# PHASIC DOPAMINE RELEASE: ITS ORIGIN AND FUNCTION IN REWARD SEEKING

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**Abstract**

**Manna Beyene: Phasic dopamine release: Its origin and function in reward seeking**

(Under the direction of Dr. R. Mark Wightman and Dr. Regina M. Carelli)

The reinforcing properties of both natural rewards and drugs of abuse are believed to be due to their action on a common neural pathway, the mesolimbic dopamine system. The mesolimbic dopamine system, extending from the ventral tegmental area (VTA) to the nucleus accumbens (NAc) and prefrontal cortex, is thought to play a pivotal role in mediating reward-seeking behaviors. Electrophysiological data suggest that midbrain dopamine neurons are activated on a subsecond timescale in response to primary rewards and their associated cues. This transient activation is believed to produce a rapid rise in extracellular dopamine concentration in the NAc that, we hypothesize, modulates goal-directed behavior. Our laboratory has recently demonstrated that subsecond, or phasic, increases in dopamine release are time-locked to key aspects of reward seeking and may be involved in mediating these behaviors. A central question that remains is if phasic activation of dopaminergic neurons in the VTA gives rise to behaviorally related, transient increases of extracellular dopamine levels in the NAc. Furthermore, the precise role of dopamine transients in reward-seeking behavior remains unclear. The experiments discussed in this thesis were designed to elucidate the origin and role of phasic dopamine release in the NAc during reward seeking. The specific behavior examined was intracranial self stimulation (ICSS), a model of reward seeking in which animals press a lever to deliver an electrical stimulation to select brain regions. The first set of experiments in this work revealed that phasic dopamine release is highly dynamic and correlates with reward-related learning. Secondly, these experiments

demonstrated that phasic dopamine release in the NAc serves as an adaptable measure of anticipated reinforcer magnitude and is integrally involved in mediating reward-seeking behavior. The third and fourth sets of experiments sought to investigate the origin of phasic dopamine release in the NAc. The results of these experiments provide unequivocal confirmation that phasic dopamine release arises from phasic VTA activity. These experiments also shed light on the afferent modulation of phasic dopamine signaling. Altogether, the work presented here provides unique insight into the plasticity and origin of phasic dopaminergic transmission and its relationship with reward seeking.

*To my parents, for instilling in me the value of education and for their unconditional love and support.*

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## List of Abbreviations

| | |
|---|---|
| ACh | acetylcholine |
| AP-5 | (±) 2-amino,5-phosphopentanoic acid |
| BNST | bed nucleus of the stria terminalis |
| [DA] | [Dopamine] |
| DHβE | dihydro-beta-erythroidine |
| FR-1 | fixed ratio 1 |
| FSCV | fast-scan cyclic voltammetry |
| GABA | γ- aminobutyric acid |
| ICS | intracranial stimulus |
| ICSS | intracranial self-stimulation |
| LDTg | laterodorsal tegmental nucleus |
| NAc | nucleus accumbens |
| NAs | nucleus accumbens shell |
| NMDA | *N*-methyl-D-aspartate |
| PPTg | pedunculopontine tegmental nucleus |
| VTA | ventral tegmental area |
| VTO | variable time-out |

# CHAPTER 1

## General Introduction

The mesolimbic dopamine system is believed to play a critical role in mediating reward-related processes (Wise, 2004). Primary rewards and their associated cues have been shown to transiently activate mesolimbic dopamine neurons and increase dopamine release in terminal regions (Di Chiara and Imperato, 1988; Schultz et al., 1997; Hyland et al., 2002; Phillips et al., 2003a; Roitman et al., 2004; Stuber et al., 2005). While it is well accepted that dopamine release is important in reward-seeking behavior, the mechanism by which dopamine functions to influence reward seeking remains unclear (Ikemoto and Panksepp, 1999; Nicola et al., 2005). Recent technological advancements have allowed us to investigate dopamine dynamics on a subsecond timescale, providing unique insight into the action of dopamine in real time. The experiments discussed in this dissertation were designed to elucidate the real-time role of dopamine in reward-seeking behavior. Therefore, this chapter will focus on reviewing the physiology, neurochemistry, and pharmacology of the mesolimbic dopamine system and will provide an overview of the techniques used in this work.

**Anatomy of the mesolimbic dopamine system**

All drugs of abuse, despite their distinct mechanisms of action, converge upon and activate the mesolimbic dopamine system (Nestler, 2005). The mesolimbic dopamine system consists of the dopaminergic projection that extends from the ventral tegmental area (VTA) to several areas of the forebrain (see Figure 1-1), including the nucleus accumbens (NAc) and prefrontal cortex (Haber and Fudge, 1997; Fields et al., 2007; Ikemoto, 2007). Each of these projection sites, in turn, is believed to play a unique role in reward-seeking behavior. The projection from the VTA to the NAc has received the most attention, however, as this projection is the densest dopaminergic projection arising from the VTA (Fields et al., 2007).

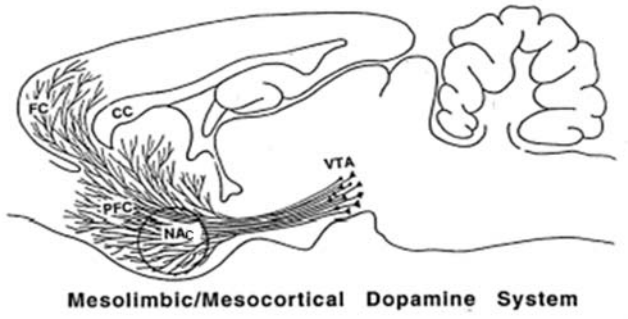


**Figure 1-1. Diagram of the mesolimbic/mesocortical dopamine system.** Dopamine neurons arising from the VTA extend into areas of the forebrain including the NAc, prefrontal cortex (PFC), and frontal cortex (FC). Figure modified from Ikemoto and Panksepp, 1999.

Dopamine neurons are believed to comprise approximately 55% of the neuronal population found in the VTA (Margolis et al., 2006). Dopamine neurons have thin, unmyelinated fibers and therefore, exhibit high thresholds of activation (Yeomans et al., 1988; Nowak and Bullier, 1998). Electrophysiologically, dopamine neurons have

traditionally been characterized by their relatively long action potentials (> 2 ms), and pronounced afterhyperpolarizations (Grace and Onn, 1989; Johnson and North, 1992; Mercuri et al., 1995). Dopamine neurons have also been shown to display inward currents in response to hyperpolarizing pulses and will exhibit hyperpolarization upon dopamine application (Grace and Onn, 1989; Johnson and North, 1992; Mercuri et al., 1995). These four criteria have been widely used in the literature to identify dopamine neurons and to characterize their patterns of activity.

*In vivo*, dopamine neurons in the VTA exhibit two distinct modes of firing (Grace and Bunney, 1984a, b). Dopamine neurons typically fire in a tonic fashion, at frequencies between 2-11 Hz, but will occasionally exhibit phasic activity ($\geq$ 20 Hz), particularly in response to salient or rewarding stimuli (Overton and Clark, 1997; Schultz, 1998). Environmental cues that have been repeatedly paired with reward delivery will also elicit phasic activation of dopamine neurons (Schultz et al., 1997; Hyland et al., 2002). As such, phasic dopamine transmission is thought to play an important role in reward-related processes. In contrast to the diverse patterns of activity observed *in vivo*, dopamine neurons in tissue slices exhibit highly regularized, pacemaker-like activity that is not observed in anesthetized or freely-moving animals (Shepard and Bunney, 1988; Grace and Onn, 1989). This suggests that the irregular, single spike, tonic patterns of activity and the phasic activity seen *in vivo* are regulated by VTA afferents.

The VTA receives both excitatory and inhibitory projections from a variety of brain structures (see (Grillner and Mercuri, 2002) for review). It is thought that excitatory and inhibitory afferents to dopamine neurons act synergistically to modulate tonic and phasic modes of dopamine activation (Grace et al., 2007). Here, a brief overview of the VTA

afferents believed to play a particularly important role in regulating dopamine neuron firing is given.

**Inhibitory VTA Input**

The primary inhibitory neurotransmitter in the brain is γ-aminobutyric acid, or GABA. GABAergic input to the VTA is thought to provide tonic inhibition of dopamine activity (Grace and Bunney, 1985). Thus, relief of this inhibition releases dopamine neurons from their hyperpolarized state and allows them to become spontaneously active (Floresco et al., 2003; Lodge and Grace, 2006b).

Dopamine neurons recorded *in vivo* appear to be tonically inhibited by inhibitory postsynaptic potentials arising from the ventral pallidum (Grace and Bunney, 1985; Floresco et al., 2001a; Floresco et al., 2003). The ventral pallidum is a subcortical brain structure comprised primarily of highly active GABAergic neurons that have been shown to innervate the VTA (Wu et al., 1996). Indeed, inactivation of the ventral pallidum has been found to relieve inhibition of dopaminergic neurons and to induce their spontaneous activity (Floresco et al., 2003). These results suggest that the GABAergic projection from the ventral pallidum to the VTA plays an important role in the activation of dopamine neurons. The NAc, via its efferent projection to the ventral pallidum is also believed to modulate spontaneous dopamine activity (Floresco et al., 2001a). Thus, the ventral pallidum and the NAc are thought to work in concert (see Figure 1-2) to mediate tonic dopamine neuron firing (Floresco et al., 2001a; Floresco et al., 2003).

## Excitatory VTA Input

Phasic activation of dopamine neurons is thought to play an important role in reward-related processes (Schultz et al., 1997; Hyland et al., 2002; Sombers et al., 2009). While tonic activation is believed to be modulated in part by inhibitory input to the VTA, phasic activation of dopamine neurons is believed to be regulated by excitatory afferents (Grace and Bunney, 1984b; Overton and Clark, 1997; Kitai et al., 1999). Both glutamatergic and cholinergic projections to dopamine neurons are excitatory in nature and have been shown to be particularly important for the induction of burst firing (Grace and Bunney, 1984b; Clarke et al., 1985; Grenhoff et al., 1986; Mereu et al., 1987; Gronier and Rasmussen, 1998; Zhang et al., 2005; Omelchenko and Sesack, 2006). An overview of these afferents is given below.

### *Glutamatergic Afferents*

A role for glutamate in the regulation of burst firing was first implicated in 1984 when it was found that iontophoretic application of glutamate onto midbrain dopamine neurons significantly increased burst firing frequency (Grace and Bunney, 1984b). It has since been shown that the ionotropic N-methyl-D-aspartate (NMDA) receptor located on dopamine neurons is critical in regulating this response (Overton and Clark, 1992; Chergui et al., 1993; Overton and Clark, 1997). Intra-VTA application of NMDA significantly increases the incidence of bursting whereas non-NMDA glutamatergic agonists do not (Johnson et al., 1992; Overton and Clark, 1992; Chergui et al., 1993). Similarly, iontophoretic ejection of NMDA receptor antagonists onto dopamine neurons potently regularizes bursting activity while non-NMDA receptor antagonists such as CNQX do not significantly affect phasic patterns of activity (Overton and Clark, 1992; Chergui et al., 1993). Altogether, these results demonstrate that glutamatergic innervation of the VTA is

critically involved in gating burst firing and that the NMDA receptor is critical to enable the switch from tonic to phasic modes of activity.

The VTA receives glutamatergic projections from the prefrontal cortex, superior colliculus, lateral hypothalamus, and the bed nucleus of the stria terminalis (Sesack and Pickel, 1992; Georges and Aston-Jones, 2002; Rosin et al., 2003; Geisler and Zahm, 2005; Fields et al., 2007). The pedunculopontine tegmental nucleus (PPTg) and the laterodorsal tegmental nucleus (LDTg) also provide substantial glutamatergic innervation of the VTA (Semba and Fibiger, 1992; Oakman et al., 1995). While all of these structures provide excitatory input to dopamine neurons, the PPTg is thought to be particularly important in modulating dopamine neuron activity.

The PPTg sends excitatory projections to the VTA that converge monosynaptically onto dopamine neurons (Scarnati et al., 1984; Futami et al., 1995). Via these projections, the PPTg is believed to directly regulate burst firing of dopamine neurons in this brain region. It has been shown that activation of the PPTg significantly increases the frequency of burst firing exhibited in the VTA (Floresco et al., 2003). In contrast, inactivation of the PPTg significantly decreases phasic activity of dopamine neurons, specifically in response to cues that predict reward availability (Pan and Hyland, 2005). Based on its afferent and efferent connections, the PPTg is well positioned to relay sensory information to dopamine neurons that may be important for associative learning. The PPTg is activated at short latencies by auditory, visual, and somatosensory stimuli and is hypothesized to be an important link in reward circuitry that integrates sensory input and in turn, modulates dopamine activity (Grunwerg et al., 1992; Reese et al., 1995; Dormont et al., 1998; Pan and Hyland, 2005).

*Cholinergic Afferents*

Cholinergic afferents to the VTA have also been shown to modulate burst firing of dopamine neurons (Gronier and Rasmussen, 1998; Floresco et al., 2003; Zhang et al., 2005). However, the specific receptor or mechanism by which this modulation occurs is unclear (Kitai et al., 1999). Within the VTA, acetylcholine (ACh) acts on two different receptor subtypes, the ionotropic nicotinic ACh receptor and the metabotropic muscarinic ACh receptor (Clarke and Pert, 1985; Cortes and Palacios, 1986; Schwartz, 1986). It has previously been shown that application of both nicotinic and muscarinic receptor agonists significantly increases the incidence of burst firing in the VTA (Grenhoff et al., 1986; Mereu et al., 1987; Gronier and Rasmussen, 1998; Zhang et al., 2005). These results suggest that both of these receptor types may be involved in regulating dopamine neuron activation.

Cholingeric innervation of midbrain dopamine neurons arises primarily from the PPTg and the LDTg (Woolf and Butcher, 1986; Hallanger and Wainer, 1988; Oakman et al., 1995; Forster and Blaha, 2000). Each of these structures is believed to play a distinct role in the modulation of dopaminergic burst firing. It has been shown that while the LDTg acts as a permissive gate, necessary for the induction of bursting, the PPTg plays a more direct role in generating phasic activation of dopamine neurons (Floresco et al., 2003; Lodge and Grace, 2006a; Grace et al., 2007). As discussed above, activation of the PPTg significantly increases burst firing of dopamine neurons in the VTA (Floresco et al., 2003). Conversely, pharmacological stimulation of the LDTg does not affect bursting activity (Lodge and Grace, 2006a). Despite this, PPTg-induced burst firing fails to occur if the LDTg is inactive (Lodge and Grace, 2006a). Thus, it appears that these two structures act together to mediate phasic dopamine activity (Figure 1-2).

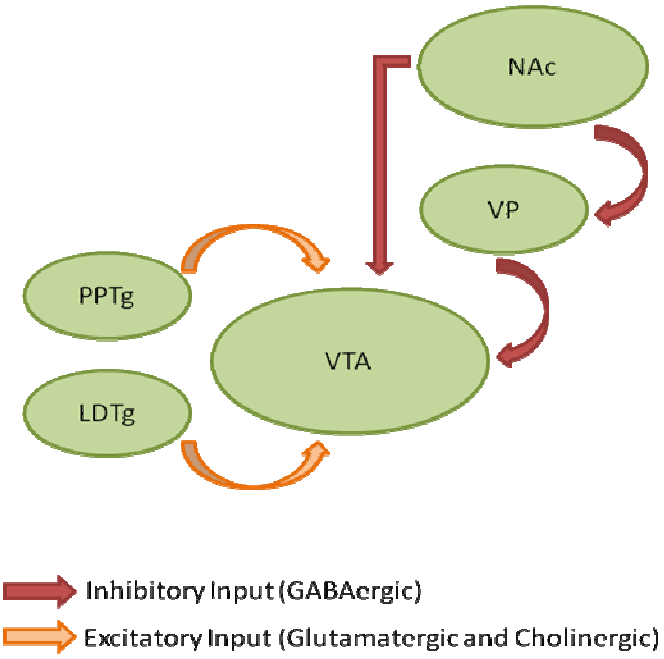


**Figure 1-2. Simplified diagram of VTA afferents that modulate dopamine neuron firing.** Excitatory projections to the VTA, particularly from the PPTg and LDTg, regulate burst firing of dopamine neurons. The NAc, via its direct projection to the VTA inhibits neural firing while its indirect projection to the VP promotes spontaneous, tonic activity in dopamine neurons via disinhibitory mechanisms.

**Dopamine Release**

Tonic and phasic patterns of dopamine cell firing produce two distinct types of dopamine release in terminal regions. Tonic activity gives rise to a low, basal concentration of dopamine that has been estimated to be between 5 – 20 nM (Parsons and Justice, 1992). In contrast, phasic activity has been shown to produce large, transient increases in dopamine release (see Table 1-1 for summary) that can reach concentrations of up to 1 µM (Garris et al., 1997; Aragona et al., 2008; Sombers et al., 2009). Transient dopamine release events

occur naturally in animals at rest and increase in frequency after administration of drugs of abuse (Robinson et al., 2002; Stuber et al., 2005; Wightman et al., 2007). Within the microenvironments of the NAc, dopamine transients exhibit marked temporal and spatial heterogeneity (Wightman et al., 2007).

| | Tonic dopamine firing | Phasic dopamine firing |
|---|---|---|
| Firing frequency | 2 – 11 Hz: Irregular, single-spike firing | > 20 Hz: Rapid, burst firing |
| Consequent release | Low-level basal concentration | Transiently high levels of dopamine |
| Type of signal | Continuous signal | Discrete signal |
| Dopamine concentration | ~ 20 nM | ~ 50 – 1000 nM |

**Table 1-1. Comparison of tonic versus phasic dopamine firing and their associated dopamine signals *in vivo***. (Grace and Bunney, 1984a; Grace and Bunney, 1984b; Parsons and Justice, 1992; Garris et al., 1997; Wightman et al., 2007; Aragona et al., 2008; Sombers et al., 2009)

While dopamine release is dependent on neural activity in the VTA, the amplitude of dopamine released is not always directly proportional to the amplitude of VTA activation. Several other factors can modulate the amount of dopamine released in response to an action potential. For example, dopamine release can exhibit facilitation or depression based on the history of recent dopamine release events (Montague et al., 2004; Kita et al., 2007). In addition, mechanisms acting at the terminal level may also modify dopamine release. Dopamine efflux in the NAc can be altered by glutmatergic inputs from the basolateral

amygdala and by opiate and nicotinic receptors on dopamine terminals (Zhou et al., 2001; Howland et al., 2002; Rice and Cragg, 2004; Britt and McGehee, 2008).

Dopamine acts as a volume neurotransmitter upon release as it readily diffuses out of the synaptic cleft to act on both presynaptic and postsynaptic targets (Garris et al., 1994). The duration and range of dopamine action are determined largely by the dopamine transporter that is responsible for transporting extracellular dopamine back into presynaptic terminals (Wightman et al., 1988; Gainetdinov et al., 1998; Cragg and Rice, 2004; Salahpour et al., 2008). The dopamine transporter is widely distributed throughout dopamine terminal regions and is a target for drugs of abuse such as cocaine and amphetamine (Ciliax et al., 1995; Kuhar et al., 1998; Jones et al., 1999). These drugs of abuse inhibit dopamine transporter function and cause a subsequent increase in extracellular levels of dopamine (Di Chiara and Imperato, 1988). Dopamine that escapes uptake is free to interact with pre- and postsynaptic dopamine receptors, located extrasynaptically (Sesack et al., 1994).

**Dopamine Receptors**

Dopamine receptors are G-protein coupled receptors that are divided into two different categories based on their intercellular function. The first class of dopamine receptors, D1-like receptors, includes D1 and D5 receptors that are positively coupled to $G_{s/olf}$. Activation of these receptors stimulates adenylate cyclase and increases intercellular levels of cyclic adenosine monophosphate, or cAMP (Brown and Makman, 1972; Kebabian et al., 1972). In addition, D1-like receptor activation has been shown to activate mitogen activated protein (MAP) kinases, stimulate phospholipase C, increase L-type $Ca^{2+}$ channel

conductances, and inhibit the $Na^+/K^+$ ATPase, along with $K^+$ and $Na^+$ channel currents (Neve et al., 2004). D2-like receptors, including the D2, D3, and D4 receptors, are $G_{i/o}$ coupled and decrease cAMP production via their inhibitory action on adenylate cyclase (De Camilli et al., 1979; Stoof and Kebabian, 1981). D2-like receptor activation also inhibits NMDA and L-, N-, and P/Q-type $Ca^{2+}$ currents and also stimulates both $K^+$ currents and the $Na^+/H^+$ exchanger, NHE1 (see Neve et al., 2004 for review). Via their distinct intercellular substrates, D1-like and D2- like receptors can modulate gene expression and influence cell excitability.

Both D1-like and D2-like receptors are expressed postsynaptically in the NAc and their activation has been shown to cause differential changes in NAc activity (Goto and Grace, 2005; Cheer et al., 2007). For example, iontophoretic delivery of the D1-like dopamine receptor antagonist, SCH23390, selectively abolishes patterned unit activity in the NAc that occurs during goal-directed behavior but iontophoretic application of a D2-like receptor antagonist does not (Cheer et al., 2007). This functional difference between the receptor types is likely to be the result of their divergent intercellular effects. In addition to being expressed postsynaptically, D2-like receptors, particularly D2 receptors, are also expressed presynaptically on dopamine terminals and function as autoreceptors to inhibit and modulate dopamine release (Kennedy et al., 1992; White, 1996; Schmitz et al., 2003; Aragona et al., 2008). Another distinguishing feature between the D1 and D2 class of receptors is their affinity for dopamine. While D1-like receptors exhibit low affinity for dopamine and require relatively high concentrations to become activated, D2-like receptors exhibit high affinity for dopamine and can be activated by relatively low dopamine concentrations (Richfield et al., 1989). Also, neurons within the NAc are believed to exhibit

distinct expression of D1-like and D2-like receptors with separate neural populations expressing either the D1 or D2 receptor type (Bertran-Gonzalez et al., 2008). In this manner, phasic and tonic dopamine release may differentially affect specific subsets of neurons within the NAc based on their dopamine receptor expression profiles. This, in turn, provides a means whereby tonic and phasic dopamine release may modulate distinct aspects of reward seeking.

### *In vivo* detection of dopamine release

Several techniques have been utilized to monitor neurotransmitter release *in vivo*. The most widely used technique to date is microdialysis, a diffusion-based sampling method that provides excellent chemical resolution and is well-suited to investigate changes in neurotransmitter concentration that occur over the course of minutes to hours (Westerink, 1995). Therefore, microdialysis has been regularly used to measure tonic changes in dopamine concentration. However, microdialysis lacks the temporal resolution to detect phasic changes in dopamine concentration that occur on a subsecond timescale (Lu et al., 1998).

Phasic dopamine release has been successfully monitored *in vivo* with electrochemical techniques (Millar et al., 1985; Lane et al., 1987; Kiyatkin and Stein, 1995; Robinson et al., 2002; Phillips et al., 2003a; Wightman et al., 2007). Electrochemical techniques can be used to measure changes in dopamine and other electroactive compounds as these analytes can be oxidized and reduced in response to voltage application. Of the electrochemical techniques, fast-scan cyclic voltammetry (FSCV) provides the best combination of chemical selectivity and temporal resolution – features that are essential for

the detection of transient changes in dopamine concentration (see (Robinson et al., 2003) for review). FSCV employs a carbon-fiber microelectrode, typically 5-6 μm in diameter, encased in a pulled-glass capillary with approximately 50-100 μm of the fiber protruding from the glass seal. These small dimensions allow for minimal tissue damage (Peters et al., 2004).

To measure subsecond fluctuations in extracellular dopamine content, the carbon-fiber microelectrode is inserted into a brain region of interest and a potential is applied to the electrode (typically every 100 ms) in a triangular fashion, ramping from a negative holding potential to a positive potential and back at a very high scan rate (see Figure 1-3A for diagram). The fast scan rate generates a large, relatively stable, background current that can be attributed to charging the double-layer capacitance of the electrode. The negative holding potential promotes adsorption of dopamine to the electrode surface and upon application of the anodic (positive) and cathodic (negative) scans, any dopamine molecules adsorbed to the surface of the carbon fiber are oxidized and subsequently reduced. The resulting faradaic current produces a small increase in the background current. Because the background current is stable over several seconds, it can be digitally subtracted from the dopamine signal to reveal changes in current due to the oxidation and reduction of dopamine, referred to as faradaic current. The faradaic current can then be plotted against the applied potential to produce a background-subtracted cyclic voltammogram, a plot that can be used to qualitatively identify dopamine and other electroactive compounds (see Figure 1-3B). Cyclic voltammograms for interferents such as pH, ascorbate, and DOPAC are sufficiently distinct from dopamine and other catecholamines and can therefore be readily resolved (Cahill et al., 1996; Heien et al., 2003; Robinson et al., 2003).

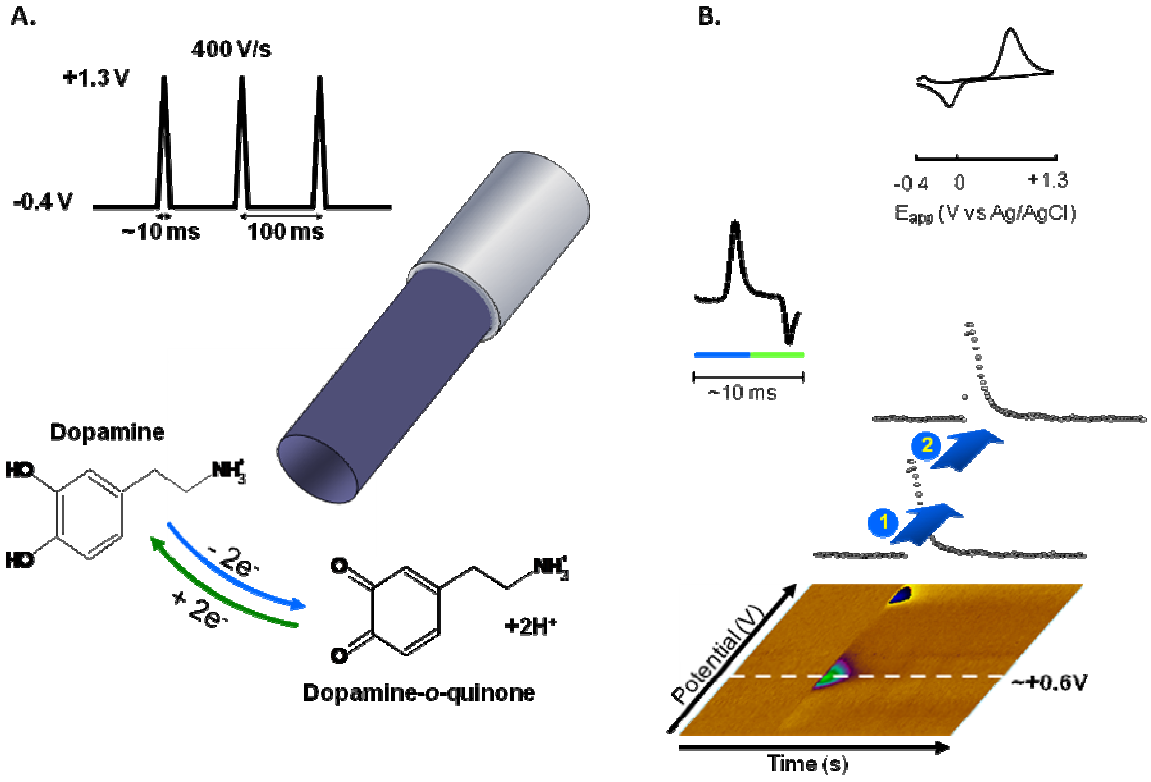


**Figure 1-3. Overview of fast-scan cyclic voltammetry.** A) A triangular waveform (-0.4 V to +1.3 V) is applied to the carbon-fiber microelectrode every 100 ms at 400 V/s. Application of this waveform causes the oxidation of dopamine into dopamine ortho-quinone (at ~0.6 V) and subsequent reduction of dopamine ortho- quinone back into dopamine (at ~-0.2 V). A representation of the faradaic current that results is shown in B. B) Faradaic current can be plotted against applied potential to generate a characteristic background-subtracted cyclic voltammogram (top). The current can also be coded in false color and plotted against time and applied potential to produce a color plot (bottom). A current versus time trace for dopamine can be extracted from the color plot (1, center) and converted into a concentration versus time trace using an in-vitro calibration factor for the electrode (2, center).

