly, in this study, unilateral intra-NAc infusions of both D1 or D2 receptor antagonists were shown to diminish the cue-evoked excitation of cell firing, but not cue-evoked behavior, whereas bilateral infusions of D1 or D2 receptor antagonists were shown to disrupt both the behavior and increased firing to the cue. These findings indicate that reduction in cue-evoked cell

firing was not attributable to the reduction in behavior itself (du Hoffmann & Nicola, 2014). Likewise, intra-NAc infusions of D1 or D2 antagonists comparably disrupted CS-potentiation of reward-seeking (Lex & Hauber, 2008). Further, conditioned place preference (CPP), a form of Pavlovian conditioning used to measure the motivational effects of rewards or experiences, induced via intra-NAc infusion of amphetamine was also reduced by intra-NAc infusion of either D1 or D2 receptor antagonists or co-infusion of the two antagonists. Numerous studies indicate that intra-NAc infusion of D1 and D2 receptors antagonists reduce instrumental responding (Nowend et al., 2001; Yun et al., 2004; Salamone et al., 2007; Lex & Hauber, 2008). Moreover, optogenetic intracranial self-stimulation of VTA DA neurons, which is considered to be extremely rewarding, was attenuated by intra-NAc infusion of D1 or D2 receptor antagonists (Steinberg et al., 2014). At first glance, the remarkably similar effect of these receptors antagonists to reduce behavior raises the question whether or not the antagonists are specific to the D1 versus D2 receptors. However, the similar effects of NAc D1 or D2 receptor blockade on cue-evoked behavior may be explained based on the emerging model of basal ganglia function.

Although the functional importance of the direct and the indirect pathways of the basal ganglia has been posited for many years (Alexander *et al.*, 1990), only recently have scientists had the technologies to study the precise contributions of these pathways to behavior. Using optogenetics to selectively stimulate D1 or D2 receptors, it was shown that activation of D1 receptor-expressing direct pathway NAc MSNs increases motor responses and attenuates freezing responses, whereas the activation of the D2 receptor expressing-indirect pathway NAc MSNs reduces motor responses and increases freezing behavior (Kravitz *et al.*, 2010). One interpretation of these findings is that activation of the direct pathway serves to promote correct movement, whereas the activation of the indirect pathway

functions to prevent competing movements. Therefore, the activation of D1 receptors on direct pathway neurons will activate D1 receptor-expressing MSNs and thereby promote the intended behavioral action, whereas the activation of D2 receptors on the indirect pathway neurons would inhibit the activity of indirect pathway neurons resulting in movements that compete with the intended actions. Consistent with this interpretation, a study using *in vivo* cell firing combined with the local infusion of DA antagonists has shown that blockade of D1 receptors decreases NAc cell firing, whereas blockade of D2 receptors increases NAc neuronal firing (West & Grace, 2002). Thus, if both pathways facilitate movement, blockade of either receptor subtype should reduce behavior directed at obtaining reward.

In addition to NAc DA's role in movement, it is important to consider that DA transmission may also play a role in mediating intracellular signaling mechanisms related to associative learning processes governed by the NAc, such as Pavlovian and instrumental conditioning, as described above. In partnership with glutamate, NAc DA serves to promote synaptic plasticity events that occur during learning (Kelley, 2004; Girault *et al.*, 2007; Shiflett & Balleine, 2011a). This idea is consistent with the observations that both D1 and NMDA antagonism in the NAc impairs the acquisition of Pavlovian learning and instrumental conditioning (Smith-Roe & Kelley, 2000; Di Ciano *et al.*, 2001; Dalley *et al.*, 2005; Hernandez *et al.*, 2005). Importantly, these foregoing studies have demonstrated that NAc D1 receptors, but not D2 receptors mediate the acquisition of reward related associative learning processes.

1.7 DOPAMINE AND ERK SIGNALING IN THE STRIATUM

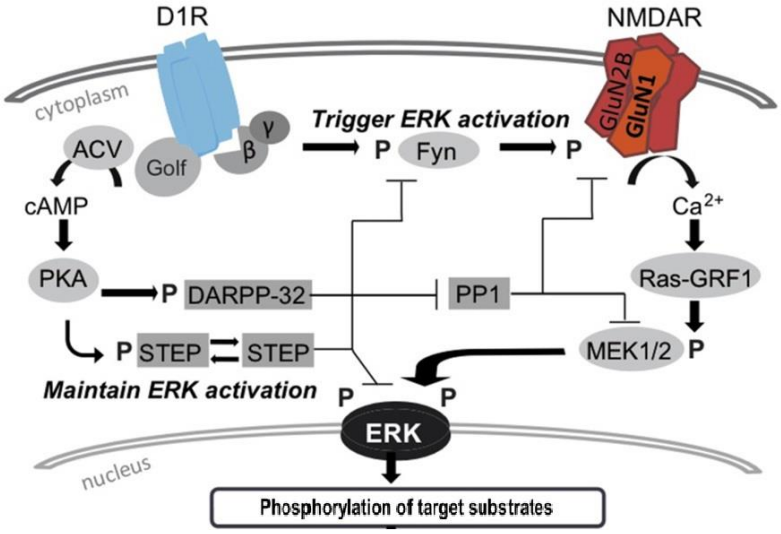

Figure 2: D1 receptor-mediated ERK activation signaling pathway (Cahill *et al.*, 2014).

D1 and D2 receptors have opposing effects on MSN physiology. D1 receptor activation serves to increase the excitability of MSNs and to promote corticostriatal synaptic plasticity (Surmeier *et al.*, 2007). In the striatum, ERK activation has been shown to occur in manner that requires the coincident activation of D1 receptors and NMDA receptors (see Figure 2) (Valjent *et al.*, 2005). Importantly, it was shown that activation of D1 receptors is not sufficient to increase ERK activation in the absence of endogenous glutamate (Pascoli *et al.*, 2011). D1 receptor activation enhances AC activity, which increases the concentration of cAMP and stimulates cAMP-dependent protein kinase A (PKA)(Greengard, 2001). PKA phosphorylates a multitude of substrates, including ion channels, neurotransmitter receptors such as NMDA receptors (Leonard *et al.*, 1999), transcription factors, and other phosphoproteins, such as dopamine- and cAMP-dependent phosphoprotein of 32 kDa (DARPP-32)(Greengard, 2001; Svenningsson *et al.*, 2004). DARPP-32 is expressed

exclusively in MSNs and the phosphorylation sites of DARPP-32 are often used as a readout of striatal signaling (Ouimet *et al.*, 1984; Sonomura *et al.*, 2007; Bertran-Gonzalez *et al.*, 2008). When DARPP-32 is phosphorylated on Thr34, it inhibits protein phosphatase 1 (PP-1), a phosphatase that inhibits the activation of ERK (Hemmings *et al.*, 1984). An important parallel signaling cascade that takes place irrespective of D1 receptor activation is glutamate-triggered NMDAR activation and the consequent activation of the Ras-Raf-MEK-cascade (Girault, 2012). The inhibition of PP-1 potentiates the ERK signal that was transduced from NMDA receptor activation. In contrast with D1 receptor activation, D2 receptor activation inhibits AC activity and reduces cAMP formation (Wickens, 2003). D2 receptor activation generally has a net effect of reducing MSN excitability (Surmeier *et al.*, 2007).

Numerous studies suggest that D1 but not D2 receptor activation mediates ERK activation *in vivo*. ERK activation evoked by drugs and drug-predictive cues was shown to occur exclusively in MSNs that contain D1 receptors in the NAc (Bertran-Gonzalez *et al.*, 2008; Borgkvist *et al.*, 2008; Fricks-Gleason & Marshall, 2011). In addition, many studies showed that systemic administration of D2 receptor antagonists *increases* ERK activation in MSNs that contain D2 receptors in the NAc (Bertran-Gonzalez *et al.*, 2008; Fricks-Gleason & Marshall, 2011). Accordingly, D2 receptors were shown to mediate the *inhibition* of ERK signaling in the striatum (Zhang *et al.*, 2004).

To further decipher contributions of the neuronal ensembles recruited in the PIT circuit, it is important to consider that there are different phenotypes of ERK immunopositive cells, each with unique afferent and efferent projections, neurochemistry, and corresponding functional implications (Valjent *et al.*, 2005). For instance, D2 receptor activation has been implicated in cocaine-evoked activation of ERK in the dorsal striatum, but in the same study administration of a D2-subtype receptor antagonist failed to block cocaine-evoked ERK

activation in the core and shell of the NAc (Valjent *et al.*, 2000). D1 receptor-mediated ERK activation also is regulated differently in the dorsal and the ventral striatum. Both stimulation of the nigrostriatal pathway or treatment with the D1 receptor agonist SKF81297 increased ERK activation in MSNs of the NAc, but only in a few interneurons in the dorsal striatum (Gerfen *et al.*, 2002). Additionally, in both D1 receptor and DARPP-32 knock-out mice, both cocaine and amphetamine-evoked ERK activation in the NAc is significantly reduced, whereas ERK activation via cocaine and amphetamine persists in the dorsal striatum of these knock-outs (Valjent *et al.*, 2005; Gerfen *et al.*, 2008). Thus, even within the striatum there are regional differences of ERK regulation.

Our lab found that ERK activation evoked by CSs predictive of natural rewards is exclusive to the ventral striatum (Shiflett *et al.*, 2008). However, it is important to consider that the knowledge of ERK regulation in the striatum is built largely from studies that have extensively investigated the neural mechanisms underlying drug-evoked ERK activation, and these patterns of ERK activation vary by the particular drug (Valjent *et al.*, 2000; Valjent *et al.*, 2004; Bertran-Gonzalez *et al.*, 2008; Borgkvist *et al.*, 2008). It is not known whether CS-evoked ERK activation during PIT is mediated by the same mechanisms as drug-evoked ERK activation, and if the pattern of ERK activation during PIT is different between the NAc core and shell, or if the phenotype of the cells in which CS-evoked ERK activation occurs is the same as drug-evoked ERK activation.

1.8 THE ROLE OF EXTRACELLULAR-SIGNAL REGULATED KINASE IN PAVLOVIAN-INSTRUMENTAL TRANSFER

Our lab previously investigated the regulation of ERK during both Pavlovian conditioning and Pavlovian-instrumental transfer (Shiflett *et al.*, 2008). We found that animals that received paired presentations of a CS and sucrose reward during training exhibited an increase in ERK activation in the NAc compared to control animals that received only CS presentations during training. The increase in ERK activation was determined to be a consequence of the CS presentation for two reasons. First, the animals were tested in the absence of the sucrose reward so the reward did not cause NAc ERK activation. Second, experimental animals that were presented with only the context during the test did not exhibit an increase in NAc ERK activation. Using both Western Blot and IHC analysis, the CS-evoked increase in ERK activation was observed in both the core and the shell subregions of the NAc, but was not observed in the dorsal striatum. Additionally, we tested whether this CS-evoked increase in ERK activation was required for PIT. Rats were implanted with bilateral cannulae directed at the NAc. First, these animals were trained in a Pavlovian conditioning paradigm followed by an instrumental conditioning paradigm. Next, the animals received bilateral intra-NAc infusions of U0126, a specific inhibitor of MEK, the kinase that activates ERK, immediately before the PIT test. Bilateral intra-NAc infusions of U0126 blocked the CS-evoked increase in ERK activation and concurrently disrupted the positive transfer that was observed in animals that received vehicle infusions during presentation of the CS. This study provides strong evidence that the ability of CSs to promote reward-seeking behaviors is dependent on increased ERK signaling within the NAc.

1.9 SUMMARY OF AIMS & RESULTS

The goals of the experiments described in this dissertation were to determine whether (1) the pattern of ERK activation during cue potentiation of reward-seeking differs between the NAc core and shell and (2) NAc ERK activation evoked by the CS during PIT is mediated by D1-like or D2-like receptors on NAc projection neurons and (3) CS-evoked ERK activation occurs in D1- or D2-expressing MSNs. To accomplish these goals, I addressed the following specific aims:

AIM 1: Which NAc subregion is responsible for processing the various stimuli that contribute to CS-potentiation of reward seeking? Following training under both Pavlovian and instrumental conditioning paradigms, rats were tested for PIT. We examined the pattern of ERK activation in both NAc subregions caused by exposure to the reward (US), and compared it to the patterns evoked by exposure to a CS during PIT versus exposure to a CS without reward-seeking opportunity. We found that presentation of the reward-predictive CS caused a significant increase in ERK activation in both subregions of the NAc. Furthermore, we found that the occurrence of reward-seeking behavior had no effect on ERK activation in either subregion of the NAc. Presentation of the reward, different from the pattern evoked by the CS, had no effect on ERK activation in the core but caused a pronounced increase in ERK activation in the shell. Taken together, our results show that during CS modulation of reward seeking, ERK activation is recruited in subsets of NAc neurons within both the core and shell, and that this ERK activation is driven by exposure to the CS irrespective of whether or not the situation permits engagement in reward-seeking behavior. Furthermore, our results show that whereas information about primary rewards is

mediated by the NAc shell, the motivational influence of the CS is mediated by both the core and the shell. Our results thereby suggest that inputs to and projection targets of cells in both the core and shell are likely elements of the neuronal circuit that underlies CS control over reward-seeking in the general PIT paradigm used here.

AIM 2: Is the ERK activation evoked by the presentation of a CS during Pavlovian instrumental transfer (PIT) mediated via D1-class or D2-class receptors on NAc projection neurons and does CS-evoked ERK activation occurs in D1- or D2-expressing MSNs? To test whether D1 or D2 receptor mediate CS-evoked NAc ERK activation during PIT, rats were fitted with bilateral cannulae aimed between the NAc core and the shell. Following surgery, rats were trained under the series of Pavlovian and instrumental conditioning paradigms, and given a unilateral infusion of SCH23390, a specific D1 receptor antagonist or raclopride, a specific D2 receptor antagonist, into the NAc of one hemisphere and vehicle solution into the other NAc immediately prior to the PIT test. Sections from these animals were examined and cell counts from the drug and vehicle infused NAc were compared to determine whether the D1 or D2 receptors antagonists altered CS-evoked ERK activation. We observed a robust activation of ERK signaling in the vehicle- and D2 receptor antagonist-treated NAc. By contrast, cue- elicited ERK activation was significantly reduced in the D1 receptor antagonist- treated NAc. To further determine whether CS-evoked ERK activation during PIT occurs in the subpopulation of MSNs that express D1 receptors, we performed dual-immunofluorescence staining for pERK and prodynorphin (PDYN). PDYN is the precursor peptide of dynorphin neuropeptides (Hokfelt *et al.*, 2000). DYN and PDYN have been shown to be contained exclusively in MSNs that express D1Rs in both the dorsal (Gerfen *et al.*, 1990; Lee *et al.*, 1997; Hara *et al.*, 2006;

Sonomura *et al.*, 2007). We found that the robust ERK signaling, which was markedly reduced upon the administration of the D1 receptor antagonist, occurred exclusively in PDYN-expressing NAc neurons. In addition, PDYN staining overlapped with staining for D1 receptors. Together, our results demonstrate that the activation of the ERK signaling pathway during PIT is mediated by activation of D1 receptors in the NAc and occurs in D1 receptor-expressing NAc neurons. One the other hand, the involvement of D2 receptors in CS potentiation seems to be ERK independent, as ERK activation in PDYN-expressing NAc neurons was unaffected by the inactivation of NAc D2 receptors and ERK activation did not occur in NAc cells that stain positive for markers of D2-expressing MSNs. These studies implicate that structures receiving input from the NAc D1 receptor-expressing MSNs are likely candidates in neuronal PIT circuit that underlies CS control over reward-seeking.

2.0 STIMULUS-SPECIFIC AND DIFFERENTIAL DISTRIBUTION OF ACTIVATED EXTRACELLULAR SIGNAL-REGULATED KINASE IN THE NUCLEUS ACCUMBENS CORE AND SHELL DURING PAVLOVIAN-INSTRUMENTAL TRANSFER

This chapter contains the published manuscript detailing the results of the studies from Aim 1. This report has been published in the following peer reviewed publication:

Remus, M. L., & Thiels, E. (2013). Stimulus-specific and differential distribution of activated extracellular signal-regulated kinase in the nucleus accumbens core and shell during Pavlovian-instrumental transfer. Brain Structure & Function, 218(4), 913–927. doi:10.1007/s00429-012-0438-x

2.1 INTRODUCTION

Stimuli that repeatedly are paired with reward, i.e., appetitive conditioned stimuli (CSs), have the remarkable ability to potentiate operant responding to obtain the reward, a behavioral phenomenon known as Pavlovian-instrumental transfer (PIT; Rescorla, 1967; Lovibond, 1983). This property of CSs generally is beneficial for survival as it enhances procurement of resources; however, it can be maladaptive when it leads to overconsumption of reward and contributes to behavioral disorders such as obesity and drug abuse (O'Brien *et al.*, 1998; Everitt & Wolf, 2002; Crombag *et al.*, 2008; Volkow *et al.*, 2009). Several lines of research, involving both animal and human subjects, have implicated the nucleus accumbens (NAc) as a key structure for mediating PIT (Corbit *et al.*, 2001; Hall *et al.*, 2001; Talmi *et al.*,

2008). Consistent with a critical role for the NAc in PIT, we found that activation of the signal transduction enzyme extracellular signal-regulated kinase (ERK) is increased in the NAc upon exposure to an appetitive CS, and that inhibition of CS-evoked ERK activation in the NAc completely abolishes PIT (Shiflett *et al.*, 2008).These findings indicate that ERK activation in the NAc is required for the ability of CSs to potentiate reward seeking.

The NAc frequently is considered to serve as a limbic-motor interface (Mogenson *et al.*, 1980; Nicola, 2007). This ventral striatal structure is not a uniform nucleus but comprises two primary subregions, the core and the shell. Anatomical, pharmacological, neurochemical, and behavioral heterogeneity has been demonstrated among the NAc subregions (Voorn *et al.*, 1989; Zahm & Brog, 1992; Groenewegen *et al.*, 1999; Zahm, 2000; Di Chiara, 2002; Baldo & Kelley, 2007). The diverse nature of the subregions' output targets, with the core projecting primarily to motor structures and the shell to limbic areas (Zahm & Brog, 1992), suggests that the two subregions may contribute differentially to, or to different aspects of, the PIT phenomenon (Shiflett & Balleine, 2011b). In our previous observations of ERK activation in the NAc during PIT, we did not differentiate between core and shell. To determine whether ERK activation is evoked in both of these subregions and to dissect out the relative contribution of the reward-seeking behavior versus the CS to the ERK activation pattern, we here examined the subregional distribution of ERK activation during PIT, as well as whether or not the opportunity to engage in reward-seeking behavior affects the subregional distribution of ERK activation in the NAc.

2.2 MATERIALS & METHODS

2.2.1 Subjects

Sixty-three male Sprague-Dawley rats (Hilltop Lab Animals, Scottdale, PA) weighing between 250 and 275 g on arrival were used in this study. Rats were handled and weighed daily. Beginning 5 days prior to training and continuing until testing was completed, rats were food-restricted to ~90% of the body weight of free-feeding age-matched rats. Rats were housed individually and maintained on a 12-h light/dark cycle with *ad libitum* access to water. All procedures were performed during the light cycle and in accordance with the NIH Guide for the Care and Use of Laboratory Animals and with the approval of the Institutional Animal Care and Use Committee of the University of Pittsburgh. The behavioral experiments were performed in the Rodent Behavior Analysis Core of the University Of Pittsburgh School Of Health Sciences.

2.2.2 Behavioral Apparatus

All procedures took place in standard rat operant chambers (30 cm x 23 cm x 23 cm; Med Associates, St. Albans, VT) equipped with a house light, a metal grid floor, and a loudspeaker. A food cup was mounted on the front wall and attached to a pellet dispenser that released a single food pellet when activated. A photobeam source and detector were mounted onto the sides of the food cup to monitor food cup approaches. One retractable lever was mounted approximately 4 cm to the left side and a second lever 4 cm to the right side of the food cup. The chambers were enclosed in light- and sound-attenuating cubicles equipped with fans that generated background noise to obscure external noise. MED-PC IV program

(Med Associates) controlled the equipment and recorded food cup approaches and lever presses.

2.2.3 Behavioral Procedures

The training protocols were adapted from Shiflett *et al.* (2008). Five behavioral groups were used in this study: TOY, TO, PIT, PAV, and REWARD (See Table 1). One day prior to Pavlovian training, all animals were habituated to the chamber in a single 30-min session. During this session, the only explicit cue was the illumination of the house light at the onset and its extinguishment at the termination of the session.

Table 1: Behavioral Paradigms for Individual Groups

Phase↓ Group→	**TOY**	**TO**	**PIT**	**PAV**	**REWARD**
Pavlovian training (6 days)	CS only	CS only	CS + reward	CS + reward	CS + reward
Instrumental training (6 days)	non-contingent reward delivery	reward contingent on lever pressing	reward contingent on lever pressing	context only	reward contingent on lever pressing
Test	CS; levers present	CS; levers present	CS; levers present	CS only	reward only

2.2.3.1 Pavlovian Conditioning

During the Pavlovian conditioning phase, animals from the PIT, PAV, and REWARD groups completed 6 daily sessions, with each daily session consisting of 8 pairings between a CS (90-s 3-kHz 80-dB tone) and delivery of 3 to 4 food pellets (45-mg dustless food pellets, BioServe, Frenchtown, NJ) dispensed on a variable time (VT) schedule of 20 s. For half of the trials, the VT schedule for pellet delivery was initiated with tone onset; for the remaining half, the VT schedule was delayed by 30 s after tone onset, to permit measurement of food cup approach in the absence of the US. The type of trial (food throughout vs. only during the last 60 s of the CS) was varied randomly between and within

sessions, with the restriction that each type of trial was presented 4 times per session. The intertrial interval (CS onset to next CS onset) varied between 210 and 330 s (mean = 270 s). For each session, the number of photobeam breaks was recorded during the first 30 s of the CS periods during which food delivery was delayed and the 30 s prior to CS onset (CS and preCS periods, respectively). The studies included two kinds of control groups, a tone-only yoked (TOY) and a tone-only (TO) group. During the Pavlovian conditioning phase, both control groups were presented with the identical number and pattern of 90-s tones as the experimental groups, but no food pellets were delivered during the sessions.

