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Causal roles for dopamine neurons in learned appetitive behaviors

by

Elizabeth E. Steinberg

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Neuroscience

in the

GRADUATE DIVISION

of the

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by Elizabeth E. Steinberg

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Through no fault of my own, I have managed to accumulate a wonderful collection of colleagues, classmates, family and friends. Without their wisdom and support, my time in graduate school would have been much less enjoyable, and less productive.

I am incredibly fortunate to have found my way to Tricia's office door as a first-year doctoral student. Tricia is (capital letters, bold, italic, underline) a ***<u>TREMENDOUS</u>*** person, mentor and scientist. Much to her credit, she has absorbed my scientific joys and sorrows with equal composure. Over the years I have managed to push through to the next project, the next experiment, and (finally) the next paper by relying on her seemingly boundless support. I am extremely grateful to have had not just an extremely capable scientific advisor but also an all-around role model to learn from.

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my mom, Donna Steinberg, who in years past would uncomplainingly stay awake and keep me company while I worked into the wee hours finishing school projects, drove me to innumerable practices, concerts, tests etc., made reasonable and unreasonable personal sacrifices for the sake of my well-being, and has generally been my #1 cheerleader in life. I am also grateful for the love and guidance of my dad, Eugene Steinberg, who taught me how to be a neurotic workaholic and still a nice person, and never lets his daughters forget that they are the center of his universe. And, after years of hair-pulling, door-slamming and petty arguments about who got to pick what to watch on TV, my little sister Susannah turned out to be a totally impressive business woman and a lot of fun to be around. I am grateful to her for still hanging out with me even though she is substantially cooler.

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Abstract of the Dissertation

Much of the behavior of humans and other animals is directed towards seeking out edible, social, cognitive or drug rewards. Dopamine, a neurotransmitter released by a handful of cells in the brain, is known to be essential for reward-seeking behavior. Yet despite decades of focused investigation, the nature of its influence remains a matter of considerable debate. The precise roles that dopamine neurons play are likely to depend on their afferent and efferent connectivity, the timing and length of neural activation, and the features of the behavior under investigation. Accordingly, it is becoming increasingly appreciated that delineating the specific contributions of dopamine neurons to cellular, circuit, and systems-level phenomena will require more sophisticated control over their patterns of activity than conventional electrophysiological and pharmacological techniques can provide.

Recently developed optogenetic tools hold great promise for disentangling these complex issues. In Chapter 2, I describe the development and characterization of a novel transgenic rat line that permits selective opsin expression in ventral tegmental area dopamine neurons, as well as other catecholamine neurons in the rat brain. I then utilize this tool to determine if temporally-precise patterns of dopamine neuron activity are causally related to appetitive behaviors. In Chapter 3, I present data from a series of experiments designed to clarify the role of ventral tegmental area dopamine neurons, or their major efferent projection to the nucleus accumbens, in positive reinforcement.

Then in Chapter 4, I describe experiments designed to determine if ventral tegmental area dopamine neurons are capable of supporting associative cue-reward learning. Collectively, my results demonstrate that ventral tegmental dopamine neurons are sufficient to reinforce instrumental responding, and that their ability to influence reward-seeking behavior is at least partly due to the fact that they can drive associative learning directly. As such, they represent an important advance in our understanding of the neural basis of learned appetitive behaviors.

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Chapter 1

Introduction: Dopamine neurons and learned appetitive behaviors

Early studies

The neural and behavioral functions of dopamine have been the focus of intense scientific investigation since the middle of the twentieth century. At this time Arvid Carlsson discovered that this molecule was indeed a neurotransmitter, and not simply a precursor for norepinephrine. He went on to demonstrate that dopamine was present at high levels in the striatum, an area of the brain important for generating movement (Carlsson et al., 1958; Carlsson, 1959). This important insight eventually led to the discovery that the movement problems that constitute the hallmark symptoms of Parkinson's disease (bradykinesia, tremor and rigidity) result from the degeneration of dopamine neurons. Experimentally induced insults to dopamine systems in rats produced aphagia, adipsia and akinesia, mimicking some aspects of Parkinson's. Further, dopamine agonists were found to ameliorate some motor difficulties in experimental animals and human patients, supporting an important role for dopamine in motor function (Carlsson, 1964; Bernheimer et al., 1973; Marshall et al., 1974; Nisenbaum et al., 1986; Zetterstrom et al., 1986; Carey, 1990).

While movement difficulties represent some of the most easily recognized symptoms in Parkinsonian patients, afflicted individuals can also develop cognitive or behavioral disturbances. This early insight suggested that dopamine had non-motor functions, a conception that was further supported by a separate line of investigation

into the acute effects of focal intracranial stimulation. In a landmark study, Olds and Milner (Olds and Milner, 1954) demonstrated that electrical stimulation of certain brain areas was highly reinforcing (Fig. 1). They found that rats would make hundreds or even thousands of responses to obtain such stimulation, a behavior referred to as intracranial self-stimulation (ICSS).

Figure 1

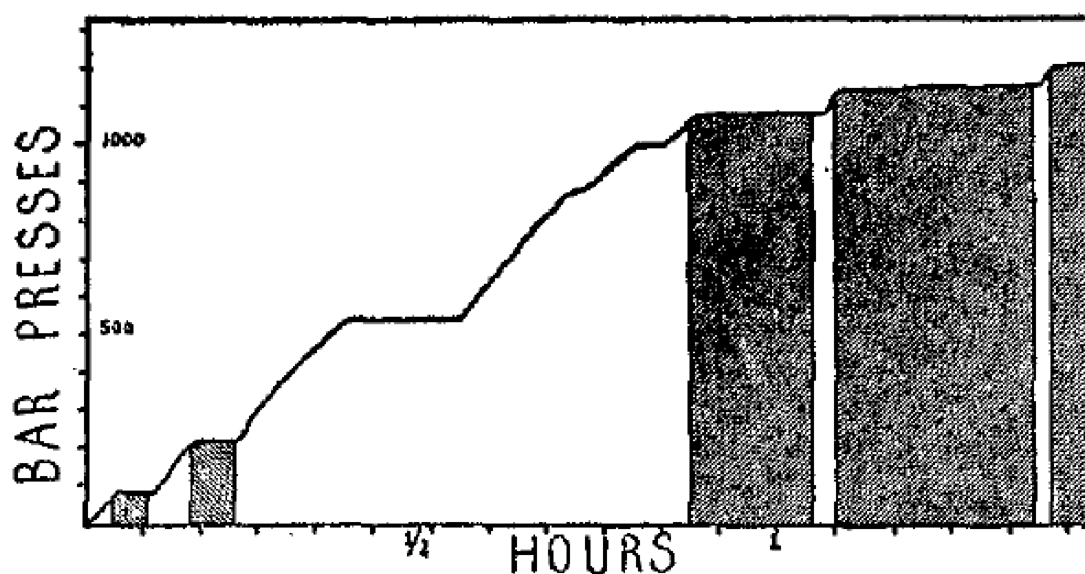

Figure 1. *Focal electrical stimulation drives vigorous operant responding.* Cumulative bar presses in a rat responding for electrical stimulation of the medial tegmentum. Open areas indicated times when stimulation was available, shaded areas indicate extinction conditions. *Reproduced from Olds and Milner 1954.*

Importantly, areas where dopamine neurons were localized, or their broad projections, were found to be particularly effective in eliciting robust ICSS (Corbett and Wise, 1980). Further, administration of dopamine antagonist caused changes in ICSS behavior that resembled extinction (Fouriezos and Wise, 1976), suggesting that

dopamine could be mediating the reinforcing effects of ICSS. Scientific investigation into the neural actions of addictive drugs also pointed to important roles for dopamine neurons in reward-related behaviors. Many drugs of abuse including cocaine, amphetamine, alcohol, morphine, marijuana and tobacco were found to evoke dopamine release (Di Chiara and Imperato, 1985; Imperato et al., 1986; Hernandez and Hoebel, 1988; Chen et al., 1990; Rada et al., 1991). Collectively, these observations triggered widespread interest in dopamine systems and the roles that they play in motivated behaviors, a trend that continues to the present day.

Current hypotheses of dopamine function in behavior

Research over the ensuing decades has led to a number of hypotheses about the specific roles that dopamine neurons play in motivated behavior. Some of the most prominent current conceptions are described briefly below.

The effort hypothesis

Early studies that employed pharmacological tools to disrupt dopamine transmission during reward-seeking documented the profound behavioral changes such treatments can cause. For example, treatment with pimozide, a dopamine receptor antagonist, dramatically reduces operant responding for food, saccharin or ICSS (Fouriezos and Wise, 1976; Wise et al., 1978b; Wise et al., 1978a). On the basis of this and other evidence, it was proposed that dopamine (specifically, dopamine acting in the NAc) serves to mediate the hedonic value of food and drug rewards. However,

subsequent studies indicated that this conception was overly simplistic. Problematic for the notion that dopamine is equivalent to hedonic value was the observation that dopamine neurons are not always activated by primary rewards such as food (Mirenowicz and Schultz, 1994) and in fact are sometimes activated by aversive stimuli such as noxious footshock (Brischoux et al., 2009; Matsumoto and Hikosaka, 2009). Taste reactivity studies, which are considered by many to measure “pure” hedonic value, reveal minimal consequences of manipulating dopaminergic transmission (Berridge et al., 1989; Wyvell and Berridge, 2000; Tindell et al., 2005). Furthermore, dopamine depletions do not impair operant responding for rewards delivered on continuous reinforcement schedules (Aberman and Salamone, 1999). Collectively, these observations do not support the hypothesis that dopamine neurons encode hedonic value.

In contrast, dopamine depletions do significantly reduce responding for natural rewards when substantial effort is required to obtain the reinforcer. While rats with dopamine lesions respond normally for food on fixed-ratio schedules with low effort requirements, their responding is significantly impaired when the effort required to obtain a reinforcer is increased (Aberman and Salamone, 1999). Similar results have been obtained in concurrent choice or cost/benefit tasks, where animals are allowed to choose between an easily-obtained but less preferable food and one that is harder to get but more preferred. Dopamine-depleted rats choose the reward that requires the least effort to obtain, whereas untreated rats will work harder to get a preferred reward (Salamone et al., 1991; Cousins et al., 1994; Cousins et al., 1996; Fig. 2). Importantly, this

difference is not due to deficits in the ability to recognize which reward is more valuable, as dopamine-depleted rats still show a strong preference when allowed to choose freely during consumption tests. Taken together, these results have led to the idea that dopamine is important for the allocation of energy resources towards more effortful tasks when this strategy is advantageous (Salamone et al., 1997).

Figure 2

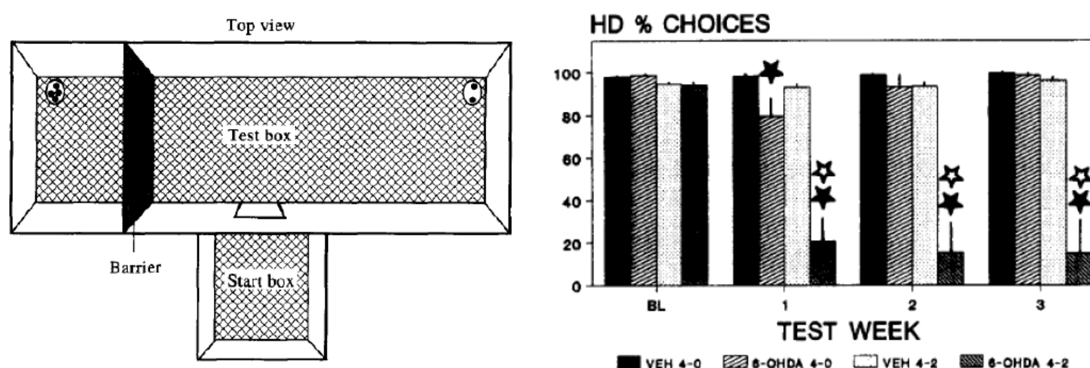

Figure 2. *Dopamine depletion impairs responding for food when response costs are high.*

Left, the t-maze apparatus used to test the effects of dopamine lesions in a cost/benefit task. Rats were allowed to freely choose between the two arms. One arm contained a high density of reward (4 pellets) but could only be accessed by climbing over a large barrier. The other arm contained a lower density of reward (2 or 0 pellets) but was easily accessible. *Reproduced from Salamone, Cousins and Snyder 1997.*

Right, mean number of choices of the high-density arm in vehicle- and 6-OHDA-treated rats. The data shown are for the last week of pre-surgical baseline testing (BL), and for the first three weeks following surgery. Dopamine-lesioned rats do not choose the large reward arm when a smaller reward is available (4-2 condition). Notably, they do choose the large reward arm when there are no alternatives (4-0 condition), indicating that they are physically capable of climbing over the barrier. *Reproduced from Cousins, Atherton, Turner and Salamone 1996.*

The reward prediction error hypothesis

Some of the most influential insights into dopamine neuron function have come from *in vivo* electrophysiological studies, where the activity of single putative dopamine neurons[1] is recorded in behaving animals. In a seminal series of experiments, Wolfram

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[1] One of the main objectives of electrophysiology experiments is to observe the physiological properties of specific neural population(s) in order to understand how these population(s) integrate into larger neural circuits. This approach is only valid to the extent that the neural populations of interest can be reliably identified.

A large number of recording studies (both *in vitro* and *in vivo*) have used electrophysiological and pharmacological criteria to identify dopamine neurons. These criteria include: (1) long duration action potentials and a slow spontaneous firing rate (2-10 Hz) with some bursting, (2) presence of a hyperpolarization-activated inward current, referred to as an I_h, (3) dopamine or D2R agonist-mediated inhibition, and (4) lack of μ opioid receptor agonist-induced inhibition (Ungless and Grace, 2012). These criteria were based on early recordings made primarily in the SNc (Bunney et al., 1973; Grace and Bunney, 1980, 1983; Lacey et al., 1987; Grace and Onn, 1989; Lacey et al., 1989) where recorded neurons were cytochemically identified *in vitro* and *in vivo*; neurons that were later confirmed to be dopaminergic had these electrophysiological and pharmacological properties.

The SNc is approximately 90% dopaminergic (Margolis et al., 2006a), and in this structure dopamine neurons can be reliably identified using these characteristics (Brown et al., 2009). However these criteria have been called into question when used to identify dopamine neurons in the VTA, where only 60% of the neurons are dopaminergic (Swanson, 1982; Margolis et al., 2006a; Nair-Roberts et al., 2008). Intriguingly, numerous exceptions to the "characteristic" electrophysiological and pharmacological properties of dopamine neurons can be found in this area. First, when considered as a population, cytochemically identified dopamine and non-dopamine neurons have overlapping firing rates and action potential widths *in vitro* and *in vivo* (Ungless et al., 2004; Margolis et al., 2006b; Margolis et al., 2006a; Margolis et al., 2012 but see Ungless and Grace, 2012). Second, some identified non-dopamine neurons exhibit an I_h (Cameron et al., 1997; Jones and Kauer, 1999; Margolis et al., 2003; Margolis et al., 2006b; Margolis et al., 2006a; Margolis et al., 2012 but see Wanat et al., 2008; Mao et al., 2011) and some identified dopamine neurons do not have an I_h (Jones and Kauer,

1999; Lammel et al., 2008; but see Margolis et al., 2006b; Margolis et al., 2006a; Margolis et al., 2012). Third, some identified non-dopamine neurons are inhibited by dopamine agonists (Johnson and North, 1992; Cameron et al., 1997; Margolis et al., 2006a; Margolis et al., 2008) and some identified dopamine neurons are not inhibited by dopamine agonists (Margolis et al., 2006a; Lammel et al., 2008; Margolis et al., 2008). In fact, up to 40% of cells that were presumed to be non-dopamine neurons based on their electrophysiological properties were inhibited by dopamine *in vivo* (Yim and Mogenson, 1980). Fourth, some dopamine neurons are actually inhibited by μ opioid agonists (Cameron et al., 1997; Margolis et al., 2003). Fifth, a significant percentage of rodent VTA, especially in the medial regions, consists of glutamatergic neurons, whose physiological properties have not been reported (Gorelova et al., 2012; Yamaguchi et al., 2007).

These findings indicate that judgments regarding the neurotransmitter content of recorded VTA neurons solely on the basis their electrophysiological and pharmacological properties are problematic. Existing solutions to this problem are juxtacellular or intracellular labeling of recorded cells with post-hoc cytochemical verification of neurotransmitter content; alternatively transgenic animals with genetically labeled dopamine or non-dopamine neurons can be used. However, these approaches are generally not practical for gathering large data sets in *in vivo* preparations where the difficulty of labeling or visualizing recorded cells is prohibitive.

Undoubtedly, some (perhaps even most) of the neurons identified with existing criteria are indeed dopaminergic. However any misattribution of response properties will make the task of understanding a complex and multi-functional circuitry more difficult; this issue will be particularly true when sample sizes are small or responses are heterogeneous (Ungless and Grace, 2012).

A recently published study used ChR2 to identify dopaminergic and GABAergic neurons in the VTA of behaving mice, circumventing the difficulties associated with identifying dopamine neurons using electrophysiological and pharmacological criteria (Cohen et al., 2012). In this study, neurons had to meet several rigorous standards to be identified as dopaminergic or non-dopaminergic. Identified neurons showed close correspondence between optically-evoked and spontaneous waveform shapes, short latency and low temporal jitter of optically-evoked spikes, and the ability to follow high-frequency (50 hz) trains of optical stimulation with high fidelity. Notably, no relationship was observed in identified neurons between neurotransmitter content and electrophysiological properties such as spike duration or spontaneous firing rate, criteria that are commonly used to identify putative dopamine neurons. However the authors of

Schultz and colleagues demonstrated that putative dopamine neurons respond strongly to the delivery of natural rewards, such as a drop of juice. However if juice delivery is preceded by another stimulus that reliably predicts its occurrence, such as a tone, these neurons will start responding to the reward-predicting tone and stop responding to the juice itself (Romo and Schultz, 1990; Ljungberg et al., 1992; Schultz et al., 1993; Mirenowicz and Schultz, 1994). Importantly, the transference of the neural response from the primary reward to its predictor happens gradually as the tone-juice association is learned (Schultz et al., 1997; Hollerman and Schultz, 1998). Early in learning, the neural response to the juice is much stronger than the response to the tone. After the tone-juice association is established, the neural response to the tone is much stronger than the response to the juice. Furthermore, once the association is well-learned, if the tone is presented but the usual juice reward is omitted, dopamine neurons briefly decrease their activity below baseline levels (Schultz et al., 1993; Fig. 3). This set of observations led to the idea that dopamine neurons encode a signed reward prediction error, increasing their activity at the earliest predictor of reward (or in the case of an unpredicted reward, coincident with the reward itself), and decreasing their activity if an expected reward fails to materialize. In part because of the close correspondence between the dopamine reward prediction error signal and 'teaching' signals called for by contemporary learning theories, dopamine neurons are suggested to drive some forms of learning directly (Montague et al., 1996; Schultz et al., 1997; Glimcher, 2011).

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this study did find that some identified dopamine neurons signaled reward prediction errors in a manner that was consistent with the firing patterns described in this section.

Figure 3

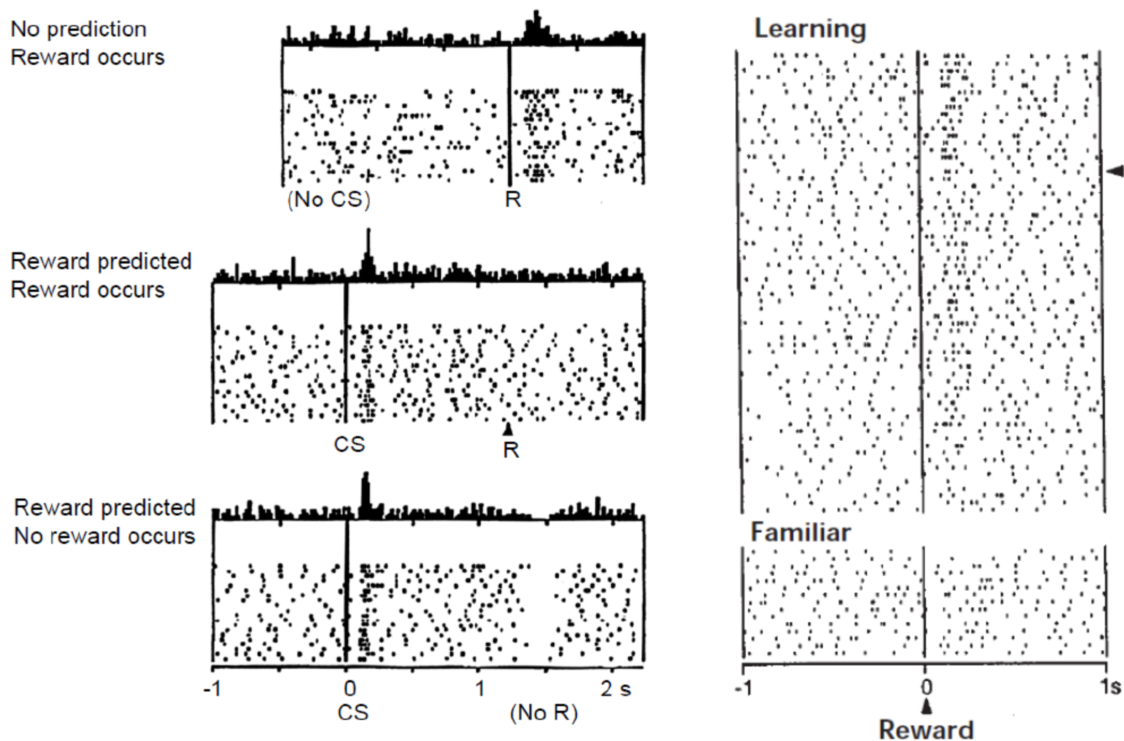


Figure 3. *Dopamine neurons encode reward prediction errors.*

Left panel: *Top,* response of a single putative dopamine neuron to unsignaled reward delivery. Each line of dots represents action potentials during a single trial. Note the strong response to reward. *Middle,* response of the same neuron to a cue that predicts reward delivery after this association has been well-learned. Note the strong response to the cue and little to no reward response. *Bottom,* response of the same neuron to the cue when the expected reward is withheld. Note that baseline firing is inhibited at precisely the time that reward would have been delivered. *Reproduced from Schultz, Dayan and Montague 1997.*

Right panel: Response of a single putative dopamine neuron to reward delivery over the course of learning. Early in the recording session, when the monkey had not learned the cue-reward association, reward delivery evokes a phasic neural response. This response progressively fades as learning progresses. *Reproduced from Hollerman and Schultz 1998.*

The incentive salience hypothesis

Other evidence suggests that dopamine functions to make primary rewards, and the environmental stimuli that signal their availability, 'wanted.' Although these concepts are closely intertwined, 'wanting' is distinct from hedonic 'liking.' And while 'wanting' triggered by a reward or a reward-associated cue requires prior experience with these stimuli (and hence is a property acquired through learning), it is not in itself a learning mechanism. Dopamine is proposed to give rewards and reward-associated cues motivational (also known as *incentive*) power, causing these stimuli to bias attentional resources by making them very *salient*. Thus, this hypothesis suggests that dopamine is important to imbue rewards and cues with *incentive salience* (Berridge and Robinson, 1998; Berridge, 2007). According to this theory, NAc dopamine is required for the ability of incentive stimuli, such as reward-paired cues, to motivate behavioral responding towards that cue, and to allow the cue to enhance behavior directed toward obtaining the reward (Berridge and Robinson, 1998).

Evidence is support of this notion has primarily come from studies that employ pharmacological or genetic tools to boost or suppress dopaminergic transmission. Sensitization of mesolimbic dopamine circuits with amphetamine causes dramatic increases in cue-triggered reward-seeking in a Pavlovian-instrumental-transfer paradigm, which the authors interpret as an elevation in cue-triggered 'wanting' (Wyvell and Berridge, 2000, 2001; Fig. 4). Conversely, suppressing dopamine transmission in the same paradigm reduced cue-triggered reward seeking (Dickinson et al., 2000).

Figure 4

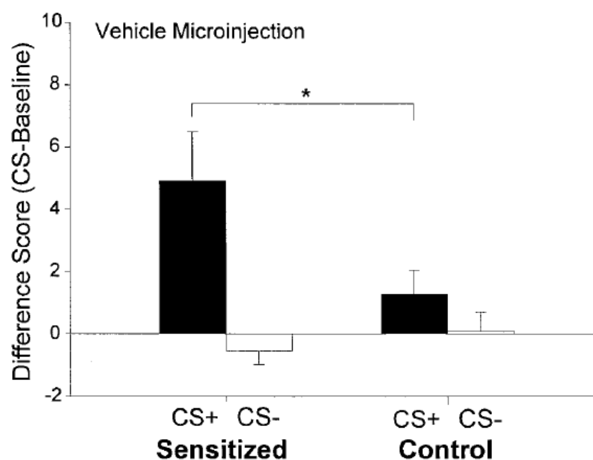


Figure 4. *Increased cue-triggered 'wanting' in amphetamine-sensitized rats.* Reward-associated cues trigger greater reward-seeking in rats with pharmacologically sensitized dopaminergic transmission (Pavlovian-Instrumental-Transfer test, performed under extinction conditions). *Reproduced from Wyvell and Berridge 2001.*

Transgenic mouse models with chronic hyper- or hypo-dopaminergic transmission (accomplished via knockdown of the dopamine transporter or interference with dopamine synthesis, respectively) also show enhanced or reduced behavior indicative of 'wanting' sweet rewards, without demonstrated impairments in 'liking' them (Cannon and Palmiter, 2003; Pecina et al., 2003; Hnasko et al., 2005; Robinson et al., 2005; Cagniard et al., 2006; Yin et al., 2006). Lastly, using a behavioral paradigm that allows a reward-associated cue's incentive and predictive properties to be examined separately (Flagel et al., 2007), a recent study demonstrated that dopamine was necessary for cues to acquire incentive value (Flagel et al., 2010).

Dopamine neurons are embedded within diverse circuits

Dopamine neurons are primarily clustered in two nuclei in the mammalian midbrain, the ventral tegmental area (VTA) and substantia nigra pars compacta (SNc). Anatomical tracing studies reveal that dopamine neurons receive inputs from many sources (Bunney and Aghajanian, 1976; Phillipson, 1979; Geisler and Zahm, 2005; Geisler et al., 2007; Zahm et al., 2011; Watabe-Uchida et al., 2012). Both VTA and SNc receive inputs from neurons in the striatum, pallidum, hypothalamus, amygdala, cortex, thalamus, hindbrain and other midbrain regions although in most cases distinct groups of neurons within these structures innervate the VTA and SNc (Watabe-Uchida et al., 2012). In addition, dopamine neurons receive input from local GABA, glutamate and dopamine neurons (Fields et al., 2007).

Just as VTA and SNc neurons receive diverse inputs, these neuronal populations also send efferent projections to many parts of the brain including the striatum, globus pallidus/ventral pallidum, subthalamic nucleus, thalamus, LHb, amygdala, bed nucleus of the stria terminalis, hippocampus, lateral septum, lateral hypothalamus, prefrontal and entorhinal cortex, raphe nuclei, parabrachial nucleus, locus coeruleus, and other midbrain regions including the periaqueductal grey (Beckstead et al., 1979; Swanson, 1982; Fields et al., 2007). Almost all of these areas also send a reciprocal projection. VTA and SN projection neurons target largely non-overlapping neural populations in these structures, especially in striatum where VTA projects primarily to ventral striatum while SNc projects primarily to dorsal striatum (Beckstead et al., 1979).

There are a significant number of non-dopamine (primarily GABAergic, with some glutamatergic) neurons in the VTA, and to a lesser extent in the SNc. These non-dopamine neurons send efferent projections both within and outside of the VTA and SNc (Swanson, 1982; Margolis et al., 2006a; Fields et al., 2007; Dobi et al., 2010; Yamaguchi et al., 2011). In the VTA, approximately 60% of the neurons are dopaminergic (Swanson, 1982; Margolis et al., 2006a; Nair-Roberts et al., 2008) and the non-dopamine content for efferent projections ranges from 20% (NAc) to >99% (lateral habenula and locus coeruleus) (Swanson, 1982; Fields et al. 2007; Fig. 5).

Figure 5

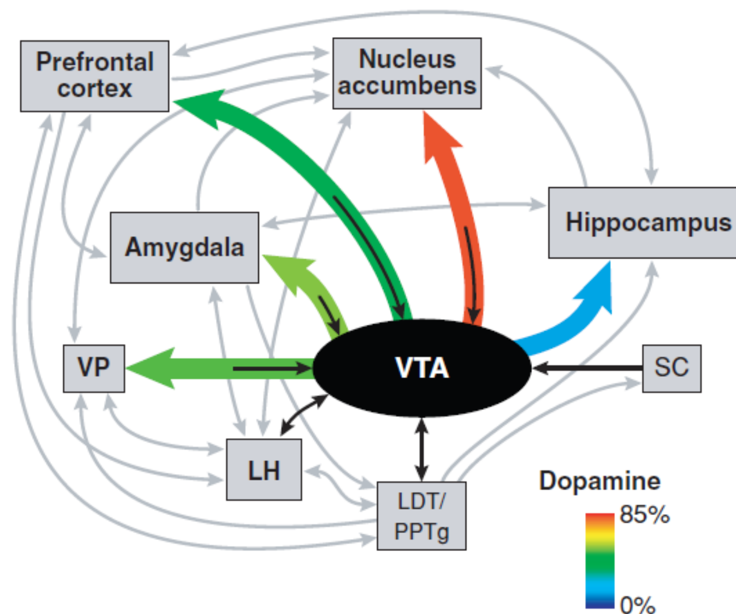


Figure 5. *Major afferent and efferent projections of the VTA. Reproduced from Fields, Hjelmstad, Margolis and Nicola 2007.*

As described in the preceding sections, dopamine neurons have been implicated in a variety of motor and cognitive functions. Given the diversity of brain circuits in which these neurons are embedded, it is unlikely that a single function can be ascribed to this neural population. The precise roles that dopamine neurons play in learned appetitive behaviors will depend on their afferent and efferent connectivity, the timing and length of neural activation, and the nature of the behavior under investigation.

Consistent with this idea, there is much evidence to suggest that dopamine release at one set of terminals may facilitate different behaviors than dopamine release at other sets of terminals (Amalric and Koob, 1993; Nicola, 2007; Robbins and Everitt, 1992; 2007; Nicola, 2010). For example, in reaction time tasks in which rats are well-trained to respond rapidly to a stimulus, dopaminergic depletions of the dorsal striatum decrease the rapidity of the response (Brown and Robbins, 1991), while depletions of the NAc have no effect (Amalric and Koob, 1987; Brown and Robbins, 1991). In contrast, NAc dopamine depletions or local dopamine antagonist infusion into the NAc impair approach behavior in response to food and drug rewards particularly for Pavlovian conditioned responses (Saunders and Robinson, 2012; Parkinson et al., 2002).

Dopamine release in other brain regions, such as the prefrontal cortex and the amygdala, has also been studied. Dopamine in the prefrontal cortex appears to affect working memory and impulsivity (Goldman-Rakic, 1990; Dagher and Robbins, 2009; Cools and D'Esposito, 2011; Dalley et al., 2011) while in the amygdala it may be critical for the modulation of emotional learning (Lamont and Kokkinidis, 1998; Rosenkranz and Grace, 2002; Fadok et al., 2010; Phillips et al., 2010; Tye et al., 2010). Taken together,

these results demonstrate that the effects of manipulation of the dopamine system clearly depend upon which dopaminergic projection is affected.

Physiological responses of putative dopamine neurons

Studies that employ electrophysiological techniques to record the activity of putative dopamine neurons have revealed that they fire in distinct temporal modes, either phasic or tonic. Within these classes, sustained activations as well as brief pauses in activity have also been observed. Phasic activity has received the most experimental attention. Recordings from behaving primates and rodents consistently show that putative dopamine neurons in VTA and SNc are briefly activated by primary rewards, reward-predictive cues, salient stimuli, and in some cases, aversive stimuli (Strecker and Jacobs, 1985; Mirenowicz and Schultz, 1994; Tobler et al., 2005; Roesch et al., 2007; Brischoux et al., 2009; Matsumoto and Hikosaka, 2009). These responses have an onset latency of 60-100 ms and typically last for less than 200 ms; they consist of a single action potential or a burst of spikes with an average frequency of 20-100 Hz (Schultz, 2007).

Rewards and reward-predictive cues also elicit phasic dopamine release in efferent targets (Roitman et al., 2004; Flagel et al., 2010; McCutcheon et al., 2012), although the relationship between dopamine neuron activation and dopamine release can be complex, and a one-to-one correspondence between dopamine neuron activation and dopamine release should not be assumed. For example, Garris and colleagues showed that electrical stimulation of the VTA evokes either robust or very

little dopamine release, depending on whether the stimulation was delivered by the experimenter or contingent on an instrumental response made by a well-trained subject (Garris et al., 1999). Similarly, Montague and colleagues demonstrated that the magnitude of an electrical stimulation-evoked dopamine transient depended strongly on the neuron's recent firing history (Montague et al., 2004). Phasic burst firing is thought to depend on glutamatergic and cholinergic excitatory synaptic drive onto dopamine neurons from a number of areas, including the pedunculopontine tegmental nucleus, laterodorsal tegmental nucleus, prefrontal cortex and subthalamic nucleus (Smith and Grace, 1992; Tong et al., 1996; Floresco et al., 2003; Lodge and Grace, 2006). In addition to stimulus-evoked bursts, most putative dopamine neurons also exhibit spontaneous bursts of comparable intensity and duration (Grace and Bunney, 1984; Hyland et al., 2002). These bursts are likely to be the source of spontaneous dopamine transients observed in target structures such as the NAc (Sombers et al., 2009).

A subset of putative dopamine neurons has been reported to exhibit sustained activity prior to reward if reward delivery is uncertain (i.e., rewards given with $p < 1$). Because the magnitude of sustained activation is greatest when reward delivery is least certain (at $p = 0.5$), this signal has been suggested to code for reward uncertainty. This signal lasts at least 5-10 times longer than the phasic activations seen to rewards and cues but only represents a modest increase (up to 40%) from baseline activity levels, much lower than the peak rate during phasic activations (Fiorillo et al., 2003, 2005; Schultz, 2010).

In the absence of specific stimuli, putative dopamine neurons mostly show low-level tonic activity that is driven by pacemaker-like membrane currents (Grace and Bunney, 1984; Grace and Onn, 1989; Hyland et al., 2002). Tonic activity is regulated by afferent structures such as the ventral pallidum (Floresco et al., 2001; Floresco et al., 2003). Tonic firing of dopamine neurons is thought to generate low-level dopamine release that constitutes a basal dopamine “tone” (Goto and Grace, 2005; Schultz, 2007), although spontaneous bursts have recently been suggested to make a substantial contribution (Owesson-White et al., 2012). Notably, phasic firing is substantially more effective than tonic firing at evoking dopamine release (Gonon, 1988; Tsai et al., 2009).