Subtraction of the background current characterizes FSCV as a differential technique that can be used to measure changes in current as opposed to absolute current values. Current measurements obtained with FSCV can be converted into concentration and resolved using an *in vitro* calibration factor for the electrode and principal component regression

(Heien et al., 2004; Heien et al., 2005). Thus, FSCV provides a quantitative, chemically selective method to probe rapid dopaminergic transmission in the brain. FSCV is currently the *only* technique with sufficient spatial, temporal, and chemical resolution to monitor phasic dopamine release *in vivo*, particularly within the microenvironments of brain regions such as the NAc (Pennartz et al., 1994; Carelli et al., 2000; Carelli and Wightman, 2004). Recent improvements in the sensitivity of FSCV at carbon-fiber microelectrodes have lowered the detection limit to the nanomolar range making this electrochemical technique well-suited for the investigation of subsecond fluctuations in dopamine concentration in freely-moving animals (Venton et al., 2002; Heien et al., 2003; Cheer et al., 2004). Transient increases in dopamine release have been observed during key aspects of reward-seeking behavior, leading to the hypothesis that phasic dopamine transmission is involved in the mediation of reward-seeking (Venton et al., 2002; Heien et al., 2003; Phillips et al., 2003a; Cheer et al., 2004; Roitman et al., 2004; Stuber et al., 2005). To examine this role more closely, FSCV will be used in this work to monitor phasic dopamine transmission in freely-moving animals performing intracranial self-stimulation.

**Intracranial Self-Stimulation**

Intracranial self-stimulation (ICSS) is a model of reward seeking in which animals are trained to operantly deliver an electrical stimulation to specific brain regions (Olds and Milner, 1954). When delivered to select brain areas, the stimulation acts as a powerful behavioral reinforcer and produces very stable responding. For instance, one of the original ICSS studies found that rats would lever press anywhere from 200 to 5,000 times in a single hour (Olds, 1962). Subsequent research has shown that rats will perform ICSS at the

expense of both food and water and can press for intracranial stimulation up to 35,000 times in one day (Annau et al., 1974; Phillips, 1989). ICSS studies done in humans have shown that humans will perform ICSS to the point of unconsciousness (Bishop et al., 1963). Together, these results demonstrate that ICSS is a potent activator of mechanisms that underlie reward-seeking.

The discovery of ICSS first led to the notion that a reward system existed in the brain (Olds and Milner, 1954; Olds, 1962; Olds and Olds, 1963). Since then, numerous studies have been conducted to try to identify brain structures involved in reinforcement (Carter and Phillips, 1975; Simon et al., 1975; Corbett and Wise, 1980; Olds and Fobes, 1981; Wise, 1981). While it has been found that stimulation of broad range of brain regions will support ICSS, stimulation along the length of the medial forebrain bundle, the VTA, and the pons have been shown to produce the most robust lever pressing behavior with the lowest levels of ambivalence (Olds and Olds, 1963; Simon et al., 1975; Wise, 1996; Waraczynski, 2006). For this reason, stimulating electrodes used in this work were placed in either the medial forebrain bundle or the VTA.

Drugs of abuse have been found to summate with ICSS, causing a potentiating effect on lever pressing behavior (Gallistel and Karras, 1984; Wise et al., 1992; Wise and Munn, 1993). As such, it has been hypothesized that both drugs of abuse and ICSS converge upon similar sites of action in the brain (Wise, 1996). One primary advantage of ICSS is that, unlike administration of drugs of abuse or natural rewards, it bypasses external inputs and directly activates reward circuitry in the brain (Shizgal, 1989). Thus, it is independent of factors such as satiety and in addition, the timing of its delivery and its precise magnitude can

be definitively measured and controlled (Wise, 1996). Therefore, ICSS provides a unique method to investigate neural regulation of reward-seeking behavior.

While several neurotransmitter systems have been implicated in ICSS, dopamine is thought to play a particularly important role in mediating the behavior (Cooper and Breese, 1975; Wise, 2004). In low doses, dopamine antagonists cause a rightward shift in rate-frequency curves, suggesting an increase in reward threshold (Gallistel and Karras, 1984). When administered in high doses, dopamine antagonists produce not only a rightward shift in rate-frequency responses but also a downward shift, implicating an attenuation in the reinforcing properties of the stimulation (Rompre and Wise, 1989). Similarly, administration of the neuroleptic pimozide has been shown to impair ICSS (Fouriezos and Wise, 1976). Although these results seem to indicate a central role for dopamine in ICSS, other findings suggest that additional systems may also be involved. Perhaps the most convincing line of evidence for this notion lies in the fact that 6-hydroxydopamine lesions of the mesolimbic dopamine system do not completely eliminate ICSS (Fibiger et al., 1987). Thus, it appears that additional, non-dopaminergic mechanisms contribute to the maintenance of ICSS.

Our research group has previously shown, using FSCV, that during continuous ICSS behavior, prolonged increases in extracellular dopamine concentration do not occur in the NAc (Garris et al., 1999). In contrast, dopamine levels (typically high during the initiation of the behavior) decline to undetectable levels as the animals acquire the behavior (Garris et al., 1999; Kilpatrick et al., 2000). As such, it has been suggested that dopamine is important for the acquisition of ICSS but not for its maintenance (Garris et al., 1999; Wightman and Robinson, 2002). Indeed, animals that do not exhibit an electrically-evoked dopamine signal prior to training will not perform the behavior, suggesting that dopamine is in fact important

for ICSS learning (Garris et al., 1999). Consistent with this, intra-accumbal injection of a D1-like receptor antagonist has been found to inhibit the initiation of ICSS (Cheer et al., 2007). Taken together, these data implicate a significant role for dopamine in the acquisition of ICSS. Indeed, it is possible that once the behavior is acquired, other neurotransmitter systems contribute to sustain behavioral responses (Shizgal, 1989).

The ability to detect sustained dopamine release throughout an ICSS session seems to depend heavily on the reinforcement schedule used. As mentioned, reinforcement schedules that allow for continuous ICSS performance reduce extracellular dopamine to undetectable (Garris et al., 1999; Kilpatrick et al., 2000; Yavich and Tiihonen, 2000). The decline in dopamine release observed with these reinforcement schedules is attributable to depletion of readily releasable stores of dopamine (Nicolaysen et al., 1988; Montague et al., 2004). Fixed or variable time-out schedules that decrease the rate of stimulation produce relatively stable amounts of electrically-evoked dopamine release that, instead of dissipating completely, decline slightly over time (Yavich and Tiihonen, 2000; Owesson-White et al., 2008). Here, a variable time-out reinforcement schedule that allowed for the detection of stimulated dopamine release was used.

In addition to electrically-stimulated dopamine release, non-stimulated dopamine release events also occur and become time-locked to cues that predict ICSS availability (Cheer et al., 2007; Owesson-White et al., 2008). Similar transient increases in dopamine concentration have been found to occur in response to cues that are associated with both natural and drug rewards (Robinson et al., 2002; Phillips et al., 2003a; Carelli and Wightman, 2004; Roitman et al., 2004; Day et al., 2007). These dopamine transients have been shown to correspond with key aspects of reward seeking and are thought to play an important role in

modulating goal-directed behavior, likely via their action in the NAc (Robinson et al., 2002; Phillips et al., 2003a; Carelli and Wightman, 2004; Stuber et al., 2005).

## Nucleus Accumbens

### *Cellular Composition*

The NAc is believed to play an integral role in mediating goal-directed behavior. The NAc is comprised primarily of GABAergic medium spiny projection neurons that make up >90% of the accumbal cell population (Groves, 1983). Medium spiny neurons have long, thin, and unmyelinated axons and exhibit spontaneous activity both *in vivo* and *in vitro* with basal firing rates between 1 – 3 Hz (Yim and Mogenson, 1982). A unique feature of medium spiny neurons in the NAc is their bistable membrane potential. Typically, medium spiny neurons are in a hyperpolarized, or "down" state at a potential of ~-85 mV but will periodically transition to a depolarized or "up" state (~-60 mV) at which time action potentials can be generated (Wilson and Kawaguchi, 1996; O'Donnell et al., 1999). Transitions from "down" states to "up" states are triggered by afferent input to medium spiny neurons from structures such as the hippocampus (O'Donnell et al., 1999; Nicola et al., 2000).

The remaining 10% of accumbal neurons are interneurons of either the GABAergic or cholinergic type (Kawaguchi, 1995; Kawaguchi et al., 1995). Cholinergic interneurons (< 5%) have short, myelinated axons and exhibit firing rates between 8 – 15 Hz (Aosaki et al., 1994). These cells have been shown to make extensive synapses within the NAc via their wide-ranging dendritic network (Berlanga et al., 2003). GABAergic interneurons in the NAc make up less than 5% of the neurons in the NAc and exhibit very high firing rates of > 20 Hz

(Koos and Tepper, 1999; Berke et al., 2004). Thus, neurons within the NAc can be readily distinguished from one another electrophysiologically.

*Afferent and Efferent Projections*

Afferent projections to the NAc arise from a number of limbic brain areas including the prefrontal cortex, basolateral amygdala, and ventral subiculum of the hippocampus (Groenewegen et al., 1987; Zahm and Brog, 1992; Wright et al., 1996). In addition to these glutamatergic inputs, the NAc also receives dense dopaminergic innervation from the VTA (Zahm and Brog, 1992). The NAc, in turn, sends efferent projections to several brain regions involved in motor function (see Figure 1-4) such as the thalamus, lateral hypothalamus, ventral pallidum and substantia nigra (Ikemoto and Panksepp, 1999; Zahm, 1999). Due to its anatomical configuration, the NAc has been referred to as a limbic-motor interface, capable of integrating limbic information and modulating behavioral output (Mogenson et al., 1980). Indeed, the NAc is well-positioned to influence behavioral response selection, integrating cognitive, contextual, and affective information encoded by limbic afferents to influence motor responses via its efferent projections (Ikemoto and Panksepp, 1999).

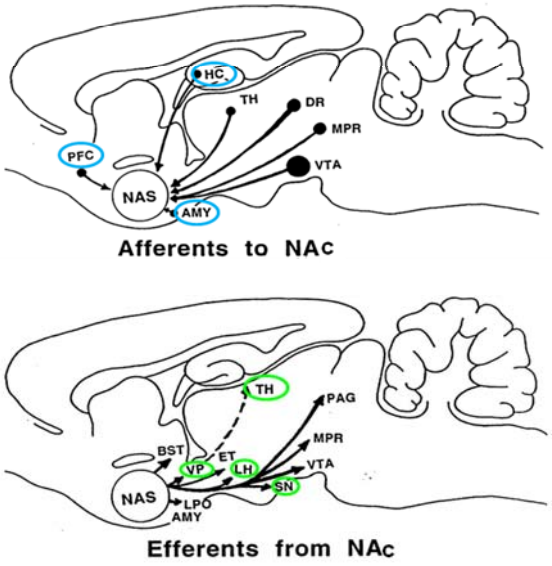


**Figure 1-4. Diagram of NAc afferents (top) and efferents (bottom).** The structures circled in blue represent the limbic inputs to the NAc (HC- hippocampus, PFC- prefrontal cortex, AMY- amygdala). The NAc is believed to integrate information from these limbic areas and in turn, influence behavior via its projections to areas of the brain involved in motor movement, circled in green (VP- ventral pallidum, LH- lateral hypothalamus, SN- substantia nigra, TH- thalamus). Figure modified from Ikemoto and Panksepp, 1999.

It has been shown that medium spiny neurons receive convergent synaptic inputs from afferents (Sesack and Pickel, 1990; Groenewegen et al., 1999). For example, glutamatergic and dopaminergic inputs have been found to synapse onto the same NAc neurons (Totterdell and Smith, 1989; Sesack and Pickel, 1990). Synaptic convergence from the prefrontal cortex and ventral subiculum onto accumbal neurons has also been demonstrated as has convergence of amygdalar and hippocampal inputs (French and Totterdell, 2002, 2003). The convergent nature of these contacts implicates an integrative role of the NAc in limbic circuitry and supports the view that the NAc serves as an interface between limbic and motor structures (Mogenson et al., 1980; Ikemoto and Panksepp, 1999; Pinto and Sesack, 2000).

*Dopamine in the Nucleus Accumbens*

As mentioned, the NAc receives a significant dopaminergic projection from the VTA (Voorn et al., 1986; Wise, 2002). This projection is a central component of the mesolimbic dopamine system and is believed to play a critical role in incentive motivation and reward (Wise, 2002). Research suggests that dopamine may act as a modulator of unit activity in the NAc, mediating input from glutamatergic afferents and ultimately influencing behavior (O'Donnell et al., 1999; Nicola et al., 2000; Goto and Grace, 2005). Anatomical evidence supports this view as dopaminergic nerve terminals are ably positioned to mediate excitatory input to medium spiny neurons (Sesack and Pickel, 1990). In the NAc, dopaminergic synapses can be found in close apposition to glutamatergic synapses, with glutamatergic afferents synapsing onto the heads of dendritic spines and dopaminergic afferents synapsing onto the necks of the same spines (Sesack and Pickel, 1990). This type of configuration allows NAc dopamine to modulate information encoded by limbic and cortical afferents. In fact, several studies have shown that dopamine mediates glutamatergic input to the NAc and influences medium spiny neuron excitability (Mogenson et al., 1988; Rebec, 1998; Floresco et al., 2001b; Floresco et al., 2001a; Goto and Grace, 2005).

Given the neuromodulatory action of dopamine in the NAc, a potential role exists for dopamine as a mediator of reward-seeking behavior. Consistent with this, it has been demonstrated that dopamine release in the NAc is both necessary and sufficient to promote behavioral responses to reward-predictive cues (Nicola et al., 2005). Furthermore, our research group has previously shown that phasic dopamine release in the NAc promotes cocaine-seeking behavior (Phillips et al., 2003a). Thus, investigation of dopamine

transmission in the NAc is important for our understanding of the neural mechanisms that govern reward seeking.

**Goals of this Dissertation:**

To date, the role of dopamine in reward seeking has been extensively studied using microdialysis procedures. However, microdialysis lacks the spatial and temporal resolution to investigate dopamine dynamics in real time (Lu et al., 1998). Electrophysiological data demonstrate that dopamine neurons are activated on a subsecond timescale in response to primary rewards and their associated cues (Schultz et al., 1997; Hyland et al., 2002). Thus, fast-scan cyclic voltammetry is the only detection technique with sufficient spatial, temporal, and chemical resolution to measure dopamine release on a subsecond, or real-time, timescale. Using FSCV, our research group has previously shown that transient dopamine release events become time-locked to cues that predict a variety of rewards (Phillips et al., 2003a; Roitman et al., 2004; Stuber et al., 2005). However, the origin of these cue-evoked transients and their role in reward seeking remains ambiguous. The work described in this dissertation was designed to elucidate the origin and function of phasic, cue-evoked dopamine release in reward-seeking behavior.

**Specific Aims:**

**1. To investigate the correlation between phasic dopamine release and associative learning in ICSS.**

It has been proposed that dopamine plays a role in reward-related learning (Dayan and Balleine, 2002; Wise, 2004). Dopamine neurons have been shown to report reward

prediction errors such that when a reward is better than expected their activation increases and when a reward is worse than predicted a decrease in firing occurs (Schultz et al., 1997). By calculating discrepancies between actual and expected rewarding events, dopamine neurons are thought to produce a prediction error signal that is consistent with reward learning theories (Montague et al., 1996; Schultz et al., 1997). The brief bursts of neural activity that signal prediction error are thought to give rise to transient increases of dopamine in the NAc. Thus, Aim 1 investigated the role of phasic dopamine release in associative learning during ICSS. Using FSCV, changes in phasic dopamine release during learning, maintenance, extinction, and reinstatement of ICSS were measured. The results from this experiment have been published by Owesson-White, Cheer, Beyene, Carelli, and Wightman, *Proceedings of the National Academy of Sciences (105)* 33, 11957-62 (2008).

**2. To assess the relationship between phasic dopamine release and reinforcer magnitude during ICSS.**

Reward-predictive cues have been shown to activate midbrain dopamine neurons and elicit time-locked, transient increases in dopamine release in terminal regions (Schultz et al., 1997; Hyland et al., 2002; Phillips et al., 2003a; Roitman et al., 2004; Day et al., 2007; Owesson-White et al., 2008). Midbrain dopamine neurons have been found to exhibit differential degrees of activation in response to cues that predict different reinforcer magnitudes (Tobler et al., 2005). Thus, cues that predict larger reinforcer magnitudes elicit greater activation of dopamine neurons than cues that predict smaller reinforcer magnitudes (Tobler et al., 2005; Roesch et al., 2007). However, whether or not these adaptive changes in neural activation were represented at the terminal level remained to be determined. Aim 2 assessed if changes in the amplitude of cue-evoked dopamine release in the NAc occurred

upon variation of reinforcer magnitude. This experiment provided unique insight into the dynamic nature of dopamine transmission. This manuscript by Beyene, Carelli, and Wightman is in preparation for submission to the *Journal of Neuroscience* as a Brief Communication.

**3. To determine if phasic activation of VTA neurons contributes to phasic dopamine release in the NAc.**

Midbrain dopamine neurons display high-frequency (> 20 Hz) burst firing in response to salient stimuli, including those associated with primary rewards (Schultz et al., 1997; Hyland et al., 2002). Research suggests that burst firing of dopaminergic neurons in the VTA is mediated *in vivo* by glutamatergic afferents via the N-methyl-D-aspartate (NMDA) receptor (Chergui et al., 1993). It has been hypothesized that brief bursts of VTA activity produce phasic dopamine release in the NAc. To determine the relationship between phasic activation of VTA neurons and phasic dopamine release in the NAc, Aim 3 employed microinjection to administer an NMDA receptor antagonist into the VTA. Subsequent changes in phasic dopamine release were then measured in the NAc during ICSS. This experiment shed unprecedented light on the correlation between phasic dopamine release and dopaminergic cell activity during goal-directed behavior. This aim was published by Sombers, Beyene, Carelli, and Wightman in *The Journal of Neuroscience 29*(6), 1735-42 (2009).

**4. To examine afferent modulation of phasic dopamine release in the NAc.**

Excitatory input to the VTA is thought be critical for the induction of dopaminergic burst firing (Overton and Clark, 1997; Kitai et al., 1999). The VTA receives excitatory projections from both glutamatergic and cholinergic afferents, each of which has been

distinctly implicated in the regulation of bursting (Grace and Bunney, 1984b; Gronier and Rasmussen, 1998; Kitai et al., 1999; Grillner and Mercuri, 2002). Aim 4 was designed to further investigate the role of excitatory afferents by examining cholinergic modulation of phasic dopamine release in the NAc. Cholinergic antagonists were microinjected into the VTA and subsequent changes in NAc dopamine dynamics were measured during ICSS. In a separate group of animals, the effect of pedunculpontine tegmental nucleus (PPTg) inactivation on phasic dopamine release was examined. The PPTg is the primary source of both glutamatergic and cholinergic inputs to the VTA and has been shown to regulate burst firing of dopamine neurons (Oakman et al., 1995; Floresco et al., 2003). Thus, Aim 4 coupled with Aim 3 provided a comprehensive view of afferent modulation of phasic dopamine transmission. A manuscript describing the results of this aim is in preparation for submission to *The European Journal of Neuroscience*.

# CHAPTER 2

## Dynamic changes in accumbens dopamine correlate with learning during intracranial self-stimulation

Published in *PNAS, 105*(33) 11957-11962 (2008)

**Abstract**

Dopamine in the nucleus accumbens (NAc) is an important neurotransmitter for reward-seeking behaviors such as intra-cranial self-stimulation (ICSS), although its precise role remains unclear. Here, dynamic fluctuations in extracellular dopamine were measured during ICSS in the rat NAc shell with fast-scan cyclic voltammetry at carbon-fiber microelectrodes. Rats were trained to press a lever to deliver electrical stimulation to the substantia nigra (SNc)/ventral tegmental area (VTA) following the random onset of a cue that predicted reward availability. Latency to respond following cue onset significantly declined across trials, indicative of learning. Dopamine release was evoked by the stimulation but also developed across trials in a time-locked fashion to the cue. Once established, the cue-evoked dopamine transients continued to grow in amplitude although they were variable from trial to trial. The emergence of cue-evoked dopamine correlated with a decline in electrically-evoked dopamine release. Extinction of ICSS resulted in a significant decline in goal-directed behavior coupled to a significant decrease in cue-evoked phasic dopamine across trials. Subsequent reinstatement of ICSS was correlated with a

return to pre-extinction transient amplitudes in response to the cue and reestablishment of ICSS behavior. The results show the dynamic nature of chemical signaling in the NAc during ICSS and provide new insight into the role of NAc dopamine in reward-related behaviors.

## Introduction

Intracranial self-stimulation (ICSS) was discovered in 1954 (Olds and Milner, 1954). In this paradigm, a rat depresses a lever to deliver an electric shock to electrodes implanted within the brain. Extensive mapping studies by Olds and Olds later showed that the neuroanatomical region supporting ICSS centered in the posterior MFB region of the lateral hypothalamus (Olds and Olds, 1963). This finding provoked considerable interest because it identified a brain reward pathway that could be centrally activated without the need for sensory stimulation (Corbett and Wise, 1980; Wise, 1981). Although a role for several neurotransmitters has been implicated in ICSS, dopamine appears to play a primary role (Cooper and Breese, 1975; Wise, 2004) leading to the view that dopaminergic signaling is essential during goal-directed behaviors. Indeed, it was postulated that increased dopaminergic neurotransmission was necessary for the reinforcement of reward-related behavior (Wise et al., 1978).

More recently, electrophysiological studies in primates have provided new insight into the role of dopaminergic neurons in reward processing (Schultz et al., 1993a). In response to unexpected rewards, dopamine neurons exhibit phasic firing. However, when an animal learns that a cue predicts reward, the burst of neuronal firing switches to the onset of the cue (Ljungberg et al., 1991, 1992; Schultz et al., 1993b; Mirenowicz and Schultz, 1994; Schultz et al., 1997b). Responses to the cue increase with repeated trials, and these paired responses of midbrain dopamine neurons follow the expectations of models of associative learning in which dopamine signaling is a reward-prediction error (Schultz et al., 1997b; Schultz, 1998). Similar responses to conditioned stimuli that predict reward have also been observed for midbrain dopaminergic neurons in rats (Pan et al., 2005).

A phasic increase in dopamine neuronal firing should lead to a dopamine concentration transient in terminal areas such as the nucleus accumbens (NAc). Indeed, using fast-scan cyclic voltammetry at carbon-fiber microelectrodes, we have previously shown that cues that predict cocaine (Phillips et al., 2003a), liquid reward (Roitman et al., 2004), and food reward (Day et al., 2007) evoke a transient increase in NAc dopamine. Dopamine transients also occur in the NAc shell during ICSS in response to conditioned stimuli that predict reward availability and to the intracranial stimulus (ICS) (Cheer et al., 2007). These responses were obtained in animals trained with a fixed time-out between trials. Here, we expand that work and examine whether this cue-evoked dopamine release correlates with behavioral indices of learning when the cues that predict the availability of ICS are presented with a variable time out between trials. Because ICSS is learned quickly in comparison with other reward-based paradigms (Wise, 2002), behavioral correlates of learning can be investigated in a single training session, thus enabling quantification of changes in dopamine release during acquisition of ICSS. Dopamine was monitored with a carbon-fiber microelectrode in the NAc shell while learning was evaluated as the rate of responding following onset of an audio-visual cue. Extracellular dopamine concentration transients, time-locked to cue onset predicting ICS availability, were monitored during regular ICSS (maintenance), extinction, and reinstatement. The results support the concept that rapid dopamine signaling is dynamic and reflects a learned association between cue-related events and ICS.

## Methods

### *Surgical procedures*

Surgery for voltammetric recordings followed previously described procedures (Phillips et al., 2003b). Briefly, a guide cannula (Bioanalytical Systems, West Lafayette, IL) was implanted above the NAc shell (1.7 mm anterior, 0.8 lateral, coordinates relative to bregma), a bipolar stimulating electrode (Plastics One, Roanoke, VA) was lowered to the substantia nigra/ventral tegmental area (VTA, 5.2 mm posterior, 1 mm lateral and 7.8 mm dorsoventral). The bipolar stimulating electrode tips were 1 mm apart. This tip separation allowed for centering in the VTA-region. These coordinates assure activation of the neurons projecting to the NAc shell (Ikemoto, 2007). An Ag/AgCl reference electrode was placed in the contralateral hemisphere (coordinates from Paxinos and Watson, 1986).

### *ICSS*

Rats (n = 9) were trained to criterion on an FR-1 schedule, lever continuously presented. Following this rats were trained to lever press on a variable time-out (VTO) schedule, FR-1 (Figure 2-1a). The VTO-schedule comprised of a maintenance and a maintenance-delay phase. When the animal depressed the lever, a stimulus train (24 biphasic pulses, 60 Hz, 125-150 μA, 2 ms per phase) was delivered to the stimulating electrode on average 150 ms later. In the maintenance phase the lever was presented with an audiovisual cue for 50 trials. In the maintenance-delay phase the audiovisual cue preceded lever-out by 2 s (trials 51-200, Figure 2-2a). Each trial finished after lever depression or if the animal failed to lever press after 15s. The inter-trial interval varied between 5 and 25 seconds.

Next, some animals (n = 8) were tested under extinction conditions. After a rest interval they were given another 30 maintenance-delay trials with the same protocol. The next 30 trials (extinction) were identical except that depression of the lever had no consequence (i.e., no electrical stimulation). Finally, the reinstatement phase followed and consisted of 0-3 operator delivered "priming" stimulations, and another 30 trials identical to those in the maintenance-delay phase.

***Fast-scan cyclic voltammetry***

Carbon-fiber microelectrodes were prepared with T650 fibers (6 μm diameter, Amoco Corporation, Greenville, SC) inserted into a pulled glass pipette (A-M Systems, Carlsborg, WA). The carbon fiber was allowed to extend 50-100 μm beyond the glass tip. The carbon-fiber electrode was held at - 0.4 V versus Ag/AgCl, and every 100 ms a cyclic voltammogram was acquired. The applied potential was ramped to + 1.3 V and back in a triangular fashion at 400 V/s (Heien et al., 2003). Timing, voltage application, and data collection was achieved with an interface board (National Instruments, Austin, Texas) in a Pentium IV computer running custom-designed LABVIEW (National Instruments) software. The interface board also controlled the stimulations.

The background-subtracted voltammograms were plotted with the abscissa as acquisition time of the cyclic voltammogram, the ordinate as the applied potential, and the current in false color (Michael et al., 1998). Dopamine oxidation occurs at approximately + 0.6 V vs. Ag/AgCl. Carbon-fiber electrodes were post-calibrated for dopamine concentration *in vitro* in a flowcell system. Principal component regression was used to extract the dopamine concentration from the voltammetric data (Heien et al., 2004; Heien et al., 2005).