2.2.3.2 Instrumental Conditioning

After completion of Pavlovian conditioning, rats in the PIT, REWARD, and TO groups underwent instrumental conditioning. Each session began with illumination of the house light and extension of both levers into the chamber. One lever, designated by the experimenter as the active lever, delivered a single food pellet into the food cup when depressed. Depression of the other lever, the inactive lever, had no programmed consequences. The active lever was randomly assigned to the left or the right lever and was counterbalanced within and across cohorts. During this first session, rats received a single pellet for every depression of the active lever (termed a fixed-ratio one, or FR1, schedule). The first session terminated after rats earned a total of 50 pellets. Rats were returned to their home cages for a 45-min intersession interval after which the second training session began. For the second session, on average three presses on the active lever were required for delivery of a pellet (random-ratio 3, or RR3). The RR3 session terminated after 50 pellets were delivered. On the subsequent five days, animals underwent two daily RR5 sessions with a 45-min intersession interval in their home cages. Each RR5 session terminated after 100

pellets had been delivered. Levers were extended for rats of the TOY group, but depression of the levers did not produce a food pellet. Instead, these control rats received a food pellet whenever a rat from the PIT group trained concurrently earned a food pellet. Rats in the PAV group were placed into the conditioning chamber during the instrumental sessions, but no stimuli other than the house light were presented.

2.2.3.3 Pavlovian Reminder & Extinction

The day before the test day, rats received a Pavlovian conditioning reminder session that was identical to prior Pavlovian training sessions, as described above. The reminder session was followed by 15 min in the home cage and then by a brief extinction session. During the extinction session, both levers were extended into the chamber for all groups, except for the PAV group, but food pellets were not delivered to any of the groups. The extinction session was terminated after instrumentally conditioned rats had pressed the active lever for 10 min or made 300 unrewarded lever presses (whichever occurred first). This extinction session served to avoid a ceiling effect during the transfer test. For animals in the PAV and TOY groups, the extinction session was terminated after 10 min.

2.2.3.4 Pavlovian-instrumental Transfer (PIT) Test

The PIT test began with the illumination of the house light and extension of both levers into the chamber for all groups, except for rats in the PAV and REWARD groups. During the first 6 min, both levers were available, but no stimuli were presented. Thereafter, four 90-s tones were presented at a fixed 270-s interstimulus interval. The number of active and inactive lever presses and food cup approaches were recorded for each 90-s CS period and the 90-s period prior to the CS (preCS period). For rats in the PAV and REWARD

groups, the levers were not extended and, therefore, only food cup approaches were recorded. Rats in the PAV group received the same series of tone presentations as described above, and food cup approaches were recorded during the preCS and CS periods. Rats in the REWARD group did not receive tone presentations but instead were given food pellets on a VT schedule of 20 s.

Immediately after the test, rats were anesthetized with an intraperitoneal (i.p) injection of chloral hydrate (300 mg/kg dissolved in 0.9% saline) and either decapitated or transcardially perfused. In the case of decapitation, brains were removed quickly and briefly immersed in 2-methyl-butane on dry ice and then stored at -80^{o} C until excision of NAc tissue in preparation for Western blot analysis. In the case of transcardial perfusion, the perfusion medium consisted of 50 ml of 0.9% saline containing heparin (Acros Organics, Antwerpen, Belgium; 20 i.u./ml) and sodium fluoride (2mM), followed by 500 ml of 4% periodate-lysine-paraformaldehyde (PLP) fixative in 0.1 M sodium phosphate buffer (PB, pH 7.4). Brains were removed and post-fixed overnight at 4^{o} C in PLP fixative, in preparation for immunohistochemistry.

2.2.4 Western Blot Analysis

NAc tissue was harvested as described previously (Shiflett *et al.*, 2008; 2009). Briefly, NAc samples were excised from 1-mm thick coronal sections by placing a tissue punch (2 mm in diameter, which yields a sufficient amount of protein for analysis; Fine Science Tools, Foster City, CA) over the core and shell, so as to include about equal parts of both subregions in each sample. Samples were homogenized in a buffer containing 150 mM NaCl, 1 mM EDTA, 50 mM Tris (pH 7.4), 0.05% SDS, 1% Trition-X-100, 1nM dithiothreitol (DTT), 2 mM sodium fluoride, 1 mM orthovandate, 2 mM sodium

pyrophosphate, 1 mg/ml pepstatin, and Protease inhibitor cocktail (Merck KGaA, Darmstadt, Germany). Homogenates were centrifuged for 15 min at 14,000 rpm. For each sample, protein concentration of the supernatant was determined in triplicates using a bicinchoninic acid assay (Pierce, Rockford, IL). Samples were diluted to a uniform concentration with homogenization buffer and sample buffer containing 2.5 M Tris (pH 6.8), 40% glycerol, 8% SDS, and 30 mg/ml DTT, and then were heated to 95° C for 5 min. Forty-five µg of protein per sample were loaded for resolution by SDS-PAGE and subsequently transferred to Immobilon membranes. Membranes were blocked for 1 h at 22° C in a Tris-buffered saline and 0.1% Tween 20 (TBST) solution containing 5% dried nonfat milk followed by an overnight incubation at 4° C in TBST containing 5% bovine serum albumin and an antibody specific for dual-phosphorylated (T202/183 and Y204/185), activated ERK1/2 (1:2500 dilution; Cell Signaling Technology, Beverly, MA). Membranes were washed in TBST solution and incubated with an HRP-linked secondary antibody (anti-rabbit, 1:5000; Cell Signaling Technology) for 1 h at 22° C. Phospho-ERK2 (pERK2) immunoreactivity was visualized on blot images captured with a CCD camera (Hamamatsu Photonics, Japan) using enhanced chemiluminescence reagent (Lumiglo; Cell Signaling Technology). To probe each sample for total ERK1/2, membranes were stripped of their antigens via incubation for 45 min at 55° C in a solution containing 62.5 mM Tris (pH 6.7), 2% SDS, and 0.62% β-mercaptoethanol. Membranes were blocked and reprobed with an antibody specific for phosphorylated and non-phosphorylated ERK1/2 (1:2500; Cell Signaling Technology), as described above. Total ERK2 (tERK2) immunoreactivity was visualized as described above. pERK2 and tERK2 immunoreactivity were analyzed using densitometry software (UVP Labworks, Upland, CA). ERK2 activation was determined for each sample by dividing pERK2 immunoreactivity by tERK2 immunoreactivity for a given sample and normalizing

the resulting ratios to similar ratios for samples from rats of the TOY group run on the same membrane.

2.2.5 Immunohistochemistry

Following overnight fixation, brains were cut into 50-µm coronal sections using a vibratome (Vibratome Series 1000) and collected in PB in four adjacent series. The fixation process was quenched by placing sections in 1% sodium borohydride in PB for 30 min and then rinsing them several times in PB. Sections were stored at -20° C in cryopreservant solution until immunohistochemical staining. In preparation for staining, free-floating sections were rinsed first in PB overnight at 4° C and then rinsed three times for 30 min in Tris-buffered saline (TBS; 50 mM Tris in 0.9% NaCl, pH 7.6). After rinsing, sections were placed in blocking buffer (5% normal goat serum and 0.3% Triton X-100 in TBS) for 1 h at 22° C, and then incubated with anti-phosphoERK antibody (1:800; Cell Signaling Technology) in blocking buffer for 24 to 48 h at 4° C. After primary antibody incubation, sections were washed in TBS and incubated with biotinylated goat anti-rabbit IgG secondary antibody (1:500; Vector Laboratories, Burlingame, CA, USA) in blocking buffer for 2 h at 22° C. Sections then were washed in TBS and incubated with avidin-biotin conjugate (1:500 for each A and B reagent; Vector Laboratories, Burlingame, CA) in blocking buffer for 90 min at 22° C. Afterwards sections were rinsed in 0.1 M sodium acetate buffer (SA, pH 6.0) three times for 10 min. Immunostaining was developed in the SA buffer containing 0.022% diaminobenzidine, 0.003% hydrogen peroxide, and 2.5% nickel sulfate. After three additional washes, sections were mounted on gelatin-coated slides, dried at room temperature, dehydrated in ethanol, cleared in xylene, and coverslipped with Cytoseal 60 (Richard-Allan Scientific).

2.2.5.1 Analysis of pERK1/2 Immunohistochemistry

Images of sections stained for pERK1/2 were captured using a 10X objective and a digital camera (Micrometrics 3.2 MP) mounted on a light microscope (Leitz Orthoplan 2). The core and shell of the NAc, and the dorsolateral and dorsomedial striatum (DLS and DMS, respectively) were identified using a rat brain atlas (Paxinos & Watson, 2007). For each subject, sections at a frequency of 200 μm between +1.1 to +2.0 mm anterior to bregma were captured for approximately 40 images (10 per subregion) per rat. Digitized images were used to estimate the number of pERK1/2-immunoreactive (pERK-IR) cells in the NAc core by placing a 200 μm x 400 μm counting window just dorsal to the anterior commissure. For the medial NAc shell, the number of pERK1/2-IR cells was estimated by placing a 400 μm x 200 μm counting window 400 μm medial to the anterior commissure between +1.1-1.2 mm anterior to bregma, 500 μm medial to the anterior commissure between +1.3-1.4 mm anterior to bregma, and 600 μm medial to the anterior commissure between +1.5-2.0 mm anterior to bregma (see Figure 5 for examples of counting window placements). For the dorsal striatum, the number of pERK1/2-IR cells was estimated by placing a 600 μm x 600 μm counting window either next to the lateral ventricle in the most medial portion of the dorsal striatum (DMS), or at the same dorsoventral level approximately 200 mm lateral to the lateral ventricle, in the most lateral portion of the dorsal striatum (DLS),. Digitized images of the counting windows were created and used to obtain cell counts with the aid of an image analysis macro written for NIH ImageJ (1.43u). To account for differences in background staining, image thresholds were adjusted prior to cell counting. Briefly, using a macro written for ImageJ, a background mask of the image was created by replacing each pixel with the median of the surrounding pixels in a 30 pixel radius. Next, the contrast of each pixel was

calculated with respect to the background from the following equation: (background - image) / (background + image). Then, a binary image was created using an arbitrary threshold of 0.10 for all images. Lastly, a watershed algorithm and a particle analysis algorithm were applied to separate any overlapping cells and to discard particles that were too small to be considered cells, respectively. Cell counts derived from ImageJ (NIH) were verified by an experimenter blind to the experimental conditions using the original image of the counting window. For each subject, the number of pERK1/2-immunopositive cells was counted in both hemispheres, yielding a total of ten estimates per region per animal.

2.2.6 Statistical Analysis

Data from Pavlovian and instrumental training were compared using analysis of variance (ANOVA) for repeated-measures with group as between-subject factor and training day as within-subject factor followed by Bonferroni post-hoc comparisons of individual groups. PIT transfer and PAV test data were compared using ANOVA for repeated-measures with group as between-subject factor and interval (preCS, CS) as within-subject factor followed by post-hoc comparisons. For experiment 1, pERK2/ tERK2 ratios were compared with a one-way ANOVA followed by post-hoc comparisons. For experiment 2, cell counts per mm2 from the TOY and TO control groups were compared using ANOVA with group as between-subject factor and area (core, shell) as within-subject factor. Comparisons between pooled controls and experimental groups were conducted using one-way ANOVAs for each subregion (core, shell, DLS, DMS) separately, followed by post-hoc comparisons. All statistical analyses were performed using the SPSS software package version 19.0 (Chicago, IL). For all statistical comparisons, $p < 0.05$ was the criterion for significance.

2.3 RESULTS

2.3.1 NAc ERK activation during Pavlovian-instrumental transfer: Does lever-pressing matter?

We previously found that active ERK in the NAc, as indicated by the density of NAc cells immunopositive for dual-phosphorylated, active ERK (pERK), is higher in rats exhibiting PIT than in control rats that were familiarized with the CS during the Pavlovian conditioning phase but that did not receive lever-press training during the instrumental conditioning phase (Shiflett *et al.*, 2008). Because control rats did not engage in lever-pressing during the PIT test, it is possible that the difference in pERK density between groups resulted, at least in part, from the difference in instrumental responding during the test. Furthermore, we did not measure total ERK levels, thus leaving it unclear whether the increase in pERK density resulted from an increase in total ERK or from higher activation of the available ERK. To address both of these issues, we here used Western blot analysis and compared both ERK activation and total ERK levels in the NAc between experimental rats (PIT group), control rats that received lever-press training (TO group), and control rats that received food pellets yoked to the experimental group but no lever-press training (TOY group; see Table 1).

2.3.1.1 Behavior

At the beginning of Pavlovian training, discriminative food cup approach rates (number of approaches/min during the CS minus number of approaches/min during the preCS period) were equally low in all three groups; however, as training progressed, these rates increased for rats in the PIT group (n=8) but not for rats in either the TO (n=6) or the TOY (n=7) control groups. ANOVA confirmed these differences, as indicated by a significant group x day interaction ($F_{(10, 90)} = 5.75$; $p < 0.01$). Post-hoc comparisons showed that the three groups did not differ from one another on the first day of training. After six days of training, the mean discriminative approach rate of the PIT group was significantly higher than that of either the TO or the TOY group (4.7 ± 0.9, 0.1 ± 0.4, and 0.8 ± 0.3 approaches/min, respectively; $p < 0.01$), whereas the rates of the two control groups did not differ significantly from one another. During instrumental training, rats in both the TO and PIT groups acquired instrumental responding at a comparable rate, and their response rates increased across training days. As expected, rats in the TOY group did not acquire lever pressing. ANOVA confirmed these trends, as indicated by a significant group x day interaction ($F_{(10, 90)} = 4.46$; $p < 0.01$). On the last day of training the mean lever-press rates for the PIT, TO, and TOY groups were 24.9 ± 3.8, 28.6 ± 8, and 0.1 ± 0.1 presses/min, respectively. Post-hoc comparisons revealed that mean rates of the PIT and the TO groups did not differ for any of the six training days, which indicates that rats in these two groups experienced similar instrumental training prior to the PIT test.

During the PIT test, rats in the PIT group pressed the lever significantly more during the CS than the preCS period, and thus demonstrated a robust PIT effect (Figure 3A). Rats in the TO group also displayed reasonably high lever-press rates; however, their response rates

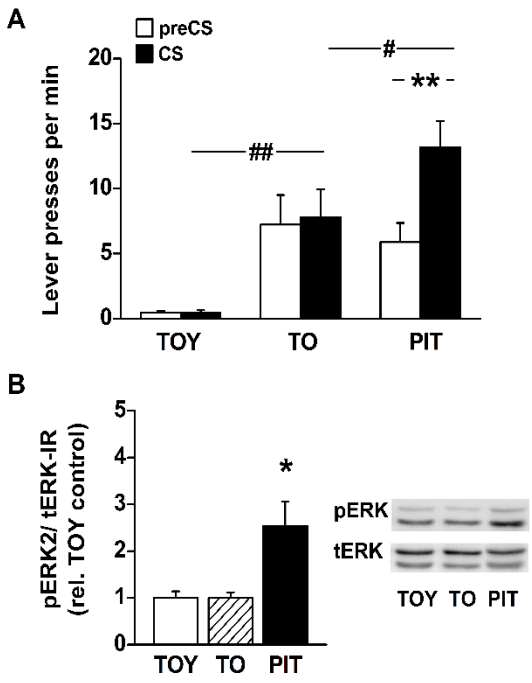

Figure 3: Pavlovian-instrumental transfer, but not lever pressing, results in increased ERK2 activation in the NAc. **a.** Mean±SEM lever presses per min for the preCS and CS periods of each group during the PIT test. Only for rats of the PIT group (n=8), but not for rats of either the TOY group (n=7) or the TO group (n=6) were response rates during the CS significantly higher than those during the preCS period (**, $p<0.01$). Lever-press rates during the preCS period did not differ between the PIT and TO groups, but lever-press rates during the CS were significantly higher for rats in the PIT group than rats in the TO group (#, $p<0.05$), whose lever-press rates, in turn, were significantly higher than those of rats in the TOY group (##, $p<0.01$). **b.** Mean±SEM normalized pERK2 immunoreactvity / tERK2 immunoreactivty in NAc samples from each group. pERK2/tERK2 immunoreactivity was significantly higher in NAc samples from rats of the PIT group than NAc samples from rats of either the TO or the TOY control group (*, $p<0.05$). Representative pERK and tERK immunoblots for each treatment group are shown on the right. The lower (p42) band was used for analysis.

were similar during the preCS and CS periods. As expected, rats in the TOY group essentially did not press the lever during either time period. ANOVA confirmed these differential trends, as indicated by a significant group x stimulus period interaction ($F_{(2, 18)} = 21.48$; $p < 0.01$). Post-hoc comparisons revealed that the mean lever-press rate during the CS was significantly higher than that during the preCS period for the PIT group ($p < 0.01$) but not for the TO or the TOY groups. The mean lever-press rate during the preCS period was significantly lower for the TOY group than for either the TO or the PIT groups ($p < 0.01$), with no difference between the latter two groups. The mean lever-press rate during the CS also was significantly lower in the TOY group than in either the TO or the PIT groups ($p < 0.01$); however, in contrast to the preCS period, the mean lever-press rate during the CS was significantly higher in the PIT than the TO group ($p < 0.05$). ANOVA of the total lever presses during the test revealed a significant effect of group ($F_{(2, 18)} = 11.03$; $p < 0.01$), and post-hoc comparisons confirmed that the total number of lever presses were significantly lower for rats in the TOY group compared with rats in the PIT or TO groups ($p < 0.05$), with

no significant difference between the latter two groups. The total number of lever presses for the PIT, TO, and TOY groups during the PIT test were 256 ± 50, 173 ± 40, and 11 ± 4, respectively.

2.3.1.2 Western Blot Analysis

Immediately after the test, rats were decapitated and the NAc was excised for determination of activation of ERK2, the ERK isoform we and others showed previously to be regulated differentially during Pavlovian conditioning (Shiflett *et al.*, 2008). As shown in Figure 3B, we found that ERK2 activation, as indicated by pERK2 immunoreactivity/tERK2 immunoreactivity (see Methods), in NAc samples from rats in the PIT group was about 2.5-fold greater than ERK2 activation in NAc samples from rats in either the TO or the TOY group. ANOVA indicated a significant effect of group ($F_{(2, 20)} = 6.34$; $p < 0.01$), and post-hoc comparisons confirmed that ERK2 activation was significantly higher in the PIT group than in either the TO or the TOY group ($p < 0.05$). Importantly, ERK2 activation did not differ between the two control groups. Thus, despite the marked difference in lever-press rates between the two control groups, NAc ERK2 activation was comparable between them. To determine whether the PIT-associated increase in ERK2 activation resulted from an increase in pERK2, rather than a decrease in tERK2, we also compared tERK2 immunoreactivity between the three groups. ANOVA revealed no significant group effect ($F_{(2, 20)}$). The lack of an effect on tERK2 level indicates that the observed changes in ERK2 activation stem from an increase in activated ERK2. By extension, these findings confirm that our previous observations of PIT-associated changes in pERK density (Shiflett *et al.*, 2008) reflected changes in ERK activation. Taken together, our results show that NAc ERK2 activation is

increased during PIT, and that this increase does not stem from conditioned lever pressing *per se*.

2.3.2 NAc ERK activation during Pavlovian-Instrumental Transfer: Subregional pattern as a function of presented stimulus

In light of demonstrated dissociations between the core and shell subregions of the NAc at the anatomical, neurochemical, and functional levels (Voorn *et al.*, 1989; Heimer *et al.*, 1991; Berendse *et al.*, 1992; Zahm & Brog, 1992; Brog *et al.*, 1993; Kelley, 1999)it is possible that the PIT-relevant molecular signal we described previously (Shiflett *et al.*, 2008) differs between NAc subregions. Furthermore, the apparent dependence of NAc ERK activation on exposure to the CS and not on lever-pressing observed in the previous experiment may not apply to the two subregions uniformly. To address these issues, we here compared the number of pERK-IR cells in the core and shell separately between the TO and TOY control groups, the PIT group, and a group of rats that received Pavlovian conditioning but no lever-press training (PAV group; see Table 1).

2.3.2.1 Behavior

During Pavlovian training, rats in the PIT (n = 8) and PAV (n = 9) groups developed discriminative food cup approach behavior, whereas control rats in either the TO (n = 8) or the TOY group (n = 9) did not. ANOVA revealed a significant group × day interaction ($F(5,150) = 3.82$; $P < 0.01$), and post hoc tests confirmed that, although there was no significant difference in approach behavior between groups at the beginning of training, rats in the PIT and PAV groups exhibited significant and similar discriminative approach behavior on the

last day of Pavlovian training, whereas rats in the TO or TOY group did not ($P < 0.01$). The mean discriminative approach rates for the PIT, PAV, TO, and TOY groups on the final day of Pavlovian conditioning were 6.3 ± 1.2, 4.9 ± 1.2, -0.2 ± 0.2, and 0.0 ± 0.4 approaches/min, respectively. During instrumental training, rats in both the PIT and the TO group acquired lever pressing at comparable rates across training sessions, whereas rats in the TOY group did not lever press. ANOVA indicated a significant group × day interaction ($F(5,110) = 8.18$; $P < 0.01$), and post hoc comparisons confirmed no differences between the PIT and TO groups for any of the instrumental training days, but both groups engaged in lever pressing at significantly higher rates than the TOY group on each of the training days ($ps < 0.01$). On the final day of instrumental conditioning, the lever-press rates were 33.6 ± 6.1, 31.5 ± 5.9, and 0.2 ± 0.1 presses/min for rats in the PIT, TO, and TOY groups, respectively. Lever-press rates during the PIT test are shown in Figure 4a. Similar to the results from the previous experiment, rats in the PIT group pressed the lever about twice as frequently during the CS compared to the preCS period. In comparison, lever-press rates for rats in the TO group did not differ between the preCS and CS periods, and rats in the TOY group did not press the lever during either the preCS or CS periods. ANOVA of the lever-press rates revealed a significant group × period interaction ($F(2,22) = 11.82$; $P < 0.01$). Post hoc tests confirmed that only the PIT group exhibited significantly enhanced lever pressing during the CS compared to the preCS period ($P < 0.01$). Lever-press rates during the preCS period did not differ between the TO and PIT groups, but were significantly higher in both of these groups compared with the TOY group ($P < 0.05$).