Under some conditions, such as when a predicted reward is unexpectedly omitted, putative dopamine neurons briefly stop firing. The resulting pause begins approximately 100 ms after reward would have been delivered and lasts approximately 400 ms (Hollerman and Schultz, 1998) creating a break in tonic activity. Because this pause in firing is a disruption in the normally continuous dopamine signal, it may represent another form of temporal coding by dopamine neurons. In fact, several studies suggest that these pauses in dopamine neuron activity are uniquely poised to impact synaptic transmission in downstream structures (Goto and Grace, 2005; Matsuda et al., 2006).

After a dopamine neuron fires an action potential, the dopamine signal is still subject to modifications that can affect its temporal profile. Dopamine release can be modulated at the level of the axon terminal; striatal afferents of varied neurotransmitter content can alter dopamine release by activating presynaptic

receptors on dopamine neuron terminals, and this influence is independent of dopamine neuron activity (Chesselet, 1984; Zhang and Sulzer, 2012). Activation of presynaptic dopamine “autoreceptors” can suppress further dopamine release, either at the level of the axon terminal or via local dopamine release in VTA/SN at somatic autoreceptors (Chesselet, 1984; Fields et al., 2007). Other types of presynaptic receptors, including metabotropic glutamate receptors, opioid receptors, acetylcholine receptors and $GABA_B$ receptors have also been shown to modulate dopamine release in the striatum, in some cases elevating dopamine tone but in most cases suppressing it (Zhang and Sulzer, 2012). Further, dopamine is actively cleared from the extracellular space by dopamine transporters located presynaptically near synaptic contacts, restricting the amount of time in which released dopamine can act upon its receptors (Giros et al., 1991; Nirenberg et al., 1996; Schultz, 2007). Importantly, the kinetics of dopamine release and reuptake vary substantially by projection target, with relatively slow clearance in the basolateral amygdala and prefrontal cortex compared to striatal regions (Garris and Wightman, 1994).

Measurements of dopamine release over brief (milliseconds to seconds) time spans can be accomplished using *in vivo* voltammetry; these experiments have supplemented findings from *in vivo* electrophysiological recordings. These measurements made in dopamine terminal regions of rodents also reveal increases in dopamine release after rewards and reward-predictive cues, and in some cases, as with neural recording, the dopaminergic response to reward disappears when fully predicted by a preceding stimulus (Roitman et al., 2004; Day et al., 2007). While rewarding tastes

increase dopamine release, aversive tastes decrease dopamine release in the NAc (Roitman et al., 2008). Voltammetric studies have additionally revealed that transient dopamine signals can precede the initiation of internally-generated behavioral responding (Phillips et al., 2003; Wassum et al., 2012) indicating that phasic dopamine activity may be important for both internally-generated and cue-evoked behavior. Interestingly, dopamine release has also been observed in striatal “reward” regions in response to stress-inducing or painful stimuli (Anstrom et al., 2009; Budygin et al., 2012), in line with observed electrophysiological responses.

Cellular actions of dopamine

After release, dopamine can bind to receptors located either pre- or postsynaptically. There are 5 subtypes of dopamine receptors that are grouped into two classes based on their ability to activate intracellular signaling pathways. The D1 class (which includes D1R and D5R) stimulate G_s and G_{olf} proteins and ultimately lead to the activation of activating protein kinase A (PKA). The D2 class (which includes D2R, D3R, and D4R) stimulates G_o and G_i proteins. These GPCRs inhibit PKA, among other functions (Gerfen and Surmeier, 2011). The distribution and relative densities of these receptor subtypes shows great regional variation in the brain.

The firing mode of the dopamine-releasing neuron strongly impacts which type of receptor is likely to be bound after release. D1Rs are less efficacious at binding dopamine than D2Rs as measured by quantitative autoradiography in rat striatum (Richfield et al., 1989). Thus, when dopamine concentrations are low, such as during

tonic firing, D2Rs will be preferentially activated because of their heightened ligand affinity. In contrast when dopamine concentrations are high, such as during phasic firing, D1Rs will be preferentially engaged (Schultz, 2007).

Actions of dopamine at D1Rs and D2Rs can have sharply divergent effects on activity and plasticity in downstream targets, presumably because of the different intracellular signaling cascades they initiate. Dopamine-mediated plasticity has been most extensively studied in the dorsal striatum, which is the major efferent target of dopamine neurons in the SNc. The principal cell type in the striatum is the medium spiny neuron (MSN), which in rodents constitute 90% of all striatal neurons. Nearly half of the MSNs exclusively express D1Rs; these neurons express substance P and dynorphin, project directly to the SNr and internal segment of the globus pallidus (GPi), and are commonly referred to as the “direct” pathway. An equivalent number of MSNs exclusively express D2Rs; these neurons express enkephalin, project to the external segment of the globus pallidus (GPe), and are referred to as the “indirect” pathway (Gerfen and Surmeier, 2011). Additionally, a minority of MSNs express both D1Rs and D2Rs (Surmeier et al., 1996).

Dopamine modulates the strength and direction of synaptic plasticity, transitions between up and down states, and the ion channels that control spiking in dorsal striatal MSNs (Gerfen and Surmeier, 2011). Importantly, the features of dopamine modulation depend on which receptor subtype is being stimulated. Dopamine acting on D1R-expressing direct-pathway neurons is required for LTP and suppresses LTD in these cells (Pawlak and Kerr, 2008; Shen et al., 2008). In contrast, dopamine acting on D2R-

expressing indirect-pathway neurons is required for LTD, and suppresses LTP (Shen et al., 2008). Additionally, dopamine modulates local inhibition in the striatum by affecting GABA release by MSNs at recurrent axon collaterals; D1R activation potentiates local inhibition while D2R activation suppresses it (Guzman et al., 2003). This type of lateral inhibition has been suggested to be a potential mechanism whereby ensembles of striatal neurons can compete for control over behavior (Groves, 1983). Thus in dorsal striatum, phasic and tonic dopamine neuron activation are poised to initiate dichotomous downstream effects by acting on different receptors, and, by virtue of the divergent anatomical connectivity and plasticity mechanisms of the cells containing these receptors, engage different neural circuits.

Less is known about how dopamine influences neural activity and plasticity in the ventral striatum, and much of the data that does exist is contradictory (Nicola et al., 2000). MSNs in dorsal and ventral striatum have significant physiological similarities, but the anatomical connectivity of these two regions is distinct. Dorsal striatum receives glutamatergic inputs from a wide array of cortical areas and thalamus, while ventral striatum receives glutamatergic inputs from amygdala, hippocampus, and prefrontal cortex (Nicola et al., 2000). As in the dorsal striatum, dopamine has divergent actions at D1 and D2Rs. In the NAc, D1R activation facilitates potentiation of hippocampal inputs, while D2 activation suppresses PFC inputs. The effect of D2R activation on PFC inputs was bidirectional, as D2R antagonism actually facilitated PFC inputs (Goto and Grace, 2005). This finding has important functional implications for pauses in dopamine neuron firing, such as when an expected reward is omitted. Reduced dopamine transmission

during pauses will transform tonic suppression of PFC inputs into facilitation, thereby enhancing PFC to NAc transmission during the pause. Dopamine also modulates lateral inhibition between MSNs via actions at D1Rs, although unlike the facilitation observed in the dorsal striatum, D1R activation attenuates lateral inhibition onto MSNs in the NAc (Taverna et al., 2005).

Tonic and phasic dopamine signals can also have divergent impacts on activity and plasticity in non-striatal efferent targets. In prefrontal cortex, low concentrations of dopamine that mimic tonic activation transform a normally LTD-eliciting stimulation into an LTP-producing one (Matsuda et al., 2006). In the basolateral amygdala, LTP induction requires activation of D2, but not D1, receptors. Dopamine acts at these receptors to suppress feed-forward inhibition onto projection neurons (Bissiere et al., 2003).

Limitations of traditional approaches and advantages of optogenetics for the study of dopamine neurons

In vivo electrophysiological studies have yielded critical insights into possible functions of dopamine neurons. However this technique is only capable of demonstrating correlations between neuronal firing patterns and perceptual or behavioral events, and cannot establish causal links between the two. In contrast, the observance of a behavioral effect after injection of a dopamine receptor antagonist indicates that endogenous dopamine actions at dopamine receptors causally contribute to that behavioral process. Accordingly, a multitude of studies that employ targeted intracranial infusions of dopamine agonists and antagonists in specific brain areas or bath application of these drugs in brain slices provide a substantial framework for

understanding how dopamine neurons impact neural activity and behavior in downstream targets. However, the limited temporal precision of these approaches leaves many questions open. For example, pharmacological approaches cause activity changes in afferent structures for minutes or hours, making it difficult to correlate particular patterns of activity in afferent structures with potential changes in dopamine neuron firing. Electrical stimulation provides greatly enhanced temporal resolution, but is likely to indiscriminately activate cells or fibers of passage in the vicinity of the stimulating electrode making the identity and spatial distribution of cells affected by this technique difficult to predict (Histed et al., 2009).

The recent development of genetically-encoded light-sensitive proteins capable of directly modifying neural activity (opsins) promises to overcome many of these technical problems. A primary advantage of optogenetic tools such as channelrhodopsin2 (ChR2) is the ability to control neural activity in mammalian cells with millisecond precision (Nagel et al., 2003; Boyden et al., 2005) without off-target actions on fibers of passage. This has immediate implications for understanding the functional effects of temporal coding by dopamine neurons, especially during discreet behavioral events. For example, as discussed, compelling electrophysiological evidence suggests that dopamine neurons signal reward prediction errors. Optogenetics should make it possible to determine if these firing patterns are indeed causal for the learned appetitive behaviors during which they are typically observed, by selectively inducing phasic dopamine neuron activity. It would also be interesting to determine if phasic

dopamine neuron activation has different effects when emitted immediately prior to, during, or immediately following reward-predictive cues or operant responses.

Unlike conventional electrical stimulation techniques, the machinery for optically-induced activity manipulation is a single protein that can be genetically targeted to desired cell populations. This represents a long sought-after experimental goal of selectively manipulating one cell population without affecting others (Zhang et al., 2007; Fig. 6).

Figure 6

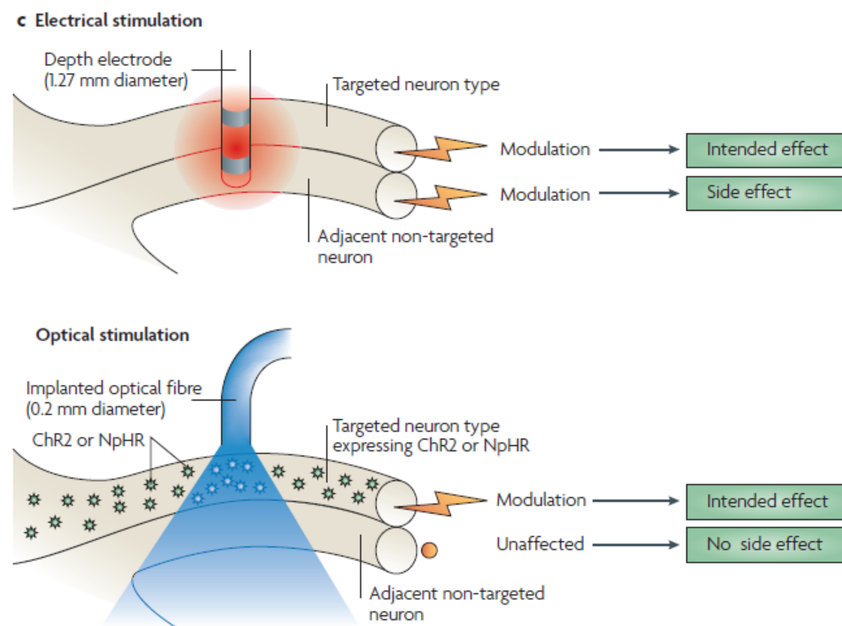


Figure 6. *Genetic targeting of opsins limits activity manipulations to neural population(s) of interest.* Top, conventional stimulating electrode activates a heterogeneous neural population. Bottom, genetic targeting of opsins such as ChR2 confines activation to targeted cell type, without directly affecting others. *Reproduced from Zhang and Deisseroth 2007.*

This approach will be particularly advantageous when applied to the study of dopamine neurons in the VTA, as the cellular heterogeneity of the VTA makes it difficult to manipulate dopamine neurons without causing concurrent changes in local GABAergic or glutamatergic neurons. Furthermore since light delivery can be targeted to axon terminals, selective control over a specific dopaminergic projection can be achieved.

Lastly, conventional electrical stimulation techniques produce large electrical artifacts at the time of stimulation, obscuring concurrent neural responses. These stimulation artifacts are virtually nonexistent with optical excitation (Zhang et al., 2007; Yizhar et al., 2011), making true dual recording and activity manipulation possible.

In summary, the advantages in temporal, neurochemical, and anatomical control make optogenetics an ideal tool to answer many outstanding questions about the neural and behavioral functions of dopamine.

Goals of this dissertation

The experiments described in this thesis were designed to establish causal relationships between phasic VTA dopamine neuron signals and learned appetitive behaviors. I chose to focus specifically on dopamine neurons in the VTA because this brain area has been extensively implicated in positive reinforcement (Fields et al., 2007). Accomplishing this experimental goal required the ability to rapidly and selectively manipulate dopamine neuron activity, a feat that is most straightforwardly accomplished with optogenetic tools. Serendipitously, I was able to initiate a

collaboration with the Deisseroth laboratory while they were in the process of developing transgenic tyrosine hydroxylase (Th)::Cre rats, and assisted with characterizing the founders they generated (Chapter 2). This animal model makes possible selective activity manipulations in dopamine neurons using the robust Cre-dependent opsin expressing viral constructs now commonly employed (Atasoy et al., 2008; Tsai et al., 2009).

With these resources in hand, I set out to determine the consequences of phasic increases in VTA dopamine neuron activity in behaving rats. An outstanding question I wanted to answer is whether the rich temporal information contained in dopamine signals (along with the distinct changes in activity and plasticity these patterns can elicit) has meaningful effects on behavior. Does it matter when dopamine neurons fire? What behavioral changes result?

The experiments presented here focus on two specific questions. First, what happens if dopamine neurons are activated as a consequence of an instrumental action? Second, what happens when dopamine neurons are activated coincidently with a natural reward? I found that these manipulations induced changes in specific aspects of reward-seeking behavior. In Chapter 3, I demonstrate that rats respond vigorously to obtain optical stimulation of VTA dopamine neurons. I also show that this behavior is partially mediated by the dopaminergic projection to the NAc, and requires activation of D1 and D2-type receptors. In Chapter 4, I demonstrate that activation of VTA dopamine neurons at the time of an expected reward can cause new learning about antecedent cues, or delay the effects of extinction if the anticipated reward is omitted. These results

are consistent with dopamine acting as a ‘teaching signal,’ as suggested by *in vivo* electrophysiological recordings.

Portions of the data presented here have been published (Witten et al., 2011) or are in the process of being submitted or reviewed for publication. Additionally, portions of this introduction are adapted from a recently published review (Steinberg and Janak, 2012).

Chapter 2

Th::Cre rats permit selective manipulation of dopamine neuron activity in behaving subjects

Abstract

Currently there is no general approach for achieving specific optogenetic control of genetically-defined cell types in rats, which provide a powerful experimental system for numerous established neurophysiological and behavioral paradigms. To overcome this challenge we have generated the first genetically-restricted recombinase-driver rat line, expressing Cre recombinase under the control of large genomic regulatory regions (200-300 Kb). Multiple tyrosine hydroxylase (Th)::Cre rat lines were produced that exhibited highly specific opsin expression in targeted cell types. We additionally developed methods for utilizing optogenetic tools in freely-moving rats. These studies complement existing targeting approaches by extending the generalizability of optogenetics to a traditionally non-genetically-tractable but vital animal model.

Acknowledgements

Efforts to develop Th::Cre rats were led by Dr. Ilana Witten during her tenure as a postdoctoral fellow in the Deisseroth lab. Other members of the Deisseroth lab (especially Soo Yeun Lee, Kay Tye, Charu Ramakrishnan and Kelly Zalocusky) and outside collaborators made critical contributions in assessing the specificity and efficacy of optogenetic tools in these rats. My contribution to this process was confined to developing methods for intracranial light delivery in behaving rats and collecting most of the histological images presented in this chapter. I then went on to demonstrate the utility of these rats for behavioral experiments; data that are presented in Chapters 3 and 4.

Introduction

While genetically-modified mice have enabled substantial advances in neuroscience and have made possible new approaches for circuit analysis with optogenetics (Gradinaru et al., 2009; Tsai et al., 2009; Kravitz et al., 2010; Lobo et al., 2010; Tye et al., 2011; Witten et al., 2011), a generalizable approach for optogenetic targeting of genetically-defined cell types in rats has proven to be elusive. This technological limitation is particularly important to address given that the substantial and flexible behavioral repertoire of rats makes these animals the preferred rodent model in many fields of neuroscience experimentation, and a wide variety of behavioral tasks have been optimized for this species (Pedersen et al., 1982; Pontecorvo et al., 1996; Phillips et al., 2003; Uchida and Mainen, 2003; Chudasama and Robbins, 2004; Vanderschuren and Everitt, 2004; Bari et al., 2008; Otazu et al., 2009). Furthermore, rats represent an essential system for *in vivo* electrophysiology, with dimensions that enable accommodation of the substantial numbers of electrodes required to obtain simultaneous data from large neuronal populations (Buzsaki et al., 1989; Wilson and McNaughton, 1993; Jog et al., 2002; Berke et al., 2009; Colgin et al., 2009; Gutierrez et al., 2010; Royer et al., 2010). Therefore, the ability to utilize population-selective genetically-targeted optogenetic tools in the rat would be a valuable technical advance.

Most efforts to target genetically-defined neurons in rats have relied on viral strategies, but given the paucity of compact and well-characterized promoters, this approach has only rarely led to highly specific targeting (Lawlor et al., 2009; Nathanson et al., 2009; Lee et al., 2010). Alternatively, transgenic rat lines can be generated to

enable use of specific larger promoter-enhancer regions (Filipiak and Saunders, 2006), but for expression of opsins in the brain this approach suffers from two serious limitations. First, this method is low-throughput and not well suited for keeping pace with the rapidly advancing opsin toolbox (requiring specific design, line generation, multi-generational breeding, and testing of each individual rat line for a particular opsin gene). Second, this approach is inconsistent with straightforward optogenetic control of single or multiple spatially distinct populations; in fact, a breakdown in specificity for control of cells or projections within a particular illuminated brain region arises because opsins traffic efficiently down axons (Gradinaru et al., 2010) and incoming afferents from other brain regions that are photosensitive will confound experiments by exhibiting optical sensitivity alongside local cell populations. For example, in a rat line expressing an opsin in all catecholamine neurons, illumination of the VTA would activate both dopamine cell bodies in the VTA and noradrenergic projections to the VTA originating from cells in the locus coeruleus.

Here, we explore an alternative approach addressing all of the above fundamental limitations, instead generating a panel of the first transgenic recombinase-driver rat lines, with regulatory information contained in 200-300 Kb of DNA upstream and downstream of the target genes, an approach that has achieved considerable success in mice (Gong et al., 2007). In these rats, large amounts of regulatory information is packaged in bacterial artificial chromosomes (BACs), whose packaging efficiency can accommodate regulatory sequences dispersed across large regions of the genome. We then adapted methods for intracranial light delivery in mice to be suitable

for larger and stronger rats. We applied these resources in combination with spatially specific injections of Cre-dependent opsin-expressing viral vectors to achieve neurochemical- and temporally-specific control of dopamine neuron activity in behaving rats.

Results

We first generated multiple BAC transgenic rat lines expressing Cre recombinase in tyrosine hydroxylase (TH) neurons (see Methods), and tested the specificity and potency of these lines for potential optogenetic experiments (Fig. 7-10). Injection of a Cre-dependent virus in dopaminergic (VTA or SNc) or noradrenergic (locus coeruleus, LC) structures in the Th::Cre rat line resulted in highly specific channelrhodopsin (ChR2) expression in catecholamine neurons (Fig. 7A-D). In the case of the VTA and SNc injection, opsin expression was confined to TH+ cell bodies and processes (Fig. 7A-C), and to projections of these cells within known target structures (e.g. ventral and dorsal striatum, Fig. 7C, bottom). Similarly, with the LC as an injection target, opsin expression was confined to the TH+ LC cell bodies and their processes; Fig. 7D). Additionally, to confirm that the VTA and LC could be targeted independently in this rat line (a potential concern because both areas expressed Cre), virus was injected in the VTA and lack of expression was confirmed in the LC (Fig. 8). Importantly, Th::Cre sublines from different founders varied quantitatively in their specificity and strength of expression (Fig. 7A). The offspring of founder 3 (line 3.1, 3.2, and 3.5) were used in all experiments in this paper, chosen for the highest specificity.

Figure 7

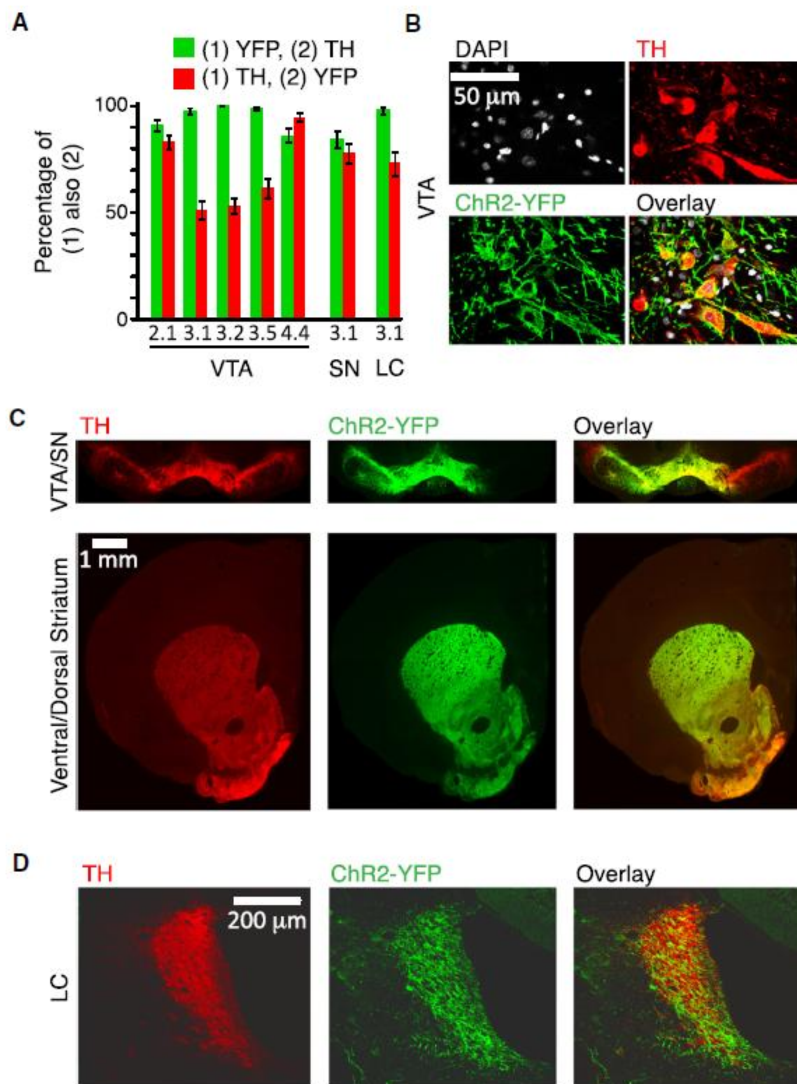


Figure 7. *Specific ChR2-YFP expression in the VTA, SNc, and LC of Th::Cre rats.* **A.** Quantification of ChR2-YFP expression profile in several Th::Cre sublines (VTA: line 2.1 *n*=113, line 3.1 *n*=150, line 3.2 *n*=190, line 3.5 *n*=122, line 4.4 *n*=126; SN: line 3.1 *n*=86; LC: line 3.1 *n*=63 where *n* refers to the number of counted cells that expressed either YFP or TH). Error bars are SEM. **B.** High magnification view of ChR2-YFP expression and DAPI staining in TH VTA cell bodies after injection of Cre-dependent virus in the VTA of a Th::Cre rat. **C.** TH staining and ChR2-YFP expression in coronal slices display colocalization in cell bodies (top: VTA and SNc) and efferents in the ventral and dorsal striatum (bottom) **D.** Colocalization of TH staining and ChR2-YFP expression in the LC after injection of Cre-dependent virus in a Th::Cre rat.

Figure 8

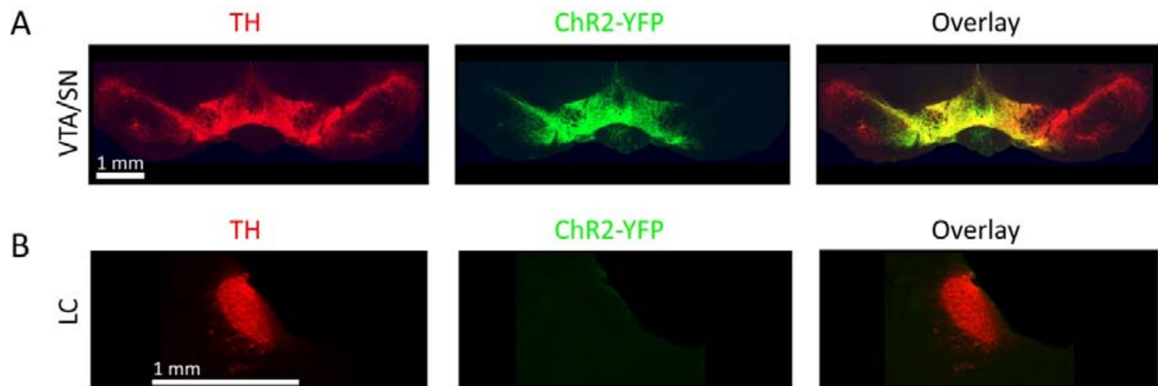


Figure 8. *ChR2-eYFP expression in the LC of Th::Cre rats injected with Cre-dependent virus in the VTA*. **A.** Sections of the VTA/ SNc demonstrating strong TH staining and ChR2-YFP expression. **B.** Section of the LC in the same rat demonstrating that virus does not spread to the LC. Red: TH antibody; Green: ChR2-YFP expression.

For example, in the VTA of line 3.5, 99+/-1% of neurons that expressed ChR2-YFP also expressed TH (a measure of specificity), while 61+/-4% of neurons that expressed TH also expressed ChR2-YFP (a measure of the proportion of targeted neurons that expressed the transgene). In the SNc of line 3.1 84+/-4% of neurons that expressed ChR2-YFP also expressed TH, while 77+/-5% of neurons that expressed TH also expressed ChR2-YFP. In the LC of line 3.1, 97+/-1% of neurons that expressed ChR2-YFP also expressed TH, while 72+/-6% of neurons that expressed TH also expressed ChR2-YFP (*n*=122 for VTA, *n*=86 for SN, *n*=63 for LC, where *n* refers to counted cells; Fig. 7A).

We performed a systematic *in vitro* electrophysiological study of the cellular and optogenetic properties of ChR2-YFP-expressing TH+ VTA neurons in Th::Cre+ rats, along with a comparison of the same properties of YFP-only-expressing neurons in Th::Cre+ littermates injected with a virus that expressed only YFP. Figure 9A shows a sample trace from a ChR2-expressing Th::Cre neuron in response to current injection steps, demonstrating the classical "sag" response induced by the hyperpolarizing pulse, the result of a hyperpolarization-activated cation current (I_h) that is present in many TH+ VTA neurons (Neuhoff et al., 2002; Margolis et al., 2006a; Lammel et al., 2008; Lammel et al., 2011). Given that the VTA TH+ neurons are heterogeneous and do not all express a prominent I_h (Margolis et al., 2006a; Lammel et al., 2008; Lammel et al., 2011), in addition to analyzing light-responses and intrinsic properties of neurons with a prominent I_h current ($I_{h/large}$ neurons), we have also included in this analysis cells without a prominent I_h ($I_{h/small}$ neurons), in either case comparing the properties of neurons that express ChR2-YFP to neurons that express YFP only.

Figure 9

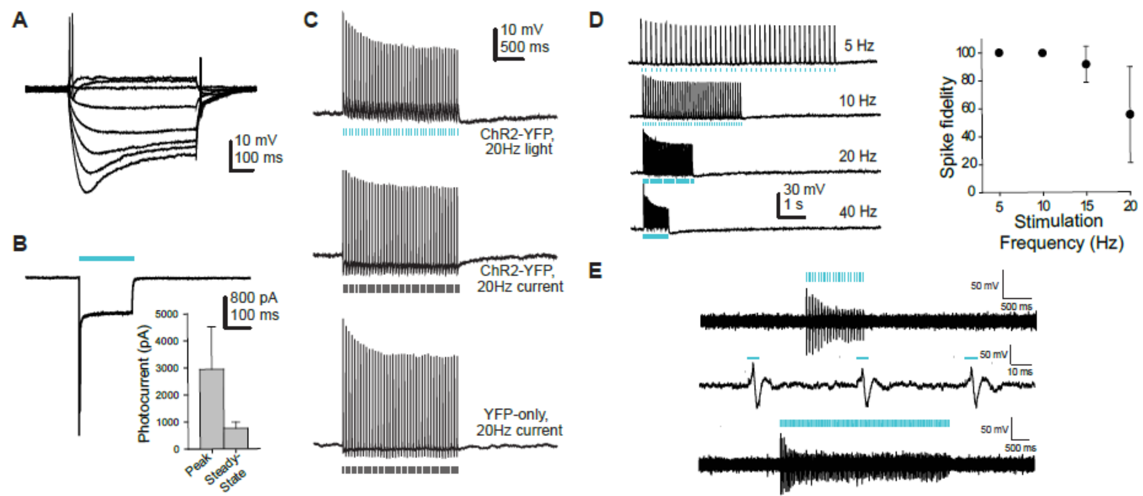


Figure 9. *In vitro and in vivo physiology of optical responses in VTA dopamine neurons in Th::Cre rats*. **A.** Example traces from a ChR2-expressing Th::Cre neuron in response to intracellular current injections (Vm: -43mV, 50pA current steps beginning at -500pA). **B.** Continuous blue light (470nm) evokes large (>500pA) inward currents in ChR2-expressing Th::Cre neurons. Inset, Summary graph of population data for photocurrent properties (n=7). **C.** Representative responses to 20Hz optical or electrical stimulation trains in ChR2-expressing and YFP-only expressing Th::Cre neurons. Spike size and shape are comparable to those previously reported for these cells. **D.** Left, ChR2-expressing Th::Cre neurons are able to reliably follow light-evoked pulse trains over a range of frequencies. Right, Summary data for spike fidelity (% of successful spikes in 40 light flashes) in ChR2-expressing Th::Cre neurons (n=7; in panels **A-D** data are from $I_{h/large}$ ChR2-YFP expressing cells). **E.** Optically evoked time-locked multi-unit neural activity recorded with an optrode *in vivo* in the VTA of anesthetized Th::Cre+ rats injected with Cre-dependent ChR2. Top: 20 Hz, 20 pulses, 5 ms pulse duration, 473 nm. Bottom: same recording site and photostimulation parameters but longer stimulation duration (100 pulses). Horizontal blue lines represent time course of optical stimulation.

Continuous blue light elicited large inward currents (peak photocurrent: -2950 ± 1574 pA for $I_{h/large}$ neurons, steady-state photocurrent: -756.5 ± 225.5 pA; n=7 $I_{h/large}$ neurons, Fig. 3B), and optical stimulation trains produced neural responses that were similar to those evoked by electrical stimulation in both ChR2-expressing or YFP-only expressing neurons (Fig. 9C); notably, the amplitude of both optically- and electrically-evoked spike trains attenuated during the course of the pulse train. Th::Cre ChR2-expressing neurons reliably responded to light-induced spike trains over a range of frequencies from 5 - 40Hz (Fig. 9D for $I_{h/large}$ neurons). Multiple spikes in response to a single light pulse were never observed during the presentation of pulse trains under these expression, illumination, and opsin (ChR2) conditions.

To complement the *in vitro* recordings and more fully characterize these new optogenetic tools, we validated tool functionality *in vivo* with electrophysiology as well. Optical stimulation of ChR2-expressing TH neurons resulted in reliable light-evoked neural activity *in vivo* assessed with optrodes; in particular, the targeted population was able to follow 20 Hz stimulation with a steady-state response level that was stable after 10 light pulses, extending to at least 100 pulses (Fig. 9E).

Next, to confirm that light-evoked neural activity resulted in neurotransmitter release, we used fast-scan cyclic voltammetry to measure dopamine release in acute brain slices of the NAc of Th::Cre rats that had been injected in the VTA with a Cre-dependent ChR2 virus (Fig. 10). 1 second of 20 Hz optical stimulation resulted in phasic transients with the characteristic dopamine current/voltage relationship (example site, Fig. 10A); across the population, mean amplitude of the transient was 0.33+/- 0.1 uM

Figure 10

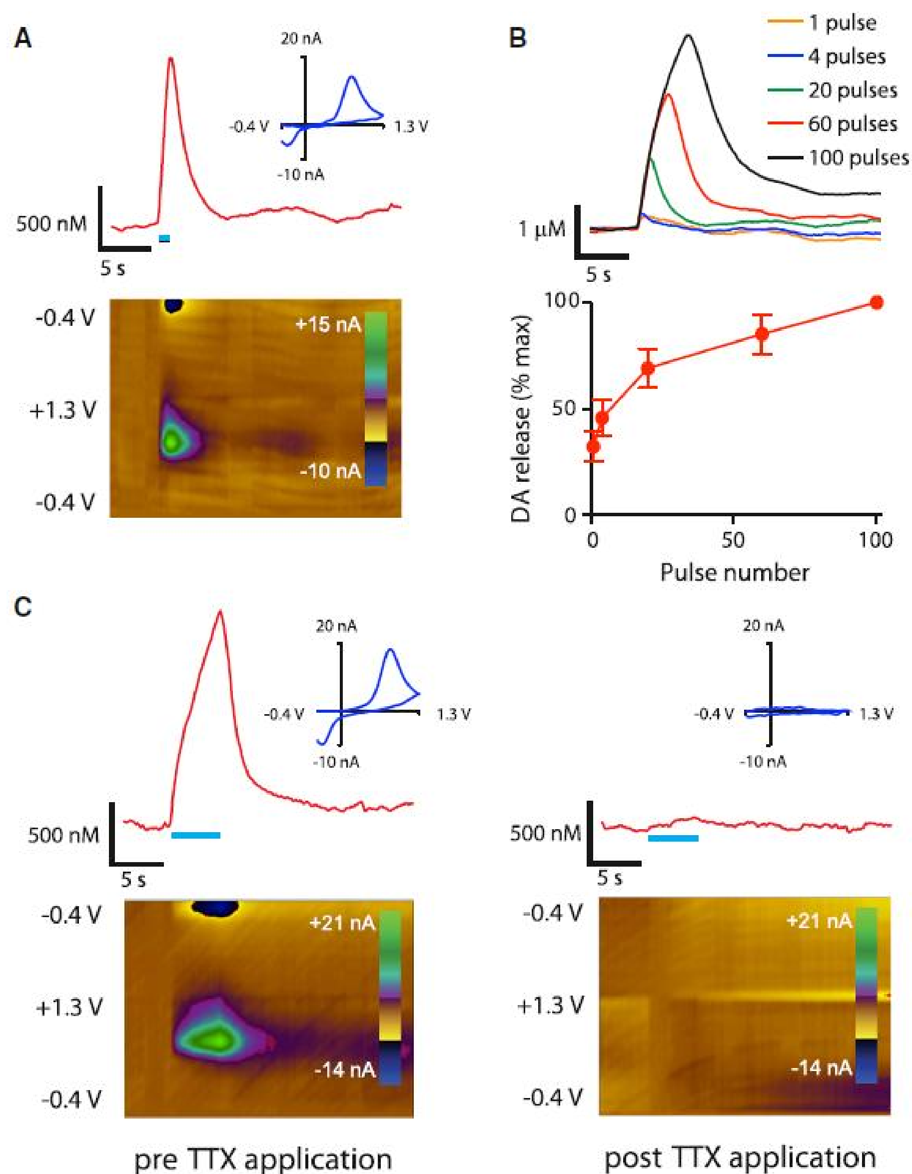


Figure 10. *Voltammetric measurements of optically evoked dopamine release in vitro in the nucleus accumbens of Th::Cre rats expressing Cre-dependent ChR2 in the VTA.* **A**. Timecourse of dopamine release at a sample site (20 pulses). **B**. Timecourse of dopamine release for various numbers of pulses at a sample site (top); peak dopamine release as a function of the number of pulses across the population (bottom, *n*=17). **C.** Bath application of TTX (1 uM) blocked optically-evoked dopamine release (left: before TTX, right: after TTX; 100 pulses. Horizontal blue lines represent time course of optical stimulation. In A-C, optical stimulation parameters: 20 Hz, 5 ms pulse duration, 473 nm.