## Results

### *Dopamine release during ICSS*

Rats (n = 9) that reached criterion responding during initial training were examined during ICSS using the VTO paradigm illustrated in Figure 2-1a. In the first VTO phase (maintenance) the lever and cues were presented simultaneously for 50 trials. As seen in the color plot for a representative trial (Figure 2-1b), the cyclic voltammetric data recorded after the lever press show that the stimulation evoked dopamine release. The dopamine concentration increase following the lever press was confirmed with principal component regression (Figure 2-1b, upper trace). Additionally, in the delay following cue-onset/lever extension but before the lever-press, a small dopamine transient was observed (Figure 2-1b, between 0-1 s).

Although not seen on every trial, cue-evoked dopamine release was observed in all animals. The mean dopamine amplitude associated with each subsequent cue/lever extension (trial) during the maintenance phase increased in a linear fashion ($r^2 = 0.047$, $p < 0.0001$, Figure 2-1c). The latency to press the lever following its extension decreased significantly over trials and was fit to a parabolic curve ($r^2 = 0.064$, 95% confidence interval, Figure 2-1d). During the first five trials the average latency to press for all animals was 5.3 s ± 0.9 s, and this latency decreased on the last five trials to 1.4 s ± 0.3 s. The decreased latency with trial number was inversely correlated with the amplitude of cue-evoked dopamine and fit to a parabolic curve with a significant linear correlation between latency to press and dopamine concentration ($r^2 = 0.022$ , 95% confidence interval, Figure 2-1e).

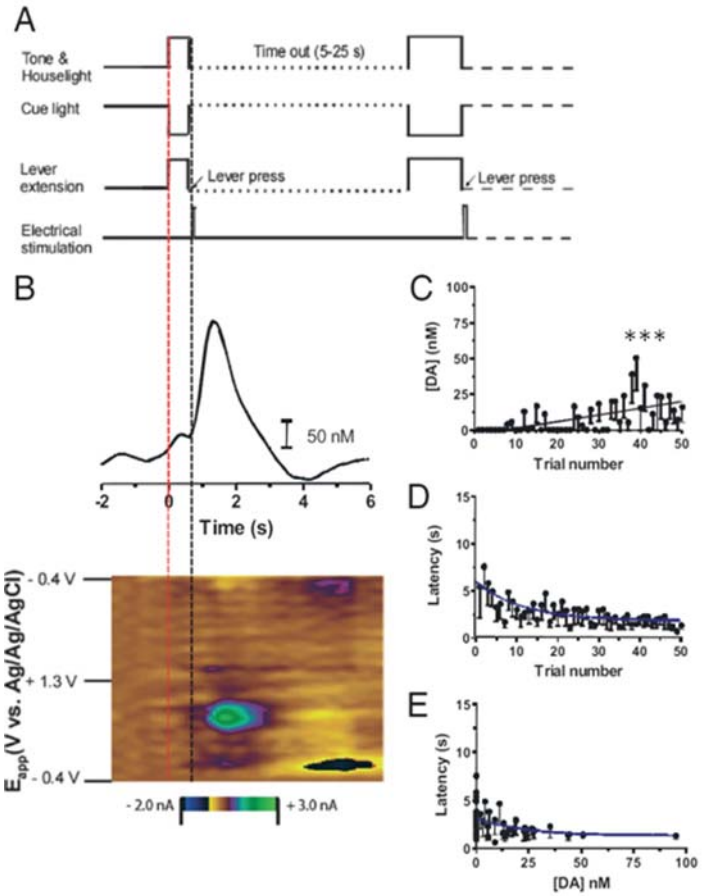


**Figure 2-1. Dopamine and behavioral changes during maintenance phase.** a) Temporal sequence during the first 50 ICSS trials (maintenance phase). b) Voltammetric response from one trial. Dopamine increased immediately after t = 0 s, the time of the cue onset/lever out (red dashed line) and again following the lever press (black dashed line). c) Maximum cue-evoked dopamine concentration increased with each cue (n = 9 animals). d) Latency to press decreased across trials. e) There was a linear correlation between latency and maximum cue-evoked dopamine concentration.

***Dopamine release during the maintenance-delay phase***

Following the 50$^{th}$ trial, the maintenance-delay phase began, with lever extension shifted so that it was delayed 2 s relative to the onset of the cues (Figure 2-2a). With this delay, cue-evoked dopamine became more clearly resolved from electrically evoked dopamine as shown for a single representative animal in Figure 2-2b. The initial increase in dopamine began immediately after 0 s, i.e. at the onset of the compound cue, reached a maximum, and then fell before the lever extension. The lever extended 2 s after cue-onset, and electrically evoked dopamine release was observed after the lever press. In this animal, cue-evoked dopamine was not seen in every trial (Figure 2-2c). However, when evaluated across the maintenance-delay phase for this animal (trials 51-200) the amplitude of cue-evoked dopamine was found to significantly increase in a linear fashion ($r^2 = 0.42$, $P < 0.0001$). This increase occurred even though there was no significant change in latency to lever-press across these trials (Figure 2-2d). In contrast to cue- evoked dopamine, extracellular dopamine following the electrical stimulation decreased significantly over trials 51-200 ($r^2 = 0.13$, $P < 0.0001$, Figure 2-2e).

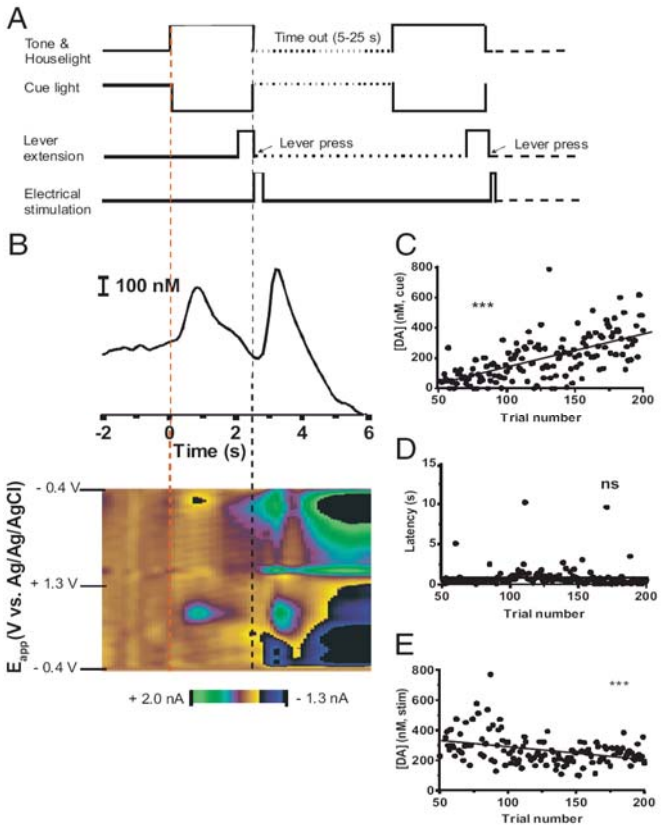


**Figure 2-2. Dopamine and behavioral changes during maintenance-delay phase.** a) Temporal sequence used for trials 51-200 (maintenance delay phase). b) Voltammetric data recorded during a single trial. The dopamine concentration rise begins at t = 0 s with cue onset (red dashed line) and again following the lever press (black dashed line). c) Maximum cue-evoked extracellular dopamine concentration increased with trial number in this animal while d) latency to press remained constant. e) Electrically evoked extracellular dopamine decreased during trials 51-200.

Similar results were obtained in 8 other animals. Figure 2-3a shows the average of all trials in another animals and the increase in dopamine following the cue is clearly observed along with the second increase following the lever-press. The average amplitude of electrically evoked dopamine release during ICSS (n = 7 rats, two ICSS rats were excluded as stimulated dopamine release was not significantly elevated following stimulation) decreased across the maintenance and maintenance-delay phases (Figure 2-3b). Although electrically evoked dopamine was initially high, there was a significant attenuation in

dopamine concentration across trials (Figure 2-3b, in two animals the stimulated release actually increased in the first 3 trials). Superimposed on the experimental data is a line computed with a neurochemical model that predicts dopamine release with repeated stimulation (Montague et al., 2004)(see Discussion).

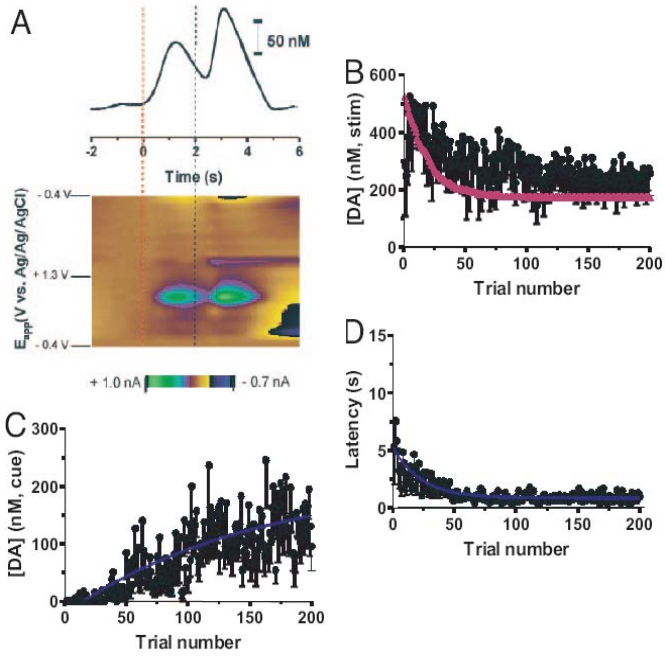


**Figure 2-3. Dopamine and behavioral changes in all trials**. a) Averaged voltammetric data recorded in the NAc shell of a single animal during trials 51-200. Dopamine concentration begins to rise at t = 0 s with cue onset (red dashed line) and following the lever press (black dashed line). b) Points: average maximal electrically evoked dopamine concentration decreases over trials 1-200. During trials 4-50, stimulated dopamine release decreased significantly. During trials 151-200, stimulated dopamine did not decrease further. Solid line: simulation of maximal dopamine release to stimulus trains repeated at 17.5 s intervals, the average between ICS trials (contributions of the cue-evoked dopamine responses were not included). Although the simulation includes terms for short term facilitation and depression, they are ineffective on this time scale. The long term depression employed an amplitude of 0.999 and a time constant of 12 minutes (Montague et al., 2004). c) Average of the maximal cue-evoked dopamine concentration increased with trial number (n = 9 animals). d) Average latency to press did not change during trials 51-200.

The average cue-evoked dopamine concentration increased over trials (1-200) and could be fit to a parabolic curve, leveling off at later trials ($r^2$ = 0.15, 95% confidence interval, Figure 2-3c). In the final trials (196-200), the dopamine concentration reached a plateau of 142 ± 22 nM. Latency to lever press (1-200) was fit to a parabolic curve ($r^2$ = 0.16, Figure 2-3d). Across all animals, the latency to lever-press following lever extension remained constant following the maintenance phase (trials 1-50), with a value of 0.8 s ± 0.2

for trials 51-55 and 0.9 s ± 0.1 s for trials 196-200, values that are not significantly different (Figure 2-3d). There was no significant relationship between latency to press and dopamine (data not shown).

***Dopamine release during extinction and reinstatement of ICSS***

Some of the rats (n = 8) that had completed the maintenance-delay VTO phase were then tested in another paradigm that consisted of 30 VTO ICSS maintenance-delay trials, followed by 30 trials in which the electrical stimulation was not delivered when the lever was pressed (extinction) and finally a reinstatement phase in which the stimulation was restored. The temporal fluctuations of dopamine concentration were extracted by principal component regression and are shown for one representative animal in Figure 2-4a, d and g. As expected, the dopamine signals at cue-onset and during the electrical stimulation were readily apparent during maintenance (Figure 2-4a). During extinction, stimulated release was eliminated and this was accompanied by a profound decrease in the amplitude of cue-evoked dopamine (Figure 2-4d). Both cue and electrically evoked dopamine were restored during reinstatement (Figure 2-4g).

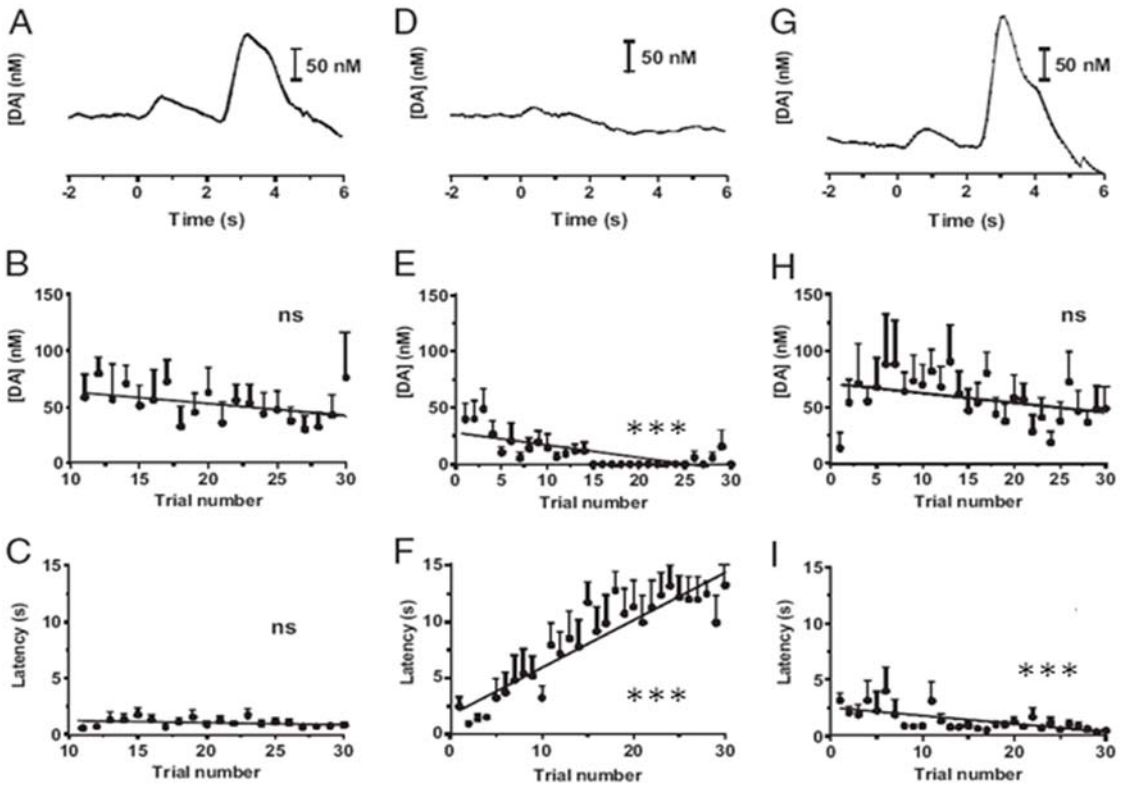


**Figure 2-4. Dopamine and behavioral changes during extinction.** a) d) g) Dopamine concentration during single trial in an animal during maintenance, extinction and reinstatement, t = 0 s is cue-onset. Remaining panels: pooled data from 8 animals. b) During maintenance, maximal amplitude of cue-evoked extracellular dopamine and c) latency to press were constant. e) During extinction, the maximal concentration of cue-evoked dopamine decreased significantly whereas f) latency to press significantly increased h) During reinstatement, the maximal concentration of cue-evoked dopamine rapidly returned to pre-extinction values as did i) the latency to press.

When examined on a trial by trial basis for all animals tested, the maximum cue-evoked dopamine concentration remained fairly constant during the maintenance trials; there was no significant correlation between dopamine concentration and trial number (Figure 2-4b, rats were allowed 30 maintenance trials, however only presses 11-30 are shown because of variability in rest time between phases which increases the variability of both response time and magnitude of cue-evoked dopamine release). During these trials, rats pressed with stable rates that did not change (mean latency following lever extension was 1.1 ± 0.1 s,

Figure 4c). During extinction, cue-evoked dopamine concentrations decreased, and the dopamine concentration plotted against trial number could be fit to a linear decline ($r^2$ = 0.140, $P < 0.0001$; Figure 2-4e). At the same time, latency to lever press rapidly increased ($r^2$ = 0.331 $P < 0.0001$, Figure 2-4f) and there was a significant, inverse, linear correlation between extracellular dopamine and latency to press ($r^2 = 0.273$, $P < 0.0001$, not shown). At the beginning of the reinstatement phase, rats were primed 0-3 times (data not shown) to resume lever pressing. Once ICSS behavior was reestablished, cue-evoked dopamine concentrations rapidly returned to pre-extinction values following trial 1 and then maintained a constant level (trial 1 differs significantly from trials 2-10 ($p < 0.05$, unpaired t-test)). At the same time, latency to press significantly decreased ($r^2 = 0.074$, $P < 0.0001$, Figure 4i).

**Discussion**

A central role for dopamine in reward-based behaviors has long been recognized (Wise, 2002). Our high speed recordings of the chemical dynamics of dopamine during ICSS resolve this into different components. Initially dopamine transients are only seen at stimulus delivery, and these closely resemble the dopamine responses to noncontingent electrical stimulation. With repeated trials, dopamine transients occur at the cues that predict reward availability, and these grow with increasing trials while the stimulus-evoked release diminishes. The development of cue-evoked dopamine correlates with a decline in latency to press the lever with repeated trials, indicative of learning. During the extinction phase, when the electrical stimulus was withheld, the cue-associated dopamine transient amplitude decreased while the latency to press dramatically increased. Upon reinstatement of the electrical stimulus, the cue-associated dopamine transients rapidly reemerged and the latency

to press diminished. The appearance of a dopamine signal associated with a random cue that predicts reward is consistent with the firing patterns of dopaminergic neurons during reward based behaviors that have been shown to follow the theories of reward-prediction error (Schultz et al., 1997a; Pan et al., 2005).

While cue-evoked dopamine transients increased in concentration with trial number, electrically evoked dopamine release gradually decreased, again resembling dopamine neuronal responses seen in reward-prediction experiments in which a switch of dopamine signaling from the reward to the cue occurs (Schultz, 2002). However, unlike natural rewards, the reinforcement in ICSS involves directly depolarizing neuronal networks. While calculations and experiments indicate direct depolarization is less likely with stimuli delivered to the cell bodies (Ranck, 1975; Anderson et al., 1996; Nowak and Bullier, 1998), electrically evoked dopamine release could arise from trans-synaptic activation of glutamatergic or cholinergic afferents in the VTA. Indeed, using much different stimulation parameters and locations, it has been shown that ICSS can be supported by stimulations that activate descending, nondopaminergic fibers and secondarily effect dopamine neurotransmission (Gallistel et al., 1981; Bielajew and Shizgal, 1986; Murray and Shizgal, 1994).

The diminished amount of dopamine release evoked by the stimulation has been reported in other ICSS studies (Garris et al., 1999). Stimulation-evoked dopamine release declines because of a restricted releasable pool of dopamine (Nicolaysen et al., 1988; Yavich and Tiihonen, 2000; Montague et al., 2004). A mathematical model proposed by Montague and coworkers predicts diminished dopamine release over the long term of the 200 ICSS trials that is quite similar to our experimental results (Figure 2-3b). Autoreceptor interactions

can also affect release amplitude of closely spaced dopamine release events (Kita et al., 2007), and thus the cue-evoked release could further modulate the stimulated release.

As the association between the cues that predict ICS availability and reward was established, the amplitude of the cue-evoked dopamine signal increased and it was inversely correlated with the latency to press (Figure 2-1). However, the relationship between dopamine and our measure of learning was not linear since the amplitude of cue-associated dopamine continued to increase during the maintenance-delay phase, eventually reaching a plateau (~150 nM, Figure 2-3). Thus, it appears that a floor effect had been achieved for the behavioral measure. During this portion of the behavioral paradigm, extracellular dopamine release following cue-onset in some cases *exceeded* levels of electrically evoked dopamine release (Figure 2-2c,e). This concentration is sufficient to activate the D1 receptors (Richfield et al., 1989) that have been shown to be important in ICSS (Cheer et al., 2007).

Dopamine neurons are activated by reward-predicting stimuli that cause phasic firing that lasts for approximately 200 ms (Tobler et al., 2005). Consistent with a burst evoking release, the initiation of the dopamine rise in response to the cue is immediate as it is in response to the electrical stimulation. Prior work using amperometry, a technique with higher temporal resolution, shows that it takes approximately 15 ms for dopamine to diffuse out of the synapse and reach the probe (Venton et al., 2003). However, when used with fast-scan cyclic voltammetry, the electrode has a delayed response to reach the peak (~ 0.2 s) as evidenced by the maximal dopamine evoked by the 0.4 s electrical stimulations at the lever press that maximizes at 0.6 s (Venton et al., 2002). Taking these delays into account, the cue-evoked dopamine transients are likely the result of burst firing observed with cues that predict reward in electrophysiological studies (Pan et al., 2005).

The increase in cue-evoked dopamine amplitude with trial number can be observed even in the results from a single animal (Figure 2-2). The variability in dopamine release between consecutive responses is striking, even though the latency to press remains constant. The fluctuations in cue-evoked dopamine release were not due to a lowered electrode sensitivity as the dopamine response to cues increased across trials. Instead, the data reveal the complexity of chemical signaling during behavior in a way never before observed. Unlike conventional chemical probes that provide an average concentration over a relatively large region, the carbon-fiber electrode reports temporal fluctuations from a microscopic local environment immediately adjacent to the electrode (Wightman et al., 2007). While the NAc shell functions as a unit that may influence behavior, the fluctuations in amplitude of dopamine release appear to indicate that the behavior is not specific to a single set of terminals. Thus, terminal release varies from trial to trial much like the firing pattern of dopaminergic neurons in response to reward predictors when examined on a trial-by-trial basis (e.g. middle panel of Figure 12 in (Ljungberg et al., 1992)). Cue-evoked chemical signaling mimics neuronal activity whereby the sum of dopamine transients across trials reflects the chemical message of cue-reward (ICS) associations.

Extinction trials were done in animals that showed stable ICSS and cue-related dopamine release. During the extinction phase, cue-evoked dopamine transients in the NAc shell rapidly diminished while the latency to press increased (Figure 2-4). Upon reinstatement of the association between cues and electrical stimulation, ICSS resumed with a partially restored, cue-evoked dopamine transient apparent at the first press. The latency to lever-press rapidly diminished while the cue-evoked dopamine returned to pre-extinction values on subsequent trials (Figure 2-4). These results are quite similar to the restoration of

cue-associated dopamine transients during reinstatement of cocaine self-administration following its extinction (Stuber et al., 2005). This rapid reacquisition of performance and dopamine signaling provides strong evidence that extinction did not eliminate all original associations between the cue, the response requirement, and the reward (Rescorla, 2001). Thus, rapid dopamine signaling in the NAc follows the expectations of reward-prediction error theory in which cue-evoked dopaminergic signals in the shell reflect "errors" when the brain failed to predict the onset of predictive cues (Schultz et al., 1997a). Consistent with this, the concentration of dopamine released in response to the cue grows during formation of the association between cue-reward and/or cue response requirement to a limiting value (Pan et al., 2005). However, when the cue is no longer associated with the ICSS reward (extinction), the acquired dopamine signal rapidly disappears.

Although dopamine's release during the acquisition of cue-evoked ICSS is revealed by this study, further studies are needed to fully understand the complete neural circuitry underlying this behavior (Wise, 2002). Cue-evoked dopamine signaling may involve activation of ascending GABAergic neurons projecting from the VTA (Steffensen et al., 2001; Lassen et al., 2007) or activation of descending neurons. Indeed, the pedunculopontine tegmentum (PPTg), a site that is a major input to dopaminergic neurons in the VTA show phasic activity to the onset of cues (Pan and Hyland, 2005). During ICSS, extracellular acetylcholine levels increase in both the PPTg and the VTA (Yeomans, 1995; Yeomans and Baptista, 1997; Chen et al., 2006). This could activate phasic firing of dopaminergic neurons leading to the dopamine transients we observe in the NAc shell. The role of cue-evoked dopamine transients may be to potentiate corticostriatal post-synaptic potentials, a function established for dopamine in rats undergoing ICSS (Reynolds et al., 2001). Future studies

will be required to evaluate dopaminergic activity in the NAc core during similar behaviors, as discussed in prior work (Cheer et al., 2007). Indeed, using a similar protocol, we previously reported stimulus evoked dopamine changes in the NAc core, but, over a limited set of trials, these were unaccompanied by cue-evoked dopamine signals (Cheer et al., 2005).

Taken together, the data presented here suggest a complex role of NAc dopamine in ICSS. As reported previously, activation of dopaminergic neurons facilitates the initiation of ICSS-behavior in tasks that do not involve a discrete audiovisual cue or extended periods between trials (Corbett and Wise, 1980; Garris et al., 1999). Our chemical measurements reveal two unrecognized aspects of dopamine signaling in the shell. First, cues that predict ICS contingent upon a response evoke transient dopamine concentrations that are high enough to activate D1 receptors. This D1 activation is highly significant because it has been linked to neural processing related to long term potentiation, a change in synaptic strength linked to learning (Reynolds et al., 2001). Second, like individual dopaminergic cell bodies, dopaminergic terminals at one location do not respond in the same way during all trials as the behavior is learned. This unprecedented finding reveals the stochastic nature of chemical signaling in the brain.

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# CHAPTER 3

## Adaptive fluctuations in cue-evoked dopamine release follow changes in reinforcer magnitude


### Abstract

The mesolimbic dopamine system is believed to be critically involved in modulating reward-seeking behavior and is transiently activated upon presentation of reward-predictive cues. It has previously been shown, using fast-scan cyclic voltammetry in freely moving rats, that cues predicting reward elicit time-locked dopamine transients in the nucleus accumbens. These dopamine transients have been found to correlate with reward-related learning and are believed to promote reward-seeking behavior. Here, we investigate the effect of reinforcer magnitude on cue-evoked dopamine release in the nucleus accumbens shell in rats performing intracranial self-stimulation and find that the amplitude of cue-evoked dopamine is an adaptable measure of anticipated reinforcer magnitude that is significantly correlated with reward seeking. We found that the concentration of cue-associated dopamine transients increased significantly with increasing reinforcer magnitude, while, at the same time, the latency to lever press decreased with reinforcer magnitude. These data support the proposed role of nucleus accumbens dopamine in the facilitation of reward-seeking behavior and provide unique insight into the plasticity of dopaminergic signaling.

## Introduction

The mesolimbic dopamine system is believed to play an essential role in modulating reward-seeking behavior and has been implicated in reinforcement learning (Hollerman and Schultz, 1998; Dayan and Balleine, 2002; Day et al., 2007; Owesson-White et al., 2008). Electrophysiological data have shown that midbrain dopamine neurons are transiently activated by the delivery of unexpected, primary rewards and also by cues that have been learned to predict rewards (Schultz et al., 1997; Hyland et al., 2002). Similarly, data from fast-scan cyclic voltammetric recordings have revealed that cues associated with a variety of rewards, including cocaine, sucrose, and intracranial stimulation, elicit time-locked, dopamine transients in the nucleus accumbens (Phillips et al., 2003a; Roitman et al., 2004; Day et al., 2007; Owesson-White et al., 2008). Indeed, the fluctuations in dopamine concentration in the extracellular fluid of the nucleus accumbens is a direct consequence of increased phasic activity in the ventral tegmental area (VTA) (Sombers et al., 2009). The dopaminergic signal resulting from the activation of dopamine neurons is thought to then guide decision making and modulate reward-seeking behavior (Goto and Grace, 2005; Nicola et al., 2005; Morris et al., 2006).

