The representation of learning parameters in neural activity patterns in the nucleus accumbens

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DEDICATION

To my mom, dad, sisters, and grandparents – this is for y'all.

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CHAPTER 1

Introduction

1.1 Adaptive behavior

The ability to adaptively navigate an environment relies on associative learning, where animals form associations between predictive cues and external stimuli in an environment. Cost-benefit evaluations of an adaptive choice rely on the ability of an organism to understand all the associated consequences – good and bad – of an action (Cardinal, 2006; Green & Myerson, 2004). While associative learning is at the core of nearly all adaptive behavior, its dysregulation is also a key feature of a wide range of disease states, including substance use disorder (SUD), gambling, depression, compulsions, and binge eating among others (Avanzi et al., 2004; S. W. C. Chang et al., 2012; Dodd et al., 2005; Everitt & Robbins, 2005; Keiflin & Janak, 2015; Redish, 2004; Schultz, 2011; Wise & Koob, 2014; Xu et al., 2020). Thus, understanding how the brain controls the ability to learn across a wide range of contexts and contingencies is a key unanswered question that has broad impact for better understanding human health. The key driving question of my dissertation has been to ask ***how neural signals, at the level of neuromodulators, individual cells, and population dynamics, encode information critical to support adaptive behavior***.

Associative learning processes form the foundation for psychological phenomenon that describe how behaviors are supported or diminished over experience. Reinforcement learning describes behaviors that are maintained or increased by a *reinforcer*, which can be both a reward (which organisms will respond to receive) or an aversive outcome (which animals will respond to

remove) (Dayan & Balleine, 2002; Dayan & Niv, 2008). A punisher is an aversive stimulus that occurs in response to a behavioral action that functions to decrease or extinguish responding (Cardinal, 2006).

Dysfunction in associative learning and reinforcement processes contributes to many psychiatric disorders (Redish, 2004). For example, in addiction stimuli that predict drug are more reinforcing whereas responses to punishment are diminished (Koob & Volkow, 2010). In effect, the cost-benefit analysis is shifted to greatly favor getting the drug even at the cost of associated negative consequences, like loss of job or loss of familial support. In depression, there is evidence as well for dysregulation in reinforcement for rewarding stimuli and punishment from negative stimuli (Eshel & Roiser, 2010). In effect, we can frame many disorders as a dysregulation in adaptive associative learning processes. In the following sections I will describe key learning parameters that underlie associative learning. In Chapters 2 and 3, I will touch on how neural and neurotransmitter signals in the ventral striatum can actually map onto these particular components.

1.1.1 Valence and valence-independent learning parameters

Valence has long been thought of as key component of adaptive behavior. Valence describes, in effect, the “goodness” or “badness” of something (Lang et al., 1997; Russell, 2003; Russell & Barrett, 1999). Its outcome for behavior is thus simple – stimuli that are positive/rewarding are sought out and stimuli that are negative are avoided. It is thus foreseeable, and true, that the brain would represent valence as a way to guide future adaptive action. Yet treating stimuli as positive or negative in valence negates that the same stimulus can elicit opposite behavioral responses depending on the context in which that stimulus is encountered. For example, an unavoidable footshock will induce freezing, but an avoidable footshock will elicit an escape response in rodents - even though the footshock in both cases has negative valence (Kutlu et al., 2020, 2021). Valence-independent factors are thus important for helping animals

navigate changing contingencies, where the same stimulus can have different value based on the context. Without understanding the relationship between stimulus processing and behavioral action our understanding of the neural circuit control of behavior and its dysregulation in disease is incomplete.

Associative learning processes are dependent on a wide range of factors including relative value and valence, but also valence-independent factors such as salience, expectation, prediction, prediction error and others (Dayan & Balleine, 2002). As such, I've defined here key factors that impact learning.

Valence

The "goodness" or "badness" of something. It is binary parameter (Lang et al., 1997; Russell, 2003; Russell & Barrett, 1999). Valence informs whether we want to approach or avoid a stimulus.

Novelty

Novelty describes the unfamiliarity of a stimulus (Bussey, 1999; Parkin, 1997). Novel stimuli are remembered more accurately than more familiar stimuli (A. Parker et al., 1998). Novelty is an important aspect of attentional allocation – we pay attention to what we have not encountered. Latent inhibition describes a psychological phenomenon in which unconditioned stimuli form stronger associations with novel cues than with familiar cues (Lubow, 1973a, 1973b).

Salience

The physical intensity of a stimulus (Ungless, 2004). It describes objective properties of the stimulus independent of an individual's experience of it. For instance, the volume of an auditory stimulus (decibels) or intensity of a shock (amps). When different outcomes

are in conflict (ex. larger reward vs smaller reward), we need a way to represent which consequence is bigger. Saliency is thus represented on a continuum. While saliency can describe neutral stimuli, valence and saliency can inform the degree to which something is good or bad.

Perceived Saliency

Perceived saliency tracks the physical intensity of a stimulus but is also dynamically modulated by how novel the stimulus is. Thus, perceived saliency is a subjective metric that can be modulated by the novelty of an environment or stimulus and can be altered over experience/learning (Schmajuk et al., 1996; Sokolov, 1978, 1990). Novelty is decreased with repeated exposure to a stimulus (Lubow, 1973a, 1973b; Lubow & Moore, 1959; Quintero et al., 2011). For example, an auditory stimulus has a set intensity (measured in decibels). The stimulus has a set physical intensity but repeated exposure results in habituation that alters how the same stimulus of the same intensity is perceived. This stimulus will feel less intense with repeated exposure to the individual experiencing it even if the physical properties of the stimulus have not changed.

Prediction/Associative Strength

If something precedes another stimulus in time or they occur together, they will form an association (R. Rescorla & Wagner, 1972). The preceding stimulus will come to predict the following stimulus. Animals form associations with stimuli in order to predict future occurrences of a stimulus in their environment.

Prediction error

Describes a mismatch between what is predicted and what occurs (Rescorla & Wagner, 1972). Adaptive behaviors require a dynamic system that can respond to changes in the

environment or external stimuli. Prediction errors serve as a way to update information about an association as one acquires new information (den Ouden et al., 2012). The classic example of a prediction error, specifically a reward prediction error, came from Schultz showing that dopamine first responds to an unexpected presentation of a reward. When a cue is presented prior to the reward, monkeys (and demonstrated across multiple species) will learn the cue predicts the reward and the dopamine signal shifts from the reward to the predictive cue. However, when the cue is presented without the reward (on a trial in which the cue has previously perfectly predicted the outcome), there is a drop in dopamine, the “error prediction” signal (Schultz et al., 1997).

One of the early pursuits of my graduate career was designing a task in which we could dissociate stimulus valence and stimulus-driven behavior with the ultimate goal that we could map a neural signal onto different learning parameters. We developed the Multidimensional Cue Outcome Action Task (MCOAT) to dissociate motivated action from cue learning and valence in mice (Kutlu et al., 2020). The MCOAT included positive reinforcement, a contingency under which a stimulus increases the probability of a response to receive an outcome (ex. sucrose) and negative reinforcement, a contingency under which a stimulus increases the probability of a response to remove an aversive outcome (ex. shock). The advantage of this task design was that it asked the mouse to perform the exact same action for a reward as for the removal of an aversive stimulus. These are considered operant conditioning tasks, a type of associative learning process which requires a response. Conversely, Pavlovian conditioning refers to an associative learning process where a stimulus elicits an instinctive response (Pavlov, 1927). Fear conditioning represents a Pavlovian paradigm where a cue predicts a shock, resulting in an instinctive freezing response as the mouse learns the association. Thus, freezing acts as a measure for how well the association has been acquired. The advantage of these tasks if that we can look at how the same

stimulus (shock) results in opposing responses (freezing in fear conditioning and a response in negative reinforcement) to rule out that an effect is driven by a motor response.

We can see these learning paradigms represented in addiction. Addiction has been described as a transition from impulsivity in the early stages to a combination of impulsivity/compulsivity in later stages. Essentially, at first an individual takes drug for the rewarding effects (positive reinforcement), then as they transition to dependence, they take drug to avoid the negative effects (withdrawal/negative affect – negative reinforcement) (Koob & Volkow, 2010; Redish, 2004). Drug-associated cue and contexts, the stimuli that come to strongly predict the drug, drive drug-taking that persists for long after the last drug experience (Everitt & Robbins, 2005; Kalivas et al., 1998; Robbins, 1997). Thus, the negative affective state induced by prolonged withdrawal coupled with the exposure to the stimuli that have come to predict the presence of drug are key mediators of relapse, independent of the presence of the drug. Thus, by understanding how the brain supports associative learning processes in adaptive states, we can better understand the factors that underlie this transition to dependence that ultimately leads to relapse.

The primary focus of my dissertation has thus been to ask how these key learning parameters, which support adaptive behavior, are represented in the brain. As such, my PhD work has focused on the ventral striatum, a key brain region involved in reward and aversive learning and also the site of maladaptive changes in SUD and other psychiatric disorders (Carlezon & Thomas, 2009; Day & Carelli, 2007; Russo & Nestler, 2013; Scofield et al., 2016). There are multiple brain regions that underlie adaptive learning and behavior, including the hippocampus and amygdala, both of which send projections to the ventral striatum. Canonically it was thought the striatum subserved valence-based learning (O'Doherty et al., 2003) whereas the hippocampus was involved in long-term episodic memory, memory retrieval, and spatial learning (FeldmanHall et al., 2021; Vikbladh et al., 2019) and the amygdala in learning and potentially storage of emotional states related to an experience (Clark, 1995). It has been

theorized that the different information (sensory, emotional, contextual, etc.) related to an experience is stored in different brain regions. Thus, it is possible that the ventral striatum is the source of valence-based information and/or is an integrator of information from these brain regions that then outputs to motor nuclei to guide behavior.

The nucleus accumbens is situated in the basal ganglia, a limbic circuit important for motivation, learning, and motor planning, and which serves as a hub that integrates sensorimotor, associative and limbic information from cortical and thalamic afferents and innervation from midbrain dopamine neurons (Bolam et al., 2000; Gerdeman et al., 2003; Gerfen, 2000; Kreitzer & Malenka, 2008; Nicola, 2007). I will now focus on the anatomical organization of this brain region and the key neuronal populations. The organization of neuronal populations within the accumbens, as well as their connectivity to various glutamatergic and dopaminergic inputs and outputs structures, has informed theories on the functional roles of neuronal populations within this brain region.

1.2 The basal ganglia

The basal ganglia are composed of subcortical nuclei that subserve motor balance and planning, procedural learning, and reward-based learning (Alexander et al., 1991; Graybiel, 2000; Kreitzer & Malenka, 2008; Wickens et al., 2003). Dysfunction in this has been linked to Parkinson's disease, Huntington's disease, and drug addiction (Albin et al., 1989; Chesselet & Delfs, 1996; DeLong, 1990; DeLong & Wichmann, 2007). The striatum contains the dorsal and ventral striatum. The dorsal striatum is further subdivided into the dorsal lateral striatum (DLS) and the dorsal medial striatum (DMS) (Lanciego et al., 2012). They are differentiated based on their glutamatergic inputs. The ventral striatum is comprised of the nucleus accumbens (NAc), which contains both the core and shell subregions, and the olfactory tubercle (Nicola, 2007).

Historically, the dorsal and ventral striatum have also been segregated based on their functional roles in behavior. The dorsal striatum is involved in motor planning and action selection

(Lanciego et al., 2012), while the ventral striatum has roles motivated behavior and reward learning (Belin et al., 2009). The NAc shell and core are functionally divergent (Ambroggi et al., 2011; Floresco et al., 2008; Li & McNally, 2015; Saddoris et al., 2011), with the core mediating goal-directed behavior and the shell modulating motivational value (West & Carelli, 2016). However, there is overlap in what information is encoded by these brain regions. It is important to note there is also evidence of a location gradient in functional roles for MSN subtypes along a ventromedial to dorsolateral trajectory (Voorn et al., 2004).

Within the striatum exists another level of organization referred to as the striasome (patches) and the matrix based histochemically on the high levels of μ-opioid receptor (MOR), substance P (SP), dopamine (DA) 1-receptor (D1R), met-enkephalin (met-ENK), calretinin, Nr4a1, pro-dynorphin, GAD-2, andEGR-1 in the striasome and the high levels of calbindin, somatostatin (SST), enkephalin (ENK), DA2-receptor (D2R), and cholinergic markers including acetylcholine esterase (AChE) and choline acetyltransferase (ChAT) in the matrix (Brimblecombe & Cragg, 2017). While I will not touch on this further, there is evidence that the striasome has consequential roles in basal ganglia function and in the pathological processes of diseases associated with deficits in striatal function (Crittenden & Graybiel, 2011; Desban et al., 1993).

1.2.1 The nucleus accumbens

The nucleus accumbens (NAc) is at the hub of learning, selecting, and executing goal-oriented behaviors (Day & Carelli, 2007; de Jong et al., 2019; Sugam et al., 2014) and has been described as a “limbic motor interface” for its limbic nuclei inputs and basal ganglia motor nuclei outputs (Mogenson et al., 1980). The NAc has long been thought of as a hub of reward learning – although there is substantial evidence it is also important in aversive learning. Work has identified a critical role for the NAc in mediating the behavioral responses governed by positive and negative reinforcement (Castro & Berridge, 2014; Everitt & Robbins, 2005; Kravitz et al., 2012). A reduction in NAc neuronal activity has been shown in major depression in humans

(Drevets et al., 1992; Mayberg et al., 2005) and this dip in activity linked to disfunction in reward processing and resulting in anhedonia (Russo & Nestler, 2013).

The ventral striatum, primary of which is the nucleus accumbens (NAc), receives projections from limbic structures, including the medial prefrontal cortex (PFC), basolateral amygdala (BLA), and hippocampus, as well as the ventral tegmental area (VTA) and anterior cingulate cortices (Britt et al., 2012; Russo & Nestler, 2013). The nucleus accumbens serves as an integrator and functions to convert limbic information from the amygdala, frontal cortex, and hippocampus to eventual motivational action through motor nuclei outputs (Kelley, 1999). Motivated behavior is thought to be encoded via two distinct subpopulations that compose the majority of neurons in the nucleus accumbens and their respective output targets. The direct pathway projects the output nuclei of the basal ganglia (ventral mesencephalon (VM) and the indirect pathway through the ventral pallidum (VP)) that ultimately projects to the VM (Bock et al., 2013a; MacAskill et al., 2014; Yawata et al., 2012).

The NAc is a heterogeneous region primarily composed of GABAergic medium spiny neurons (MSN) that can be classified based on their expression of D1 or D2 dopamine receptors, their projection targets (Albin et al., 1989; Gerfen, 1992; Gerfen et al., 1990; Kupchik et al., 2015), and their peptide expression (Steiner & Gerfen, 1998; Surmeier et al., 1996). They comprise 95% of the neurons in this brain region, of which approximately each represent about half of that total (Graveland & DiFiglia, 1985; Surmeier et al., 2007). The D1 and D2 MSNs were thus thought to represent the direct and indirect pathways, respectively. Dopamine receptors are G-protein coupled receptors (GPCRs). D1 receptors are Gs- or Golf-coupled, resulting in increased cAMP and PKA downstream, and D2 receptors are Gi- or Go-coupled, resulting in decreased cAMP and PKA activity (Gerfen & Surmeier, 2011; Surmeier et al., 2007). These are largely distinct populations, with only about 6-7% of MSNs expressing both D1 and D2 receptors in the NAc core in the adult striatum (Bertran-Gonzalez et al., 2010; Gagnon et al., 2017). D1-MSNs also selectively express M4 cholinergic receptors, dynorphin, and substance P, while D2-MSNs co-

express A2a adenosine receptors, enkephalin, and neurotensin (Bertran-Gonzalez et al., 2010; Gerfen et al., 1990; Graybiel, 2000; Surmeier et al., 2007).

MSNs exist in a what is termed a down-state, in which they are inhibited. Glutamatergic excitation of MSNs releases them from this down-state. Dopamine, which acts on the D1 and D2 receptors, modulates the excitatory glutamatergic input differentially based on the associated G-protein. D1 activation supports an up-state transition, as increased PKA results in enhanced Cav1 L-type Ca2+ channel activity, decreased somatic K+ channel activity, and downregulated Cav2 Ca2+ channels results in increased excitability of D1 MSNs. D2 receptor signaling has the converse effect, where decreased PKA inhibits this up-state transition (Gerfen & Surmeier, 2011; Surmeier et al., 2007).

The remaining 5% of neurons in the ventral striatum are interneuron populations (cholinergic and GABAergic interneurons). The GABAergic interneurons can be further categorized into the fast-spiking (FSI), low-threshold spiking (LTSI), and the calretinin expressing (CR) interneurons (Garas et al., 2018; Schall et al., 2020). Cholinergic interneurons (CIN), which comprise only about 0.5-1% of neurons, are tonically active neurons that release acetylcholine and have extensive local axonal arborizations. For their small number, interneurons, via there dense axonal arborizations, are able to exert extensive control over accumbal output (Tepper et al., 2010; Tepper & Bolam, 2004). As an example of interneuron-mediated control over striatal output, parvalbumin-expressing fast-spiking interneurons (PV-INs) contribute to motivational behavior through feedforward microcircuits that shape excitatory gain from glutamatergic inputs into the NAc. This microcircuitry is targeted by acute cocaine exposure, which decreases glutamatergic drive onto PV-INs, thus releasing the feedforward mechanism that integrates glutamatergic input to support goal-directed behavior (Manz et al., 2019).