(n=17 recording sites). The amplitude of the NAc dopamine transient increased monotonically but not linearly with the number of 20 Hz stimulation pulses; this quantitative relationship is illustrated in Figure 10B. Light-evoked phasic dopamine release was TTX-dependent (Fig. 10C), implicating presynaptic activation of voltage-gated Na^+ channels in optically-evoked dopamine release.

Methods for Optogenetic Stimulation in Freely-Moving Rats

In order to capitalize on these new reagents, we developed a system for optogenetic stimulation in freely behaving rats (Fig. 11). The essential components of this system are (1) an implantable optical fiber to reduce fiber breakages that result from repeatedly connecting to a light source over multiple behavioral sessions, (2) a secure connection between the implanted fiber and optical cable, (3) a protective spring encasing the optical patch cable to improve durability, (4) a counterbalanced lever arm to reduce tension associated with the attached cable, and (5) an optical commutator to allow the optical cable attached to the rat to rotate freely during behavioral sessions. The design and use of these optogenetic rat-optimized tools are described in the Materials and Methods.

Figure 11

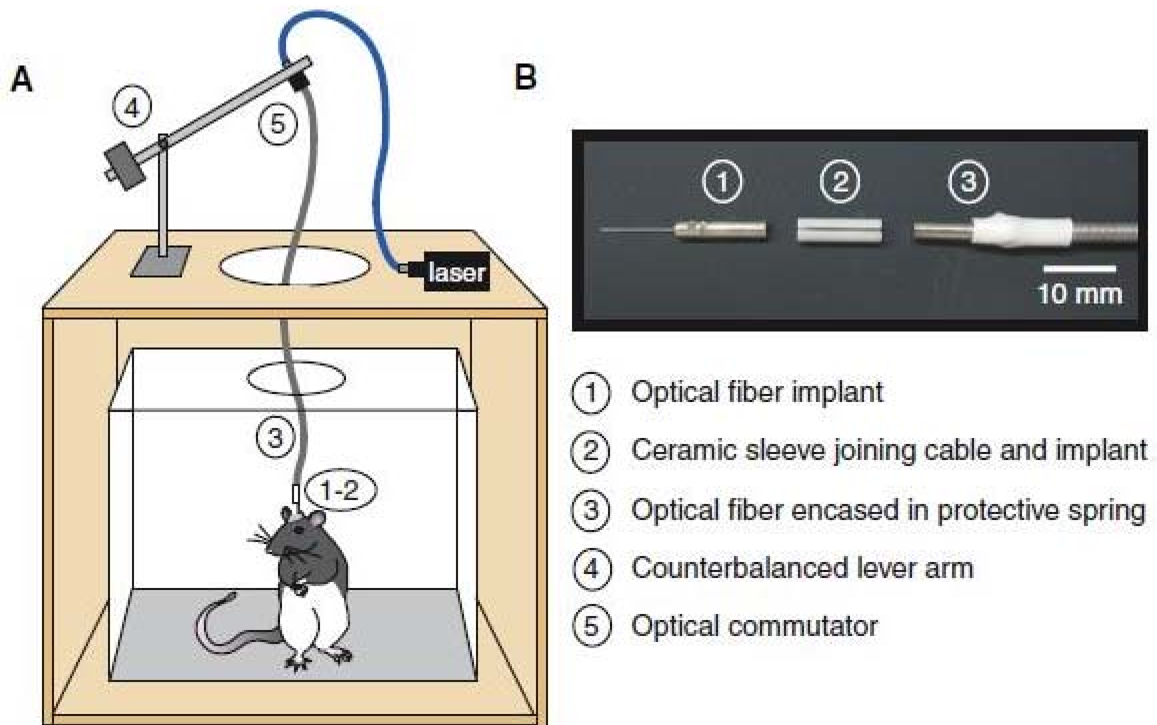


Figure 11. *Rat-optimized integration of optogenetic photostimulation with freely-moving behavior in an operant conditioning chamber*. **A**. Schematic of the behavioral set-up, which was optimized to facilitate freely-moving behavior along with photostimulation (while minimizing the chance of fiber breakage or disconnection). An optical fiber in a metal ferrule was surgically implanted over the targeted brain area (1), and connected with a ceramic sleeve (2) to a patch cable encased in a protective spring (3). A counterbalanced lever arm compensated for accumulation of slack in the patch cable during rearing (4), and an optical commutator (5) enabled the rat to move freely in the chamber. **B**. A close-up view of the implantable fiber in the metal ferrule (1), the ceramic sleeve (2), and the patch cord encased in the protective spring (3).

Discussion

Here, we introduce a panel of the first genetically-targeted recombinase-driver rat lines and validate these lines as a versatile and effective tool to target opsins to genetically-defined cell types in this essential animal model. Most efforts to target genetically-defined subpopulations of neurons in rats have relied on viral strategies (Lawlor et al., 2009; Lee et al., 2010), but since compact promoters are rare and viral vectors have limited packing capacity, published attempts often result in only partial specificity for the targeted cell type (Wang et al., 1999; Tan et al., 2008; Nathanson et al., 2009). In contrast, we found that BAC Cre transgenic rats offer an attractive alternative for precise optogenetic targeting. We were able to achieve 98% and 84% specific opsin expression in dopamine neurons of the VTA and SNc respectively, as well as 97% opsin specificity in noradrenergic neurons of the LC in Th::Cre rats.

These lines thus offer a powerful means to selectivity target dopaminergic and noradrenergic neurons in rats, providing long-sought experimental control of neuronal populations that are likely to influence a wide variety of neural and behavioral functions (Nader and LeDoux, 1999; Montague et al., 2004; Shen et al., 2008; Surmeier et al., 2009; Gerfen and Surmeier, 2011) in this important animal system. When combined with optogenetics, these tools now enable selective control of neuromodulatory function with exceptional temporal precision in genetically-defined subpopulations and their projections, and we expect this approach to be readily generalizable to other cell types in rats. This approach capitalizes on BAC technology that had been developed for the generation of transgenic mice (Gong et al., 2007); coupling these constructs with

recent advances in pronuclear injection technology in rats (Filipiak and Saunders, 2006) results in a versatile approach that will enable targeting of a virtually unlimited array of genetically-defined cell-types of interest. Our success in achieving cell-type specific expression in rats was fundamentally related to the very large regulatory/promoter element that we employed (the BACs allowed for a regulatory region of 200-300 kB), which contrasts to the much smaller promoter regions that typically can be packaged in viruses (typically 2-5 kB promoter region, depending on the type of virus and the size of the proteins being expressed by the virus). We were able to achieve high levels of specificity, although not all founders generated offspring with highly specific expression. In fact, only 1 Th::Cre founder (out of 7) resulted in a very high (>90%) specificity line. Thus, a primary challenge in the generation of transgenic rat lines is the extensive characterization required to validate transgene specificity.

Chapter 3

Selective activation of VTA dopamine neurons drives positive reinforcement

Abstract

Actions that lead to beneficial outcomes are more likely to be repeated than those that do not. This phenomenon, arguably at the root of much of human and animal behavior, is referred to as positive reinforcement. The neural basis of positive reinforcement is often studied in the laboratory using intracranial self-stimulation (ICSS), a simple behavioral model in which subjects perform an action in order to obtain exogenous electrical stimulation of a specific brain area. These studies have implicated dopamine neurons or their broad projections as a neural substrate capable of supporting ICSS. However, ambiguities inherent to this methodology have precluded determining whether dopamine neurons are indeed sufficient to drive this behavior. Here, we address this question by leveraging the neurochemical selectivity of recently developed Th::Cre rats in order to optogenetically activate VTA dopamine neurons, or their axons innervating the NAc. We demonstrate that these neural populations can indeed support ICSS, and further find that ICSS driven by optical activation of VTA dopamine neurons is significantly attenuated by intra-NAc injections of D1 or D2 receptor antagonists. These data establish a causal relationship between dopamine neurons and ICSS and identify essential receptor subtypes through which dopamine acts to promote this behavior, ultimately helping to refine our understanding of the neural circuitry that mediates positive reinforcement.

Acknowledgements

Some of the experiments described in this chapter (dopamine antagonist infusions) were performed with assistance from Josiah Boivin, a graduate student in the Janak lab. I am additionally grateful for the technical assistance of Saemi Cho and Lacey Sahuque.

Introduction

Rewards are often used to encourage particular behaviors: politicians are re-elected when their actions please voters, a child's good conduct elicits parental praise, companies offer bonuses when employees perform well. This process, whereby the probability of a behavioral response increases as a consequence of a beneficial outcome following that response, is referred to as positive reinforcement. Positive reinforcement facilitates the selection of behaviors that promote survival; however this normally adaptive learning process can go awry in disease states such as addiction where drug-seeking persists despite negative consequences (Redish, 2004; Everitt and Robbins, 2005). Despite its importance for normal and pathological behavior, our understanding of the neural circuits that mediate positive reinforcement remains incomplete.

Over the course of the last half-century, electrical intracranial self-stimulation (ICSS) has emerged as a powerful approach to identify brain areas that serve as positive reinforcement sites (Olds and Milner, 1954; Olds and Olds, 1963; Corbett and Wise, 1980). In ICSS paradigms, subjects make operant responses in order to deliver stimulation to a specific part of the brain. An extensive literature suggests that dopamine neurons play an important role in electrical ICSS, as altering levels of dopamine or lesioning dopamine neurons dramatically affects ICSS thresholds (German and Bowden, 1974; Fouriezos and Wise, 1976; Mogenson et al., 1979; Fibiger et al., 1987; Wise and Rompre, 1989) and effective sites for ICSS, including the medial forebrain bundle, closely parallel the anatomical location of dopamine neurons or their broad projections (Corbett and Wise, 1980). However, several studies have suggested

that powerful ICSS sites may have a non-dopaminergic component, or perhaps not even require dopamine neurons at all. Robust ICSS has been demonstrated behaviorally without metabolic activation of major dopaminergic projection targets (Gallistel et al., 1985), and rats with near-complete lesions of the dopamine system show reduced but still significant ICSS behavior (Fibiger et al., 1987). Additionally, the electrophysiological properties of axons thought to be necessary for sustaining ICSS were shown to be inconsistent with the conduction velocity of dopamine axons (Bielajew and Shizgal, 1986). Further, studies employing *in vivo* voltammetry during ICSS have found that dopamine release in the NAc is only rarely observed in well-trained animals (Garris et al., 1999; Owesson-White et al., 2008). Interestingly, a recent optogenetic study in mice found that dopamine-neuron stimulation by itself was not sufficient for the acquisition of ICSS (Adamantidis et al., 2011).

The challenge in linking dopamine neuron activation to ICSS may be rooted in the complexity and heterogeneity of the rat ventral tegmental area (VTA), coupled with ambiguities inherent to electrical stimulation. Less than 60% of neurons in the rat VTA are dopaminergic (Swanson, 1982; Margolis et al., 2006a; Fields et al., 2007; Nair-Roberts et al., 2008). The sizeable population of GABAergic and to a lesser extent glutamatergic neurons that constitute the remainder send extensive efferent projections both within and outside of the VTA (Dobi et al., 2010; Yamaguchi et al., 2011). An additional concern arises from recent imaging experiments demonstrating that electrical stimulation activates a sparse and scattered neural population with a spatial distribution that is difficult to predict (Histed et al., 2009). This issue is

particularly significant given the wide array of brain areas that support electrical ICSS (Olds and Olds, 1963; German and Bowden, 1974; Wise, 1996). Electrical stimulation of the VTA therefore undoubtedly activates a complex and heterogeneous circuitry. To circumvent this issue we leveraged the specificity of transgenic Th::Cre rats in order to test the hypotheses that direct activation of VTA dopamine neurons, or their major efferent projection to the nucleus accumbens (NAc), will be sufficient to acquire and sustain ICSS in freely-moving rats.

Results

Th::Cre+ rats and their wild-type littermates received identical injections of Cre-dependent ChR2 virus in the VTA, as well as optical fiber implants targeted dorsal to this structure (Fig. 12A; see Figure 13 for placement summary and fluorescence images). All rats were given the opportunity to respond freely at two identical nosepoke ports. A response at the active port resulted in a 1 second train of light pulses (20 Hz, 20 pulses, 5 ms pulse duration) delivered on a fixed-ratio 1 (FR1) schedule, while responses at the inactive port were without consequence. Th::Cre+ rats made significantly more responses at the active port relative to the inactive port on all 4 days of training (Fig. 12B, 2-tailed Wilcoxon signed rank test with Bonferroni correction, $p<0.05$ on days 1-4; see also Fig. 12C for cumulative active nosepoke responding across all days of training for a representative rat) indicating rapid acquisition of dopamine ICSS. By the third and fourth training day, Th::Cre+ rats performed more than 4,000 nosepokes on average at the active port, compared to less than 100 at the inactive port (Fig. 12B). Variability in

Figure 12

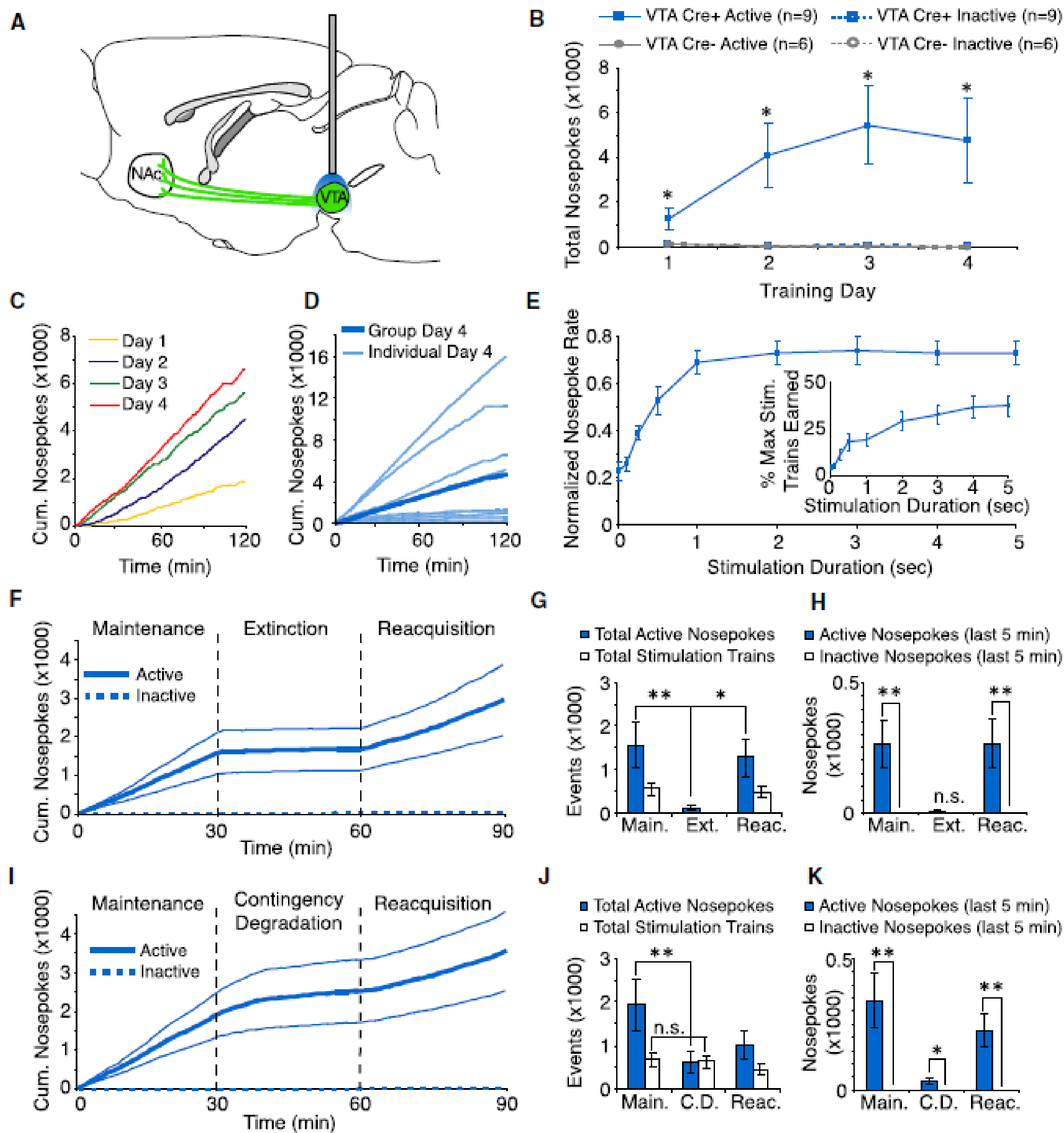


Figure 12. *Optical stimulation of VTA dopamine neurons supports robust self-stimulation*. **A.** The VTA was injected with a Cre-dependent ChR2 virus, and an optical fiber was implanted above the VTA. **B.** Nosepokes during 4 days of FR1 training (120 min sessions), in which nosepokes at the active port resulted in photostimulation (20 Hz, 20 pulses, 5 ms duration, 473 nm), while nosepokes at the inactive port were without consequence. Th::Cre+ rats performed significantly more active than inactive nosepokes (2-tailed Wilcoxon signed rank test with Bonferroni correction; $p<0.05$ on days 1-4) **C.** Cumulative responding at the active nosepoke port across all 4 days of training for a representative Th::Cre+ rat. **D.** Cumulative responding at the active nosepoke port on Day 4 of FR1 training for all Th::Cre+ rats. Dark blue: population average; light blue: individual rats. **E.** Normalized nosepoke rate at the active port for Th::Cre+ rats for duration-response test in which the relationship between stimulation duration and response rate was mapped systematically. Before averaging across rats, the nosepoke rate for each rat was normalized to the maximum rate across all stimulus durations. Response rate depended on stimulation duration (Kruskal-Wallis Test, $p<0.0001$; 20 Hz, 1-100 pulses, 5 ms pulse duration). Inset: Percent of the maximum possible stimulation trains earned as a function of stimulus duration for the same data set. **F.** Cumulative responding at the active port for Th::Cre+ rats for the within-session extinction test during maintenance, extinction and reacquisition. **G.** Quantification of total active nosepoke responses and stimulation trains delivered during maintenance, extinction and reacquisition. Response rate decreased during extinction and then increased during reacquisition (2-tailed Wilcoxon signed rank test; $p<0.01$ for average nosepokes during maintenance vs. extinction, $p<0.05$ for extinction vs. reacquisition). **H.** Quantification of responses at the active and inactive nosepoke ports for the last 5 minutes of maintenance, extinction and reacquisition. Th::Cre+ rats responded preferentially at the active nosepoke port at the end of maintenance and reacquisition, but not extinction (2-tailed Wilcoxon signed rank test; $p<0.01$). **I.** Cumulative responding at the active port for Th::Cre+ rats for the within-session contingency degradation test during maintenance, contingency degradation and reacquisition. **J.** Quantification of total active nosepoke responses and stimulation trains delivered during maintenance, contingency degradation, and reacquisition. Response rate decreased during contingency degradation (2-tailed Wilcoxon signed rank test, $p<0.01$ for average nosepokes during maintenance vs. extinction) **K.** Quantification of responses at the active and inactive nosepoke ports for the last 5 minutes of maintenance, contingency degradation and reacquisition. Th::Cre+ rats responded preferentially at the active nosepoke port at the end of all 3 phases (2-tailed Wilcoxon signed rank test; $p<0.01$ for maintenance and reacquisition; $p<0.05$ for contingency degradation). In all panels, error bars indicate SEM.

Figure 13

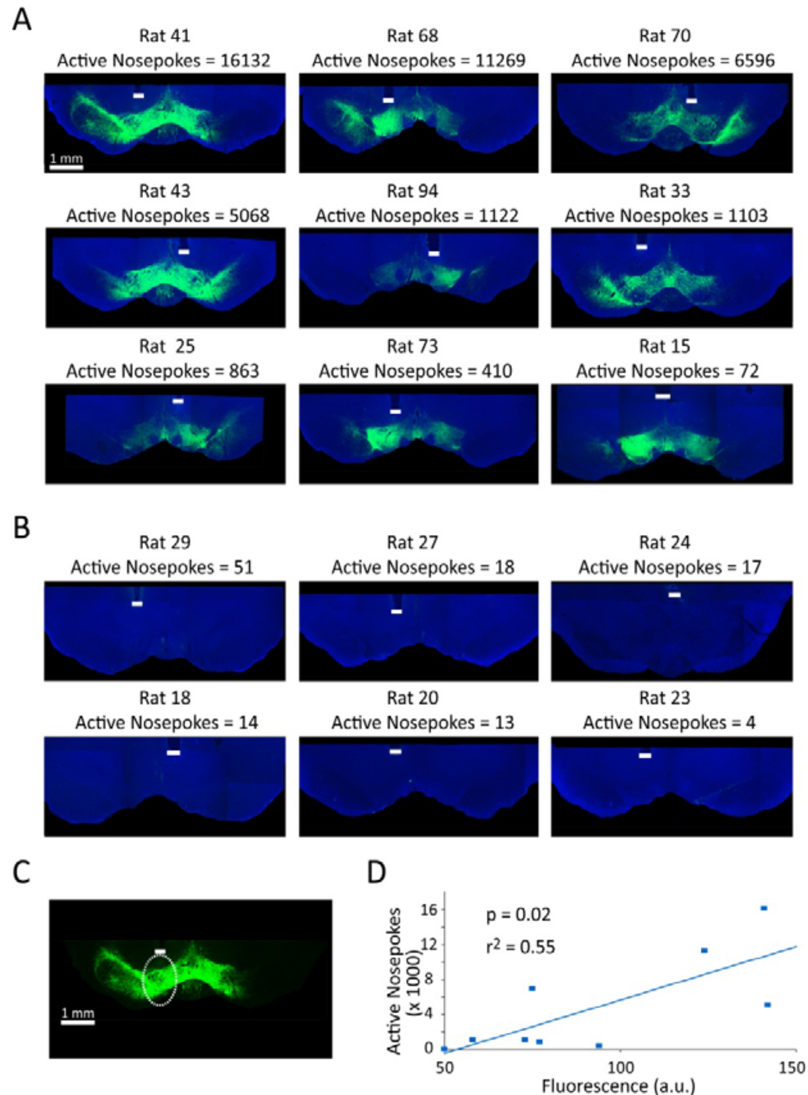


Figure 13. *Histology of all rats that were used in behavior*. **A.** Sections of the VTA in *Th::Cre*+ rats, depicting fiber placement and ChR2-YFP expression, along with active nosepokes on Day 4 of FR1 training. Green: ChR2-YFP expression; Blue: DAPI nuclear counterstain; Horizontal white line: termination of fiber track. **B.** Same as **A**, but for *Th::Cre*- littermates. **C.** In each *Th::Cre*+ rat, YFP fluorescence was quantified within a selected region that was placed directly below the fiber tip (an oval that was 1mm x 1.5mm), as depicted in this example (Rat 41). Fluorescence was determined based on the average pixel intensity within the selected region using ImageJ software. **D.** A statistically significant relationship between fluorescence in the selected region and active nosepokes on day 4 of FR1 training was observed (*t*-test, p=0.02, r^2=0.55).

the vigor of responding between subjects (Fig. 12D) could explained by differences in the strength of virus expression directly beneath the implanted optical fiber tip (t-test, $p<0.05$, $r^2=0.55$; see Fig. 13A-C for placement summary and fluorescence quantification). Additionally, Th::Cre- rats made significantly fewer nosepokes at the active port than Th::Cre+ rats on all 4 days (2-tailed Mann- Whitney test with Bonferroni correction, $p<0.05$ on day 1, $p<0.005$ on days 2-4). Notably, responding of Th::Cre- rats at the active port was indistinguishable from responding at the inactive port (2-tailed Wilcoxon signed rank test with Bonferroni correction; $p>0.05$), indicating that active port responses in Th::Cre- rats were not altered by optical stimulation.

We then systematically varied the duration of optical stimulation that was provided for each single active nosepoke response in order to investigate the relationship between the magnitude of dopaminergic neuron activation and the vigor of behavioral responding (“duration-response test”). We chose to vary stimulation duration, having already established that altering this parameter results in corresponding changes in evoked dopamine transients *in vitro* (Chapter 1; Fig. 10B). Further, varying this parameter allowed us to confirm that subsequent spikes in a stimulation train are propagated faithfully to generate dopamine release in behaving rat (in agreement with our *in vitro* confirmation, Chapter 1; Fig. 10B). The rate of responding of Th::Cre+ rats at the active nosepoke port depended powerfully on the duration of stimulation received (Fig. 12E, Kruskal-Wallis Test, $p<0.0001$). Response rate increased more than threefold as the duration of a stimulation train increased from 5 ms to 1 second, and saturated for durations above 1 second. This saturation could not

be explained by a ceiling effect on the number of reinforcers that could be earned, since even for the longest stimulation train durations, rats earned on average less than 50% of the possible available optical stimulation trains (Fig. 12E, inset).

We further applied two classical behavioral tests to confirm that rats were responding to obtain response-contingent optical stimulation, rather than showing non-specific increases in arousal and activity subsequent to dopamine neuronal activation. First, we tested the effect of discontinuing stimulation during the middle of a self-stimulation session. Rats were allowed to respond over 30 min for 1-second stimulation trains (20 Hz, 20 pulses, 5 ms pulse duration; "maintenance"). Subsequently, stimulation was discontinued and responses at the active port had no effect ("extinction"). After a further 30 minutes had elapsed, brief "priming" stimulation trains were delivered to indicate to the rat that stimulation was once again available ("reacquisition"). We found that Th::Cre+ rats rapidly extinguished and then reacquired responding for dopamine ICSS, performing significantly fewer active nosepokes during extinction as compared to both maintenance and reacquisition (2-tailed Wilcoxon signed rank test; $p<0.01$ for maintenance vs. extinction, $p<0.05$ for extinction vs. reacquisition, Fig. 12F,G). The extinction of active responding was rapid; within 5 minutes after extinction onset, rats had decreased their average rate of responding at the active nosepoke to less than 10% of the rate sustained during maintenance. Importantly, by the last 5 minutes of the extinction phase Th::Cre+ rats no longer responded preferentially at the active nosepoke (Fig. 12H), instead responding at equivalently low levels at both active and inactive nosepoke ports.

Next, we asked if the contingency between behavioral responses and dopamine ICSS was required to sustain responding. Rats were allowed to respond for stimulation over 30 minutes ("maintenance"), followed by a period of contingency degradation ("CD") during which stimulation trains were delivered pseudorandomly at intervals matched to the average rate at which they were earned by each rat during FR1-responding in a previous session. Rats could continue to respond at the active port during this phase, but the delivery of stimulation trains occurred independently of these responses. After 30 minutes had passed, non-contingent stimulation ceased and reinforcement was once again made contingent on responses in the active port ("reacquisition"). We found that Th::Cre+ rats were sensitive to degradation of the contingency between response and reinforcement, as they performed significantly fewer active nosepokes during CD than they had during maintenance (2-tailed Wilcoxon signed rank test, $p<0.01$; Fig. 12I,J) despite the fact that the number of stimulation trains delivered did not differ across the two epochs (2-tailed Wilcoxon signed rank test, $p>0.05$, Fig. 12J). Interestingly, by the last 5 minutes of the CD phase Th::Cre+ rats still showed a small but significant preference for responding at the active nosepoke (Fig. 12K). Additionally, on average rats increased responding at the active port during reacquisition, although when summed across the 30-minute epoch this change was not statistically significant (2-tailed Wilcoxon signed rank test, $p>0.05$; Fig. 12J). Together, the extinction and contingency degradation manipulations demonstrate that the robust maintenance of Th::Cre+ rat responding at the active port was the product of response-contingent optical stimulation of dopamine neurons.

If the reinforcement produced by optical stimulation of VTA dopamine neurons is perceived to be positive, rats should demonstrate a preference for the location where they received reinforcement, even when tested in the absence of the stimulation itself. We gave a separate group of Th::Cre+ rats and their wild-type littermates 4 days of FR1 ICSS training (60 min sessions), during which Th::Cre+ rats rapidly developed a preference for the active nosepoke, confirming our prior result (2-tailed Wilcoxon signed rank test with Bonferroni correction, $p=0.008$ on days 1-4, Fig. 14A). 24 hours after the last training session, rats were returned to the behavioral chambers where ICSS training had taken place and briefly given the chance to respond at either the active or inactive nosepoke ports. All responses were recorded during this 10 min test, but no stimulation was delivered. We found that Th::Cre+ rats retained a significant active nosepoke preference during this test, while Th::Cre- rats did not (2-tailed Wilcoxon signed rank test Cre+ $p=0.002$, Cre- $p=0.250$; Fig. 14B). This indicates that optical stimulation was perceived to be positive in Th::Cre+ rats, supporting our assertion that activation of VTA dopamine neurons is sufficient to mediate positive reinforcement.

Having established that activation of VTA dopamine neurons could sustain ICSS, we next turned to the question of which efferent projection was likely to mediate this effect. A primary region of interest is the NAc, which receives a substantial dopaminergic projection from the VTA. Dopamine acting in the NAc has been extensively implicated in the reinforcing effects of food and drug rewards, although the exact nature of this involvement remains a matter of active debate (Fields et al., 2007; Nicola, 2010; Salamone and Correa, 2012). Dopamine exerts its actions in the NAc via

Figure 14

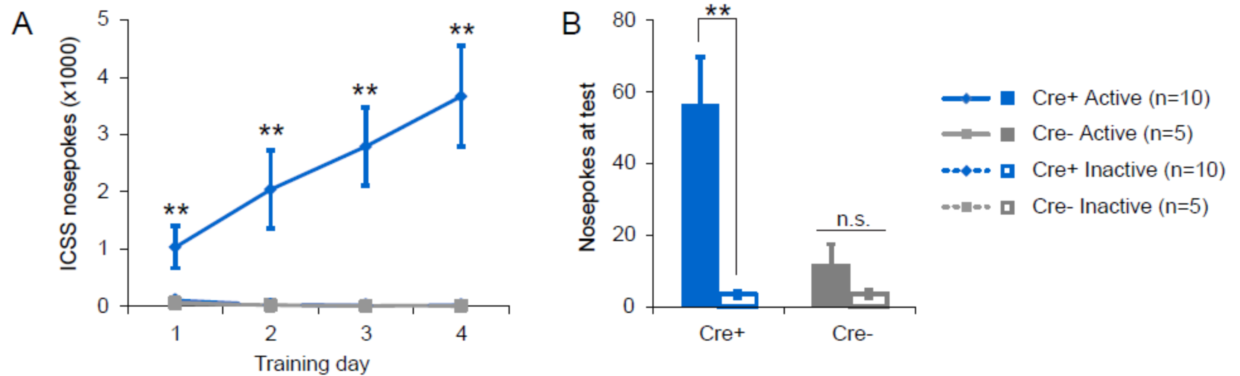


Figure 14. *Th::Cre+ rats prefer active nosepoke 24 hours after ICSS training has ended.* (**A**) Nosepokes during 4 days of FR1 training (60 min sessions), in which nosepokes at the active port resulted in photostimulation (20 Hz, 20 pulses, 5 ms duration, 473 nm), while nosepokes at the inactive port were without consequence. Th::Cre+ rats performed significantly more active than inactive nosepokes (2-tailed Wilcoxon signed rank test with Bonferroni correction; p=0.008 on days 1-4). (**B**) 24 hours after the last day of ICSS training, rats were returned to the chambers where training had been conducted and given the opportunity to respond freely at either nosepoke for 10 min. No optical stimulation was available during this test session. Th::Cre+ rats responded preferentially at the active nosepoke during test even though these responses were not reinforced (2-tailed Wilcoxon signed rank test, p=0.002). In contrast, Th::Cre- rats responded at equivalently low levels at each nosepoke port (2-tailed Wilcoxon signed rank test, p=0.250).

multiple receptors, most notably D1 and D2 receptors. The relationship between striatal dopamine release, receptor activation and behavior is complex. Pharmacological tools reveal that D1 and D2 receptors can act in concert or independently in subjects engaged in motivated behaviors (Ikemoto et al., 1997; Nowend et al., 2001; Yun et al., 2004; Cheer et al., 2007), and selective activation of D1 and D2 receptor-expressing striatal neurons produces opposing behavioral effects (Kravitz et al., 2010; Lobo et al., 2010; Kravitz et al., 2012; Tai et al., 2012). The roles of these receptor classes in the NAc in maintaining VTA dopamine neuron-specific ICSS is unknown. We sought to determine whether VTA dopamine neuron-specific ICSS was mediated by the NAc and, if so, which dopamine receptors were involved. We relied on two complementary experimental approaches to address these questions. First, we optogenetically activated VTA dopamine neuron axon terminals innervating the NAc to determine if selective activation of this pathway would support ICSS. Next, we used targeted infusions of dopamine receptor antagonists into the NAc during ICSS behavior driven by optogenetic activation of VTA dopamine neuron cell bodies.