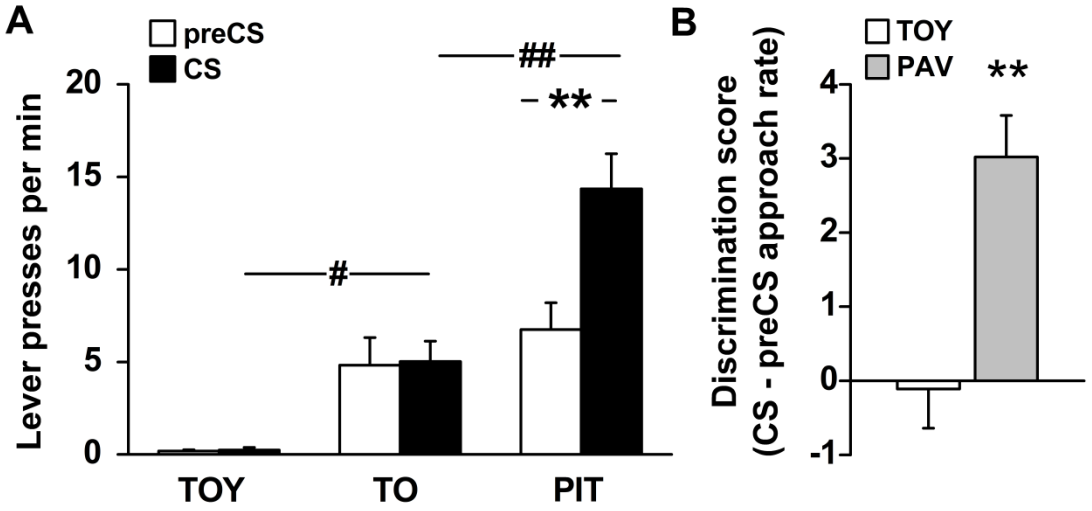

Figure 4: Appetitive conditioning leads to PIT and discriminative food cup approach during testing. **A,** Mean±SEM lever presses per min for the preCS and CS periods of the TOY group (n=9), the TO group (n=8), and the PIT group (n=8) during the PIT test. Only for rats of PIT group, but not for rats of either the TOY or the TO group, were response rates during the CS significantly higher than those during the preCS period (**, $p<0.01$). Lever-press rates during the preCS period did not differ between the PIT and TO groups, but lever-press rates during the CS were significantly higher for rats in the PIT group than rats in the TO group (##, $p<0.01$), whose lever-press rates, in turn, were significantly higher than those of rats in the TOY group (#, $p<0.05$). **B,** Mean±SEM discriminative food cup approach for rats of the TOY group and rats of the PAV group (n=9). Discriminative food cup approach was significantly higher for rats in the PAV group than rats in the TOY control group (*$p<0.01$).

In contrast, lever-press rates during the CS were significantly higher in the PIT group compared to the TO group ($P < 0.01$), which, in turn, was significantly higher than in the TOY group ($P < 0.05$). The total number of lever presses for the PIT, TO, and TOY groups during the PIT test were 218 ± 32, 152 ± 33, and 6 ± 2, respectively, and did not differ between the PIT and TO groups, but both of these groups pressed significantly more than rats in the TOY group ($F(2,22) = 18.55$; $P < 0.01$; post hoc test, $P < 0.01$). Discriminative food cup approach rates during the PIT test for rats in the PAV and TOY groups are shown in Figure 4b. Whereas rats in the PAV group exhibited pronounced discriminative approach behavior, rats in the TOY group showed no differential response. A t test for independent groups confirmed that discriminative responding was significantly higher in the PAV group compared to the TOY control group ($t(16) = 4.06$; $P < 0.01$). However, the total number of

food cup approaches during the test did not differ between the two groups (51 ± 8 and 45 ± 4 approaches/min for the PAV and TOY groups, respectively; $t(16) < 1$).

2.3.2.2 pERK1/2 Immunohistochemistry

Immediately after the test, animals were perfused to assess pERK1/2 immunostaining in the core and shell. As illustrated in Figure 5b, Figure 6b and d, pERK immunoreactivity was observed throughout the cell body and in the larger dendritic branches of accumbal neurons in both subregions. The great majority of immunopositive cells appeared to have a medium-sized soma (~14 μm). To determine whether lever pressing in the absence of a CS influences the pattern of pERK immunolabeling across the two NAc subregions, we first compared the number of pERK1/2-IR cells in the core and the shell between the TO and TOY control groups. pERK-IR cells were observed throughout the NAc core of rats in both groups, and the distribution appeared equally sparse in the two groups. In comparison, pERK immunolabeling appeared to be populous in the medial shell (~500 μm medial to the ac). Again, this effect was observed similarly in the two control groups (Figure 5b; see Table 2). ANOVA of the number of pERK-IR cells confirmed these observations, as there was a significant effect of area ($F(1, 15) = 32.20$; $P < 0.01$), but neither the main effect of group nor the group × area interaction was significant. Interestingly, we observed comparable numbers of pERK-IR cells in these

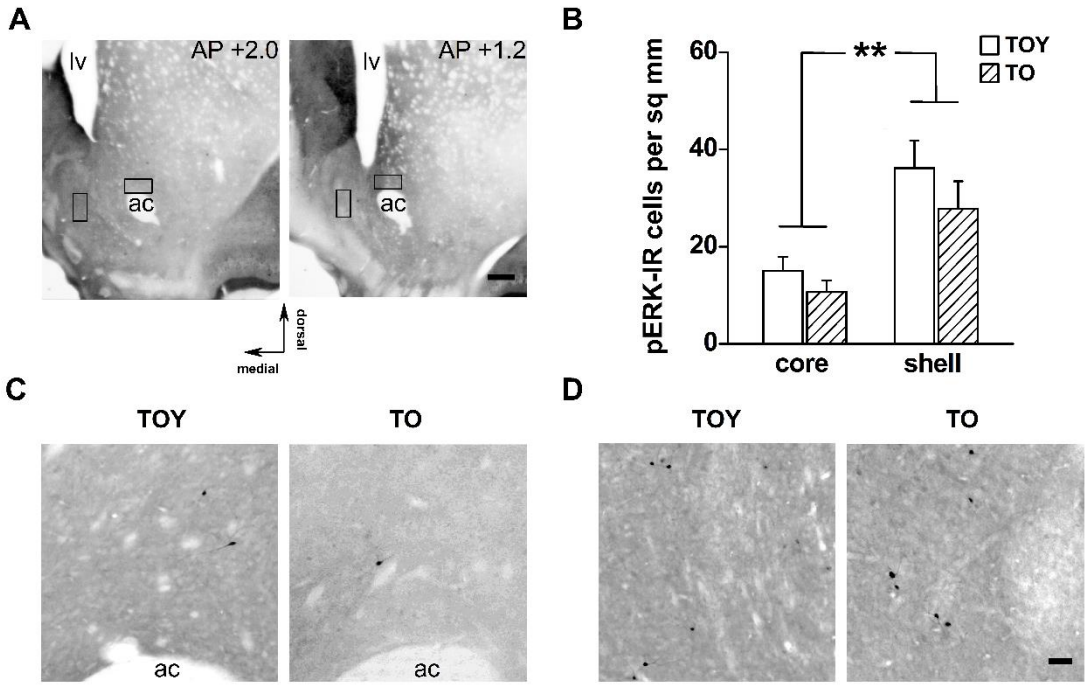

Figure 5: Reward-seeking behavior alone does not affect the level of active ERK in either the core or the shell. **A,** Representative low-magnification photomicrographs of a section taken from the most anterior end of or counting range (AP +2.0; left) and the most posterior end of our counting range (AP +1.2; right). Black rectangles illustrate placements of the counting window for pERK-IR cell counts in the core and the shell, respectively. The same counting window placements were used for the data depicted in Figures 3 through 5. **B,** Mean±SEM number of pERK1/2-IR cells per mm^2 in each subregion for the TOY (n=9) and the TO (n=8) groups. The number of pERK1/2-IR cells were comparable between the two control groups in both the core and the shell, and were similarly greater in the shell than the core (**, $p<0.01$). **C,** Representative high-magnification photomicrographs of pERK-IR cells in the core from a rat of the TOY group and a rat of the TO group (scale bar, see panel D; ac, anterior commissure). **D,** Similar representative high-magnification photomicrographs of pERK-IR cells in the shell from a rat of each of the two control groups (scale bar, 40 µm; applies to C and D).

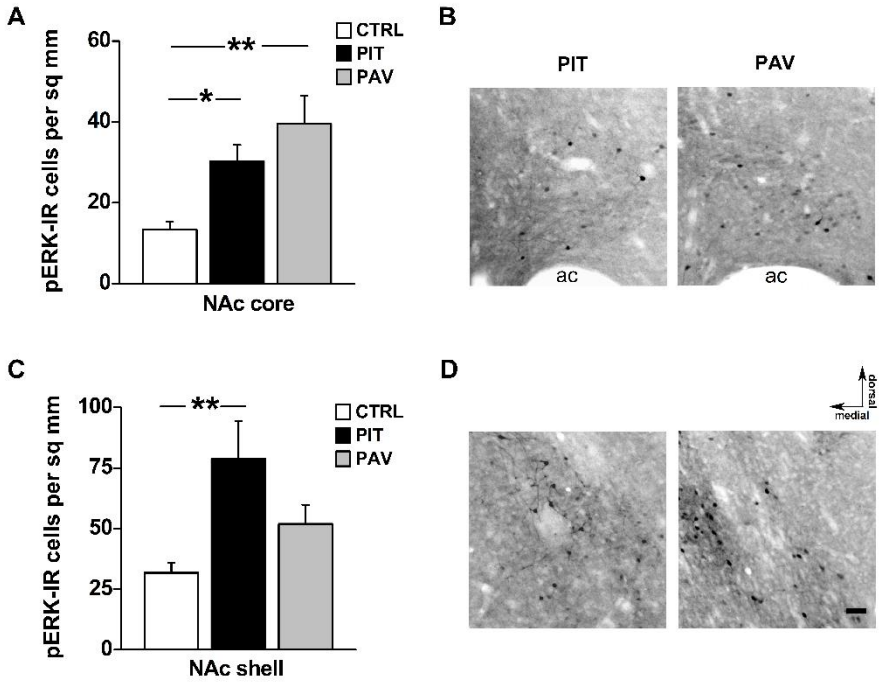

Figure 6: Presentation of a CS during PIT increases the level of active ERK in both the core and the shell. **A,** Mean±SEM number of pERK1/2-IR cells in the NAc core for the PIT (n=8), the PAV (n=9), and the pooled CTRL group (n=17; pooled data depicted in **Figure 5**). The number of pERK1/2-IR cells was significantly greater in both experimental groups than in the CTRL group (*, $p<0.05$, **, $p<0.01$). **B,** Representative photomicrographs of pERK-labeled cells in the core from a rat of the PIT group (left) and a rat of the PAV group (right) (scale bar, see panel D; ac, anterior commissure). **C,** Similar data as shown in panel A here depicted for the NAc shell. The number of pERK1/2-IR cells was significantly greater for the PIT group than the CTRL group (**, $p<0.01$). A similar, albeit less pronounced trend was observed for the PAV group (see text for more details). **D,** Representative photomicrographs of pERK-labeled cells in the shell from a rat of the PIT group (left) and a rat of the PAV group (right) (scale bar, 40 µm).

Table 2: Summary of behavioral and immunohistochemical results (group means ± SEM) for the individual groups in Experiment 2.

Measure↓ Group→	**Handled**	**TOY**	**TO**	**PIT**	**PAV**	**REWARD**
Behavior during test	n/a	-0.1 ± 0.5[#]	0.2 ± 1.7[&]	7.6 ± 1.5[&]	3.0 ± 0.6[#]	2.8 ± 0.5[$]
pERK$^+$ cell count CORE	9.6 ± 1.7	15.0 ± 2.9	10.7 ± 2.4	30.3 ± 4.0	39.5 ± 6.9	11.1 ± 2.0
pERK$^+$ cell count SHELL	34.2 ± 3.6	36.2 ± 5.7	27.8 ± 5.7	78.8 ± 15.6	52.0 ± 7.9	138.3 ± 16.3
pERK$^+$ cell count DMS	n.d.	6.9 ± 2.8	6.5 ± 1.5	9.8 ± 3.5	11.6 ± 5.1	n.d.
pERK$^+$ cell count DLS	n.d.	21.0 ± 9.1	16.5 ± 4.5	19.9 ± 4.1	31.2 ± 5.9	n.d.

[#] discrimination rate of Pavlovian conditioned response; rate of food cup approaches during the CS minus rate of food cup approaches during the preCS
[&] PIT score; rate of active lever presses during the CS minus rate of active lever presses during the preCS
[$] food cup approaches during the period of intermittent food delivery (coincident with the CS period for the other groups) minus rate of food cup approaches during a time period equivalent to the preCS period. See text for further details.
n/a; not applicable
n.d.; not determined

two subregions of the NAc of rats that merely received daily handling but no conditioning (n = 3; 9.6 ± 1.7 and 34.2 ± 3.6 pERK-IR cells per mm2 for the core and the shell, respectively; see Table 2). In light of these findings, it is reasonable to conclude that the level of pERK immunoreactivity observed in the TO and TOY control groups reflects basal pERK immunoreactivity. Because the results did not differ between our two control groups, we pooled their data for comparison with the experimental groups below.

Similar to our observations with the controls, pERK-IR cells were distributed relatively sparsely in the core of rats from the PIT and PAV groups. Further, compared with immunolabeling in the dorsal, ventral, and medial regions of the core, labeling was scant in the lateral core in both trained groups, and this pattern was observed throughout the rostral–caudal extent of the NAc (see Figure 6b). Overall, the density of pERK immunolabeling was greater in the two trained groups compared to the controls (Figure 6a; see Table 2). ANOVA of the pERK-IR cell counts in the NAc core of rats from the PIT, PAV, and pooled control (CTRL) groups confirmed a significant group effect ($F(2,31) = 13.26$; $P < 0.01$), and post hoc comparisons revealed that the number of pERK-IR cells was significantly higher for both experimental groups compared to the CTRL group ($P \leq 0.01$). Importantly, the number of pERK-IR cells did not differ between the PIT and the PAV groups. The difference in the number of pERK-IR cells between the experimental and control groups tended to be more pronounced in the anterior compared to posterior portion of the core because of relatively lower immunolabeling in the anterior (>+1.7 AP) compared to the posterior core of control rats. To determine whether this trend was statistically significant, we compared pERK-IR cell counts from the two most anterior sections versus those from the two most posterior sections collected from each rat between the three groups. However, ANOVA using groups as between-subject factor and anterior versus posterior subdivision as within-subject factor did

not reveal a significant group × subdivision interaction ($F(2, 31) < 1$), which suggests that the differential patterns of pERK labeling were overall comparable across the anterior–posterior extent examined here.

In the NAc shell, the great majority of pERK-IR cells were located in the medial shell, and very few pERK-IR cells were found in the lateral portion of the shell in rats from both the PIT and PAV groups. The distribution of pERK-IR cells within the medial shell was characterized by patches of sparse and dense staining (see Figure 6d). Although clusters of pERK-IR cells were found in rats from all four groups, they appeared to be more frequent and often contained more immunopositive cells in rats from the PIT and PAV groups than in control rats. Consistent with this trend, pERK-IR cells overall appeared to be denser in the shell of experimental than control rats (Figure 6c; see Table 2). ANOVA of the pERK-IR cell counts in the NAc shell confirmed a significant group effect ($F(2,31) = 8.22$; $P < 0.01$); however, post hoc comparisons revealed that only the PIT group expressed significantly more pERK-IR cells than the CTRL group ($P < 0.01$). Nevertheless, the number of pERK-IR cells did not differ between the two experimental groups, similar to what we observed in the core. Moreover, as suggested by the trend for a greater number of pERK-IR cells in the PAV compared to the CTRL group (see Figure 6c), a separate comparison between these two groups indicated a significantly greater number of pERK-IR cells in the PAV than the CTRL group (Student's t test for independent groups, $t(24) = 2.49$, $P < 0.02$, two-tailed), and this difference remained statistically significant after adjustment of the P value for a second comparison (e.g., with the PIT group). In contrast, a similar separate comparison between the PIT and PAV groups confirmed the lack of a significant difference between the two experimental groups ($t(15) = 1.59$, $P > 0.1$). Similar to the rostro-caudal pattern noted in the core, the difference in the number of pERK-IR cells in the shell between the experimental

and control groups was more prominent in the anterior compared to the posterior portion of the shell; however, different from the core, the gradient in effect size resulted from overall stronger immunolabeling in the anterior compared to the posterior portion of the shell in experimental rats. To determine whether this qualitative trend in the shell was borne out statistically, we compared pERK-IR cell counts from the two most anterior sections versus those from the two most posterior sections collected from each rat, as described for the core above. ANOVA confirmed a group-dependent difference in pERK-IR cell count between the anterior and the posterior portion of the shell, as indicated by a significant group × subdivision interaction ($F(2, 31) = 3.99$, $P < 0.05$), and post hoc comparisons indicated significantly higher immunolabeling in the anterior compared to the posterior subdivision of the shell in rats from the PIT and PAV groups ($P < 0.01$), but not the shell of rats from the CTRL group.

Taken together, the results indicate a robust increase in the number of pERK-IR cells in both the core and the shell of rats from the PIT group and the core of rats from the PAV group, and a mild increase in the shell of rats from the PAV group. To place these increases in pERK immunolabeling upon exposure to a reward-predictive CS into perspective, not just relative to controls but also relative to ERK signaling evoked by the reward itself, we also examined the number of pERK-IR cells in the core and shell of rats that were trained identically to the PIT group, but they received food pellets in place of tone presentations during the test (REWARD group; $n = 8$; see Table 1). Similar to controls but different from both experimental groups, the number of pERK-IR cells was very sparse in the core of rats from the REWARD group (see Figure 7b; Table 2). In contrast, the number of pERK-IR cells in the shell of rats from the REWARD group was very pronounced and, similar to the pattern observed in the two experimental groups, located predominately in the medial shell (see

Figure 7d; Table 2). This distribution pattern within the shell was observed throughout the rostral–caudal extent we examined, although the density of pERK-IR cells was greater in the anterior than the posterior portion of the shell, similar to the trend we observed in the PIT and PAV groups. Comparing numbers of pERK-IR cells in the core of rats from the three experimental groups in terms of fold change from control level, ANOVA indicated a significant group effect ($F(2,24) = 8.46$, $P < 0.01$), and post hoc comparisons revealed that the fold change from control level for rats from the REWARD group was significantly lower than that for rats from either the PIT or the PAV group ($P < 0.05$; Figure 7a), with no significant difference between the latter two groups. Indeed, the number of pERK-IR cells in the core of rats from the REWARD group (11.1 ± 2.0 cells) did not present a significant fold change from control level (Student's t test assuming a population mean of 1.0 or no difference from control, $t(7) < 1.0$), whereas the fold change for both the PIT and the PAV group was significant ($t(7) = 4.35$ and $t(8) = 3.84$, respectively, $P < 0.01$, two-tailed). Similar comparisons of fold changes in the number of pERK-IR cells in the shell also indicated a significant group effect ($F(2,24) = 10.86$; $P < 0.01$); however, in the case of the shell, the fold change from the control level was significantly greater for rats from the REWARD group than rats from either the PIT or the PAV group (post hoc comparison, $P < 0.05$; Figure 7c), again with no significant difference between the latter two groups. Furthermore, the number of pERK-IR cells in the shell of rats from the REWARD group (138.3 ± 16.3 cells) presented a significant fold change from control level (REWARD: $t(7) = 6.52$, $P < 0.01$, two-tailed), as did the changes observed in the PIT and PAV groups ($t(7) = 2.98$ and $t(8) = 2.51$, respectively, $P < 0.05$, two-tailed).

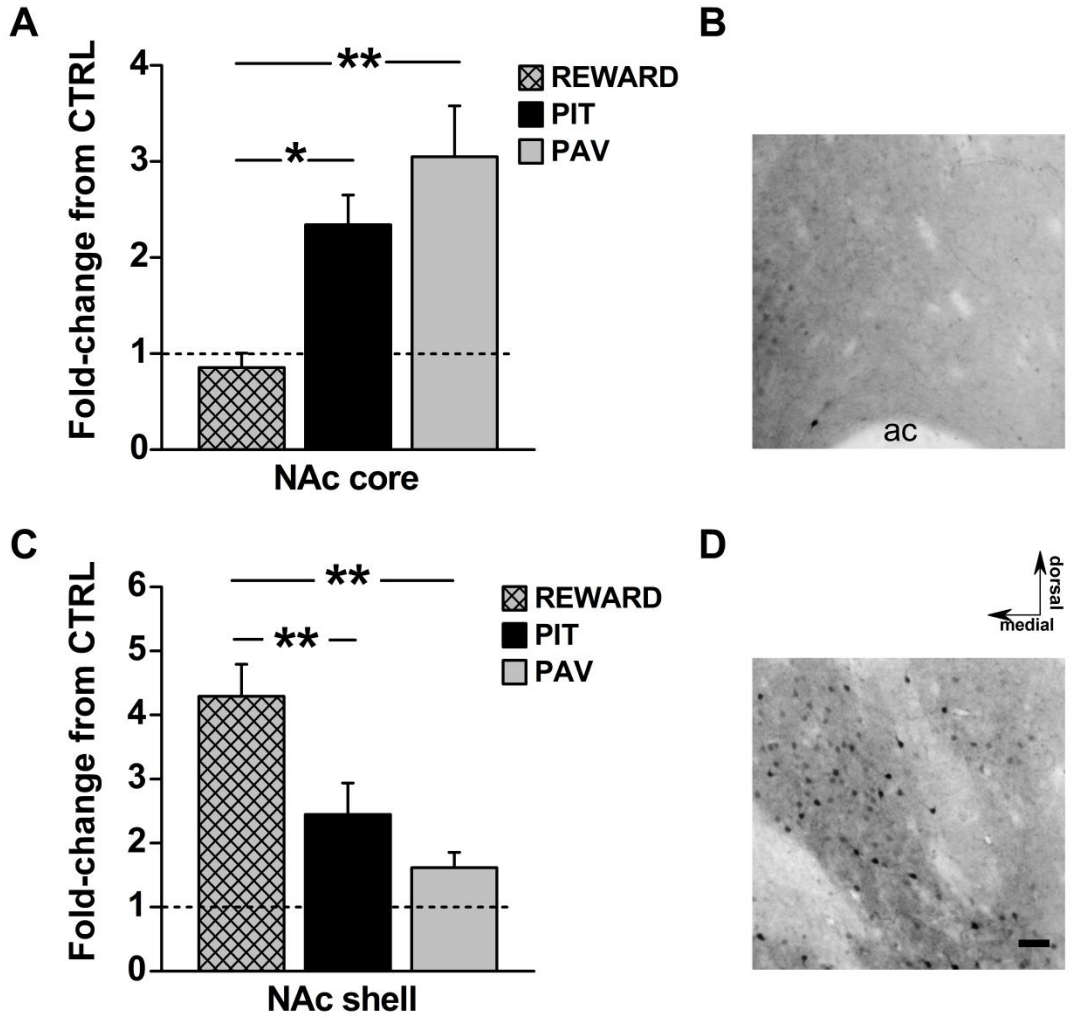

Figure 7: Presentation of food reward evokes a different subregional pattern of active ERK than does a CS predicting the reward. **A,** Mean±SEM fold-change from CTRL level in the number of pERK1/2-IR cells in the NAc core for the REWARD group (n=8), the PIT group (n=8), and the PAV group (n=9). Data for the PIT and PAV groups are based on those depicted in **Figure 6** and expressed relative to the mean count of pERK1/2-IR cells in the pooled CTRL group. The fold-changes were significantly higher for the PIT and the PAV groups than the REWARD group (*, $p<0.05$ and **, $p<0.01$, respectively), for which pERK-IR cells counts did not differ from CTRL level. **B,** Representative photomicrograph of pERK-lR cells in the core of a rat from the REWARD group (scale bar, see panel D; ac, anterior commissure). **C,** Similar data as shown in panel A here depicted for the NAc shell. The fold-change was significantly higher for the REWARD group than either the PIT or the PAV group (**, $p<0.01$). **D,** Representative photomicrograph of pERK-lR cells in the shell of a rat from the REWARD group (scale bar, 40 µm).