Fascinatingly, the degree of dopamine activation in response to reward-predictive cues has been found to reflect salient information about predicted rewards, including the probability and timing of these rewards (Fiorillo et al., 2003; Kobayashi and Schultz, 2008). Furthermore, tt has been found that cues that predict larger reinforcer magnitudes elicit greater activation of dopamine neurons than cues that predict smaller reinforcer magnitudes, suggesting that dopamine neurons encode important information related to anticipated

rewards (Tobler et al., 2005; Roesch et al., 2007). However, it remains to be shown whether these adaptive changes in neural activation manifest themselves at the terminal level.

In this work, we use fast-scan cyclic voltammetry at carbon-fiber microelectrodes to examine the effect of reinforcer magnitude on cue-evoked dopamine release in the shell of the nucleus accumbens (NAs), a key dopamine terminal region. This electrochemical technique enables detection of dopamine release with subsecond temporal resolution (Robinson et al., 2003). The behavioral paradigm employed is intracranial self-stimulation (ICSS), in which a rat depresses a lever to electrically stimulate its dopamine neurons (Wise, 2004). We have previously shown that cue-evoked dopamine release in the NAs is associated with ICSS and that the cue-evoked signal is a learned response (Owesson-White et al., 2008). Here we show that the concentration of cue-associated dopamine transients increases significantly with increasing reinforcer magnitude. Furthermore, increases in cue-evoked dopamine were significantly correlated with shorter latencies to lever press, further establishing a role for dopamine in the facilitation of reward-seeking behavior (Nicola et al., 2005). Taken together, our results give an unprecedented view of the plasticity of phasic dopamine release in the NAs, demonstrating that cue-evoked dopamine is an adaptable measure of reinforcer magnitude that plays an important role in reward seeking.

## Methods

### *Animals and Surgery*

Male, Sprague-Dawley rats (n = 6) were individually housed on a 12:12 hour light:dark cycle and allowed *ad libitum* access to food and water. Animals were anesthetized with intra-peritoneal injections of ketamine (100 mg/kg) and xylazine (20 mg/kg). Stereotaxic surgeries were performed using aseptic, flat-skull technique. Bupivicaine was used as a local analgesic. Guide cannulae (Bioanalytical Systems, West Lafayette, IN) were cut to 2.5 mm and implanted above the nucleus accumbens shell (1.7 mm anterior, 0.8 mm lateral, relative to bregma). Bipolar, stainless-steel stimulating electrodes (Plastics One, Roanoke, VA) were ipsilaterally implanted in the lateral hypothalamus (2.8 mm posterior, 1.7 mm lateral relative to bregma, and 8.4 mm ventral from dura) and Ag/AgCl reference electrodes were placed in the contralateral hemisphere. All coordinates used were from (Paxinos, 2007) and surgical procedures are described in (Phillips et al., 2003b). Skull screws and cranioplastic cement were used to secure electrode placements. All animals were given at least 3 days to recover. Animal procedures used in this experiment were all in accordance with guidelines set forth by The University of North Carolina at Chapel Hill Animal Care and Use Committee.

### *Behavioral Procedures*

Animals were placed in an operant chamber and allowed 5 min to acclimate. They were then attached, via a flexible cable, to a commutator secured to the top of the chamber that allowed for both voltammetric recording and electrical stimulation. An additional acclimation period of 5 min was given at this time before the first phase of ICSS training

commenced. During the first phase of training, rats were trained to press a continuously available lever on a fixed-ratio 1 (FR-1) reinforcement schedule for ICSS. Once rats exhibited stable responding (30-50 consecutive lever presses), the session was stopped and the lever was retracted.

Next, reinforcer magnitude was determined for each animal by measuring the number of reinforced presses in a 1 min interval for different stimulation currents. Stimulation currents (60 Hz, 24 biphasic pulses, 2 ms/phase) were varied randomly between sessions and ranged from 16 μA - 200 μA in 0.1 log unit increments. This phase of the experiment was used to determine a low, medium, and high reinforcer magnitude (i.e., stimulation current) for each individual animal (n = 6). A medium reinforcer magnitude was set as the current that produced maximal responding while low and high reinforcer magnitudes were set as the currents that produced half-maximal responding to the left and to the right of the maximal point, respectively.

Rats were then trained to perform ICSS on an FR-1, variable time-out (5 s- 25 s) reinforcement schedule. For the first 50 trials of training, lever extension was accompanied by simultaneous presentation of an audio-visual cue (a 67 dB, 1 kHz tone coupled with a change in the lighting of the experimental chamber). During the first 50 trials, lever extension was accompanied by simultaneous presentation of an audio-visual cue (a 67 dB, 1 kHz tone coupled with a change in the lighting of the experimental chamber). Depression of the lever delivered the stimulus train. For the next 150 trials, the audio-visual cue preceded lever extension by 2 s. When training for the VTO schedule was complete, a fresh carbon-fiber microelectrode was lowered into the NAs for voltammetric recording and an optimal recording site was identified (see below). The animal was then allowed to perform ICSS on

the same VTO schedule with the audiovisual cue preceding lever extension by 2 s. Each rat was given three distinct ICSS sessions comprised of 70 trials that used low, medium, or high stimulation currents. These sessions were randomly ordered (i.e. low-medium-high, low-high-medium, etc) and were separated by 5 minutes. In this manner, each animal served as a within-subject control.

***Voltammetric Recording***

Microelectrodes were prepared by aspirating T650 carbon fibers (6 μm diameter, Amoco) into thin glass capillaries (0.6 mm outer diameter, 0.4 mm inner diameter, A-M Systems). Filled glass capillaries were then pulled in a vertical puller (Narashige, Tokyo, Japan) and the exposed carbon fiber tip was cut to 75 μm- 100 μm. For electrochemical recordings, a triangular waveform (-0.4 V to +1.3 V vs. Ag/AgCl) was applied at 400 V/s to the carbon-fiber microelectrode every 100 ms. Optimal recording sites within the NAs were sought by incrementally lowering the electrode through the NAs and measuring electrically-evoked dopamine release, ensuring placement in an area rich in active dopamine terminals. After use, electrodes were calibrated in an *in vitro* flowcell system to enable conversion of dopamine current measurements into concentration units. Principal component regression was used to resolve recorded dopamine signals from interfering species, namely pH (Heien et al., 2004; Heien et al., 2005). Amplitudes of released dopamine were characterized using MiniAnalysis software (Synaptosoft, version 6.03).

## Results

### *Current plays an influential role in reward-seeking behavior*

As shown in Figure 3-1, stimulation current amplitude was found to play an important role in lever pressing behavior during ICSS. When given free access to a permanently extended lever, lever presses were infrequent at stimulation currents of 40 μA or less. The lever pressing rate increased at 50 μA and reached a plateau at higher stimulation currents. At the highest currents evaluated, 200 μA, lever pressing rate decreased. However, the decrease in lever press rate was accompanied by a strong physical reaction of the rat to the stimulation. Reflexively, the animal was moved away from the lever. Based on these data we selected three stimulation intensities for further evaluation using the VTO paradigm: 67 μA $\pm$ 8.71, 115 μA $\pm$ 11.4, and 186.67 μA $\pm$ 8.43, which were defined as low, medium, and

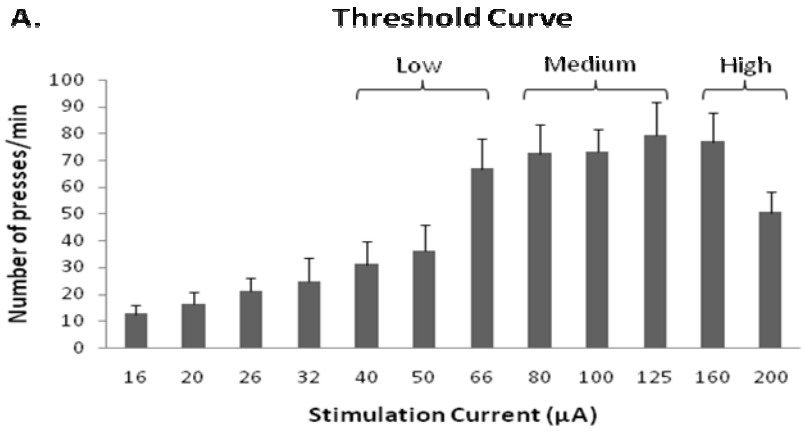


**Figure 3-1. Reinforcer magnitude influences response rates for ICSS.** The threshold curve shows the number of presses in a 1 min interval for a range of stimulation currents. The average currents chosen for the low, medium and high reinforcer magnitudes were 67 μA $\pm$ 8.71, 115 μA $\pm$ 11.4, and 186.67 μA $\pm$ 8.43, respectively. For each animal, medium magnitudes were set at currents that produced maximal responding. Currents that produced half-maximal responding to the left and right of the maximum were chosen as the low and high magnitudes, respectively.

high, respectively.

### *Dynamic changes in dopamine release occur in response to reward-predictive cues during ICSS*

Following threshold evaluation, we evaluated cue-evoked and stimulated dopamine responses during ICS using the low, medium and high currents using the VTO schedule. Fast-scan cyclic voltammetry was used to measure changes in cue-evoked dopamine release while animals performed ICSS for the low, medium, and high reinforcer magnitudes. A representation of the VTO reinforcement schedule is displayed in Figure 3-2A (top). Each trial began with the onset of an audio-visual cue that preceded lever extension by 2 s. Once the lever was pressed, it retracted and the audio-visual cue was terminated. Trials were separated by a VTO of 5 s - 25 s. As shown in the representative color plot from a single trial in a single animal, in well-trained animals cue onset elicited a time-locked increase in dopamine release (Figure 3-2A, center). Electrically stimulated dopamine release was also readily observed in response to the lever press. A corresponding trace of the dopamine concentration with time (resulting from principal component regression of these data) is shown just below the color plot (Figure 3-2A, bottom).

Individual trials within each reinforcer magnitude session were averaged to examine the mean dopaminergic response in each condition for each animal. An average electrochemical signal observed in a high reinforcer magnitude session is shown in a representative color plot in Figure 3-2B. The corresponding concentration trace for dopamine is also displayed. Cue-evoked dopamine release occurred in a tightly time-locked fashion with the initial rise beginning directly after cue onset. Electrically evoked dopamine

release was also observed following the lever press. Thus, consistent with our prior reports, we found that a transient, time-locked increase in dopamine release occurs in the NAs upon presentation of a cue that predicts ICSS availability (Owesson-White et al., 2008; Sombers et al., 2009)

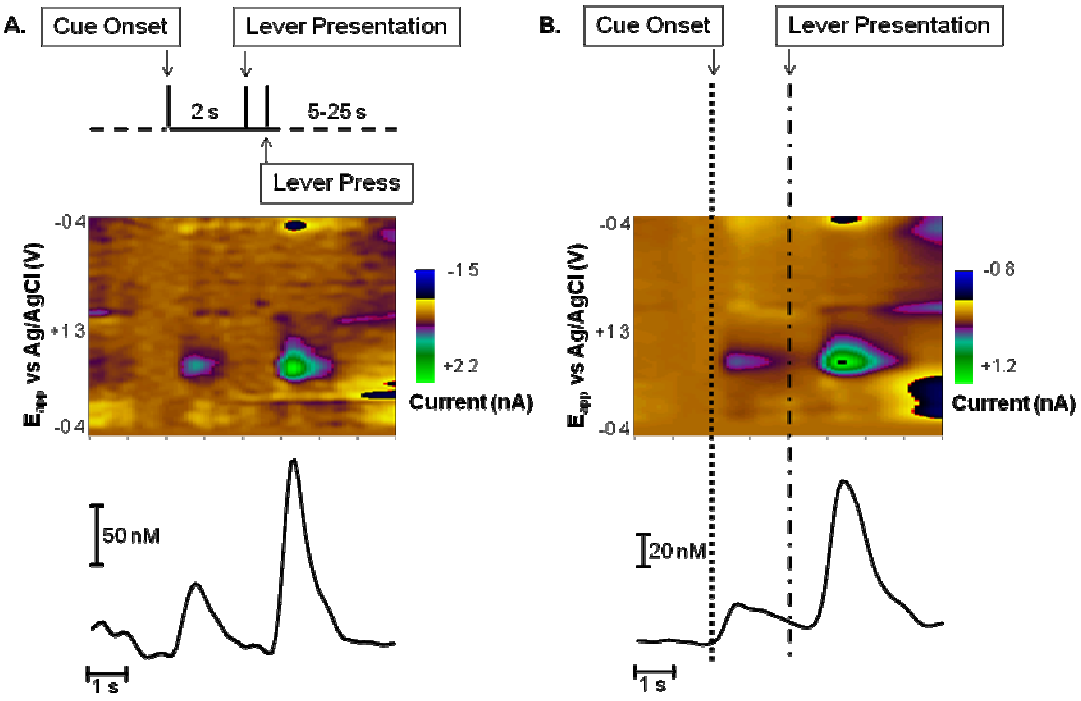


**Figure 3-2. Transient increases in dopamine release follow cue onset during ICSS.** A) *Top,* The behavioral paradigm used to investigate the effect of reinforcer magnitude on dopamine release in the NAs consisted of an audio-visual cue that preceded lever presentation by 2 s. Each trial began with cue onset and ended upon stimulation delivery. Trials were separated by a random interval of 5 s – 25 s (as indicated by the dashed line). *Center*, a color plot representing a single trial in a single animal exhibits the transient dopamine activity observed in response to cue onset and stimulation during a high reinforcer magnitude condition. Changes in dopamine current are color-coded and occur along the oxidation potential for dopamine (~0.6 V vs. Ag/AgCl). *Bottom*, the corresponding concentration versus time trace is shown. B) The color plot and corresponding concentration versus time trace represent the averaged response seen over 70 trials in a single animal during a high reinforcer magnitude session.

***Adaptations in cue-evoked dopamine release closely follow transitions in reinforcer magnitude***

The amplitude of cue-evoked dopamine release in the NAs reflects the magnitude of the anticipated reinforcer (Figure 3-3). Figure 3-3A displays representative average dopamine concentration traces for the low, medium, and high reinforcer magnitude sessions from a single animal. Individual trial maximal concentration values can be found in Figure 3-3B. To avoid the effects of the contrast that occurs around the lever press at the beginning of a new session with a different reinforcer magnitude, we discarded the results from the first 10 trials. Note that the amount of dopamine released, both in response to cue onset and the electrical stimulation, increases significantly with increasing reinforcer magnitude (cue-evoked dopamine: High > Medium > Low, $p < 0.001$, one-way ANOVA $F(2, 59) = 1.19$, stimulated dopamine: High > Medium > Low, $p < 0.001$ one-way ANOVA $F(2, 69) = 1.80$). The average values for cue-evoked and stimulated dopamine release are given in Table 3-1 and are shown in Figure 3-4.

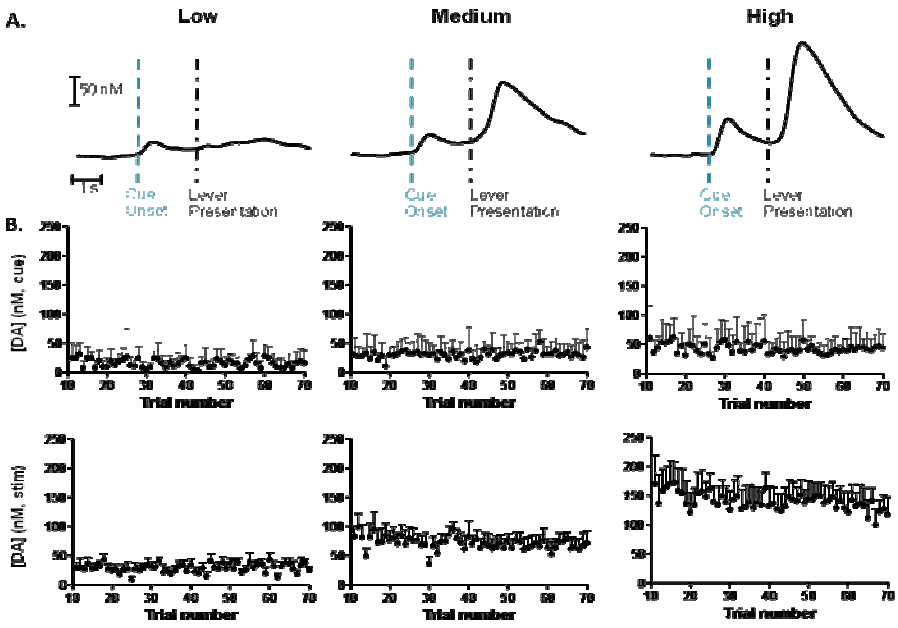


**Figure 3-3. Cue-evoked dopamine release in the NAs varies as a function of reinforcer magnitude.** A) Representative concentration traces are shown for the low, medium, and high reinforcer magnitude conditions. Cue onset is indicated by the first dotted line and lever presentation is indicated by the second dashed line. B) Individual trial concentrations for cue-evoked (top) and stimulated (bottom) dopamine were averaged across animals (n = 6).

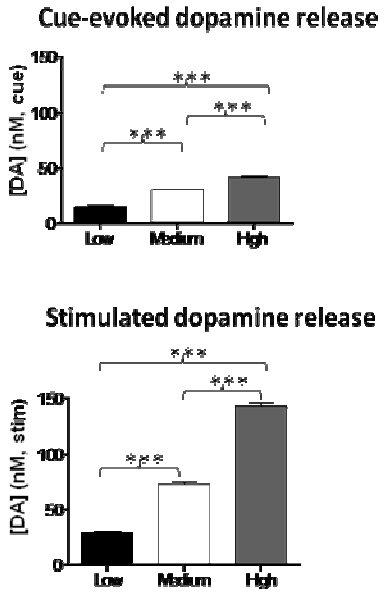


**Figure 3-4. Reinforcer magnitude significantly affects the amplitude of dopamine release in the NAs.** The average amplitude of cue-evoked (top) and stimulated (bottom) dopamine were found to increase significantly with increasing reinforcer magnitude (***$p < 0.001$).

Data from the first 10 trials immediately following changes in reinforcer magnitude were discarded because the amplitude of cue-evoked dopamine fluctuated considerably in this time interval. Figure 3-5 shows data taken from a subset of animals that exhibits the variability of cue-evoked dopamine release observe in the 10 trials immediately following transitions in reinforcer magnitude. The most notable shift in the amplitude of cue-evoked dopamine occurred in animals that experienced a switch from a low reinforcer magnitude to a high reinforcer magnitude (Figure 3-5, bottom right panel). The initial concentration value after this transition differs significantly from the second trial (~9.3 nM vs ~127.3 nM, $p < 0.05$, unpaired *t* test). This significant increase resembles the increase seen in the amplitude of cue-evoked dopamine release following reinstatement of ICSS after extinction (Owesson-White et al., 2008).

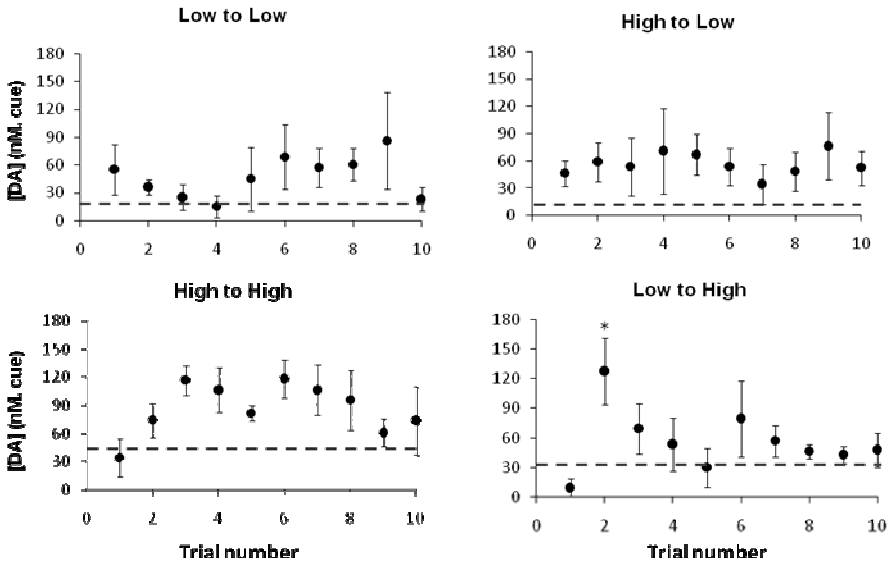


**Figure 3-5. Transient fluctuations in cue-evoked dopamine concentration closely follow changes in reinforcer magnitude.** Individual trial concentrations for the first 10 trials following various switches in reinforcer magnitude session are displayed. The values shown represent data obtained in a subset of animals (n = 3). The rapid nature of the adaptive changes in dopamine release that occur following changes in reinforcer magnitude are particularly apparent in the low to high transition (bottom right panel) where the amplitude of cue-evoked release differed significantly between trial 1 and trial 2 (~9.3 nM vs ~127.3 nM, respectively. *$p < 0.05$, unpaired *t* test). The dashed line in all the graphs represents the average concentration of cue-evoked dopamine ultimately reached in the given magnitude session (a total of 70 trials).

***Cue-evoked dopamine release in the NAs is positively correlated with reward seeking***

In addition to modulating rapid dopamine signaling, reinforcer magnitude also significantly affected the average latency to lever press. The average latency to press decreased significantly with increasing reinforcer magnitude (low > medium, $p < 0.001$, medium > high, $p < 0.05$, one-way ANOVA $F(2, 69) = 1.28$, Table 3-1). Moreover, the latency to lever press was found to significantly correlate with the amplitude of cue-evoked dopamine release in an inverse manner ($r^2 = 0.337$, $p = 0.023$). The latency to lever press was found to significantly correlate with the amplitude of cue-evoked dopamine release in an inverse manner ($r^2 = 0.337$, $p = 0.023$, Figure 3-6). Thus, higher levels of cue-associated dopamine were correlated with shorter latencies to press and vice versa.

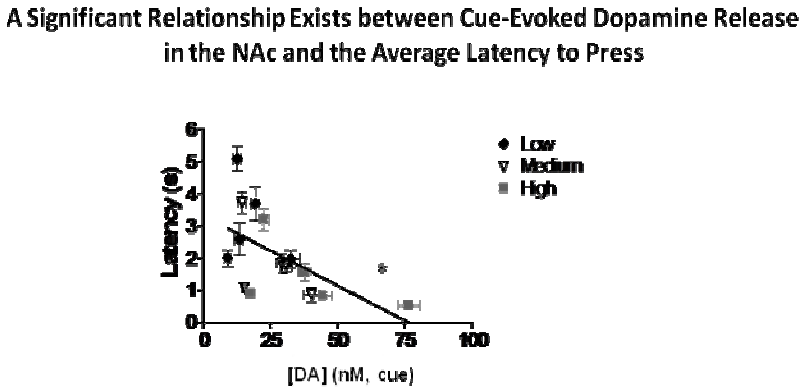


**Figure 3-6. Cue-evoked dopamine in the NAs significantly influences reward-seeking behavior.** Cue-evoked dopamine was significantly correlated with the latency to press in an inverse manner ($r^2 = 0.337$,*$p < 0.05$).

| Reinforcer Magnitude | Cue-evoked Dopamine (nM) | Stimulated Dopamine (nM) | Latency to Lever Press (s) |
|---|---|---|---|
| Low | 15.7 ± 0.90 | 29.4 ± 0.93 | 2.9 ± 0.91 |
| Medium | 30.2 ± 0.83*** | 72.9 ± 1.38*** | 1.8 ± 0.11*** |
| High | 42.1 ± 1.07*** | 143.7 ± 2.24*** | 1.4 ± 0.10* |

**Table 3-1. Reinforcer magnitude significantly affects dopamine release in the NAs and the average latency to lever press.** The amplitude of cue-evoked and electrically-stimulated dopamine release varied as a function of reinforcer magnitude (H>M>L). Increases in reinforcer magnitude also lead to a decrease in the average latency to lever press (H<M<L) (*$p<0.05$, ***$p<0.001$, One-way ANOVA).

## Discussion

Reward-predictive cues activate midbrain dopamine neurons and elicit time-locked, transient increases in dopamine release in terminal regions (Schultz et al., 1997; Hyland et al., 2002; Phillips et al., 2003a; Roitman et al., 2004; Day et al., 2007; Owesson-White et al., 2008). Midbrain dopamine neurons have been found to exhibit differential degrees of activation in response to different reinforcer magnitudes and their associated cues (Tobler et al., 2005). Thus, cues that predict larger reinforcer magnitudes produce more pronounced activation of dopamine neurons than cues that predict smaller reinforcer magnitudes (Tobler et al., 2005; Roesch et al., 2007). Thus, dopamine neurons appear to be conditional output neurons that reflect salient reward-related information in their patterns of activity. Here, we show for the first time that the amplitude of cue-evoked dopamine release in the NAs, a key dopamine terminal region, reflects anticipated reinforcer magnitude. We also found a significant correlation between the amplitude of cue-evoked dopamine release in the NAs

and reward-seeking behavior. Altogether, our results provide unique insight into the adaptable nature of dopaminergic signaling and demonstrate an integral role of cue-evoked dopamine in reward-seeking behavior.

The model of reward seeking used in this work was ICSS. Discovered in 1954, ICSS has been widely used to investigate reward-seeking behavior and to identify brain structures involved in mediating reinforcement (Olds and Milner, 1954; Olds and Olds, 1963; Simon et al., 1975; Corbett and Wise, 1980; Wise, 1996; Waraczynski, 2006). In this behavioral paradigm, animals are trained to perform an operant response, typically a lever press, to deliver an electrical stimulation to select brain areas. An advantage of ICSS is that it directly activates neural circuits involved in reward, bypassing normal physiological inputs (Wise, 1996). Thus, the precise timing and magnitude of reward delivery can be tightly controlled and measured. Several pioneering studies in the field of ICSS have shown that the magnitude of experienced reinforcement during ICSS is a function of both stimulation current and pulse frequency (Gallistel and Leon, 1991; Gallistel et al., 1991; Simmons and Gallistel, 1994; Arvanitogiannis and Shizgal, 2008). It has been hypothesized that the maximum possible reward during ICSS can be attained by manipulating stimulation current (Waraczynski and Kaplan, 1990; Gallistel et al., 1991; Sax and Gallistel, 1991).

Using a free-access reinforcement schedule for ICSS, we found that stimulation current played an influential role in lever pressing behavior. This approach enabled assessment of relative reinforcer value for each animal. As shown in the threshold curve in Figure 3-1, the response rate for ICSS was initially relatively low at the low stimulation currents and peaked at ~125μA before declining slightly. However, the low lever press rate at the highest currents evaluated appeared to be controlled in part by the animals reflexive

response to the stimulation and not to a decrease in the reinforcing properties of the stimulation. Indeed, the significant difference in the latency to lever press between the high (~187 μA) and the medium (~115 μA) reinforcer magnitude conditions, observed with the variable time-out schedule, indicates that the subjective reward value for the high reinforcer magnitude was in fact higher (see Table 3-1).

With the VTO paradigm we have shown that in a well-trained animal, presentation of an audio-visual cue that predicts ICSS produces an increase in dopamine concentration in the NAs. Cue-evoked dopamine release develops with repeated trials during acquisition of ICSS (Owesson-White et al., 2008). This increase in cue-evoked dopamine concentration was correlated with learning of the behavior, indicated by a significant decrease in the latency to lever press in early trials. In addition to growing in with repeated stimulus-reward pairings during the course of learning, cue-evoked dopamine release disappears with extinction of ICSS- suggesting that cue-evoked dopamine release is dependent on a learned stimulus-reward association (Owesson-White et al., 2008). Thus, these results provide strong support for the proposed role of error prediction, as it relates to transient elevated concentrations of dopamine that are time locked to cues, in reward-related learning (Hollerman and Schultz, 1998; Dayan and Balleine, 2002).