We set out to determine whether selective activation of dopaminergic axon terminals innervating the NAc would be sufficient to support ICSS. Subjects received intra-VTA injections of a Cre-dependent virus encoding ChR2; after virus injection, Th::Cre+ rats or their wild-type (Th::Cre-) littermates were chronically implanted with an optical fiber targeting the NAc (Fig. 15A, 16A). Four weeks later, all subjects were given ICSS training sessions. During ICSS training, each response at an active nosepoke port resulted in a 1-second (20 pulses, 5ms duration, 20 Hz) optical stimulation train

Figure 15

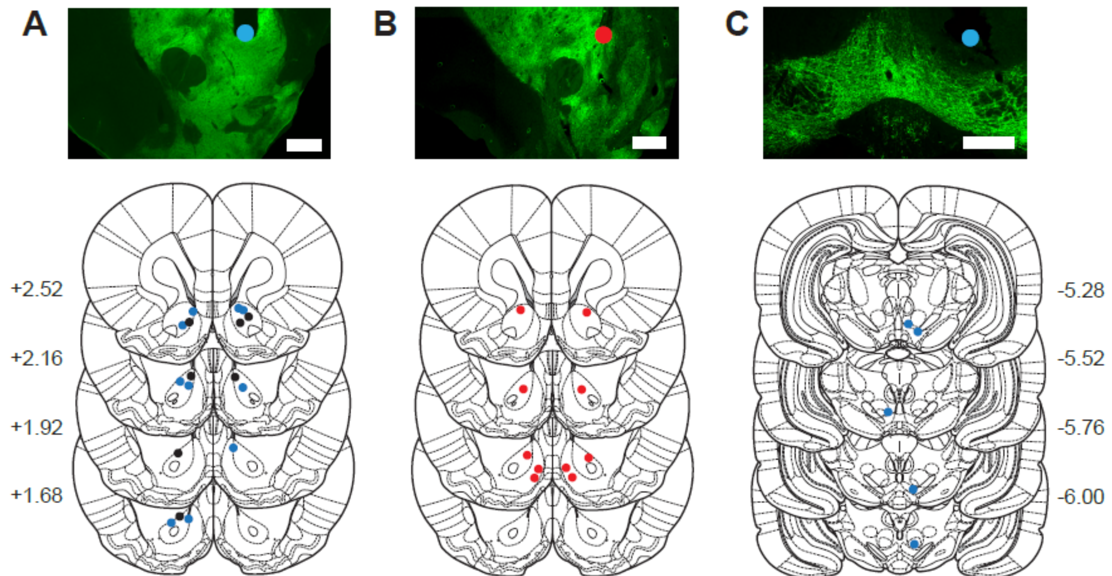


Figure 15. *Example and group histology.* (**A**) Top; representative striatal ChR2-eYFP expression and NAc optical fiber placement for experiment 1 (blue dot indicates fiber tip). Bottom; histological reconstruction of optical fiber tip placements for subjects receiving intra-NAc optical stimulation. Blue dots indicate tip placement in Th::Cre+ rats; black dots indicate tip placement in Th::Cre- rats. (**B**) Top; representative striatal ChR2-eYFP expression and NAc infuser tip placement for experiment 2 (red dot indicates infuser tip). Bottom; histological reconstruction of infuser tip placements for subjects receiving intra-NAc drug infusions. (**C**) Top; representative VTA ChR2-eYFP expression and optical fiber placement for experiment 2 (blue dot indicates fiber tip). Bottom; histological reconstruction of optical fiber tip placements for subjects receiving intra-VTA optical stimulation. In C-E scale bars = 500 μm.

Figure 16

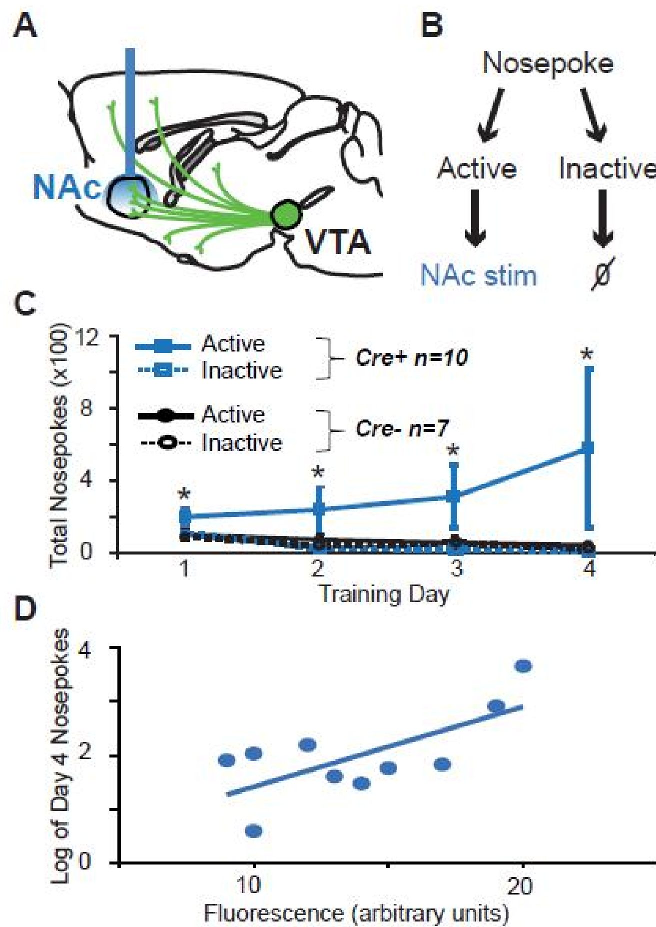


Figure 16. *Optical stimulation of VTA dopamine efferents to NAc supports self-stimulation.* (**A**) Virus was infused into the VTA, and an optical fiber was implanted targeting the NAc. (**B**) Schematic of ICSS task. A response at the active nosepoke port was reinforced with optical stimulation (20 pulses, 20 Hz, 5ms duration, 473nm) on an FR1 schedule. Responses at the inactive nosepoke port had no consequence. (**C**) Active and inactive nosepoke responding for Th::Cre+ and Th::Cre- rats during 4 days of FR1 training (120 min sessions). Th::Cre+ rats performed significantly more active than inactive nosepokes on all 4 days (2-tailed Wilcoxon Rank test with Bonferroni correction; *$p<0.05$) (**D**) YFP fluorescence in the VTA correlates with the log of FR1 response rate on Day 4 of FR1 training ($p=.026$; $r^2=0.482$).

delivered intracranially to the NAc, while responses at an otherwise identical inactive nosepoke port had no consequence (Fig. 16B). Th::Cre+ rats made more active than inactive nosepoke responses on all 4 training days (Fig. 9C, 2-tailed Wilcoxon Rank test with Bonferroni correction; days 1-4 $p=0.016$, 0.016, 0.04 and 0.008 respectively), while Th::Cre- rats did not (Fig. 16C, 2-tailed Wilcoxon Rank test with Bonferroni correction; days 1-4 $p=1.0$, 0.064, 1.0 and 0.876 respectively). Interestingly, ChR2 expression strength in the VTA of Th::Cre+ rats was significantly correlated with total responses made at the active port (Fig. 16D; $p=0.026$, $r^2=0.482$), and the Th::Cre+ rats that displayed above-average expression of ChR2 ($n=4$) performed significantly more active nosepokes than Th::Cre- rats on day 4 ($p=0.024$; 2-tailed Mann-Whitney test). Thus, optical activation of the dopamine neuron projection to the NAc is sufficient to support ICSS, confirming an important role for this pathway in the neural basis of positive reinforcement.

Next, we combined our optogenetic approach with pharmacological tools that allowed us to assess the contribution of specific dopamine receptor subtypes to ICSS behavior. Th::Cre+ rats were injected with Cre-dependent ChR2 virus unilaterally into the VTA, and an optical fiber was implanted dorsal to this structure (Fig. 15C, 17A). Additionally, bilateral cannulae were implanted targeting the NAc (Fig. 15B, 17A). After a recovery period, subjects were initially allowed to acquire ICSS behavior where each response at the active nosepoke resulted in a 1-second (20 pulses, 5ms duration, 20 Hz) optical stimulation train delivered intracranially to dopamine somata in the VTA. Once robust ICSS behavior had been established (>4 training sessions), subjects received test

Figure 17

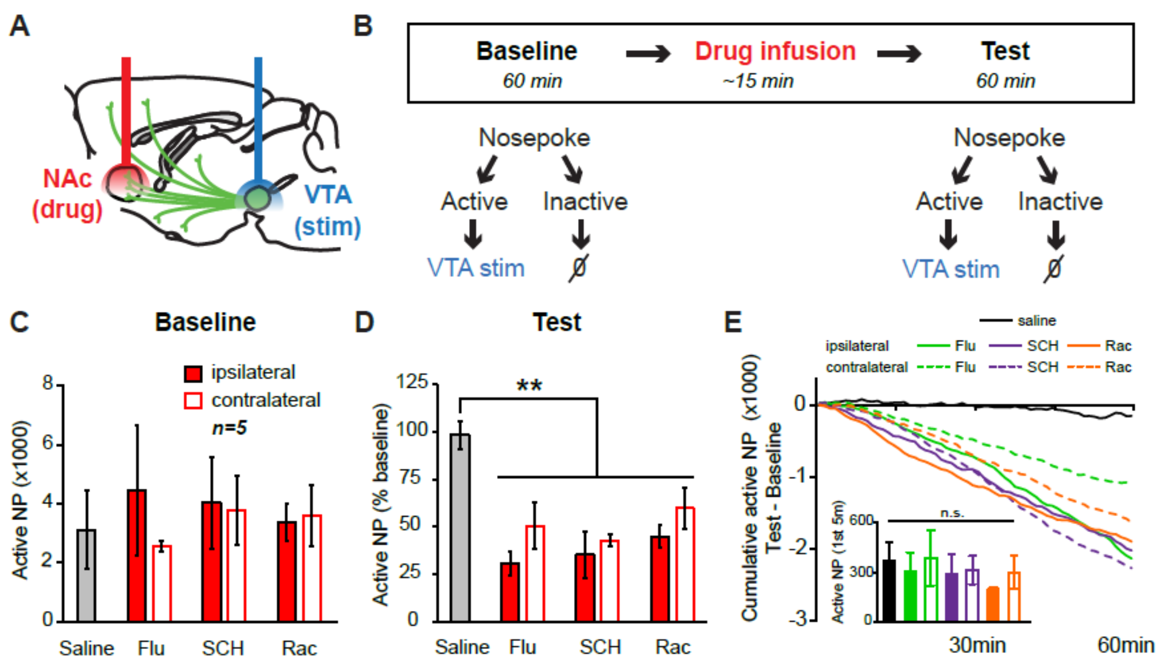


Figure 17. *Self-stimulation driven by VTA dopamine neurons is attenuated by intra-NAc D1 and D2 receptor antagonists.* (**A**) Virus was injected into the VTA and an optical fiber was targeted to this region; cannulae were targeted to the NAc. (**B**) Schematic of ICSS task with drug infusions. A 60 min baseline ICSS session was administered where responses at the active nosepoke port were reinforced with optical stimulation (20 pulses, 20 Hz, 5ms duration, 473nm) on an FR1 schedule. After intra-NAc drug infusion, a 60 min test ICSS session was administered that was identical to the baseline session. (**C**) Active nosepoke responding during baseline (pre-drug) sessions. There were no differences in responding (1-way RM ANOVA, main effect of future treatment p=0.648) (**D**) Active nosepokes during test (post-drug) sessions quantified as a percentage of baseline responding. Relative to saline, all drug treatments significantly reduced responding (1way RM ANOVA, main effect of treatment p<0.001, **post-hoc test vs. saline p<0.01). (**E**) Cumulative active nosepokes made during the 60 min test session, with the corresponding value from baseline sessions subtracted to highlight differential responding. Note that responding from saline sessions remains close to the baseline value (i.e., close to 0) while responding after drug treatment steadily decreases. Data represent the average of all rats (n=5), SEM not shown. Inset, total number of active nosepokes made in the first 5 minutes of each test session without baseline subtraction. There were no differences in this measure (one-way RM ANOVA, main effect of treatment p=0.316).

sessions where dopamine antagonists were infused into the NAc prior to ICSS training. We used a within-session, within-subject experimental design. Subjects were allowed to respond for dopamine-neuron ICSS during a 1-hour baseline session. Then, dopamine antagonists were infused into the NAc unilaterally (either ipsilateral or contralateral to the optical fiber implanted above the VTA), and subjects were returned to the behavioral chambers where they received an additional 1-hour ICSS test session (Fig. 17B). Drug effects were assessed by comparing post-drug active nosepoke responding to the same subject's pre-drug baseline value. All subjects maintained robust ICSS behavior during baseline sessions *prior* to drug infusion (1-way repeated measures ANOVA, main effect of future treatment p=0.648, Fig. 17C). We found that administration of dopamine antagonists into the NAc significantly reduced ICSS behavior during test sessions (1-way repeated measures ANOVA, main effect of treatment p<0.001, Fig. 17D). Planned post-hoc comparisons revealed that unilateral infusions injection of saline vehicle (all p's vs. saline <0.007). Decreased ICSS behavior observed in drug- treated rats was unlikely to result from motor impairments, as active nosepoke responding was similar under all treatment conditions during the first 5 minutes of the test session (one-way RM ANOVA, main effect of treatment p=0.316; Fig. 17E, inset).

In order to determine whether the location of drug infusions (ipsilateral vs. contralateral to the optical fiber) affected ICSS behavior similarly, we performed a targeted analysis of behavior following dopamine antagonist injections. A two-way RM ANOVA revealed a significant effect of side ($F_{8,29}$=11.16, p=0.029; main effect of drug and drug x side interaction p>0.385), indicating that ipsilateral drug infusions attenuated

behavioral responding to a greater extent than contralateral treatments. Yet while ipsilateral drug treatments were more efficacious, dopamine antagonists infused into either hemisphere significantly reduced ICSS behavior when compared to saline vehicle (Fig. 17D). This result was surprising since the dopaminergic projection from the VTA to the NAc is thought to be almost exclusively unilateral (Nauta et al., 1978; Phillipson and Griffiths, 1985; Brog et al., 1993; Hasue and Shammah-Lagnado, 2002). We hypothesized that the pronounced effects of contralateral drug infusions were a consequence of optical activation of VTA dopamine neurons in the contralateral hemisphere during ICSS. This hypothesis is supported by the observation that our unilateral virus injections resulted in bilateral ChR2 expression in VTA neurons (Fig. 15C), likely because of the VTA's midline location and the large volume of virus used to ensure robust infection. This observation is significant given the fact that recent efforts to quantify the propagation of light in living neural tissue demonstrate that the width of light spread in an intact brain is quantitatively similar to its depth, indicating that light may have reached ChR2-expressing dopamine neurons in the contralateral hemisphere (Yizhar et al., 2011).

We used immunohistochemical detection of Fos, a common marker of recently active neurons, in order to determine if contralateral VTA dopamine neurons were activated in rats engaged in ICSS behavior. A subset of subjects used in the antagonist study just described were sacrificed immediately after a 2-hour ICSS session, during which no drugs were infused, and their brains were subsequently processed for Fos, TH, and ChR2 triple-immunohistochemistry. VTA Fos labeling from these subjects was

compared to a control condition; control subjects had identical surgical treatments and prior ICSS training, however on the day of sacrifice they were placed in the behavioral chambers and no training program was initiated. Thus, control subjects did not experience ICSS prior to sacrifice. Figure 18 depicts representative images from a rat that underwent ICSS immediately prior to sacrifice (Fig. 18 A-C) and a control rat that did not (Fig. 18 D-F). Both subjects showed robust expression of ChR2-YFP and appropriately placed optical fiber dorsal to the VTA (Fig. 18 A,D). Notably, robust, bilateral Fos labeling was only observed in the subjects that experienced ICSS (Fig. 18B-C vs. E-F). Importantly, Fos+ cells observed in subjects that experienced ICSS often co-expressed TH and ChR2-YFP (Fig. 18G), indicating that these cells are likely to be light-activated dopamine neurons.

Figure 18

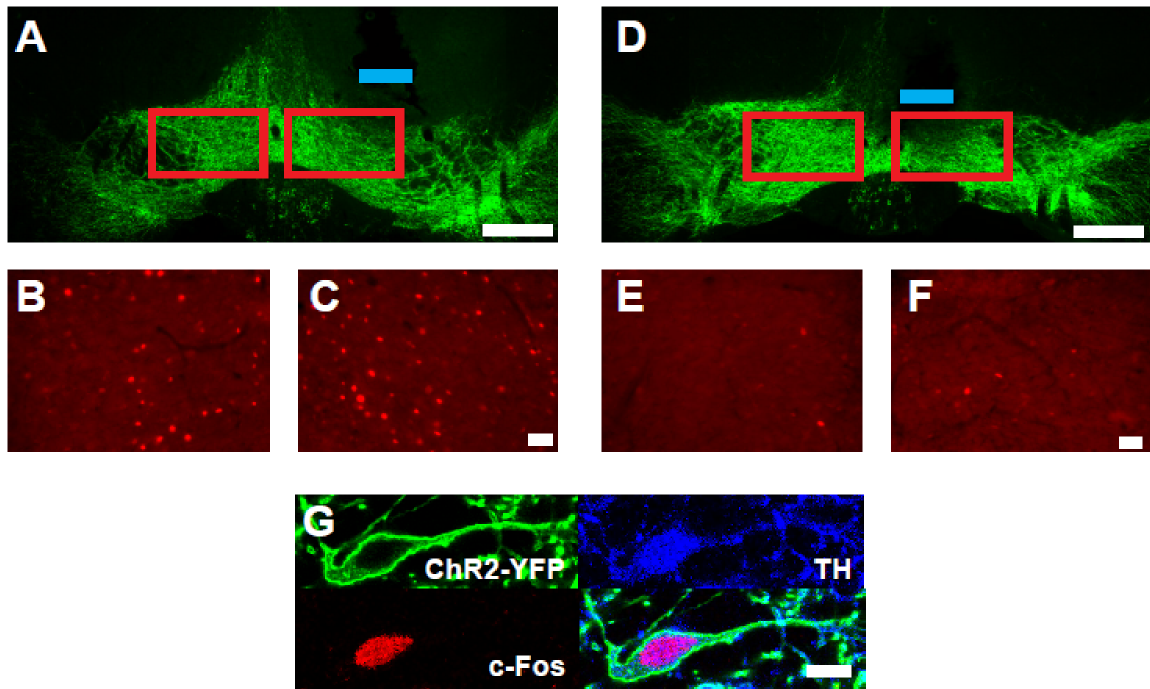


Figure 18. *Unilateral optical stimulation causes bilateral activation of VTA dopamine neurons.* (**A**) VTA ChR2-eYFP expression and optical fiber placement in a Th::Cre+ rat given a 120 min ICSS session immediately before being sacrificed for double- or triple-labeling immunohistochemistry. Blue line indicates location of optical fiber tip. Note virus spread into contralateral VTA. Red boxes correspond to areas magnified in B and C. (**B**) Fos immunohistochemical staining showing neural activation both contralateral and ipsilateral (**C**) to the optical fiber. (**D**) VTA ChR2-eYFP expression and optical fiber placement in a Th::Cre+ rat that did not undergo ICSS prior to sacrifice. Blue line indicates location of optical fiber tip. Red boxes correspond to areas magnified in E and F. (**E**) Fos immunohistochemical staining showing a lack of robust neural activation both contralateral and ipsilateral (**F**) to the optical fiber. (**G**) High-magnification view of ChR2-eYFP and TH immunohistochemical staining in a representative Fos-positive neuron. Scale bar = 500 µm in A and D; 50 µm in B-C and E-F; and 10 µm in G.

Discussion

Using Cre-dependent opsin expression in transgenic rats, we were able to manipulate dopamine neuron activity with genetic, anatomical and temporal precision. We leveraged these tools to clarify the relationship between dopamine neuron activation and positive reinforcement, and found that brief optical stimulation of dopaminergic VTA neurons was sufficient to drive vigorous ICSS. Electrical ICSS experiments have been difficult to interpret in the context of phasic dopamine activation since electrical stimulation activates a heterogeneous and complex population of neurons (Swanson, 1982; Margolis et al., 2006a; Fields et al., 2007; Lammel et al., 2008; Nair-Roberts et al., 2008; Histed et al., 2009; Dobi et al., 2010) and fails to elicit reliable dopamine release in well-trained animals (Garris et al., 1999; Owesson-White et al., 2008). Our studies show that phasic dopamine stimulation can support both the acquisition and maintenance of instrumental responding, significantly extending the recent finding that optogenetic stimulation of dopamine neurons can support conditioned place preference (a form of Pavlovian learning;(Tsai et al., 2009). We also found that activation of the VTA dopamine neuron projection to the NAc was sufficient to support ICSS, and that ICSS behavior mediated by VTA dopamine neurons was significantly reduced by antagonism of either D1 or D2 receptors in the NAc. Taken together, these results add to an influential body of evidence implicating dopamine neurons, and more specifically dopaminergic transmission in the NAc, as important elements of the neural circuitry mediating positive reinforcement.

Interestingly, our characterization of dopamine ICSS reveals that this behavior has much in common with electrical self-stimulation: rats rapidly acquire responding, with some rats responding at remarkably high rates; responding scales with the duration of stimulation; responding is extinguished very rapidly upon cessation of stimulation, and requires contingency between response and reinforcement. Although the current results do not preclude an involvement of other non-dopaminergic cell types in mediating electrical ICSS, our findings clearly demonstrate that activation of VTA dopamine neurons is sufficient, and the strong parallels with electrical self-stimulation are consistent with a major role of dopamine neuron activation in ICSS.

Our data also demonstrate that the dopaminergic projection to the NAc plays a major role in positive reinforcement. We found that selective activation of dopaminergic terminals innervating the NAc was sufficient to support ICSS. Although excitation of axon terminals in the NAc may have triggered back-propagating action potentials in activated cells, prior studies have demonstrated that the vast majority of VTA dopamine neurons have one projection target (Fallon, 1981; Swanson, 1982; Lammel et al., 2008). Thus, we are confident that our optical manipulation was highly selective in activating NAc-projecting dopamine neurons. In addition, we found that ICSS behavior driven by optical activation of dopamine somata in the VTA was significantly attenuated by localized infusion of dopamine antagonists into the NAc. Dopamine neuron-specific ICSS required activation of both D1 and D2 receptors, as antagonists of either receptor reduced ICSS behavior to a similar extent.

These results are in accord with a long literature implicating VTA dopamine neurons, and their major efferent projection to the NAc, in reward-related behaviors (Fields et al., 2007; Salamone and Correa, 2012; Steinberg and Janak, 2012). Interestingly, prior studies that used electrical stimulation of the VTA to drive ICSS found that intra-NAc antagonism of D1 receptors, but not D2 receptors, attenuated ICSS (Kurumiya and Nakajima, 1988; Cheer et al., 2007). In contrast, our results demonstrate that D1 and D2 receptors act in concert to support this behavior. It is important to note that D2 receptors are found both pre- and postsynaptically within the NAc (Zhang and Sulzer, 2012), and receptor activation at these sites can produce divergent effects. Because our pharmacological manipulations cannot distinguish between these sites of action, the cellular localization of the receptors responsible for generating the behavioral effects we observed remains to be demonstrated.

Another interesting feature of our data is the order-of-magnitude difference in ICSS behavior evoked by stimulation of dopamine neuron somata in the VTA (e.g., Fig. 6B) and stimulation of dopamine neuron terminals within the NAc (e.g., Fig. 9C). This could be due to anatomical differences in the density of dopamine neurons/axons within the area of illumination or, alternatively, may indicate that VTA dopamine neurons also support reinforcement via connections with other brain regions. However, the substantial reductions in somata-driven ICSS behavior induced by intra-NAc dopamine antagonist infusions (which presumably impact a larger volume of tissue) suggest that limited light penetration within a large structure is a likely, if partial, explanation for the discrepancy.

Positive reinforcement is typically adaptive, facilitating the selection of actions that produce beneficial outcomes such as procuring food or avoiding danger. However it can become pathological in disease states such as addiction (Redish, 2004; Everitt and Robbins, 2005), where drug-seeking behavior persists despite deleterious consequences (2000). Because both natural rewards (McCutcheon et al., 2012) and drugs of abuse (Phillips et al., 2003) evoke dopamine release, dopamine neuron-specific ICSS can be viewed as a model for either normal or maladaptive reward-seeking behavior. Thus the data presented here may serve to refine our understanding of the neural circuitry that mediates both of these processes.

Chapter 4

Selective activation of VTA dopamine neurons drives cue-reward learning

Abstract

Situations where rewards are unexpectedly obtained or withheld represent opportunities for new learning. Often, this learning includes identifying antecedent cues that reliably predict reward availability. Unexpected rewards strongly activate midbrain dopamine neurons. This phasic signal is proposed to support learning about antecedent cues by signaling discrepancies between actual and expected outcomes, termed a reward prediction error. However, it is unknown whether dopamine neuron prediction error signaling and cue-reward learning are causally linked. Here we show that optogenetic activation of dopamine neurons concurrent with reward delivery, mimicking a prediction error, is sufficient to cause long-lasting increases in reward-seeking behavior. We manipulated dopamine neuron activity in two behavioral procedures, associative blocking and extinction, that illustrate the essential function of prediction errors in learning. Our findings establish a causal role for temporally-precise dopamine neuron signaling in linking environmental cues with subsequent reward, bridging a critical gap between experimental evidence and influential theoretical frameworks.

Acknowledgements

The experiments described in this chapter were conceived, executed and interpreted in close collaboration with Dr. Ronald Keiflin, a postdoctoral fellow in the Janak lab. His insight and expertise were invaluable and he made significant contributions that shaped this line of investigation. Josiah Boivin assisted with the flavor preference study while he was a rotating graduate student in the Janak lab under my supervision. I am also grateful for the technical assistance of Lacey Sahuque, Rebecca Reese, Irene Grossrubatcher and Maike Olsman.

Introduction

Much of the behavior of humans and other animals is directed towards seeking out rewards. Learning to identify environmental cues that provide information about where and when natural rewards can be obtained is an adaptive process that allows this behavior to be distributed efficiently. Theories of associative learning have long recognized that simply pairing a cue with reward is not sufficient for learning to occur. In addition to contiguity between two events, learning also requires the subject to detect a discrepancy between an expected reward and the reward that is actually obtained (Rescorla and Wagner, 1972).

This discrepancy, or 'reward prediction error' (RPE), acts as a teaching signal used to correct inaccurate predictions. Presentation of unpredicted reward or reward that is better than expected generates a positive prediction error and strengthens cue-reward associations. Presentation of a perfectly predicted reward does not generate a prediction error and fails to support new learning. Conversely, omission of a predicted outcome generates a negative prediction error and leads to extinction of conditioned behavior. The error correction principle figures prominently in psychological and computational models of associative learning (Rescorla and Wagner, 1972; Sutton and Barto, 1981; Montague et al., 1996; Schultz et al., 1997; Schultz and Dickinson, 2000; Glimcher, 2011) but the neural bases of this influential concept remain to be definitively demonstrated.

In vivo electrophysiological recordings in non-human primates and rodents reveal that putative dopamine neurons in the ventral tegmental area (VTA) and the

substantia nigra pars compacta respond to natural rewards such as palatable food (Schultz et al., 1993; Roesch et al., 2007; Cohen et al., 2012). Notably, the sign and magnitude of the dopamine neuron response is modulated by the degree to which the reward is expected. Surprising or unexpected rewards elicit strong increases in firing rate, while anticipated rewards produce little or no change (Mirenowicz and Schultz, 1994; Hollerman and Schultz, 1998; Cohen et al., 2012). Conversely, when an expected reward fails to materialize, neural activity is depressed below baseline (Hollerman and Schultz, 1998; Roesch et al., 2007; Cohen et al., 2012). Reward-evoked dopamine release at terminal regions *in vivo* is also more pronounced when rewards are unexpected (Day et al., 2007). On the basis of this parallel between RPE and dopamine responses, a current hypothesis suggests that dopamine neuron activity at the time of reward delivery acts as a teaching signal and causes learning about antecedent cues (Montague et al., 1996; Schultz et al., 1997; Glimcher, 2011). This conception is further supported by the observation that dopamine neurons are strongly activated by primary rewards before cue-reward associations are well-learned. As learning progresses and behavioral performance nears asymptote, the magnitude of dopamine neuron activation elicited by reward delivery progressively wanes (Schultz et al., 1993; Hollerman and Schultz, 1998).

Though the correlative evidence linking reward-evoked dopamine neuron activity with learning is compelling, little causal evidence exists to support this hypothesis. Previous studies that attempted to address the role of prediction errors and phasic dopamine neuron activity in learning employed pharmacological tools, such as

targeted inactivation of the VTA (Takahashi et al., 2009), or administration of dopamine antagonists (Iordanova et al., 2006) or indirect agonists (O'Tuathaigh et al., 2003). Such studies suffer from the major limitation that pharmacological agents alter the activity of neurons over long timescales and thus cannot determine the contribution of specific patterns of dopamine neuron activity to behavior. Genetic manipulations that chronically alter the actions of dopamine neurons by reducing or eliminating the ability of dopamine neurons to fire in bursts (Zweifel et al., 2009; Parker et al., 2010) do alter learning, but suffer from similar problems, as the impact of dopamine neuron activity during specific behavioral events (such as reward delivery) cannot be evaluated. Other studies circumvented these issues using optogenetic tools that permit temporally-precise control of dopamine neuron activity; however these studies failed to utilize behavioral paradigms that explicitly manipulate reward expectation (Tsai et al., 2009; Adamantidis et al., 2011; Domingos et al., 2011; Witten et al., 2011), involve natural rewards (Tsai et al., 2009; Witten et al., 2011), or are suitable to assess cue-reward learning (Domingos et al., 2011). Thus, despite the prevalence and influence of the hypothesis that RPE signaling by dopamine neurons drives associative cue-reward learning, a direct link between the two has yet to be established.

To address this unresolved issue we capitalized on the ability to selectively control the activity of dopamine neurons in the awake, behaving rat with temporally-precise and neuron-specific optogenetic tools (Boyden et al., 2005; Zhang et al., 2006; Witten et al., 2011) in order to simulate naturally-occurring dopamine signals. We sought to determine whether activation of dopamine neurons in the VTA timed with the

delivery of an expected reward would mimic a RPE and drive cue-reward learning using two distinct behavioral procedures, blocking and extinction.

First, we employed blocking, the associative phenomenon that best demonstrates the role of prediction errors in learning (Kamin, 1968, 1969a, b). In a blocking procedure, the association between a cue and a reward is prevented (or 'blocked') if another cue present in the environment at the same time already reliably signals reward delivery (Holland, 1984). It is generally argued that the absence of an RPE, supposedly encoded by the reduced or absent phasic dopamine response to the reward, prevents further learning about the redundant cue (Schultz et al., 1997; Waelti et al., 2001). We reasoned that artificial VTA dopamine neuron activation paired with reward delivery would mimic a positive prediction error and facilitate learning about the redundant cue. Next, we tested the role of dopamine neuron activation during extinction learning. Extinction refers to the observed decrease in conditioned responding that results from the reduction or omission of an expected reward. The negative prediction error, supposedly encoded by a pause in dopamine firing, is proposed to be responsible for extinction of behavioral responding (Schultz et al., 1997; Schultz, 1998). We reasoned that artificial VTA dopamine neuron activation timed with the reduced or omitted reward would interfere with extinction learning. In both procedures, optogenetic activation of dopamine neurons at the time of expected reward delivery affected learning in a manner that was consistent with the hypothesis that dopamine neuron prediction error signaling drives associative learning.

Results

The blocking procedure provides an illustration of the essential role of RPEs in associative learning. Consider two cues (e.g. a tone and a light) presented simultaneously (in compound) and followed by reward delivery. It has been shown that conditioning to one element of the compound is reduced (or 'blocked') if the other element has already been established as a reliable predictor of the reward (Kamin, 1968, 1969a, b; Holland, 1984). In other words, despite consistent pairing between a cue and reward, the absence of a prediction error prevents learning about the redundant cue. In agreement with the conception that dopamine neurons encode prediction errors, putative dopamine neurons recorded *in vivo* exhibit little to no reward-evoked responses within a blocking procedure (Waelti et al., 2001). The lack of dopamine neuron activity, combined with a failure to learn in the blocking procedure, is considered to be a key piece of evidence (albeit correlative) linking dopamine RPE signals to learning. On the basis of this evidence, we determined that the blocking procedure would provide an ideal environment in which to test the hypothesis that RPE signaling by dopamine neurons can drive learning. According to this hypothesis, artificially activating dopamine neurons during reward delivery in the blocking condition when dopamine neurons normally do not fire would mimic a naturally-occurring prediction error signal and allow subjects to learn about the otherwise 'blocked' cue.

We first demonstrated associative blocking of reward-seeking (Fig. 19) using parameters suitable for subsequent optogenetic neural manipulation. Two groups of rats were initially trained to respond for a liquid sucrose reward (unconditioned

Figure 19

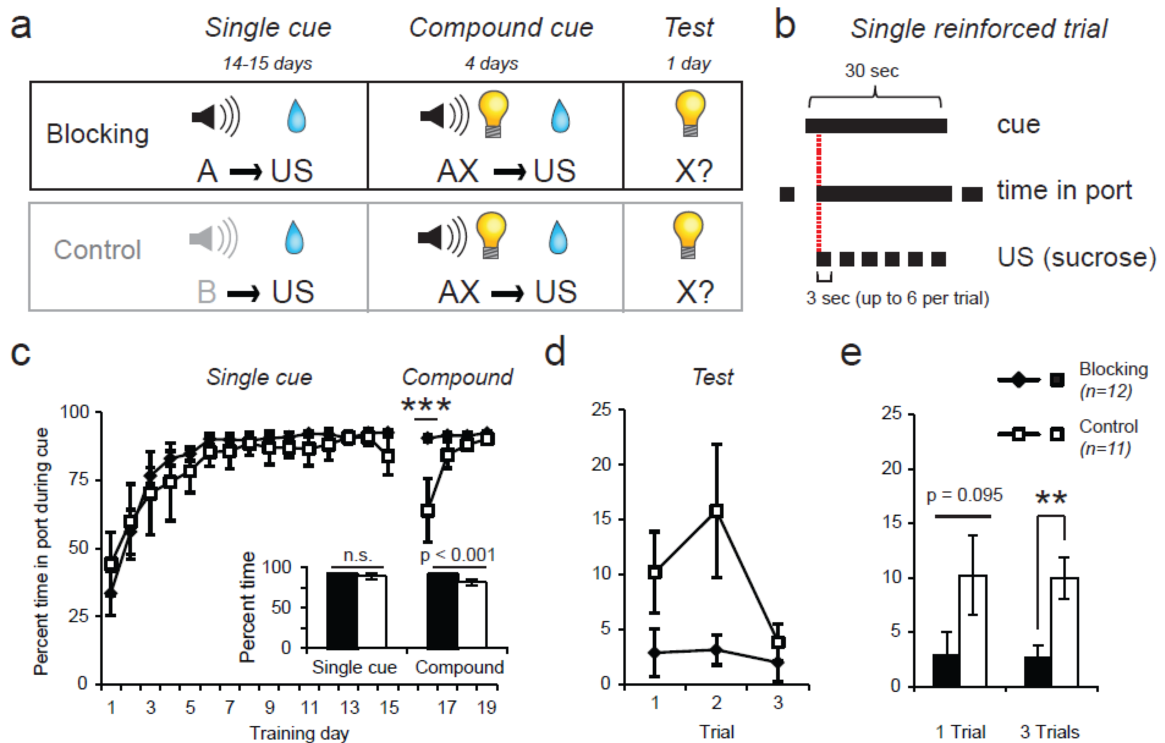


Figure 19. *Behavioral demonstration of the blocking effect.* (**a**) Experimental design of the blocking task. (**b**) During reinforced trials, sucrose delivery was contingent upon reward port entry during the 30s cue. After entry, sucrose was delivered for 3s followed by a 2s timeout. Up to 6 sucrose rewards could be earned per trial, depending on the rats' behavior. (**c**) Performance across all single cue and compound training sessions. Inset, mean performance among groups over the last four days of single-cue training did not differ; controls showed reduced behavior during compound training. (**d**) Performance during visual cue test. The blocking group exhibited reduced responding to the cue at test relative to controls (main effect of group, p=0.003, group x trial interaction, p=0.286). (**e**) Visual cue test performance for the first trial and the average of all three trials. The blocking group showed reduced cue responding for the 3-trial measure (**p=0.003) but were not different on the first trial (p=0.095). For all figures, values depicted are means and error bars represent SEM.

stimulus, US) during an auditory cue in a “single cue” training phase. Subsequently, a combined auditory and visual cue was presented in a “compound” training phase and the identical sucrose US was delivered. For subjects assigned to the Blocking group, the same auditory cue was presented during single and compound phases, whereas distinct auditory cues were used for Control group subjects (Fig. 19a); in both phases, US delivery was contingent on the rat’s presence in the reward port during the cue (Fig. 19b). Hence, the critical difference between experimental groups is US predictability during the compound phase: because of its prior association with the previously-trained auditory cue, the US is expected for the Blocking group, whereas, for the Control group, its occurrence is unexpected. Both groups showed equivalently high levels of conditioned responding, measured as an increase in percent time in the port during the cue relative to baseline, at the end of the single cue phase (two-way repeated-measures (RM) ANOVA, no effect of group or group x day interaction, all p’s >0.05), but differed in their performance when the compound cue was introduced (two-way RM ANOVA, main effect of group, $F_{1,21}=21.15$, $p<0.001$, and group x day interaction, $F_{3,63}=11.63$, $p<0.001$), consistent with the fact that the association between the compound cue and US had to be learned by the Control group (Fig. 19c).