MSN activity on short timescales can thus be modulated by multiple factors, including GABAergic interneurons (which inhibit activity of local MSNs), glutamatergic inputs, and collateral transmission (MSN-MSN connectivity I will discuss in the discussion) (Dobbs et al., 2016;

Edwards et al., 2017; Freeze et al., 2013; Kalivas, 2009; Taverna et al., 2008; Tepper & Bolam, 2004; Turner et al., 2018), and on longer timescales by peptide release which alters excitatory input (Steiner & Gerfen, 1998; Surmeier et al., 2007).

1.3 D1 and D2 Medium Spiny Neurons (MSNs)

1.3.1 D1 and D2 MSN organization

Our understanding of D1 and D2 MSNs has largely been derived from work in the dorsal striatum showing that these two populations are segregated into the direct (D1 MSNs) and indirect (D2 MSNs) pathways that drive action initiation and inhibition, respectively (Cui et al., 2013; Kravitz et al., 2010, 2012; J. G. Parker et al., 2018). In support of this classical model, Kravitz et al. (2010) showed that bilateral excitation of indirect-pathway MSNs increased freezing and bradykinesia while decreasing initiations of locomotion, behaviors associated with a parkinsonian state. Conversely, activating the direct pathway had the opposing outcome for freezing and locomotion initiations. When this was then tested in a mouse model of Parkinson's disease, stimulating the direct pathway excitation restored the deficits in freezing, bradykinesia, and locomotor initiation (Kravitz et al., 2010). These findings point to a bidirectional role for direct and indirect pathway striatal neurons in behavioral responding.

In a second paper from Kravitz et al., (2012) in the dorsal striatum, the authors showed these bidirectional functional roles were conserved in reinforcement learning. As noted, reinforcement describes behaviors that maintain or increase a behavior (Cardinal, 2006). dMSN-ChR2 mice (D1-Cre mice expressing the excitatory opsin channelrhodopsin) showed a bias for the laser-paired trigger, while iMSN-ChR2 mice showed a bias away from the laser paired trigger (Kravitz et al., 2012). Interestingly, this behavior is independent of the dopamine signal as a dopamine antagonist fails to alter these response biases once acquired or during acquisition of the laser-trigger bias, a point I will touch on later in regard to the relationship between dopamine and D1 and D2 MSNs in the accumbens. These findings on bidirectional roles for direct and

indirect pathway MSNs has informed a general theory on the relationship of D1 and D2 MSNs across brain regions.

It was long thought that D1 and D2 MSNs in the ventral striatum conserved these opposing roles in action initiation and inhibition seen in the dorsal striatum via their downstream impact on thalamic activity. D1 direct pathway neurons inhibit the substantia nigra pars reticulata (SNr) and the internal globus pallidus, resulting in release of thalamic inhibition (Deniau et al., 2007; Graybiel, 2000). Conversely, D2 indirect pathway neurons project to the globus pallidus (GP), which releases inhibition onto the subthalamic nucleus (STN) and sends a glutamatergic input to the SNr. The release of inhibition of thalamic activity promotes behavior while its inhibition suppresses behavior. However, pathway segregation is less clear in the NAc than in the dorsal striatum. Canonically, D1 MSNs project directly to midbrain structures like the VTA and D2 MSNs to the ventral pallidum (VP). However, Kupchick et al. showed that the ventral pallidum receives projections from both D1 and D2 MSNs. Approximately 50% of the VP neurons received D1 MSN input and ~90% received D2 MSN input (Kupchik et al., 2015).

For the above reasons, it was thought that D1 and D2 MSNs had opposing roles. This view was additionally supported in other areas, even so far as suggesting that D1 and D2 MSNs encode opposing valence. I will review the literature in depth that supported this theory and the literature that has suggested a more complex role in the next sections. This theory has formed the basis for my research describing the nature of what D1 and D2 MSNs encode across multiple behavioral contingencies. Thus, it is important that I characterize the body of work that has supported these viewpoints. In Chapter 4: Discussion, I will reevaulate some of the data used to support the opposing valence-hypothesis. These findings may in fact support a more complex view of the functional roles of D1 and D2 MSNs in adaptive behavior.

1.3.2 D1 and D2 MSNs in reward and aversive learning

The NAc has been canonically linked to reward learning and motivation. However, NAc activity is implicated in associative learning for aversive and rewarding stimuli (Correia et al., 2022; Day & Carelli, 2007; de Jong et al., 2019; Haralambous & Westbrook, 1999; Li & McNally, 2015; McDannald et al., 2011; McGinty et al., 2013; Ray et al., 2020; Schwienbacher et al., 2004; Setlow et al., 2003; Stuber et al., 2011; Sugam et al., 2014) and these signals evolve over learning at the single cell and population level (Bin Saifullah et al., 2018; Day & Carelli, 2007, 2007; de Jong et al., 2019; Ray et al., 2020; Roitman et al., 2005; Sugam et al., 2014). Learning-related plasticity within the NAc has also been observed ex vivo where numerous studies have shown that repeated administration of drugs of abuse, presentation of rewards, or experience with aversive stimuli leads to robust plasticity within neuronal populations within the NAc (Campioni et al., 2009; Grueter et al., 2013; Kourrich et al., 2007; Lüscher & Bellone, 2008; Russo et al., 2010; Vega-Villar et al., 2019; Wolf & Ferrario, 2010). Further, through a variety of activation/inhibition strategies, work has shown that neural activity in the NAc is critical for both the acquisition and expression of learned behavior, including responses to conditioned cues (Ambroggi et al., 2011; Calipari et al., 2016; Cruz et al., 2014; Floresco et al., 2008; Pothuizen et al., 2005; Schwienbacher et al., 2004).

1.3.3 D1 and D2 MSNs in addiction

A clear role for the nucleus accumbens in addiction has been defined by dopamine, through which all drugs of abuse, via different mechanisms, activate the mesolimbic dopamine system (Di Chiara and Imperato, 1988; Pascoli et al., 2015; Koob and Bloom, 1988). But work has demonstrated that reinforcement in the nucleus accumbens can be dopamine-independent (Koob, 1992). The fundamental organizational differences noted above between the dorsal and ventral striatum have precluded our ability to infer NAc cell function on behavior and in addiction. Currently, much of our understanding of the role these populations in the NAc play in behavioral

control has been largely based on their role in reward-based behaviors, and in particular, addiction (Day & Carelli, 2007; Lobo & Nestler, 2011; Russo & Nestler, 2013). Together these studies that span physiology, transcriptional plasticity, and pharmacological and optical manipulations have led to the hypothesis that D1 MSNs promote reward and D2 MSNs promote aversion (Lobo et al., 2010a; Lobo & Nestler, 2011).

Further support for this notion of opposing roles was supported by the differential effects of drugs of abuse on D1 and D2 MSNs. Considerable work has identified synaptic plasticity in D1 MSNs in response to cocaine (Creed et al., 2016; Lobo & Nestler, 2011; Pascoli et al., 2014) and furthermore, that plasticity at defined inputs onto D1-MSNs supports sensitization (Pascoli et al., 2011; Robinson & Berridge, 2003) and is key for cue-associated cocaine seeking (Kalivas et al., 1998; Pascoli et al., 2014, 2015). Thus, D1 MSNs are key in relapse. Cocaine, ethanol, and THC induced ΔFosB expression only in D1 MSNs in the NAc core, shell, and dorsal striatum (Lobo et al., 2013). Overexpression of ΔFosB only in D1 MSNs enhanced cocaine sensitization and conditioned place preference (Grueter et al., 2013). Additionally, acute exposure to psychostimulants selectively induced FosB, ERK, c-Fos, and Zif268 in D1 MSNs, but not in D2 MSNs. These effects also shape at the synaptic level, with repeated cocaine altering GABA receptor and other ion channel subunits selectively in D1 MSNs. In mice that underwent >2 weeks of cocaine self-administration, the ratio of AMPA/NDMA excitatory postsynaptic (EPSCs) currents was increased in D1 MSNs, but not in D2 MSNs (Bock et al., 2013b). These findings support the notion that D1 MSNs are primarily responsible for the reinforcing and sensitizing effects of drugs of abuse.

Conversely, D2 MSNs are linked to aversion (Danjo et al., 2014; Hikida et al., 2010). In models of cocaine addiction, D1 and D2 MSNs show differential transcriptional adaptations (Chandra et al., 2015; Lobo et al., 2010a). When Egr3, a transcription factor targeted by cocaine-mediated signaling pathways, was overexpressed in D1 MSNs, it increases rewarding and locomotor responses. Overexpression in D2 MSNs decreases these effects. Low levels of D2

receptors in the striatum has also been linked to vulnerability to and maintenance of compulsive behaviors towards stimulant drugs in rodents and nonhuman primates (Belin et al., 2008; Ersche et al., 2011; Nader et al., 2006; Volkow et al., 2002, 2009).

However, for the evidence that exists regarding the selective effects of drugs of abuse on D1 and D2 MSNs, these affects can be drug dependent. D-Amphetamine treatment induces a conditioned place-preference. However, inhibition of either D1 or D2 MSNs by selective D1 and D2 receptor antagonists blocked the development of a conditioned place preference (Liao, 2008). Downstream, as noted, the ventral pallidum (VP) receives input from both D1 and D2 MSNs, though it was previously thought to solely be the target of the indirect pathway (Kupchik et al., 2015). The VP responds to rewards and reward-predictive cues (Tindell et al., 2005) and a subset projects back to the VTA where it can modulate drug seeking (Mahler et al., 2014). This functional role for the VP, as a major target of the NAc, is part of the body of literature that has reinforced the view of nucleus accumbens involvement in reward learning.

1.3.4 Current theory – bidirectional effects of D1 and D2 MSNs

In the core

A large body of literature has suggested opposing roles for D1 and D2 MSNs in reward and aversion (Calipari et al., 2016; Francis & Lobo, 2017; Lobo & Nestler, 2011; Macpherson et al., 2014), where activating D1 MSNs promotes reward-related outcomes while activating D2 MSNs promotes aversive outcomes or blunts reward. Taken in the context of drugs of abuse, in which D1 and D2 MSNs have been predominantly investigated, D1 MSN activation supports the motivation for drug while D2 MSN activation supports the inhibition of cocaine-seeking.

One of the primary experiments that supported this theory of opposing roles showed that optogenetic activation (10Hz, ChR2) of D1 MSNs enhanced cocaine reward in a conditioned place preference (CPP) experiment. Conversely, activation of D2 MSNs resulted in a significant attenuation of the cocaine/stimulation preference (Lobo et al., 2010b). This supported the

conclusion that D1 MSNs support the rewarding effects of cocaine whereas the D2 MSNs have an antagonistic role on cocaine reward.

Via a reversible neurotransmission blocking technique, Hikida et al, 2010 addressed the roles of the direct (D1 MSNs, striatonigral) and the indirect (D2 MSNs, striatopallidal) pathways in adaptive rewarding and aversive learning and in the context of stimulant drugs. It is important to note; however, that this study did not appear to target a subregion of the striatum. Hikida showed that selective transmission blockade of the direct pathway (D1 MSNs) reduced locomotor sensitization following prolonged cocaine administration. Transmission blockade of the indirect pathway (D2 MSNs) showed delay but eventual hyperlocomotion. In a conditioned place preference experiment direct pathway blockade attenuated cocaine-induced CPP while indirect pathway blockade did not alter levels of cocaine-induced CPP as compared to controls. This indicates D1 MSNs are important for the locomotor sensitizing effects of stimulant drugs. Perhaps more interestingly, this trend was preserved across a food reinforcer. Blockade of D1 MSN transmission impaired the chocolate-food induced CPP while not altering CPP in the D2 MSNs. The authors then probed this in an aversive learning task and demonstrated that blockade of the direct pathway had no effect as compared to wild-type controls in the latency to enter a preferred dark chamber paired with electrical shock after a prior session in which the mice had encountered the shock upon entering the dark chamber. However, blockade of the indirect pathway dramatically decreased the time mice took to enter the shock-paired dark chamber, suggesting D2 MSNs are key for aversive learning and D1 MSNs for reward learning (Hikida et al., 2010).

As noted, cocaine self-administration increased the ratio of AMPA/NMDA EPSC currents, which was not observed in D2 MSNs. Interestingly, individuals that did not develop compulsive behaviors towards cocaine had higher AMPA/NMDA ratios at excitatory inputs onto D2 MSNs (Bock et al., 2013b). This led the authors to conclude that weakening of the indirect pathway increased vulnerability to compulsion for cocaine and so probed this utilizing inhibitory DREADDs. Inhibition of D2 MSNs in mice that had acquired cocaine self-administration and then were placed

on a progressive ratio schedule, showed a higher motivation to acquire cocaine (assessed by breakpoint). The authors then assessed how stimulation of this pathway altered cocaine self-administration. They showed that optogenetic stimulation (ChR2; 10ms pulses at 16.6 Hz, 10 min OFF, 5 min ON throughout session) of D2 MSNs following acquisition of self-administration reduced the mean number of earned rewards during the laser-ON periods and when normalized to the OFF sessions, suppressed cocaine self-administration. This was a schedule-independent effect, as the same was seen across an FR1, FR2, and FR3 schedules – although this was not tested with progressive ratio (Bock et al., 2013b). In effect, D2 MSNs have an antagonistic role on cocaine reward.

This was further supported by work from Danjo et al., 2014 who addressed the dopamine-MSN connection. As noted, increased dopamine in response to reward signals is thought to primarily activate D1Rs and the direct pathway, while decreased dopamine levels in response to aversive stimuli is thought to predominantly activate the D2Rs and indirect pathway (I will touch on tonic and phasic dopamine and their relationship to dopamine 1 and 2 receptors in more depth in a later section “Dopamine and D1 and D2 MSNs). The authors thus posited that suppressing dopamine neuron firing would indirectly activate D2 MSNs. Optical inactivation of DA neurons in the VTA resulted in aversion to a dark room, which mice show an innate tendency to prefer. This effect was dependent on the D2R, thus supporting the notion that D2 MSNs mediate processing of aversive stimuli or aversive learning (Danjo et al., 2014).

As expected by the differential G-proteins associated with dopamine 1 and 2 receptors, dopamine elicited by a single cocaine injection increased D1 MSN calcium transient frequency and decreased D2 MSN transient frequency. Physiologically, D2 MSNs are more active at baseline (Calipari et al., 2016). When calcium transients in D1 and D2 MSNs were then recorded in a conditioned place preference task, Calipari et al., 2016, identified a biphasic neural signal in which an increase in D1 activity preceded entry into the drug-paired chamber and a decrease in D2 MSN activity followed entry. Inhibiting D1 MSNs (hM4Di DREADD) both before the cocaine

pairing and on a choice test day blocked the preference for the drug-paired chamber. Inhibiting D2 MSNs activity did not alter the cocaine place preference (Danjo et al., 2014).

This bidirectional effect on behavior has been observed in other models. In one study, mice underwent chronic social defeat stress (CSDS), a model of depression, to assess how manipulation of D1 and D2 MSN signaling altered depression-like outcomes (Francis et al., 2015). In this protocol, mice were exposed to an aggressive mouse daily. On the 11th day, mice underwent a social interaction test where experimenters quantified the amount of time mice spent time interacting with a novel social target. The outcome of this protocol was a group of stress susceptible mice (display depression-like behaviors) and a group of resilient mice. In the group of susceptible mice, repeated high-frequency stimulation (>50Hz; ChR2(E123A)-enhanced yellow fluorescent protein (EYFP), also known as ChETAA-EYFP) of D1 MSNs over days rescued the social interaction time to control levels. Furthermore, repeated high frequency activation also resulted in increased preference for rewarding stimuli in a sucrose preference task. Inhibiting D1 MSNs (inhibitory DREADD) shifted resilient mice to susceptible in the CSDS task. Neither repeated high frequency stimulation or inhibition altered the social interaction time or sucrose preference in susceptible or resilient D2-cre mice following CSDS (Francis et al., 2015).

Because D2 stimulation (ChETAA-EYFP) did not alter social interaction after chronic social defeat stress, the authors proposed that repeated activation of D2 MSNs in stress naïve mice would promote susceptibility to a single subthreshold social defeat stress – and it did induce social avoidance. It is interesting to note that acute inhibition (halorhodopsin) of D1 MSNs did not impact phenotype. Again, we see a context under which D1 and D2 MSNs demonstrate bidirectional effects on behavioral outcome (Francis et al., 2015).