Here, we show that cue-evoked dopamine release in the NAs reflects salient information about predicted reinforcer magnitude. It is important to note that the audio-visual cue presented in all three of the reinforcer magnitude conditions was identical. Thus, the *same* audio-visual cue was able to elicit differential amounts of dopamine release based on the magnitude of the reinforcer it predicted. Interestingly, we found a high degree of variability in dopamine release amplitudes immediately following transitions in reinforcer

magnitude, particularly within the first 10 trials of a new reinforcer magnitude session. The etiology of this response, however, is unclear. These results demonstrate that dopaminergic transmission is highly adaptable in the encoding of important information about future rewarding events.

One alternative explanation for the changes in cue-evoked dopamine release we saw in response to reinforcer magnitude may be that the observed fluctuations in cue-evoked dopamine release were strictly a physiological function of the electrical stimulations delivered. For example, in the high reinforcer magnitude condition, where a high stimulation current was delivered, it is possible that the intensity of the stimulation applied left the stimulated neurons in an 'up', or more excitable state. Therefore, in the trial immediately following this stimulation, neurons would have an increased aptitude to fire in response to cue onset, resulting in higher amplitudes of cue-evoked dopamine release. To investigate this possibility, we examined the relationship between cue-evoked and stimulated dopamine release in each reinforcer magnitude condition. If cue-evoked dopamine were solely a physiological function of the electrical stimulation, one would expect a significant correlation to exist between the two types of release. However, this was not the case. While the amplitudes of cue-evoked and stimulated dopamine exhibited similar patterns in response to reinforcer magnitude, the two were not found to be significantly correlated (data not shown). Furthermore, the slopes of the lines through cue-evoked and stimulated dopamine release values for all three magnitudes were significantly different from one another ($p < 0.05$, data not shown). Thus, we believe that the changes in the amplitude of cue-associated dopamine transients seen here reflected changes in incentive reward value.

Our research group has previously shown that cue-evoked dopamine release during ICSS is dependent on phasic activation of dopamine neurons in the VTA (Sombers et al., 2009). Midbrain dopamine neurons have been shown to report reward prediction errors such that when a reward is better than predicted, the dopaminergic response in positive and an increase in firing occurs (Schultz et al., 1997). Conversely, when a reward is worse than predicted (or is omitted altogether) the dopaminergic response is negative and a depression in firing occurs. Consistent with this, it has been found that the degree of phasic activation in midbrain dopamine neurons varied monotonically with reinforcer magnitude (Tobler et al., 2005). Similarly, cues that predicted larger reinforcer magnitudes produced greater activation of dopamine neurons than did cues predicting smaller reinforcer magnitudes (Tobler et al., 2005). Our results suggest that dopamine release in the NAs reflects these changes in neural firing, as cues that predicted higher reinforcer magnitudes consistently produced greater amplitudes of dopamine release than cues that predicted smaller reinforcer magnitudes. Taken together, these results give an unequivocal view that phasic dopaminergic transmission acts as a sensitive measure of reinforcer magnitude.

Phasic activation of dopamine neurons is believed to be modulated by the pedunculpontine tegmental nucleus, or PPTg (Floresco et al., 2003). Therefore, the changes in cue-associated dopamine release observed in this study may, in part, be attributable to changes in PPTg activity. In fact, PPTg neurons have been shown to report reward prediction errors (Kobayashi and Okada, 2007). Therefore, the PPTg may play a role in the computation of reward prediction errors observed in dopamine neurons that are ultimately reflected in dopamine concentration fluctuations in the NAs. As such, Chapter 5 of this dissertation discusses PPTg-mediated changes in cue-evoked dopamine release during ICSS.

In addition to the PPTg, it has recently been shown that neurons in the midbrain reticular formation also exhibit reward prediction error (Puryear and Mizumori, 2008). Together, these two brain structures may provide an excitatory contribution to the reward prediction error signal. Indeed, neural regulation of such a highly complex phenomenon is likely to involve multiple brain areas.

In summary, our results show that cue-evoked dopamine release in the NAs reports information about predicted reinforcer magnitude. Moreover, the data demonstrate an important role of cue-associated dopamine transients in facilitating reward-seeking behavior. Further investigation of the precise mechanism by which dopamine acts to promote reward seeking would increase our understanding of the brain's regulation of reinforcement and would provide profound insight into the mechanisms that underlie addiction.

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# CHAPTER 4

## Synaptic Overflow of Dopamine in the Nucleus Accumbens Arises from Neuronal Activity in the Ventral Tegmental Area

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**Abstract**

Dopamine concentrations fluctuate on a subsecond time scale in the nucleus accumbens (NAc) of awake rats. These transients occur in resting animals, are more frequent following administration of drugs of abuse, and become time–locked to cues predicting reward. Despite their importance in various behaviors, the origin of these signals has not been demonstrated. Here we show that dopamine transients are evoked by neural activity in the ventral tegmental area (VTA), a brain region containing dopaminergic cell bodies. The frequency of naturally occurring dopamine transients in a resting, awake animal was reduced by a local ventral tegmental area (VTA) microinfusion of either lidocaine or (±)2-amino,5-phosphopentanoic acid (AP-5), an *N*-methyl-D-aspartate (NMDA) receptor antagonist that attenuates phasic firing. When dopamine increases were pharmacologically evoked by noncontingent administration of cocaine, intra-VTA infusion of lidocaine or AP-5 significantly diminished this effect. Dopamine transients acquired in response to a cue during intracranial self-stimulation (ICSS) were also attenuated by intra-VTA microinfusion

of AP-5, and this was accompanied by an increase in latency to lever press. The results from these three distinct experiments directly demonstrate, for the first time, how neuronal firing of dopamine neurons originating in the VTA translates into synaptic overflow in a key terminal region, the NAc shell.

## Introduction

Dopaminergic neurons provide a critical modulatory influence in reward seeking (Everitt and Robbins, 2000; Phillips et al., 2003a), prediction error (Schultz et al., 1997) and reinforcement (Wise, 2004). Real-time dopamine neurotransmission in awake animals, monitored with fast-scan cyclic voltammetry at carbon-fiber microelectrodes, has revealed naturally occurring, subsecond dopamine concentration fluctuations (transients) in the NAc (Robinson et al., 2002; Wightman et al., 2007). Under basal conditions these transients occur at highly variable frequencies with amplitudes of ~ 50 nM and durations of ~ 1 s (Wightman et al., 2007). They are enhanced upon administration of drugs of abuse (Stuber et al., 2005), and become time-locked to cues that predict reward availability (Phillips et al., 2003a; Roitman et al., 2004; Day et al., 2007; Owesson-White et al., 2008). Despite their importance, the origin of dopamine transients in the NAc is unclear.

The most likely cause of dopamine transients is phasic firing of dopaminergic neurons in the VTA. These neurons normally fire in a tonic pattern (~5 Hz) and periodically discharge in short bursts (~20 Hz). Bursts are particularly apparent at presentation of primary rewards or their associated cues (Schultz et al., 1997; Hyland et al., 2002). The activity of dopaminergic neurons is regulated by multiple inputs (Floresco et al., 2003; Lodge and Grace, 2006), and in brain slices that lack these inputs, phasic activity is not observed (Overton and Clark, 1997). In the intact brain the transition from tonic to phasic firing is caused by excitatory amino acids in the VTA (Overton and Clark, 1992; Chergui et al., 1993). Consistent with this, microdialysis studies revealed that activation of NMDA receptors in the VTA causes an increase in NAc extracellular dopamine (Karreman et al.,

1996; Kretschmer, 1999). However, a direct assessment of the release consequence of phasic firing in the VTA requires rapid dopamine measurements.

Although VTA cell firing is a likely origin of dopamine transients, other factors may contribute. First, dopamine release is not always directly proportional to the degree of VTA activation, but can exhibit facilitation or depression (Montague et al., 2004; Kita et al., 2007). Moreover, terminal mechanisms may alter release. For example, glutamatergic inputs from the basolateral amygdala to the NAc modulate dopamine efflux (Howland et al., 2002), and nicotinic and opiate receptors on dopamine terminals can locally influence dopamine release probability (Zhou et al., 2001; Rice and Cragg, 2004; Britt and McGehee, 2008). Reverse transport via the dopamine transporter could also generate extracellular dopamine (Falkenburger et al., 2001). Here, we investigate the origin of dopamine transients in the NAc shell, a region that exhibits dopamine transients in animals at rest (Wightman et al., 2007), following pharmacological manipulation (Stuber et al., 2005; Cheer et al., 2007), and in response to cues that predict reward (Phillips et al., 2003a; Roitman et al., 2004; Stuber et al., 2005; Cheer et al., 2007; Owesson-White et al., 2008). Intra-VTA microinfusion of neuronal firing inhibitors establishes that dopamine transients in the NAc shell require ongoing phasic activity in the VTA.

## Methods

### *Electrodes*

Glass-encased, carbon-fiber microelectrodes were constructed as previously described with T-650 carbon fiber (Phillips et al., 2003b). The reference electrodes were chloridized silver wires (0.5 mm diameter, Sigma-Aldrich) in 0.1 N HCl. All potentials reported are vs. Ag/AgCl.

### *Animals and surgery*

Male Sprague-Dawley rats (n = 22; Charles River Laboratories, Wilmington, MA; 250-350 g) some of which were implanted with a jugular vein catheter (n = 11) were individually housed on a 12:12 hour light cycle with *ad libitum* access to food and water. Rats were anesthetized with ketamine hydrochloride (100 mg/kg i.p.) and xylazine hydrochloride (20 mg/kg i.p.) and stereotaxic surgeries were performed as described previously (Phillips et al., 2003b). The Ag/AgCl reference electrode was placed in the forebrain, and a guide cannula (Bioanalytical Systems, West Lafayette, IN) was positioned above the contralateral NAc (1.7 mm AP, 0.8 mm ML, -2.5 mm DV relative to bregma). A combination bipolar stimulating electrode/steel guide cannula (26 gauge; Plastics One, Roanoke, VA) was implanted unilaterally into the VTA at a $6^{o}$ angle toward the midline to avoid the midline sinus (5.4 mm posterior, 1.2 mm lateral, 7.8 mm ventral relative to bregma). The components were permanently affixed with dental cement. The animals recovered for 3 days. All procedures were performed in accordance with the University of North Carolina Animal Care and Use Committee.

***Data acquisition***

The cyclic voltammetric waveform was generated and the resulting signal was collected using LabVIEW (National Instruments, Austin, TX) and a multifunction data acquisition board (PCI-6052E, National Instruments). PCI-6711E and PCI-6601 (National Instruments) boards were used to synchronize waveform acquisition, data collection, and stimulation delivery. Waveform processing and current transduction employed custom-built instrumentation (University of North Carolina, Department of Chemistry Electronics Facility).

***Recording sessions***

A fresh carbon-fiber microelectrode was lowered into the NAc. The electrodes were connected to a head-mounted amplifier attached to a commutator (Crist Instrument Company, Hagerstown, MD). Electrodes were conditioned at 60 Hz for 15 minutes with a triangular waveform (-0.4 V to 1.3 V vs. Ag/AgCl, 400 V/s), followed by 15 minutes of cycling at 10 Hz. The microelectrode position was optimized by monitoring naturally occurring and electrically evoked (biphasic, 2 ms/phase, 24 pulses, 60 Hz, 125 μA) dopamine release. All data included in this work were from subjects whose electrically evoked dopamine release exhibited a signal to noise ratio of at least 30. Stimulated dopamine release was evoked at the end of each session to ensure neuronal viability, and the electrode response was calibrated *in vitro.* A separate set of animals exhibited naturally occurring dopamine transients but minimal or no stimulated release, suggesting a surgical misplacement of the

combination bipolar stimulating electrode/steel guide microinjection cannula in the VTA. These were used as misplacement controls. Drugs (Sigma Aldrich) were administered with a syringe pump (Kent Scientific Corporation, CT, USA, 0.5 μL for 60 s) unilaterally via an infusion cannulae (33 gauge) inserted into the implanted guide.

*Experiment 1:*

Experiments consisted of two minutes of baseline collection, four minutes of recording during and after microinfusion, and an electrical stimulation that evoked dopamine release (n = 6). The first microinfusion into the VTA consisted of saline (0.9%), and the process was repeated one hour later with microinfusion of lidocaine (350 nmol/0.5 μL, dissolved in sterile saline; pH 6). On the next day, a similar experiment was done with these animals to evaluate NMDA receptor effects. Microinfusions of saline into the VTA were followed one hour later by microinfusion of NMDA (0.2 nmol) or AP-5 (5 nmol) (dissolved in 0.5 μL sterile saline), randomly selected. Two hours later the other NMDA active compound was microinfused in the same way. Values were expressed as a ratio of post-infusion to pre-infusion measurements. The onset of behavioral activation served as the initial time for the frequency measurement following NMDA or AP-5 microinfusion.

*Experiment 2:*

After 2 minutes of baseline collection saline was microinfused into the VTA (n = 11). Thirty seconds into the microinfusion the animal received a computer-controlled, 3.0 mg/kg (i.v.) cocaine administration and recording continued for 90 s. The microinfusion needle was then removed. Following a two hour rest period, the experiment was repeated with a second 0.5 μL microinfusion of saline (n = 5) or lidocaine (n = 6, 350 nmol/0.5 μL, dissolved in

saline; pH 6) and an identical systemic cocaine administration. On the next day, a similar experiment was done with the saline control animals to evaluate the effects of NMDA receptors on the cocaine-elicited dopamine release. The first part of the experiment was identical to that done on the previous day. The second part of the experiment (following the two hour rest period) consisted of an intra-VTA microinfusion of AP-5 (5 nmol, dissolved in 0.5 µL sterile saline), paired with the systemic cocaine administration

*Experiment 3:*

Rats (n = 5) were trained to perform ICSS on an FR-1 reinforcement schedule as described previously (Owesson-White et al., 2008). The stimulation current was selected to optimize operant responding (100 µA- 150 µA, 60 Hz, 24 biphasic pulses, 2 ms/phase). Initially, the lever was continuously extended and the rats pressed freely. Once criterion responding was achieved (30 consecutive presses), the lever was retracted. Rats were then trained to perform ICSS on an FR-1, variable time-out reinforcement schedule. Lever extension was accompanied by simultaneous presentation of an audiovisual cue (67 dB (1 kHz) tone coupled with a change in the lighting of the experimental chamber) for the first 50 trials. The audiovisual cue was then set to precede lever extension by 2 s for the next 150 trials (Owesson-White et al., 2008) with a random time-out between trials (5 - 25 s). This paradigm was used for the recording session.

Following training, a carbon-fiber microelectrode was lowered into the NAc and an optimal recording site was found as described above. Animals were allowed to resume operant responding for ICSS. Once a reproducible, cue-evoked increase in dopamine was detected the behavioral session was briefly stopped and saline (0.9%, 0.5µL) was

microinfused into the VTA over 60 s. The needle was removed 60 s after the end of the infusion and the behavioral session resumed for 50 trials. Next, AP-5 (5 nmol, 0.5 μL) was microinfused in an identical manner.

### *Data Analysis*

Background subtraction and digital filtering were done with locally written programs. A non-linear color scale was used to represent the current (Wightman et al., 2007). Substances were resolved with principal component regression using MATLAB (The MathWorks, Natick, MA (Heien et al., 2005)). Dopamine concentration transients were events with a signal-to-noise ratio greater than five, and were characterized with Mini Analysis Software (Synaptosoft, Decatur, GA).

### *Statistical Analysis*

Two-tailed paired student's t tests were used to determine statistical differences in all experiments except, in Experiment 3, where dopamine fluctuations were analyzed using a one-way ANOVA with Tukey's post hoc test for repeated measures. Statistical significance was designated at $p < 0.05$. Statistical analyses were carried out using GraphPad Prism 4 Software Version 4.03 for Windows (Graphpad Software, San Diego, CA).

### *Histology*

Animals were anesthetized with sodium urethane (2g $kg^{-1}$, i.p.). NAc recording locations were marked via an electrical lesion. 0.5 μL of a 2% Chicago Sky Blue solution was microinfused into the VTA. Animals were transcardially perfused with saline followed by 10% formalin. Brains were removed, frozen, coronally sectioned at 40 μm, stained with thionin and visualized under a microscope.

## Results

### *Dopamine Signals in the NAc shell*

Transient changes in dopamine concentration that occur following electrical stimulation of the VTA, and in absence of such a stimulation, can be measured using FSCV (Wightman et al., 2007). Figure 4-1 shows an example of voltammetric recordings obtained in the NAc shell of a resting, awake rat. At this recording site, frequent dopamine transients were observed that resemble dopamine release evoked by a 4 pulse, 20 Hz stimulation of the VTA (Figure 4-1A,B). Principal component analysis with a training set made up of representative, background-subtracted cyclic voltammograms for dopamine and pH was used to extract the data from the color plot shown. The low residual (Figure 4-1C) shows that the retained principal components describe the data well. The current fluctuations due to dopamine release are evident on the positive portion of the voltage scan at the potential where dopamine is oxidized (~0.6 V vs. Ag/AgCl). Here, both naturally occurring (indicated by the inverted white triangle) and electrically evoked (red arrow) dopamine fluctuations are evident in the area surrounding the sensor, as well as a delayed and longer-lasting basic pH shift (white asterisk). The current fluctuation due to the pH shift is evident as a blue patch spanning ~ -0.3 V to +0.3 V. pH shifts are typically seen after electrical stimulation of dopaminergic neurons (Cheer et al., 2006).

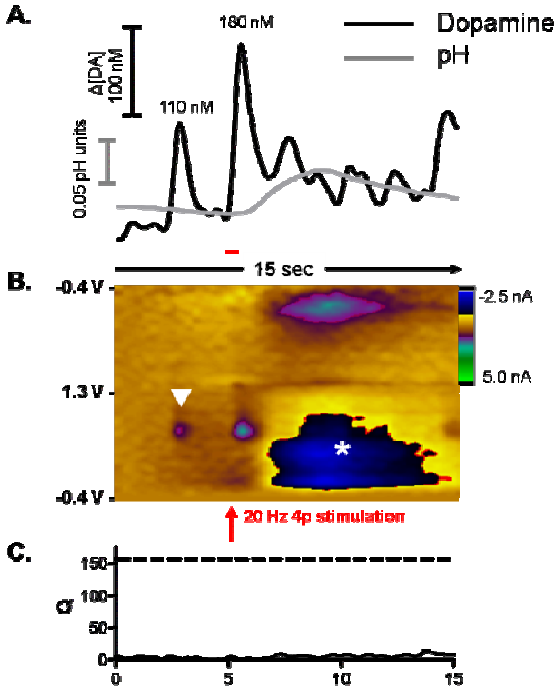


**Figure 4-1. Voltammetric recording from an awake rat showing both naturally occurring (indicated with an inverted white triangle) and electrically evoked (arrow) dopamine release, and a pH shift (white asterisk).** A) Dopamine concentration changes and pH shift extracted from the voltammetric data using principal component analysis. B) The color plot contains 150 background-subtracted cyclic voltammograms recorded over 15 s. The ordinate is the potential applied to the carbon fiber electrode, the abscissa is time, and the current is depicted in falsecolor. 4 p, Four pulse. C) The residual shows the principal components describe the data well. The dashed line is the threshold for noise predicted by the principal components.

***Experiment 1: Dopamine signaling in the NAc shell is dependent on neuronal activity in the VTA***

After the electrode was positioned at a site where transients occurred and dopamine was electrically elicited, we assessed the effects of inactivation of the VTA on these signals. When the sodium channel blocker lidocaine was microinfused into the VTA (350 nmol), both

electrically evoked release ($p < 0.05$) and naturally occurring dopamine transients ($p < 0.001$) were significantly attenuated relative to their values following saline infusions. Lidocaine eliminates dopamine fluctuations as shown in a representative location in Figure 4-2, and the averaged effects are quantified and reported as ratios in Table 4-1.

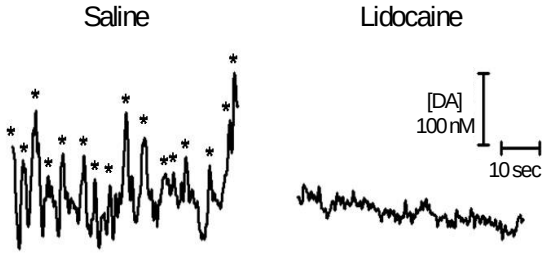


**Figure 4-2. Dopamine transients in the NAc shell of an awake rat are suppressed or enhanced by select agents microinfused into the VTA (experiment 1).** Representative dopamine signals after microinfusion of saline (left) and after microinfusion of lidocaine (350 nmol, right). Dopamine transients (with a signal-to-noise ratio > 5, asterisks) are eliminated after microinfusion of lidocaine. Dopamine concentration changes were extracted from background-subtracted voltammograms.

Intra-VTA lidocaine also reduced the electrically evoked, basic pH shifts in all animals tested (Table 4-1, $p < 0.05$). In the absence of electrical stimulation, pH shifts are less obvious and were not quantitated further. Dopamine and pH signals recovered in 1-2 hours (data not shown). In a separate set of animals ($n = 3$) that did not exhibit electrically stimulated release and were thus used as misplacement controls, the effects of lidocaine microinfusion on naturally occurring dopamine transients were not significantly different from saline control (data not shown).

| Treatment | Stimulated dopamine | Stimulated pH | Relative frequency of spontaneous dopamine |
|---|---|---|---|
| Saline | 1.12 ± 0.28 | 0.92 ± 0.09 | 0.94 ± 0.07 |
| Lidocaine | 0.01 ± 0.01* | 0.15 ± 0.07* | 0.08 ± 0.04*** |

**Table 4-1. Effects of VTA inactivation on spontaneous and stimulated responses in the NAc shell (experiment 1).** Average effects of intra-VTA saline or lidocaine ($n$ = 5 for each microinfusion) on electrically stimulated dopamine release, electrically stimulated basic pH shifts, and relative frequency of naturally occurring dopamine transients (right, averaged over 2 min epochs). Values are expressed as a ratio of postinfusion to preinfusion measurements (*$p < 0.05$, ***$p < 0.001$, Student's $t$ test).

To investigate whether NMDA receptors in the VTA modulate the occurrence of dopamine transients in the NAc, we applied NMDA receptor-specific agents to the VTA. Local administration of NMDA into the VTA induces locomotor activity; thus we used doses shown to be most effective in generating locomotion to ensure an effective dose (Cornish et al., 2001; Ikemoto, 2004). In Figure 4-3A, four transients (indicated by *) occurred during the 60 s interval shown prior to intra-VTA microinfusion. Following treatment with the selective NMDA receptor antagonist AP-5 (5 nmol, right panel) dopamine transients were abolished. The recording shown in Figure 4-3B shows data collected from the same animal 2 hours later. Three dopamine transients are evident prior to intra-VTA microinfusion (left), and 9 dopamine transients are evident immediately following the microinfusion of NMDA (right, 0.2 nmol). Figure 4-3C quantifies these effects for all animals following microinfusion of saline (n = 6), AP-5 (n = 6), or NMDA (n = 5). The frequency of transients

was unchanged by saline infusions. In contrast, AP-5 significantly decreased the frequency of dopamine transients ($p < 0.05$), whereas the trend following NMDA was an increase in transients that did not achieve statistical significance. For both drugs, the neurochemical and behavioral effects were temporally coincident, with the peak effects lasting for 2 min prior to neurochemical rebound (data not shown). In the misplacement control subjects (n = 3), the effects of AP-5 or NMDA microinfusion on naturally occurring dopamine transients or behavior were not significantly different from saline control (data not shown).

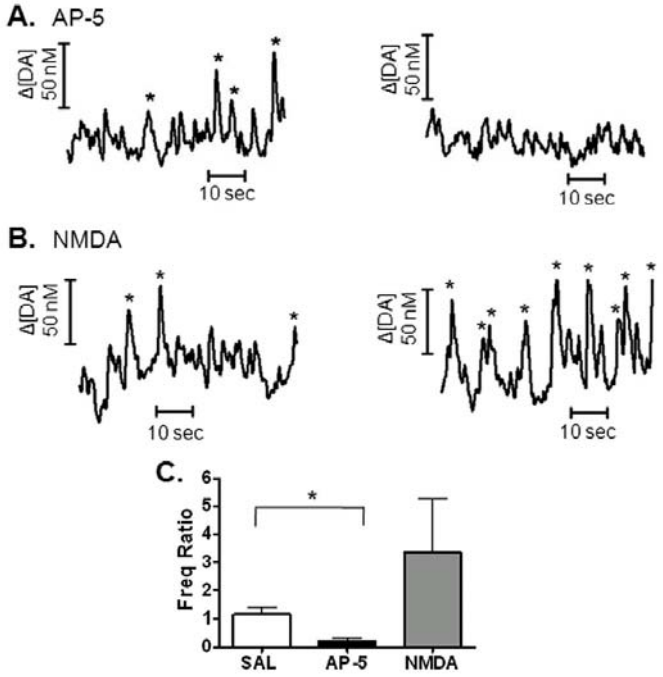


**Figure 4-3. Phasic dopamine signaling is modulated by NMDA receptor-dependent agents in the VTA (experiment 1).** A, B,) Representative voltammetric data collected in the NAc shell of an awake animal at rest before (left) and immediately after (right) an intra-VTA microinfusion. Dopamine fluctuations are indicated by the asterisks, and are attenuated by microinfusion of AP-5 (A) The effects of microinfusion of NMDA are shown in panel B. Dopamine concentration changes were extracted from the voltammetric data. C) Average dopamine transient frequency ratio (measured over 2 min) before and after microinfusions of saline ($n$ = 6), AP-5 (5 nmol, $n$ = 6, *$p < 0.05$), and NMDA (0.2 nmol, $n$ = 5).

***Experiment 2: NMDA receptors in the VTA regulate tonic and phasic dopamine fluctuations elicited by cocaine***

A carbon-fiber electrode was positioned in the NAc shell at a site that exhibited both naturally occurring dopamine transients occurring at >1 per min and electrically evoked dopamine release. A one minute microinfusion of saline vehicle was administered, and thirty seconds later cocaine was administered i.v. at a dose (3 mg/kg) demonstrated previously to have robust effects both on dopamine extracellular concentrations and behavior (Di Chiara and Imperato, 1988; Heien et al., 2005).

Administration of cocaine caused a gradual increase in extracellular dopamine concentration in the NAc that appeared to plateau during the 90-s observation time with superimposed phasic dopamine transients. An example is shown in Figure 4-4A (gray); it resembles that reported for an i.v. cocaine injection without an intra-VTA saline microinfusion (Heien et al., 2005). The increase in frequency and amplitude of dopamine transients is termed the phasic response while the more gradual increase in dopamine concentration is termed the tonic response. One hour later, a second intra-VTA saline microinfusion was administered followed 30 s later by a second cocaine administration (n = 5). Dopamine concentration changes due to this second cocaine challenge (black, Figure 4-4A) are the same as those following the initial i.v. cocaine injection. Figure 4-4B and 4-4C show representative examples using the same protocol but the second VTA microinfusion was of either lidocaine (n = 6, 350 nmol, Figure 4-4B) or AP-5 (n = 5, 5 nmol, Figure 4-4C). In both cases the dopamine responses were attenuated. In a separate set of misplacement

control subjects (n = 2), the effects of AP-5 or lidocaine microinfusion on the cocaine-evoked dopamine response were not significantly different from saline control (data not shown).