In light of our findings from experiment 1 that tERK levels are unaffected by prior conditioning history and remain stable during PIT, our observations described here likely reflect differences in ERK activation. Thus, the cross-regional pattern of ERK activation evoked by a reward-predicting CS differs markedly from that evoked by the reward itself, with the signal being stronger for a CS than the reward in the core and stronger for a reward than the CS that predicts it in the shell.

To examine whether the increases in pERK immunolabeling upon exposure to a reward-predictive CS are specific to the ventral striatum or also occur in the dorsal striatum at the same anterior–posterior level as the NAc, we also counted the number of pERK-IR cells in the DMS and the DLS in a subset of the rats. We chose to distinguish between the DLS and DMS, as these subregions of the dorsal striatum were shown to play distinct roles in PIT (Corbit & Janak, 2007; 2010). Compared to the NAc, the staining in the dorsal striatum was sparser. Within the dorsal striatum, the density of pERK-IR cells was higher in the DLS compared to the DMS (see Table 2). We first compared the number of pERK-IR cells in the DMS and the DLS between the TO (n = 4) and the TOY (n = 4) control groups. ANOVA revealed a significant effect of area ($F(1,6) = 7.79$, $P < 0.05$), indicative that pERK labeling was higher in the DLS than the DMS in rats from both groups. However, neither the effect of group nor the group × area interaction was significant. Therefore, we pooled the results from the two control groups for comparison with the PIT (n = 6) and the PAV groups (n = 6), and conducted separate analyses for the DMS and the DLS. Different from the pattern we observed in the NAc, the number of pERK-IR cells in the dorsal striatum was comparable across groups (see Table 2), and ANOVA confirmed the lack of a significant group effect for both the DMS ($F(2,17) < 1$) and the DLS ($F(2,17) = 1.45$; $P < 0.2$). Taken together, these results suggest that CS-evoked increases in ERK signaling are restricted to the NAc and do

not extend to the dorsal striatum, at least not within the anterior–posterior range examined here.

2.4 DISCUSSION

Our examination of the subregional pattern of ERK activation in the NAc during PIT revealed that in both the core and the shell, ERK activation is increased about two- to threefold above control levels. The increase in ERK activation in both subregions was similar regardless of whether animals were presented with the CS only or also given the opportunity to display PIT. In fact, our data indicate that reward-seeking behavior itself does not contribute to ERK activation during PIT in either the core or the shell. Interestingly, exposure to the reward alone caused a pronounced increase in ERK activation in the shell while leaving ERK activation in the core equivalent to control levels.

We previously demonstrated that blockade of ERK activation in the NAc abolishes PIT (Shiflett *et al.*, 2008). Our present observations of increased ERK activation in both the core and the shell suggest that both subregions are recruited during PIT. In support of this suggestion, lesions of the core were found to interfere with general PIT (Hall et al. 2001) and lesions of the shell with stimulus-specific PIT (Corbit *et al.*, 2001; Shiflett & Balleine, 2010a; Corbit & Balleine, 2011). General PIT refers to the potentiating effect of a CS on reward seeking regardless of whether the CS previously was paired with the same reward as the one for which the animal learned to emit a particular seeking behavior (e.g., lever press). On the other hand, stimulus-specific PIT refers to the potentiating effect of a CS when the situation affords multiple reward-seeking actions (e.g., pressing the left lever vs. the right lever), each of which leads to a different reward, and a CS enhances selectively the seeking behavior for

the reward with which the CS previously was paired. Although the PIT paradigm we used here aligns more closely with paradigms for general PIT, the inclusion of an active and inactive lever in our paradigm may also have led to the recruitment of elements operative during specific PIT. Indeed, dopamine receptor blockade in either the core or the shell was found to disrupt PIT using a paradigm similar to the one we used here (Lex & Hauber, 2008).

Our findings of increased ERK activation in both the core and the shell during PIT suggest that inputs to and outputs from both subregions are part of the neural circuit that underlies PIT. The ventral tegmental area (VTA) provides dopaminergic input to both the core and the shell (Brog *et al.*, 1993), and manipulations that disrupt VTA innervation to the NAc were found to abolish both forms of PIT (Murschall & Hauber, 2006; Corbit *et al.*, 2007). In light of evidence of positive coupling between dopamine D1 receptor activation and the ERK signaling cascade in NAc neurons (Valjent *et al.*, 2004; Valjent *et al.*, 2005; Borgkvist *et al.*, 2008; Fricks-Gleason & Marshall, 2011), the pattern of PIT-associated ERK activation we described here may be mediated, at least in part, by input to both NAc subregions from the VTA. The basolateral amygdala (BLA) also has been implicated in PIT (Blundell *et al.*, 2001; Corbit & Balleine, 2005) and sends glutamatergic afferents to both subregions (Kelley *et al.*, 1982; Brog *et al.*, 1993; Groenewegen *et al.*, 1999). Activation of N-methyl-d-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors was shown to drive ERK signaling in striatal neurons (Mao & Abdel-Rahman, 1994; Perkinton *et al.*, 1999; Haberny & Carr, 2005; Valjent *et al.*, 2005); the ERK activation pattern observed here may thus also be attributable to input from the BLA, potentially to the same cells as those innervated by the VTA (Johnson *et al.*, 1994; Floresco *et al.*, 2001a; Valjent *et al.*, 2005). Much less is known about the role of structures downstream of the NAc in PIT. Whereas the core projects primarily to the dorsolateral

division of the VP, the subthalamic nucleus, and the medial substantia nigra pars reticulata, the shell projects primarily to the ventromedial division of the VP, the VTA, and the lateral hypothalamus (Heimer *et al.*, 1991; Zahm & Brog, 1992; Groenewegen *et al.*, 1999; Tripathi *et al.*, 2010). Accordingly, CS-evoked ERK activation in the core is likely to serve a different behavioral aspect of PIT than does CS-evoked ERK activation in the shell. For instance, in light of our findings of a marked increase in ERK activation in the shell upon exposure to the food reward, increased ERK activation in the shell during PIT may reflect a CS-evoked molecular representation of the reward. Consistent with this idea, stimulus-specific PIT, in which the CS is thought to trigger retrieval of specific sensory properties of the reward, relies on intact NAc shell functioning (Corbit *et al.*, 2001; Corbit & Balleine, 2011). Future studies will serve to pinpoint which of the various output targets critically participate in PIT, and the particular behavioral aspects mediated by the pERK immunopositive cells in the NAc projecting to these targets.

Our findings of comparable ERK activation in the NAc of rats from the PAV and PIT groups, with the increase above control levels being more robust in the core than the shell, suggest that exposure to the CS, and not the occurrence of reward-seeking behavior, is responsible for the increase in ERK activation during PIT. This conclusion is consistent with our findings of no difference in NAc ERK activation and no increase relative to handled controls between control rats that did (TO controls) and control rats that did not engage in reward seeking (TOY controls). Taken together with our previous findings that inhibition of NAc ERK activation blocks PIT but not reward-seeking per se (Shiflett *et al.*, 2008), our present findings suggest that CS-evoked ERK activation in the NAc core and shell is necessary but not sufficient for CS-potentiation of reward seeking. One possible mechanism through which CS-evoked ERK activation may enable enhanced reward seeking is through

ERK action on voltage-gated channels that regulate NAc cell excitability (Tkatch *et al.*, 2000; Yuan *et al.*, 2002; Day, 2008; Shiflett & Balleine, 2011a).

We found the pattern of increased ERK activation to vary differentially across NAc subregions depending on the type of stimulus to which animals were exposed. Presentation of reward caused no change in ERK activation in the core but a very pronounced increase in the shell; in contrast, presentation of the CS predictive of the reward caused a more reliable increase in the core than in the shell. Similar to our observations with a food reward, some investigators found that the psychostimulant cocaine causes a substantial increase in ERK activation predominately in the shell (Bertran-Gonzalez *et al.*, 2008). However, others found that a range of psychostimulants and other addictive drugs greatly elevate ERK activation in both the core and the shell (Valjent *et al.*, 2000; Valjent *et al.*, 2004; Corbille *et al.*, 2007; Ibba *et al.*, 2009). The difference between the subregional activation patterns we observed and the ones observed in these latter studies may reflect a difference in the magnitude of reward, or they may indicate that natural rewards and addictive drugs recruit different groups of NAc cells. In support of the latter explanation, different groups of NAc neurons were found to exhibit a change in firing rate during cocaine-reinforced responding versus food- or water-reinforced responding (Pennartz *et al.*, 1994; Carelli, 2002; Carelli *et al.*, 2003; Wheeler & Carelli, 2009). In agreement with our observations of a robust CS-evoked increase in ERK activation in the core, numerous studies support the involvement of the core in mediating the incentive salience of a CS (Di Ciano *et al.*, 2001; Cardinal *et al.*, 2002b; Fuchs *et al.*, 2004; Hollander & Carelli, 2007). Furthermore, complementary to our observations of a preferential but not an exclusive CS-evoked increase in ERK activation in the core, neuronal firing has been reported as increased more frequently, but by no means exclusively, in the core than in the shell upon exposure to a CS (Day *et al.*, 2006;

Cacciapaglia *et al.*, 2011; Saddoris *et al.*, 2011). It is tempting to speculate that the NAc neurons whose firing rate is increased during presentation of a CS are the same neurons that exhibit a CS-evoked increase in ERK activation. Enhanced pERK immunoreactivity might, thus, serve as a useful marker to identify the phenotype of cells that respond to presentation of a CS in an excitatory fashion.

Various groups recently have begun to examine the involvement of the dorsal striatum in PIT (Corbit & Janak, 2007; Homayoun & Moghaddam, 2009; Corbit & Janak, 2010; Pielock *et al.*, 2011).We previously found no evidence for CS-evoked ERK activation in the dorsal striatum (Shiflett *et al.*, 2008), which suggested to us that the recruitment of ERK signaling during PIT is specific to the NAc and does not extend to the dorsal striatum, similar to the requirement of dopamine signaling in the NAc but not the dorsal striatum during PIT (Wyvell & Berridge, 2000; Lex & Hauber, 2008; Pielock *et al.*, 2011). Our present findings of no significant effect of CS exposure on the ERK signal detected in either the DMS or the DLS confirm and extend our previous conclusion by showing that ERK signaling in the anterior medial and lateral striatum dorsal to the NAc is not differentially engaged upon exposure to a reward-predictive CS or during PIT. Similar to our findings in the NAc, we did not detect a change in pERK immunoreactivity in either the DMS or the DLS as a function of instrumental responding, as indicated by a lack of a significant difference between the TO and TOY groups, or between the PIT and PAV groups (see Table 2). In each case, the former of the two groups engaged in lever pressing during the test, whereas the latter did not. The lack of an effect of instrumental responding on ERK signaling in the dorsal striatum was surprising in light of recent observations that ERK activation is increased in both the DMS and the DLS after instrumental training, and that ERK inhibition in either of these striatal subregions interferes with the reduction of instrumental responding

upon reward devaluation (Shiflett & Balleine, 2010b). The apparent discrepancy between observations may be attributable to differences in the anterior–posterior level at which ERK activation was examined: whereas we focused on the striatum dorsal to the NAc and thus the anterior dorsal striatum, Shiflett and Balleine (2010b) examined and blocked ERK signaling in more posterior regions of the dorsal striatum. Additional procedural differences, such as the amount of instrumental training and the absence/presence of food reward during the test, may also have contributed to the difference in findings between studies. It should be noted that our observations of no change in ERK signaling in the anterior dorsal striatum during PIT do not preclude a specific role for either the DLS or the DMS in PIT (Corbit & Janak, 2007). They merely suggest that the PIT-critical intracellular signaling steps in these subregions are unlikely to involve ERK.

In summary, we demonstrate that ERK signaling during PIT encompasses ensembles of neurons in both the core and the shell but not the anterior dorsal striatum, and that the CS is the principal stimulus responsible for the ERK activation pattern during PIT. Whereas the ERK signal in the core appears to reflect CS-specific properties, the ERK signal in the shell may reflect CS-evoked retrieval of reward-specific properties. CS-evoked ERK activation can serve as a useful molecular marker to study further the neuronal ensembles implicated in PIT, including their terminal regions and distinct roles in PIT.

3.0 INCREASED ERK SIGNALING IN THE NUCLEUS ACCUMBENS DURING CONDITIONED CUE-POTENTIATED REWARD SEEKING IS MEDIATED BY DOPAMINE D1 RECEPTORS ON PRODYNORPHIN-EXPRESSING NEURONS

3.1 INTRODUCTION

Neutral stimuli in the environment that have been paired with rewards (unconditioned stimuli, USs) acquire predictive value for the reward and, as a consequence, facilitate behaviors directed toward acquisition of the reward. The excitatory modulation of reward-seeking behavior by conditioned stimuli (CSs) is known as Pavlovian-instrumental transfer (PIT; Estes, 1948; Rescorla & Solomon, 1967; Lovibond, 1983). CSs play an important role in learning and influence decisions about what behaviors are most advantageous for an organism's survival (Dickinson & Balleine, 2002). Although normally beneficial, CS modulation of reward seeking is detrimental when CSs facilitate maladaptive behaviors. PIT-like effects are thought to contribute to CS-induced relapse in drug addiction and exaggerated food seeking in obesity (O'Brien *et al.*, 1998; Hyman, 2005). Therefore, an understanding of the mechanisms and the neural circuitry that underlie PIT is important for the development of treatments to curb excessive and maladaptive reward-seeking behaviors.

PIT depends crucially on the nucleus accumbens (NAc), a key component of the brain's reward circuitry (Corbit *et al.*, 2001; Hall *et al.*, 2001). We previously found that the

presentation of an appetitive CS induces an increase in the extracellular signal-regulated kinase (ERK) activation in the NAc both after Pavlovian conditioning and during PIT (Shiflett *et al.*, 2008; Remus & Thiels, 2013). Furthermore, we demonstrated that transient blockade of ERK signaling in the NAc abolishes PIT, without effect on Pavlovian conditioned responding or instrumental responding for reward in the absence of the CS (Shiflett et al., 2008). Recently, we identified two synaptic mediators of accumbal ERK activation after Pavlovian conditioning: N-methyl-d-aspartate glutamate receptors (NMDA receptors) and D1-type dopamine receptors (D1Rs). Local blockade of either NMDA receptors or D1Rs abolished CS-evoked ERK activation in the NAc (Kirschmann *et al.*, 2014). Several investigators showed that PIT depends on accumbal dopamine receptor activation (Lex & Hauber, 2008; Ostlund & Maidment, 2012; Laurent *et al.*, 2014). Disruption of PIT by D1R blockade maybe be attributable to a disruption in NAc ERK signaling. Interestingly, activation of both D1Rs and D2-type dopamine receptors (D2Rs) in the NAc was shown to be critical for PIT (Lex & Hauber, 2008). Therefore, our first goal was to determine the role of D1Rs and D2Rs in the activation of ERK by CSs during PIT.

The major cell type in the NAc are medium-sized spiny neurons (MSNs); they constitute the output neurons of the NAc (Meredith, 1999). MSNs have generally been segregated into two populations based on the type of dopamine receptor and neuropeptides expressed and axonal projection pathways (Gerfen *et al.*, 1990; Le Moine *et al.*, 1991; Le Moine & Bloch, 1995; Zahm *et al.*, 1996; Lu *et al.*, 1998). MSNs of the direct pathway express D1Rs and the neuropeptides dynorphin (DYN) and substance P (SP), and they project to substania nigra reticular and the internal segment of the globus pallidus (GPi) (Groenewegen & Russchen, 1984; Curran & Watson, 1995; Hara *et al.*, 2006). In contrast, MSNs of the indirect pathway express D2Rs and the neuropeptide enkephalin (ENK), and

they project to the external segment of the globus pallidus (GPe) (Yang *et al.*, 1977; Groenewegen & Russchen, 1984). Interestingly, NAc ERK activation triggered either by drugs of abuse and drug-predictive cues was shown to occur via a D1R-dependent mechanism (Valjent *et al.*, 2004; Ibba *et al.*, 2009) and to be restricted to D1R-expressing MSNs (Bertran-Gonzalez *et al.*, 2008; Borgkvist *et al.*, 2008; Fricks-Gleason & Marshall, 2011). Therefore, our second goal was to determine the NAc cell type in which CS-evoked ERK during PIT occurs.

3.2 MATERIALS AND METHODS

3.2.1 Subjects

Thirty-eight male Sprague-Dawley rats (Hilltop Lab Animals, Scottdale, PA) weighing between 250 and 275 g on delivery were used in this study. Rats were handled and weighed daily, housed separately, and maintained on a 12-h light/dark cycle with ad libitum access to food and water unless specified otherwise. All experiments were conducted during the light cycle, in accordance with the National Institutes of Health guidelines, and with approval of the Institutional Animal Care and Use Committee of the University of Pittsburgh. All behavioral data were collected in the Rodent Behavior Analysis Core of the University of Pittsburgh Schools of Health Sciences.

3.2.2 Surgical Procedures

The surgical procedures were based on our previous studies (Meredith, 1999; Shiflett et al., 2008; Kirschmann et al., 2014). Briefly, rats were anesthetized with an intraperitoneal (i.p.) injection of a combination of ketamine (Ketaset; 80 mg/kg; Fort Dodge Animal Health, Fort Dodge, IA) and xylazine (AnaSed; 8 mg/kg; Lloyd Laboratories, Shenandoah, IA), and positioned in a stereotaxic apparatus equipped with blunt ear bars. The skull surface was exposed, and small holes were drilled bilaterally targeting the NAc using the following stereotaxic coordinates relative to bregma: AP + 1.3 mm; ML ± 1.5), and two 26-gauge stainless-steel cannula guides (6 mm length; Plastics One, Roanoke, VA) were lowered bilaterally into the NAc, 5.4 mm ventral relative to dura. The guides were fastened to the skull via self-curing dental cement. Stainless steel stylets were placed into the guides to prevent occlusion. Rats were given 5 to 7 days to recover from surgery and then placed on a food-restricted diet to bring their body weights to ~90% of the body weight of free-feeding age-matched rats.

3.2.3 Behavioral Apparatus

Training and testing took place in rat operant chambers (30 cm x 23 cm x 23 cm; Med Associates, St. Albans, VT, USA) furnished with a house light, metal grid flooring, and a loudspeaker. The chambers were enclosed in light- and sound-attenuating shells equipped with fans that generated background noise to obfuscate external sound. A food cup was fastened to the front wall and attached to a pellet dispenser that released a single food pellet when depressed. A photobeam source and sensor were mounted onto the sides of the food

cup to register food cup approaches. A retractable lever was mounted approximately 4 cm to both the left side and the right side of the food cup. MED-PC IV program (Med Associates) was used to control the equipment and record food cup approaches and lever presses.

3.2.4 Behavioral Procedures

The conditioning procedures were essentially as described by (Remus & Thiels, 2013). Rats were habituated to the conditioning chambers in a single 30-min session one day prior to Pavlovian conditioning**.**

3.2.4.1 Pavlovian conditioning

Rats in the Pavlovian-Instrumental Transfer (PIT) group completed 6 daily conditioning sessions, with each daily session consisting of 8 pairings between a CS (90-s 3-kHz 80-dB tone) and delivery of 3 to 4 food pellets (45-mg dustless food pellets, BioServe, Frenchtown, NJ) on a variable time (VT) schedule of 20 s. For half of the trials, the VT schedule for pellet delivery was initiated with tone onset; for the remaining half, the VT schedule was delayed by 30 s after tone onset, to permit recording of food cup approaches in the absence of the US. The type of trial (food throughout vs. only during the last 60 s of the CS) was varied between and within sessions. The intertrial interval (CS onset to next CS onset) varied between 210 and 330 s (mean = 270 s). For each session, number of photobeam breaks was recorded during the first 30 s of the CS periods during which food delivery was delayed, and the 30 s prior to CS onset (CS and preCS periods, respectively). Rats in the control group, named tone only (TO group), were presented with the identical number and

pattern of 90-s tones as the PIT group, but no food pellets were delivered during the Pavlovian conditioning sessions.

3.2.4.2 Instrumental conditioning

Following Pavlovian conditioning, rats in both the PIT and TO groups underwent instrumental conditioning. Each session began with illumination of the house light and extension of both levers into the chamber. One lever, designated as the active lever, delivered a single food pellet into the food cup when depressed. Depression of the second lever, designated as the inactive lever, result in no consequences. The active lever was randomly assigned to the left or the right lever, and was counterbalanced within and across cohorts. During this first instrumental conditioning session, rats received a single pellet for every depression of the active lever (fixed-ratio one, or FR1, schedule). The first session terminated after rats earned a total of 50 pellets. Rats were returned to their home cages for a 45-min intersession interval after which the second training session began. For the second session, on average three presses on the active lever were required for delivery of a pellet (random-ratio 3, or RR3). The RR3 session terminated after 50 pellets were delivered. On the subsequent five days, rats underwent two daily RR5 sessions, separated by a 45-min intersession interval in their home cages. Each RR5 session was terminated after delivery of 100 pellets.