In the shell

I have focused on the nucleus accumbens core, but the ventral striatum also includes the nucleus accumbens shell, a distinct functional structure (Ambroggi et al., 2011; Floresco et al.,

2008; Li & McNally, 2015). I will not exhaustively review the literature here but want to illustrate that the theory of bidirectionality in the ventral striatum has also been supported by work in the shell. High frequency stimulation (HFS) induces LTP at D1 MSN-to-VP synapses while HFS induces LTD at D2 MSN-to-VP. D1 MSN outputs to the VP were potentiated, while D2 MSN outputs were weakened, by repeated cocaine experience. The effect of this D1 MSN-to-VP circuit on locomotor sensitization to cocaine was abolished by in vivo depotentiation of D1 MSN output to the VP. Manipulating D2 MSN-to-VP transmission did not alter locomotor sensitization but did normalize performance in a progressive ratio task in mice with repeated cocaine experience. Thus, it appears cocaine's rewarding and sensitizing effects are mediated through D1 MSNs and its affects on motivation and induction of a negative affective state are mediated through D2 MSNs (Creed et al., 2016).

1.3.5 In opposition to bidirectional roles of D1 and D2 MSNs

In the core

For the literature that has supported opposing roles for D1 and D2 MSNs, work has emerged in opposition to this theory. Several papers have shown that mice will self-stimulate for activation of both D1 and D2 MSNs (Cole et al., 2018; Soares-Cunha et al., 2016, 2018, 2020). Cole et al. (2018) showed that in a spout-touch self-administration task, both D1- and D2-cre mice will make many more contacts on the side that gives illumination of the NAc laser (1s constant, 1mW). The authors did identify some sub-type specific patterns in the spout self-stimulation. D2 ChR2 self-stimulation occurred at a slower rate than D1 ChR2 self-stimulation in session 1 before reaching a stable rate across sessions that was significantly greater than the EYFP control rate but modest as compared to the rate of D1 ChR2 self-stimulation (We cannot imply what this difference in rate means, only that both D1 and D2 MSNs support reinforcement). Furthermore, D2 ChR2 mice could track the changes in location of the spout that gave stimulation, but at a slower rate than D1 ChR2 mice. It is also important to note that once the active spout no longer

gave laser stimulation (extinction) both D1- and D2-Cre mice reduced their responding (Cole et al., 2018). Another critical note to this set of experiments is that it did not selectively target the core or shell – the authors utilized histology to assess targeting of their optical fibers. Both groups of mice contained medial shell and core targets but when the data was evaluated by localization of the optic fiber tips, both D1- and D2-cre mice showed similar levels of self-stimulation independent of whether the stimulation occurred in the medial shell or the core.

Soares-Cunha in their 2016 paper evaluated the role of D1 and D2 MSNs in motivation. In a progressive ratio (PR) task, which measures the willingness to work for a reward (breakpoint), D1- and D2-cre mice expressing ChR2 underwent progressive ratio training where D1 and D2 MSNs were stimulated (12.5 ms light pulses at 40 Hz, during 1 s of cue exposure) to the cue. Both D1 and D2 MSNs increased the cumulative presses and breakpoint in a PR session, indicating both D1 and D2 MSN activation correlates positively with motivation (Soares-Cunha et al., 2016). Natsubori et al. 2017 showed in the ventrolateral striatum, that both D1 and D2 MSNs show a calcium response to the presentation of levers (cue), which initiates the trial in a progressive ratio task and to the food pellet reward (Natsubori et al., 2017).

The above data led to the conclusion that the stimulation paradigm for activation of D2 MSNs may shape the behavioral outcome, whether mice show reinforcement or aversion, and may explain the discrepancy in results seen previously. As such, Soares-Cunha et al., (2019) sought to tease this effect apart by looking at reinforcement contingencies with different stimulation paradigms. They started with a conditioned place preference experiment, in which entering the laser-paired side of the chamber resulted in either brief (1s, 12.5ms at 40Hz, every minute; experiment 1) or prolonged stimulation (60s, 12.5ms at 40Hz, every 2 min; experiment 2). They demonstrated that the stimulation paradigm was responsible for the place preference or aversion in both D1- and D2-ChR2 mice. Brief stimulation resulted in a place preference in both, prolonged stimulation in a place aversion in both. This effect was conserved across real-time place preference, in which mice entering the laser-paired chamber resulted in continuous

stimulation until they leave the ON side. Both D1- and D2-ChR2 mice induced aversion, likely because, on average, mice received 60s of stimulation per entry into the laser-paired side (Soares-Cunha et al., 2020)

The outcome of these experiments is key when you consider the experimental design for the CPP experiment in the Lobo et al. (2010) paper. Soares-Cunha et al. (2020) performed this experiment using their brief (1s) and prolonged (60s) stimulation parameters and showed that a sub-threshold dose of cocaine (which on its own will not induce CPP) only induced a conditioned place preference with brief optical activation of D1 and D2 MSNs, and not prolonged. Prolonged stimulation in D2 MSNs decreased cocaine reward. Prolonged stimulation of D1 MSNs did not change the reinforcing properties of cocaine, but it also did not enhance these effects as seen with brief stimulation (Soares-Cunha et al., 2020). In the Lobo et al., (2010) paper, prolonged stimulation parameters were used (10 Hz blue light pulses over 3 min; intensity 2-4 mW). Experimenters should thus consider how the stimulation parameters can differentially affect behavioral responses and is thus a key consideration for what are most physiologically relevant stimulation parameters. Divergent behavioral outcomes may actually reflect different activation parameters rather than a reflect subpopulation differences.

In the shell

Other work in the NAc shell has indicated bidirectionality, but in a sub-region dependent manner. Al-hasani et al., (2015) showed dynorphinergic cells in the NAc shell can drive both aversion and reward dependent on ventral or dorsal localization in the shell. They showed that drd1-positive neurons colocalize with dynorphin mRNA-positive neurons in both the ventral and dorsal shell. There was no difference in the number of dyn mRNA-expressing neurons between these two regions, suggesting this bidirectional effect on behavior is a result of sub-region localization, independent of D1 receptor expression. Dyn mRNA-expressing neurons did not colocalize with Drd2-positive neurons (Al-Hasani et al., 2015).

On another note, it was thought that valence could be transmitted by specific glutamatergic projections to the NAc. However, it has since been shown that PFC, vHip, and BLA inputs to the NAc are all reinforcing and furthermore, that these inputs synapse on both NAc D1 and D2 MSNs. (Britt et al., 2012; Otis et al., 2017; Stuber et al., 2011). Activation of glutamatergic inputs from the paraventricular nucleus (PVN) of the thalamus actually does cause aversion; however, as with the PFC, vHip, and BLA inputs, it synapses on both NAc D1 and D2 MSNs (Zhu et al., 2016). Thus, the specific glutamatergic input driving NAc MSN activity may not be functionally relevant and does not appear responsible for transmitting valence-based information.

Despite the evidence that D1 and D2 MSNs do not have opposing roles, the question of what they do actually encode is left to be defined. This brings us to the first question of my PhD work: *what information do D1 and D2 MSNs encode in basal, adaptive states?* The ultimate goal would be to provide a framework for how drugs of abuse and disease acts on these neural populations to subvert adaptive behavior. I will cover these results in Chapter 2: “Accumbal D1 and D2 medium spiny neurons have distinct and valence-independent roles in learning”.

1.4 Dopamine

For the extensive work focusing on what is on the receiving end of the dopamine signal, an even larger body of work has aimed to define the precise information encoded by dopaminergic projections to the nucleus NAc and the role of dopamine in learning and memory (Bayer & Glimcher, 2005; Dayan & Balleine, 2002; Dayan & Niv, 2008, 2008; Fiorillo et al., 2008; Flagel et al., 2011; Glimcher, 2011; Iordanova et al., 2021; Kutlu et al., 2021).

Dopamine has long been thought to be the biological substrate for reward prediction error (RPE) (Schultz, 2016; Steinberg et al., 2013). This originated from the Pavlovian conditioning model, the Rescorla-Wagner model (R. Rescorla & Wagner, 1972), which was then applied to dopamine’s action by Schultz (Schultz et al., 1997) and characterized as a reward prediction error due to dopamine’s response to rewarding stimuli. First dopamine neurons fire to the unpredicted

reward, then firing shifts to a cue that signals reward will come, and then dopamine neurons reduce their firing rate when a predicted reward is not given. This effect has been seen across species (Bayer & Glimcher, 2005; Eshel et al., 2016; Lak et al., 2014; Starkweather et al., 2018; Takahashi et al., 2016) and in the NAc (Day & Carelli, 2007; Flagel et al., 2011; Hart et al., 2014; Howe et al., 2013; Phillips et al., 2003; Saddoris, Cacciapaglia, et al., 2015; Saddoris, Sugam, et al., 2015).

However, we showed in our work (Kutlu et al., 2021) that dopamine only resembles RPE predictions under certain scenarios, and that in fact, valence-independent perceived saliency better describes the dopamine signal in the accumbens core across contingencies, including positive and negative reinforcement and fear conditioning. Perceived saliency accounts for the physical intensity of a stimulus, which can be perceived differently based on situation and contingency (Schmajuk et al., 1996; Sokolov, 1978, 1990). Thus, novelty is a key factor that can shape the subjective experience of a stimulus. Together, perceived saliency provides an explanation for how the dopamine signal can both respond to stimulus intensity and change with learning.

We first showed that dopamine does in fact look like a reward prediction error in a positive reinforcement task where mice can nosepoke during a discriminative stimulus for a sucrose reward. This pattern is not replicated in negative reinforcement. This is sensible if dopamine only responds to rewards; however, dopamine responds in an intensity dependent fashion to both rewarding (sucrose) and aversive (shock and quinine) stimuli. To illustrate that the dopamine signal is not in fact an unsigned prediction error (rather than a reward prediction error), mice underwent fear conditioning training where a cue was paired with a shock. On omission trials (cue presented without the shock in a subset of trials), there was a dopamine response, although smaller than the dopamine response to the shock itself. If dopamine signals a signed prediction error, the response to the omitted but expected shock should be negative instead of positive. If dopamine signals an unsigned prediction error, the response to the expected but omitted shock

should be larger than the response to the expected shock, which it was not. Lastly, introducing a novel stimulus to the cue presentation in the fear conditioning task increased the dopamine response to the cue. This is termed external inhibition, where the prediction of the shock did not change but the addition of a novel stimulus at the time of the cue reduced the freezing response while resulting in a robust dopamine response only to the cue + novel stimulus but not to the cue alone. As such, dopamine responds to valence-free stimuli but does not signal shifts in associative strength (Kutlu et al., 2021).

We thus sought to address two questions – one of which we addressed in our manuscript "Dopamine signaling in the nucleus accumbens core mediates latent inhibition" included in this dissertation and one which will continue to be an evolving goal of this work.

1. *Does dopamine respond to novelty in a task that probes associations with new and familiar stimuli?*
2. *How and what information does dopamine transmit to D1 and D2 MSNs?*

This second question has long been a question of the field and while my PhD work did not directly probe this question – it is a natural extension of this work facilitated by tools we now have that allow us to simultaneously record MSNs and manipulate dopamine release in vivo.

1.4.1 Dopamine and D1 and D2 MSNs

One of the key acknowledgments that should be made regarding dopamine is that it is a neuromodulator. It is not inducing activity in MSNs, instead, via G-protein coupled receptors, it is modulating MSN excitability to glutamatergic input. Dopamine neurons show two distinct firing patters: phasic firing and tonic firing (Grace et al., 2007; Schultz, 2007). The D1 receptor has low-affinity for dopamine as compared to the high-affinity D2 receptor. The phasic firing, which activates the D1 receptor, is thought to be the key signal in reward-related behavior. Alternatively,

the tonic firing modulates the D2 receptor and has been shown to be suppressed by aversive stimuli (Grace et al., 2007; Mirenowicz & Schultz, 1994, 1996; Ungless et al., 2004).

One of the critical considerations of the relationship between MSNs and dopamine is that the glutamatergic inputs that excite MSNs are consequential to dopamine's effect on D1 and D2 MSNs. Furthermore, activation of D1 and D2 MSNs can be dopamine-independent – which begs the question under what scenarios is dopamine shaping MSN output? I will cover the results that address Question 1 above in Chapter 3: "Dopamine signaling in the nucleus accumbens core causally mediates latent inhibition". I provide my theory on the connection between D1 and D2 MSN and dopamine activity in the discussion.

CHAPTER 2

Accumbal D1 and D2 medium spiny neurons have distinct and valence-independent roles in learning

2.1 Summary

At the core of value-based learning is the nucleus accumbens (NAc). D1- and D2-receptor containing neurons (MSNs) in the NAc are hypothesized to have opposing valence-based roles in behavior. Using optical imaging and manipulation approaches, we show that neither D1 or D2 MSNs signal valence. D1 MSNs responses were evoked by stimuli regardless of valence, contingency, or learning. D2 MSNs were evoked by both cues and outcomes, were dynamically changed with learning, and tracked valence-free prediction error at the population and individual neuron level. Finally, D2 MSN responses to cues were necessary for associative learning. Thus, D1 and D2 MSNs work in tandem, rather than in opposition, by signaling specific properties of stimuli to control learning.

2.2 Introduction

The ability to adaptively navigate an environment relies on learning, where animals form associations between predictive cues and external stimuli (Cardinal, 2006; Green & Myerson, 2004). This process is at the core of nearly all adaptive behavior and its dysregulation is also a key feature of a wide range of psychopathologies (Avanzi et al., 2004; C.-J. Chang et al., 2021; Dodd et al., 2005; Keiflin & Janak, 2015; Redish, 2004; Schultz, 2011; Wise & Koob, 2014). Thus,

understanding how the brain controls the ability to learn across contexts and contingencies has broad implications for human health.

As described in Chapter 1, our understanding of D1 and D2 MSNs has been largely derived from work in the dorsal striatum showing that these two populations are segregated into the direct (D1 MSNs) and indirect (D2 MSNs) pathways that drive action initiation and inhibition, respectively (Bateup et al., 2010; Kravitz et al., 2010, 2012; J. G. Parker et al., 2018). However, this pathway segregation is less clear in the NAc (Kupchik et al., 2015). As such, these fundamental organizational differences preclude our ability to infer NAc cell function and behavioral control based on this previous work. Specifically, in the NAc, much of our understanding of the role these populations play in behavioral control has been based on their role in reward-based behaviors. Together these studies that span physiology, transcriptional plasticity, and pharmacological and optical manipulations have led to the hypothesis that D1 MSNs promote reward and D2 MSNs promote aversion/prevent reward seeking (Bock et al., 2013a; Calipari et al., 2016; Creed et al., 2016; Danjo et al., 2014; Francis et al., 2015; Lobo et al., 2010a; Lobo & Nestler, 2011).

However, as noted, emerging evidence is beginning to show that the functions of these neurons extend beyond simple reward-based coding (Al-Hasani et al., 2015; Cole et al., 2018; Nishioka et al., 2021; Soares-Cunha et al., 2016, 2018, 2020, 2022; Zalocusky et al., 2016) and it is currently unclear exactly what environmental factors elicit responses in these populations and how these temporal dynamics are linked to behavioral control. Here we utilized optical approaches to record from and manipulate D1 and D2 MSNs during ongoing behavior in response to both appetitive and aversive stimuli. Further, by combining these recording/manipulation approaches with behaviors that include both operant and Pavlovian contingencies, we show the precise valence-independent role that these populations play in associative learning.

2.3 Materials and Methods

Subjects. Male and female 6- to 8-week-old C57BL/6J mice were obtained from Jackson Laboratories (Bar Harbor, ME; SN: 000664) and housed five animals per cage. All animals were maintained on a 12h reverse light/dark cycle. Animals were food restricted to 90% of free-feeding weight for the duration of the studies. Mice were weighed every other day to ensure that weight was maintained. All experiments were conducted in accordance with the guidelines of the Institutional Animal Care and Use Committee (IACUC) at Vanderbilt University School of Medicine, which approved and supervised all animal protocols.

Apparatus. Mice were trained and tested daily in individual Med Associates (St. Albans, Vermont) operant conditioning chambers fitted with two illuminated nose pokes on either side of an illuminated sucrose delivery port, all of which featured an infrared beam break to assess head entries and nose pokes as well as a lickometer to record lickbouts**.** One nose poke functioned as the active and the other as the inactive nose poke depending on the phase of the experiment (described below). Responses on both nose pokes were recorded throughout the duration of the experiments. Chambers were fitted with additional visual stimuli including a standard house light and two yellow LEDs located above each nose poke. Auditory stimuli included a white noise generator (which were used at 85 dB in these experiments) and a 16-channel tone generator capable of outputting frequencies between the range of 1 and 20 kHz (also presented at 85 dB).