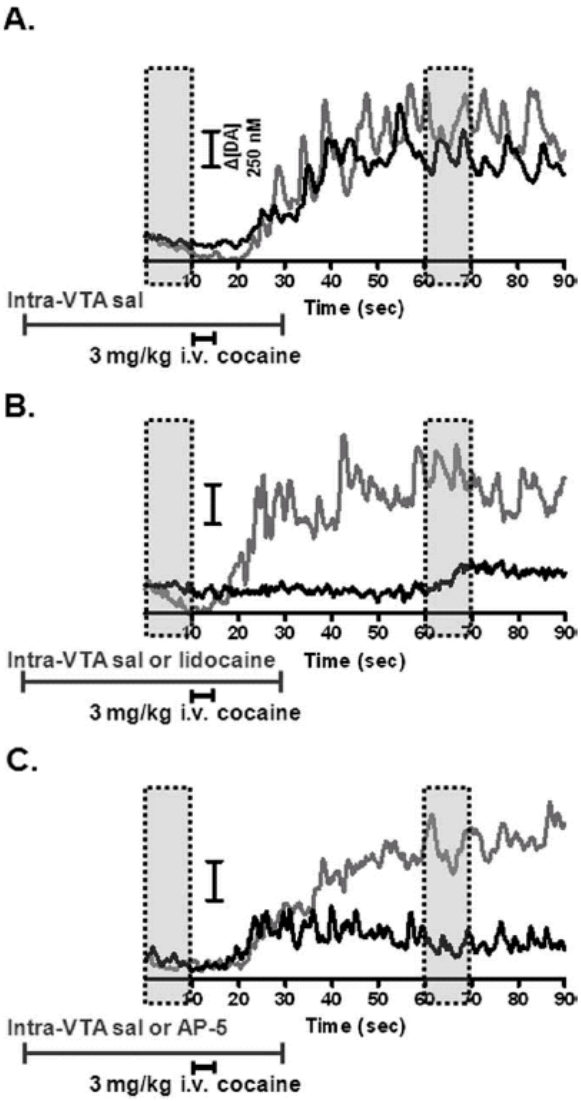


**Figure 4-4. NMDA receptors in the VTA regulate phasic and tonic dopamine in the NAc shell elicited by intravenous cocaine administration (experiment 2).** A–C, Each panel contains two superimposed concentration traces collected in a single animal. The animals first received an intra-VTA saline microinfusion during which cocaine was administered intravenously (gray). A second systemic cocaine administration was given 2 h later while saline ($n$ = 5) (A), lidocaine (350 nmol, $n$ = 5) (B), or AP-5 (5 nmol, $n$ = 6) (C) was infused into the VTA (black). Scale bar is the same in all traces.

The tonic increase in dopamine concentration ($[DA]_T$) was quantified as the average cocaine-induced increase in concentration during a 10 s epoch at the end of the sampling period (50-60 s after cocaine administration) relative to that measured in the 10 s epoch immediately prior to cocaine (epochs boxed in gray, left panels, Figure 4-4). Phasic fluctuations in dopamine concentration were quantitated as the number of dopamine transients measured during the second epoch. The averaged tonic and phasic dopamine responses for all animals (n = 5 saline, n = 5 AP-5, n = 6 lidocaine) are shown in Table 4-2. Both types of cocaine-induced dopamine responses were significantly diminished by intra-VTA microinfusion of lidocaine or AP-5, but not saline.

| Treatment | $[DA]_T$ (nM) | Transient count / 10 s |
|---|---|---|
| Saline 1 | 396 ± 140 | 2.5 ± 0.6 |
| Saline 2 | 262 ± 61 | 2.3 ± 0.9 |
| Saline | 266 ± 73 | 3.6 ± 0.6 |
| Lidocaine | 36 ± 27* | 0.6 ± 0.4** |
| Saline | 371 ± 58 | 2.6 ± 0.5 |
| AP-5 | 74 ± 59* | 1.0 ± 0.4* |

**Table 4-2. NMDA receptors in the VTA regulate phasic and tonic dopamine in the NAc shell elicited by intravenous administration of cocaine (experiment 2).** Intra-VTA microinfusion of AP-5 or lidocaine significantly decreased both the average dopamine concentration increase ($[DA]_T$) and the number of phasic events elicited by cocaine compared with microinfusion of saline (*$p < 0.05$, **$p < 0.01$, Student's *t* test).

***Experiment 3: NMDA receptors in the VTA regulate cue-induced phasic dopamine fluctuations***

We previously reported enhanced rapid dopamine signaling relative to cues during ICSS (Owesson-White et al., 2008). Rats were trained to depress a lever to deliver an electrical stimulation (60 Hz, 24 biphasic pulses, 125 μA each phase) to their VTA. Lever extension was preceded by 2 s with an audio-visual cue. Consistent with our prior reports, presentation of the cue predicting ICSS availability elicits a time-locked, transient increase in extracellular dopamine concentration in the NAc shell that precedes the electrically stimulated dopamine response (Figure 4-5A). Intra-VTA microinfusion of saline did not significantly alter the cue-evoked dopamine release (Figure 4-5A, left) while intra-VTA microinfusion of AP-5 dramatically decreased it (Figure 4-5A, right). Interestingly, intra-VTA application of AP-5 also decreased electrically stimulated dopamine release in the NAc (Figure 4-5A, right panel). Across all animals, ($n = 5$), intra-VTA microinfusion of AP-5 produced a significant attenuation ($p < 0.001$, Figure 4-5B) in the cue-evoked response that exhibited post-infusion recovery and a significant attenuation in stimulated release ($p < 0.001$ data not shown). The average latency to lever press following lever extension was significantly increased after microinfusion of AP-5 relative to saline values ($p < 0.01$) (Table 4-3).

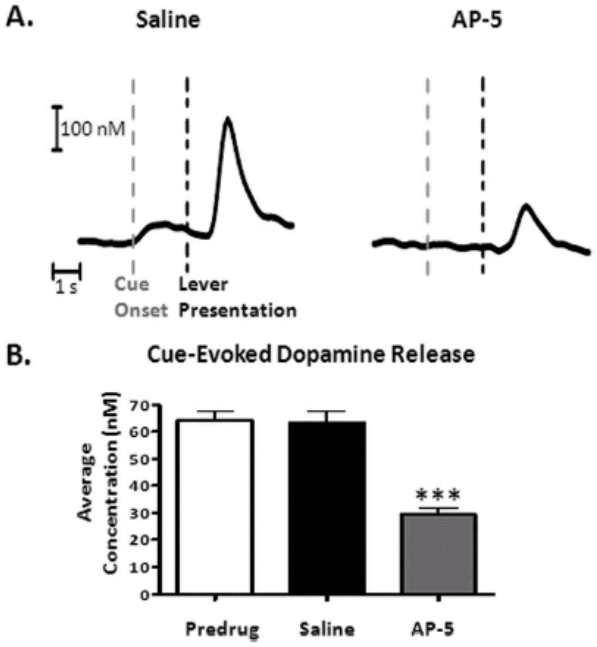


**Figure 4-5. NMDA receptors in the VTA regulate phasic dopamine elicited by reward-predictive cues (experiment 3).** A) Representative dopamine concentration trace after microinfusion of saline (left) is attenuated after AP-5 microinfusion (right). B) Average cue-evoked dopamine concentration changes for 50 trials, $n = 5$ rats. Intra-VTA microinfusion of AP-5, but not saline, significantly decreased the dopamine concentration change elicited by the cue (***$p < 0.001$).

| Treatment | Latency |
|---|---|
| Saline | $0.89 \pm 0.18$ |
| AP-5 | $1.89 \pm 0.38$** |

**Table 4-3. Average latency to press for ICSS.** The average latency to lever press was significantly increased by intra-VTA AP-5 (**$p < 0.01$, Student's $t$ test) but not saline. Values are expressed as a ratio of postinfusion to preinfusion measurements.

***Histology***

Histological examination of electrode placements in select animals revealed that recordings were made in the NAc shell; see Figure 4-6A for details. For visualization of the region of the VTA affected by microinfusion, 0.5 µL of a 2% Chicago Sky Blue solution was microinfused immediately prior to fixation. The location of each cannula tip is shown in Figure 6B. Subjects included in this work (n = 14, black) showed dye covering ~ 70% of the VTA, defined as the paranigral, the parainterfascicular, the parabrachial pigmented nuclei and the rostral VTA (Paxinos and Watson, 2005). In misplacement control subjects that did not exhibit stimulated dopamine release (n = 5, gray), dye covered less than ~10% of the VTA.

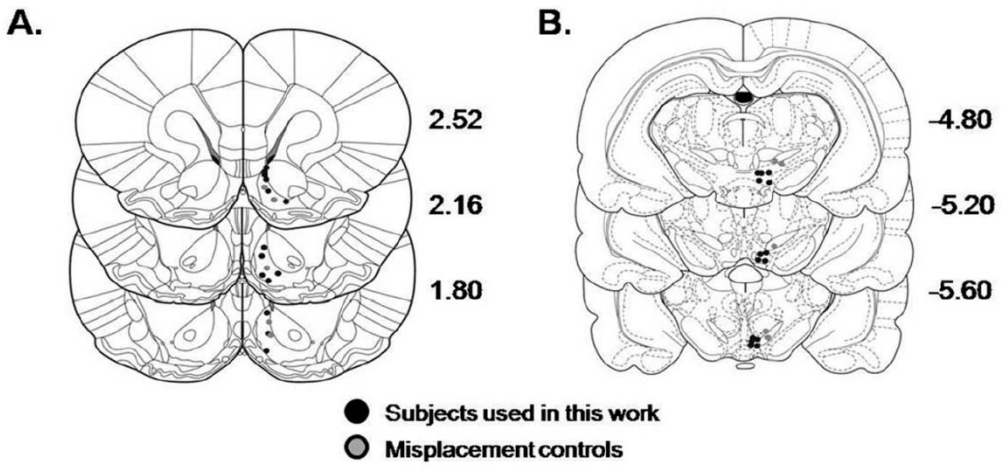


**Figure 4-6. Histological verification of electrode placements.** A) Distribution of carbon fiber microelectrode placements in the NAc shell. Coronal diagrams show electrode tip locations for 14 subjects used in this study (black) and for 5 VTA misplacement control subjects (gray). B) Distribution of combination bipolar stimulating electrode/steel guide cannulae placements in the VTA for the same animals. Numbers to the right indicate the anteroposterior coordinates (± 0.2 mm) relative to bregma. Coordinates and drawings were taken from a stereotaxic atlas (Paxinos and Watson, 1997).

**Discussion**

Using fast-scan cyclic voltammetry at carbon-fiber microelectrodes in freely-moving rats, subsecond, dopamine-concentration fluctuations (transients) can be detected in select locations of the NAc (Wightman et al., 2007). Dopamine transients become more pronounced following administration of drugs of abuse (Cheer et al., 2007), and they become time locked to cues that predict reward availability (Phillips et al., 2003a; Roitman et al., 2004; Stuber et al., 2005; Day et al., 2007; Owesson-White et al., 2008). Here, we establish that dopamine transients observed during these three distinct conditions depend on neuronal activity in the VTA. Although factors such as neuronal firing history and presynaptic mechanisms may modulate synaptic dopamine overflow, this work confirms that NMDA receptor-mediated firing of dopamine neurons in the VTA is a major factor underlying rapid dopamine neurotransmission in the NAc shell.

We previously hypothesized that dopamine transients arise from phasic firing of dopaminergic neurons (Wightman et al., 2007). Modeling of dopamine terminal activity during phasic firing predicts increased extracellular dopamine (Arbuthnott and Wickens, 2007) due to a decreased time between action pulses that allows less time for uptake (Venton et al., 2003), consistent with the results of Figure 4-1. Such an accumulation of dopamine enables activation of both low affinity as well as high affinity dopamine receptors (Richfield et al., 1989), and suggests functionally distinct roles arising from tonic and phasic firing modes. Further support that dopamine transients arise from phasic firing comes from the similarity between the rate of transients observed and the rate of action potential bursts exhibited by the majority of dopaminergic neurons in the VTA of ambulant rats (Hyland et

al., 2002). Consistent with this evidence, naturally occurring dopamine transients, as well as electrically stimulated release, were abolished by nonspecific inactivation of the VTA via lidocaine microinfusion in Experiment 4-1 (Figure 4-2, Table 4-1). Furthermore, the pH changes that are evoked by electrical stimulation, and are a consequence of changes in local blood flow and metabolism (Cheer et al., 2006), were also abolished by microinfusions of lidocaine (Figure 4-2, Table 4-1). pH changes occur independently of dopamine release (Cheer et al., 2006); however, the present data suggests that they also originate from presynaptic activity of VTA neurons that project to the NAc.

As reviewed by Fields et al. (Fields et al., 2007), excitatory projections to the VTA include glutamatergic inputs from the lateral hypothalamus (LH) (Rosin et al., 2003), bed nucleus of the stria terminalis (Georges and Aston-Jones, 2002), the superior colliculus (Geisler and Zahm, 2005) and a large excitatory input from the prefrontal cortex (PFC) (Sesack and Pickel, 1992). Additionally, two groups of mesopontine tegmental area neurons provide a large excitatory input to the VTA: the pedunculopontine tegmental nucleus (PPTg) and the laterodorsal tegmental nucleus (LDT) (Semba and Fibiger, 1992). The NMDA receptors located on dopaminergic neurons are major targets of these inputs, and electrophysiological studies have shown that application of NMDA into the VTA produces phasic firing in putative dopamine neurons (Johnson et al., 1992; Chergui et al., 1993). *In vitro* studies in VTA slices have shown that the non-NMDA agonists kainate or quisqualate do not induce bursts (Johnson et al., 1992), and have also shown that application of NMDA antagonists block glutamate-induced firing rate increases (Wang and French, 1993). Iontophoretic ejections of NMDA receptor antagonists into the VTA potently regularized the discharge pattern of phasic firing cells (Overton and Clark, 1992; Chergui et al., 1993).

However similar ejections of CNQX, a competitive AMPA/kainate glutamate receptor antagonist, were unable to affect the firing pattern (Chergui et al., 1993). The effects of microinfusion of NMDA were compared to those induced by non-NMDA excitatory agonists. While all agonists increased the firing rate of putative dopaminergic neurons; only NMDA evoked a phasic firing pattern (Suaud-Chagny et al., 1992; Chergui et al., 1993). Using differential pulse amperometry in anesthetized rats treated with pargyline, NMDA was shown to be twice as potent as quisqualate at evoking NAc dopamine release (Suaud-Chagny et al., 1992).

Since the control of bursting activity by NMDA receptors is well documented, we microinfused the NMDA antagonist, AP-5 into the VTA. Like lidocaine, AP-5 inhibited the occurrence of dopamine transients (Figure 4-3). This result is consistent with prior microdialysis studies in freely moving animals that showed a decrease in extracellular dopamine levels following intra-VTA administration of AP-5 (Karreman et al., 1996; Kretschmer, 1999). The diminished transient frequency after AP-5 microinfusions demonstrates that NMDA receptors are occupied with endogenous ligand in freely moving animals, and the lack of significance in our NMDA microinfusion results may indicate that further activity is difficult to promote with NMDA microinfusion alone. Indeed, only small dopamine increases were found with microdialysis using similar doses of NMDA (Karreman et al., 1996; Kretschmer, 1999).

Dopamine transients are significantly enhanced when the dopamine transporter is inhibited (Stuber et al., 2005; Cheer et al., 2007; Aragona et al., 2008). Specifically, i.v. injection of cocaine increases the transient frequency in the NAc and also causes a gradual increase in extracellular dopamine (Heien et al., 2005). The gradual increase is consistent

with uptake inhibition accompanying continued tonic firing (Venton et al., 2003). While it has long been known that dopamine release by cocaine requires neuronal action potentials (Nomikos et al., 1990), the cocaine-induced increase in rapid dopamine transients was not originally anticipated because cocaine tends to decrease the firing rate of VTA neurons (Einhorn et al., 1988), presumably due to D2 autoreceptor-mediated inhibition of firing (Bunney et al., 2001; Shi et al., 2004). The results of Experiment 2 show that both tonic ($[DA]_T$) and phasic (transients) cocaine-induced increases in dopamine concentrations in the NAc shell are virtually eliminated with VTA inactivation by lidocaine (Figure 4-4B, Table 4-2). Thus, ongoing firing of VTA neurons is necessary for cocaine to elevate dopamine concentrations in the NAc. Similarly, NMDA-receptor blockade attenuated the effects of cocaine on dopamine concentrations, demonstrating that glutamatergic activation of the VTA is a necessary component of cocaine-induced dopamine changes in the NAc (Figure 4-4C, Table 4-2). Indeed, it has been shown by microdialysis that acute cocaine administration elevates extracellular VTA glutamate (Kalivas and Duffy, 1995). Consistent with this, intra-VTA application of ionotropic glutamate receptor antagonists reduces the rewarding effects of cocaine (Harris and Aston-Jones, 2003; Sun et al., 2005; You et al., 2007). Even a single exposure to cocaine potentiates NMDA receptor function in the VTA (Schilstrom et al., 2006), and with repeated cocaine treatments or cocaine self-administration this effect may become even more prominent.

Like the burst firing of dopaminergic neurons (Schultz, 1998), dopamine transients in the NAc become time-locked to cues that predict reward availability in well trained animals (Phillips et al., 2003a; Roitman et al., 2004; Stuber et al., 2005; Cheer et al., 2007; Day et al., 2007; Owesson-White et al., 2008). Furthermore, NMDA receptors in the VTA play a key

role in the acquisition of reward-related learning (Zellner et al., 2008). Thus, Experiment 3 examined whether cue-evoked dopamine signals and behavior during ICSS were altered by pharmacological inhibition of VTA phasic firing. Intra-VTA microinfusion of AP-5 produced a significant decrease in cue-evoked dopamine release in the NAc (Figure 4-5A, B). At the same time the latency to lever press for ICSS was increased (Table 4-3). The NAc is thought to act as a limbic-motor interface, integrating information from limbic and cortical afferents and influencing goal-directed behavior via its efferent projections (Goto and Grace, 2005; Nicola et al., 2005). Thus, fluctuations in NAc dopamine are anticipated to influence behavioral output. The simultaneous attenuation of cue-induced dopamine transients and delayed lever pressing behavior caused by AP-5 microinfusion provide direct support for this hypothesis. The application of AP-5 to the VTA also significantly attenuated electrically stimulated dopamine release in the NAc, suggesting that electrically stimulated dopamine release is an indirect response resulting from glutamatergic innervation of the VTA. Indeed, direct activation of dopamine neurons is unlikely to occur with electrical stimulation to the cell bodies because they have thin, unmyelinated axons and exhibit high thresholds of activation (Ranck, 1975; Yeomans et al., 1988; Yeomans, 1989; Anderson et al., 1996; Nowak and Bullier, 1998).

Much of our knowledge of the role of dopamine has come from single-unit electrophysiological recordings. However, identification of VTA dopamine neurons by electrophysiological characteristics can be ambiguous (Margolis et al., 2006). Here we provide unequivocal confirmation that dopamine release in terminal fields follows the expectations of the classic electrophysiological studies. In the NAc shell, extracellular dopamine levels fall when phasic activity in the VTA is disrupted or when the VTA is

inactivated. Even more intriguing, this relationship is maintained whether dopamine fluctuations in the NAc are spontaneous in animals at rest, pharmacologically induced by cocaine administration, or evoked by ICSS reward predictive cues. This suggests that NMDA-dependent phasic firing of dopamine cells is a mechanism that is broadly operant and not solely associated with reward-related stimuli. Dopamine neurons are conditional output neurons capable of switching between tonic and phasic firing patterns (Floresco et al., 2003), and this work shows that activation of NMDA receptors is necessary to enable the switch. Further research into other neuronal mechanisms underlying phasic firing will increase our understanding of reward-related behaviors and disease states, including addiction.

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# CHAPTER 5

## VTA afferents play a critical modulatory role in cue-evoked dopamine release and reward-seeking behavior


### Abstract

Phasic dopamine transmission has been shown to play an important role in reward-related processes. Excitatory afferents to the ventral tegmental area (VTA) are believed to regulate phasic activation of dopamine neurons. The pedunculopontine tegmental nucleus (PPTg), a structure that sends both glutamatergic and cholinergic projections to the VTA, has been heavily implicated in the regulation of phasic dopamine activity. In previous work, we showed that blocking glutamatergic receptors in the VTA significantly attenuated phasic, cue-associated dopamine release in the nucleus accumbens (NAc). Here, we show that blocking cholinergic receptors in the VTA also significantly attenuates cue-evoked dopamine release in the NAc. We additionally demonstrate that inactivation of the PPTg- a primary source of glutamatergic and cholinergic input to the VTA- causes a significant decrease in the amplitude of cue-associated dopamine transients and a concurrent decrease in reward-seeking behavior. Together, our results show that excitatory VTA afferents modulate cue-evoked dopamine release in the NAc and provide unique insight into the subcortical modulation of phasic dopamine activity and reward seeking.

## Introduction

Phasic activation of dopamine neurons is thought to play an integral role in reward-related processes. Midbrain dopamine neurons in the ventral tegmental area (VTA) have been shown to exhibit phasic activation in response to primary rewards and presentation of their associated cues (Schultz et al., 1997; Hyland et al., 2002). Pharmacological attenuation of phasic activity in the VTA has been found to significantly decrease reward-seeking behavior, suggesting that this mode of firing is in fact important in reinforcement (Sombers et al., 2009).

Excitatory afferents are believed to mediate phasic activation of dopamine neurons in the VTA (Overton and Clark, 1997; Kitai et al., 1999). The VTA receives excitatory projections from a variety of brain structures that send glutamatergic or cholinergic projections to dopamine neurons (Oakman et al., 1995; Grillner and Mercuri, 2002; Fields et al., 2007; Geisler et al., 2007). Indeed, it has been shown that application of either glutamatergic agonists or cholinergic agonists will induce burst firing in midbrain dopamine neurons (Grace and Bunney, 1984; Gronier and Rasmussen, 1998; Zhang et al., 2005). Thus, these neurotransmitters are thought to be particularly critical in regulating phasic dopamine activity.

Using fast-scan cyclic voltammetry (FSCV) at carbon-fiber microelectrodes, our research group has previously demonstrated that phasic activation of dopamine neurons in the VTA gives rise to transient increases of dopamine release in the nucleus accumbens (NAc), a key dopamine terminal region (Sombers et al., 2009). We found that blocking

glutamatergic input to the VTA significantly decreased both naturally-occurring and cue-associated dopamine transients in the NAc (Sombers et al., 2009). We believe that this finding was the result of a glutamate-mediated attenuation of phasic dopamine activity.

As mentioned, in addition to glutamatergic afferents, the VTA also receives excitatory cholinergic inputs that are thought to play an important role in phasic dopamine activity (Gronier and Rasmussen, 1998; Zhang et al., 2005). To comprehensively investigate the role of excitatory VTA afferents in dopamine transmission and reward seeking, we applied cholinergic antagonists to the VTA. We also inactivated the pedunculopontine tegmental nucleus (PPTg), one of the primary sources of both glutamatergic and cholinergic projections to the VTA, and measured subsequent changes in dopamine release and reward-seeking behavior (Woolf and Butcher, 1986; Hallanger and Wainer, 1988; Clements and Grant, 1990; Oakman et al., 1995). Using FSCV and intracranial self-stimulation (ICSS), a model of reward seeking, we found that intra-VTA microinjection of cholinergic receptor antagonists significantly decreased cue-evoked dopamine release in the NAc and altered reward-seeking behavior. In addition, unilateral inactivation of the PPTg also produced a significant attenuation in the amplitude of cue-associated dopamine transients. This decrease was accompanied by a significant reduction in reward-seeking behavior. Taken together, these results suggest that excitatory afferents to the VTA play a critical role in mediating phasic dopamine transmission and reward-seeking behavior.

## Methods

### *Animals and Surgery*

Male, Sprague-Dawley rats (n = 13, 250 g – 300 g, Charles River Laboratories) were housed individually with *ad libitum* access to food and water on a 12:12 light:dark cycle. Ketamine hydrochloride (100 mg/kg, i.p.) and xylazine hydrochloride (20 mg/kg, i.p.) were used to anesthetize the animals for stereotaxic surgery. Guide cannula (Bioanalytical Systems, West Lafayette, IN), cut to 2.5 mm in length, were placed above the NAc shell (1.7 mm anterior and 0.8 mm lateral, relative to bregma). For rats receiving intra-VTA microinjections (n = 10), bipolar, stainless-steel stimulating electrodes coupled with 26 gauge injection cannula (Plastics One, Roanoke, VA) were implanted ipsilaterally in the VTA (5.4 mm posterior to bregma, 1.2 mm lateral to bregma, 7.8 mm ventral from dura) as described previously (Sombers et al., 2009). For rats receiving intra-PPTg injections (n = 3), bipolar stainless-steel stimulating electrodes (Plastics One) were placed in the lateral hypothalamus (2.8 mm posterior to bregma, 1.7 mm lateral to bregma, 8.4 mm ventral from dura) and 26 gauge microinjection cannula (Plastics One) were implanted above the PPTg (7.8 mm posterior to bregma, 2 mm lateral to bregma, 6.5 mm ventral from dura). All Ag/AgCl reference electrodes were positioned in the contralateral hemisphere. Skull screws and cranioplastic cement were used to secure electrode placements and animals were given 3 days to recover. All procedures performed were in accordance with The University of North Carolina at Chapel Hill Animal Care and Use Committee guidelines.

### *ICSS*

Rats were trained to perform ICSS in 3 phases. First, rats were trained to press a continuously available lever for intracranial stimulation on a FR-1 reinforcement schedule. Once criterion responding was achieved (30 - 50 consecutive presses), the session was stopped and rats were then trained to perform ICSS on a FR-1, variable-time out (VTO) reinforcement schedule. During this second phase of training, comprised of 50 trials, lever presentation was accompanied with simultaneous presentation of an audio-visual cue (consisting of a 67 dB, 1 kHz tone and a change in the lighting of the operant chamber). After 50 trials, the audio-visual cue was set to precede lever extension by 2 s (Owesson-White et al., 2008). This third phase of training consisted of 150 trials. Trials were separated by a random time-out interval (5 s - 25 s). Stimulation currents typically ranged from 125 μA - 150 μA (60 Hz, 24 biphasic pulses, 2 ms/phase).

***Voltammetric Recording***

Carbon-fiber microelectrodes were made by aspirating T650 carbon fibers (6 μm diameter, Amoco) into thin glass capillaries (0.6 mm outer diameter, 0.4 inner diameter, A-M Systems, Sequim, WA) and pulling the filled glass capillaries in a vertical puller (Narashige, Tokyo, Japan). The exposed carbon-fiber tip was cut to 50 μm -100 μm in length and the electrodes were loaded into micromanipulators to allow for careful insertion of the electrodes into the NAc shell.

A triangular waveform (-0.4 V to +1.3 V versus Ag/AgCl) was applied to the carbon-fiber microelectrode at 400 V/s with a 10 Hz acquisition rate. Recording sites within the NAc were optimized by measuring electrically-stimulated (60 Hz, 24 biphasic pulses, 2 ms/phase) dopamine release. After experiments were completed, electrodes were calibrated in an *in vitro* flowcell system. Principal component regression was used to resolve recorded

dopamine signals from interfering species, namely pH (Heien et al., 2004; Heien et al., 2005). Amplitudes of released dopamine were then quantified using MiniAnalysis software (Synaptosoft, version 6.03).