3.2.4.3 Pavlovian Reminder & Extinction

One day prior to the PIT test, rats received a Pavlovian conditioning reminder session that was essentially identical to the Pavlovian training sessions described above, except that the reminder session consisted of only 4 trials. The reminder session was followed by 15 min

in the home cage and then a brief extinction session. During the extinction session, both levers were extended into the chamber, and the session was terminated after rats had pressed the active lever for 10 min or made 250 unrewarded lever presses (whichever occurred first). The purpose of the extinction session was to avoid a ceiling effect during the PIT test.

3.2.4.4 Pavlovian-instrumental Transfer (PIT) Test

For all rats, the PIT test began with the illumination of the house light and extension of both levers into the chamber. No stimuli were presented for the first six minutes of the test. Thereafter, four 90-s tones were presented at a fixed 270-s intertrial interval. Number of active and inactive lever presses and food cup approaches were recorded for each 90-s CS period and the 90-s period prior to each CS (preCS period).

3.2.5 Drugs and Intra-NAc Infusions

To familiarize rats to the infusion procedure prior to testing for PIT, rats were given a single mock infusion shortly before the training session on day 4 or 5 of instrumental conditioning. The wire stylets were removed from the guides, and short infusion cannulae (33-gauge, Plastics One) that projected 0.5mm past the ventral tip of the guide cannulae and were attached to a 5-µl Hamilton syringe (Reno, NV) were inserted, and 0.5µl of sterile saline was infused bilaterally at a rate of 0.5µl/min. Infusion cannulae were left in the NAc for one additional minute before they were removed and the stylets were replaced.

Prior to testing, animals received either unilateral or bilateral intra-NAc microinjections of either the selective D1-type receptor antagonist R(+)-SCH-23390 hydrochloride (SCH; Sigma-Aldrich; 0.3 µg) or the selective D2-type receptor antagonist S-

(--)-raclopride (+)-tartrate salt (RAC; Sigma-Aldrich; 0.5 µg) dissolved in 0.5 µl of 0.9% (w/v) sterile saline. The side infused with the antagonist vs. vehicle solution was counterbalanced across subjects. The infusions were conducted essentially as described above, except that the infusion cannulae projected 2.2mm beyond the tip of the guides, to extend into the NAc.

3.2.6 Experimental Design

To determine whether NAc D1Rs play a role in the CS-evoked ERK activation during PIT we described previously, we administered to PIT rats (n=7) and TO controls (n=6) an infusion of SCH23390 into the NAc on one side and of vehicle solution into the NAc on the other side. To determine whether NAc D2Rs contribute to the CS-evoked ERK activation during PIT, we administered to different PIT rats (n=7) and TO controls (n=5) a unilateral infusion of raclopride into the NAc on one side and vehicle solution into the NAc on the other side. Rats were transcardially perfused immediately after the PIT test, and coronal sections of their brains immunostained for activated ERK (pERK; see details below). To determine whether CS-evoked ERK activation during PIT occurs in the subpopulation of MSNs that express D1Rs, some sections were immunostained for pERK and prodynorphin (PDYN; see details below). PDYN is the precursor peptide of DYN neuropeptides (Hokfelt *et al.*, 2000) and was shown in the soma and dendrites of MSNs that express D1Rs, but not D2Rs (Gerfen *et al.*, 1990; Lee *et al.*, 1997; Sonomura *et al.*, 2007).

To confirm that doses of SCH23390 and raclopride used were sufficient to disrupt PIT, but not overall motor behavior (lever pressing), we used separate cohorts of rats (biSCH, n= 7; biRAC; n= 8) and infused bilaterally either SCH23390 (dose and volume as described

above) or saline immediately before the PIT test. After the first microinfusion and PIT test, rats received two additional days of instrumental training and one additional Pavlovian reminder session before being tested for PIT a second time, this time shortly after bilateral infusion of either vehicle or SCH23390, i.e., the infusate they did not receive before the first PIT test. The order of microinfusions (i.e., vehicle vs. drug first) was counterbalanced across subjects. Essentially the same procedure was applied with additional cohorts of rats to test the appropriateness of the dose of raclopride we used. As an additional test to rule out 'non-specific' effects of the doses of SCH23390 and raclopride we used, we tested in additional sets of rats the effect of bilateral infusion of these agents versus saline on instrumental responding on a RR5 schedule (n=4 per group). All animals were perfused after the last test, for histological verification of cannula placement and additional analyses (see below).

3.2.7 Tissue Preparation

Immediately following the final PIT test, rats were anesthetized with an i.p. injection of chloral hydrate (300 mg/kg, i.p.; Sigma-Aldrich; dissolved in 150 mM saline) and transcardially perfused with 50 ml of 0.9% saline containing heparin (Acros Organics, Antwerpen, Belgium; 20 i.u./ml) and sodium fluoride (2mM), followed by 500 ml of 4% periodate-lysine-paraformaldehyde (PLP) fixative in 0.1 M sodium phosphate buffer (PB, pH 7.4). Brains were removed and post-fixed overnight at 4o C in fixative, in preparation for immunoperoxidase (IHC) or immunofluorescence (IF). The next day, brains were placed into a 20% sucrose solution until sunken and then cut into 50-µm coronal sections using a cryostat. Sections were collected in PB in four adjacent series. Additional series were stored at -20° C in cryopreservant solution until IHC, IF, or cresyl violet staining.

3.2.8 Histological Assessment

From one series of sections, Nissl bodies were stained with cresyl violet, and cannula tip locations were determined using light microscopy and mapped using a Rat Brain Atlas (Paxinos & Watson, 2007). Rats included in the behavioral and IHC analyses had cannulae placed bilaterally within the NAc (Figure 8B and Figure 10B). Subjects with inaccurate cannula placement (biSCH/RAC; n=2: uniSCH/RAC n=3) were excluded from analyses.

3.2.9 Immunohistochemistry

Sections were processed as described previously (Remus & Thiels, 2013). Briefly, sections were rinsed first in 0.1 M phosphate buffer and then several times in Tris-buffered saline (TBS; 50 mM Tris in 0.9% NaCl, pH 7.6). Sections were placed in blocking buffer (5% normal goat serum and 0.3% Triton X-100 in TBS) for 1 h at 22° C, and then incubated with anti-dual-phosphorylated, active ERK (pERK) antibody (1:800; Cell Signaling Technology) in blocking buffer for 24 to 48 h at 4° C. After primary antibody incubation, sections were washed in TBS and incubated with biotinylated goat anti-rabbit IgG secondary antibody (1:500; Vector Laboratories, Burlingame, CA, USA) in blocking buffer for 24 h at 4° C. Sections then were washed in TBS, incubated with avidin-biotin conjugate (1:500 for each A and B reagent; Vector Laboratories, Burlingame, CA) for 90 min at 22° C, and rinsed in 0.1 M sodium acetate buffer (NAOAc, pH 6.0) three times. Immunostaining was developed in NAOAc buffer containing 0.022% diaminobenzidine, 0.003% hydrogen peroxide, and 2.5% nickel sulfate. After additional washes, sections were mounted on

gelatin-coated slides, dried at room temperature, dehydrated in ethanol, cleared in xylene, and coverslipped with Cytoseal 60 (Richard-Allan Scientific).

3.2.9.1 Analysis of pERK1/2 Immunohistochemistry

For rats that received unilateral infusions of SCH23390 or raclopride, cell counts for number pERK1/2-immunoreactive (pERK-IR) cells were generated following procedures described previously (Remus & Thiels, 2013). Briefly, images of sections stained for pERK1/2 were acquired using a 10X objective and a digital camera (Micrometrics 3.2 MP) mounted on a light microscope (Leitz Orthoplan 2). Digitized images were used to estimate the number of pERK-IR cells in the NAc core by placing a 200 μm x 400 μm counting window just dorsal to the anterior commissure, and in the medial NAc shell by placing a 400 μm x 200 μm counting window 400 μm medial to the anterior commissure between +1.1-1.2 mm anterior to bregma, 500 μm medial to the anterior commissure between +1.3-1.4 mm anterior to bregma, and 600 μm medial to the anterior commissure between +1.5-2.0 mm anterior to bregma. Cell counts were obtained with the aid of an image analysis macro written for ImageJ (1.43u; NIH, Bethesda, MD) and verified by an experimenter blind to experimental conditions. Number of pERK1/2-immunopositive cells was counted in both hemispheres, yielding a total of 5 estimates per region per infusate per animal.

3.2.10 Immunofluorescence

To determine whether the cue-evoked NAc ERK activation during PIT occurs in MSNs that express D1 receptors, a series of sections from rats that received unilateral infusion of SCH23390 or raclopride (n= 2-3 per group) was double-labeled for pERK and

PDYN. Sections (n=3) were prepared as described above, and incubated overnight with both anti-phosphoERK antibody (1:400; Cell Signaling Technology) and guinea pig anti-ProDynorphin antibody (1:500; Abcam) in blocking buffer for 24 to 48 h at 4° C. Following incubation with the primary antibodies, sections were rinsed three times in TBS and incubated overnight with, respectively, goat anti-Rabbit DyLight 549 and goat anti-guinea pig DyLight 488 secondary antibody (1:500; Jackson ImmunoResearch) at 4° C. Sections were rinsed three times before mounting and coverslipping with Cytoseal 60 (Richard-Allan Scientific). Single- and double-labeled images from each NAc core and NAc shell were obtained bilaterally using sequential laser scanning confocal microscopy (Nikon A1). The colocalization of pERK with PDYN was determined using a 60X objective.

To demonstrate specificity of the PDYN antibody, we performed an immunizing blocking peptide experiment. Briefly, before staining NAc sections for PDYN, the PDYN antibody was incubated with the immunizing peptide (1: 500; Abcam; ab38201) in blocking solution for 30 min at RT. Additionally, NAc sections were dual-labeled with the PDYN antibody and rat anti-D1R antibody (1:500; Sigma Aldrich). Sections were prepared as described above. Following incubation with primary antibodies, sections were rinsed three times in TBS and incubated overnight with, respectively, goat anti-Rat DyLight 549 and goat anti-guinea pig DyLight 488 secondary antibodies (1:500; Jackson ImmunoResearch) at 4° C. Double-labeled images from NAc were obtained using confocal microscopy, as described above. The colocalization of D1R with PDYN was determined using a 60X objective.

3.2.11 Statistical Analysis

For Pavlovian conditioning, number of photo beam breaks during the CS and preCS periods was summed for each session and divided by time to calculate approach rates. For instrumental conditioning, number of lever presses per minute was recorded. Data from Pavlovian and instrumental training were compared using repeated-measures analyses of variance (ANOVAs) with group as between-subject factor and training day as within-subject factor followed by Bonferroni post-hoc comparisons of individual groups. For the PIT test, both photo beam breaks and lever press rates were recorded for the preCS and CS periods, and these data were compared using repeated-measures ANOVAs with group as between-subject factor and interval (preCS, CS) as within-subject factor followed by post-hoc comparisons. To evaluate the effect of D1R blockade and, separately, D2R blockade on pERK immunoreactivity, pERK immunopositive cell counts per mm^2 from the NAc core and, separately, the NAc shell were compared using repeated-measures ANOVAs with group as between-subject factor and infusate (vehicle, drug) as within-subject factor followed by post-hoc comparisons. All statistical analyses were performed using the SPSS software package version 19.0 (Chicago, IL). For all statistical comparisons, $p \leq 0.05$ was the criterion for significance.

3.3 RESULTS

3.3.1 NAc ERK activation during Pavlovian-instrumental transfer is mediated via activation of D1Rs

To determine whether D1R activation is required for CS-evoked NAc ERK activation during PIT, rats received unilateral infusions of SCH23390 into the NAc on one side and vehicle solution into the other NAc immediately prior to the PIT test in extinction. The rats' brains were harvested immediately after the test, and coronal sections were prepared and stained for pERK.

3.3.1.1 Behavior

One the first day of Pavlovian training, the discriminative food cup approach rates (number of approaches/min during the CS minus number of approaches/min during the preCS period) were similar for both groups; however, as training progressed, these rates increased for rats in the PIT group (n=7) but not for rats in the TO (n=6) control group. ANOVA confirmed these observations, as indicated by a significant group x day interaction ($F_{(5, 55)} = 5.50$; $p = 0.00$). Following six days of training, the mean discriminative approach rate of the PIT group was significantly higher than that of the TO group (6.1 ± 1.1 verses -0.3 ± 1.2 approaches/min, respectively; $p < 0.01$). During instrumental training, rats in both the PIT and the TO group acquired instrumental responding at a comparable rate, and their response rates increased across training days. ANOVA confirmed these trends, as indicated by a significant effect of training day ($F_{(5, 55)} = 77.32$; $p = 0.00$), but no effect of group ($F_{(1, 11)} = 0.99$; $p = 0.34$), and a lack of a group x day interaction ($F_{(5, 55)} = 1.44$; $p = 0.23$). On the

last day of training the mean lever-press rates for the PIT and TO groups were 48.4 ± 3.4 and 39.5 ± 3.7 presses/min, respectively.

Following unilateral intra-NAc infusion of SCH23390, rats in the PIT group pressed the lever significantly more during the CS than the preCS period, and thus demonstrated a robust PIT effect during testing (Figure 8A). Rats in the TO group that had received unilateral infusion of SCH23390 into the NAc also displayed reasonably high lever-press rates during testing; however, unlike the PIT group, their response rates during the preCS and CS periods were comparable. ANOVA confirmed these differential trends, as indicated by a significant group x stimulus period interaction ($F_{(1, 11)} = 4.98$; $p < 0.05$). Post-hoc comparisons revealed that the mean lever-press rate during the CS was significantly higher than that during the preCS period for the PIT group ($p < 0.01$), but not for the TO group ($p = 0.65$). There was no significant difference between the mean lever-press rate during the preCS for the two groups ($p = 0.78$). The total number of lever presses for the PIT and TO groups during the PIT test were 285 ± 52 and 263 ± 62, respectively, and did not differ statistically form one another ($t_{(11)} = 0.20$; $p = 0.84$). Together, these results indicate that unilateral D1R blockade in the NAc did not interfere with either discriminative responding to the CS or general lever-pressing behavior.

3.3.1.2 pERK1/2 Immunohistochemistry

Immediately after the PIT test, rats were perfused to assess pERK1/2 immunostaining in the NAc core and shell. To determine whether blockade of D1 receptor transmission influences the pattern of pERK immunostaining that we previously observed during PIT (Remus and Thiels, 2013), we used a within-subject design. This design allowed us to compare the number of pERK1/2-IR cells between the SCH23390- and vehicle-infused NAc

of the same rats. We also compared the number of pERK-IR cells between subjects in the PIT and TO groups to determine the effect of intra-NAc D1R blockade on CS-evoked versus basal ERK activation.

The effect of unilateral intra-NAc D1 receptor blockade on NAc ERK activation evoked during PIT is shown in Figure 8. On the vehicle-infused side of the NAc, we found that the number of pERK1/2-IR cells in the core was about three-fold higher in PIT subjects than in TO control rats (Figure 8B-D, Table 3). In contrast, on the SCH23390-infused side of the NAc, we found that the number of pERK1/2-IR cells in the core of PIT rats and TO rats was comparable as a result of a marked reduction (~70%) in ERK1/2-IR cells upon intra-NAc infusion of SCH23390 in PIT rats. ANOVA of pERK-IR cell counts confirmed these observations, revealing a significant group effect ($F_{(1, 11)} = 8.55$; $p = 0.01$), a significant effect of infusate ($F_{(1, 11)} = 33.32$; $p < 0.01$) and, importantly, a significant infusate x group interaction ($F_{(1, 11)} = 25.33$; $p < 0.01$). Post-hoc comparisons indicated that infusion of SCH23390 significantly reduced the number of pERK-IR cells in the core of PIT subjects ($p < 0.01$), but not of TO subjects ($p = 0.62$), and that the number of pERK-IR cells in the core was significantly higher for the PIT compared to the TO group on the vehicle-infused side ($p < 0.01$) but not the SCH23390-infused side ($p = 0.75$). A similar pattern of results was observed on the NAc shell (Figure 8F-H, Table 3). The number of pERK1/2-IR cells on the vehicle-infused side was about two-fold higher in PIT subjects when compared with TO controls whereas the group difference was abolished on the SCH23390-infused side because of a dramatic reduction in the number of pERK1/2-IR cells of PIT subjects. ANOVA confirmed these trends, as there was a significant effect of infusate ($F_{(1, 11)} = 23.33$; $p < 0.01$) and a significant infusate x group interaction ($F_{(1, 11)} = 13.44$; $p < 0.01$). Post-hoc comparisons revealed a significant effect of the infusate treatment for the PIT group ($p <$

0.01) but not the TO group ($p > 0.44$), and the number of pERK1/2-IR cells in the shell was greater for PIT than TO subjects on the vehicle-infused ($p < 0.05$) but not the SCH23390-infused side ($p = 1$).

To confirm that the dose of SCH23390 used here was sufficient to disrupt PIT, a different set of rats (n=7) received bilateral infusions of either vehicle or SCH23390. When rats underwent vehicle infusion prior to PIT, their lever press rates were 7.1±2 and 13.8±3 presses/min during the preCS and CS periods, respectively, indicative of a robust positive transfer. In contrast, when the same rats were treated with SCH23390 prior to PIT, their lever press rates were 4.3±1.6 and 3.5±1.1 presses/min, during the preCS and CS periods, respectively. ANOVA indicated a significant stimulus period x infusate interaction ($F_{(1, 6)} = 12.46$; $p = 0.01$), and post-hoc comparisons confirmed that press rates differed between the preCS and the CS period after infusion of vehicle ($p < 0.01$) but not after infusion of SCH23390 ($p > 0.5$). Total numbers of lever presses during the PIT test did not differ significantly as a function of drug treatment for the SCH23390 and vehicle treatments and were (309 ± 66 and 211 ± 43 pressed for the vehicle and the SCH23390 condition, respectively; paired *t*-test $t_{(7)} = 1.62$; $p = 0.16$).

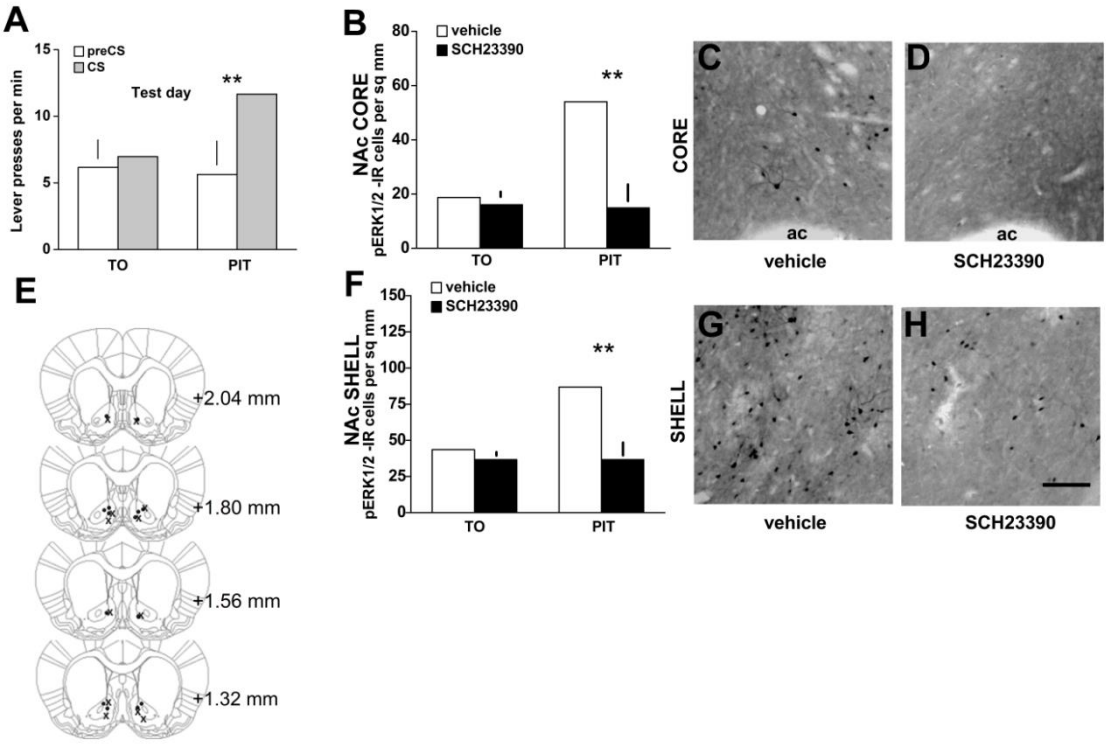

Figure 8: Unilateral infusion of SCH23390 into the NAc blocks the CS-evoked increase in the number of pERK immunopositive cells in the core and the shell observed during PIT. A, Rats in the PIT group displayed positive transfer, whereas rats in the TO control group did not. Both groups received unilateral intra-NAc infusion of SCH3390 before the transfer test. Bars represent the average active lever press rates from the preCS and CS periods during the PIT test for rats in the TO and PIT groups. ** Repeated measures ANOVA, p <0.01, significantly different from preCS responding. Scale bar = +1 standard error of the difference (SED), a measure of within-subject variability. E, The schematic depicts the location of the tip of the injection cannulae within the NAc for PIT (•) and TO subjects (x). Cannula placement was estimated based on observed tissue damage from the guide cannulas in histologically prepared sections. The number beside each plate corresponds to millimeters (mm) anterior to bregma. The schematic was reproduced with permission from Paxinos and Watson (2007). C, Mean±SED number of pERK1/2-IR cells in the NAc core for the TO control (n=6) and the PIT (n=7) groups. Intra-NAc infusion of SCH23390 significantly reduced the number of pERK1/2-IR cells in the NAc core of PIT rats (**, p<0.01) but had no significant effect on the number of pERK immunopositive cells in the NAc core of TO control rats (left panels B and F). D-E, Representative photomicrographs of pERK-labeled cells in the core from a rat of the PIT group that received infusion of vehicle (C) on one side and SCH23390 (D) on the other (scale bar, see panel H; ac, anterior commissure). F, Similar data as shown in panel C is depicted for the NAc shell. Intra-NAc infusion of SCH23390 significantly reduced the number of pERK1/2-IR cells in the NAc shell of PIT rats (**, p<0.01). G-H, Representative photomicrographs of pERK-labeled cells in the shell from a rat of the PIT group that received infusion of vehicle (G) on one side and SCH23390 (H) on the other (scale bar, 100 μm)

3.3.2 NAc ERK activation during Pavlovian-instrumental transfer is not mediated via activation of D2Rs

To test whether D2R activation is required for CS-evoked NAc ERK activation during PIT, we repeated the experiment as described above except that rats received unilateral infusions of raclopride, into the NAc on one side and vehicle solution into the other NAc immediately prior to the PIT test.