To determine whether learning about the visual cue introduced during compound training was affected by the predictability of reward, conditioned responding to unreinforced presentations of the visual cue alone was assessed one day later. Conditioned responding was reduced in the Blocking group as compared to Controls (Fig. 19 d-e; two-way RM ANOVA, main effect of group, $F_{1,21}=11.27$, $p=0.003$, no group x

trial interaction, $F_{2,42}$=1.29, p=0.286), indicating that new learning about preceding environmental cues occurs after unpredicted, but not predicted, reward in this procedure, in accord with prior findings (Waelti et al., 2001; Burke et al., 2008).

Reward-paired dopamine neuron activation drives new learning

Putative dopamine neurons recorded in monkeys are strongly activated by unexpected reward, but fail to respond to the same reward if it is fully predicted (Mirenowicz and Schultz, 1994; Hollerman and Schultz, 1998), including when delivered in a blocking condition (Waelti et al., 2001). The close correspondence between dopamine neural activity and behavioral evidence of learning in this task suggests that positive RPEs caused by unexpected reward delivery activate dopamine neurons and lead to learning observed under control conditions. To test this hypothesis, we optogenetically activated VTA dopamine neurons at the time of US delivery on compound trials in our blocking task to drive learning under conditions in which learning normally does not occur. We used parameters that we have previously established elicit robust, time-locked activation of dopamine neurons and neurotransmitter release (Witten et al., 2011). We predicted that phasic dopamine neuron activation delivered coincidently with fully-predicted reward would be sufficient to cause new learning about preceding cues.

Female transgenic rats expressing Cre recombinase under the control of the tyrosine hydroxylase (Th) promoter (Th::Cre+ rats), or their wild-type littermates

(Th::Cre- rats) were used to gain selective control of dopamine neuron activity as described previously (Witten et al., 2011). Cre+ and Cre- subjects received identical injections of a Cre-dependent virus expressing channelrhodopsin-2 (ChR2) in the VTA; chronic optical fiber implants were also targeted dorsal to this region to allow for selective unilateral optogenetic dopamine neuron activation (Fig. 20, Fig. 21a). Three groups of rats were trained under conditions that normally result in blocked learning to the light cue (cue X; Fig. 21b). The behavioral performance of an experimental group (PairedCre+), consisting of Th::Cre+ rats receiving optical stimulation (1s train, 5ms pulse, 20 Hz) paired with the US during compound training (see Methods), was compared to the performance of two control groups that received identical training but differed either in genotype (PairedCre-) or the time at which optical stimulation was delivered (UnpairedCre+; optical stimulation during the intertrial interval; ITI) (Fig. 21c). Groups performed equivalently during single cue and compound training (Fig. 14d), suggesting that all rats learned the task and that the optical stimulation delivered during compound training did not disrupt ongoing behavior (two-way RM ANOVA revealed no significant effects of group or group x day interaction; all p's >0.111).

The critical comparison among groups occurred when the visual cue introduced during compound training was tested alone in an unreinforced session. PairedCre+ subjects responded more strongly to the visual cue on the first test trial than subjects from either control group (Fig. 21e-f), indicating greater learning. A two-way RM ANOVA revealed a significant interaction between group and trial ($F_{4,50}$=3.819, p=0.009) and a trend towards a main effect of group ($F_{2,25}$=3.272, p=0.055). Planned post-hoc

Figure 20

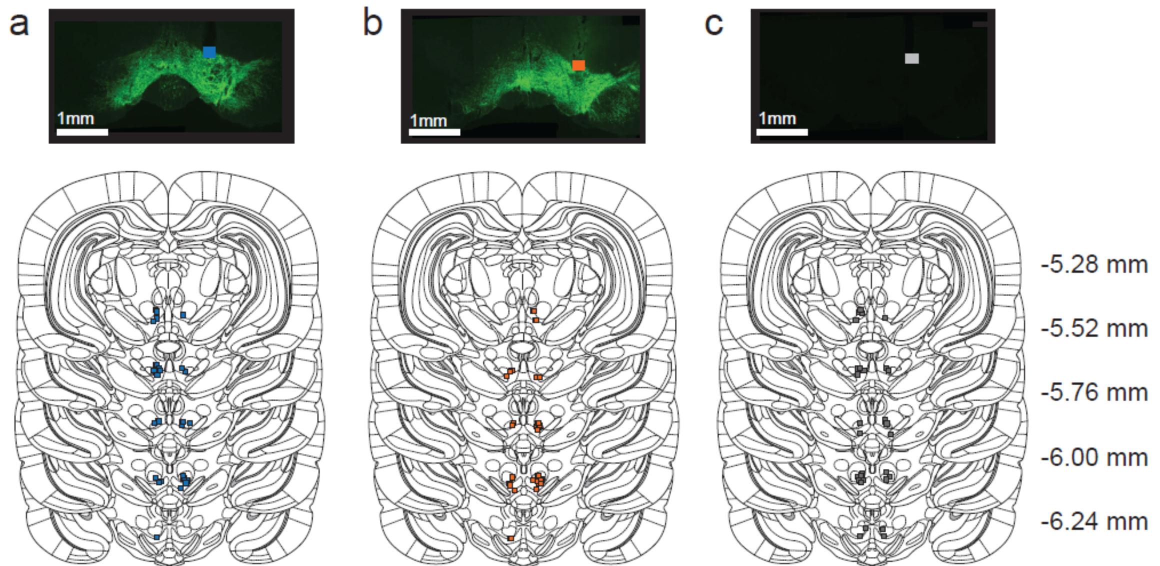


Figure 20. *Histological reconstruction of optical fiber placements for subjects in all studies.* (**a**) Top, ChR2-YFP expression for a representative PairedCre+ rat. Blue line denotes the location of the optical fiber tip in this rat. Note that virus injection and optical fiber placements were unilateral in this and all other studies. Bottom, optical fiber tip placement for all rats in this group (n=37). (**b**) Top, ChR2-YFP expression for a representative UnpairedCre+ rat. Orange line denotes optical fiber tip location in this rat. Bottom, optical fiber tip placement for all rats in this group (n=36). (**c**) Top, ChR2-YFP expression for a representative PairedCre- rat. Grey line denotes optical fiber tip location in this rat. Bottom, optical fiber tip placement for all rats in this group (n=41).

Figure 21

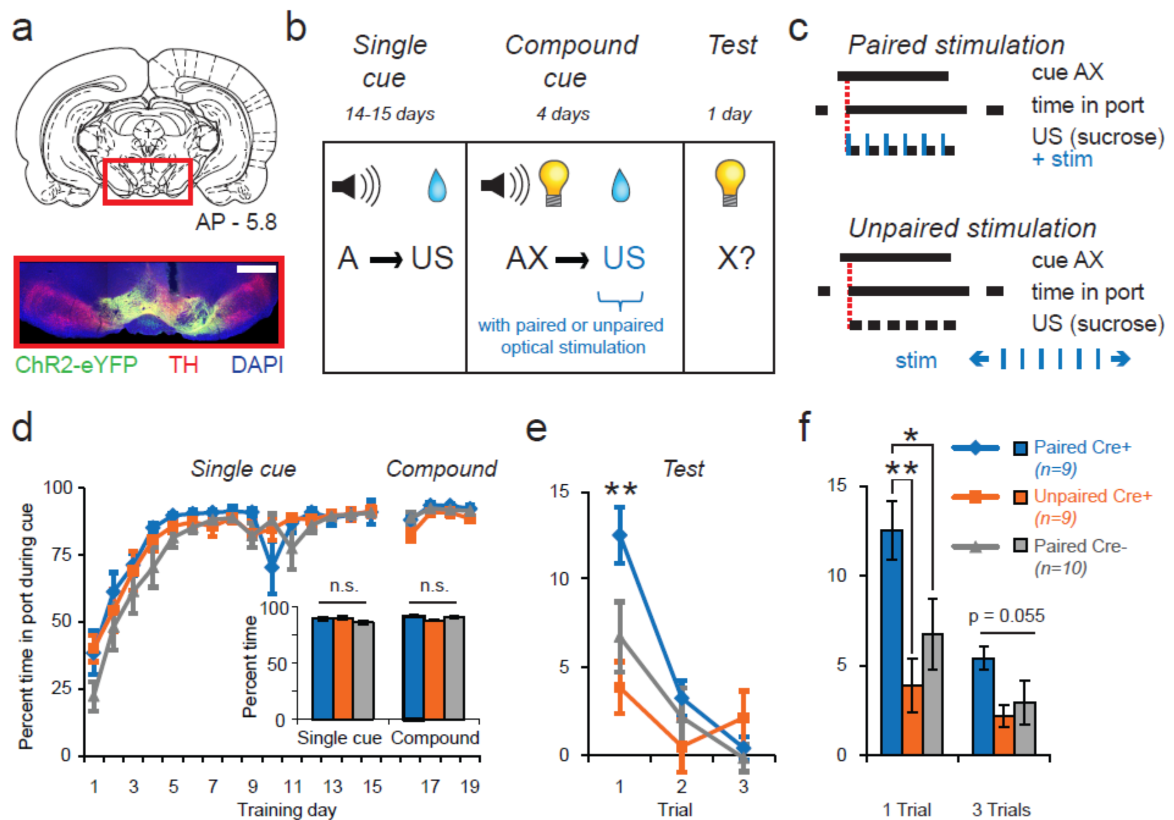


Figure 21. *Dopamine neuron stimulation drives new learning.* (**a**) Example histology from a Th::Cre+ rat injected with a Cre-dependent ChR2-containing virus. Vertical track indicates optical fiber placement above VTA. Scale bar = 1mm. (**b**) Experimental design for blocking task with optogenetics. All groups received identical behavioral training according to the “blocking” group design in Fig. 1a. (**c**) Optical stimulation (1s train, 5ms pulse, 20 Hz, 473nm) was synchronized with sucrose delivery in Paired (Cre+ and Cre-), but not Unpaired (Cre+), groups. (**d**) Performance across all single cue and compound training sessions. Inset, no group differences over last four days of single cue training or during compound training. (**e**) Performance during visual cue test. The PairedCre+ group exhibited increased responding to the cue relative to both control groups at test on the first trial (**p<0.005). (**f**) Visual cue test performance for the first trial and all three trials averaged. The PairedCre+ group exhibited increased cue responding relative to controls for the 1-trial measure (PairedCre+ vs. UnpairedCre+, **p=0.005, PairedCre+ vs. PairedCre-, *p=0.025, PairedCre- vs. UnpairedCre+, p=0.26); there was a trend for a group effect for the 3-trial average (main effect of group, p=0.055).

comparisons showed a significant difference between the PairedCre+ group and PairedCre- ($p=0.005$) or UnpairedCre+ ($p<0.001$) controls on the first test trial, while control groups did not differ (UnpairedCre+ vs. PairedCre- $p=0.155$; Fig. 21e-f). This result demonstrates that unilateral VTA dopamine neuron activity at the time of US delivery is sufficient to cause new learning about preceding environmental cues. The observed dopamine neuron-induced learning enhancement was temporally specific, as responding to the visual cue was blocked in the UnpairedCre+ group receiving optical stimulation outside of the cue and US periods. Importantly, PairedCre+ and UnpairedCre+ rats received equivalent stimulation and this stimulation was equally reinforcing (Fig. 22a-c), so discrepancies in the efficacy of optical stimulation between Cre+ groups cannot explain the observed behavioral differences.

One possible explanation for the behavioral changes we observed in the blocking experiment is that optical stimulation of dopamine neurons during compound training served to increase the value of the paired sucrose reward. Such an increase in value would result in a RPE (although not encoded by dopamine neurons) and unblock learning. We found however that the manipulation of dopamine neuron activity during the consumption of one of two equally-preferred, distinctly-flavored sucrose solutions did not change the relative value of these rewards, measured as reward preference; Fig. 23). This suggests that the unblocked learning about the newly-added cue X was not the result of increased reward value induced by manipulating dopamine neuron activity.

Figure 22

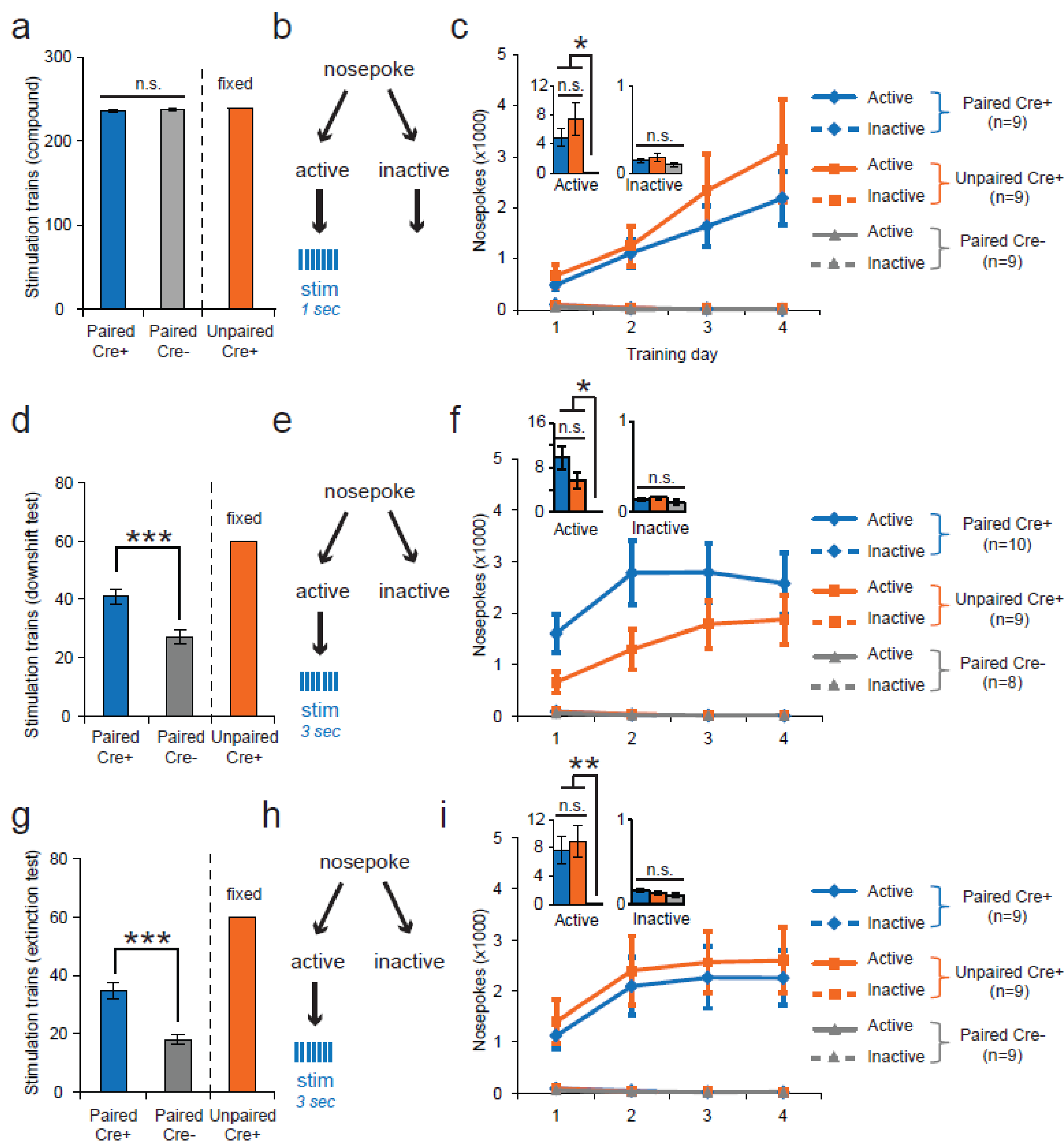


Figure 22. *Optical activation of dopamine neurons is equally reinforcing in Cre+ groups in all experiments.* (**a**) Stimulation trains delivered to all groups across four days of compound training in the Blocking study (Fig. 2). PairedCre+ and PairedCre- rats received equivalent amounts of optical stimulation (t-test, p=0.723) because of

equivalent behavioral performance during these sessions. UnpairedCre+ rats received a fixed number of stimulation trains during the ITIs, set at the maximum number of trains that could be earned by Paired groups. (**b**) Schematic of intracranial self-stimulation (ICSS) task for rats in the Blocking study (see Methods). (**c**) Responding across four daily ICSS sessions for rats in the Blocking study. PairedCre+ and UnpairedCre+ groups made significantly more active nosepoke responses than PairedCre- controls (two-way RM ANOVA, main effect of group and group x day interaction $F>4.589$, $p<0.008$; SNK post-hoc test PairedCre+ vs. PairedCre- $p=0.018$, UnpairedCre+ vs. Paired Cre-$p=0.005$) but did not differ from each other ($p=0.355$). There were no group differences in inactive nosepoke responding (two-way RM ANOVA, main effect of group and group x day interaction, $F<2.17$, $p>0.056$). Inset, summed responding across all 4 sessions. (**d**) Stimulation trains delivered to all groups during the downshift test session (Fig. 3). PairedCre+ rats earned more stimulation trains than PairedCre- rats (t-test, $p<0.001$) because of differences in behavioral performance during this test. (**e**) Schematic of ICSS task for rats in Downshift study. (**f**) As in C, but for rats in the Downshift study. PairedCre+ and UnpairedCre+ groups made significantly more active nosepoke responses than PairedCre- controls (two-way RM ANOVA, main effect of group and group x day interaction $F>3.711$, $p<0.003$; SNK post-hoc test PairedCre+ vs. PairedCre- $p<0.001$, UnpairedCre+ vs. PairedCre- $p=0.025$) but did not differ from each other ($p=0.068$). There were no group differences in inactive nosepoke responding (two-way RM ANOVA, main effect of group and group x day interaction, $F<1.7$, $p>0.154$). (**g**) Stimulation trains delivered to all groups during the extinction test session (Fig. 4). PairedCre+ rats earned more stimulation trains than PairedCre- rats (t-test, $p<0.001$) because of differences in behavioral performance during this test. (**h**) Schematic of ICSS task for rats in Extinction study. (**i**) As in C, but for rats in the Extinction study. PairedCre+ and UnpairedCre+ groups made significantly more active nosepoke responses than PairedCre- controls (two-way RM ANOVA, main effect of group and group x day interaction $F>3.041$, $p<0.01$; SNK post-hoc test PairedCre+ vs. PairedCre- $p=0.003$, UnpairedCre+ vs. PairedCre- $p=0.004$) but did not differ from each other ($p=0.612$). There were no group differences in inactive nosepoke responding (two-way RM ANOVA, main effect of group and group x day interaction, $F<2.187$, $p>0.054$). Note that the same behavioral data was used in F and I, but data from Cre+ rats was sorted differentially in each case according to each subject’s group assignment in Downshift and Extinction studies, as the same rats were used for both experiments. *$p<0.05$; ***$p<0.001$.

Figure 23

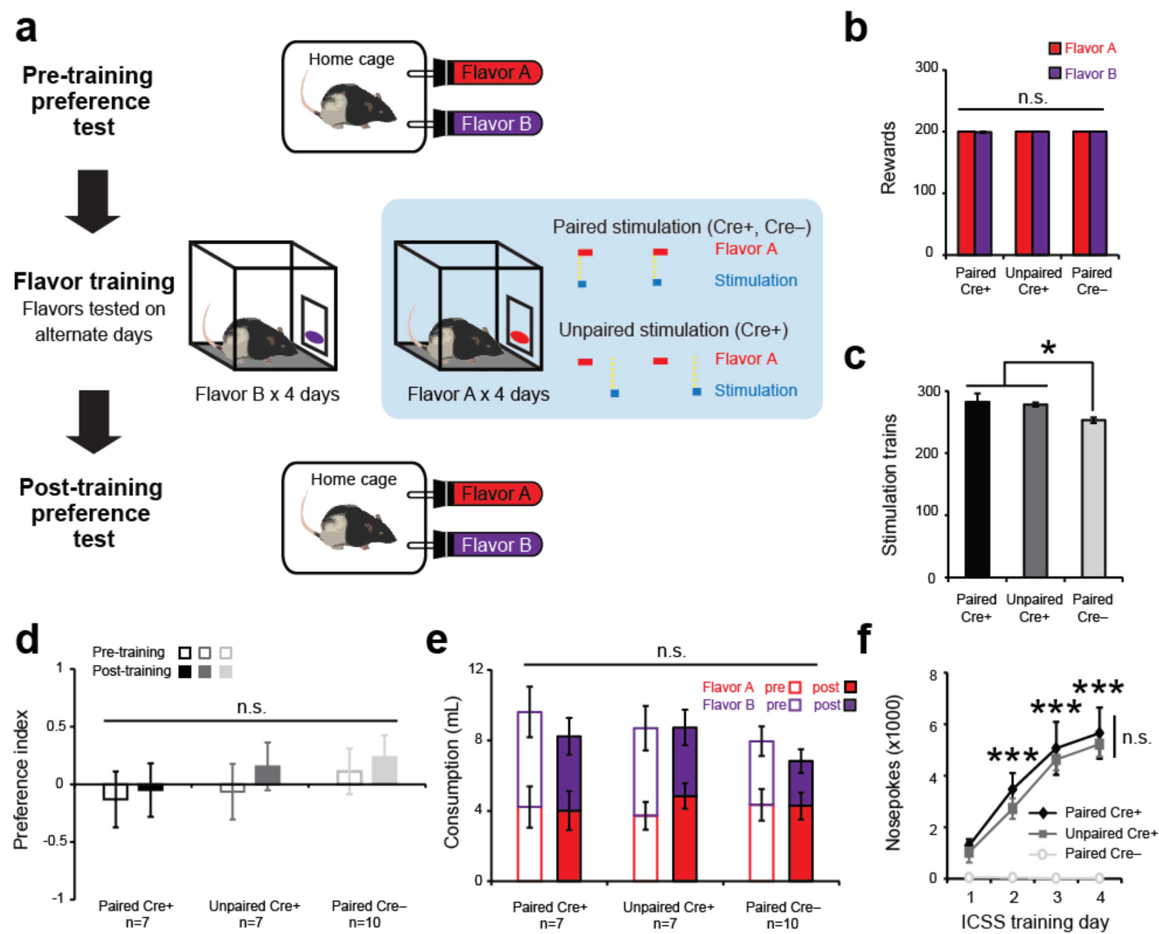


Figure 23. *Dopamine neuron activation does not alter preference for a paired natural reward.* (**a**) Experimental design for flavor preference study. Th::Cre+ rats, or their Th::Cre- littermates, were exposed to distinctly-flavored sucrose solutions in their home cage to quantify initial preference between the two flavors during a brief (10 min) pre-training test. The next day, flavor training commenced. Flavor training sessions were conducted in operant chambers; subjects received 50 reward deliveries on a VI-30s schedule. Only one flavor was available per day; flavor A and flavor B training days were interleaved. On days where flavor A was available, rats also received optical stimulation (20 pulses, 5ms duration, 20 Hz) of VTA dopamine neurons either paired or unpaired with reward delivery (see Supplemental Methods). 24 hours after the last flavor training session, subjects were again simultaneously exposed to both flavors during a brief (10 min) post-training test in their home cage to assess flavor preference. (**b**) Total number of rewards (flavor A and flavor B) consumed by each group during flavor training. There were no differences between groups or between flavors (two-way RM ANOVA, main effect of group, flavor and group x flavor interaction $F<1.296$, $p>0.268$). (**c**) Total number of stimulation trains delivered during flavor A training sessions. Note similarity to stimulation parameters used in the blocking experiment (Fig. S2A). PairedCre+ and UnpairedCre+ rats received more stimulation trains than PairedCre- rats (one-way ANOVA, main effect of group $F_{2,21}=4.869$, $p=0.018$; SNK post-hoc test PairedCre+ vs. PairedCre- $p=0.027$, UnpairedCre+ vs. PairedCre- $p=0.026$) because of slight differences in behavior during training sessions (see Supplemental Methods). (**d**) Pre-training and post-training flavor preference, quantified using a preference index. Positive values indicate a preference for flavor A, and negative values indicate a preference for flavor B. There were no group differences in flavor preference before or after flavor training (two-way RM ANOVA, main effect of group, time and group x time interaction $F<0.815$, $p>0.453$). (**e**) Total consumption of flavor A and flavor B during 10 min home cage tests. There were no group differences in the consumption of either flavor before or after flavor training (flavor A; two-way RM ANOVA, main effect of group, time and group x time interaction $F<0.137$, $p>0.715$; flavor B; two-way RM ANOVA, main effect of group, time and group x time interaction $F<2.061$, $p>0.152$). (**f**) Although no group differences were observed in the flavor preference study, subsequent ICSS training revealed that optical stimulation was highly reinforcing in the Cre+ rats used in these experiments (two-way RM ANOVA, main effect of group and group x day interaction $F>14.738$, $p<0.001$; SNK post-hoc test PairedCre+ vs. PairedCre-, $p<0.001$; UnpairedCre+ vs. PairedCre-, $p<0.001$; PairedCre+ vs. UnpairedCre+, $p=0.406$). *$p<0.05$; ***$p<0.001$.

Dopamine neuron activation at the time of expected reward slows extinction

Negative prediction errors also drive learned behavioral changes. For example, after a cue-reward association has been learned, decrementing or omitting the expected reward results in decreased reward-seeking behavior. dopamine neurons show a characteristic pause in firing in response to reward decrements or omissions (Hollerman and Schultz, 1998; Roesch et al., 2007; Cohen et al., 2012), and this pause is proposed to contribute to decreased behavioral responding to cues after reward decrement (Schultz et al., 1997; Schultz, 1998). Having established that optogenetically activating dopamine neurons can drive new learning about cues under conditions in which dopamine neurons normally do not change their firing patterns from baseline levels, we next tested whether similar artificial activation at a time when dopamine neurons normally *decrease* firing could counter decrements in behavioral performance produced by US value reductions. Th::Cre+ and Th::Cre- rats that received unilateral ChR2-containing virus infusions and optical fiber implants targeted to the VTA (Fig. 20), were trained to respond for sucrose whose availability was predicted by an auditory cue. One day after the last training session, the auditory cue was presented but water was substituted for the sucrose US (Downshift test; Fig. 24a). PairedCre+ and PairedCre- rats received dopamine neuron optical stimulation (3s train, 5ms pulse, 20 Hz) concurrent with water delivery when they entered the reward port during the cue; UnpairedCre+ rats received stimulation during the ITI. One day later, rats were given a downshift recall session identical to the first downshift test except that no optical stimulation was given. The purpose of the recall session was to determine if optical

stimulation had caused long-lasting behavioral changes. In this study, we measured cue responding in two ways, as the percent time spent in the reward port during the cue (Fig. 24b-d), and as the latency to enter the reward port after cue onset (Fig. 24e-g). As can be seen in Fig. 24b and 24e, all groups acquired the initial cue-US association; a two-way RM ANOVA revealed no significant effects of group or group x day interactions at the end of training (all p's>0.277). During the downshift test, PairedCre- and UnpairedCre+ group performance rapidly deteriorated. This was evident on a trial-by-trial basis (Fig. 24c,f, left) and when cue responding was averaged across the entire downshift test session (Fig. 24c,f, right). In contrast, PairedCre+ rats receiving optical stimulation concurrent with water delivery showed much reduced (Fig. 24c) or no (Fig. 24f) decrement in behavioral responding. Two-way RM ANOVAs revealed significant effects of group and group x trial interactions for both time spent in the port during the cue ($F_{2,28}$=11.12, p<0.001 and $F_{18,252}$=1.953, p=0.013) and latency to respond after cue onset ($F_{2,28}$=12.463, p<0.001 and $F_{18,252}$=4.394, p<0.001). Planned post-hoc comparisons demonstrated that PairedCre+ rats differed significantly from controls in both time and latency (p<0.001) while control groups did not differ from each other (p>0.375). Notably, some group differences persisted into the downshift recall session wherein no stimulation was delivered (latency: main effect of group, $F_{2,28}$=4.597, p=0.019; Fig. 24g). These data indicate that phasic VTA dopamine neuron activation can partially counteract performance decrements associated with reducing reward value.

We next determined if our optical manipulation would be effective if the expected reinforcer were omitted entirely (Fig. 25a). Rats used in the Downshift

Figure 24

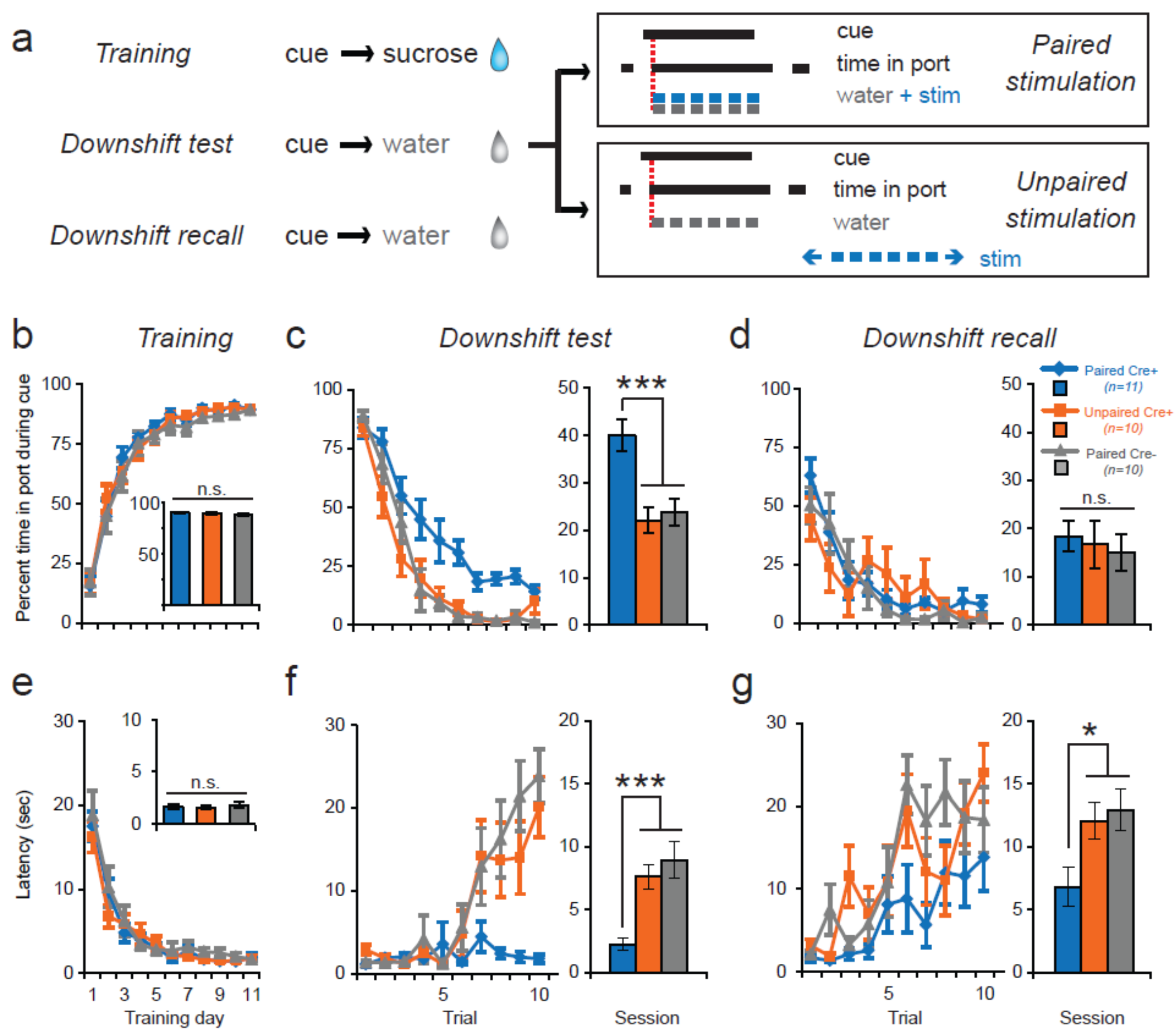


Figure 24. *Dopamine neuron stimulation attenuates behavioral decrements associated with a downshift in reward value.* (**a**) Experimental design for reward downshift test. Optical stimulation (3s train, 5ms pulse, 20 Hz, 473nm) was either paired with the water "reward" (PairedCre+ and Cre- groups) or explicitly unpaired (UnpairedCre+). (**b**) Percent time in port during the cue across training sessions. Inset, no difference in average performance during the last two training sessions. (**c**) Percent time in port during the cue for the downshift test. Data are displayed for single trials (left) and as a session average (right). PairedCre+ rats exhibited increased time in port compared to controls (PairedCre+ vs. UnpairedCre+, ***p<0.001, PairedCre+ vs. PairedCre-, ***p<0.001, PairedCre- vs. UnpairedCre+, p=0.691). (**d**). Percent time in port during the cue for downshift recall. Data are displayed for single trials (left) and as a session average (right). There were no group differences during this phase (2-way RM ANOVA, main effect of group p=0.835). (**e**) Latency to enter the reward port after cue onset. Inset, no group differences during last two training sessions. (**f)** As in C, but for latency. PairedCre+ rats responded faster to the cue compared to controls during the downshift test (PairedCre+ vs. UnpairedCre+, ***p<0.001, PairedCre+ vs. PairedCre-, ***p<0.001, PairedCre- vs. Unpaired Cre+, p=0.375). (**g**) As in D, but for latency. PairedCre+ rats responded faster to the cue compared to controls during downshift recall (PairedCre+ vs. UnpairedCre+, *p=0.024, PairedCre+ vs. PairedCre-, *p=0.025, PairedCre- vs. UnpairedCre+, p=0.706).

experiment (see Materials and Methods) were trained on a new cue-reward association. All rats learned the new association (Fig. 25b, e); a two-way RM ANOVA revealed no significant effects of group or group x day interactions at the end of training (all p's>0.242). Subsequently all rats were given an extinction test, during which the expected sucrose reward was withheld. Instead, PairedCre+ and PairedCre- rats received optical stimulation (3s train, 5ms pulse, 20 Hz) of dopamine neurons at the time of expected US delivery, while UnpairedCre+ rats received optical stimulation during the ITI. One day later, rats were given an extinction recall session where neither the US nor optical stimulation was delivered to determine if prior optical stimulation caused long-lasting behavioral changes.