Surgical Procedure. Ketoprofen (5mg/kg; subcutaneous injection) was administered at least 30 mins before surgery. Under Isoflurane anesthesia, mice were positioned in a stereotaxic frame (Kopf Instruments) and the NAc core (bregma coordinates: anterior/posterior, **+ 1.2 mm**; medial/lateral, + 1.5 mm; dorsal/ventral, −4.3 mm; 10° angle) for fiber photometry and optogenetics or NAc core **(**bregma coordinates: anterior/posterior, + 1.4 mm; medial/lateral, + 1.0 mm; dorsal/ventral, −3.8 for viral injection**,** 0° angle**)** for **miniscope e**xperiments were targeted

(unilateral for fiber photometry and optogenetic stimulation experiments and bilaterally for optogenetic inhibition experiments). Ophthalmic ointment was applied to the eyes. Using aseptic technique, a midline incision was made down the scalp and a craniotomy was made using a dental drill. A 10-mL Nanofil Hamilton syringe (WPI) with a 34-gauge beveled metal needle was used to infuse viral constructs. Virus was infused at a rate of 50 nL/min for a total of 500 nL. Following infusion, the needle was kept at the injection site for seven minutes and then slowly withdrawn. Permanent implantable 2.5 mm fiber optic ferrules (Doric) were implanted in the NAc. Ferrules were positioned above the viral injection site (bregma coordinates: anterior/posterior, + 1.4 mm; medial/lateral, + 1.5 mm; dorsal/ventral, −4.2 mm; 10° angle) and were cemented to the skull using C&B Metabond adhesive cement system. For Miniscope surgeries, following viral injection, a **26**gauge needle was slowly lowered into the brain to create a tract for the scope. Once removed, the lense was positioned above the viral injection site (**bregma coordinates: anterior/posterior, + 1.4 mm; medial/lateral, + 1.0 mm; dorsal/ventral, −3.7 mm; 0° angle**) and were cemented to the skull using C&B Metabond adhesive cement system. Follow up care was performed according to IACUC/OAWA and DAC standard protocol. Animals were allowed to recover for a minimum of six weeks to ensure efficient viral expression before commencing experiments.

Histology: Subjects were deeply anesthetized with an intraperitoneal injection of Ketamine/Xylazine (100mg/kg/10mg/kg) and transcardially perfused with 10 mL of PBS solution followed by 10 mL of cold 4% PFA in 1x PBS. Animals were quickly decapitated, the brain was extracted and placed in 4% PFA solution and stored at 4 °C for at least 48-hours. Brains were then transferred to a 30% sucrose solution in 1x PBS and allowed to sit until brains sank to the bottom of the conical tube at 4 °C. After sinking, brains were sectioned at 35µm on a freezing sliding microtome (Leica SM2010R). Sections were stored in a cryoprotectant solution (7.5% sucrose + 15% ethylene glycol in 0.1 M PB) at -20 °C until immunohistochemical processing. We immunohistochemically stained all NAc slices with an anti-GFP antibody (chicken anti-GFP;

Abcam #AB13970, 1:2000) for GCaMP6f, GCaMP6m, channelrhodopsin, and halorhodopsin for the validation of viral placement. Sections were then incubated with secondary antibodies [gfp: goat anti-chicken AlexaFluor 488 (Life Technologies #A-11039)] for 2 h at room temperature. After washing, sections were incubated for 5 min with DAPI (NucBlue, Invitrogen) to achieve counterstaining of nuclei before mounting in Prolong Gold (Invitrogen). Following staining, sections were mounted on glass microscope slides with Prolong Gold antifade 2 reagent. Fluorescent images were taken using a Keyence BZ-X700 inverted fluorescence microscope (Keyence), under a dry 10x objective (Nikon). The injection site location and the fiber implant placements were determined via serial imaging in all animals.

Fiber Photometry. For all fiber photometry experiments, to record specific activity in D1 and D2 MSNs, we expressed a Cre-recombinase-dependent virus (double-floxed inverse open reading frame (DIO) adeno-associated viral (AAV)) carrying the fluorescent calcium indicator GCaMP6f (AAV5.hSyn.Flex.GCaMP6f.WPRE.SV40) in the NAc core of transgenic mice that express cre-recombinase in either D1- or D2- MSNs (D1-Cre or Adora2a, A2A,-Cre mice). The fiber photometry recording system uses two light-emitting diodes (LED, Thorlabs) controlled by an LED driver (Thorlabs) at 490nm (run through a 470nM filter to produce 470nM excitation - the excitation peak of dLight1.1) and 405nm (an isosbestic control channel 54). Light was passed through a number of filters and reflected off of a series of dichroic mirrors (Fluorescence MiniCube, Doric) coupled to a 400µm 0.48 NA optical fiber (Thorlabs, 2.5mm ferrule size, optimized for low autofluorescence). LEDs were controlled by a real-time signal processor (RZ5P; Tucker-Davis Technologies) and emission signals from each LED stimulation were determined via multiplexing. The fluorescent signals were collected via a photoreceiver (Newport Visible Femtowatt Photoreceiver Module, Doric). Synapse software (Tucker-Davis Technologies) was used to control the timing and intensity of the LEDs and to record the emitted fluorescent signals. The

LED intensity was set to 125µW for each LED and was measured daily to ensure that it was constant across trials and experiments. For each event of interest (e.g., predictive cue, headentries, licks, shock), transistor-transistor logic (TTL) signals were used to timestamp onset times from Med-PC V software (Med Associates Inc.) and were detected via the RZ5P in the synapse software (see below).

Fiber Photometry Analysis. The analysis of the fiber photometry data was conducted using a custom Matlab pipeline. Raw 470nm (F470 channel) and isosbestic 405nm (F405 channel) traces were collected at a rate of 1000 samples per second (1kHz) and used to compute Δf/f values via polynomial curve fitting. For analysis, data was cropped around behavioral events using TTL pulses and for each experiment 2s of pre-TTL and 18s of post-TTL Δf/f values were analyzed. Δf/f was calculated as F470nm-F405nm/F405nm. This transformation uses the isosbestic F405nm channel, which is not responsive to fluctuations in calcium, to control for calcium-independent fluctuations in the signal and to control for photobleaching. Z-scores were calculated by taking the pre-TTL Δf/f values as baseline (z-score = (TTLsignal - b_mean)/b_stdev, where TTL signal is the Δf/f value for each post-TTL time point, b_mean is the baseline mean, and b_stdev is the baseline standard deviation). This allowed for the determination of dopamine events that occurred at the precise moment of each significant behavioral event. We also provided baselines as well as raw 405nm and 470nm traces used to calculate Δf/fs for the critical experiments as supplementary figures (**fig. S2**). For statistical analysis, we also calculated peak heights and area under the curve (AUC) values for each individual calcium peak via trapezoidal numerical integration on each of the z-scores across a fixed timescale which varied based on experiment. The duration of the peak height and AUC data collection was determined by limiting the analysis to the z-scores between 0 time point (TTL signal onset) and the time where the calcium peak goes back to baseline.

Single-photon calcium imaging via miniscopes. For calcimimaging at the single cell level, we used endoscopic miniature scopes (nVista miniature microscope, Inscopix) combined with a calcium indicator, GCaMP6m (GCamP (AAV5.CAG.Flex.GCaMP6m.WPRE.SV40) in order to record single cell activity in the NAc core in vivo. During each behavioral session, the miniscope was attached to the integrated lens baseplates implanted previously. The imaging parameters (gain, LED power, focus) were determined for each animal to ensure recording quality and kept constant throughout the study. The imaging videos were recorded at 20 frames per second (fps). At the end of the recording session, the miniscope was removed and the baseplate cover was replaced. During each session, important events such as stimulus or outcome presentation times were recorded via transition-transition-logic (TTL) signals sent from the MedPC behavioral box to the Inscopix data acquiring computer.

Single-photon calcium imaging analysis. Data was acquired at 20 frames per second using an nVista miniature microscope (Inscopix). TTLs from MedPC were directly fed to the nVista system, which allowed alignment to behavioral timestamps without further processing. The recordings were spatially down-sampled by a factor of 2 and corrected for motion artifacts using the Inscopix Data Processing Software (IDPS v1.3.1). The df/f values were computed for the whole field view as the output pixel value represented as a relative percent change from the baseline. We used Nonnegative Matrix Factorization - Extended (CNMF-e, Pnevmatikakis et al., 2016; Zhou et al., 2018) to identify and extract calcium traces from individual cells (CNMF-e cell detection parameters: patch_dims = 50, 50; K = 20; gSiz = 20; gSig = 12; min_pnr = 20; min_corr = 0.8; max_tau = 0.400). Raw CNMFe traces were used for all analyses. The spatial mask and calcium time series of each cell were manually inspected using the IDPS interface. Cells found to be duplicated or misdetected due to neuropils or other artifacts were discarded. The raw Δf/f data

were exported and used for TTL analysis, in which we cropped the data around each significant event (cue presentations; TTL) and z-scored it in order to normalize for baseline differences. Z-scores were calculated by taking the pre-TTL Δf/f values as baseline (z-score = (TTLsignal - b_mean)/b_stdev, where TTL signal is the Δf/f value for each post-TTL time point, b_mean is the baseline mean, and b_stdev is the baseline standard deviation).

Using the z-scored traces, we then calculated whether the cell response to the cues and shock outcomes were significant in order to determine responsive and non-responsive cells as well as the direction of the response (positive, negative, or bimodal). For this analysis, we calculated averaged peak heights of the cue or shock cell responses as the maximum z-score achieved during a 2 second post-TTL window. We then ran two separate one-tailed independent t-tests to determine the response was significantly higher than +1.96 or lower than -1.96; critical z-score for significance at p=0.05. The cells that showed an averaged maximum response through 6 trials higher than the threshold were labeled as “Positive” cells. The cells that showed a significant negative response were labeled “Negative” cells. All other cells were determined as “No response” cells. We conducted this analysis for the cue and shock cell responses.

Longitudinal Registration: We used the longitudinal registration pipeline, defined in the Inscopix Data Process Software (IDPS) Guide, to identify the same cell across recording sessions in longitudinal series. Cell sets are preprocessed to generate a cell map which is then aligned to the first cell map (the reference). The images of the first cell set are defined as the global cell set against which the other cell sets are matched. We then find the pair of cell images between the global cell set and other aligned cell sets that maximizes the normalized cross correlation (NCC). The program then generates an output that aligns the same cell from across sessions.

METHOD DETAILS

Behavioral Experiments: A series of behavioral experiments were run throughout this study to link activity within D1- and D2-MSNs to behavioral responding in Pavlovian and reinforcement contexts. They are outlined in detail below:

Positive Reinforcement. Mice were trained to nose poke on an active nose poke – denoted by its illumination – for delivery of sucrose in a trial-based fashion. Following a correct response, the sucrose delivery port was illuminated for 5 seconds and sucrose was delivered (1s duration of delivery, 10% sucrose w/v, 10ul volume per delivery). To create a trial-based procedure, a discriminative stimulus (Sd, sucrose) was presented signaling that responses during the presentation of Sd, sucrose resulted in the delivery of sucrose. Responses made during any other time in the session were recorded, but not reinforced. The discriminative stimulus was an auditory tone that consisted of 85dB at 2.5 kHz or white noise in a counterbalanced fashion. During the initial training, Sd, sucrose was presented throughout the entirety of each 1-hour session and animals could respond for sucrose without interruption. When animals reached ≥ 60 active responses in a single session, they were then moved to a discrete trial-based structure in subsequent 1-hour sessions, wherein Sd, sucrose was presented for 30 seconds at the beginning of each trial with a variable 30 second inter-trial interval (ITI). Each trial ended following a correct response and associated sucrose delivery or at the end of a 30 second period with no active response. At the end of the trial both the trial and Sd, sucrose were terminated. Animals that exhibited active responses in ≥ 80% of trials during a session then proceeded to the final phase of training wherein the duration of Sd, sucrose was reduced to 10 seconds. Upon reaching the 80% criterion during this phase (i.e., acquisition), post-training calcium responses in D1- and D2-MSNs were recorded over a 30 min session.

Negative Reinforcement. Mice were trained to nose poke on the opposite, non-sucrose-paired nose poke for shock avoidance.. A second auditory discriminative stimulus (Sd, shock) was presented at the beginning of each trial following a variable ITI as described above. In each trial the discriminative stimulus was presented for 30 seconds after which a series of 20 footshocks (1mA, 0.5 second duration) was delivered with a 15 second inter-stimulus interval. Trials ended when animals responded on the correct nose poke or at the end of the shock period. The end of the shock period was denoted by the presentation of a house light cue that signaled the end of the trial and was illuminated for one second. During these trials, mice could respond during the initial 30 second Sd, shock period to avoid shocks completely, respond any time during the shock period to terminate the remaining shocks, or not respond at all. If mice did not respond to both the trial and Sd, shock were terminated after all 20 shocks had been presented (330 seconds total). Acquisition during negative reinforcement training was defined as receiving fewer than 20% of total shocks in a single one-hour session.

Varying footshock intensities: A total of **8 footshocks** were delivered in a non-contingent and inescapable fashion over a 12-minute period. Shocks were delivered at 0.3mA and 1mA intensities (4 presentations for each shock intensity). Shocks were delivered in a pseudo-random order with variable inter-stimulus intervals (mean ITI = 30 sec). All shock intensities were presented within the same test session.

Sucrose delivery: Mice were given ad libitum access to the port, which was manually filled with sucrose prior to the session. The timing of the lickbouts were recorded.

Fear conditioning, fear extinction. For fiber photometry experiments, mice received a single footshock (1mA, 0.5 second duration) immediately following a 5 second auditory cue (5kHz

tone; 85dB) for 6 pairings. Mice underwent 4 fear conditioning sessions, followed by 4 extinction sessions in which the cue was presented, but shocks were omitted entirely. For cellular resolution imaging experiments, the cue was presented for 10s, instead of 5, during the fear conditioning and fear extinction sessions. This was due to the reduced movement of the mice in response to the sizeable scope.

Optogenetic stimulation and inhibition of D1- and D2- MSNs.

Intracranial Self-Stimulation (ICSS) optogenetic excitation of D1- and D2-MSNs via channelrhodopsin (ChR2). In a separate group of **D1 or A2A-Cre**, we unilaterally expressed channelrhodopsin [excitatory opsin (AAV5-DIO-ChR2-YFP)], or **eYFP [control vector (AAV5-DIO-eYFP)]** in the NAc core using the strategies previously described. A 200um fiber optic implant was placed into the NAc core. This allowed for the selective stimulation of D1- and D2-MSNs. For these experiments, mice were placed in an operant chamber where they could nose poke on active nosepoke to receive laser stimulation (470nm, 2s, 20Hz, 8mW). We recorded the number of active nosepokes (nosepokes that resulted in stimulation) and inactive nosepokes (nose pokes on the opposite nose poke that did not result in stimulation). We counterbalanced which nosepoke was considered the active nosepoke.

Inhibition of D1- and D2-MSNs during fear learning.

We bilaterally expressed halorhodopsin [inhibitory opsin (AAV5-DIO-NpHR3.0-YFP)] or eYFP [control vector (AAV5-DIO-eYFP)] in D1-Cre and A2A-Cre mice using the strategies previously described. In a fear conditioning session, mice received a single footshock (1mA, 0.5 second duration) immediately following a 5 second auditory cue for 3 pairings. The cue was a tone (5kHz tone; 85dB). During this initial training session, each presentation of the cue was optically inhibited (5 sec, 8 mw, continuous laser)for the duration of the cue presentation. We hand scored freezing

behavior for the 5 second pre-footshock cue period for each trial in a blind fashion. The freezing response was defined as the time (seconds) that mice were immobile (lack of any movement including sniffing) during the tone period and calculated as percentage of total cue time.

Statistical analysis. Statistical analyses were performed using GraphPad Prism (version 8; GraphPad Software, Inc, La Jolla, CA) and Matlab (Mathworks, Natick, MA). Z-scores were calculated as explained above (see Fiber photometry analysis). Unpaired t-tests and one-way ANOVAs were employed for analysis where fiber photometry AUCs had two and three levels, respectively. Repeated measures ANOVAs were used for the behavioral data from reinforcement studies (positive and negative reinforcement). For all repeated measures ANOVA analysis, we used the Geisser-Greenhouse correction for sphericity. We also calculated maximum z-scores for event fiber photometry traces and analyzed to see if these were significantly different from the critical z-score at p=0.05 level (1.645) using independent-t-tests. Alpha was 0.05 for all statistical analysis. All data were depicted as group mean ± standard error of the mean (S.E.M.). Outliers were determined using the Grubbs's test for outliers (alpha=0.0001). The exclusion criterion was established a priori. We assumed normal distribution of sample means for all t and F statistics.

Statistical analysis. Statistical analyses were performed using GraphPad Prism (version 9; GraphPad Software, Inc, La Jolla, CA) and Matlab (Mathworks, Natick, MA). We used nested-ANOVAs as well as paired and unpaired t-tests where appropriate for analyzing peak height z-scores. For multiple comparisons between peak height values and number of detected cells from different sessions, we used Repeated Measures ANOVA followed by Tukey post-hocs.