3.3.2.1 Behavior

During Pavlovian training, rats in the PIT group (n=7) developed discriminative food cup approach behavior, whereas rats in the TO group (n=5) did not. ANOVA confirmed these observations, as indicated by a significant group x day interaction ($F_{(5, 50)} = 4.37$; $p < 0.01$). Following six days of training, the mean discriminative approach rate of the PIT group was significantly higher than that of the TO group (6.4 ± 0.9 and -0.3 ± 1.1 approaches/min, respectively; $p < 0.01$). During instrumental training, rats in both the PIT and the TO group acquired instrumental responding at a comparable rate, and their response rates increased across training days. ANOVA confirmed these trends, as indicated by a significant effect of training day ($F_{(5, 50)} = 42.86$; $p < 0.01$), but no group x day interaction ($F_{(5, 50)} = 0.37$; $p = 0.86$). On the last day of instrumental conditioning, the lever-press rates were 42.9 ± 6.1 and 40.9 ± 7.3 presses/min for rats in the PIT and TO groups, respectively.

Following unilateral intra-NAc infusion of raclopride, rats in the PIT group pressed the lever significantly more during the CS than the preCS period, and thus demonstrated a robust PIT effect (Figure 9A). Rats in the TO group that had received unilateral infusion of raclopride into the NAc also displayed reasonably high lever-press rates during testing;

however, unlike the PIT group, their response rates during the preCS and CS periods were comparable. ANOVA confirmed these trends, as revealed by a significant group x stimulus period interaction ($F_{(1, 10)} = 6.98$; $p < 0.05$). Post-hoc comparisons revealed that the mean lever-press rate during the CS was significantly higher than that during the preCS period for the PIT group ($p < 0.01$), but not for the TO group ($p = 0.71$). There was no significant difference in mean lever-press rate during the preCS period between the two groups ($p = 0.96$). Total number of lever presses for the PIT and TO groups during the PIT test were 338 ± 67 and 269 ± 46, respectively, and did not differ statistically from each other, ($t_{(10)} < 1$).

3.3.2.2 pERK1/2 Immunohistochemistry.

The effect of unilateral intra-NAc D2R blockade on NAc ERK activation evoked during PIT is shown in Figure 9 and Table 3. Different from the experiment above, we found that the number of pERK1/2-IR cells in the core was about three-fold higher in PIT subjects than in TO control rats on both the vehicle-infused side of the NAc and the raclopride-infused side of the NAc. In fact, the number of pERK1/2-IR cells in the NAc core appeared to be comparable between the vehicle-treated NAc and the raclopride-treated NAc, a trend in both groups (Figure 9 B-D, Table 3). ANOVA of pERK-IR cell counts confirmed these observations, revealing a significant group effect ($F_{(1, 10)} = 12.10$; $p < 0.01$), but no significant effect of infusate ($F_{(1, 10)} < 1$) and no significant group x infusate interaction ($F_{(1, 10)} < 1$). Post-hoc comparisons revealed that the number of pERK-IR cells in the core was significantly higher for the PIT group compared with the TO group ($p s \leq 0.01$) irrespective of the infusate treatment. Similar results were observed for the NAc shell (Figure 9F-H, Table 3). ANOVA of pERK-IR cell counts in the NAc shell revealed a significant group effect ($F_{(1, 10)} = 10.43$; $p < 0.01$), but no significant effect of infusate ($F_{(1, 10)} < 1$) and no significant

group x infusate interaction ($F_{(1, 10)} < 1$). Post-hoc comparisons revealed that PIT group expressed significantly more pERK-IR cells than the TO group in both vehicle and raclopride infused NAc (ps < 0.01) indicative that raclopride infusion did not reduce CS-evoked ERK activation during PIT.

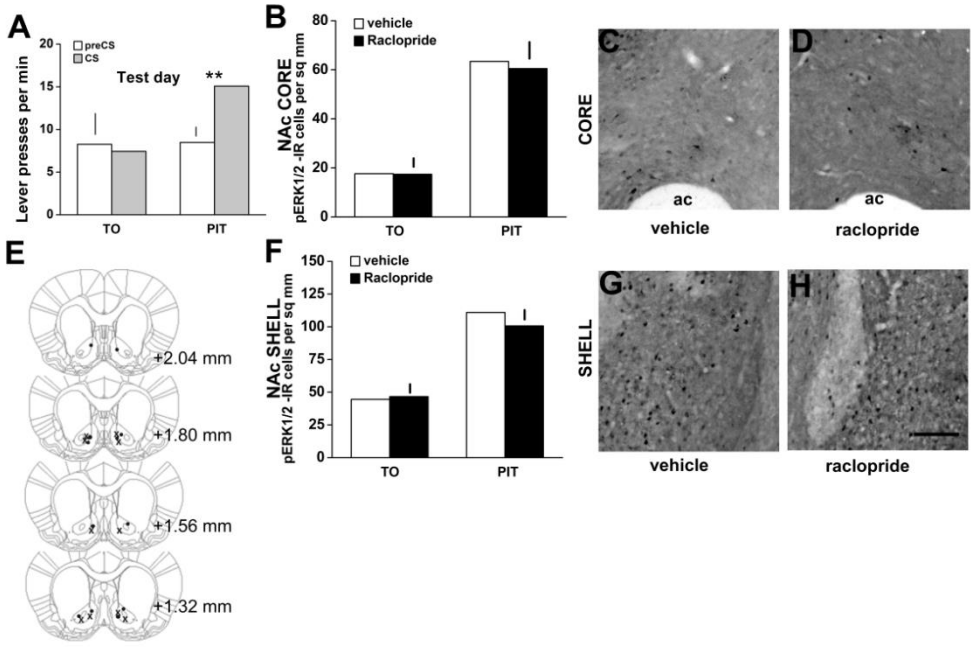

Figure 9: Unilateral infusion of raclopride into the NAc has no effect on the CS-evoked increase in the number of pERK immunopositive cells in the core and shell observed during PIT. A, When rats in the PIT group received unilateral intra-NAc infusions of raclopride before the transfer test, they displayed positive transfer, whereas rats in the TO control group that also received unilateral intra-NAc infusions of raclopride before test showed no evidence of transfer. Bars represent the average active lever press rates from the preCS and CS periods during the PIT test for rats in the TO and PIT groups. ***p* <0.01, significantly different from preCS responding. Scale bar = +1 SED. E, The schematic depicts the location of the injection cannulae tips within the NAc for PIT (•) and TO subjects (x). Cannula placement was estimated based on observed tissue damage from the guide cannulas in histologically prepared sections. The number beside each plate corresponds to mm anterior to bregma. The schematic was reproduced with permission from Paxinos and Watson (2007). **B,** Mean±SED number of pERK1/2-IR cells in the NAc core for the TO control (n=5) and the PIT (n=7) groups. Intra-NAc infusion of raclopride (black bars) had no significant effect on the number of pERK1/2-IR cells in the NAc core of PIT or TO rats. When rats received intra-NAc infusion of vehicle (white bars), the number of pERK1/2-IR cells was significantly greater in the NAc core of the rats in the PIT group (p<0.01). **C-D,** Representative photomicrographs of pERK-labeled cells in the core from a rat of the PIT group that received infusion of vehicle (**C**) on one side and raclopride (**D**) on the other (scale bar, see panel H; ac, anterior commissure). **F**, Similar data as shown in panel B is depicted for the NAc shell. Intra-NAc infusion of raclopride (black bars) had no significant effect on the number of pERK1/2-IR cells in the NAc shell of PIT or TO rats. Comparison of the vehicle-treated NAc showed that the number of pERK1/2-IR cells in the NAc shell was significantly greater for the PIT group than the TO group (p<0.01). G-H, Representative photomicrographs of pERK-labeled cells in the shell from a rat of the PIT group that received infusion of vehicle (**E**) on one side and raclopride (**F**) on the other (scale bar, 100 µm).

To confirm that the dose of raclopride used here was sufficient to disrupt PIT, a different set of rats (n=8) received bilateral infusions of either vehicle or raclopride. When rats underwent vehicle infusion prior to PIT, their lever press rates were 7.9±2 and 15.6±3 presses/min during the preCS and CS periods, respectively, indicative of a robust positive transfer. In contrast, when the same rats were treated with raclopride prior to PIT, their lever press rates were 7.44±2.69 and 7.50±2.14 presses/min during the preCS and CS periods, respectively. ANOVA indicated a significant stimulus period x infusate interaction ($F_{(1, 7)} = 21.46$; $p < 0.01$), and post-hoc comparisons confirmed that press rates differed between the preCS and the CS period after infusion of vehicle ($p< 0.01$) but not after infusion of raclopride ($p= 0.96$). Total number of lever presses during the PIT test did not differ significantly as a function of infusate treatment for the raclopride and vehicle treatments, and were 374 ± 40 and 245 ± 57 presses/min for the vehicle and the raclopride condition, respectively (paired *t*-test ($t_{(7)} = 1.71$; $p = 0.13$).

Although bilateral infusion of SCH23390 and raclopride produced similar effects on PIT, in both cases without affecting total lever press rates, the two antagonists had different effects on discriminative food cup approach during the PIT test (Figure 10). Interestingly, bilateral intra-NAc infusion of SCH23390 reduced discriminative approach. That is the discriminative approach rates were significantly different when rats received bilateral intra-NAc infusion of SCH23390 compared to when the same rats received vehicle bilateral intra-NAc infusion of vehicle just prior to the PIT test (paired *t*-test ($t_{(7)} = 3.00$; $p < 0.05$). Unlike the disruptive effect of SCH23390, bilateral infusion of raclopride did not disrupt discriminative approach behavior. There was no difference between the discriminative approach rates of the rats when they received bilateral intra-NAc infusions of raclopride

compared to when the same animals received bilateral intra-NAc infusions of vehicle (paired *t*-test ($t_{(7)} = 0.19$; $p > 0.1$) (Figure 10).

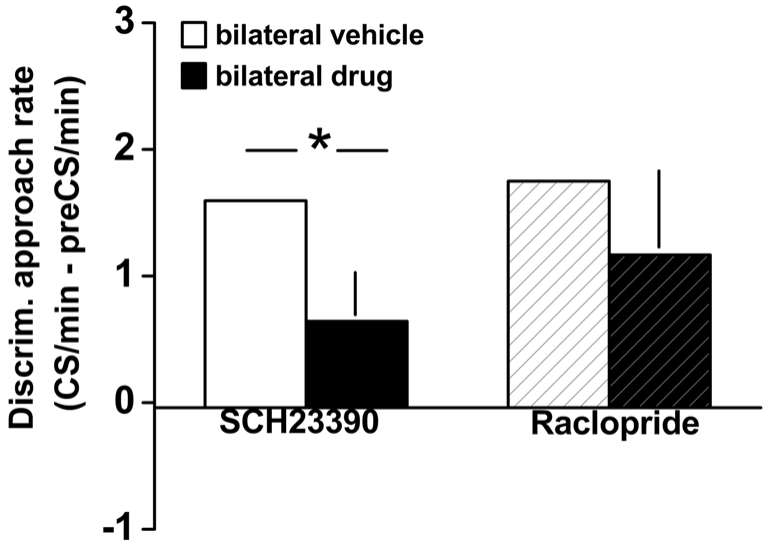

Figure 10: Bilateral intra-NAc infusion of SCH23390 disrupts discriminative approach during the PIT test, whereas bilateral intra-NAc infusion of raclopride does not. Left bars, When rats in the PIT group received intra-NAc infusions of vehicle (white bar), they displayed discriminative food cup approach behavior (more food cup approaches during the CS compared with the preCS period), whereas when the same rats received bilateral intra-NAc infusions of SCH23390 before the transfer test (black bar), approaches during the CS were attenuated, and therefore discriminative food-cup approach was not observed. **Right bars,** When rats in the PIT group received intra-NAc infusions of vehicle, they exhibited discriminative conditioned approach behavior (white bar), which persisted even after the same rats received bilateral intra-NAc infusions of raclopride before the transfer test (black bar). Bars represent the mean difference of the food cup approaches during the CS compared to the preCS period of the PIT test for rats in PIT groups. *p <0.05, significantly different from discriminative approach rate following the bilateral intra-NAc infusion of SCH23390.

Table 3: Summary of the immunohistochemistry results (group means ± SEM) for the unilateral intra-NAc infusions of SCH23390 or raclopride.

Experiment →	**Unilateral Intra NAc D1 receptor antagonist**				**Unilateral Intra NAc D2 receptor antagonist**			
Treatment → Cell Count ↓	**TO Vehicle (n=6)**	**TO SCH23390 (n=6)**	**PIT Vehicle (n=7)**	**PIT SCH23390 (n=7)**	**TO Vehicle (n=5)**	**TO Raclopride (n=5)**	**PIT Vehicle (n=7)**	**PIT Raclopride (n=7)**
pERK$^+$cell count CORE	16.1 ± 2.3	18.8 ± 3.6	54.0 ± 7.1	14.9 ± 2.3	17.4 ± 4.0	17.6 ± 2.5	63.4 ± 11.9	60.5 ± 10.3
pERK$^+$cell count SHELL	43.5 ± 3.5	36.7 ± 4.6	86.9 ± 15.1	36.7 ± 7.1	44.6 ± 3.9	46.6 ± 5.3	110.9 ± 18.0	100.7 ± 13.5

3.3.3 NAc ERK activation during Pavlovian-instrumental transfer occurs in putative D1-R expressing neurons

To determine the phenotype of the cells that exhibit pERK immunoreactivity following CS-potentiation of reward-seeking, we applied dual-immunofluorescence staining for pERK and PDYN, a precursor of DYN and marker of D1R-expressing medium spiny neurons (Gerfen *et al.*, 1990; Lee *et al.*, 1997; Missale *et al.*, 1998; Sonomura *et al.*, 2007). We conducted dual-immunofluorescence on sections from rats that received unilateral infusions of either SCH23390 or raclopride immediately before PIT testing and were included in the behavioral and immunohistochemical analyses of the experiments described above. First, we examined whether pERK immunopositive cells in the core and shell of the NAc that received vehicle treatment in each group (n=3 per group) also contained PDYN. Then, we applied dual-immunofluorescence staining for PDYN and D1Rs to confirm that PDYN immunolabeling overlaps with staining for D1Rs. We also qualitatively compared the vehicle-treated NAc with the corresponding SCH23390- or raclopride- treated NAc to determine whether disruption of D1 or D2 receptor activation alters the phenotype of CS-evoked ERK activation.

Focusing first on the side that was infused with vehicle solution before PIT testing, we observed copious amounts of neurons with somata diameters ranging from 8-14 µm throughout the NAc core and shell that stained positive for PDYN (See left panels in Figure 11 and Figure 12). Both small clusters of PDYN-IR cells and single PDYN-IR cells were homogeneously distributed in both the core and the shell throughout the rostrocaudal extent of the NAc we examined (2.1 through 1.1 mm relative to Bregma). For the vast majority of PDYN-IR cells, staining was very strong in the cytoplasm surrounding the nucleus and very

weak or absent in the nucleus. All cells that stained for pERK were found to also be stained for PDYN (See right panels in Figure 11 and Figure 12). Consistent with our previous work (Remus & Thiels, 2013), the somata of cells that stained positive for pERK ranged from 8-14 µm in diameter and thus were of similar size as PDYN-IR cells.

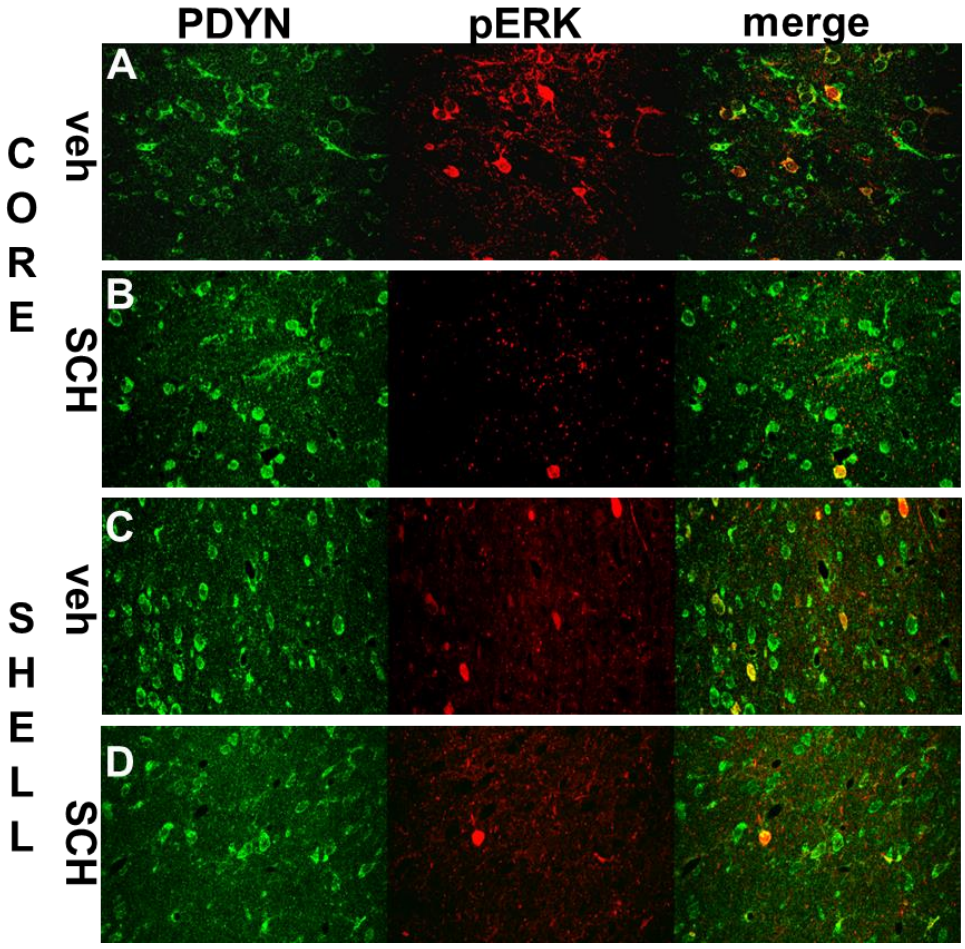

Figure 11: CS-evoked NAc ERK activation during PIT occurs in NAc cells that express PDYN and intra-NAc infusion of SCH23390 reduces the number of pERK-IR-IR cells. Panels A-D Representative confocal images showing labeling for PDYN (left), pERK (middle), and the colocalization of PDYN and pERK (right) from the NAc of a PIT rat. A, PDYN-IR cells in the core of a PIT rat that received vehicle treatment also stained positive for pERK1/2 B, In the presence of SCH23390, the number of pERK-labeled cells in the core is reduced significantly. C, Similar to A, PDYN-IR cells in the shell of a PIT rat that received vehicle treatment also stained positive for pERK1/2 D, Similar to panel B, In the presence of SCH23390, the number of pERK-labeled cells in the shell is reduced significantly. (For scale bar see Figure 12, 10 µm). A similar pattern of overlap in pERK and PDYN staining was observed in other sections from this PIT rat and in sections from the other two PIT rats as was examined here.

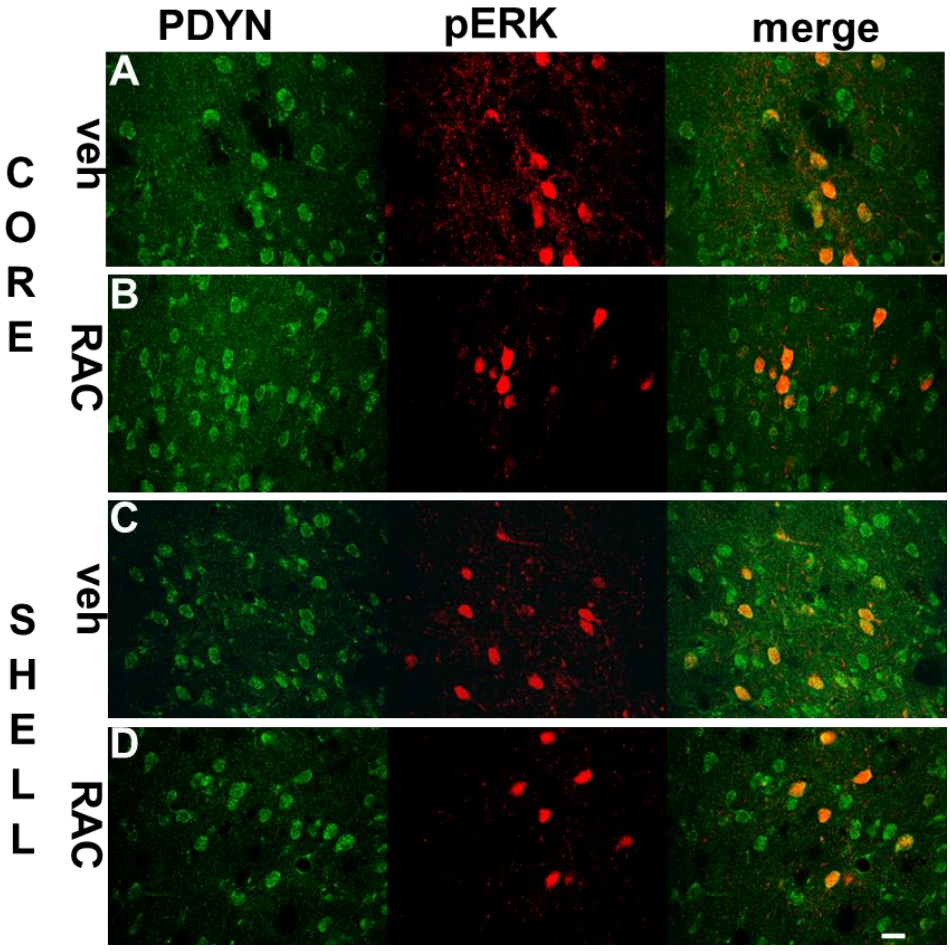

Figure 12: Infusion of raclopride into the NAc does not alter the number or the phenotype of the cells that exhibit ERK activation by CSs during PIT. Panels A-D Representative confocal images showing labeling for PDYN (left), pERK (middle), and the colocalization of PDYN and pERK (right) from the NAc of a PIT rat. A, PDYN-IR cells in the core of a PIT rat that received vehicle treatment also stained positive for pERK1/2. B, In the presence of raclopride, the number of pERK-IR cells in the NAc core is similar to vehicle treatment C, Similar to A, PDYN-IR cells in the shell of a PIT rat that received vehicle treatment also stained positive for pERK1/2. D, Similar to panel B, In the presence of raclopride, the number of pERK-IR cells in the NAc core is similar to vehicle treatment (scale bar, 10 μm). A similar pattern of overlap in pERK and PDYN staining was observed in other sections from this PIT rat and in sections from the other two PIT rats as was examined here.