During the extinction test, PairedCre+ rats spent more time in the reward port during the cue and responded to the cue more quickly as compared to both PairedCre- and UnpairedCre+ rats (Fig. 25c,f); two-way RM ANOVAs revealed significant effects of group and/or group x trial interactions for both measures (percent time: $F_{2,28}$=40.054, p<0.001 and $F_{18,252}$=0.419, p=0.983; latency: $F_{2,28}$=3.827, p=0.034 and $F_{18,252}$=2.047, p=0.008), and these behavioral differences persisted into the extinction recall session (Fig. 25d,g; two-way RM ANOVAs, significant main effects of group and group x trial interactions (F>2, p<0.01 in all cases). Hence, VTA dopamine neuron activation at the time of expected reward is sufficient to sustain conditioned behavioral responding when expected reward is omitted. For both reward downshift and omission, the behavioral effects of dopamine neuron stimulation were temporally specific, as UnpairedCre+ rats responded less than PairedCre+ rats despite receiving more stimulation during the test

Figure 25

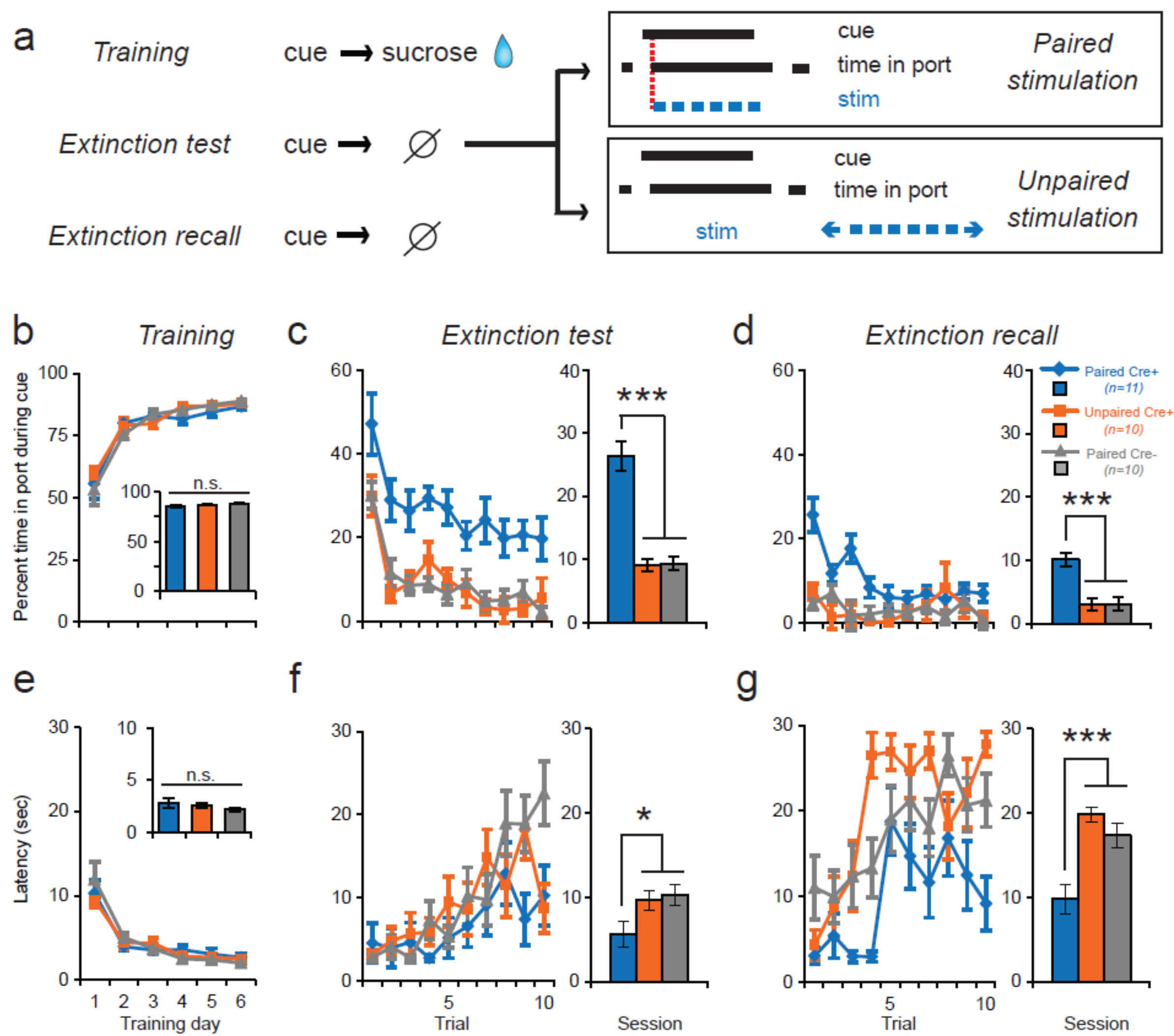


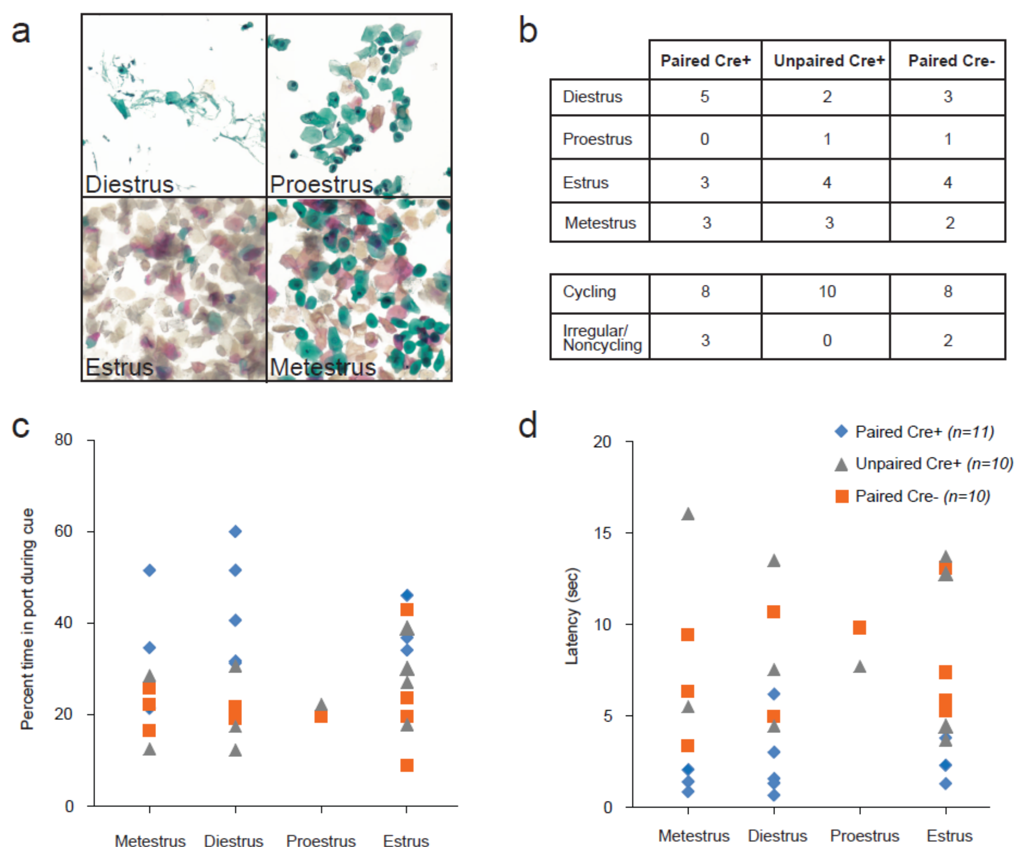
Figure 25. *Dopamine neuron stimulation attenuates behavioral decrements associated with reward omission.* (**a**) Experimental design for extinction test. Note that the same subjects from the downshift experiment were used for this procedure, with Cre+ groups shuffled between experiments (see Methods). Optical stimulation (3s train, 5ms pulse, 20 Hz, 473nm) was delivered at the time of expected reward for Paired groups and during ITI for UnpairedCre+ rats. (**b**) Percent time in port during the cue across training sessions. Inset, no difference in average performance during the last two training sessions. (**c**) Percent time in port during the cue for the extinction test. Data are displayed for single trials (left) and as a session average (right). PairedCre+ rats exhibited increased time in port compared to controls (PairedCre+ vs. UnpairedCre+, ***$p<0.001$, PairedCre+ vs. PairedCre-, ***$p<0.001$, PairedCre- vs. UnpairedCre+, $p=0.920$). (**d**). Percent time in port during the cue for extinction recall. Data are displayed for single trials (left) and as a session average (right). PairedCre+ rats exhibited increased time in port compared to controls (PairedCre+ vs. UnpairedCre+, ***$p<0.001$, PairedCre+ vs. PairedCre-, ***$p<0.001$, PairedCre- vs. UnpairedCre+, $p=0.984$). (**e**) Latency to enter the reward port after cue onset. Inset, no group differences during last two training sessions. (**f)** As in C, but for latency. PairedCre+ rats responded faster to the cue compared to controls during the extinction test (PairedCre+ vs. UnpairedCre+, *$p=0.038$, PairedCre+ vs. PairedCre-, *$p=0.04$, PairedCre- vs. UnpairedCre+ $p=0.727$). (**g**) As in D, but for latency. PairedCre+ rats responded faster to the cue compared to controls during extinction recall (PairedCre+ vs. UnpairedCre+, ***$p<0.001$, PairedCre+ vs. PairedCre-, ***$p<0.001$, PairedCre- vs. UnpairedCre+, $p=0.211$).

sessions (Fig. 22d,g), and despite verification that this stimulation is equally reinforcing in both Cre+ groups (Fig. 22e,f & h,i).

Despite causing significant behavioral changes during extinction, optogenetic activation of dopamine neurons failed to maintain reward-seeking behavior at pre-extinction levels. This may be due to the inability of our dopamine neuron stimulation to fully counter the expected decrease in dopamine neuron firing during reward omission or downshift. Alternatively, this may reflect competition between the artificially-imposed dopamine signal and other neural circuits specialized to inhibit conditioned responding when this behavior is no longer advantageous, as has been previously proposed (Daw et al., 2002; Peters et al., 2009).

Estrus cycle can modulate dopamine transmission under some circumstances (Becker, 1999). Notably, although female rats were used in these studies, we tracked estrus stage during a behavioral session where dopamine neurons were stimulated and failed to observe correlations between estrus cycle stage and behavioral performance (Fig. 26).

Figure 26



	Paired Cre+	Unpaired Cre+	Paired Cre-
Diestrus	5	2	3
Proestrus	0	1	1
Estrus	3	4	4
Metestrus	3	3	2

Cycling	8	10	8
Irregular/ Noncycling	3	0	2

Figure 26. *Estrus cycle stage is not related to performance during a behavioral test session with dopamine neuron activation.* (**a**) Typical cytology observed during diestrus, proestrus, estrus and metestrus from cell samples collected from the vaginal wall. Stages were classified according to established criteria (see Methods). (**b**) Vaginal cytology results from all rats in the Downshift experiment during the downshift test when optical stimulation was delivered. Assessments of cycle regularity were based on an examination of cytology samples over five consecutive days. (**c**) Scatterplot of behavioral performance and estrus cycle stage for all rats during the downshift test. A one-way ANOVA revealed no main effect of estrus cycle stage on performance (pooled data from all subjects; main effect of stage, $F_{3,27}$=0.502, p=0.684). Behavioral performance was measured as percent time spent in the reward port during the cue. (**d**). As in C, but with behavioral performance measured as the latency to enter the reward port after cue onset. A one-way ANOVA revealed no main effect of estrus cycle stage on performance (pooled data from all subjects; main effect of stage, $F_{3,27}$=0.41, p=0.747).

Discussion

Here we demonstrate that reward prediction error signaling by dopamine neurons is causally related to cue-reward learning. We leveraged the temporal precision afforded by optogenetic tools to mimic endogenous RPE signaling in VTA dopamine neurons in order to determine how these artificial signals impact subsequent behavior. Using an associative blocking procedure, we observed that increasing dopamine neuron activity during reward delivery could drive learning about antecedent cues that would not normally guide behavior. Using extinction procedures, we observed that reductions in conditioned responding that normally accompany decreases in reward value are attenuated when dopamine neuron activity is increased at the time of expected reward. Importantly, the behavioral changes we observed in all experiments were long-lasting, persisting twenty-four hours after dopamine neurons were optogenetically activated, and temporally-specific, failing to occur if dopamine neurons were activated at times outside of the reward consumption period. Taken together, our results demonstrate that RPE signaling by dopamine neurons is sufficient to support new cue-reward learning and modify previously-learned cue-reward associations.

Our results clearly establish that artificially activating VTA dopamine neurons at the time that a natural reward is delivered (or expected) supports cue-elicited responding. A question of fundamental importance is *why* this occurs. In particular, for the blocking study, one possibility is that dopamine stimulation acted as an independent reward, discriminable from the paired sucrose reward that allows the formation of a parallel association between the reward-predictive cue and dopamine stimulation itself.

However this explanation assumes cue independence and would require the rat to compute two simultaneous yet separate prediction errors controlling, respectively, the strength of two separate associations (cue A → sucrose; cue X → dopamine stimulation). Indeed, the assumption of cue independence was challenged (Rescorla and Wagner, 1972) specifically because separate prediction errors cannot account for the phenomenon of blocking. If each cue generated its own independent prediction error, then the preconditioning of one cue would not affect the future conditioning of other cues; but it does, as the blocking procedure shows. Blocking demonstrates that cues presented simultaneously interact and compete for associative strength. Hence, it is unlikely that a parallel association formed between reward-predictive cues and dopamine stimulation can account for our results. Of interest, putative dopamine neurons do not appear to encode a sensory representation of reward since they do not discriminate among rewards based on their sensory properties (Schultz, 1998), thus it is not obvious how dopamine neuron activation at the same time as natural reward delivery could be perceived as distinct from that reward.

Although previous studies suggest otherwise (Berridge and Robinson, 1998; Wassum et al., 2011), another related possibility is that optical activation of dopamine neurons induces behavioral changes by *directly* enhancing the value of the paired natural reward. To address this possibility, we conducted a control study based on the conception that high-value rewards are preferred over less valuable alternatives. We paired dopamine stimulation with consumption of a flavored, and thus discriminable, sucrose solution; we reasoned that if dopamine stimulation served to increase the value

of a paired reward, this should manifest as an increased preference for the stimulation-paired reward over a distinctly-flavored but otherwise identical sucrose solution. However, we observed that reward that was previously paired with dopamine stimulation was preferred equivalently to one that was not. This result does not support the interpretation that optical dopamine stimulation supported learning in our experiments by increasing the value of the sucrose reward.

Alternatively, the behavioral changes we observed in PairedCre+ rats could reflect the development of a conditioned place preference for the location where optical stimulation was delivered (i.e., the reward port), as has been previously demonstrated (Tsai et al., 2009). If this were the case, we should have observed generalized increases in reward-seeking behavior across the entire test session. Critically, our primary behavioral metric (time spent in the reward port during the cue) was normalized to pre-cue baseline levels. If optical stimulation had induced non-specific increases in reward-seeking behavior, our normalized measure should have approached zero. However, we found that reward-seeking was specifically elevated during cue presentation. While we observed robust group differences in our normalized measures, a separate analysis of the absolute percent time spent in the port in the pre-cue baseline period during any test session revealed no significant group differences in baseline responding (all p's>0.17). Together, these findings indicate that the behavioral changes we observed are unlikely to be the result of a conditioned place preference.

Instead, the most parsimonious explanation for our results is that dopamine stimulation reproduced a RPE. Theories of associative learning hold that simple pairing, or contiguity, between a stimulus and reward or punishment is not sufficient for conditioning to occur; learning requires the subject to detect a discrepancy or 'prediction error' between the expected and actual outcome which serves to correct future predictions (Rescorla and Wagner, 1972). Although compelling correlative evidence indicated that dopamine neurons are well-suited to provide such a teaching signal, remarkably little proof existed to support this notion. For this reason, our results represent an advance over previous work. While prior studies that also utilized optogenetic tools to permit temporally-precise control of dopamine neuron activity demonstrated that dopamine neuron activation is reinforcing, these studies did not establish the means by which this stimulation can reinforce behavior. Because we used behavioral procedures in which learning is driven by reward prediction errors, our data establishes the critical behavioral mechanism (RPE) through which phasic dopamine signals timed with reward cause learning.

Through which cellular and circuit mechanisms could this dopamine signal cause learning to take place? Though few in number, VTA dopamine neurons send extensive projections to a variety of cortical and subcortical areas and are thus well-positioned to influence neuronal computation (Beckstead et al., 1979; Swanson, 1982; Fields et al., 2007; Glimcher, 2011). Increases in dopamine neuron firing during unexpected reward could function as a teaching signal used broadly within efferent targets to strengthen neural representations that facilitate reward receipt (Reynolds and Wickens, 2002;

Wickens et al., 2007), possibly via alterations in the strength and direction of synaptic plasticity (Reynolds et al., 2001; Fields et al., 2007; Tye et al., 2010; Gerfen and Surmeier, 2011). Because our artificial manipulation of dopamine neuron activity produced behavioral changes that lasted at least twenty-four hours after the stimulation ended, such dopamine-induced, downstream changes in synaptic function may have occurred; additionally, both natural cue-reward learning (Stuber et al., 2008) and optogenetic stimulation of dopamine neurons (Brown et al., 2010) alter glutamatergic synaptic strength onto dopamine neurons themselves, providing another possible basis for the long-lasting effects of dopamine neuron activation on behavior. One or both of these synaptic mechanisms may underlie the behavioral changes reported here.

Here we focused on the role of dopamine neuron activation at the time of reward. Another hallmark feature of dopamine neural firing during associative learning is the gradual transfer of neural activation from reward delivery to cue onset. Early in learning when cue-reward associations are weak, dopamine neurons respond robustly to the occurrence of reward and weakly to reward-predictive cues. As learning progresses this relationship is reversed as neural responses to the cue become more pronounced and reward responses diminish (Hollerman and Schultz, 1998). While our current results support the idea that reward-evoked dopamine neuron activity drives conditioned behavioral responding to cues, the function(s) of cue-evoked dopamine neuron activity remain a fruitful avenue for future investigation.

Possible answers to this question have already been proposed. This transfer of the dopamine teaching signal from the primary reinforcer to the preceding cue is

predicted by temporal-difference models of learning (Suri, 2002). In such models, the back-propagation of the teaching signal allows the earliest predictor of the reward to be identified, thereby delineating the chain of events leading to reward delivery (Sutton and Barto, 1981; Suri, 2002; Glimcher, 2011). Alternatively, or in addition, cue-evoked dopamine may encode the cue's incentive value, endowing the cue itself with some of the motivational properties originally elicited by the reward, thereby making the cue desirable in its own right (Berridge and Robinson, 1998). Using behavioral procedures that allow a cue's predictive and incentive properties to be assessed separately, a recent study provided support for a role for dopamine in the acquisition of cue-reward learning for the latter, but not the former process (Flagel et al., 2010). Such behavioral procedures could also prove useful to determine in greater detail how learning induced by mimicking RPE signals impacts cue-induced conditioned responding. These and other future attempts to define behaviorally-specific roles for dopamine neuron activity during cues and rewards will further refine our conceptions of the role of dopamine RPE signaling in associative learning.

Chapter 5

Significance of this dissertation and remaining questions

Significance

Dopamine neurons have received a great deal of experimental attention since they were first identified in the middle of the last century. They feature prominently in theories that describe how experimental animals (and presumably humans) move, eat, learn, remember, and experience natural and drug rewards. Dopamine neurons have even been suggested to play a major role in the neural basis of consciousness (Palmiter, 2011). Despite a wealth of knowledge gained from thousands of dopamine studies that have been conducted to date, many significant questions remain. Many of these questions could be best answered by directly modifying the activity of dopamine neurons or other elements of the circuits in which they participate.

The experiments presented in this dissertation stemmed directly from this idea. After helping to develop the tools necessary to selectively manipulate VTA dopamine neuron activity in behaving rats (Chapter 2), I used these tools to clarify the relationship between dopamine neuron activity and positive reinforcement (Chapter 3). I chose a simple behavioral model (ICSS) in order to facilitate interpretation of the results in the context of the question I had set out to address. I found that activation of VTA dopamine neurons was sufficient to support ICSS, and further found that the same behavior (albeit at reduced levels) could be elicited by selectively activating the major efferent projection of these neurons. I also demonstrated that dopamine-specific ICSS

was significantly reduced by intra-NAc administration of D1 or D2 antagonists. These data unambiguously establish dopamine neurons as a mediator of positive reinforcement. However, they did not provide insight into *why* this occurs.

To address this question, I conducted a series of experiments in collaboration with Dr. Ronald Keiflin designed to determine whether dopamine neuron activation was sufficient to drive associative cue-reward learning by signaling errors in reward prediction (Chapter 4). We used Associative Blocking as our behavioral model, as this paradigm provides a strong test of the role of prediction errors in learning. We found that optogenetically activating dopamine neurons coincidently with a fully-expected reward was sufficient to drive learning about an antecedent cue, as evidenced by subsequent increases in cue-elicited reward-seeking behavior. We went on to confirm this effect in a different behavioral paradigm, Extinction. Here, we found that optogenetically activating dopamine neurons coincident with an expected (but not actually delivered) reward was sufficient to slow the normally observed reductions in cue-elicited reward-seeking behavior. These results demonstrate that at least one reason why dopamine neurons support reinforcement is because they directly drive learning.

While these data represent an important advance in our understanding of how dopamine neurons contribute to appetitive behaviors, a significant interpretational caveat merits some discussion. Although dopamine neurons are commonly referred to as just that – dopamine neurons – many of these cells also co-release peptides and other neurotransmitters. Thus, when dopamine neurons are optogenetically activated,

release of these molecules will also be modulated. Some dopamine neurons co-release other neurotransmitters such as glutamate (Stuber et al., 2010; Tecuapetla et al., 2010), or GABA (Tritsch et al., 2012) and contain several peptides which also have the potential to potently modulate neural activity in downstream structures. These include cholecystokinin (Seroogy et al., 1989; Hamilton et al., 2000) and neurotensin (Seroogy et al., 1988). Activity-dependent release of neurotrophic factors, such as neurotrophin3 (Seroogy and Gall, 1993), and brain-derived neurotrophic factor (Seroogy and Gall, 1993) could also occur following optogenetic activation of dopamine neurons. Under some conditions, dopamine neurons can even co-release serotonin (Zhou et al., 2005). It should be noted that this confound is not specific to optogenetic techniques, as any manipulation (such as electrical stimulation or pharmacological inhibition) that acts to enhance or suppress dopamine neuron activity will cause corresponding changes in the release of other signaling molecules present in dopamine neurons. However, unless specific antagonists are used to verify that dopamine acting at its receptors is causally contributing to the observed behavioral effects, conclusions can only be drawn about the consequences of dopamine neuron activity, and not specifically about the actions of dopamine. Thus, it is notable that the vigorous ICSS behavior that we observed in Chapter 3 was substantially reduced by targeted infusions of dopamine antagonists into the NAc, providing evidence that at least this behavior was linked to the post-synaptic actions of dopamine.

Remaining questions

This is an exciting time to be a scientist investigating the neural and behavioral functions of dopamine. Advancements in the ability to manipulate, visualize and measure dopaminergic transmission in a variety of *in vivo* and *ex vivo* preparations open the door for new and exciting research opportunities. Going forward, some currently-held conceptions are likely to be supported by the insights gained from the next generation of research, and others may eventually seem less tenable.

The results presented in this dissertation address only a small fraction of the questions currently ripe for exploration. For example, dopamine neurons are strongly activated by reward-predictive cues, in addition to rewards themselves. What are the consequences of manipulating dopamine neuron activity during salient cues? Does the answer to this question depend on whether the cues are associated with food or drug delivery, or are merely novel? Some dopamine neurons are also activated by aversive stimuli, such as footshock. What are the consequences of manipulating phasic dopamine neuron activity during paradigms in which learning is motivated by stimuli with negative valence? Dopamine neurons also have low-level tonic activity that is proposed to be important for enabling motor function, particularly in the dorsal striatum. What are the consequences of manipulating tonic dopamine neuron activity during defined time periods? Another critical, though admittedly broad question is how specific afferent or efferent projections to/from dopamine neurons impact neural function and behavior. Do the answers to any of the above questions vary by projection

target? With time, the answers to these and other pressing questions are sure to be forthcoming.

Materials and Methods

General Methods

Surgical procedures

Standard stereotaxic surgical procedures were used to unilaterally infuse Cre-dependent virus (AAV5 Ef1α-DIO-ChR2-eYFP, titer 1.5-4 x 10^{12} particles/mL, University of North Carolina viral vector core) and implant optical fibers. Two small burr holes were drilled over the VTA at the following coordinates: AP -5.4 & -6.2; ML ± 0.7. A custom-made 31 gauge infuser was used to deliver 1.0 ul of virus at two depths in each hole (DV -8.4 and -7.4 for males, -8.1 and -7.1 for females) for a total of 4.0 ul virus delivered unilaterally to the VTA. Each 1.0 ul of virus was infused at a speed of 0.1 ul per minute using a syringe pump (Harvard Apparatus). The virus infuser was left in place for an additional 10 minutes following each injection before it was slowly removed. A third burr hole was drilled (AP -5.8; ML ± 0.7) for the insertion of an implantable optical fiber targeted just dorsal to the VTA (DV -7.4 for males, -7.1 for females) in most rats. Alternatively, optical fiber implants or bilateral guide cannulae (26 gauge, Plastics1) were targeted to the NAc (AP +1.6, ML ± 1.4 , DV -6.5 (fiber) or -6.8 (infuser tip)) in some subjects. Chronically implantable optical fibers were made in-house with optical fiber (BFL37-300, Thorlabs) and a metal ferrule (F10061F360, Fiber Instrument Sales) and were secured to the skull surface with 5 jeweler's screws and dental cement. All coordinates are in mm relative to bregma and skull surface.

Optical activation

Prior to training sessions, rats were gently attached to patch cables made in-house with optical fiber (BFL37-200, Thorlabs) encased in a durable metal spring covering (PS95, Instech). These cables terminated with a metal ferrule connector (F10061F250, Fiber Instrument Sales) that was secured to the rats' cranial implant with a fitted ceramic sleeve (F18300SSC25, Fiber Instrument Sales), and were attached at the other end to an optical commutator (Doric Lenses). This commutator was connected via a second optical patch cable to a 100-150 mW DPSS 473nm laser (OEM Laser Systems). The commutator was affixed to a counter-balanced lever arm (Med Associates) to minimize cable weight and provide lift when rats were rearing. Optical stimulation was controlled by a computer running Med PC IV (Med Associates) software, which also recorded behavioral responses.

Peak light output during photostimulation was estimated to be ~ 1.5-2 mW at the tip of the implanted fiber for each session, and ~0.45-0.6 mW/mm^2 at the targeted tissue 500 um from the fiber tip. This peak light power was based on measuring the *average* light power for the pulsed light parameters used during experiments (20 Hz, 5 ms duration), and then correcting for the duty cycle to arrive at the peak power (in this case by dividing by 0.1). The power density estimate was based on the light transmission calculator at www.optogenetics.org/calc.

Histological procedures

Rats were deeply anesthetized with sodium pentobarbital and perfused transcardially with saline followed by 4% paraformaldehyde. Immunohistochemical detection of TH and YFP was performed in all subjects; in addition Fos immunohistochemistry was performed on a subset of subjects (Chapter 3). For Fos immunohistochemistry, subjects were sacrificed immediately after experiencing one of two behavioral conditions: experimental condition, 120 min ICSS session during which no drugs were infused; or control condition, 120 min spent in the behavioral chambers where no behavioral program was loaded and no stimulation was delivered.

In all cases, brains were removed, post-fixed in paraformaldehyde for up to 24 hours, cryoprotected in 25% sucrose for >48 hours and sectioned at 40-60 µm. Free-floating coronal sections were washed in PBS and then incubated with bovine serum albumin (BSA) and Triton X-100 (each 0.2%) for 20 min. 10% normal donkey serum (NDS) was added for a further 30 min incubation. Primary antibody incubations were performed overnight at 4°C in PBS with BSA and Triton X-100 (each 0.2%). Double and in some cases triple immunohistochemistry was performed; concentrations and sources for primary antibodies were: mouse anti-GFP (1:1500, Invitrogen) rabbit anti-TH (1:1500, Fisher Scientific), and goat anti-Fos (1:500, Santa Cruz). Sections were then washed and incubated with 2% NDS in PBS for 10 minutes and secondary antibodies were added (1:200) conjugated to Alexa Fluor 488 or 594 dyes (Invitrogen) or CF633 (Biotium Inc.) for 2 hours at room temperature. Sections were mounted onto microscope slides in phosphate-buffered water, coverslipped with Vectashield mounting

medium (Fisher Scientific) and visualized on a confocal microscope. Although optical fiber placements and virus expression varied slightly between subjects, none were excluded based on histology.

Methods, Chapter 2

BAC transgenic rat production *(Dr. Ilana Witten and Deisseroth lab)*

The Th::Cre construct consisted of a Cre gene introduced immediately before the ATG of the mouse Th gene (BAC address RP23-350E13) as described previously (Gong et al, 2007). The BAC construct was purified using NucleoBond® BAC 100 from ClonTech. BAC DNA was verified by sequencing and by pulse field electrophoresis of a Not1 digest. It was then resuspended in microinjection buffer (10 mM Tris-HCl pH 7.5 0.1 mM EDTA 100 mM NaCl + 1 x polyamine) at a concentration of 1.0 ng/ul. The construct was injected into the nucleus of fertilized eggs (derived from mating Long Evans rats) and transferred to a pseudopregnant recipient (University of Michigan transgenic core). This procedure resulted in 7 Th::Cre founder lines with transgene incorporation into the genome, as determined by Cre genotyping.

Of the initial founders, 3 Th::Cre founders exhibited robust expression of Cre-dependent opsin virus in the VTA. The breeding procedure consisted of mating Cre-positive founders or their offspring with wild-type rats from a commercial source to obtain heterozygous (as well as wild-type) offspring. The advantage of using heterozygous offspring was twofold. First, it is easier to create a large, stable colony of heterozygous animals without risking in-breeding; second, heterozygous rats are less likely than homozygous rats to exhibit unwanted side-effects of expressing the transgene since they express one wild-type chromosome. The expression profile of YFP was quantified in relationship to TH expression for the F2 generation (or later) in these lines to allow for stabilization of the transgenes, and specificity was found to be greater

than 90% for the offspring of 1 Th::Cre (Founder 3). No differences in ChR2-YFP expression profile were observed between F2s originating from the same founder.

Acute slice *in vitro* electrophysiology *(Dr. Soo Yeun Lee)*

Coronal slices (325µm) from adult rats (3 months or older) previously injected with virus were prepared after intracardial perfusion with ice-cold sucrose-containing artificial cerebrospinal fluid (ACSF; in mM: 85 NaCl, 75 sucrose, 2.5 KCl, 25 glucose, 1.25 NaH_2PO_4, 4 $MgCl_2$, 0.5 $CaCl_2$, and 24 $NaHCO_3$). Slices were incubated for 1 hr at 32-34ºC, and then at room temperature until transferred to an oxygenated standard ACSF solution (in mM: 126 NaCl, 2.5 KCl, 26 $NaHCO_3$, 2 $CaCl_2$, 2 $MgCl_2$, 1.25 NaH_2PO_4, and 10 glucose) for electrophysiological recordings performed at 32-34°C. Slices were visualized with an upright microscope equipped with a 40X water-immersion objective (DM-LFSA, Leica Microsystems) and infrared differential interference contrast (IR-DIC) optics. Individual neuron recordings were obtained after identifying fluorescent protein expression under constant ACSF perfusion. Filtered light from a broad-wavelength xenon lamp source (Sutter Instruments DG-4) was coupled to the fluorescence port of the microscope and used both to view fluorescence and deliver light pulses for opsin activation. Power density of the blue light was 4.3-5 mW/mm^2, measured with a power meter (ThorLabs). Whole-cell recordings were obtained with patch pipettes (3-5 MΩ) pulled from borosilicate glass capillaries (Sutter Instruments) with a horizontal puller (P-2000, Sutter Instruments) and contained the following internal solution (in mM: 135 K-gluconate, 5 KCl, 10 HEPES, 0.1 EGTA, 2 MgCl2, 2 MgATP, 0.2 NaGTP). Recordings were

made using a MultiClamp700B amplifier (Molecular Devices). Signals were filtered at 1 kHz and digitized at 10-20 kHz with a Digidata 1440A analog–digital interface (Molecular Devices). pClamp10.3 software (Molecular Devices) was used to record and analyze data.

In the analysis of *in vitro* electrophysiology, listed membrane potential refers to the initial potential measured immediately after attaining whole-cell configuration. To measure the magnitude of the hyperpolarization-activated inward I_h current, cells were held at -40mV, and a 500ms voltage step to -120mV was applied. I_h was measured as the difference between the initial capacitative response to the voltage step (usually ~20-40 ms after the beginning of the voltage step) and the final steady-state current the end of the 500ms pulse; responses greater than 115pA were classified as $I_{h/large}$. The apparent input resistance was calculated from the linear portion of the steady-state I-V curve obtained by applying 500ms hyperpolarizing current pulse steps. Action potential threshold was measured as the voltage at which the first-order derivative of the membrane potential (dV/dt) exhibited a sharp transition (typically >10mV/ms). The action potential threshold was also used to set the threshold in determining spike fidelity (% of successful action potential after various light stimulation frequencies). Peak and steady-state photocurrents were measured from a 1s light pulse in voltage-clamp mode. Series resistances were carefully monitored and recordings were not used if the series resistance changed significantly (by >20%) or reached 20 MΩ. Statistical

analysis was performed with a two-tailed Student's t-test, with a level of significance set at $p < 0.05$.