2.4 Results

D1 and D2 MSNs do not signal valence

To record temporally defined neural activity that occurred to valence-based stimuli, the genetically encoded calcium indicator, GCaMP6f, was selectively expressed in either D1 or D2 MSNs in the NAc core (using D1-cre or A2A-cre mice, respectively). Because D2 receptors are expressed in some interneuron populations in the NAc, A2A-Cre mice were used to select for D2 MSNs, specifically (Gallo et al., 2018). Using fiber photometry, population-level calcium transients were monitored in awake and behaving animals (**Fig. 1A**). First, D1 and D2 MSNs were recorded during a positive reinforcement task where mice nose poked during an auditory cue to for sucrose reinforcer. In line with previous valence-based predictions (Lobo et al., 2010a; Lobo & Nestler, 2011), at the time of sucrose collection D1 MSNs showed a positive response (**Fig. 1B**), while D2 MSNs showed a small, but significant, decrease (**Fig. 1C**). However, when mice were presented with unsignaled footshocks at random intervals of varying intensity (0.3 or 1.0 mA in random order) both D1 MSNs (**Fig. 1D**) and D2 MSNs (**Fig. 1E**) showed positive responses that tracked shock intensity (see **fig. S1** for area under the curve values). When the change in response between the high versus low intensity shock was assessed, there was a trend towards a larger change in response to changing intensities in D1 MSNs as compared to D2 MSNs (**Fig. 1F**). Next, the excitatory opsin (channelrhodopsin) was expressed in D1 or D2 MSNs as described above and mice were given the opportunity to nose poke for optical stimulation (470nm, 2s, 20Hz, 8mW) (**Fig. 1G,H**). Interestingly, both D1 and D2 MSNs supported reinforcement and elicited responding above eYFP controls during training (**Fig. 1I**), showed discrimination of the active vs inactive operanda (**Fig. 1J**), and increased their response rates over training (**Fig. 1K**). Together, these data showed that there is no clear demarcation between D1and D2 MSNs in the NAc based on valence alone. D1 MSN responses (in the positive direction) were evoked by both appetitive and aversive stimuli, rather than decreasing to aversive stimuli as would be predicted for a reward-based signal (Roitman et al., 2005; Schultz et al., 1997). Further, D2 MSNs supported

reinforcement, suggesting that they were not transmitting an aversive signal or preventing motivated responding.

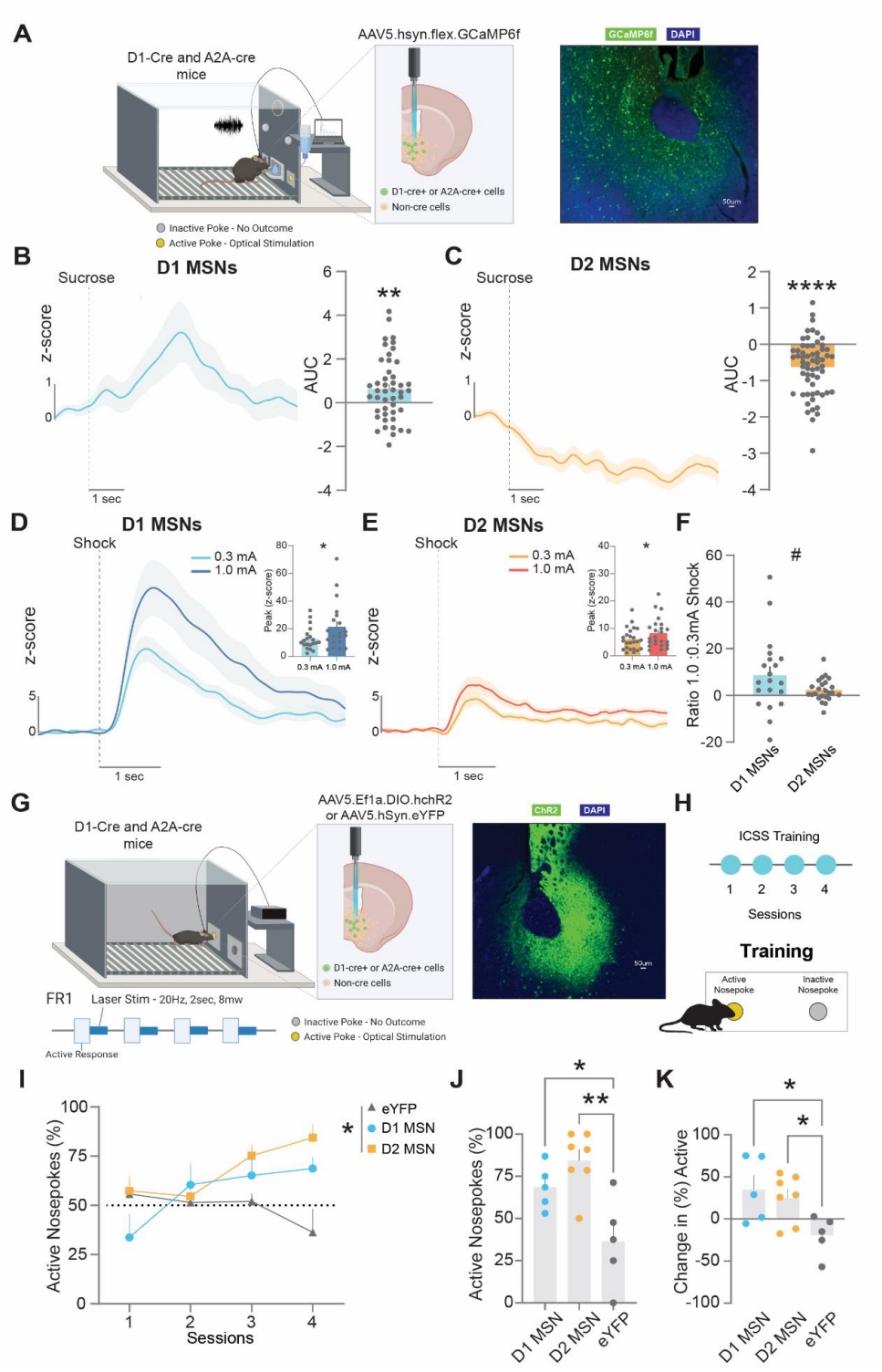

Fig. 1. D1 and D2 MSNs do not track valence.

(**A**) Experimental design. Cre-dependent GCaMP6f (AAV5.hsyn.flex.CGaMP6f) was expressed in D1 (D1-cre mice) or D2 (A2A-cre mice) MSNs. (right) Example of GCaMP6f expression in NAc core. (**B**) D1 MSNs showed a positive response to sucrose retrieval in a positive reinforcement operant task (two-tailed independent sample t-test, $t_{45} = 4.02$, $p = 0.0058$, n = 6 mice). (**C**) D2 MSNs showed a decrease to sucrose retrieval in the same task (two-tailed independent sample t-test, $t_{60} = 6.287$, $p < 0.0001$, n = 6 mice). (**D**) D1 MSNs showed an intensity-dependent positive response to unsignaled shock (nested ANOVA $F_{(1,39)} = 6.53$, $p = 0.0159$, n = 5 mice). (**E**) D2 MSNs showed an intensity-dependent positive response to unsignaled shock (nested ANOVA $F_{(1,47)} = 5.04$, $p = 0.031$, n = 6 mice). (**F**) The increased response to shock over intensities (ratio of 1 mA to 0.3 mA response) trended towards being larger in D1 MSNs (two-tailed independent sample t-test, $t_{42} = 1.896$, $p < 0.0648$). (**G**) Intracranial self-stimulation (ICSS) task design. An excitatory opsin (ChR2; AAV5.Ef1a.DIO.hchR2) or a control vector (eYFP; AAV5.hSyn.eYFP) was expressed in D1 or D2 MSNs in the NAc core. Nosepokes resulted in laser illumination (14Hz, 2s, 8mW, 470nM). Viral expression of ChR2 in the NAc core (**H**) Stimulation of both D1 and D2 MSNs supported reinforcement. (**I**) D1- (D1 MSN) and A2A-Cre (D2 MSN) mice showed a preference for the active nosepoke as compared to eYFP controls (repeated measures ANOVA trial × group interaction $F_{(6,42)} = 3.168$, $p = 0.0118$). (**J**) D1-cre (n = 5 mice) and A2A-Cre (D2 MSNs, n = 7 mice) showed a greater percentage of total responses on the active operanda as compared to the eYFP controls (n = 5 mice, one-way ANOVA $F_{(2,14)} = 8.955$, $p = 0.0031$; Dunnett's post-hoc eYFP versus D1, $p = 0.0360$; eYFP verses D2, $p = 0.0016$). (**K**) Training-dependent increase in responses in D1 and A2A-Cre mice as compared to eYFP (one-way ANOVA $F_{(2,14)} = 4.602$, $p = 0.0291$; Dunnett's post-hoc eYFP versus D1, $p = 0.0248$; eYFP verses A2A, $p = 0.0486$). Data represented as mean ± S.E.M.; * $p < 0.05$; ** $p < 0.01$, ****$p < 0.0001$, # $p = 0.0648$.

D2, but not D1, MSNs scale with learning

While the studies above suggest that D1 and D2 MSN responses cannot be explained by stimulus valence alone, it was still possible that differential dynamics in these populations could signal reward or aversive learning more broadly or could signal when opposing behavioral action is necessary. For example, if D1 MSNs were involved in reward seeking and motivated behavior, one would expect a dynamic increase over learning as animals learned to respond for a reinforcer. Conversely, an associated decrease would be expected in the D2 MSN population under similar conditions. We specifically tested this hypothesis.

One of the difficulties with defining the precise role of neural populations in behavioral control is the ability to dissociate multiple behavioral factors from one another. To this end, we first utilized a behavioral task developed in our laboratory (the MCOAT task; (Kutlu et al., 2020)) to delineate the relationship between multiple task parameters (e.g., valence, action initiation, prediction) and the resultant neural signals. In the first phase of this task, mice were trained in

positive reinforcement where an auditory cue predicted that an operant nose-poke response would result in delivery of sucrose (**Fig. 2A-C**). In the next phase of MCOAT, mice were trained in negative reinforcement task where a distinct auditory cue indicated that a response on a second nose-poke prevented the delivery of a series of footshocks (**Fig. 2D-F**). Importantly, the operant response in the two phases are the same (nose-poke), and both outcomes (sucrose retrieval or removal of shock) are positive; however, the maintaining stimulus has opposite valence (shock - negative, sucrose - positive).

In the positive reinforcement task, mice learned quickly to respond on the active nosepoke [acquisition >= 60 correct responses; (**Fig. 2B**)] and responded with greater probability during the cue then during the inter-trial interval (**Fig. 2C;** also see **fig. S2**) indicating that not only did they learn the task but were using the cue to complete the task. During negative reinforcement, mice avoided greater than 80% of potential shocks within a session and had a higher probability of responding during the cue than during the inter-trial interval (**Fig. 2E, F**; also see **fig. S2**). D1 and D2 MSNs responses to the discriminative cues that predicted positive or negative reinforcement were assessed during the first training session and again in the same mice after they had met acquisition criteria.

D1 MSNs responded to the discriminative cue for positive reinforcement; however, this response did not change over training. The same pattern was present in the negative reinforcement task, where D1 MSNs responded to the discriminative cue and the responses were not sensitive to training history (**Fig. 2G-J**; see **fig. S3** for area under the curve). Conversely, D2 MSNs responded to both the discriminative cue that signaled positive reinforcement and the discriminative cue that signaled negative reinforcement and both responses increased with performance (**Fig. 2K-N**; see **fig. S3** for area under the curve). Thus, even regarding motivated action, we did not observe opposing responses in these populations. Further, it was D2 MSNs, not D1 MSNs, that were most likely to predict changing performance on reinforcement tasks associated with positive outcomes. Together, these data - combined with the data above - suggest

that D1 MSNs respond to stimuli and do not change over learning, while D2 MSNs dynamically respond to changes in predictive cues.

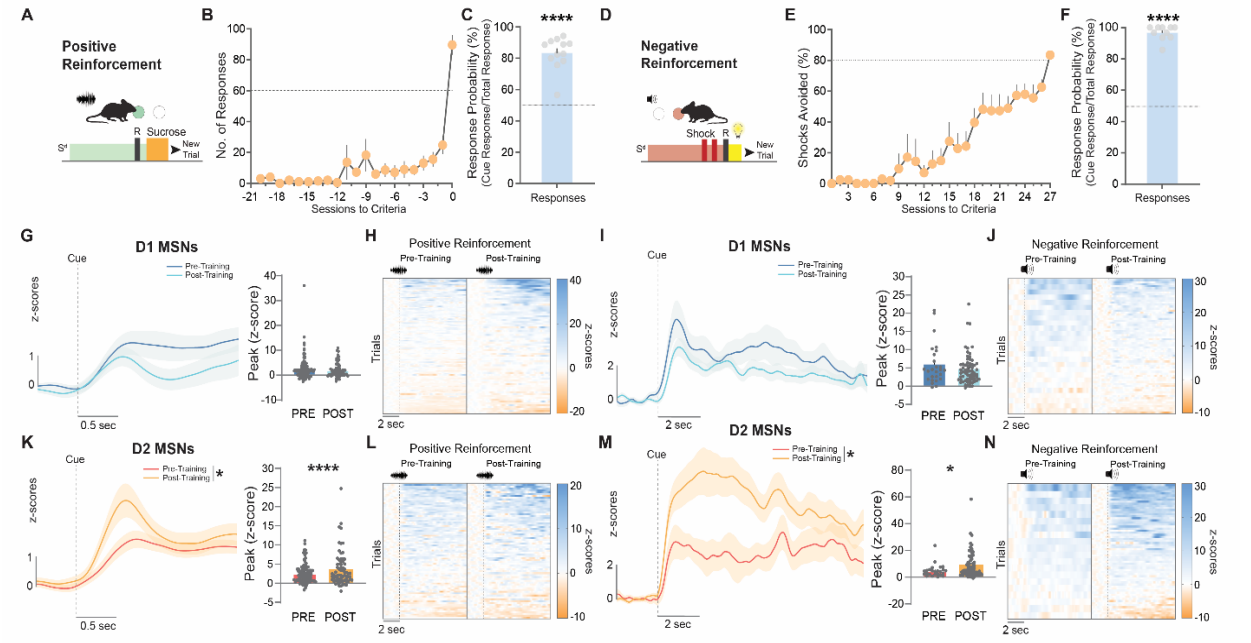

Fig. 2. D2 MSN responses to predictive cues scale with associative strength, while D1 MSN responses do not change.

(**A**) Positive reinforcement. A discriminative cue (S^d) indicated that responses on a fixed-ratio 1 schedule resulted in sucrose delivery. (**B**) Mice acquired this task (>60 responses on the active operanda). (**C**) Nearly all responses were made during the cue period, indicating that mice learned the value of this cue (two-tailed independent sample t-test, $t_{11} = 11.23$, $p < 0.0001$, n = 10 mice; critical value = 50%). (**D**) Negative reinforcement. Mice responded during an S^d to prevent shock presentation. (**E**) Mice avoided almost all possible shocks. (**F)** Almost alll responses were made during the cue period, indicating that mice learned the cue value (two-tailed independent sample t-test, $t_9 = 31.79$, $p < 0.0001$, n = 12 mice; critical value = 50%). (**G**) D1 MSNs showed a response to the S^d that signaled positive reinforcement. This response did not change with training (nested ANOVA F(1,176) = 10.1413, p = 0.427, n = 6 mice). (**H**) Heatmap of D2 MSN responses pre-training and post-training. (**I**) D2 MSNs showed an increase in response to the S^d signaling positive reinforcement between pre- and post-training (nested ANOVA F(1,166) = 16.38, p < 0.0001, n = 6 mice). (**J**) Heatmap of D2 MSN responses pre-training and post-training. (**K**) The same recordings were done during the negative reinforcement task. D1 MSNs showed a response to the S^d signaling negative reinforcement. This did not change with experience (nested ANOVA F(1,91) = 1.21, p = 0.2747, n = 5 mice). (**L**) Heatmap of D1 MSN responses. (**M**) D2 MSNs showed a learning-dependent increase in response to the cue (nested ANOVA $F_{(1,88)} = 5.35$, $p = 0.0234$, n = 5 mice). These data indicate that D2 MSNs, but no D1 MSNs, scale with learning across task types. (**N**) Heatmap of trial responses pre-training and post-training. Data represented as mean ± S.E.M. * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$.

MSN responses are consistent across tasks

The results reported above rule out valence-specific information encoding by NAc D1 and D2 MSNs. They also suggest that D1 MSNs respond to stimuli (neutral cues, footshocks, sucrose) but do not change as animals learn. Conversely, D2 MSN activity patterns change with learning; however, it was not clear if these signals were specific to motivated action or were signaling information about cues that were not sensitive to behavioral contingencies/actions - for example, simply if how strongly the cue predicted an outcome. We hypothesized that D2 MSN responses dynamically change with the predictive value of cues, regardless of the task requirements. Further, D1 MSN activity will be insensitive to these changes, signaling only the absence/presence of stimuli.

We tested our hypothesis using a Pavlovian fear conditioning paradigm. Mice were presented with a 5s cue followed by a brief, unavoidable footshock. Mice were trained over four fear conditioning sessions (6 trials per session) followed by four sessions of extinction where the cue, but no shock, was presented (**Fig. 3A**). Freezing was analyzed during **1)** fear conditioning session 1 (FC1) when learning of the cue-shock association was forming, **2)** in the final session [fear conditioning session 4 (FC4)], when the association was well established, and **3)** on the final session of extinction, when the cue-shock association was extinguished (EXT4). Both groups of mice (D1- and A2A-cre) increased freezing during acquisition and decreased freezing following extinction (**Fig. 3B**; see **fig. S4** for D1- and A2A-cre mice freezing separately) as expected.