Since PDYN was shown to occur in NAc MSNs that express D1Rs (Gerfen *et al.*, 1990; Lee *et al.*, 1997; Sonomura *et al.*, 2007), we assessed whether staining with the PDYN antibody used in this study also colocalized with staining for D1 receptor (n=2). We found that D1R immunolabeling was present in both the cytoplasm and the nucleus. PDYN immunostaining was present predominantly in the cytoplasm, as described above. Therefore, the dual-labeled D1R/PDYN-IR cells consisted of green cytoplasmic outline with orange to red centers (Figure 13). When we examined the degree of overlap, we found that every D1R-IR cell also stained positive for PDYN, which confirms that use of the PDYN antibody selectively identifies D1R-containing MSNs. By extension, these findings indicate that the pERK-IR cells we described above (Figures Figure **11** and Figure **12**) are D1R-expressing cells. We also performed a blocking peptide experiment to confirm binding of the PDYN antibody to the specific peptide sequence for rat PDYN. Comparison of the staining patterns between the un-blocked and blocked antibodies yielded PDYN staining and no staining, respectively (see Figure 14) The lack of PDYN stain on blocked sections suggests that the PDYN antibody used here binds specifically the 235/248 amino acid sequence of rat PDYN.

Next, we compared the drug-treated NAc with the respective vehicle treated NAc, to determine whether blockade of NAc D1 or D2 receptors alters the phenotype of the pERK-IR cells. When we compared the SCH23390-treated NAc with the vehicle-treated NAc in PIT rats, we observed that SCH23390 reduced the number of pERK-IR cells in both the core and the shell, but the drug appeared to have no effect on the number of PDYN-IR cells. When we compared the raclopride-treated NAc with the vehicle-treated NAc, many pERK-IR cells remained in both the core and the shell despite the fact that bilateral infusion of raclopride reduced PIT. In addition, the number of PDYN-IR cells were similar between the vehicle- and raclopride-treated NAc. The pERK immunofluorescence observations were consistent

with the IHC analyses described above. Similar to the IHC experiments, both core and shell of the SCH23390-treated NAc of PIT rats contained much fewer pERK-IR cells than the vehicle-treated NAc (Figure 11. panels B and D), whereas, following NAc treatment with raclopride, several pERK-IR cells remained in the core and shell of PIT rats (Figure 12 panels B and D). Taken together, these results show that inactivation of NAc D1 receptors markedly attenuated CS-evoked ERK activation, and that the inactivation of NAc D1 or D2 receptors did not alter the phenotype of the NAc cells in which CS-evoked ERK activation occurs.

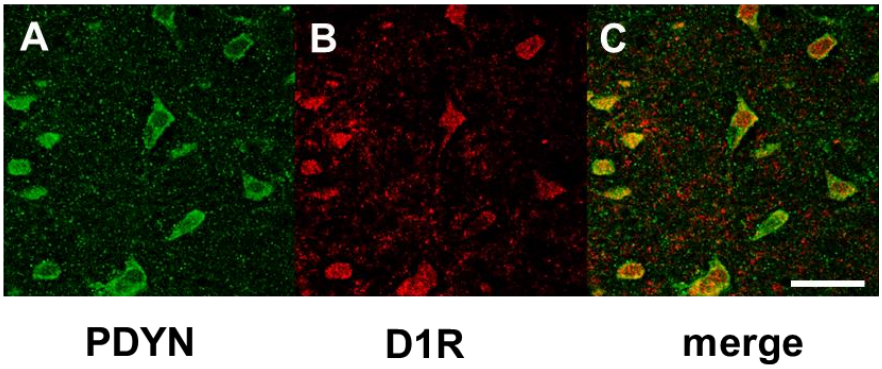

Figure 13: D1R immunostaining colocalizes with staining for PDYN. Panels A-C Representative confocal images showing labeling for PDYN **(A),** D1R **(B),** and the colocalization of PDYN and D1R **(C)** from the NAc of a PIT rat. (scale bar, 10 µm). A similar pattern of overlap in PDYN and D1R staining was observed in two additional sections from this rat and in sections examined from an additional rat.

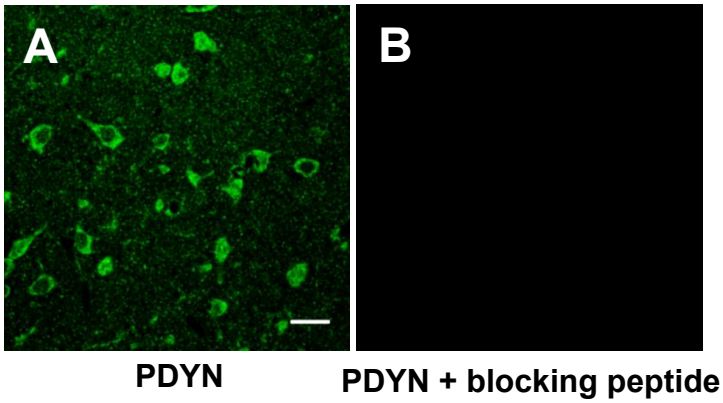

Figure 14: PDYN immunostaining is blocked following pre-incubation with cognate peptide. A-B, Confocal images showing immunolabeling for PDYN (**A**), and immunolabeling for PDYN after addition of the antibody blocking peptide. Sections were taken from the same brain (**B**). (scale bar, 10 µm).

3.4 DISCUSSION

We previously showed that exposure to an appetitive CS after Pavlovian conditioning causes an increase in ERK activation in the NAc core and NAc shell and that this signaling event is dependent on both D1R and NMDAR activation in the NAc (Shiflett *et al.*, 2008; Kirschmann *et al.*, 2014). Furthermore, we showed that exposure to a CS that enhances seeking for the reward it predicts and causes an increase in ERK activation in the NAc core and NAc shell (Remus & Thiels, 2013), and this effect of CS-potentiation of reward seeking fails to occur if NAc ERK activation is blocked (Shiflett *et al.*, 2008). Here, we extended our previous work by demonstrating that NAc D1Rs also play a critical role in ERK activation in the core and the shell during PIT, and that ERK activation during PIT occurs in putative D1R-expressing MSNs.

Ours results implicate activation of NAc D1Rs, but not D2Rs, in CS-evoked ERK activation during PIT. We found that disruption of D1R signaling in the NAc, via local infusion of SCH23390, abolished CS-evoked ERK activation required for PIT. This finding was revealed by a marked reduction in the number of pERK-IR cells in both the core and the shell subregions of SCH23390-treated NAc compared with the number of pERK immunopositive cells in vehicle-treated NAc. The D1 receptor antagonist had no significant effect on ERK activation in the NAc of TO controls, which suggests that the dose of SCH23390 infused here did not disrupt basal ERK signaling. One the other hand, we found that disruption of D2R signaling in the NAc, via local infusion of raclopride, had no effect on CS-evoked ERK activation, as illustrated by similar numbers of pERK-IR cells in both the core and the shell of raclopride-treated NAc compared with the number of pERK-IR cells in vehicle-treated NAc. Importantly, the lack of an effect of raclopride infusion on the number

of pERK-IR cells cannot be attributed to a lack of increased ERK activation because the number of pERK-IR cells in the raclopride-treated NAc of PIT subjects was significantly greater than the number of pERK immunopositive cells in the raclopride-treated NAc of TO control subjects.

The role of dopamine signaling in the NAc in PIT has been studied extensively. Dopaminergic input from the VTA was shown to be critical to PIT (Murschall & Hauber, 2006; Corbit & Janak, 2007), and altered dopamine signaling was shown to interfere with the ability of a CS to invigorate reward seeking (Dickinson *et al.*, 2000; Wyvell & Berridge, 2000; Lex & Hauber, 2008; Wassum *et al.*, 2011; Wassum *et al.*, 2013). In agreement with this previous work, we found that both D1- and D2-receptor blockade in the NAc disrupt PIT. With respect to the D1R, our results extend previous studies by specifying a molecular mechanism through which activation of this dopamine receptor type in the NAc potentiates reward seeking in the presence of a CS. While our studies indicate that D2 receptor activation is necessary for PIT, they suggest that D2 receptors act through a molecular mechanism other than ERK.

Numerous studies have demonstrated that NAc ERK activation is mediated through a D1R-dependent mechanism (Valjent *et al.*, 2004; Borgkvist *et al.*, 2008; Ibba *et al.*, 2009; Fricks-Gleason & Marshall, 2011; Kirschmann *et al.*, 2014). Our findings indicate that increased ERK signaling in the NAc induced by exposure to a reward-predictive CS is dependent on activation of D1Rs, but not D2Rs. These results are in agreement with studies demonstrating that ERK activation elicited by drugs and cues predictive of drugs is dependent on D1R activation, but not D2 activation, in the NAc (Valjent *et al.*, 2004; Borgkvist *et al.*, 2008; Fricks-Gleason & Marshall, 2011). In the case of psychostimulant-triggered activation of ERK, coincident activation of NMDA receptors and D1Rs has been

shown to be required to activate ERK in the striatum (Valjent *et al.*, 2004; Valjent *et al.*, 2005; Ibba *et al.*, 2009; Cahill *et al.*, 2014). Likewise, we recently demonstrated that CSs after appetitive Pavlovian conditioning evoke ERK signaling and that the CS-evoked ERK signaling due to natural rewards is dependent on the activation of both NMDAR and D1Rs in the NAc (Kirschmann *et al.*, 2014). In light of these findings, it is compelling to speculate that ERK activation induced by exposure to the CS during PIT also is mediated by convergent activation of D1Rs and NMDA receptors in the NAc (Day, 2008; Shiflett & Balleine, 2011a). In support of this proposal, glutamatergic inputs from cortical, limbic, and thalamic projections and dopaminergic inputs from the VTA synapse in close proximity on MSNs (Hara & Pickel, 2005). Moreover, D1Rs were shown to modulate responses activated by NMDA receptors on MSNs (Levine *et al.*, 1996; Price *et al.*, 1999; Charara & Grace, 2003). Complementary findings showed that physical interactions between NMDA receptors and D1Rs affect the functionality of each other. For example, exposure to NMDA results in the recruitment of more D1Rs to the spines of MSNs (Scott *et al.*, 2002; Scott *et al.*, 2006), and D1R activation was shown to induce trafficking of NMDA receptors (Dunah & Standaert, 2001). Thus, D1Rs on MSNs may regulate the ability of NMDA receptors to facilitate enhancement of reward-seeking by CSs. Future experimentation is required to determine the role of NAc NMDA receptors in PIT.

A major goal of this study was to determine the phenotype of the NAc cells that exhibit CS-evoked ERK activation during PIT. We discovered that all cells that stained positively for pERK also stained positively for PDYN, the precursor peptide of DYN. PDYN is expressed selectively in D1R-containing MSNs (Gerfen & Young, 1988; Gerfen et al., 1990; Le Moine & Bloch, 1995; Hara & Pickel, 2005; Sonomura et al., 2007). Consistent with such an expression pattern, we showed that NAc cells that stained positively for PDYN

also stained positively for D1Rs. Previous studies have demonstrated that cues predictive of drug reward activate ERK signaling in D1R-expressing MSNs, but not D2Rs-expressing MSNs expressing (Borgkvist *et al.*, 2008; Fricks-Gleason & Marshall, 2011). Taken together, our results suggest that CSs activate ERK signaling in the NAc during PIT via a D1R-dependent mechanism in a subpopulation of NAc cells that express PDYN and D1Rs.

We did not address whether neurons that exhibit ERK activation during PIT also contain proenkephalin (PENK), the precursor peptide of ENK, which was shown to be contained in MSNs that express D2Rs (Hong *et al.*, 1977; Gerfen *et al.*, 1990; Sonomura *et al.*, 2007). Recently, a small subset of NAc neurons that contain the D1R-D2R heteromer and express both PDYN and PENK has been reported (Surmeier *et al.*, 1992; Aizman *et al.*, 2000; Perreault *et al.*, 2010), which raises the possibility that some of the cells recruited during PIT based on increased ERK activation by CSs may belong to this third subpopulation. However, in a pilot study we did not observe an overlap of staining between pERK and PENK (Appendix A: Figure 17). Moreover, infusion of raclopride in this study did not affect NAc CS-evoked ERK activation. Furthermore, activation of D2Rs was shown to either have no effect or decrease ERK activation in striatal cells, whereas blockade of D2Rs was shown to increase ERK activation (Valjent *et al.*, 2004; Bertran-Gonzalez *et al.*, 2008; Fricks-Gleason & Marshall, 2011). In contrast, we did not observe an increase in ERK activation following intra-NAc infusion of raclopride. This apparent discrepancy between our study and studies that reported increased ERK activation in D2R-expressing MSNs is likely due to the application of D2R antagonist. Increased ERK activation in D2R-expressing NAc cell was shown following the systemic administration of D2R antagonist whereas in this study we infused the D2R antagonist locally into the NAc. Taking the aforementioned studies

and our work into account, it seems reasonable to conclude that NAc D2R-expressing MSNs do not exhibit ERK activation upon exposure to a CS.

Little is known about the neural circuitry involved in PIT downstream of the NAc. It was shown that D1R and D2Rs are largely segregated. D1R-expressing MSNs comprise the direct pathway; however, the indirect pathway includes both D1R- and D2R- expressing MSNs (Lu *et al.*, 1998; Smith *et al.*, 2013). Our findings of increased ERK activation in D1R-expressing MSNs in both the core and the shell during PIT suggest that NAc outputs receiving input from D1R-expressing MSNs are implicated in the neural circuit that underlies PIT. Principal recipients of input from D1R-expressing MSNs originating in the NAc core include the substantia nigra and the dorsolateral VP, whereas principal recipients of input from D1R-expresing MSNs originating in the shell include the VTA and the medial VP (Groenewegen & Russchen, 1984; Zahm & Heimer, 1990; Lu *et al.*, 1997; Lu *et al.*, 1998; Zhou *et al.*, 2003). Future retrograde tracing experiments combined with immunofluorescence for pERK are suggested to determine which of the output structures from the NAc core and shell are implicated in PIT.

In conclusion, our data indicate that activation of D1Rs in the NAc is required for CS-evoked ERK activation during PIT and suggest that the cellular events regulated by CS-evoked ERK activation take place in D1R-expressing MSNs. Furthermore, our results suggest that although both D1R and D2R mechanisms contribute to the CS's motivational influence over behavior, the CS-evoked ERK signaling during PIT is mediated exclusively via activation of D1Rs, whereas the mechanism(s) by which D2Rs mediate PIT seem to be ERK-independent. By extension, these findings suggest that inputs to and projections targets from D1R-expressing MSNs are critical elements of the circuit underlying CS-potentiation of reward seeking.

4.0 GENERAL DISCUSSION

4.1 COMPARTMENTALIZATION OF NUCLEUS ACCUMBENS FUNCTIONS DURING PAVLOVIAN-INSTRUMENTAL TRANSFER

To identify the contributions of the NAc core and shell with regard to activated ERK during CS modulation of behavior, we examined the expression of activated ERK in response to various behavioral elements during PIT. In Chapter 2, we observed both similar and differential expression of activated ERK immunoreactivity when comparing the core and shell subregions of the NAc. The pattern of ERK signal was dependent on the type of stimulus presented to the animal. Presentation of a reward-predictive cue during PIT caused a significant increase in ERK activation in both subregions. These results were surprising considering studies that implicated the NAc core, but not the shell in general PIT, which we employed here (Hall *et al.*, 2001; Corbit & Balleine, 2011). Moreover, considering that several studies have revealed dissociable functionalities of the NAc subregions, we hesitated to conclude that the CS-evoked ERK activation in the core and shell served identical functions. To further address the role of ERK activation in the NAc shell, we trained another cohort of animals using the same protocols, but exposed them solely to the reward during the PIT test. Presentation of the reward had no effect on ERK activation in the core, but caused a pronounced increase in ERK activation in the shell. Consistent with our findings, a

subsequent report demonstrated that palatable food reward triggered ERK activation in the NAc shell, but not the core (Guegan *et al.*, 2013). Although the data describing ERK activation triggered by natural rewards is sparse, thus far, the available evidence suggests the exclusive involvement of the NAc shell. The region-specific differences we observed in the activation of the NAc ERK pathway during reward processing can be explained by at least two, not mutually exclusive hypotheses. One possibility is that the temporal kinetics of ERK activation may differ between the NAc core and shell, such that our observations of differential ERK immunoreactivity are merely a reflection of the differential kinetics underlying the afferent circuitry to the subregions. This view is strongly supported by the different anatomical input between the core and shell subregions (van Dongen *et al.*, 2005; van Dongen *et al.*, 2008), which could function as a hierarchy of information flow. A second possibility is that NAc ERK activation is a reflection of dissociable functional roles underlying the neuronal ensembles engaged during CS modulation of behavior. In support of the latter proposal, a growing body of literature has implicated that the NAc core is responsible for the encoding of CSs (Day *et al.*, 2006; Aragona *et al.*, 2009; Ambroggi *et al.*, 2011; Cacciapaglia *et al.*, 2012); whereas the shell is preferentially implicated in the processing of rewards themselves (Bassareo & Di Chiara, 1999; Ikemoto *et al.*, 2005; Pecina & Berridge, 2005). Therefore, we suggest that NAc ERK activation during a CS presentation reflects the preferential processing roles akin to core and shell, as a function of the stimulus type (CS or reward), respectively.

If the core and shell have rather dissociable roles in promoting appropriate behavioral responses in responses to stimuli, how do we explain our finding that CS presentation increased ERK activation comparatively in both the core and the shell? The results from studies designed to assess the roles of the core and the shell in PIT are largely

incontrovertible and have ascribed distinguishable functional roles to the core and the shell. The general and stimulus-specific forms of PIT are generally observed under different training parameters and engage different neural circuitries (Corbit & Balleine, 2005; Murschall & Hauber, 2006; Corbit & Balleine, 2011). General PIT, which relies on the integrity of the NAc core, was demonstrated to be necessary for the general motivating properties of CSs which have been posited to increase "wanting" and pursuit of reward (Hall *et al.*, 2001; Berridge, 2009). On the other hand, stimulus-specific PIT, which depends on the integrity of the NAc shell, was demonstrated to be necessary for the subject to use specific sensory information about the reward to guide behavior to obtain the reward (Corbit *et al.*, 2001). Although it was previously demonstrated that blockade of ERK signaling in the NAc disrupts PIT (Shiflett *et al.*, 2008), whether ERK signaling in the core or the shell alone is required for CS-potentiation of reward-seeking behavior has not yet been addressed. While it is extremely likely that CS-evoked ERK activation in the NAc core is responsible for the CS enhancement of responding during general PIT employed here, the role of CS-evoked ERK activation in the NAc shell is less apparent. Given that the function of the NAc shell in stimulus-specific PIT is to bias instrumental responding towards specific outcomes, one possibility is that the presentation of the CS evokes a cognitive representation of the reward which is then used to guide behavior toward the appropriate instrumental response. Interestingly, a recent study, which employed stimulus-specific PIT, showed increased ERK activation in the NAc shell of animals that have exhibited PIT (Laurent *et al.*, 2014). Unfortunately, this study did not report whether ERK activation was observed in the NAc core and whether ERK activation in the NAc shell is a requirement stimulus-specific PIT. In our studies, we can neither confirm nor rule out the possibility that NAc shell CS-evoked ERK activation is associated with the reward-specific or response-biasing effect of CSs

because our animals were not provided a choice of CSs and responses. Relevant to our findings that CSs evoked ERK activation in both the core and the shell, Berridge and colleagues reported that local microinfusions of either amphetamine or DAMGO in the NAc core or the shell resulted in comparably sized plumes of elevated c-Fos expression accompanied by equivalent enhancement of general PIT (Pecina & Berridge, 2013). From these results, the authors concluded that both core and shell mediate neurochemical enhancements of PIT. In view of our results and the literature discussed herein, it is clear that both the core and the medial shell contain substrates able to boost reward-seeking behavior. This conclusion is compatible with anatomical evidence for crosstalk between NAc subregions (van Dongen *et al.*, 2005; van Dongen *et al.*, 2008). A localized and pointed examination of the requirement of ERK signaling within the core and shell is needed to further discern the precise contributions of core and shell ERK signaling to general PIT.

4.2 CONTRIBUTIONS OF D1 AND D2 EXPRESSING MSNS

DA signaling has long been implicated as the driving force in the ability of CS to potentiate behavior (Wise & Rompre, 1989; Berridge & Robinson, 1998; Berridge, 2004; Wise, 2004). Earlier studies showed that DA within the NAc plays a role in CS-driven behavior by inactivating dopaminergic inputs from the VTA (Murschall & Hauber, 2006; Corbit *et al.*, 2007) or systemically applying dopamine antagonists (Dickinson *et al.*, 2000; Ostlund & Maidment, 2012) and observing a disruption of PIT. In addition, manipulations that augment NAc dopamine were shown to enhance PIT (Wyvell & Berridge, 2000; Pecina

& Berridge, 2013). Our lab previously identified that ERK activation in the NAc is a critical molecular mechanism for PIT (Shiflett *et al.*, 2008). Numerous studies have demonstrated that D1 receptors mediate ERK signaling by drugs of abuse (Valjent *et al.*, 2000; Valjent *et al.*, 2004; Miller & Marshall, 2005; Valjent *et al.*, 2005; Ibba *et al.*, 2009; Fricks-Gleason & Marshall, 2011). Our goal was to determine whether NAc ERK activation evoked by *natural* rewards during PIT is also mediated by the activation of NAc D1 receptors. The activation of both NAc D1 and D2 receptors were previously shown to play a role in general PIT (Lex & Hauber, 2008), therefore, we also examined the effects of NAc blockade of D2 receptors on CS-evoked ERK activation.

We observed severely blunted ERK activation in both the NAc core and the shell when the D1 receptor antagonist, but not the D2 receptor antagonist, was infused into the NAc just prior to the PIT test. In our hands, bilateral NAc infusion of either the D1 receptor or the D2 receptor antagonist was sufficient to disrupt PIT; these findings replicated the main findings of Lex and Hauber (2008). Importantly, our results demonstrate that NAc D2 receptor blockade is sufficient to disrupt PIT, but leaves NAc ERK activation unscathed. These results highlight that while NAc CS-evoked ERK is a requirement for expression of PIT, NAc ERK activation on its own is not sufficient for the manifestation of PIT. Additionally, our results extend those of previous studies in that we provide a mechanism for CS-evoked ERK activation during PIT. Importantly, while both D1 and D2 receptor activation contribute to the ability of CSs to influence behavior, our results implicate that these receptors exert their involvement in PIT through different molecular pathways. Thus, while D1 receptors exert their influence through the regulation of the ERK signaling cascade to facilitate CS-potentiation of reward seeking, D2 receptors appear to exert their influence through an ERK-independent mechanism.