***In vivo* optrode recording** *(Dr. Ilana Witten)*

Simultaneous optical stimulation and extracellular electrical recording were performed in anesthetized rats as described previously (Gradinaru et al., 2007). Optrodes consisted of a tungsten electrode (1 MΩ; .005 in; parylene insulation) glued to an optical fiber (300 µm core diameter, 0.37 N.A.), with the tip of the electrode projecting beyond the fiber by 300-500 mm, and the optical fiber was coupled to a 473 nm laser. The light power was 1-3 mW at the fiber tip, which corresponds to a density at the tip of the electrode of about ~0.3-0.9mW/mm^2. Signals were amplified and band-pass filtered (300Hz low cut-off, 10 kHz high cut-off) before digitizing and recording to disk.

Fast-scan cyclic voltammetry *(Dr. Kay Tye)*

Coronal brain slices (300-400 µm) were prepared from adult rats with virus injected 5-7 weeks prior. Slices were maintained in artificial cerebral spinal fluid (ACSF) containing (in mM): 123 NaCl, 26 $NaHCO_3$, 3 KCl, 1.25 $NaH2PO_4$*H_2O, 1 $MgCl_2$*6H_2O, 2 $CaCl_2$*2H_2O, and 11 glucose. ACSF was bubbled with 95% O_2/5% CO_2 and slices were heated to 32-33ºC during recordings. A carbon fiber glass electrode (with the carbon fiber protruding beyond the glass tip by 200-300 um) was positioned in the NAc under fluorescent guidance. Voltammetric measurements were made every 100 ms by

application of a triangular waveform (-0.4 V to +1.4 V, at 400 V/s) to the carbon fiber electrode vs. an Ag/AgCl reference electrode. Data was acquired and analyzed using custom software written in LabVIEW. Dopaminergic terminals were activated with an optical fiber connected to a 473nm laser (20 Hz, 5ms pulse duration). Slices were allowed to recover 5 min between each stimulation train. To estimate changes in dopamine release, background current at the electrode was subtracted from the current measured immediately following optical stimulation. Background subtracted cyclic voltammogram showed peak oxidation and reduction currents at ~650 mV and -200 mV respectively, indicating the signals were due to the detection of evoked dopamine release, and consistent with previous results. Following slice recordings, electrodes were calibrated in 1 µM dopamine solution.

Methods, Chapter 3

Subjects

37 male Th::Cre rats, 300-550g at the start of the experiment, were individually housed in a light-regulated (12h light/dark cycle, lights on at 07:00) colony room. Food and water were available *ad libitum* throughout the experiment. Animal husbandry and all experimental procedures were in accordance with the guidelines from the National Institutes of Health and were approved in advance by the Gallo Center Institutional Animal Care and Use Committee.

Behavioral procedures

All behavioral tests were conducted > 5 weeks post-surgery. The tests are described in the order in which they were performed. For all test sessions, the start of a session was indicated to the rat by the illumination of a white house light and the onset of low-volume white noise (65 dB) to mask extraneous sounds.

Apparatus

Experimental sessions were conducted in operant conditioning chambers (Med Associates Inc.) contained within sound-attenuating cubicles. The left panel was fitted with two nosepoke ports, each with 3 LED lights at the rear.

Fixed-ratio 1 (FR1) ICSS training

During the first training session, both active and inactive nosepoke ports were baited with a crushed cereal treat to facilitate initial investigation. Rats were given four daily sessions of two hours each in which they could respond freely at either nosepoke port. For all rats (Th::Cre+ and Th::Cre-), a response at the active port resulted in the delivery of a 1 sec train of light pulses (20 Hz, 20 pulses, 5 ms duration). Concurrently, the LED lights in the recess of the active port were illuminated, providing a visible cue whenever stimulation was delivered. Responses at the active port made during the 1 sec period when the light train was being delivered were recorded but had no consequence. Responses at the inactive port were always without consequence.

Duration-response test

The duration-response test measured the rats' response to stimulation trains that varied systematically in length. As before, all stimulation trains consisted of pulses of 20 Hz frequency and 5 ms duration. The test was organized into 9 trials, and in each trial nosepokes at the active port were rewarded with stimulation trains of a specific length (100, 80, 60, 40, 20, 10, 5, 3, or 1 pulse/ train). The first trial consisted of the longest stimulation length (100 pulses); the next trial consisted of the next longest stimulation length (80 pulses), and so on in descending order. A series of all 9 trials was considered to be a “sweep.” A session consisted of four consecutive sweeps. The data presented is an average of all 8 sweeps from two consecutive days of testing. The start of a trial was

signaled by the illumination of the house light and the onset of low-level white noise as described above. Three “priming” trains of stimulation were then delivered non-contingently to inform the rat of the stimulation parameters that would be available on the upcoming trial. The separation between these trains was equal to the length of stimulation or 1 second, whichever longer. Once the priming trains had finished, the subject then had the opportunity to earn up to 60 stimulation trains during that trial; this was true regardless of the length of stimulation delivered. The length of an individual trial was 60 x the length of stimulation train or 60 x 1 second, whichever longer. For stimulation lengths of 20 pulses or greater, a new stimulation train could be earned as soon as the previous train had finished. For stimulation lengths of 10 pulses or less, there was a short timeout (1 sec – length of stimulation train) before the next train could be earned. This was to ensure that it was physically possible for subjects to respond for all available trains regardless of the stimulation length used. At the end of each trial, the house light and the white noise were turned off. After an inter-trial interval of approximately 1 minute, the next trial began.

Extinction and reacquisition

During this 1.5 hour test session subjects were initially given 30 min of regular FR1 training. Subsequently, a within-session extinction period began. During this phase, responses at the active port were recorded but had no consequence. After 30 min of extinction had elapsed, 5 stimulation trains (each train 1 sec

long, inter-train interval = 1 sec) were delivered to signal the renewed availability of reinforcement at the active port. For the rest of the session rats were reinforced on an FR1 basis for responses at the active port.

Contingency degradation

On the day before the contingency degradation test, subjects were given an additional 1 hour FR1 training session. The number of stimulation trains delivered during this session (dependent on subjects' responses at the active port) was used to calculate the average rate of stimulation for the contingency degradation portion of the experiment. The next day, rats were given 30 min of regular FR1 training. Subsequently, a within-session contingency degradation period began. During this phase, stimulation trains were delivered pseudorandomly and non-contingently at a rate that was matched to each subjects' performance during FR1 training on the previous day. After a further 30 min had elapsed, a reacquisition phase was initiated. During this phase, stimulation was once again contingent on a response made at the active port. The total length of this test was 1.5 hours. During this experiment, whenever a stimulation train was delivered (regardless of whether it had been earned by a nosepoke response or was delivered non-contingently) the LED lights in the active port were concurrently illuminated.

Dopamine antagonist infusions

Subjects in that received intra-NAc drug infusions underwent FR-1 ICSS training as described above except that instead of single daily 120 min sessions, subjects were given a 60 min baseline session, removed from the chamber for drug infusions, and then returned for a further 60 min test session to assess drug effects on behavior. This was done because while ICSS responding was stable within a single day, the absolute magnitude of behavior emitted was variable across multiple days.

Subjects received targeted intracranial drug infusions into the NAc once ICSS behavior was well-established (>4 training sessions prior to drug administration). The following drugs were used: (1) flupenthixol, a non-selective dopamine receptor antagonist dissolved in water (10 µg; F114, Sigma); (2) SCH23390, a D1-selective antagonist dissolved in saline (1 µg; D054, Sigma); (3) Raclopride, a D2-selective antagonist dissolved in saline (1 µg; R121, Sigma) or (4) saline control. All drug infusions were unilateral, delivered ipsilateral or contralateral to the hemisphere where VTA dopamine neurons were optogenetically stimulated, with the exception of control saline infusions which were bilateral. Doses indicate the amount delivered per hemisphere in 0.5 µL. All solutions were infused at a rate of 0.25 µL/min via 33 gauge infusers inserted into the guide cannulae; infusers were left in place for an additional 2 min to allow for drug diffusion. Subjects were then placed in their home cages for 10 minutes to allow the drugs to take effect before being returned to the behavioral chambers for testing. All subjects experienced all 7 treatments. The order of drugs was randomized and drug

infusion testing days were preceded and followed by at least one recovery session where no treatment was given.

Data analysis

The behavioral data in Figs. 12, 14 and 16 were analyzed with non-parametric tests as specified (e.g. Wilcoxon signed rank rest for paired data, Mann Whitney test for unpaired data) coupled with Bonferroni tests to control for multiple comparisons where appropriate. This statistical approach resulted in a conservative measure of statistical significance. Given that the behavioral data was distributed in a non-Gaussian manner, and the different behavioral conditions varied greatly in their variance (e.g. Fig 12B), a parametric ANOVA would not have been an appropriate statistical choice for this data set. For the duration-response test (Fig. 12E), before averaging across rats, an individual rat's response rate was divided by the response rate for the condition with the maximum responding. Since the condition that corresponded to the maximum rate was not the same for all rats, on average this resulted in a maximum normalized response rate below 1.

The behavioral data in Fig. 17 was analyzed with parametric tests (one-way RM ANOVA followed by post-hoc Student-Newman-Keuls tests) because in most cases these data met assumptions of normality and equal variance. In cases where data did not meet the assumptions of normality and/or equal variance, both parametric and non-parametric analyses were performed and in every case returned the same result.

Methods, Chapter 4

Subjects

A total of 115 female transgenic rats (on a Long-Evans background) were used in these studies; 68 rats expressed Cre Recombinase under the control of the tyrosine hydroxylase promoter (Cre+), and 47 rats were their wild-type littermates (Cre-). All rats weighed >225g at the time of surgery, and were individually housed beginning at least one week prior to the start of experiments. During testing, rats were mildly food-restricted to 18g of lab chow per day given after the conclusion of daily behavioral sessions; on average rats maintained >95% free-feeding weight under these conditions. Water was always available ad libitum in the home cage. Light/dark cycle was 12h on /12h off, with lights on at 7am. The majority of behavioral experiments were conducted during the light cycle. Animal care and all experimental procedures were in accordance with guidelines from the National Institutes of Health and were approved in advance by the Gallo Center Institutional Animal Care and Use Committee.

Behavioral procedures

All behavioral experiments were conducted >2 weeks post-surgery; sessions that included optical stimulation were conducted >4 weeks post-surgery.

Apparatus

Behavioral sessions were conducted in conditioning chambers (Med Associates Inc.) contained within sound-attenuating cubicles. The left and right walls were

fitted with reward delivery ports; computer-controlled syringe pumps located outside of the sound-attenuating cubicle delivered sucrose solution or water to these ports when appropriate. For the blocking and extinction experiments, the left reward port was designated to be the active port; for the downshift experiment the right reward port was active. Entry into the reward ports was tracked via an infrared beam positioned at the front of the reward port. The left wall also contained two nosepoke ports flanking the central reward delivery port; each nosepoke port had three LED lights at the rear. Chambers were also outfitted with 2700 Hz pure tone and white noise auditory stimuli, both delivered at 70 dB, as well as a 28V chamber light located above the left reward port. During behavioral sessions, the pure tone was “pulsed” at 3 Hz (0.1s on/0.2s off) to create a stimulus that was easily distinguished from continuous white noise. For Blocking experiments, the identity of cues A and B (pulsed tone or white noise) was counterbalanced across subjects. The white noise cue was used for the Downshift study, and the pulsed tone cue was used for the Extinction study.

Reward delivery for all experiments

All experiments (except the preference test, see below) involved the delivery of a liquid sucrose solution (15% w/v) during the presentation of auditory or combined auditory-visual cues. During each cue, entry into the active port triggered a 3-sec delivery of sucrose solution (0.1 mL). After a 2-sec time-out,

another entry into the port (or the rat's continued presence at the port) triggered an additional 3-sec reward delivery. This 5-sec cycle could be repeated up to 6 times per 30-sec trial, depending on the rat's behavior. For sessions where optical stimulation was delivered, the laser was activated each time sucrose was delivered (or expected) as illustrated in Fig. 21C, 24A, 25A. This method of reward delivery, where reward and optical stimulation were both contingent on the rat's presence in the active port, was used for all experiments as it allowed for the coincident delivery of natural rewards and optical stimulation.

Blocking procedure

Rats received a one-day habituation session where all auditory and visual cues used during future training sessions, as well as the liquid sucrose reward, were presented individually (3 presentations of each cue, 5min ITI; ~60 reward deliveries, 1min ITI). This session was intended to minimize unconditioned responses to novel stimuli and shape reward-seeking behavior to the correct (left) reward port. Next, rats underwent single-cue training where one of two auditory cues (white noise or pulsed tone, counterbalanced across subjects in each group) was presented for 30 sec on a variable interval (VI) 4-min schedule for 10 trials per session. Sucrose was delivered during each cue as described above. After 14-15 sessions of single cue training, compound cue training commenced. During this phase either the same auditory cue used in single cue

training (Blocking groups) or a new auditory cue (Control group) was presented simultaneously with a visual cue. The visual cue consisted of the chamber light, which was the sole source of chamber illumination, flashing on/off at 0.3 Hz (1 sec on, 2 sec off). Sucrose reward was delivered as described above during this phase. After 4 days of compound training, a probe test was administered 24 hours later to assess conditioned responding to the visual cue. During this session the visual cue was presented alone in the absence of sucrose, auditory cues or optical stimulation.

Downshift procedure

Rats received one session where sucrose reward was delivered to the active (right) port (50 deliveries, VI-30 sec) to shape reward-seeking behavior to this location. Subsequently, rats were trained to respond for sucrose during presentations of an auditory cue (white noise) as described above in 11 daily sessions. 24 hours later, a downshift test session was administered that was identical to previous training sessions except that water was substituted for the usual sucrose solution and optical stimulation was delivered coincidently. 24 hours later, a downshift recall test was given, in which water was delivered during the cue, but optical stimulation did not occur.

Extinction procedure

This experiment was conducted two weeks after the end of the Downshift experiment with the same subjects; group assignment for Cre+ rats was shuffled between experiments. Rats received one session of sucrose reward delivery to the opposite (left) port used in the downshift test, to shape reward-seeking behavior to this location. Subsequently rats were trained to respond for sucrose during presentations of an auditory cue (pulsed tone) as described above in 6 daily training sessions. 24 hours later, an extinction test session was administered that was identical to previous training sessions except that no reward was given and optical stimulation was delivered at the time that the sucrose reward had been available in previous training. 24 hours later, an extinction recall test was administered in which the auditory cue was presented but no reward or optical stimulation was delivered.

Fixed-ratio 1 ICSS Training

Upon completion of the behavioral experiments described above, all rats were given 4 daily one-hour sessions of self-stimulation training. Food restriction ceased at least 24 hours before the start of these experiments. During the first training session, both nosepoke ports were baited with a crushed cereal treat to facilitate initial investigation. A response at the nosepoke port designated as “active” resulted in the delivery of a train of light pulses matched to the stimulation parameters used in that subject’s previous behavioral experiment

(1s/20Hz for rats in Blocking study, 3s/20Hz for rats in Downshift or Extinction studies). The LED lights in the recess of the active port were illuminated for the same length of time that stimulation was delivered (1s or 3s). Responses made at the active nosepoke port were reinforced with optical stimulation on a fixed-ratio 1 schedule, with the exception that a new stimulation train could not be earned until any ongoing train finished. Responses at the inactive nosepoke port were recorded but were without consequence.

Flavor preference test

Pre-exposure and pre-training

Rodent home cages were equipped with two bottle slots; prior to the start of the experiment both slots were occupied by water bottles to reduce possible side bias. Approximately four weeks after surgery, rats were trained to drink unflavored 15% (w/v) sucrose solution from the reward port in the conditioning chambers (0.1 ml of sucrose solution delivered on a VI-30s schedule; 50 deliveries). Rats were then given overnight access to 40 ml each of two of flavored sucrose solutions in their home cage to ensure that all subjects had sampled both flavors before critical consumption tests. Flavored solutions consisted of 15% sucrose (w/v) + 0.15% Kool-aid (w/v, tropical punch or grape flavors).

Home-cage consumption tests

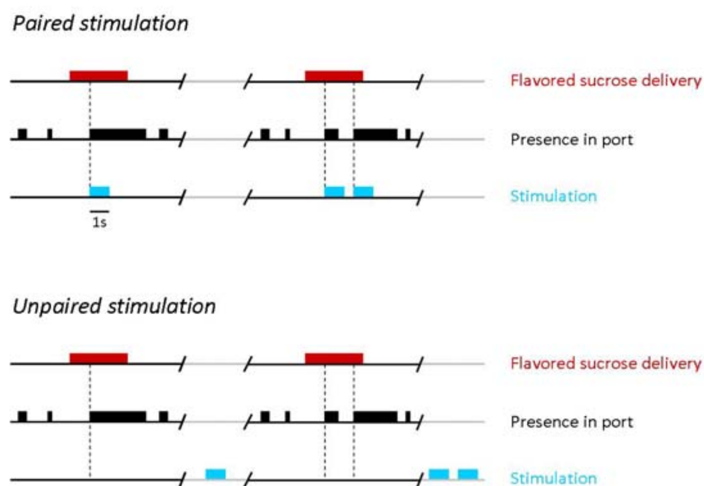
Water bottles were removed from the home cage 15-30 min prior to the start of consumption tests. A standardized procedure was used to ensure that rats briefly sampled both flavors before free access to the flavored solutions began. The purpose of this procedure was to make sure that rats were aware that both flavors were available, so that any measured preference reflected true choice. The experimenter placed a flavor bottle on the left side of the cage until the rat consumed the solution for 2-3s. This bottle was removed, and a second bottle containing the other flavored solution was placed on the right side of the cage until the rat consumed the new solution for 2-3s. The second bottle was then removed, and both bottles were simultaneously placed on the home cage to start the test. 10 minutes later, both bottles were removed at the amount consumed was recorded. The cage side assigned to each flavor (left or right) alternated between consumption tests to control for possible side bias.

Flavor training with optical stimulation

24 hours after the baseline consumption test in the home cage, daily flavor training began in the conditioning chambers (8 sessions total). Only one flavored sucrose solution was available per day; training days with each flavor were interleaved. One of the two flavors was assigned to be the “stimulated” flavor; the identity of the “stimulated” flavor, grape or

tropical punch, was randomly assigned for each rat. On training days where this “stimulated” flavor was available, optical stimulation (20 pulses, 5ms pulse duration, 20 Hz, 473 nm) was either paired with reward consumption for rats assigned to PairedCre+ and PairedCre- groups, or explicitly unpaired (presented during the ITI at times when no reward was available) for UnpairedCre+ rats. Flavored sucrose was delivered to a reward port on a VI-30s schedule, with the exception that each reward had to be consumed before the next would be delivered. A reward was considered to be consumed if the rat maintained presence in the port for 1s or longer. For rats assigned to Paired groups, optical stimulation was delivered the first time the rat entered the reward port once a new reward was available. If the rat did not maintain presence in the port for 1s or longer, subsequent port entries would trigger additional stimulation trains until the 1s requirement was met to ensure that reward consumption and optical stimulation were always experienced coincidently (*see trial examples below*). Because of this, more stimulation trains were delivered than rewards (Fig. 23b,c). To ensure that UnpairedCre+ rats received equivalent amounts of stimulation as their PairedCre+ counterparts, the number of stimulation trains delivered to the UnpairedCre+ rats was determined using the same criteria, but instead of being delivered during flavored sucrose consumption, the stimulation trains were delivered during the ITI, when no sucrose solution

was present. Sessions lasted until the maximum of 50, 0.1ml rewards were consumed or one hour elapsed, whichever occurred first. The final preference test was conducted 24 hours after the last flavor training session.



Optical activation

For all experiments, 5-ms light pulses were delivered with a 50-ms inter-pulse interval (i.e., 20 Hz). For blocking experiments, 20 pulses were used (1s of stimulation). For downshift and extinction experiments, 60 pulses were used (3 s). Data from sessions where light output was compromised because of broken or disconnected optical cables was discarded and these subjects were excluded from the study. This criterion led to the exclusion of 1 rat from each of the Blocking and Extinction experiments, and 4 rats from the self-stimulation protocol.

Assessment of Estrus Cycle

Stage of estrus cycle was assessed by vaginal cytological examination using well-established methods (Karim et al., 2003). After daily behavioral sessions (Downshift study), the tip of a moistened cotton applicator swab was gently inserted into the exterior portion of the vaginal canal and then rotated to dislodge cells from the vaginal wall. The swab was then immediately rolled onto a glass slide, and the sample preserved with spray fixative (Spray-Cyte, Fisher Scientific) without allowing the cells to dry. Samples were collected over five consecutive days to ensure that multiple stages of the estrus cycle would be observed, as the typical estrus cycle lasts 4-5 days in the rat. This was done to improve the accuracy of determining estrus cycle stage on any *single* day of the experiment. Slides were then stained with a modified Papanicolau staining procedure as follows: 50% ethyl alcohol, 3 min; tap water, 10 dips (x2); Gill’s hematoxylin 1, 6 min; tap water, 10 dips (x2); Scott’s water, 4 min; tap water, 10 dips (x2); 95% ethyl alcohol, 10 dips (x2); modified orange-greenish 6 (OG-6), 1 min; 95% ethyl alcohol, 10 dips; 95% ethyl alcohol 8 dips; 95% ethyl alcohol 6 dips; modified eosin azure 36 (EA-36), 20 min; 95% ethyl alcohol, 40 dips; 95% ethyl alcohol, 30 dips; 95% ethyl alcohol, 20 dips; 100% ethyl alcohol, 10 dips (x2); xylene, 10 dips (x2); coverslip immediately. All staining solutions (Gill’s hematoxylin 1, OG-6, and EA-36) were sourced from Richard Allen Scientific. After staining was complete, consecutive daily samples from each subject were examined using an inverted microscope. Estrus cycle stage was determined by identifying cellular morphology characteristic to each phase (diestrus, proestrus, estrus, metestrus) according to previously described criteria (Karim et al.,

2003). Rats were considered to be cycling if consecutive daily samples represented three stages out of four. If morphology consistent with diestrus, metestrus, or a combination of both was observed for four consecutive days, the rat was considered to be non-cycling. If estrus was observed but further samples did not indicate progression through successive stages, the rat's cycle was considered to be irregular.

Data analysis

Conditioned responding was measured as the amount of time spent in the reward port during cue presentation, normalized by subtracting the time spent in the port during a pre-cue period of equal length. Note that this measure during training is not a pure measure of learning since the time spent in the port during the cue is also directly affected by the time spent consuming the reward after delivery. We focus on this measure as the test results were robust and, critically, in the test, responding only reflects conditioning as reward is not delivered at this time. In addition, for some experiments the latency to enter the reward port after cue onset was also measured. In cases where behavioral data from individual subjects varied from the group mean by more than two standard deviations (calculated with data from all subjects included), these subjects were excluded as statistical outliers (2 rats from the blocking experiment and 3 each from the downshift and extinction experiments) and their data were not further analyzed. Behavioral measures were analyzed using a mixed factorial ANOVA with the between-subjects factor of experimental group and the within-subjects factor

of session or trial as appropriate, followed by post-hoc Student Newman-Keul's tests as indicated, with $\alpha=0.05$.

References

(2000) Diagnostic and statistical manual of mental disorders, 4th Edition. Washington DC: American Psychiatric Association.

Aberman JE, Salamone JD (1999) Nucleus accumbens dopamine depletions make rats more sensitive to high ratio requirements but do not impair primary food reinforcement. Neuroscience 92:545-552.

Adamantidis AR, Tsai HC, Boutrel B, Zhang F, Stuber GD, Budygin EA, Tourino C, Bonci A, Deisseroth K, de Lecea L (2011) Optogenetic interrogation of dopaminergic modulation of the multiple phases of reward-seeking behavior. J Neurosci 31:10829-10835.

Amalric M, Koob GF (1987) Depletion of dopamine in the caudate nucleus but not in nucleus accumbens impairs reaction-time performance in rats. J Neurosci 7:2129-2134.

Amalric M, Koob GF (1993) Functionally selective neurochemical afferents and efferents of the mesocorticolimbic and nigrostriatal dopamine system. Prog Brain Res 99:209-226.

Anstrom KK, Miczek KA, Budygin EA (2009) Increased phasic dopamine signaling in the mesolimbic pathway during social defeat in rats. Neuroscience 161:3-12.

Atasoy D, Aponte Y, Su HH, Sternson SM (2008) A FLEX switch targets Channelrhodopsin-2 to multiple cell types for imaging and long-range circuit mapping. J Neurosci 28:7025-7030.

Bari A, Dalley JW, Robbins TW (2008) The application of the 5-choice serial reaction time task for the assessment of visual attentional processes and impulse control in rats. Nat Protoc 3:759-767.

Becker JB (1999) Gender differences in dopaminergic function in striatum and nucleus accumbens. Pharmacol Biochem Behav 64:803-812.

Beckstead RM, Domesick VB, Nauta WJ (1979) Efferent connections of the substantia nigra and ventral tegmental area in the rat. Brain Res 175:191-217.

Berke JD, Breck JT, Eichenbaum H (2009) Striatal versus hippocampal representations during win-stay maze performance. J Neurophysiol 101:1575-1587.

Bernheimer H, Birkmayer W, Hornykiewicz O, Jellinger K, Seitelberger F (1973) Brain dopamine and the syndromes of Parkinson and Huntington. Clinical, morphological and neurochemical correlations. J Neurol Sci 20:415-455.

Berridge KC (2007) The debate over dopamine's role in reward: the case for incentive salience. Psychopharmacology (Berl) 191:391-431.

Berridge KC, Robinson TE (1998) What is the role of dopamine in reward: hedonic impact, reward learning, or incentive salience? Brain Res Brain Res Rev 28:309-369.

Berridge KC, Venier IL, Robinson TE (1989) Taste reactivity analysis of 6-hydroxydopamine-induced aphagia: implications for arousal and anhedonia hypotheses of dopamine function. Behav Neurosci 103:36-45.

Bielajew C, Shizgal P (1986) Evidence implicating descending fibers in self-stimulation of the medial forebrain bundle. J Neurosci 6:919-929.

Bissiere S, Humeau Y, Luthi A (2003) Dopamine gates LTP induction in lateral amygdala by suppressing feedforward inhibition. Nat Neurosci 6:587-592.

Boyden ES, Zhang F, Bamberg E, Nagel G, Deisseroth K (2005) Millisecond-timescale, genetically targeted optical control of neural activity. Nat Neurosci 8:1263-1268.

Brischoux F, Chakraborty S, Brierley DI, Ungless MA (2009) Phasic excitation of dopamine neurons in ventral VTA by noxious stimuli. Proc Natl Acad Sci U S A 106:4894-4899.

Brog JS, Salyapongse A, Deutch AY, Zahm DS (1993) The patterns of afferent innervation of the core and shell in the "accumbens" part of the rat ventral striatum:

immunohistochemical detection of retrogradely transported fluoro-gold. J Comp Neurol 338:255-278.

Brown MT, Henny P, Bolam JP, Magill PJ (2009) Activity of neurochemically heterogeneous dopaminergic neurons in the substantia nigra during spontaneous and driven changes in brain state. J Neurosci 29:2915-2925.

Brown MT, Bellone C, Mameli M, Labouebe G, Bocklisch C, Balland B, Dahan L, Lujan R, Deisseroth K, Luscher C (2010) Drug-driven AMPA receptor redistribution mimicked by selective dopamine neuron stimulation. PLoS One 5:e15870.

Brown VJ, Robbins TW (1991) Simple and choice reaction time performance following unilateral striatal dopamine depletion in the rat. Impaired motor readiness but preserved response preparation. Brain 114 (Pt 1B):513-525.

Budygin EA, Park J, Bass CE, Grinevich VP, Bonin KD, Wightman RM (2012) Aversive stimulus differentially triggers subsecond dopamine release in reward regions. Neuroscience 201:331-337.

Bunney BS, Aghajanian GK (1976) The precise localization of nigral afferents in the rat as determined by a retrograde tracing technique. Brain Res 117:423-435.

Bunney BS, Walters JR, Roth RH, Aghajanian GK (1973) Dopaminergic neurons: effect of antipsychotic drugs and amphetamine on single cell activity. J Pharmacol Exp Ther 185:560-571.

Burke KA, Franz TM, Miller DN, Schoenbaum G (2008) The role of the orbitofrontal cortex in the pursuit of happiness and more specific rewards. Nature 454:340-344.

Buzsaki G, Bickford RG, Ryan LJ, Young S, Prohaska O, Mandel RJ, Gage FH (1989) Multisite recording of brain field potentials and unit activity in freely moving rats. J Neurosci Methods 28:209-217.

Cagniard B, Balsam PD, Brunner D, Zhuang X (2006) Mice with chronically elevated dopamine exhibit enhanced motivation, but not learning, for a food reward. Neuropsychopharmacology 31:1362-1370.

Cameron DL, Wessendorf MW, Williams JT (1997) A subset of ventral tegmental area neurons is inhibited by dopamine, 5-hydroxytryptamine and opioids. Neuroscience 77:155-166.

Cannon CM, Palmiter RD (2003) Reward without dopamine. J Neurosci 23:10827-10831.

Carey RJ (1990) Dopamine receptors mediate drug-induced but not Pavlovian conditioned contralateral rotation in the unilateral 6-OHDA animal model. Brain Res 515:292-298.

Carlsson A (1959) The occurrence, distribution and physiological role of catecholamines in the nervous system. Pharmacol Rev 11:490-493.

Carlsson A (1964) Evidence for a Role of Dopamine in Extrapyramidal Functions. Acta Neuroveg (Wien) 26:484-493.

Carlsson A, Lindqvist M, Magnusson T, Waldeck B (1958) On the presence of 3-hydroxytyramine in brain. Science 127:471.

Cheer JF, Aragona BJ, Heien ML, Seipel AT, Carelli RM, Wightman RM (2007) Coordinated accumbal dopamine release and neural activity drive goal-directed behavior. Neuron 54:237-244.

Chen JP, Paredes W, Li J, Smith D, Lowinson J, Gardner EL (1990) Delta 9-tetrahydrocannabinol produces naloxone-blockable enhancement of presynaptic basal dopamine efflux in nucleus accumbens of conscious, freely-moving rats as measured by intracerebral microdialysis. Psychopharmacology (Berl) 102:156-162.

Chesselet MF (1984) Presynaptic regulation of neurotransmitter release in the brain: facts and hypothesis. Neuroscience 12:347-375.

Chudasama Y, Robbins TW (2004) Dopaminergic modulation of visual attention and working memory in the rodent prefrontal cortex. Neuropsychopharmacology 29:1628-1636.

Cohen JY, Haesler S, Vong L, Lowell BB, Uchida N (2012) Neuron-type-specific signals for reward and punishment in the ventral tegmental area. Nature 482:85-88.

Colgin LL, Denninger T, Fyhn M, Hafting T, Bonnevie T, Jensen O, Moser MB, Moser EI (2009) Frequency of gamma oscillations routes flow of information in the hippocampus. Nature 462:353-357.

Cools R, D'Esposito M (2011) Inverted-U-shaped dopamine actions on human working memory and cognitive control. Biol Psychiatry 69:e113-125.

Corbett D, Wise RA (1980) Intracranial self-stimulation in relation to the ascending dopaminergic systems of the midbrain: a moveable electrode mapping study. Brain Res 185:1-15.

Cousins MS, Wei W, Salamone JD (1994) Pharmacological characterization of performance on a concurrent lever pressing/feeding choice procedure: effects of dopamine antagonist, cholinomimetic, sedative and stimulant drugs. Psychopharmacology (Berl) 116:529-537.

Cousins MS, Atherton A, Turner L, Salamone JD (1996) Nucleus accumbens dopamine depletions alter relative response allocation in a T-maze cost/benefit task. Behav Brain Res 74:189-197.

Dagher A, Robbins TW (2009) Personality, addiction, dopamine: insights from Parkinson's disease. Neuron 61:502-510.

Dalley JW, Everitt BJ, Robbins TW (2011) Impulsivity, compulsivity, and top-down cognitive control. Neuron 69:680-694.

Daw ND, Kakade S, Dayan P (2002) Opponent interactions between serotonin and dopamine. Neural Netw 15:603-616.

Day JJ, Roitman MF, Wightman RM, Carelli RM (2007) Associative learning mediates dynamic shifts in dopamine signaling in the nucleus accumbens. Nat Neurosci 10:1020-1028.

Di Chiara G, Imperato A (1985) Ethanol preferentially stimulates dopamine release in the nucleus accumbens of freely moving rats. Eur J Pharmacol 115:131-132.

Dickinson A, Smith J, Mirenowicz J (2000) Dissociation of Pavlovian and instrumental incentive learning under dopamine antagonists. Behav Neurosci 114:468-483.

Dobi A, Margolis EB, Wang HL, Harvey BK, Morales M (2010) Glutamatergic and nonglutamatergic neurons of the ventral tegmental area establish local synaptic contacts with dopaminergic and nondopaminergic neurons. J Neurosci 30:218-229.

Domingos AI, Vaynshteyn J, Voss HU, Ren X, Gradinaru V, Zang F, Deisseroth K, de Araujo IE, Friedman J (2011) Leptin regulates the reward value of nutrient. Nat Neurosci 14:1562-1568.

Everitt BJ, Robbins TW (2005) Neural systems of reinforcement for drug addiction: from actions to habits to compulsion. Nat Neurosci 8:1481-1489.

Fadok JP, Darvas M, Dickerson TM, Palmiter RD (2010) Long-term memory for pavlovian fear conditioning requires dopamine in the nucleus accumbens and basolateral amygdala. PLoS One 5:e12751.

Fallon JH (1981) Collateralization of monoamine neurons: mesotelencephalic dopamine projections to caudate, septum, and frontal cortex. J Neurosci 1:1361-1368.

Fibiger HC, LePiane FG, Jakubovic A, Phillips AG (1987) The role of dopamine in intracranial self-stimulation of the ventral tegmental area. J Neurosci 7:3888-3896.

Fields HL, Hjelmstad GO, Margolis EB, Nicola SM (2007) Ventral tegmental area neurons in learned appetitive behavior and positive reinforcement. Annu Rev Neurosci 30:289-316.

Filipiak WE, Saunders TL (2006) Advances in transgenic rat production. Transgenic Res 15:673-686.

Fiorillo CD, Tobler PN, Schultz W (2003) Discrete coding of reward probability and uncertainty by dopamine neurons. Science 299:1898-1902.

Fiorillo CD, Tobler PN, Schultz W (2005) Evidence that the delay-period activity of dopamine neurons corresponds to reward uncertainty rather than backpropagating TD errors. Behav Brain Funct 1:7.

Flagel SB, Watson SJ, Robinson TE, Akil H (2007) Individual differences in the propensity to approach signals vs goals promote different adaptations in the dopamine system of rats. Psychopharmacology (Berl) 191:599-607.

Flagel SB, Clark JJ, Robinson TE, Mayo L, Czuj A, Willuhn I, Akers CA, Clinton SM, Phillips PE, Akil H (2010) A selective role for dopamine in stimulus-reward learning. Nature 469:53-57.

Floresco SB, Todd CL, Grace AA (2001) Glutamatergic afferents from the hippocampus to the nucleus accumbens regulate activity of ventral tegmental area dopamine neurons. J Neurosci 21:4915-4922.