As hypothesized, D1 MSNs responded to both the cue and the shock in early fear conditioning (FC1) and late fear conditioning (FC4; **Fig. 3C-E,O**; see **fig. S5** for are under the curve values). The magnitude of this response did not change over learning. Conversely, D2 MSNs exhibited a response to the cue that increased with learning (**Fig. 3F-H,P**; see **fig. S5** for are under the curve values). While we observed no significant difference in the overall peak of the shock-evoked responses in D2 MSNs (when normalized to the inter-trial period preceding the trial), there was a shift in the baseline that occurred at the onset of the cue that remained elevated

until shock presentation, suggesting a temporally defined, prediction-based signal that likely influenced the relative shock responses over learning (this is specifically tested below).

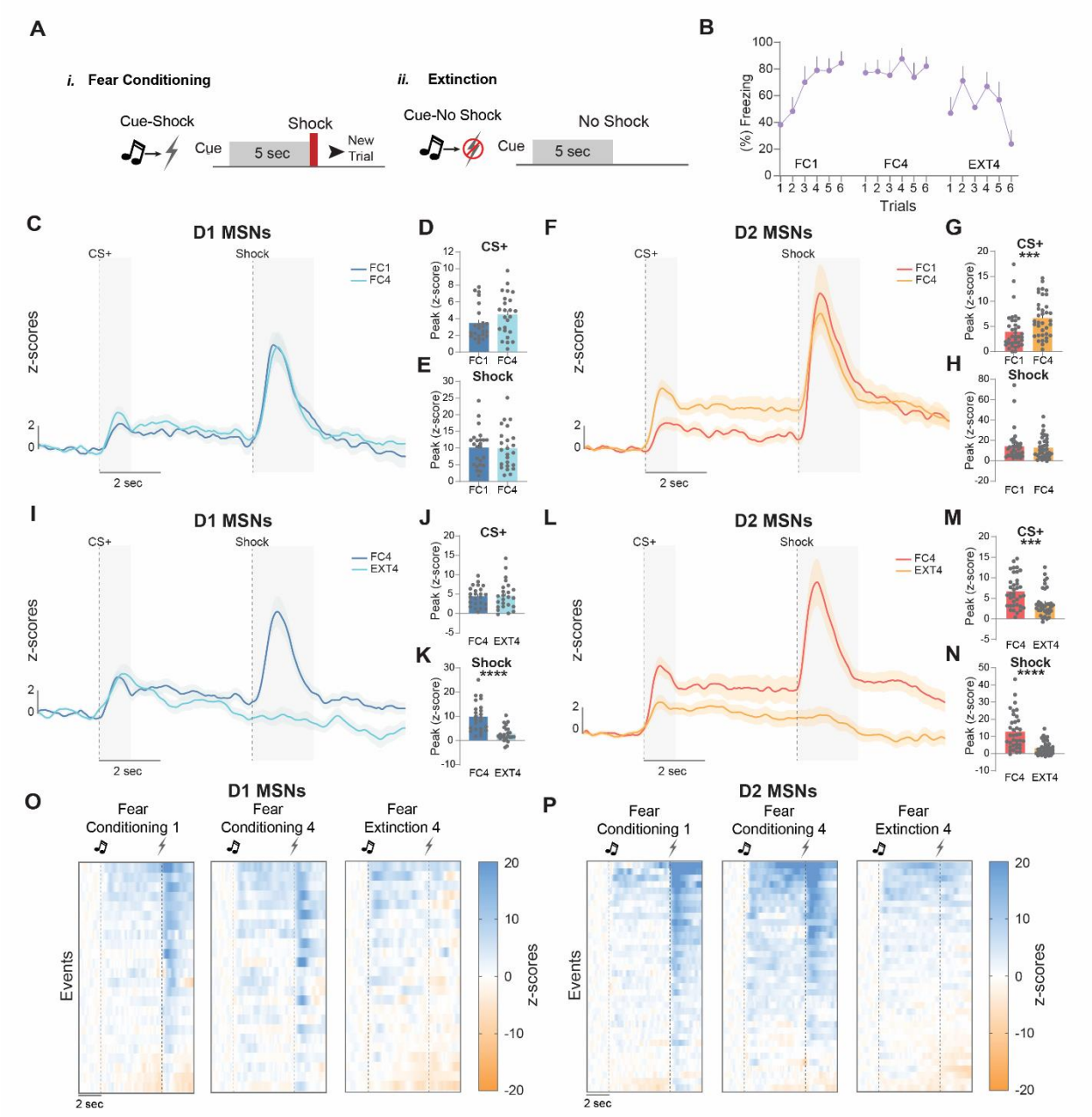

Fig. 3. D2 MSN responses to cues increase over learning, while D1 MSN responses do not change, in Pavlovian tasks.

(**A**) Experimental design. Mice received a five second cue followed by a half-second shock in a Pavlovian fear conditioning task. During extinction, the cue was presented for five seconds but the shock was not delivered. (**B**) Freezing across trials during fear conditioning session 1 (FC1), fear conditioning session 4 (FC4), and extinction session 4 (EXT4) (repeated measures ANOVA trial × group interaction $F_{(4.263,38.37)} = 3.370$, $p = 0.0168$). (**C**) D1 MSN response to the cue in FC1

and FC4. (**D**, **E**) D1 MSNs showed no difference in response to the cue (nested ANOVA $F_{(1,47)}$ = 2.85, p = 0.099, n = 4 mice) or the shock (nested ANOVA $F_{(1,47)}$ = 0.02, p = 0.8945, n = 4 mice) over sessions. (**F**) D2 MSN response to the cue in FC1 and FC4. (**G**) D2 MSNs showed an increase in response to the cue over session (nested ANOVA $F_{(1,71)}$ = 11.28, p = 0.0014, n = 6 mice). (**H**) The peak responses to the shock in D2 MSNs normalized to the pre-trial baseline (nested ANOVA $F_{(1,71)}$ = 0.42, p = 0.5218, n = 6 mice). (**I**) D1 MSN cue and shock responses in the last fear conditioning session (FC4) compared to the last session of extinction (data from FC4, replotted from panel C). (**J**) There was no change in the D1 MSN response to the cue following extinction (nested ANOVA $F_{(1,47)}$ = 0.03, p = 0.8733, n = 4 mice). (**K**) D1 MSN responses to shock period. In FC4 the shock was presented and in extinction (EXT4) it was not (nested ANOVA $F_{(1,47)}$ = 53.09, p < 0.0001, n = 4 mice). (**L**) D2 MSN response to the cue and shock in FC4 as compared to extinction (FC4 data plotted from panel F). (**M**) There was a decrease in the cue response following extinction (nested ANOVA $F_{(1,71)}$ = 16.32, p = 0.0002, n = 6 mice). (**N**) The response during the shock period was also reduced (nested ANOVA $F_{(1,71)}$ = 56.31, p < 0.0001, n = 6 mice). In FC4 the shock was presented and in extinction (EXT4) it was not. (**O, P**) Heatmap of D1 MSN (**O**) and D2 MSN (**P**) responses to task parameters during early (FC1) and late (FC4) fear conditioning and late fear extinction (EXT4). Data represented as mean ± S.E.M. * p < 0.05, ** p < 0.01, **** p < 0.0001. [fear conditioning session 1 [FC1]; fear conditioning session 4 [FC4]; extinction session 4 [EXT4]].

When mice went through extinction, freezing was reduced (**Fig. 3B**). However, even as the association between the cue and the shock was weakened during extinction, we observed no difference in the D1 MSN response to the cue (**Fig. 3I-J,O**; see **fig. S5** for are under the curve values). At the time in the task when the shock was presented previously (but the shock was absent in extinction), the D1 MSN response also disappeared (**Fig. 3I,K**). D2 MSNs responses that were evoked by the cue were reduced with extinction (**Fig. 3L,M,P**; see **fig. S5** for are under the curve values). Further, there was no response at the time of the omitted shock during the final extinction session when shock was not expected (**Fig. 3L,N**).

Combined, these data suggest that D2 MSN responses track the strength of the association between cues and outcomes. This occurs regardless of whether the outcome is positive or negative (**Fig 2, 3**) and does not depend on the action required, as this same pattern was present for both reinforcement (motivated action, **Fig 2**) and fear conditioning (freezing, **Fig 3**). Thus, D2 MSNs appear to track predictions in an environment, while D1 MSNs respond to stimuli and are not changed with learning.

D2 MSNs track predictions error

Based on the data above, we hypothesized that D2 MSN responses tracked a valueless prediction error, where, as cues acquire predictive value responses are increased (regardless of valence) and decreased as associations are weakened. One key feature of prediction-error signals is that in addition to cue responses changing over learning, relative stimulus responses also change based on prior predictions. That is, as the cue becomes more predictive, the stimulus response is less unexpected and represents a smaller error signal (R. Rescorla & Wagner, 1972). In this framework, the cue response increases as associations become stronger and the stimulus response (to the sucrose or shock) becomes smaller.

Indeed, while we observed in **Fig 1C** that D2 MSN responses were reduced at the time of sucrose collection in well-trained animals, when unexpected sucrose was given to untrained animals D2 MSN responses to sucrose were increased (**Fig. 4A**). Thus, the D2 MSN response to an unexpected reward was positive (**Fig. 4B**; see **fig. S6A** for area under the curve values) and this response to the same rewarding stimulus is reduced as the reward delivery becomes more predicted (**Fig. 1C**). We observed similar D2 MSN responses that occurred in response to unpredicted vs predicted aversive stimuli. D1 and D2 MSN responses to the shock were compared during the first 2 trials of fear conditioning (FC1, when animals did not expect to receive the footshock) and during the first 2 trials of the final fear conditioning session (FC4, when the footshock was anticipated). We analyzed around the onset of the shock (instead of the cue as in **Fig. 3H**) to probe the shock response relative to baseline. D1 MSN response to the shock did not change with expectation (**Fig. 4C**; see **fig. S6** for are under the curve values). However, the D2 MSN response to the shock was smaller when it was expected (**Fig. 4D**; see **fig. S6** for are under the curve values). Thus, D2 MSN, but not D1 MSN, responses to outcomes are reduced as they become more predicted, regardless of whether the outcome is appetitive or aversive.

Finally, we directly manipulated the probability of shock presentation in two separate sessions: one where the shock was predicted by the cue 10% of the time, and another where the

shock was predicted by the cue 75% of the time (**Fig. 4E**). Accordingly, the size of the cue response in the D2 MSNs reflected how well the shock was predicted, where the higher probability cue resulted in a stronger D2 MSN cue response (**Fig. 4F**) and a reduced response to the shock. Thus, as the cue becomes more predictive the cue response is largest and the outcome response is smallest, similar to what has been observed by other prediction-based signals (Fiorillo et al., 2003, 2003; Tobler et al., 2005). These results give further support to the hypothesis that D2 MSNs, but D1 MSNs, are sensitive to predictions/prediction errors.

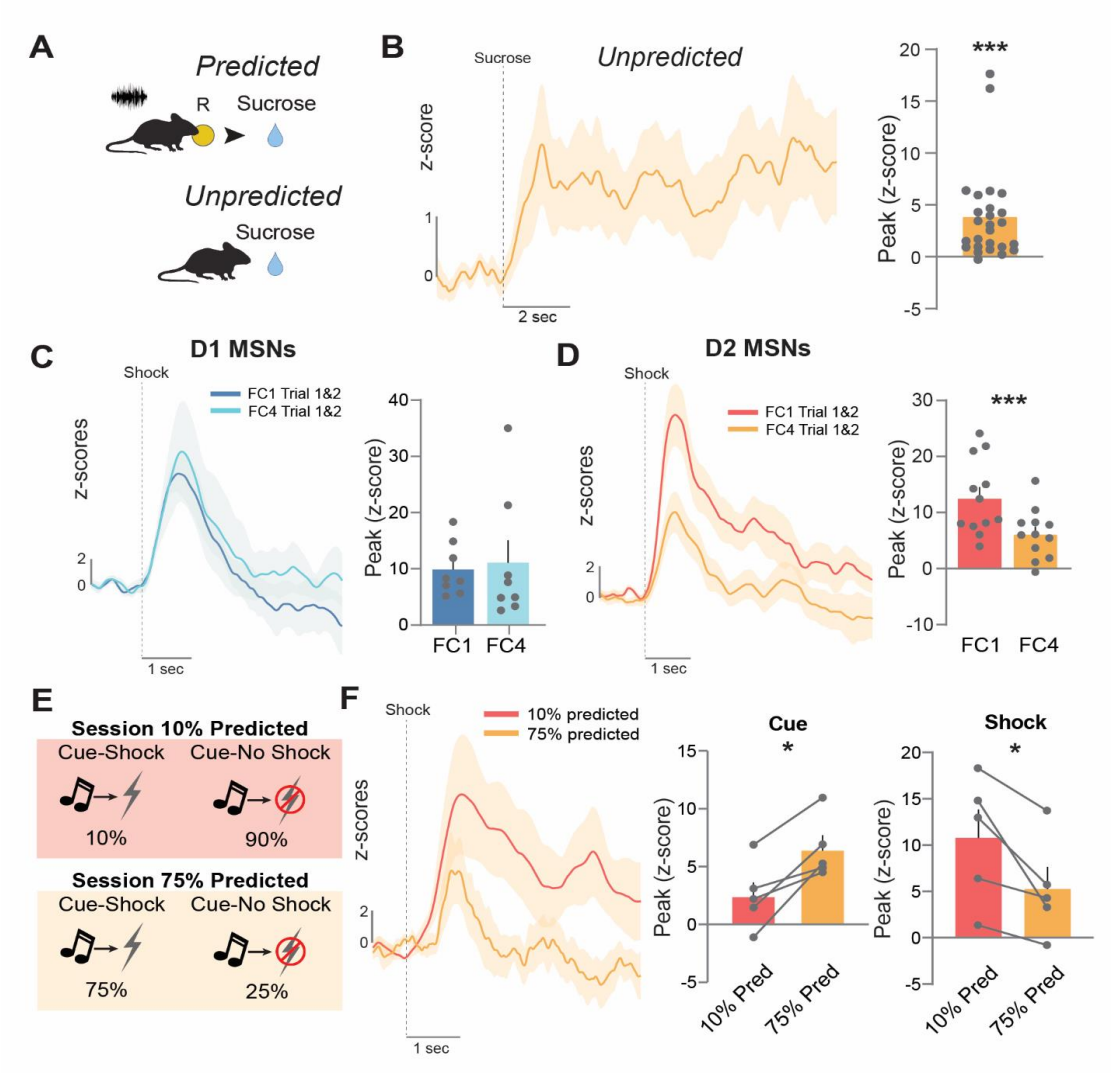

Fig. 4. D2 MSN responses to stimuli are modulated by prior predictions.

(**A**) To test whether the directionality of this D2 MSN responses to sucrose changed based on prior predictions we designed an experiment where mice received unsignaled sucrose. In the

unpredicted condition, mice were given ad libitum access to sucrose in the delivery port and signals were analyzed around the first lick in a lick bout. (**B**) When sucrose was not predicted, D2 MSN response were increased (independent sample t-test, t_{25} = 4.408, p = 0.0002, n = 4 mice), rather than decreased (**Fig 1C**) . We then wanted to determine if D1 or D2 responses to footshocks were changed based on prediction (**C**) Signals were z-scored around the baseline preceding the onset of the shock in the first two trials of fear conditioning session 1 (FC1), when the mouse experiences the cue and the shock for the first and second time) and fear conditioning session 4 (FC4) when the mouse has extensive experience with the cue-shock association. There was no effect on D1 MSNs responses to the shock under these conditions (nested ANOVA $F_{(1,15)}$ = 0.4, p = 0.5456, n = 4 mice). (**D**) However, D2 MSN responses to the footshock became smaller as the prediction between cue and shock became stronger (nested ANOVA $F_{(1,23)}$ = 18.22, p < 0.0011, n = 6 mice). (**E**) Experimental design for a shock probability experiment in which the likelihood of a cue-shock pairing was manipulated (shock occurs 10% of the trials in the session or 75% of the trials in the session). (**F**) D2 MSNs showed a marked increase in the cue response (paired t-test, t_4 = 4.540, p = 0.0105, n = 5 mice) and decrease in the magnitude of the response to the shock (paired t-test, t_4 = 3.117, p = 0.0356, n = 5 mice) with greater predictability of the shock outcome . Data represented as mean ± S.E.M. * p < 0.05, *** p < 0.001. [fear conditioning session 1 [FC1]; fear conditioning session 4 [FC4].

Learning increases D2 MSN recruitment

To further understand how these signals developed over learning and how these population-level signals related to individual neuronal responses, we employed microendoscopic cellular resolution calcium imaging. GCaMP6m was expressed in either D1 or D2 MSNs as described above, and using a GRIN lens for optical access, we recorded single cell calcium transients in these identified populations during fear conditioning (**Fig. 5A-C**; see **fig. S7** for freezing response by genotype; **fig. S8** for whole field calcium traces).

Replicating our results, we found that D1 MSNs - when represented as a whole field trace or the average of all identified single cell responses - responded to both the cue and the footshock during fear conditioning session 1 (FC1; **Fig. 5D-F; fig. S8**, for whole field calcium traces; **fig. S9** for area under the curve). While there was heterogeneity in the responses (**Fig. 5G, left**), a large majority of cells responded positively (55.41%, of all identified cells recorded during the session) to the shock (**Fig. 5G, right**). Following extended training, when behavioral responses were asymptotic (fear conditioning session 4, FC4), the proportionality of D1 MSN responses did not change (**Fig. 5H**). Consistent with the idea that D1 MSNs respond to the presence of stimuli, the

number D1 MSNs that were active at the time of the previous footshock were reduced when the shock was not present in extinction (to 23.81%; **fig. S10A-E**). These data both support the assertion that D1 MSNs track stimulus presence and show that the population-level responses in the NAc core are representative of a majority of the population.