### *Microinjections*

Following ICSS training, a fresh carbon-fiber microelectrode was lowered into the NAc and an optimal recording site was found as described above. Animals were then allowed to resume operant responding for ICSS on the VTO schedule. Once a reproducible, cue-evoked dopamine transient was observed, the behavioral session was briefly stopped and saline (0.9%, 0.5 µL or 1.0 µL depending on drug volume) was infused at a rate of 0.5 µL/min. The needle was removed 60 s after the end of the injection and a new behavioral session was started. At the end of the session, one of three drugs (described below) was then infused in the same manner as saline. All microinjections were performed using a microsyringe pump (Kent Scientific Corporation, Torrington, CT) fitted with a 100 µL Hamilton syringe (Hamilton Company, Reno, NV) and a 33 gauge injection needle (Plastics One). All drugs were obtained from Sigma-Aldrich (St. Louis, MO) and dissolved in sterile saline (0.9%).

*Dihydro-beta- erythroidine.*

Dihydro-beta-erythroidine (DHβE), a competitive nicotinic acetylcholine (ACh) receptor antagonist, was infused into the VTA at 0.5 µL/min (30 µg, 0.5 µL, n = 5). The behavioral session began immediately following removal of the needle, 60 s after the end of the infusion. Each behavioral session was comprised of 50 trials.

*Scopolamine.*

The nonselective, competitive muscarinic ACh receptor antagonist, scopolamine (200 μg, 1.0 μL), was infused into the VTA at a rate of 0.5 μL (n= 5). After the injection, the needle was left in place for 60 s prior to removal. Once the needle was removed, the animal was allowed to resume ICSS. Each session consisted of 50 trials.

*Lidocaine.*

A 1% Lidocaine hydrochloride solution (0.5 μL) was microinjected into the PPTg at 0.5 μL/min (n = 3). The needle was removed 60 s after the injection, at which time the behavioral session commenced. Each session consisted of 50 trials.

## Results

### *Nicotinic acetylcholine receptors in the VTA contribute to cue-evoked dopamine release in the NAc*

Consistent with our prior reports, we found that in well-trained animals, presentation of an audio-visual cue that predicted ICSS availability elicited a time-locked, transient increase in dopamine release in the NAc (Owesson-White et al., 2008; Sombers et al., 2009). Rats that exhibited this response had been trained to perform ICSS on a VTO (variable time-out) reinforcement schedule in which an audio-visual cue preceded lever extension by 2 s. The average dopamine response seen following microinjection of saline into the VTA is shown in Figure 5-1A (left). Unilateral microinjection of DHβE into the VTA significantly attenuated not only the cue-evoked dopamine response but the stimulated dopamine response as well (Figure 5-1A, right). Altogether (n = 5), intra-VTA microinjection of DHβE produced a significant decrease in both cue-evoked dopamine (from 37.1 $\pm$ 1.62 nM to 28.6

± 1.29 nM, $p < 0.001$, student's *t* test) and stimulated dopamine (from 162.1 ± 2.62 nM to 111.3 ± 2.65 nM, $p < 0.001$, student's *t* test) release (Figure 5-1B). Both of these values exhibited recovery following drug infusion (see Table 5-1). The average latency to lever press after lever extension was not found to be significantly affected by intra-VTA microinjection of DHβE.

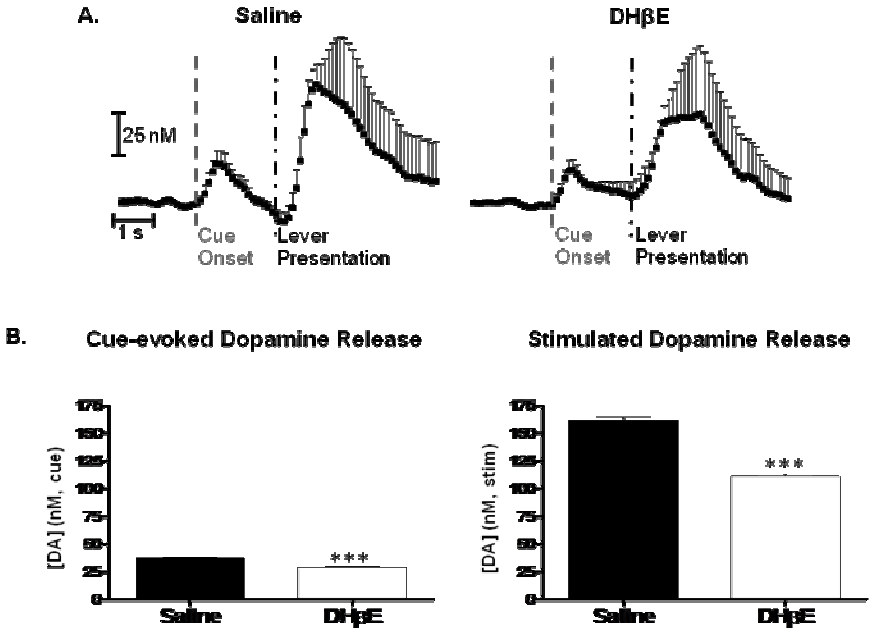


**Figure 5-1. Nicotinic receptors in the VTA modulate cue-evoked dopamine release in the NAc.** A) Average dopamine traces following saline (left) and DHβE (right) microinjections into the VTA are shown (n = 5). B) Bar graphs display the average amplitudes of cue-evoked and stimulated dopamine release. Microinjection of DHβE into the VTA caused a significant attenuation of dopamine release in the NAc (***$p < 0.001$).

### *Muscarinic acetylcholine receptors in the VTA mediate cue-evoked dopamine release in the NAc and modulate reward-seeking behavior*

Similar to the results obtained with DHβE microinjection, microinjection of scopolamine into the VTA decreased the amplitude of both cue-evoked dopamine and

stimulated dopamine release relative to saline values (Figure 5-2A). Across all animals (n = 5), intra-VTA microinjection of scopolamine produced a significant reduction of cue-evoked dopamine (from 47.4 ± 1.84 nM to 33.3 ± 1.43 nM, $p < 0.001$, student's *t* test) and stimulated dopamine (from 240.9 ± 5.49 nM to 214.3 ± 4.45 nM, $p < 0.001$, student's *t* test) release (Figure 5-2B) that did not exhibit post-injection recovery (see Table 5-2).

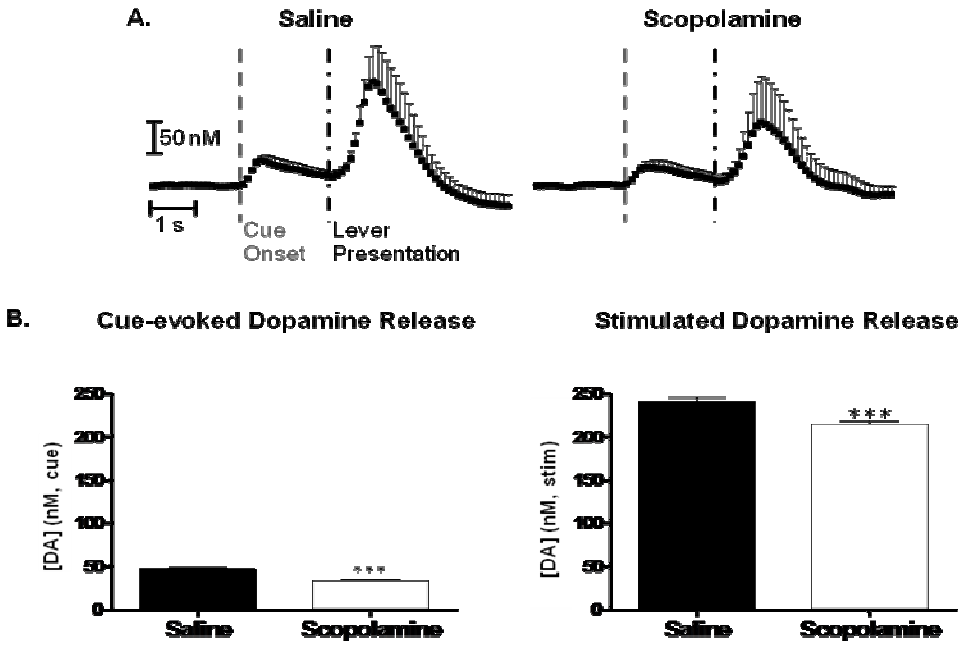


**Figure 5-2. Muscarinic receptors in the VTA modulate cue-evoked dopamine release in the NAc.** A) Average dopamine concentration traces for saline and scopolamine ICSS sessions are shown (n = 5). B) Average dopamine concentration changes following microinjections are shown in the bar graphs. Intra-VTA microinjection of scopolamine significantly reduced NAc dopamine release (***$p < 0.001$).

Analysis of behavioral data revealed that intra-VTA microinjection of scopolamine significantly increased the average latency to lever press (from 0.66 s to 1.83 s, $p < 0.001$, student's *t* test) (Figure 5-3A). To determine if the scopolamine-induced increase in the

latency to lever press was due to potential side effects of the drug on motor function, pressing rates were measured after unilateral microinjection of either saline or scopolamine into the VTA. Intriguingly, intra-VTA microinjection of scopolamine did not significantly affect lever pressing behavior at any stimulation current tested (Figure 5-3B).

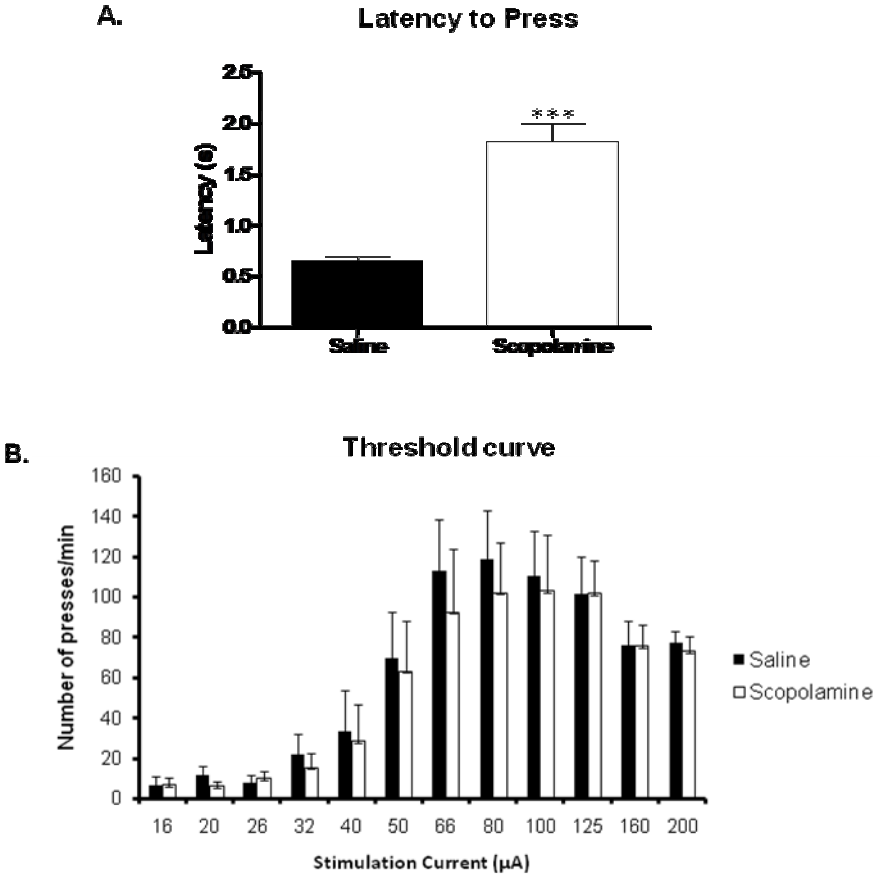


**Figure 5-3. Intra-VTA microinjection of scopolamine significantly affects reward seeking.** A) The average latency to press increased significantly with microinjection of scopolamine into the VTA (***$p < 0.001$). B) A threshold curve was generated by allowing rats to press a continuously available lever for a range of stimulation currents, presented randomly. As exhibited by the number of presses in a 1 min interval, intra-VTA infusion of scopolamine did not affect rats' ability to physically perform the behavior at any stimulation current tested.

***The PPTg plays an important role in modulating phasic dopamine release and reward-seeking behavior***

The PPTg is a principal source of cholinergic innervation in midbrain dopamine neurons (Woolf and Butcher, 1986; Hallanger and Wainer, 1988; Oakman et al., 1995; Forster and Blaha, 2000). It has been shown that inactivation of the PPTg significantly attenuates cue-induced activation of dopamine neurons in the midbrain (Pan and Hyland, 2005). To determine the role of the PPTg in the modulation of cue-evoked dopamine release in the NAc, we microinjected lidocaine into the PPTg and measured subsequent changes in dopamine release. As shown in Figure 5-4, unilateral intra-PPTg microinjection of lidocaine significantly decreased the amplitude of cue-evoked dopamine release in the NAc (from 48.1 $\pm$ 2.28 nM to 35.6 $\pm$ 1.69 nM, $p < 0.001$, student's *t* test, $n = 3$). Stimulated dopamine release was also found to decrease significantly (from 90.5 $\pm$ 2.50 nM to 68.7 $\pm$ 1.65 nM) with microinjection of lidocaine into the PPTg ($p < 0.001$, student's *t* test) (Figure 5-4B). Neither cue-evoked nor stimulated dopamine release exhibited postdrug recovery (see Table 5-3).

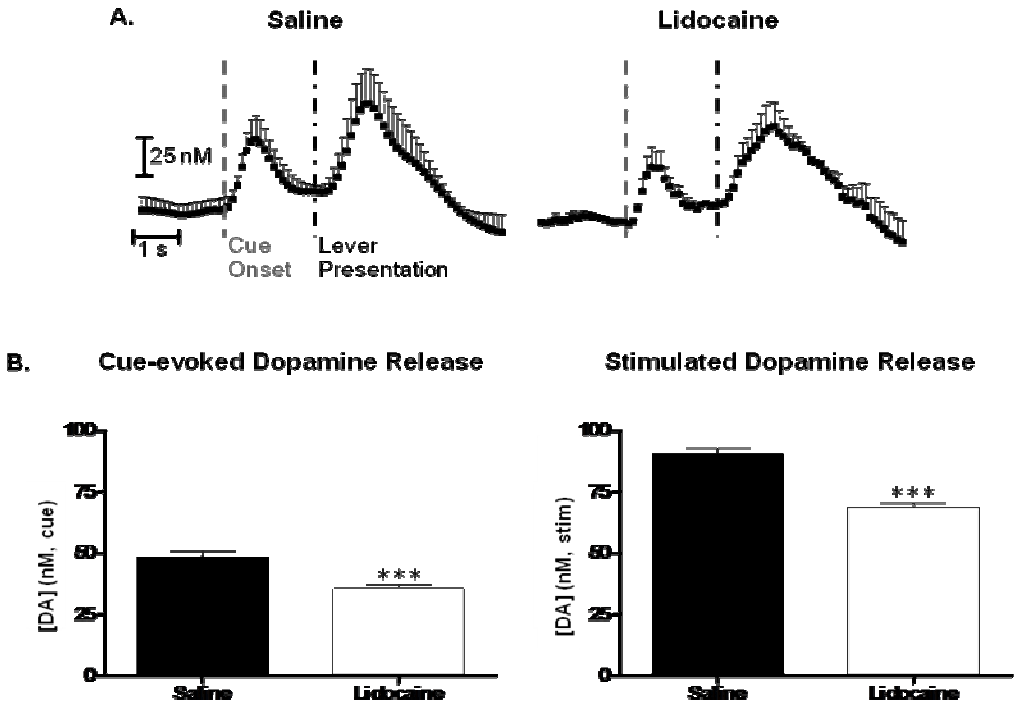


**Figure 5-4. Unilateral inactivation of the PPTg significantly attenuates cue-evoked dopamine release in the NAc.** A) Average dopamine concentration traces following intra-PPTg microinjection of saline and lidocaine are shown. B) Intra-PPTg injections of lidocaine significantly reduced cue-evoked (***$p < 0.001$) and stimulated (***$p < 0.001$) dopamine release in the NAc (n=3).

Intra-PPTg microinjection of lidocaine was found to produce a significant increase ($p < 0.001$, student's *t* test) in the average latency to lever press (Figure 5-5A). To assess whether or not the increase in latency seen after lidocaine microinjection was a result of potential behavioral side effects associated with PPTg inactivation, a threshold curve was built to examine lever pressing behavior after injection of either saline or lidocaine into the PPTg. As displayed in Figure 5-5B, unilateral intra-PPTg microinjection of lidocaine did not significantly affect response rates for any stimulation current given.

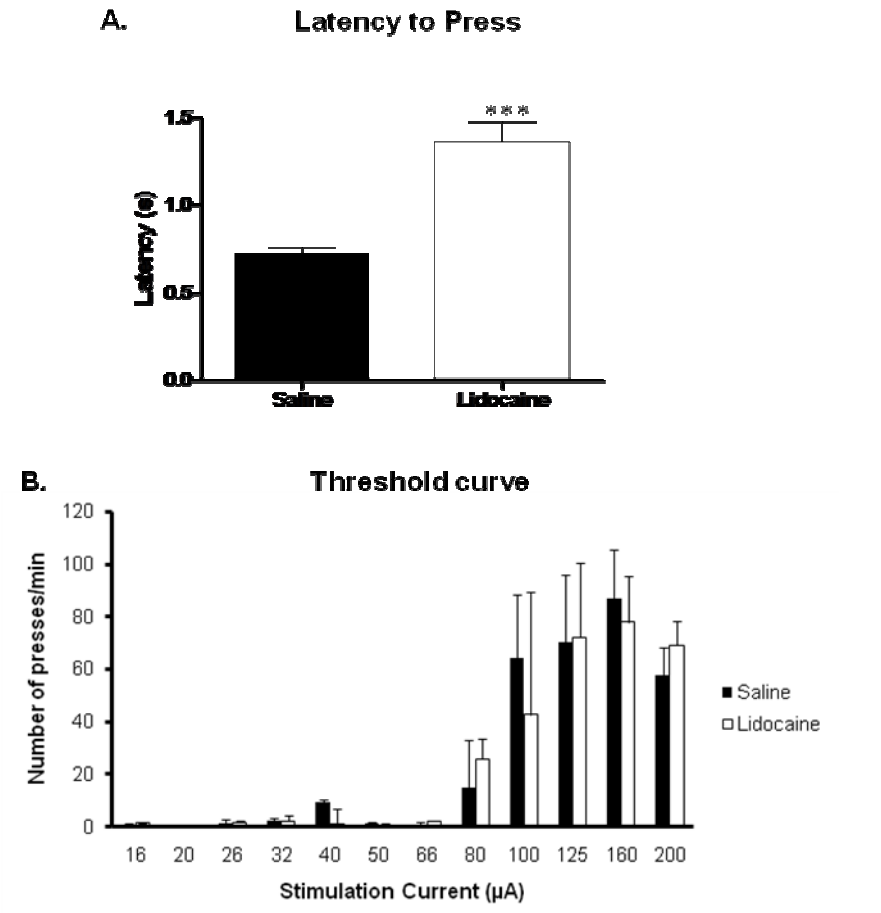


**Figure 5-5. The PPTg modulates reward-seeking behavior.** A) Inactivation of the PPTg produced a significant increase in the average latency to lever press (***$p < 0.001$). B) Threshold curve indicates that motor function was not affected by intra-PPTg injections of lidocaine.

## Discussion

Our research group has previously shown that transient dopamine release events closely follow presentation of rewards and reward-predictive cues (Phillips et al., 2003a; Roitman et al., 2004; Day et al., 2007; Owesson-White et al., 2008). It is believed that these dopamine transients arise from burst firing of dopamine neurons in the VTA (Wightman et al., 2007). Excitatory VTA afferents have been shown to play a critical role in regulating

phasic dopamine activity and have been implicated in the modulation of reward processes (Inglis et al., 2000; Floresco et al., 2003; Pan and Hyland, 2005). In this work, we establish that the PPTg and its excitatory cholinergic projection to the VTA contribute significantly to cue-evoked dopamine release in the NAc. We also show that the PPTg-VTA projection mediates reward-seeking behavior. These results lend unique insight into afferent control of phasic dopamine transmission and motivated behavior.

The PPTg provides a major cholinergic projection to the VTA (Oakman et al., 1995). The VTA has been shown to project, via both dopamine and γ-aminobutyric acid (GABA) projection neurons, to the NAc and prefrontal cortex (Swanson, 1982; Van Bockstaele and Pickel, 1995; Carr and Sesack, 2000). Data suggest that mesoaccumbens and mesoprefrontal dopamine neurons are segregated and functionally distinct from one another (Deutch et al., 1991; White, 1996; Tzschentke, 2001). Detailed investigation of cholinergic input to the VTA has revealed that cholinergic neurons preferentially synapse onto mesoaccumbens dopamine neurons (Omelchenko and Sesack, 2006). These findings are in line with data that show that administration of cholinergic agonists to the VTA produce significant increases in dopamine release in the NAc (Miller and Blaha, 2005).

Nicotinic acetylcholine (ACh) receptors exhibit diverse expression in the VTA (Wada et al., 1989; Charpantier et al., 1998; Klink et al., 2001; Azam et al., 2007). They are comprised of 5 subunits, with α4β2 nicotinic receptors representing the majority of functional, heteromeric nicotinic ACh receptors located on dopamine cell bodies (McGehee and Role, 1995; Champtiaux et al., 2003). Nicotinic ACh receptor-mediated currents in the VTA are thought to be mediated primarily by β2 containing subunits as β2-null mice exhibit a significant decrease in these currents (Picciotto et al., 1998). Moreover, it has been shown

that in α4-null mice, nicotine-induced increases in striatal dopamine release do not occur (Marubio et al., 2003). Therefore, the α4 subunits are thought to be important for cholinergic modulation of dopamine transmission. Consistent with this, we found that intra-VTA microinjection of DHβE, a specific α4β2 receptor antagonist, significantly attenuated cue-evoked dopamine release in the NAc of rats performing ICSS. This is likely attributable to an attenuation of phasic firing of VTA dopamine neurons. Indeed, administration of the nonselective cholinergic agonist carbachol to dopamine neurons has been shown to promote burst firing in the VTA and to produce transient increases of dopamine concentration in the NAc (Miller and Blaha, 2005; Zhang et al., 2005). These findings implicate a potential role of the nicotinic ACh receptor in the VTA in the mediation of dopamine dynamics. Our results support this notion.

Interestingly, administration of DHβE into the VTA also attenuated stimulated dopamine release in the NAc. Dopamine neurons exhibit high thresholds of activation due to their thin, unmyelinated axons (Yeomans et al., 1988; Yeomans, 1989). Thus, direct activation of dopamine neurons via electrical stimulation of their cell bodies is unlikely (Nowak and Bullier, 1998). Excitatory projections to dopamine neurons are believed to be particularly important in regulating dopamine neuron activity (Overton and Clark, 1997; Kitai et al., 1999). It has been shown that the cholinergic projections to mesoaccumbens neurons in the VTA form primarily asymmetric, presumably excitatory, synapses (Omelchenko and Sesack, 2006). Therefore, it is likely that the dopaminergic excitation produced by electrical stimulation in this study was an indirect result of cholinergic activation.

We have previously reported that pharmacological attenuation of phasic VTA activity with a glutamatergic antagonist produces significant decreases in cue-evoked dopamine release that are associated with significant decreases in reward-seeking behavior (Sombers et al., 2009). Here, we found that intra-VTA administration of DHβE attenuated cue-evoked dopamine release but did not significantly affect reward seeking, as measured using latency to lever press. This may be due the fact that while previously, administration of a glutamatergic receptor antagonist produced a >50% decrease in the amplitude of cue-evoked dopamine release, microinjection of DHβE produced approximately a 25% decrease, which may not have been sufficient to produce a functional behavioral effect. Consistent with our findings, (Bruijnzeel and Markou, 2004) found that intra-VTA microinjection of DHβE, at a dose similar to the one used in this work, did not produce shifts in lever pressing behavior for rats performing ICSS.

In addition to nicotinic ACh receptors, dopamine neurons also express muscarinic ACh receptors (Vilaro et al., 1990; Weiner et al., 1990). Muscarinic receptor agonists have been found to directly excite dopamine neurons in the VTA and increase extracellular levels of dopamine in the NAc (Lacey et al., 1990; Gronier et al., 2000). Moreover, administration of muscarinic agonists has been shown to significantly increase burst firing of dopamine neurons in the VTA (Gronier and Rasmussen, 1998). Of the 5 muscarinic receptors, M1-M5, dopamine neurons are believed to specifically express the M5 receptor (Yeomans et al., 2001). m5 mRNA and M5-selective binding are lost when 6-hydroxydopamine is used to selectively lesion dopamine neurons, suggesting that M5 receptors are the muscarinic receptor type expressed on dopamine neurons (Yeomans et al., 2001).

To date, no specific antagonists for the M5 muscarinic receptor have been described. Therefore, in this work, we used scopolamine, a non-selective muscarinic receptor antagonist, with documented affinity for the M5 receptor that has previously been used in the literature to investigate muscarinic receptor-mediated changes in dopaminergic activity (Ferrari-Dileo et al., 1994; Reever et al., 1997; Gronier and Rasmussen, 1998; Gronier et al., 2000; Miller and Blaha, 2005). Here, we show that microinjection of scopolamine into the VTA significantly reduces cue-evoked dopamine release in the NAc. We believe that this attenuation is due to a decrease in phasic dopamine activity in the VTA. In support of this hypothesis, it has been shown that scopolamine significantly reduces burst firing of VTA dopamine neurons *in vivo* (Gronier and Rasmussen, 1998). As seen with intracranial DHβE injections, microinjection of scopolamine into the VTA also produced a decrease in stimulated dopamine release, further suggesting that dopaminergic activation is an indirect result of afferent innervation (see Sombers et al., 2009).

Intra-VTA microinjection of scopolamine produced a substantial (> 1 s) increase in the latency to lever press. This result is consistent with previous findings that demonstrate that intra-VTA administration of scopolamine raises reward thresholds for rats performing ICSS (Kofman and Yeomans, 1988). The increase in the latency to lever press observed in this experiment is independent of any potential effects of scopolamine on motor function. Figure 5-3B clearly illustrates that microinjection of scopolamine into the VTA does not significantly affect the animals ability to physically perform the behavior, implying that blockade of muscarinic ACh receptors in the VTA affects motivational- not physical-processes. These results also lend support to the notion that NAc dopamine is important in

mediating reward-seeking behavior because the increase in latency was accompanied by a simultaneous decrease in NAc dopamine (Phillips et al., 2003a; Nicola et al., 2005).

Our current results with cholinergic drugs, coupled with our previous results (Sombers et al., 2009), suggest that both glutamatergic and cholinergic inputs to the VTA play a significant role in the modulation of phasic dopamine release and reward-seeking behavior. The PPTg is a primary source of both of these afferents to the VTA (Woolf and Butcher, 1986; Hallanger and Wainer, 1988; Clements and Grant, 1990; Oakman et al., 1995). Thus, in our last set of experiments, we investigated the effect of PPTg inactivation on cue-evoked dopamine release in the NAc and reward-seeking behavior. We found that unilateral inactivation of the PPTg with a lidocaine solution significantly reduced the amplitude of cue-associated dopamine transients in the NAc. Intra-PPTg microinjection of lidocaine, at the same concentration used in this work, has previously been shown to significantly attenuate cue-induced burst firing of dopamine neurons in freely-moving rats (Pan and Hyland, 2005). Thus, together these results provide an irrefutable link between PPTg activity and phasic dopamine transmission.