Floresco SB, West AR, Ash B, Moore H, Grace AA (2003) Afferent modulation of dopamine neuron firing differentially regulates tonic and phasic dopamine transmission. Nat Neurosci 6:968-973.

Fouriezos G, Wise RA (1976) Pimozide-induced extinction of intracranial self-stimulation: response patterns rule out motor or performance deficits. Brain Res 103:377-380.

Gallistel CR, Gomita Y, Yadin E, Campbell KA (1985) Forebrain origins and terminations of the medial forebrain bundle metabolically activated by rewarding stimulation or by reward-blocking doses of pimozide. J Neurosci 5:1246-1261.

Garris PA, Wightman RM (1994) Different kinetics govern dopaminergic transmission in the amygdala, prefrontal cortex, and striatum: an in vivo voltammetric study. J Neurosci 14:442-450.

Garris PA, Kilpatrick M, Bunin MA, Michael D, Walker QD, Wightman RM (1999) Dissociation of dopamine release in the nucleus accumbens from intracranial self-stimulation. Nature 398:67-69.

Geisler S, Zahm DS (2005) Afferents of the ventral tegmental area in the rat-anatomical substratum for integrative functions. J Comp Neurol 490:270-294.

Geisler S, Derst C, Veh RW, Zahm DS (2007) Glutamatergic afferents of the ventral tegmental area in the rat. J Neurosci 27:5730-5743.

Gerfen CR, Surmeier DJ (2011) Modulation of striatal projection systems by dopamine. Annu Rev Neurosci 34:441-466.

German DC, Bowden DM (1974) Catecholamine systems as the neural substrate for intracranial self-stimulation: a hypothesis. Brain Res 73:381-419.

Giros B, el Mestikawy S, Bertrand L, Caron MG (1991) Cloning and functional characterization of a cocaine-sensitive dopamine transporter. FEBS Lett 295:149-154.

Glimcher PW (2011) Understanding dopamine and reinforcement learning: the dopamine reward prediction error hypothesis. Proc Natl Acad Sci U S A 108 Suppl 3:15647-15654.

Goldman-Rakic PS (1990) Cellular and circuit basis of working memory in prefrontal cortex of nonhuman primates. Prog Brain Res 85:325-335; discussion 335-326.

Gong S, Doughty M, Harbaugh CR, Cummins A, Hatten ME, Heintz N, Gerfen CR (2007) Targeting Cre recombinase to specific neuron populations with bacterial artificial chromosome constructs. J Neurosci 27:9817-9823.

Gonon FG (1988) Nonlinear relationship between impulse flow and dopamine released by rat midbrain dopaminergic neurons as studied by in vivo electrochemistry. Neuroscience 24:19-28.

Gorelova N, Mulholland PJ, Chandler LJ, Seamans JK (2012). The glutamatergic component of the mesocortical pathway emanating from different subregions of the ventral midbrain. Cereb Cortex 22:327-336.

Goto Y, Grace AA (2005) Dopaminergic modulation of limbic and cortical drive of nucleus accumbens in goal-directed behavior. Nat Neurosci 8:805-812.

Grace AA, Bunney BS (1980) Nigral dopamine neurons: intracellular recording and identification with L-dopa injection and histofluorescence. Science 210:654-656.

Grace AA, Bunney BS (1983) Intracellular and extracellular electrophysiology of nigral dopaminergic neurons--1. Identification and characterization. Neuroscience 10:301-315.

Grace AA, Bunney BS (1984) The control of firing pattern in nigral dopamine neurons: single spike firing. J Neurosci 4:2866-2876.

Grace AA, Onn SP (1989) Morphology and electrophysiological properties of immunocytochemically identified rat dopamine neurons recorded in vitro. J Neurosci 9:3463-3481.

Gradinaru V, Mogri M, Thompson KR, Henderson JM, Deisseroth K (2009) Optical deconstruction of parkinsonian neural circuitry. Science 324:354-359.

Gradinaru V, Zhang F, Ramakrishnan C, Mattis J, Prakash R, Diester I, Goshen I, Thompson KR, Deisseroth K (2010) Molecular and cellular approaches for diversifying and extending optogenetics. Cell 141:154-165.

Groves PM (1983) A theory of the functional organization of the neostriatum and the neostriatal control of voluntary movement. Brain Res 286:109-132.

Gutierrez R, Simon SA, Nicolelis MA (2010) Licking-induced synchrony in the taste-reward circuit improves cue discrimination during learning. J Neurosci 30:287-303.

Guzman JN, Hernandez A, Galarraga E, Tapia D, Laville A, Vergara R, Aceves J, Bargas J (2003) Dopaminergic modulation of axon collaterals interconnecting spiny neurons of the rat striatum. J Neurosci 23:8931-8940.

Hamilton ME, Redondo JL, Freeman AS (2000) Overflow of dopamine and cholecystokinin in rat nucleus accumbens in response to acute drug administration. Synapse 38:238-242.

Hasue RH, Shammah-Lagnado SJ (2002) Origin of the dopaminergic innervation of the central extended amygdala and accumbens shell: a combined retrograde tracing and immunohistochemical study in the rat. J Comp Neurol 454:15-33.

Hernandez L, Hoebel BG (1988) Food reward and cocaine increase extracellular dopamine in the nucleus accumbens as measured by microdialysis. Life Sci 42:1705-1712.

Histed MH, Bonin V, Reid RC (2009) Direct activation of sparse, distributed populations of cortical neurons by electrical microstimulation. Neuron 63:508-522.

Hnasko TS, Sotak BN, Palmiter RD (2005) Morphine reward in dopamine-deficient mice. Nature 438:854-857.

Holland PC (1984) Unblocking in Pavlovian appetitive conditioning. J Exp Psychol Anim Behav Process 10:476-497.

Hollerman JR, Schultz W (1998) Dopamine neurons report an error in the temporal prediction of reward during learning. Nat Neurosci 1:304-309.

Hyland BI, Reynolds JN, Hay J, Perk CG, Miller R (2002) Firing modes of midbrain dopamine cells in the freely moving rat. Neuroscience 114:475-492.

Ikemoto S, Glazier BS, Murphy JM, McBride WJ (1997) Role of dopamine D1 and D2 receptors in the nucleus accumbens in mediating reward. J Neurosci 17:8580-8587.

Imperato A, Mulas A, Di Chiara G (1986) Nicotine preferentially stimulates dopamine release in the limbic system of freely moving rats. Eur J Pharmacol 132:337-338.

Iordanova MD, Westbrook RF, Killcross AS (2006) Dopamine activity in the nucleus accumbens modulates blocking in fear conditioning. Eur J Neurosci 24:3265-3270.

Jog MS, Connolly CI, Kubota Y, Iyengar DR, Garrido L, Harlan R, Graybiel AM (2002) Tetrode technology: advances in implantable hardware, neuroimaging, and data analysis techniques. J Neurosci Methods 117:141-152.

Johnson SW, North RA (1992) Two types of neurone in the rat ventral tegmental area and their synaptic inputs. J Physiol 450:455-468.

Jones S, Kauer JA (1999) Amphetamine depresses excitatory synaptic transmission via serotonin receptors in the ventral tegmental area. J Neurosci 19:9780-9787.

Kamin LJ (1968) "Attention-like" processes in classical conditioning. Coral Gables, FL: University of Miami Press.

Kamin LJ (1969a) Selective association and conditioning. Halifax, NS: Dalhousie University Press.

Kamin LJ (1969b) Predictability, surprise, attention and conditioning. New York, NY: Appleton-Century-Crofts.

Karim BO, Landolfi JA, Christian A, Ricart-Arbona R, Qiu W, McAlonis M, Eyabi PO, Khan KA, Dicello JF, Mann JF, Huso DL (2003) Estrous cycle and ovarian changes in a rat mammary carcinogenesis model after irradiation, tamoxifen chemoprevention, and aging. Comp Med 53:532-538.

Kravitz AV, Tye LD, Kreitzer AC (2012) Distinct roles for direct and indirect pathway striatal neurons in reinforcement. Nat Neurosci 15:816-818.

Kravitz AV, Freeze BS, Parker PR, Kay K, Thwin MT, Deisseroth K, Kreitzer AC (2010) Regulation of parkinsonian motor behaviours by optogenetic control of basal ganglia circuitry. Nature 466:622-626.

Kurumiya S, Nakajima S (1988) Dopamine D1 receptors in the nucleus accumbens: involvement in the reinforcing effect of tegmental stimulation. Brain Res 448:1-6.

Lacey MG, Mercuri NB, North RA (1987) Dopamine acts on D2 receptors to increase potassium conductance in neurones of the rat substantia nigra zona compacta. J Physiol 392:397-416.

Lacey MG, Mercuri NB, North RA (1989) Two cell types in rat substantia nigra zona compacta distinguished by membrane properties and the actions of dopamine and opioids. J Neurosci 9:1233-1241.

Lammel S, Ion DI, Roeper J, Malenka RC (2011) Projection-specific modulation of dopamine neuron synapses by aversive and rewarding stimuli. Neuron 70:855-862.

Lammel S, Hetzel A, Hackel O, Jones I, Liss B, Roeper J (2008) Unique properties of mesoprefrontal neurons within a dual mesocorticolimbic dopamine system. Neuron 57:760-773.

Lamont EW, Kokkinidis L (1998) Infusion of the dopamine D1 receptor antagonist SCH 23390 into the amygdala blocks fear expression in a potentiated startle paradigm. Brain Res 795:128-136.

Lawlor PA, Bland RJ, Mouravlev A, Young D, During MJ (2009) Efficient gene delivery and selective transduction of glial cells in the mammalian brain by AAV serotypes isolated from nonhuman primates. Mol Ther 17:1692-1702.

Lee JH, Durand R, Gradinaru V, Zhang F, Goshen I, Kim DS, Fenno LE, Ramakrishnan C, Deisseroth K (2010) Global and local fMRI signals driven by neurons defined optogenetically by type and wiring. Nature 465:788-792.

Ljungberg T, Apicella P, Schultz W (1992) Responses of monkey dopamine neurons during learning of behavioral reactions. J Neurophysiol 67:145-163.

Lobo MK, Covington HE, 3rd, Chaudhury D, Friedman AK, Sun H, Damez-Werno D, Dietz DM, Zaman S, Koo JW, Kennedy PJ, Mouzon E, Mogri M, Neve RL, Deisseroth K, Han MH, Nestler EJ (2010) Cell type-specific loss of BDNF signaling mimics optogenetic control of cocaine reward. Science 330:385-390.

Lodge DJ, Grace AA (2006) The laterodorsal tegmentum is essential for burst firing of ventral tegmental area dopamine neurons. Proc Natl Acad Sci U S A 103:5167-5172.

Mao D, Gallagher K, McGehee DS (2011) Nicotine potentiation of excitatory inputs to ventral tegmental area dopamine neurons. J Neurosci 31:6710-6720.

Margolis EB, Hjelmstad GO, Bonci A, Fields HL (2003) Kappa-opioid agonists directly inhibit midbrain dopaminergic neurons. J Neurosci 23:9981-9986.

Margolis EB, Lock H, Hjelmstad GO, Fields HL (2006a) The ventral tegmental area revisited: is there an electrophysiological marker for dopaminergic neurons? J Physiol 577:907-924.

Margolis EB, Fields HL, Hjelmstad GO, Mitchell JM (2008) Delta-opioid receptor expression in the ventral tegmental area protects against elevated alcohol consumption. J Neurosci 28:12672-12681.

Margolis EB, Toy B, Himmels P, Morales M, Fields HL (2012) Identification of rat ventral tegmental area GABAergic neurons. PLoS One 7:e42365.

Margolis EB, Lock H, Chefer VI, Shippenberg TS, Hjelmstad GO, Fields HL (2006b) Kappa opioids selectively control dopaminergic neurons projecting to the prefrontal cortex. Proc Natl Acad Sci U S A 103:2938-2942.

Marshall JF, Richardson JS, Teitelbaum P (1974) Nigrostriatal bundle damage and the lateral hypothalamic syndrome. J Comp Physiol Psychol 87:808-830.

Matsuda Y, Marzo A, Otani S (2006) The presence of background dopamine signal converts long-term synaptic depression to potentiation in rat prefrontal cortex. J Neurosci 26:4803-4810.

Matsumoto M, Hikosaka O (2009) Two types of dopamine neuron distinctly convey positive and negative motivational signals. Nature 459:837-841.

McCutcheon JE, Beeler JA, Roitman MF (2012) Sucrose-predictive cues evoke greater phasic dopamine release than saccharin-predictive cues. Synapse 66:346-351.

Mirenowicz J, Schultz W (1994) Importance of unpredictability for reward responses in primate dopamine neurons. J Neurophysiol 72:1024-1027.

Mogenson GJ, Takigawa M, Robertson A, Wu M (1979) Self-stimulation of the nucleus accumbens and ventral tegmental area of Tsai attenuated by microinjections of spiroperidol into the nucleus accumbens. Brain Res 171:247-259.

Montague PR, Dayan P, Sejnowski TJ (1996) A framework for mesencephalic dopamine systems based on predictive Hebbian learning. J Neurosci 16:1936-1947.

Montague PR, Hyman SE, Cohen JD (2004) Computational roles for dopamine in behavioural control. Nature 431:760-767.

Nader K, LeDoux JE (1999) Inhibition of the mesoamygdala dopaminergic pathway impairs the retrieval of conditioned fear associations. Behav Neurosci 113:891-901.

Nagel G, Szellas T, Huhn W, Kateriya S, Adeishvili N, Berthold P, Ollig D, Hegemann P, Bamberg E (2003) Channelrhodopsin-2, a directly light-gated cation-selective membrane channel. Proc Natl Acad Sci U S A 100:13940-13945.

Nair-Roberts RG, Chatelain-Badie SD, Benson E, White-Cooper H, Bolam JP, Ungless MA (2008) Stereological estimates of dopaminergic, GABAergic and glutamatergic neurons in the ventral tegmental area, substantia nigra and retrorubral field in the rat. Neuroscience 152:1024-1031.

Nathanson JL, Jappelli R, Scheeff ED, Manning G, Obata K, Brenner S, Callaway EM (2009) Short Promoters in Viral Vectors Drive Selective Expression in Mammalian Inhibitory Neurons, but do not Restrict Activity to Specific Inhibitory Cell-Types. Front Neural Circuits 3:19.

Nauta WJ, Smith GP, Faull RL, Domesick VB (1978) Efferent connections and nigral afferents of the nucleus accumbens septi in the rat. Neuroscience 3:385-401.

Neuhoff H, Neu A, Liss B, Roeper J (2002) I(h) channels contribute to the different functional properties of identified dopaminergic subpopulations in the midbrain. J Neurosci 22:1290-1302.

Nicola SM (2007) The nucleus accumbens as part of a basal ganglia action selection circuit. Psychopharmacology (Berl) 191:521-550.

Nicola SM (2010) The flexible approach hypothesis: unification of effort and cue-responding hypotheses for the role of nucleus accumbens dopamine in the activation of reward-seeking behavior. J Neurosci 30:16585-16600.

Nicola SM, Surmeier J, Malenka RC (2000) Dopaminergic modulation of neuronal excitability in the striatum and nucleus accumbens. Annu Rev Neurosci 23:185-215.

Nirenberg MJ, Vaughan RA, Uhl GR, Kuhar MJ, Pickel VM (1996) The dopamine transporter is localized to dendritic and axonal plasma membranes of nigrostriatal dopaminergic neurons. J Neurosci 16:436-447.

Nisenbaum ES, Stricker EM, Zigmond MJ, Berger TW (1986) Long-term effects of dopamine-depleting brain lesions on spontaneous activity of type II striatal neurons: relation to behavioral recovery. Brain Res 398:221-230.

Nowend KL, Arizzi M, Carlson BB, Salamone JD (2001) D1 or D2 antagonism in nucleus accumbens core or dorsomedial shell suppresses lever pressing for food but leads to compensatory increases in chow consumption. Pharmacol Biochem Behav 69:373-382.

O'Tuathaigh CM, Salum C, Young AM, Pickering AD, Joseph MH, Moran PM (2003) The effect of amphetamine on Kamin blocking and overshadowing. Behav Pharmacol 14:315-322.

Olds J, Milner P (1954) Positive reinforcement produced by electrical stimulation of septal area and other regions of rat brain. J Comp Physiol Psychol 47:419-427.

Olds ME, Olds J (1963) Approach-avoidance analysis of rat diencephalon. J Comp Neurol 120:259-295.

Otazu GH, Tai LH, Yang Y, Zador AM (2009) Engaging in an auditory task suppresses responses in auditory cortex. Nat Neurosci 12:646-654.

Owesson-White CA, Cheer JF, Beyene M, Carelli RM, Wightman RM (2008) Dynamic changes in accumbens dopamine correlate with learning during intracranial self-stimulation. Proc Natl Acad Sci U S A 105:11957-11962.

Owesson-White CA, Roitman MF, Sombers LA, Belle AM, Keithley RB, Peele JL, Carelli RM, Wightman RM (2012) Sources contributing to the average extracellular concentration of dopamine in the nucleus accumbens. J Neurochem 121:252-262.

Palmiter RD (2011) Dopamine signaling as a neural correlate of consciousness. Neuroscience 198:213-220.

Parker JG, Zweifel LS, Clark JJ, Evans SB, Phillips PE, Palmiter RD (2010) Absence of NMDA receptors in dopamine neurons attenuates dopamine release but not conditioned approach during Pavlovian conditioning. Proc Natl Acad Sci U S A 107:13491-13496.

Parkinson JA, Dalley JW, Cardinal RN, Bamford A, Fehnert B, Lachenal G, Rudarakanchana N, Halkerston KM, Robbins TW, Everitt BJ (2002) Nucleus accumbens dopamine depletion impairs both acquisition and performance of appetitive Pavlovian approach behaviour: implications for mesoaccumbens dopamine function. Behav Brain Res 137:149-163.

Pawlak V, Kerr JN (2008) Dopamine receptor activation is required for corticostriatal spike-timing-dependent plasticity. J Neurosci 28:2435-2446.

Pecina S, Cagniard B, Berridge KC, Aldridge JW, Zhuang X (2003) Hyperdopaminergic mutant mice have higher "wanting" but not "liking" for sweet rewards. J Neurosci 23:9395-9402.

Pedersen CA, Ascher JA, Monroe YL, Prange AJ, Jr. (1982) Oxytocin induces maternal behavior in virgin female rats. Science 216:648-650.

Peters J, Kalivas PW, Quirk GJ (2009) Extinction circuits for fear and addiction overlap in prefrontal cortex. Learn Mem 16:279-288.

Phillips GD, Salussolia E, Hitchcott PK (2010) Role of the mesoamygdaloid dopamine projection in emotional learning. Psychopharmacology (Berl) 210:303-316.

Phillips PE, Stuber GD, Heien ML, Wightman RM, Carelli RM (2003) Subsecond dopamine release promotes cocaine seeking. Nature 422:614-618.

Phillipson OT (1979) Afferent projections to the ventral tegmental area of Tsai and interfascicular nucleus: a horseradish peroxidase study in the rat. J Comp Neurol 187:117-143.

Phillipson OT, Griffiths AC (1985) The topographic order of inputs to nucleus accumbens in the rat. Neuroscience 16:275-296.

Pontecorvo MJ, Sahgal A, Steckler T (1996) Further developments in the measurement of working memory in rodents. Brain Res Cogn Brain Res 3:205-213.

Rada P, Mark GP, Pothos E, Hoebel BG (1991) Systemic morphine simultaneously decreases extracellular acetylcholine and increases dopamine in the nucleus accumbens of freely moving rats. Neuropharmacology 30:1133-1136.

Redish AD (2004) Addiction as a computational process gone awry. Science 306:1944-1947.

Rescorla RA, Wagner AR (1972) A theory of Pavlovian conditioning: Variations in the effectiveness of reinforcement and nonreinforcement. in Classical Conditioning II: Current Research and Theory (eds Black, A H & Prokasy, W F) 64-99 (Appleton Century Crofts, New York, 1972).

Reynolds JN, Wickens JR (2002) Dopamine-dependent plasticity of corticostriatal synapses. Neural Netw 15:507-521.

Reynolds JN, Hyland BI, Wickens JR (2001) A cellular mechanism of reward-related learning. Nature 413:67-70.

Richfield EK, Penney JB, Young AB (1989) Anatomical and affinity state comparisons between dopamine D1 and D2 receptors in the rat central nervous system. Neuroscience 30:767-777.

Robbins, T.W., and Everitt, B.J. (1992). Functions of dopamine in the dorsal and ventral striatum.

Seminars in Neuroscience 4, 119–127.

Robbins TW, Everitt BJ (2007) A role for mesencephalic dopamine in activation: commentary on Berridge (2006). Psychopharmacology (Berl) 191:433-437.

Robinson S, Sandstrom SM, Denenberg VH, Palmiter RD (2005) Distinguishing whether dopamine regulates liking, wanting, and/or learning about rewards. Behav Neurosci 119:5-15.

Roesch MR, Calu DJ, Schoenbaum G (2007) Dopamine neurons encode the better option in rats deciding between differently delayed or sized rewards. Nat Neurosci 10:1615-1624.

Roitman MF, Wheeler RA, Wightman RM, Carelli RM (2008) Real-time chemical responses in the nucleus accumbens differentiate rewarding and aversive stimuli. Nat Neurosci 11:1376-1377.

Roitman MF, Stuber GD, Phillips PE, Wightman RM, Carelli RM (2004) Dopamine operates as a subsecond modulator of food seeking. J Neurosci 24:1265-1271.

Romo R, Schultz W (1990) Dopamine neurons of the monkey midbrain: contingencies of responses to active touch during self-initiated arm movements. J Neurophysiol 63:592-606.

Rosenkranz JA, Grace AA (2002) Dopamine-mediated modulation of odour-evoked amygdala potentials during pavlovian conditioning. Nature 417:282-287.

Royer S, Zemelman BV, Barbic M, Losonczy A, Buzsaki G, Magee JC (2010) Multi-array silicon probes with integrated optical fibers: light-assisted perturbation and recording of local neural circuits in the behaving animal. Eur J Neurosci 31:2279-2291.

Salamone JD, Correa M (2012) The mysterious motivational functions of mesolimbic dopamine. Neuron 76:470-485.

Salamone JD, Cousins MS, Snyder BJ (1997) Behavioral functions of nucleus accumbens dopamine: empirical and conceptual problems with the anhedonia hypothesis. Neurosci Biobehav Rev 21:341-359.

Salamone JD, Steinpreis RE, McCullough LD, Smith P, Grebel D, Mahan K (1991) Haloperidol and nucleus accumbens dopamine depletion suppress lever pressing for food but increase free food consumption in a novel food choice procedure. Psychopharmacology (Berl) 104:515-521.

Saunders BT, Robinson TE (2012). The role of dopamine in the accumbens core in the expression of Pavlovian-conditioned responses. Eur J Neurosci 36:2521-2532.

Schultz W (1998) Predictive reward signal of dopamine neurons. J Neurophysiol 80:1-27.

Schultz W (2007) Multiple dopamine functions at different time courses. Annu Rev Neurosci 30:259-288.

Schultz W (2010) Dopamine signals for reward value and risk: basic and recent data. Behav Brain Funct 6:24.

Schultz W, Dickinson A (2000) Neuronal coding of prediction errors. Annu Rev Neurosci 23:473-500.

Schultz W, Apicella P, Ljungberg T (1993) Responses of monkey dopamine neurons to reward and conditioned stimuli during successive steps of learning a delayed response task. J Neurosci 13:900-913.

Schultz W, Dayan P, Montague PR (1997) A neural substrate of prediction and reward. Science 275:1593-1599.

Seroogy K, Ceccatelli S, Schalling M, Hokfelt T, Frey P, Walsh J, Dockray G, Brown J, Buchan A, Goldstein M (1988) A subpopulation of dopaminergic neurons in rat ventral mesencephalon contains both neurotensin and cholecystokinin. Brain Res 455:88-98.

Seroogy K, Schalling M, Brene S, Dagerlind A, Chai SY, Hokfelt T, Persson H, Brownstein M, Huan R, Dixon J, et al. (1989) Cholecystokinin and tyrosine hydroxylase messenger RNAs in

neurons of rat mesencephalon: peptide/monoamine coexistence studies using in situ hybridization combined with immunocytochemistry. Exp Brain Res 74:149-162.

Seroogy KB, Gall CM (1993) Expression of neurotrophins by midbrain dopaminergic neurons. Exp Neurol 124:119-128.

Shen W, Flajolet M, Greengard P, Surmeier DJ (2008) Dichotomous dopaminergic control of striatal synaptic plasticity. Science 321:848-851.

Smith ID, Grace AA (1992) Role of the subthalamic nucleus in the regulation of nigral dopamine neuron activity. Synapse 12:287-303.

Sombers LA, Beyene M, Carelli RM, Wightman RM (2009) Synaptic overflow of dopamine in the nucleus accumbens arises from neuronal activity in the ventral tegmental area. J Neurosci 29:1735-1742.

Steinberg EE, Janak PH (2012) Establishing causality for dopamine in neural function and behavior with optogenetics. Brain Res.

Strecker RE, Jacobs BL (1985) Substantia nigra dopaminergic unit activity in behaving cats: effect of arousal on spontaneous discharge and sensory evoked activity. Brain Res 361:339-350.

Stuber GD, Hnasko TS, Britt JP, Edwards RH, Bonci A (2010) Dopaminergic terminals in the nucleus accumbens but not the dorsal striatum corelease glutamate. J Neurosci 30:8229-8233.

Stuber GD, Klanker M, de Ridder B, Bowers MS, Joosten RN, Feenstra MG, Bonci A (2008) Reward-predictive cues enhance excitatory synaptic strength onto midbrain dopamine neurons. Science 321:1690-1692.

Suri RE (2002) TD models of reward predictive responses in dopamine neurons. Neural Netw 15:523-533.

Surmeier DJ, Song WJ, Yan Z (1996) Coordinated expression of dopamine receptors in neostriatal medium spiny neurons. J Neurosci 16:6579-6591.

Surmeier DJ, Plotkin J, Shen W (2009) Dopamine and synaptic plasticity in dorsal striatal circuits controlling action selection. Curr Opin Neurobiol 19:621-628.

Sutton RS, Barto AG (1981) Toward a modern theory of adaptive networks: expectation and prediction. Psychol Rev 88:135-170.

Swanson LW (1982) The projections of the ventral tegmental area and adjacent regions: a combined fluorescent retrograde tracer and immunofluorescence study in the rat. Brain Res Bull 9:321-353.

Tai LH, Lee AM, Benavidez N, Bonci A, Wilbrecht L (2012) Transient stimulation of distinct subpopulations of striatal neurons mimics changes in action value. Nat Neurosci 15:1281-1289.

Takahashi YK, Roesch MR, Stalnaker TA, Haney RZ, Calu DJ, Taylor AR, Burke KA, Schoenbaum G (2009) The orbitofrontal cortex and ventral tegmental area are necessary for learning from unexpected outcomes. Neuron 62:269-280.

Tan W, Janczewski WA, Yang P, Shao XM, Callaway EM, Feldman JL (2008) Silencing preBotzinger complex somatostatin-expressing neurons induces persistent apnea in awake rat. Nat Neurosci 11:538-540.

Taverna S, Canciani B, Pennartz CM (2005) Dopamine D1-receptors modulate lateral inhibition between principal cells of the nucleus accumbens. J Neurophysiol 93:1816-1819.

Tecuapetla F, Patel JC, Xenias H, English D, Tadros I, Shah F, Berlin J, Deisseroth K, Rice ME, Tepper JM, Koos T (2010) Glutamatergic signaling by mesolimbic dopamine neurons in the nucleus accumbens. J Neurosci 30:7105-7110.

Tindell AJ, Berridge KC, Zhang J, Pecina S, Aldridge JW (2005) Ventral pallidal neurons code incentive motivation: amplification by mesolimbic sensitization and amphetamine. Eur J Neurosci 22:2617-2634.

Tobler PN, Fiorillo CD, Schultz W (2005) Adaptive coding of reward value by dopamine neurons. Science 307:1642-1645.

Tong ZY, Overton PG, Clark D (1996) Stimulation of the prefrontal cortex in the rat induces patterns of activity in midbrain dopaminergic neurons which resemble natural burst events. Synapse 22:195-208.

Tritsch NX, Ding JB, Sabatini BL (2012) Dopaminergic neurons inhibit striatal output through non-canonical release of GABA. Nature 490:262-266.

Tsai HC, Zhang F, Adamantidis A, Stuber GD, Bonci A, de Lecea L, Deisseroth K (2009) Phasic firing in dopaminergic neurons is sufficient for behavioral conditioning. Science 324:1080-1084.

Tye KM, Tye LD, Cone JJ, Hekkelman EF, Janak PH, Bonci A (2010) Methylphenidate facilitates learning-induced amygdala plasticity. Nat Neurosci 13:475-481.

Tye KM, Prakash R, Kim SY, Fenno LE, Grosenick L, Zarabi H, Thompson KR, Gradinaru V, Ramakrishnan C, Deisseroth K (2011) Amygdala circuitry mediating reversible and bidirectional control of anxiety. Nature 471:358-362.

Uchida N, Mainen ZF (2003) Speed and accuracy of olfactory discrimination in the rat. Nat Neurosci 6:1224-1229.

Ungless MA, Grace AA (2012) Are you or aren't you? Challenges associated with physiologically identifying dopamine neurons. Trends Neurosci 35:422-430.

Ungless MA, Magill PJ, Bolam JP (2004) Uniform inhibition of dopamine neurons in the ventral tegmental area by aversive stimuli. Science 303:2040-2042.

Vanderschuren LJ, Everitt BJ (2004) Drug seeking becomes compulsive after prolonged cocaine self-administration. Science 305:1017-1019.

Waelti P, Dickinson A, Schultz W (2001) Dopamine responses comply with basic assumptions of formal learning theory. Nature 412:43-48.

Wanat MJ, Hopf FW, Stuber GD, Phillips PE, Bonci A (2008) Corticotropin-releasing factor increases mouse ventral tegmental area dopamine neuron firing through a protein kinase C-dependent enhancement of Ih. J Physiol 586:2157-2170.

Wang Y, Yu L, Geller AI (1999) Diverse stabilities of expression in the rat brain from different cellular promoters in a helper virus-free herpes simplex virus type 1 vector system. Hum Gene Ther 10:1763-1771.

Wassum KM, Ostlund SB, Maidment NT (2012) Phasic mesolimbic dopamine signaling precedes and predicts performance of a self-initiated action sequence task. Biol Psychiatry 71:846-854.

Wassum KM, Ostlund SB, Balleine BW, Maidment NT (2011) Differential dependence of Pavlovian incentive motivation and instrumental incentive learning processes on dopamine signaling. Learn Mem 18:475-483.

Watabe-Uchida M, Zhu L, Ogawa SK, Vamanrao A, Uchida N (2012) Whole-brain mapping of direct inputs to midbrain dopamine neurons. Neuron 74:858-873.

Wickens JR, Horvitz JC, Costa RM, Killcross S (2007) Dopaminergic mechanisms in actions and habits. J Neurosci 27:8181-8183.

Wilson MA, McNaughton BL (1993) Dynamics of the hippocampal ensemble code for space. Science 261:1055-1058.

Wise RA (1996) Addictive drugs and brain stimulation reward. Annu Rev Neurosci 19:319-340.

Wise RA, Rompre PP (1989) Brain dopamine and reward. Annu Rev Psychol 40:191-225.

Wise RA, Spindler J, Legault L (1978a) Major attenuation of food reward with performance-sparing doses of pimozide in the rat. Can J Psychol 32:77-85.

Wise RA, Spindler J, deWit H, Gerberg GJ (1978b) Neuroleptic-induced "anhedonia" in rats: pimozide blocks reward quality of food. Science 201:262-264.

Witten IB, Steinberg EE, Lee SY, Davidson TJ, Zalocusky KA, Brodsky M, Yizhar O, Cho SL, Gong S, Ramakrishnan C, Stuber GD, Tye KM, Janak PH, Deisseroth K (2011) Recombinase-driver rat lines: tools, techniques, and optogenetic application to dopamine-mediated reinforcement. Neuron 72:721-733.

Wyvell CL, Berridge KC (2000) Intra-accumbens amphetamine increases the conditioned incentive salience of sucrose reward: enhancement of reward "wanting" without enhanced "liking" or response reinforcement. J Neurosci 20:8122-8130.

Wyvell CL, Berridge KC (2001) Incentive sensitization by previous amphetamine exposure: increased cue-triggered "wanting" for sucrose reward. J Neurosci 21:7831-7840.

Yamaguchi T, Sheen W, Morales M (2007) Glutamatergic neurons are present in the rat ventral tegmental area. Eur J Neurosci 25:106-118.

Yamaguchi T, Wang HL, Li X, Ng TH, Morales M (2011) Mesocorticolimbic glutamatergic pathway. J Neurosci 31:8476-8490.

Yim CY, Mogenson GJ (1980) Electrophysiological studies of neurons in the ventral tegmental area of Tsai. Brain Res 181:301-313.

Yin HH, Zhuang X, Balleine BW (2006) Instrumental learning in hyperdopaminergic mice. Neurobiol Learn Mem 85:283-288.

Yizhar O, Fenno LE, Davidson TJ, Mogri M, Deisseroth K (2011) Optogenetics in neural systems. Neuron 71:9-34.

Yun IA, Nicola SM, Fields HL (2004) Contrasting effects of dopamine and glutamate receptor antagonist injection in the nucleus accumbens suggest a neural mechanism underlying cue-evoked goal-directed behavior. Eur J Neurosci 20:249-263.

Zahm DS, Cheng AY, Lee TJ, Ghobadi CW, Schwartz ZM, Geisler S, Parsely KP, Gruber C, Veh RW (2011) Inputs to the midbrain dopaminergic complex in the rat, with emphasis on extended amygdala-recipient sectors. J Comp Neurol 519:3159-3188.

Zetterstrom T, Herrera-Marschitz M, Ungerstedt U (1986) Simultaneous measurement of dopamine release and rotational behaviour in 6-hydroxydopamine denervated rats using intracerebral dialysis. Brain Res 376:1-7.

Zhang F, Wang LP, Boyden ES, Deisseroth K (2006) Channelrhodopsin-2 and optical control of excitable cells. Nat Methods 3:785-792.

Zhang F, Aravanis AM, Adamantidis A, de Lecea L, Deisseroth K (2007) Circuit-breakers: optical technologies for probing neural signals and systems. Nat Rev Neurosci 8:577-581.

Zhang H, Sulzer D (2012) Regulation of striatal dopamine release by presynaptic auto- and heteroreceptors. Basal Ganglia 2:5-13.

Zhou FM, Liang Y, Salas R, Zhang L, De Biasi M, Dani JA (2005) Corelease of dopamine and serotonin from striatal dopamine terminals. Neuron 46:65-74.

Zweifel LS, Parker JG, Lobb CJ, Rainwater A, Wall VZ, Fadok JP, Darvas M, Kim MJ, Mizumori SJ, Paladini CA, Phillips PE, Palmiter RD (2009) Disruption of NMDAR-dependent burst firing by dopamine neurons provides selective assessment of phasic dopamine-dependent behavior. Proc Natl Acad Sci U S A 106:7281-7288.

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