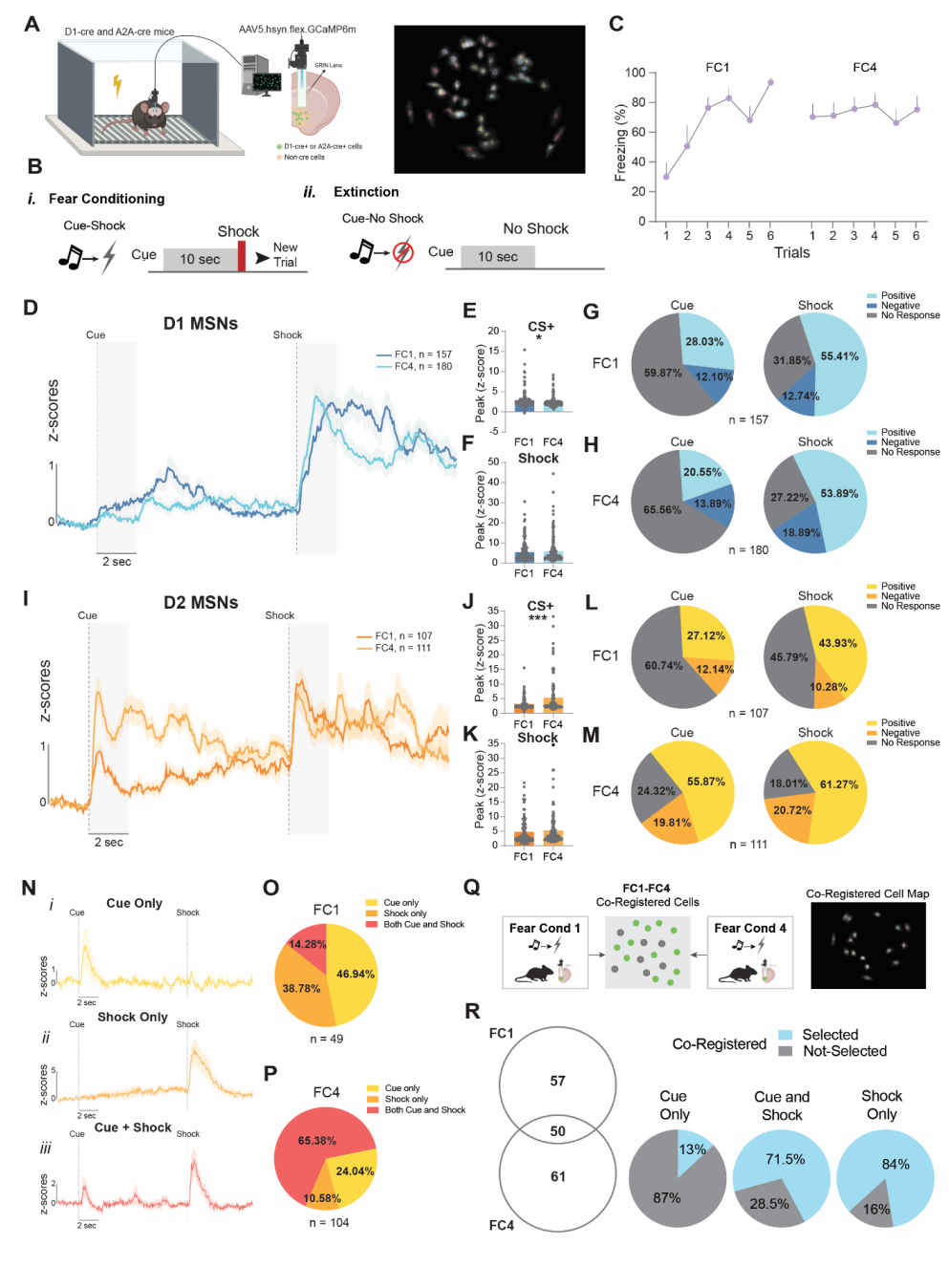

Fig. 5. D2 MSNs recruitment is increased over learning and individual D2 MSNs respond to both the cue and the shock.

(A) D1 and D2 MSN responses were recorded via cell-type specific expression of GCaMP6m as described. A GRIN lens was implanted above the NAc core for optical access. (**B**) Fear conditioning. Mice received a ten second cue followed by a 0.5s shock. (**C**) Freezing responses increased over training (repeated measures ANOVA trial × group interaction $F_{(3.990,35.91)} = 4.212$, $p = 0.0068$). Session 1 (FC1), Session 4 (FC4). (**D**) D1 MSNs responses across detected cells (157 cells in FC1, 180 cells in FC4). (**E**) There was a moderate decrease in the peak response to the cue in the last session as compared to the first (independent sample t-test, $t_{335} = 2.505$, $p = 0.0127$, n = 5 mice). (**F**) The shock response did not change (independent sample t-test, $t_{335} = 0.6697$, $p = 0.5035$, n = 5 mice). (**G, H**) Percentage of the total D1 MSNs detected in each session that increased (positive), decreased (negative), or showed no response (no response) to the cue or shock in first (**G,** FC1) **or** last session (**H,** FC4). D1 MSN responses did not change over learning. (**I**) D2 MSNs responses (107 cells in FC1, 111 cells in FC4). (**J**) D2 MSNs responses to the cue were increased over sessions (independent sample t-test, $t_{216} = 3.435$, $p = 0.0007$, n = 5 mice). (**K**) No difference in the shock response (independent sample t-test, $t_{216} = 0.7109$, $p = 0.4779$, n = 5 mice). (**L, M**) Percentage of D2 MSNs that increased their response (positive), decreased their response (negative), or did not respond (no response) to the cue and the shock in the first session (**L,** FC1) and the last (**M**, FC4) showing that the number of D2 MSNs that responded to the cue changed over learning. (**N**) D2 MSN were categorized based on observed activity patterns in the NAc during the initial fear conditioning session (FC1) in the following categories: (*i*) response only to the cue; (*ii*) response only to the shock (*iii*) response both to the cue and shock. (**O**) Initially, in the first session, only a small percentage (14.28%) responded to both the cue and shock. (**P**) In fear conditioning session 4 (FC4), a majority of cells responded to both the cue and shock (65.38%). (**Q**) D2 MSNs were recorded on the first session (FC1) and cells detected during this session were longitudinally co-registered with cells in the last session (FC4) based on detected activity during each session. (**R**) A majority of the cells that only responded to the cue in FC1 were not detected as active during the final fear conditioning session (FC4, only 13% co-registered). The majority of D2 MSNs that responded to the shock (either shock alone, or both cue and shock) were re-recruited in FC4. Data represented as mean ± S.E.M. * $p < 0.05$, *** $p < 0.001$. [fear conditioning session 1 [FC1]; fear conditioning session 4 [FC4].

Analysis with cellular-resolution also gave further support to the hypothesis that D2 MSNs serve as a prediction-based signal. D2 MSNs showed the same population response patterns observed above – both when represented as field of view and when presented as the average of all identified single cell responses (**Fig. 5I-K; fig. S8**, for whole field calcium traces; **fig. S9** for area under the curve analysis). The increase in population-level response to the cue over training was explained by an increase in the size of the D2 MSN ensemble that was evoked by the presentation of the predictive cue, which was largest on the final fear condition session (FC4) compared to the initial fear conditioning session (FC1). Indeed, the percentage of cue responsive

D2 MSNs increased from 39.26% to 75.68% of identified cells between FC1 and FC4 (**Fig. 5L,M, left**). Shock responsive D2 MSNs were also increased, and went from 54.21% to 81.99% of the population (**Fig. 5L,M, right**). Critically, the opposite was observed when animals moved from fear conditioning to fear extinction where the percentages of responsive cells were reduced to 42.86% for the cue and to 34.92% for the footshock (**fig. S10F-J**). These results support the hypothesis that D2 MSNs signal predictions as this signal progressively develops with learning (over fear conditioning) and is updated when new information is encountered (during extinction). More specifically, the D2 MSN response to the cue becomes stronger as the animals learn the association between the cue and the footshock and weaken as this association is extinguished (**Fig. 5I-K; fig. S10F-J**).

D2 MSNs bridge cues and outcomes

For D2 MSNs to function as a prediction error signal, both the cue and the outcome response would need to be represented in the same cells, rather than two separate populations. Analysis of the single cell responses during the first fear conditioning session (FC1) revealed 3 groups of D2 MSNs: **1)** D2 MSNs that showed a response only to the cue (Cue only); **2)** D2 MSNs that showed a response only to the shock (Shock only) and **3)** D2 MSNs that showed a response to both the cue and shock (Both Cue and Shock; **Fig. 5N**). Initially only a small percentage of the D2 MSNs respond to both the cue and shock (14.28%) while the rest of the cells respond only to the cue (46.94%) or only to the shock (38.78%; **Fig. 5O**). However, when animals learned the association between the cue and shock - in the fourth fear conditioning session (FC4) - the majority of the D2 MSNs responded to both the cue and the shock (65.38%; **Fig. 5P**).

Next, we used activity-based co-registration to determine if cells showing one of the three activity signatures were more or less likely to be active in subsequent sessions. In this approach, co-registered cells were identified based on activity during the first fear conditioning session (FC1) and compared against activity signatures in the last fear conditioning session (FC4). Thus, only

cells that were active in both FC1 and FC4 were considered co-registered (**Fig. 5Q**; see **fig. S11-12** for co-registration approach). Critically, cells that responded to the cue only were not likely to be identified in FC4 (13% co-registered), while a majority of the D2 MSNs that responded only to the shock (84% co-registered) or both to the cue and shock (71.5% co-registered) were re-recruited and active in FC4 (**Fig. 5R**). Overall, these results suggest that the shock response within the NAc D2 MSN population may drive associative learning via the same cells developing a response to the predictive cue and potentially bridging the predictive cue and the outcome in an associative manner.

D2 MSN response to the predictive cue is necessary for associative learning

Finally, if the recruitment of D2 MSN responses to a predictive cue that occurs over time is a prediction error signal, then D2 MSN activity at the time of a cue should be necessary for associative learning. To test this hypothesis, we expressed halorhodopsin, an inhibitory opsin, selectively in D1 or D2 MSNs, using D1- and A2A-cre mice, respectively (**Fig. 6A**). Mice underwent fear conditioning, as described above, where a 5s cue was paired with a brief shock. On each trial, D1 and D2 MSNs were inhibited for the duration of the cue presentation (590nm, 8mW, constant; **Fig. 6B**). Inhibiting D2 MSNs during the cue presentation resulted in a deficit in learning of the cue-outcome association as compared to the D1-cre and eYFP controls (**Fig. 6C**). D1 MSN inhibition at the time of the predictive cue did not have any effect on the trajectory of associative learning (**Fig. 6C**). In the larger context of our study, these results strongly suggest that the D2 MSN signal at the time of the predictive cue in the NAc causally mediates cue-outcome associations.

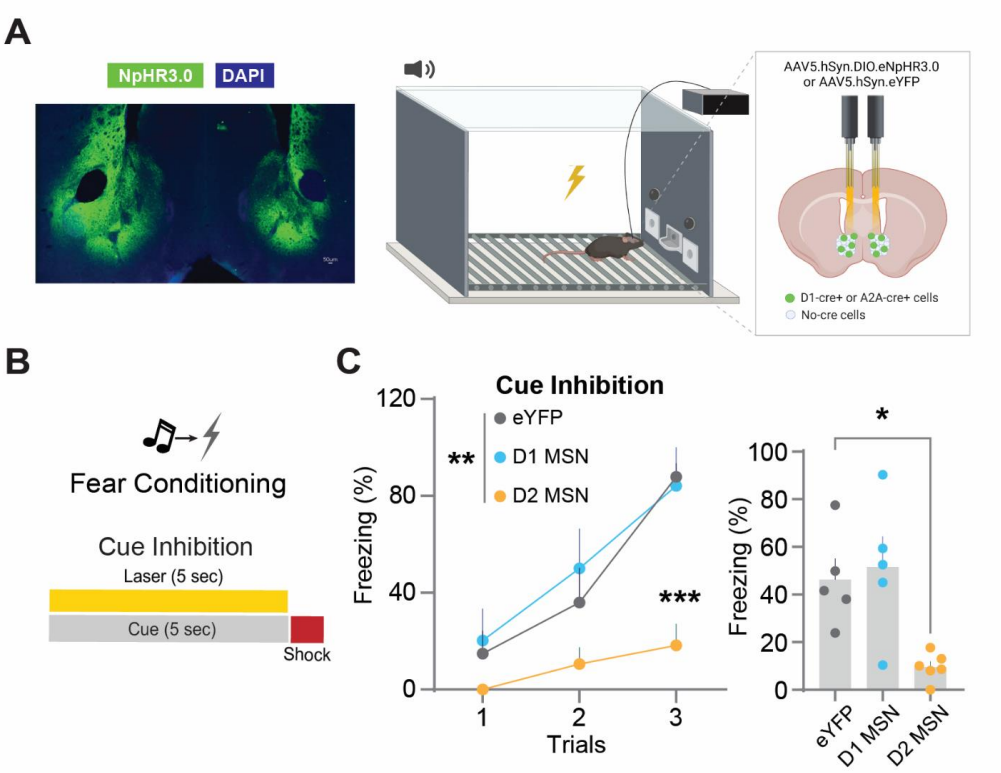

Fig. 6. Optogenetic inhibition of D2 MSN responses during the cue slows associative learning.

(**A**) The inhibitory opsin halorhodopsin (AAV5.hSyn.DIO.eNpHR3.0) was selectively expressed in D1 or D2 MSNs. Representative histology. (**B**) A laser (constant, 5s, 8mW, 590nM) was illuminated at the time of cue onset for five seconds. **(C)** When D2, but not D1 MSNs, were inhibited mice developed a freezing response at a slower rate (RM ANOVA trial × group interaction $F_{(2,18)}$ = 8.174, p = 0.0030; multiple comparison D2 MSN versus eYFP session 3, p = 0.0005). **(D)** D2 MSN inhibition reduced averaged freezing during the 3 training trials as compared to eYFP animals (one-way ANOVA $F_{(2,13)}$ = 7.524, p = 0.0067; Bonferroni's post-hoc eYFP versus D1, p > 0.9999; eYFP verses A2A, p = 0.0180)**.** Data represented as mean ± S.E.M. * p < 0.05, ** p < 0.01.

2.5 Discussion

Here, using a wide range of paradigms that result in behavioral responses to stimuli of both positive and negative valence, we characterized the precise roles that NAc core D1 and D2 MSNs play in behavioral control within the brain. At both the population and single cell level, we showed that D1 MSNs in the NAc core responded to the presence of unconditioned stimuli – regardless of valence. Conversely, D2 MSNs MSNs in the NAc core responded in a prediction-based fashion, increasing with learning, scaling with prediction, and causally controlling the trajectory of associative learning. Overall, these data show that D1 and D2 MSNs in the NAc do not have opposing roles in valence coding or behavioral control. Rather, these results suggest that D1 and D2 MSNs work in tandem to provide information regarding specific valence-independent aspects of associative learning.

While the NAc has been referred to as a reward-associated brain region (Day & Carelli, 2007; Nicola, 2007; Russo & Nestler, 2013; Willmore et al., 2022), these studies are not the first to show that signaling the NAc is causally related to both aversive and appetitive stimulus processing (Bin Saifullah et al., 2018; Day & Carelli, 2007; de Jong et al., 2019; Iordanova et al., 2021; Kutlu et al., 2020, 2021; Li & McNally, 2015; Ray et al., 2020, 2020; Roitman et al., 2005; Sugam et al., 2014; Wenzel et al., 2018). While some of these studies have still explained these results based on the idea of bidirectional valence coding, these data suggest that signaling in the NAc - especially the core region (Ambroggi et al., 2011; Corbit et al., 2001; Floresco et al., 2008; Li & McNally, 2015; West & Carelli, 2016) - may best be explained by valence-independent factors that allow for adaptive behavioral control across contexts and conditions. This would also be consistent with data across a range of experiments and fields showing that stimulation of inputs from the PFC, vHip, or BLA inputs are reinforcing (Britt et al., 2012; Otis et al., 2017; Stuber et al., 2011), even though all of these populations have been shown to synapse on both D1 and D2 MSN populations to a similar extent and show overlapping inputs of sensory and cortical systems in both populations of neurons (Guo et al., 2015). Indeed, we and others show that the stimulation

of both D1 and D2 MSNs supports reinforcement (Cole et al., 2018; Soares-Cunha et al., 2016, 2018, 2020), indicating that these populations do not track valence or opposing motivational drive.