Recent evidence from optogenetic studies enable us to interpret a mechanism by which the activation of both D1 and D2 receptors contributes to CS-potentiation of reward seeking. Using optogenetic stimulation of D1 and D2 receptors in the NAc, Kravitz and colleagues (2010) demonstrated that in wild-type mice, the activation of D1 receptor-expressing direct pathway MSNs increased motor responses and attenuated freezing responses, whereas the activation of the D2-receptor expressing indirect pathway reduced motor responses and increased freezing behavior. Interestingly, the activation of the direct pathway was able to alleviate the locomotor impairments of bradykinesia and freezing in mice exhibiting a model of Parkinson's disease (Kravitz *et al.*, 2010), which leads to interpretation that activation of the direct pathway may result in promotion of the goal-directed behavior, whereas the activation of the indirect pathway may function to prevent competing actions. This interpretation is in agreement with other models of basal ganglia function (Alexander *et al.*, 1990; Nicola, 2007). In light of this functional demonstration of the direct and indirect pathways, we can consider the roles of direct and indirect pathways in the context of PIT. Consistent with the hypothesis that the direct pathway promotes behavior, the function of the direct pathway may be to facilitate appropriate behavior towards obtaining rewards. Activation of D1 receptors on direct pathway MSNs tends to enhance the excitability of the direct pathway MSNs (West & Grace, 2002; Surmeier *et al.*, 2007), thus the activation of D1 receptors on the direct pathway MSNs may function to invigorate CS-directed behavior during PIT. Consistent with the hypothesis that the indirect pathway inhibits competing behaviors, the function of the indirect pathway would be to inhibit behaviors that compete with behaviors to obtain reward. Activation of D2 receptors on indirect pathways reduces the excitability of the indirect pathway MSNs (West & Grace, 2002; Surmeier *et al.*, 2007), thus the activation of D2 receptors would attenuate actions that

compete with obtaining the reward. This interpretation provides a mechanism by which the stimulation of both D1 and D2 receptors on NAc MSNs enables CS-potentiation of reward-seeking: D1 receptor activation on direct pathway MSNs serves to promote the instrumental response to obtain the reward, whereas D2 receptor activation serves to attenuate alternative behavioral responses that may be in competition with instrumental responding to obtain the reward. This interpretation is supported by our data showing that bilateral intra-NAc inactivation of either the D1 or D2 receptors interferes with the ability of the CS presentation to facilitate reward-seeking behavior.

The mediation of PIT by D1 receptors on direct pathway MSNs supports the hypothesis that activation of the direct pathway promotes behavior (Kravitz *et al.,* 2010). However, it is important to consider that the disruption of PIT is not likely to be a direct effect of the intra-NAc D1 receptor blockade. The activation of D1 receptors in the NAc has been shown to increase the excitability of D1 receptor expressing neurons through the trafficking and increased expression of NMDA (Hallett *et al.*, 2006) and AMPA receptors (Snyder *et al.,* 2000). These consequences would render these cells more excitable to glutamatergic inputs. In addition, the activation of D1 receptors has been shown to enhance excitatory currents in NAc neurons (Charara & Grace, 2003). Thus, disruption of PIT by intra-NAc blockade of D1 receptors is likely to be explained by a reduction in the excitability in MSNs.

If the foregoing hypothesis of basal ganglia pathways in the preceding paragraphs is applicable to our results, intra-NAc blockade of D2 receptors should result in the disruption of PIT as a result of a failure to suppress competing behaviors. Interestingly, we found that bilateral blockade of D2 receptors in the NAc disrupted PIT, but not discriminative conditioned food cup approach. This result was different from the effect of bilateral NAc D1

receptor blockade, which uniformly disrupted both PIT and Pavlovian approach behavior. These data hint at the possibility that D1 and D2 receptor populations support different roles in PIT. We found the discriminative approach rates (food cup approach rate during the CS minus food cup approach rate during the preCS) were actually higher during intra-NAc blockade of D2 receptors than when these same animals received intra-NAc infusion of saline; unfortunately, these data were not significantly different from each other. Thus, disrupted PIT as a result of intra-NAc D2 receptor blockade cannot be easily explained based only on the increased food cup approaches during the CS. In addition, intra-NAc D2 receptor blockade did not significantly increase the overall number of head insertions or lever presses. From the perspective of watching the animals, intra-NAc blockade of D2 receptors appeared to increase bouts of locomotion and stereotypic behaviors such as licking the levers. However, our attempt to characterize differences in behavioral responses between intra-NAc D1 and D2 receptor blockade using activity monitors did not substantiate the observations of increased locomotion. Further experimentation with the inclusion of video monitoring would be required to substantiate our observations of increased licking behavior during the CS presentation.

Numerous studies showed that ERK activation in the striatum occurs in D1 receptor-expressing neurons (Bertran-Gonzalez *et al.*, 2008; Borgkvist *et al.*, 2008; Ibba *et al.*, 2009; Guegan *et al.*, 2013; Laurent *et al.*, 2014). Here, we showed that ERK activation by CSs predictive of *natural rewards* during PIT occurs in PDYN expressing cells. Our findings support the role of NAc D1/PDYN-expressing neurons in CS-evoked ERK activation, but leave unresolved a potential role for D2/PENK-expressing neurons in PIT. Importantly, other work has addressed whether cue-evoked ERK activation occurs in D2/PENK-expressing neurons. Cues predictive of cocaine reward were shown to occur exclusively in D1 receptor-

expressing MSNs. Moreover, complementary to our studies, Laurent *et al.* (2014) recently used ERK as marker to determine which cells in the NAc shell were involved in *stimulus-specific PIT*. Strikingly similar to our findings, ERK activation during stimulus-specific PIT was shown to occur in putative D1 receptor-expressing cells and was shown to be dependent on the activation of D1 receptors in the NAc shell. Collectively, the foregoing findings with our data support the view that NAc ERK activation induced by CSs is mediated by D1 receptors on D1 receptor-expressing MSNs.

4.3 PIT: RELEVANCE TO HUMAN BEHAVIOR AND CONCLUDING REMARKS

Although PIT has been most frequently demonstrated in rodents, it is now well-established in humans. To date, at least 15 studies have demonstrated PIT in humans subjects in laboratory settings using paradigms similar to those used to study PIT in rodents (Hogarth *et al.*, 2007; Bray *et al.*, 2008; Talmi *et al.*, 2008; Huys *et al.*, 2011; Trick *et al.*, 2011; Hogarth & Chase, 2012; Garbusow *et al.*, 2014). These studies have established PIT in situations of overeating, gaming, smoking, drug taking, and alcohol dependence. Importantly, many of the brain structures that have been implicated in PIT through animal studies are now being confirmed in humans using non-invasive technologies such as functional magnetic resonance imaging (fMRI). For instance, it has been demonstrated that presentation of a CS enhanced human participants' motivation to work for a monetary reward (Talmi *et al.*, 2008). During the performance of this task, the blood oxygenation level-dependent (BOLD) signal in the NAc of participants increased with increased performance upon CS presentation, i.e.,

PIT. In addition, the strength of a participant's PIT effect was correlated with the strength of their BOLD signal in the amygdala. Addiction and obesity are clinical conditions were PIT-like behavior has been implicated.

Relapse, the resumption of drug-seeking and drug-taking behavior after a period of abstinence, has been shown to be promoted by both the presence of environmental stimuli and contexts that have been previously paired with drug use (Crombag *et al.*, 2008). Although several factors contribute to relapse and drug-seeking behaviors, PIT is one way in which environmental stimuli promote craving and seeking for drugs (O'Brien *et al.*, 1998; Everitt & Wolf, 2002; Hyman *et al.*, 2006). For instance, Hogarth and colleagues (2007) have demonstrated that presentation of cues previously paired with cigarettes increased smokers' responding for cigarettes. Further, craving and seeking for cigarettes persisted after participants had been informed about the considerable health risks of smoking (Hogarth & Chase, 2011) or had undergone medicinal replacement of nicotine (Hogarth, 2012), whereas the same participants significantly decreased their responding for cigarettes in the absence of cues following the aforementioned interventions.

Obesity is another clinical condition where heightened reward seeking due to exposure of cues is maladaptive. During the past 20 years, there has been a dramatic increase in obesity rates in the United States. Currently, more than one-third of U.S. adults (34.9%) and approximately 17% (or 12.7 million) of children and adolescents aged 2—19 years exhibit obesity (CDC, 2014). Although several factors contribute to obesity, there is much agreement that reward pathways in the brain are involved in promoting the consumption of palatable and calorically dense foods (Palmiter, 2007; Wang *et al.*, 2009; Bruce *et al.*, 2010; Johnson & Kenny, 2010; Kenny, 2011b; a; Stice *et al.*, 2013). Remarkably similar to what has been demonstrated in rodent studies; stimulus-specific PIT effects in humans have been

shown to be insensitive to devaluation of the reward. That is, when individuals were satiated on food rewards such as chocolate, they nevertheless still increased their behavior to pursue more chocolate in the presence of cue that has been previously paired with chocolate (Hogarth & Chase, 2011).

The relevance of PIT to human conditions highlights the importance of continued study of this behavioral phenomenon to further elucidate the neurobiological mechanism of cue potentiated reward-seeking. Our results demonstrate that conditioned cue-evoked ERK activation is mediated by D1 receptors and takes place in D1 receptor-expressing NAc projection cells. Taken together with findings that D1 receptor antagonism in the NAc abrogates the ability of conditioned cues to potentiate reward seeking, our results elucidate a molecular mechanism (ERK signaling) through which dopamine mediates enhanced reward seeking in the presence of reward-predictive cues. Furthermore, the results presented here suggest that inputs to and projection targets of D1 receptor-expressing NAc output cells are candidate elements of the neural circuit underlying cue-potentiation of reward seeking. Collectively, our findings may lead to the development of targeted interventions for the treatment of conditions characterized by maladaptive reward seeking, such as drug addiction and obesity.

5.0 FUTURE DIRECTIONS

The experiments described in the preceding chapters comprise initial experiments designed to begin investigations of the role DA in mediating ERK signaling during cue potentiation of reward-seeking behaviors. The results from these experiments generated new questions that will provide the basis for future research. Below are two suggestions for additional experiments that will help to clarify the role of NAc DA and ERK signaling, specifically in cue potentiation of reward-seeking behaviors.

5.1 ERK ACTIVATION DURING CS-EVOKED REWARD SEEKING: A ROLE FOR PHASIC DA RELEASE?

NAc neurons were shown to exhibit increases in cell firing upon CS presentation (Day *et al.*, 2006; Ambroggi *et al.*, 2011). Interestingly, these modulations in firing in response to CSs are eliminated following the inactivation of NMDA receptors in the VTA (Cacciapaglia *et al.*, 2011). Importantly, inactivation of NMDA receptors in the VTA was shown to selectively attenuate the phasic but not tonic firing of DA neurons (Overton & Clark, 1992; Chergui *et al.*, 1993; Deister *et al.*, 2009). Likewise, intra-VTA infusion of a NMDA receptor antagonist, was shown to attenuate cue-evoked DA release in the NAc (Sombers *et al.*, 2009). Here we have shown that D1 receptors on NAc MSNs are required

for CS-evoked ERK activation. Moreover, phasic DA release was shown to occur during PIT upon presentation of the CS, and phasic release correlated with CS-potentiation of behavior (Wassum *et al.*, 2013). These forgoing studies strongly suggest that phasic DA released in the NAc by CSs may stimulate D1 receptors on MSNs which results in increased activation of ERK in those cells to facilitate PIT (Day, 2008; Shiflett & Balleine, 2011a); however, this interpretation requires experimental support.

One approach to determine whether ERK activation elicited by CSs is mediated by burst firing of VTA DA neurons would be to pharmacologically inactivate the VTA using the NMDA receptor antagonist, APV. This procedure was shown to selectively decrease phasic but not tonic firing of DA neurons (Overton & Clark, 1992; Deister *et al.*, 2009). Therefore, I hypothesize that inactivation of the VTA via local infusion of APV would significantly blunt ERK activation in the NAc elicited by CSs. Furthermore, I hypothesize that bilateral microinfusions of APV to the VTA immediately prior to the test would disrupt PIT. In support of this proposal, previous work demonstrated that VTA inactivation disturbs CS-potentiation of reward seeking (Murschall & Hauber, 2006; Corbit & Janak, 2007). However, in these previous studies, the VTA was inactivated via the application of baclofen and muscimol. This general inactivation procedure inhibits both tonic and phasic dopamine firing so that neither D1 nor D2 receptors can be activated. The proposal above would extend these studies by providing evidence for a role of phasic DA release in the mediation of NAc ERK activation by CSs.

5.2 ERK ACTIVATION DURING CS-EVOKED REWARD SEEKING: A ROLE FOR NUCLEUS ACCUMBENS NMDA RECEPTORS?

Although dopamine constitutes the leading source of synaptic modulation in the NAc, other transmitters regulate MSN function in coordination with DA. For example, dopamine-dependent enhancement of glutamatergic transmission may promote the activity of select ensembles of NAc neurons (potentially those in which CS-evoked activation occurs) that receive glutamatergic input coincidently as dopamine is released. This process would enable dopamine released in the NAc to increase the excitability of MSNs in response to glutamatergic input and thereby facilitate the vigor of reward-seeking responses in the presence of CSs such as during PIT. An abundance of evidence from the literature supports this proposal. First, cue-evoked changes in firing of MSNs are blocked not only by dopamine antagonists, but also by the inactivation of either the BLA (Ambroggi *et al.*, 2008) or the PFC (Ishikawa *et al.*, 2008), both of which send glutamatergic projections to the NAc (Brog *et al.*, 1993). Second, anatomical evidence demonstrates that dopaminergic and glutamatergic inputs converge onto the dendrites of MSNs (Hara & Pickel, 2005). Further, dendritic spines of MSNs were shown to recruit more D1 receptors upon exposure to NMDA (Scott *et al.*, 2006). In addition, ERK has been named a coincidence detector, as its activation requires the simultaneous activation of glutamatergic input from cortical, thalamic or limbic inputs and dopaminergic input from the VTA (Goto & Grace, 2005; Valjent *et al.*, 2005; Girault *et al.*, 2007). Moreover, a recent study demonstrated that dopaminergic terminals in the NAc shell are capable of releasing glutamate as well as dopamine (Stuber *et al.*, 2010). Furthermore, activation of both D1 receptors and NMDA receptors was shown to be necessary for CS-evoked ERK activation in the NAc (Kirschmann *et al.*, 2014). A role for NMDA receptors in

PIT has not yet been investigated. Future experimentation is required to determine whether the inactivation of NMDA receptors in the NAc disrupts PIT.

APPENDIX A

6.0 VALIDATION OF THE PDYN ANTIBODY

Using a commercial antibody from Abcam, we have shown that CS-evoked ERK during PIT occurs in NAc cells that express PDYN. However, this interpretation strongly relies on the specificity of the PDYN antibody used to determine the phenotype of the ERK immunopositive cells. We have demonstrated that expression of PDYN can be blocked by pre-incubation with the associated peptide, although the results of this control experiment only demonstrate that the antibody is capable of recognizing the 235-248 amino acid sequence of rat PDYN. However, a BLAST® search did not reveal other proteins with a similar peptide sequence. Moreover, we have shown that our PDYN staining overlaps with immunostaining for D1 receptors. Furthermore, several other studies have used a PDYN antibody consisting of a similar peptide sequence (229-249), which by comparison is the same c-terminal sequence, but six amino acids longer. In these studies, it was shown that PDYN is expressed in D1 receptor expressing MSNs (Hara *et al.*, 2006; Sonomura *et al.*, 2007). In a pilot study, we also examined the phenotype of the pERK-IR cells using the same PDYN and PENK antibodies from Dr. Takeshi Kaneko's lab. We observed overlap of pERK and PDYN immunostaining, but not overlap of immunostaining for pERK and PENK; however, the PDYN and PENK antibodies were not available to complete our study. Since the sample size was small (n=1-2), we had to conduct our experiments with a different antibody against PDYN. The results from our pilot dual-immunofluorescence experiments are shown in Figure 17. We also attempted to further characterize the antibody using Western blot analysis. Although we observed a band at the predicted molecular weight 28 kDA, we

also observed a strong band at a high molecular weight. Several different variables of the western blotting protocol were modified, but the high molecular weight band persisted. This western blot result raises concerns that the PDYN antibody used in the experiments detailed in Chapter 3 is not specific; however, it should be noted that the conditions for antibody binding to denatured tissue in the western blot analysis are different from the antibody binding to fixed tissue. Thus, the high molecular weight band in the western blot result may not necessarily contribute to the immunofluorescence signal in the fixed tissue. In order to demonstrate conclusively the specificity of the antibody, results demonstrating the lack of PDYN immunostaining in NAc tissue from PDYN knock-out mice would be required (Saper, 2005). Nevertheless, our PDYN staining results together with the results from our dual-immunofluorescence pilot experiments using the antibodies from Dr. Kaneko's lab and our DA receptor pharmacology results, strongly suggest that CS-evoked ERK activation occurs in D1 receptor-expressing MSNs.

7.0 ADDITIONAL FIGURES

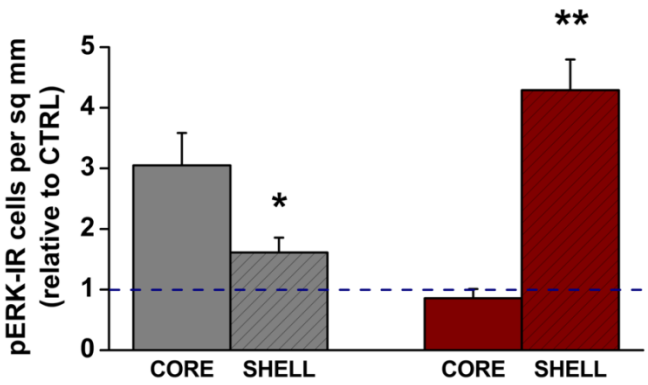

Figure 15: A CS and a US evoke different subregional patterns of ERK signaling in the NAc. Number of pERK-IR cells for the PAV group (grey) and the REWARD group (red), expressed relative to the number of pERK-IR cells for the CTRL group, in the NAc core and shell. * $p < 0.05$ **, $p < 0.01$, within-group core vs shell. Dotted line indicates CTRL level.

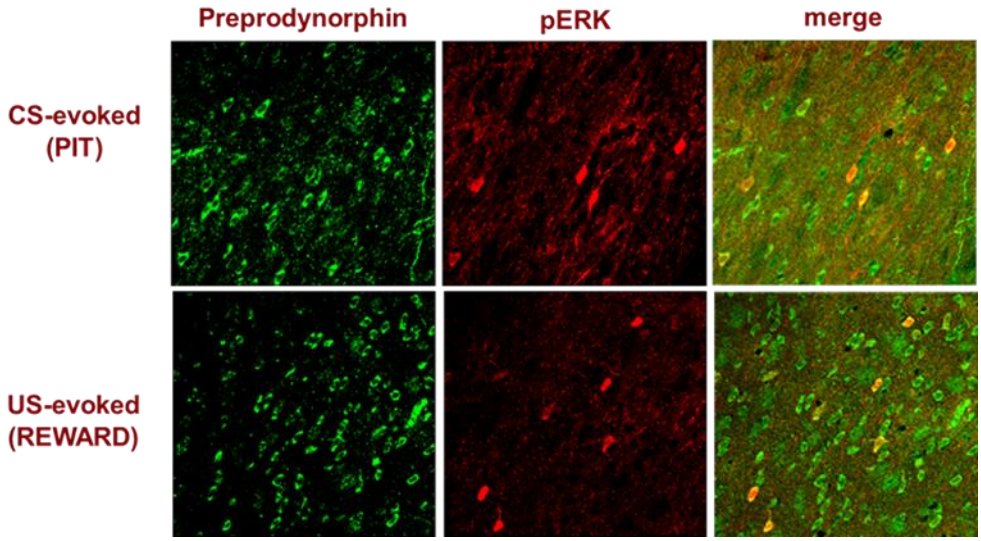

Figure 16: Both the CS and the US increase ERK signaling in NAc shell neurons that are immunoreactive for preprodynorphin, a marker for D1R-expressing MSNs.

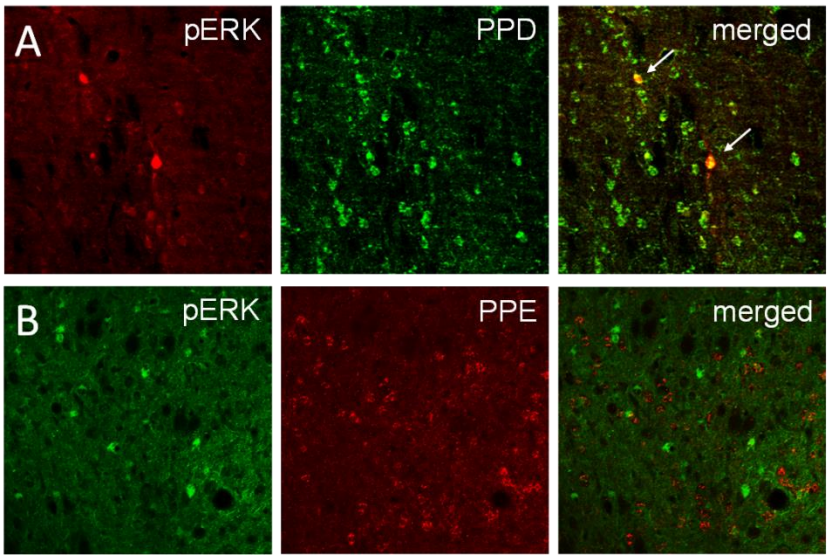

Figure 17: Immunofluorescence labeling for pERK and PPD or PENK from antibodies generously donated by Dr. Kanero's lab used in pilot experiments for the PDYN phenotyping. We observed overlap of perk and PPD in (panel A), In contrast, there was no overlap between pERK and pENK (panel B). In the characterization of these antibodies, the PDD antibody was shown to occur exclusive in D1 receptor containing MSNs, whereas the PENK antibody was shown to occur exclusively in D2 receptor containing MSNs (Sonomura *et al.*, 2007).

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