Stimulated dopamine release was also attenuated by PPTg inactivation, suggesting that the PPTg may provide a substrate by which dopamine neurons are indirectly activated upon stimulation. In addition to affecting dopamine release in the NAc, unilateral inactivation of the PPTg significantly increased the average latency to lever press. PPTg inactivation did not significantly influence lever pressing behavior in a threshold test - ruling out the possibility that the increase in the latency to press was due to motor or behavioral deficits brought about by PPTg inactivation.

In this work, postdrug sessions were used to examine recovery of dopamine release amplitudes following drug administration. Recovery of dopamine dynamics varied based on the drug infused (see Tables 5-1 through 5-3). Postdrug measurements, taken 70 min after DHβE infusion, revealed recovery in the amplitudes of both cue-associated and stimulated dopamine. These results suggest that DHβE has a relatively short duration of action at nicotinic receptors. In a nicotine-induced conditioned taste aversion test, it was shown that while co-administration of DHβE with nicotine blocked acquisition of the conditioned taste aversion, administration of DHβE 30 minutes prior to testing failed to block conditioning (Shoaib et al., 2000). Together, these results imply that the effects of DHβE on the nicotinic receptor are short lasting.

In contrast to the results seen with DHβE microinjection, cue-evoked and stimulated dopamine release amplitudes did not exhibit recovery following intra-VTA microinjection of scopolamine. The fact that neither cue-evoked nor stimulated dopamine release recovered suggests that scopolamine may still have, in part, been bound to the muscarinic receptor during the time postdrug measurements were taken. Indeed, dissociation of 3H-N-methylscopolamine occurs with a half-life of 20.5 min for the M5 receptor, meaning that approximately 10% of the drug's original concentration was still bound to the receptor when the postdrug measurements were made (Ferrari-Dileo et al., 1994; Yeomans et al., 2001). It is interesting to note that not only did the amplitude of stimulated dopamine release fail to recover, but it decreased significantly during the postdrug collection period. A loss of electrode sensitivity is unlikely to explain these results because the amplitude of cue-evoked dopamine release remained stable and did not decrease further during the postdrug session. Also, animals receiving DHβE microinjections exhibited recovery in their dopamine release

profiles, which argues against a global loss of electrode sensitivity over time. One possible explanation of the results may involve intracellular mechanisms that modulate cell excitability properties such as afterhyperpolarizations (Madison and Nicoll, 1984; Constanti and Sim, 1987; Krause et al., 2002; Ishii and Kurachi, 2006). As with microinjections of scopolamine into the VTA, neither cue-evoked nor stimulated dopamine release amplitudes exhibited post-PPTg inactivation recovery. Unpublished results from our laboratory suggest that recovery of neural function following lidocaine microinjection can take up to 2 hrs-which exceeds the recovery time allowed here.

Across all drug manipulations, a significant decrease in stimulated dopamine was observed. In addition to pharmacological effects, one factor that likely contributed to the reduction of stimulated dopamine release is a limited pool of readily releasable dopamine (Nicolaysen et al., 1988; Yavich and Tiihonen, 2000; Montague et al., 2004). Consistent with this, decreases in electrically-stimulated dopamine release have been reported elsewhere (Garris et al., 1999; Owesson-White et al., 2008). These results suggest a mechanism whereby electrically-stimulated dopamine exhausts over time. An additional contributing factor may have been potential modulation of stimulated dopamine release by cue-evoked dopamine release via interaction with the dopamine autoreceptor (Kita et al., 2007).

Phasic dopamine transmission has been heavily implicated in reward-related processes (Schultz et al., 1997; Hyland et al., 2002; Phillips et al., 2003a; Roitman et al., 2004; Day et al., 2007; Owesson-White et al., 2008). We have previously shown that glutamatergic innervation of the VTA, via its modulation of phasic dopamine activity, plays a critical role in phasic dopamine release in the NAc (Sombers et al., 2009). The data presented here expand upon our previous findings and demonstrate that cholinergic

innervation of the VTA also plays a significant role in mediating phasic dopamine release. Together with our results from the PPTg inactivation, our data provide a unique view of the neural mechanisms underlying phasic dopamine transmission and reward-seeking behavior.

| | Cue-evoked Dopamine (nM) | Stimulated Dopamine (nM) |
|---|---|---|
| Saline | 37.1 ± 1.62 | 162.1 ± 2.62 |
| DHβE | 28.6 ± 1.29 | 111.3 ± 2.65 |
| Postdrug | 42.4 ± 1.69 | 131.2 ± 2.67 |

**Table 5-1. Intra-VTA microinjection of DHβE significantly attenuates dopamine release in the NAc.** Average amplitude of cue-evoked and stimulated dopamine decreased significantly with DHβE infusion ($p < 0.001$) and recovered significantly during postdrug collection periods ($p < 0.001$).

| | Cue-evoked Dopamine (nM) | Stimulated Dopamine (nM) |
|---|---|---|
| Saline | 47.4 ± 1.84 | 240.9 ± 5.49 |
| Scopolamine | 33.3 ± 1.43 | 214.3 ± 4.45 |
| Postdrug | 34.1 ± 1.47 | 147.6 ± 3.24 |

**Table 5-2. Intra-VTA microinjection of scopolamine significantly decreases dopamine release in the NAc.** Microinjection of scopolamine into the VTA produced significant decreases in cue-evoked and stimulated dopamine release in the NAc ($p < 0.001$). While the amplitude of cue-evoked dopamine release remained stable during postdrug collection, the amplitude of stimulated dopamine decreased significantly ($p < 0.001$).

| | Cue-evoked Dopamine (nM) | Stimulated Dopamine (nM) |
|---|---|---|
| Saline | 48.1 ± 2.28 | 90.5 ± 2.50 |
| Lidocaine | 35.6 ± 1.69 | 68.7 ± 1.65 |
| Postdrug | 33.3 ± 1.11 | 65.9 ± 2.10 |

**Table 5-3. Microinjection of lidocaine into the PPTg significantly reduces dopamine release in the NAc.** Intra-PPTg infusion of lidocaine caused a significant attenuation of both cue-evoked and stimulated dopamine release ($p < 0.001$) that did not exhibit postdrug recovery.

**Acknowledgements:**

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# CHAPTER 6

# General Discussion

## Summary of Findings

The experiments discussed in this dissertation were designed to elucidate the origin of phasic, cue-evoked dopamine release and to assess its role in reward-seeking behavior. The results demonstrate that cue-evoked dopamine release in the NAc is highly dynamic and is an important modulator of reward seeking. The data reveal that cue-associated dopamine develops with reward-related learning, that it is dependent on a learned stimulus-reward association and that it reflects salient information about predicted rewards. Furthermore, the results establish that phasic dopamine release arises from phasic activity in the VTA and that excitatory VTA afferents play an influential role in this response. A short summary of each research aim is given below.

### *Dynamic changes in accumbens dopamine correlate with learning during ICSS*

Research Aim 1 (Chapter 2) was designed to investigate phasic dopamine transmission during the course of reward-related learning. Using a VTO ICSS paradigm, we found that the amplitude of cue-evoked dopamine release in the NAc grew in with repeated stimulus-reward pairings, reaching a plateau in later trials. The increase in cue-evoked dopamine release in early trials was found to significantly correlate with a decrease in the

latency to lever press, indicating learning. Extinction of ICSS abolished cue-associated dopamine transients and resulted in a significant decrease in lever pressing behavior. Subsequent reinstatement of ICSS restored cue-associated dopamine release to pre-extinction values and re-established ICSS behavior. Together, these results demonstrate that cue-evoked dopamine release in the NAc correlates with learning during ICSS and is dependent on a learned stimulus-reward association. These results also suggest that phasic dopamine release in the NAc plays an important role in modulating reward-seeking behavior.

*Adaptive fluctuations in cue-evoked dopamine release follow changes in reinforcer magnitude*

The objective of Research Aim 2 (Chapter 3) was to assess the relationship between reinforcer magnitude and cue-evoked dopamine release in the NAc. In this experiment, rats were allowed to perform ICSS on a VTO reinforcement schedule for three distinct reinforcer magnitudes (low, medium and high) and the amplitude of cue-associated dopamine transients was measured in each condition. Cue-evoked dopamine release was found to vary as a function of reinforcer magnitude with levels of cue-associated dopamine increasing significantly with increasing reinforcer magnitude. Conversely, the average latency to lever press decreased significantly with increasing reinforcer magnitude. Intriguingly, the amplitude of cue-associated dopamine release correlated with the latency to lever press in an inverse manner. These results demonstrate that cue-evoked dopamine release in the NAc is an adaptable measure of anticipated reinforcer magnitude that is significantly correlated with reward seeking.

*Synaptic overflow of dopamine in the nucleus accumbens arises from neuronal activity in the ventral tegmental area*

The goal of Research Aim 3 (Chapter 4) was to determine if phasic activity in the VTA gives rise to phasic dopamine release in the NAc. As such, pharmacological agents were used to block either burst firing and/or overall neural activity in the VTA and subsequent changes in phasic dopamine release were measured 1) under basal conditions 2) following cocaine administration, and 3) during ICSS. Intra-VTA microinfusion of either lidocaine or the NMDA receptor antagonist, AP-5, significantly attenuated the amplitude of phasic dopamine release in the NAc in all three conditions. Furthermore, attenuation of phasic, cue-evoked dopamine during ICSS was associated with a significant increase in the latency to lever press. These data provide unequivocal confirmation that phasic dopamine release in the NAc arises from neural activity in the VTA and further support a facilitative role for phasic dopamine in reward-seeking behavior.

*Ventral tegmental area afferents play a critical modulatory role in cue-evoked dopamine release and reward-seeking behavior*

Research Aim 4 (Chapter 5) was designed to examine afferent modulation of cue-evoked dopamine release in the NAc. Excitatory afferents to the VTA, which include both glutamatergic and cholinergic projections, are critical in regulating phasic dopamine neuron activity. Thus, Aim 4 was designed to further investigate the role of excitatory VTA afferents by examining cholinergic modulation of phasic dopamine release in the NAc. Cholinergic antagonists were microinjected into the VTA and subsequent changes in cue-evoked dopamine were measured during ICSS. Intra-VTA microinfusion of both nicotinic and muscarinic ACh receptor antagonists significantly attenuated the amplitude of cue-

associated dopamine transients in the NAc. Furthermore, infusion of a muscarinic ACh receptor antagonist simultaneously attenuated reward-seeking behavior. In a separate group of animals, the primary source of both glutamatergic and cholinergic inputs to the VTA- the pedunculpontine tegmental nucleus (PPTg)- was pharmacologically inactivated and the effect on phasic dopamine release was examined. PPTg inactivation was found to significantly decrease both cue-evoked dopamine release in the NAc and reward seeking. Altogether, these results show that excitatory VTA afferents modulate cue-evoked dopamine release in the NAc and shed light on the subcortical modulation of phasic dopamine activity and reward seeking.

**General Discussion and Significance of Findings**

Altogether, the findings presented in this dissertation provide unique insight into the function of the mesolimbic dopamine system by giving an unprecedented view of the origin and role of phasic dopamine release in reward-related learning and reward-seeking behavior. While the significance of the experimental results are discussed at the conclusion of each research chapter, this section will focus on incorporating the present findings into the broader scheme of existing data in the field.

*Neuromodulatory role of dopamine*

As demonstrated in this work, cues associated with ICSS availability elicit time-locked, transient increases in NAc dopamine concentration. Several additional studies have demonstrated that cues predicting a variety of rewards produce similar increases in phasic dopamine release in the NAc (Robinson et al., 2002; Phillips et al., 2003a; Roitman et al., 2004; Stuber et al., 2005). A central topic that arises from these findings is the neuromodulatory role of dopamine in the NAc. Thus, this subject is discussed below.

Given the integral role of the NAc in goal-directed behavior and the correlation between dopamine release and reward seeking, the postsynaptic effect of dopamine in the NAc has received much attention in addiction research. This topic is controversial however as some findings demonstrate that dopamine inhibits neural activity in the NAc while others show that dopamine has an excitatory effect on neural firing in the NAc (Calabresi et al., 1997; Hernandez-Lopez et al., 1997). One integrative theory that explains the discrepancies in these reports is that the postsynaptic effect of dopamine depends on a variety of factors, including postsynaptic expression of dopamine receptors and the membrane potential of the postsynaptic cell (O'Donnell et al., 1999; Nicola et al., 2000; Goto and Grace, 2005). For example, it has been suggested that dopamine release in the NAc increases signal-to-noise ratios by inhibiting firing in medium spiny neurons receiving weak coincidental afferent input and promoting firing in neurons receiving strong coincidental input (Nicola et al., 2000). In this manner, dopamine would serve to enhance evoked firing while suppressing spontaneous, or "noise", firing. In fact, this dopamine-induced enhancement of evoked activity relative to spontaneous neural activity has been previously documented (Rolls et al., 1984; Kiyatkin and Rebec, 1996). Thus, it is possible that the phasic dopamine release

discussed in this work functioned to decrease system level noise and to augment evoked, potentially behaviorally-relevant, activity in the NAc.

Dopamine has also been shown to be necessary for the induction of long-term potentiation, or LTP, in medium spiny neurons (Calabresi et al., 1997). LTP is a form of plasticity believed to support associative learning and the D1 dopamine receptor is critical for the generation of this response (Reynolds et al., 2001). As mentioned, D1-like receptors typically exist in a low-affinity state, requiring relatively high concentrations of dopamine to become activated (Richfield et al., 1989). The concentrations reported in this work were sufficient to activate the D1 receptor and as a result, may have contributed to changes in synaptic strength. In fact, preliminary results from ICSS extinction experiments support this notion. It was found that rats that had undergone extinction (as described in Chapter 2) would lever press for ICSS for up to ten days following the initial extinction session *without* reinstatement. This finding suggests a long-term change in the neural networks that support ICSS.

*Dopamine and reward seeking*

As demonstrated in each research chapter, changes in the amplitude of phasic, cue-evoked dopamine release were consistently associated with changes in reward-seeking behavior. Higher concentrations of cue-associated dopamine transients were typically associated with shorter latencies to lever press and vice versa. Therefore, this section will focus on discussing potential mechanisms whereby dopamine may influence behavioral output.

Similar to the results presented in this work, several other studies have also reported dopamine-induced facilitation of reward seeking. For example, it has been shown that increasing extracellular levels of dopamine in the NAc significantly increases reward-seeking behavior while attenuating dopamine function in the NAc significantly decreases it (Nicola et al., 2005). Other studies have shown that blocking dopamine receptors, particularly the D1-like dopamine receptor, will abolish reward seeking completely (Cheer et al., 2007). Together, these results demonstrate that dopamine is both necessary and sufficient to promote reward seeking (Nicola et al., 2005).

The NAc sends projections to several brain regions that are involved in regulating motor movement, including the ventral pallidum and lateral hypothalamus (Mogenson et al., 1980). Due to the efferent projections of the NAc and the neuromodulatory role of dopamine in the NAc, a potential role exists for dopamine in the modulation of goal-directed behavior. Recent technological advancements that have allowed for simultaneous measurement of both phasic dopamine signals and single unit activity in the NAc have shown that transient, accumbal dopamine release is predominantly associated with inhibitions in neural firing (Cheer et al., 2005; Cheer et al., 2007). As mentioned in Chapter 1, the majority (> 90%) of neurons in the NAc are GABAergic. Thus, an inhibition of firing would ultimately disinhibit afferent targets such as the ventral pallidum. A potential dopamine-mediated excitation of the ventral pallidum is particularly relevant to the studies presented here because stimulation of the ventral pallidum supports ICSS (Panagis et al., 1995; Panagis and Spyraki, 1996). Furthermore, intra-pallidal infusions of GABAergic agonists attenuate locomotor activity (Klitenick et al., 1992). Therefore, one distinct mechanism by which dopamine may potentially modulate behavior is via a disinhibitory influence in the ventral pallidum,

mediated via its postsynaptic action in the NAc. Another mechanism may involve the efferent projection from the NAc to the lateral hypothalamus. Stimulation of the lateral hypothalamus induces behavioral activation and- as demonstrated in Chapters 3 and 5- also supports ICSS (Olds and Olds, 1963). Thus, phasic dopamine release in the NAc may indirectly stimulate lateral hypothalamic activity and promote behavioral responses by inhibiting accumbal firing.

*Modulation of dopaminergic activity in the ventral tegmental area*

One primary goal of the experiments described in this work was to elucidate the origin of phasic dopamine signals. Chapter 4 established that transient dopamine release events arise from phasic activity in the VTA while Chapter 5 assessed afferent modulation of this response. As discussed, excitatory afferents to the VTA are believed to play a critical role in the induction of dopaminergic burst firing and subsequent increases in phasic dopamine release in terminal regions. In addition to the excitatory projection from the PPTg, electrophysiological and neuroanatomical data indicate that excitatory projections to the VTA also arise from the prefrontal cortex, the bed nucleus of the stria terminalis and the LDTg, suggesting that dopaminergic activity is regulated primarily by few structures (Sesack and Pickel, 1992; Georges and Aston-Jones, 2002; Floresco et al., 2003; Omelchenko and Sesack, 2005; Lodge and Grace, 2006; Omelchenko and Sesack, 2007). Each of these afferents is believed to play a distinct role in mediating dopamine neuron activity and will be discussed below.

The prefrontal cortex provides one of the major glutamatergic projections to the VTA (Sesack and Pickel, 1992). Within the VTA, there are two populations of dopaminergic and GABAergic projection neurons- mesoaccumbens neurons, projecting to the NAc and mesoprefrontal neurons, targeting the prefrontal cortex. These two groups are believed to be segregated and functionally distinct from one another (Deutch et al., 1991; White, 1996; Tzschentke, 2001). Research has shown that glutamatergic projections from the prefrontal cortex to the VTA are highly specific in their synaptic targets, synapsing onto mesoprefrontal VTA dopamine neurons and mesoaccumbal VTA GABA neurons (Carr and Sesack, 2000). However, prefrontal neurons were not found to innervate mesoprefrontal GABA neurons or mesoaccumbal dopamine neurons in the VTA (Carr and Sesack, 2000). Consistent with this anatomical framework, low-frequency, physiological stimulation of the prefrontal cortex has been found to decrease dopamine levels in the NAc (Jackson et al., 2001). Furthermore, stimulation of the prefrontal cortex can inhibit dopamine neuron firing (presumably mesoaccumbal dopamine firing) in the VTA (Tong et al., 1998). Therefore, despite the excitatory nature of the projection from the prefrontal cortex to the VTA, its functional effect in the NAc is an inhibition of dopamine signaling.

Another structure that provides substantial excitatory innervation of the VTA is the LDTg (Woolf and Butcher, 1986; Oakman et al., 1995). The LDTg sends both glutamatergic and cholinergic projections to midbrain dopamine neurons and preferentially innervates the VTA as opposed to the substantia nigra (Oakman et al., 1995; Mena-Segovia et al., 2008). As discussed in Chapter 1, the primary role of the LDTg in regulating dopamine firing is to gate bursting activity (Lodge and Grace, 2006; Grace et al., 2007). While stimulation of the LDTg alone does not significantly affect burst firing of VTA dopamine neurons, burst firing

cannot be induced when this structure is inactivated (Lodge and Grace, 2006). Thus, it is believed that the LDTg acts as a permissive gate allowing for the induction of bursting. The LDTg also appears to play a role in the regulation of spontaneous dopamine activity as LDTg inactivation produces highly regular, pacemaker-like firing, similar to what is observed *in vitro* (Grace and Onn, 1989; Lodge and Grace, 2006). The LDTg sends efferent projections to the VTA in addition to a number of brain regions, including the PPTg, that innervate the VTA (Semba and Fibiger, 1992; Oakman et al., 1995). The precise mechanism by which the LDTg regulates dopamine activity, however, is presently unknown.

Another primary excitatory projection to the VTA arises from the bed nucleus of the stria terminalis, or BNST (Georges and Aston-Jones, 2001, 2002; Fields et al., 2007). The BNST projects directly to VTA dopamine neurons and has been implicated in stress output and reward-seeking behavior (Herman and Cullinan, 1997; Georges and Aston-Jones, 2001; Dumont et al., 2005). Single-pulse stimulation of the BNST has been shown to activate >80% of dopamine neurons in the VTA (Georges and Aston-Jones, 2002). Interestingly, stimulation of the BNST does not significantly affect GABAergic neurons in the VTA suggesting that, similar to afferents from the prefrontal cortex, projections from the BNST also exhibit target specificity (Carr and Sesack, 2000; Georges and Aston-Jones, 2002; Sesack et al., 2003). In addition to increasing general dopaminergic activity in the VTA, the BNST has also been shown to modulate phasic bursting activity (Georges and Aston-Jones, 2002). Microinfusion of glutamate into the BNST significantly increases burst firing of dopamine neurons while intra-BNST infusion of GABA selectively attenuates bursting activity (Georges and Aston-Jones, 2002). Thus, the BNST plays an important excitatory role in the regulation of both spontaneous and phasic dopamine neuron activity.

Of the four primary structures that provide excitatory innervation of the VTA, the structure selected for investigation in this work was the PPTg. The PPTg, like the LDTg, provides both glutamatergic and cholinergic innervation of the VTA and has been shown to regulate burst firing of dopamine neurons directly (Semba and Fibiger, 1992; Oakman et al., 1995; Floresco et al., 2003). To date, the PPTg is the only structure, among those described here, which has been demonstrated to regulate phasic dopamine activity in awake, freely moving rats. More specifically, the PPTg has been shown to mediate cue-induced, phasic activation of dopamine neurons in rats performing in reward-seeking behavior (Pan and Hyland, 2005). Due to the similarity in experimental design, the PPTg was chosen here to assess afferent modulation of cue-evoked dopamine release during ICSS.

**Future Directions**

While the findings presented in this dissertation answer a multitude of questions concerning regulation of phasic dopamine transmission and its relationship with reward seeking, they also give rise to a number of questions that will hopefully drive future research. This section will focus on outlining potential experiments that would further elucidate the functional role of dopamine and expand our understanding of the systems or mechanisms that govern its release.

*The role of phasic dopamine in modulating accumbal firing*

Combined electrophysiological/electrochemical techniques have allowed us to measure both cell firing and phasic dopamine release simultaneously in freely moving rats. Results from these experiments have revealed remarkable correlations between patterned unit activity and transient dopamine release events in the NAc during reward-seeking behaviors (Cheer et al., 2005; Owesson-White et al., 2009). During ICSS, phasic dopamine release in the NAc is typically associated with an inhibition of cell firing, although increases have been reported in a small number of cells (Cheer et al., 2005; Cheer et al., 2007). Future experiments could utilize iontophoresis, coupled with the combined electrophysiology/electrochemistry technique, to establish a causative link between dopamine release and changes in neural firing. Iontophoretic application of D1-like and D2-like receptor antagonists would provide unprecedented insight into the precise mechanism by which dopamine functions to alter unit activity in the NAc.

*Phasic dopamine release and synaptic strength in the nucleus accumbens*

Changes in synaptic strength have been shown to occur in the VTA during the course of reward-related learning and are thought to underlie the conversion of environmentally neutral stimuli into reward-predictive stimuli (Stuber et al., 2008). In addition, dopamine-dependent changes in synaptic plasticity have been found to occur in the striatum (Calabresi et al., 1997). One study, in particular, demonstrated that ICSS induces potentiation of corticostriatal synapses (Reynolds et al., 2001). This potentiation was found to be dependent on the D1-like dopamine receptor and was correlated with the amount of time it took the rats

to learn the behavior (Reynolds et al., 2001). Similar enhancements in synaptic strength may also occur in the NAc during ICSS learning. However, investigations of how phasic dopamine release affects synaptic plasticity in the NAc remain to be done. While it has been shown that tonic NAc dopamine does not significantly influence synaptic strength, tonic dopamine levels- reported to be between 5-20 nM- are unlikely to activate the D1-like dopamine receptor which, in turn, is critical for the induction of LTP (Richfield et al., 1989; Parsons and Justice, 1992; Pennartz et al., 1993; Reynolds et al., 2001). Conversely, phasic dopamine release can produce concentrations sufficient to activate the D1-like dopamine receptor and may therefore contribute to changes in synaptic strength in the NAc. Therefore, future investigation of the role of phasic dopamine release in accumbal synaptic plasticity may reveal a novel role of phasic dopamine transmission.

*Neural control of reward seeking*

It has been proposed that the NAc exerts its effects on goal-directed behaviors via its efferent projections to motor areas (Mogenson et al., 1980). As discussed above, both the lateral hypothalamus and ventral pallidum are NAc targets involved in mediating behavior (Ikemoto and Panksepp, 1999). Electrophysiological data suggests that these structures may become activated upon phasic dopamine release in the NAc (Cheer et al., 2005). To get a better idea as to how, or if, each of these target structures regulates ICSS, microinjection techniques may be employed to alter neural activity in these structures during ICSS. Elucidation of the contributions that these structures make to the maintenance of operant

behavior would give us a clearer picture of how phasic dopamine release in the NAc operates on a system level to alter reward-seeking behavior.

*Afferent mechanisms that modulate phasic dopamine release*

In addition to the PPTg, the BNST also sends excitatory projections to the VTA that have been shown to modulate burst firing of dopamine neurons (Georges and Aston-Jones, 2002). As such, it would be interesting to evaluate the role of the BNST in modulating phasic dopamine release. Similar to the experiments done in Chapter 5, BNST activity could be attenuated via microinjection of either lidocaine or a baclofen/muscimol cocktail and subsequent changes in phasic, cue-evoked dopamine release could be assessed during ICSS. Such an experiment could provide more information about the afferent mechanisms that mediate phasic dopamine release. Indeed, data presented in Chapter 5 suggest that additional afferents, besides the PPTg, also contribute to cue-evoked dopamine release in the NAc. Unilateral inactivation of the PPTg produced approximately a 35% decrease in the amplitude of cue-associated dopamine transients while unilateral intra-VTA administration of a glutamatergic antagonist produced greater than a 50% decrease in phasic dopamine signals (Chapter 4, experiment 3). Therefore, it is likely that excitatory inputs from additional brain regions contribute to cue-evoked dopamine release in the NAc. Of the 3 uninvestigated brain structures that provide glutamatergic innervation of the VTA, electrophysiological data suggest that the BNST is the most likely candidate for this role (Georges and Aston-Jones, 2001, 2002).

*Additional receptors involved in mediating burst firing*

While glutamatergic and cholinergic drugs have been extensively implicated in the regulation of dopaminergic burst firing, other classes of drugs have also been shown to promote bursting. For example, apamin, a $Ca^{2+}$ gated $K^{+}$ channel antagonist, can facilitate burst firing *in vitro* where deafferented dopamine neurons typically fire in a highly regular, pacemaker-like fashion (Grace and Onn, 1989; Seutin et al., 1993). In addition, L-type $Ca^{2+}$ channels are also believed to facilitate burst firing of dopamine neurons (Grillner and Mercuri, 2002; Zhang et al., 2005). Thus, both of these channels may provide targets for future research concerning the regulation of phasic dopamine transmission.

**Implications**

As mentioned previously, all drugs of abuse converge upon and activate the mesolimbic dopamine system (Nestler, 2005). Thus, despite their distinct mechanisms of action, drugs of abuse activate the same neural circuits discussed here. As such, the findings presented in this dissertation have substantial implications for addiction research. Further investigation of the precise role of phasic dopamine release in the NAc, in addition to elucidation of the interactions between dopaminergic and other transmitter systems that promote reward seeking will greatly enhance our understanding of the neural mechanisms that regulate addiction.

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