The framework presented within this manuscript is consistent with a large amount of emerging data on the role of these populations in behavioral control. Recent work has shown that manipulating D2 MSNs alters behavior, especially under unpredictable conditions (risky choice) and when behavioral updating is necessary. Zalocusky et al. (2016) showed that in a model of risk preference in rats (which identifies both risk-seeking and risk-averse rats), stimulation of D2 MSNs during a decision period that initiated trial onset resulted in fewer risky choices only in the risk-seeking group (Zalocusky et al., 2016). Here we show that D2 MSNs track the predictability of cues and are engaged during similar types of operant task in a fashion that tracks performance. Nishioka et al. showed that optogenetic inhibition of D2 MSNs in the NAc during trials where there were errors in outcomes negatively impacted performance on the subsequent trial. Here we show that D2 MSNs are modulated by predictions and signals when outcomes are unexpected, something that would be necessary for error-based updating. Data in the current manuscript is also consistent with previous work using optogenetics, where activation of D2 MSNs during a reward-predictive cue enhances motivation, but triggering activity during the food pellet delivery reduces motivation (Soares-Cunha et al., 2016, 2018). This would be predicted based on the idea that D2 MSNs are transmitting a prediction/prediction error signal. This is also consistent with other recent work showing that both D1 and D2 MSN activity is evoked in response to food rewards (Baldo & Kelley, 2007; Carlezon & Thomas, 2009; Natsubori et al., 2017), where we show here that unexpected sucrose delivery results in increased D1 responses (signaling stimulus presence) and D2 MSN responses (signaling an unexpected outcome). Together, these data and data from others converge to show that D2 MSN activity is critical in situations where updating is necessary. Overall, the ability of D1 and D2 MSNs to modulate precise types of information along with the fact that there is evidence of collateral transmission between MSNs thus suggests an

ongoing relationship between D1 and D2 MSNs (Burke et al., 2017; Dobbs et al., 2016), which allows activation in one pathway to regulate the output of the other, further refining signaling.

Finally, these data are critical for conceptualizing how experience-dependent changes within these two cellular populations gives rise to behavioral maladaptation associated with disease states. There has been extensive characterization of the effects of drugs of abuse on D1 and D2 MSNs at the molecular and cellular level. Acute drug exposure has been shown to enhance D1, while suppressing D2 activity - likely through activation of dopamine receptors on these populations (Calipari et al., 2016; Luo et al., 2011). Additionally, repeated drug exposure has been shown to result in long-lasting enhancement of synaptic activity in D1 MSNs relative to D2 MSNs (Calipari et al., 2016; Creed et al., 2016; MacAskill et al., 2014; Pascoli et al., 2014). D2 MSNs have also been linked to drug-associated plasticity and seeking, especially as it relates to cue-driven behavior like drug seeking, or drug-taking under more variable reinforcement schedules (Bock et al., 2013a; Heinsbroek et al., 2017), a result that would be predicted based on the data presented in the current manuscript. Given the critical role we have identified here for D1 and D2 MSNs in associative learning, it is plausible that drugs of abuse may disrupt the "natural balance" critical for forming associations between predictive cues and outcomes in the environment (Bateup et al., 2010; Bock et al., 2013a; Durieux et al., 2009; Kravitz et al., 2010, 2012; Lobo et al., 2010a). There is a growing appreciation for the greater complexity of the relationship between striatal D1 and D2 MSNs and how it is not necessarily a push-and-pull relationship but a coordination of activity that is disrupted in diseased states.

By experimentation that integrates learning across contexts, behavioral action, and valence, we provide a framework for accumbal MSNs in valence-independent behavioral control. We conclude that NAc core MSNs help coordinate learning of associations to drive adaptive behavior in all cases. The roles that D1 and D2 MSNs play in adaptive behavior thus provide new insights into many of the psychopathologies associated with disruptions in this brain region – including substance use disorder (SUD), gambling, and depression among others (Avanzi et al.,

2004; C.-J. Chang et al., 2021; Dodd et al., 2005; Redish, 2004; Schultz, 2011; Xu et al., 2020). In effect, we can frame many of these disorders as a dysregulation in adaptive associative learning processes, a perspective that can ultimately reshape how we treat them.

Limitations and future directions

In figure 1, we saw an increase in both the D1 and D2 MSNs to the greater magnitude shock, a salient, negatively-valenced stimuli. We did not assess how D1 and D2 MSNs would respond to a positively-valenced stimuli of various intensities. To address this, we could offer the same stimuli in different quantities or various levels of sweetness to tease apart whether D1 and D2 MSNs show this intensity-dependent response across positive and negative stimuli. I hypothesize that D2 MSNs would not necessarily show an intensity-dependent response to a positively-valenced stimuli. They may instead be responding to the unexpectedness of a higher intensity shock rather than the intensity.

Another consideration is that positive and negative reinforcement were reinforced by stimuli in different modalities (positive reinforcement – sucrose; negative reinforcement – shock). This was a confound of our study design. In negative reinforcement, mice cannot avoid the shock unless they make a nosepoke response. With a bitter tastant like quinine (same modality as sucrose) presented in a port, mice can choose never to interact with the stimuli (or they can taste it and not approach again). Negative reinforcement relies on a predictable, unavoidable outcome if the mouse does not make an action to remove it. They thus have no pressure to learn an association to avoid this outcome. To resolve this, we would have to set up an oral gavage that would deliver quinine directly into the subject's mouth. Mice could then learn to nosepoke to avoid delivery of the quinine. I anticipate the same results we see with negative reinforcement reinforced by a shock where the D2 MSN response to the cue increases with learning of the association between the cue and the quinine.

The final experiment I would propose is to show that inhibition of D2 MSNs in a reward learning task would slow learning of the contingency, similar to the reduced freezing following inhibition to the cue in the fear conditioning task (**Fig. 6**). This would provide substantial evidence for D1 and D2 MSN roles in valence-independent associative learning.

2.6 Supplementary figures

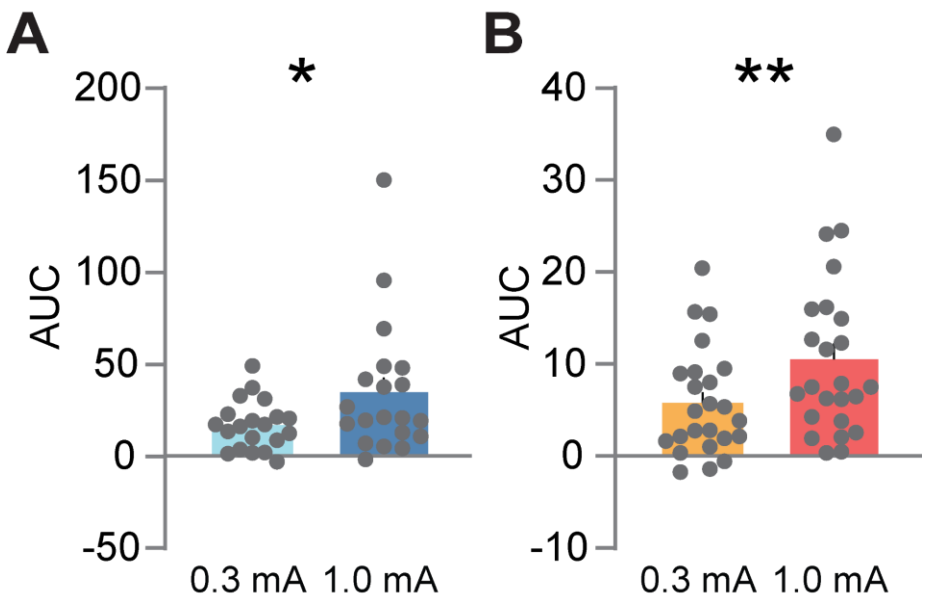

Fig. S1. D1 and D2 MSNs respond to shock intensity.

Fig. S1. D1 and D2 MSNs respond to shock intensity.

(**A**) Mice were presented with shocks of varying intensity randomly presented within the same session. D1 MSNs showed an intensity-dependent response to the shock (nested ANOVA $F(1,39) = 6.13$, $p = 0.0191$). (**B**) D2 MSNs also responded to shock and exhibited an intensity-dependent response (nested ANOVA $F(1,47) = 8.24$, $p = 0.0068$). Data represented as mean ± S.E.M. * $p < 0.05$, ** $p < 0.01$.

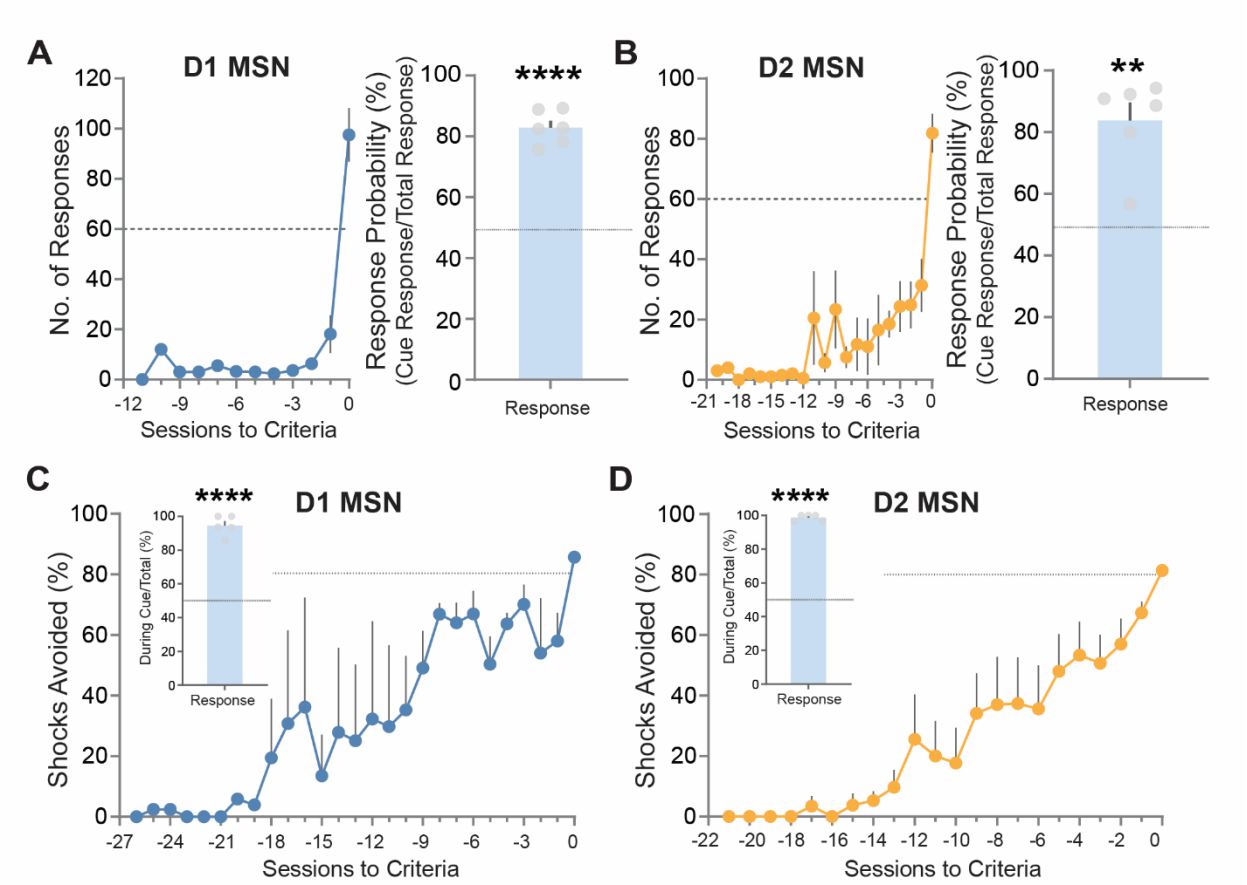

Fig. S2. D1 and A2A-cre mice acquired positive and negative reinforcement.

(**A** and **B**) D1- and A2A cre mice met performance criteria of greater than 60 responses on the FR1 schedule for sucrose. They made more responses during the cue (correct) than during the inter-trial interval (ITI) (D1-cre; (independent sample t-test, $t_5 = 14.69$, $p < 0.0001$, n = 6 mice; critical value = 50%); A2A-cre; independent sample t-test, $t_5 = 5.823$, $p = 0.0021$, n = 6 mice). (**C** and **D**) D1-cre and A2A-cre mice met learning criterion for avoiding greater than 80% of total possible shocks in negative reinforcement task. They made more responses during the cue (correct) than during the inter-trial interval (ITI) (D1-cre; (independent sample t-test, $t_4 = 16.91$, $p < 0.0001$, n = 5 mice; critical value = 50%); A2A-cre; independent sample t-test, $t_4 = 59.60$, $p < 0.0001$, n = 5 mice). Data represented as mean ± S.E.M. **** $p < 0.0001$.

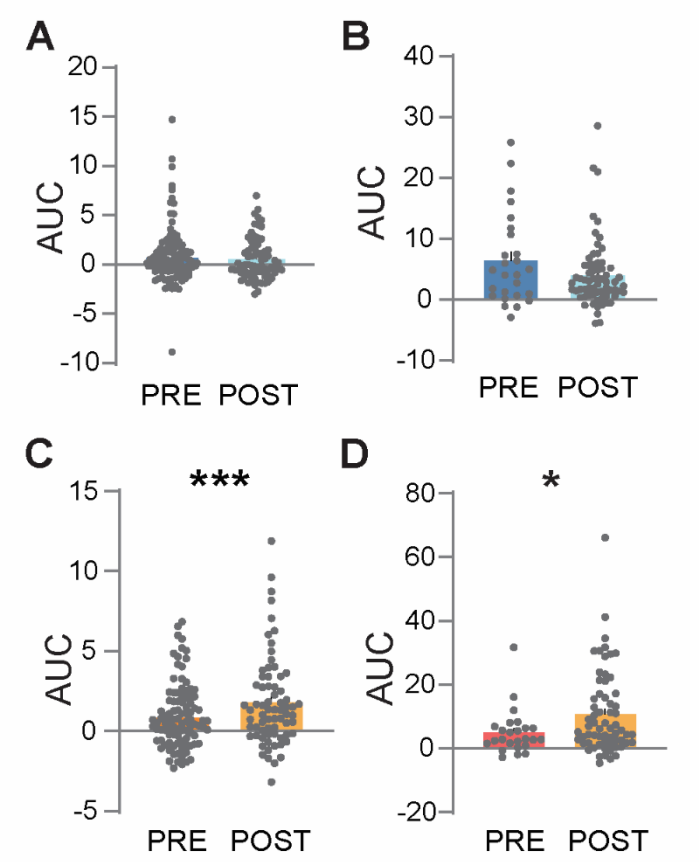

Fig. S3. D2 MSNs, and not D1 MSNs, respond to the cue for positive and negative reinforcement.

There was no difference in D1 MSN response to the cue for (**A**) positive reinforcement (nested ANOVA $F_{(1,176)} = 0.66$, $p = 0.4193$) or (**B**) negative reinforcement (nested ANOVA $F_{(1,91)} = 2.56$, $p = 0.1135$). D2 MSN response to cue for (**C**) positive reinforcement (nested ANOVA $F_{(1,166)} = 12.16$, $p = 0.0006$) and (**D**) negative reinforcement (nested ANOVA $F_{(1,88)} = 6.26$, $p = 0.0144$) was significantly increased following learning of the cue-outcome association. Data represented as mean ± S.E.M. *** $p < 0.001$.

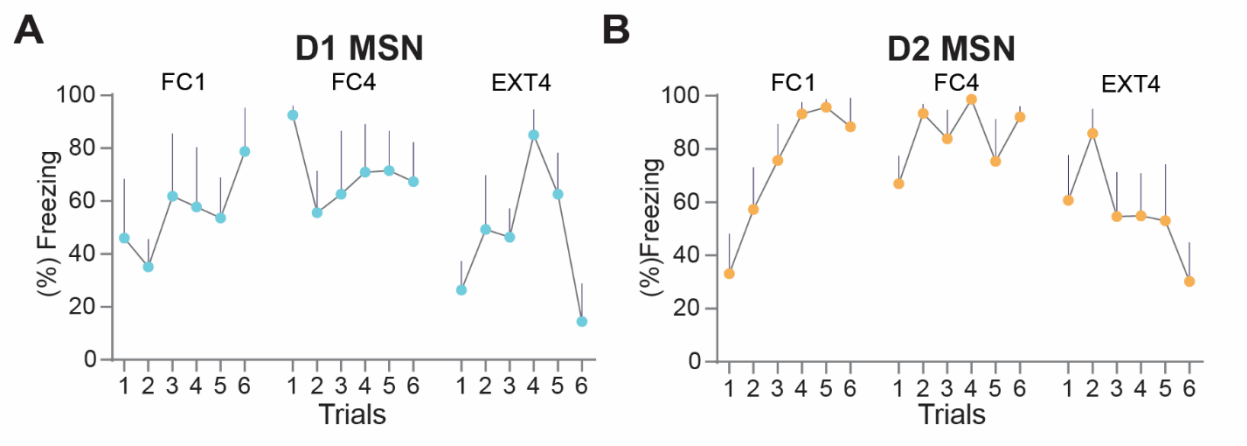

Fig. S4. Freezing data by genotype.

(**A)** D1- and (**B**) A2A-cre freezing response during fear conditioning session 1 (FC1), fear conditioning session 4 (FC4), and extinction session 4 (EXT4).

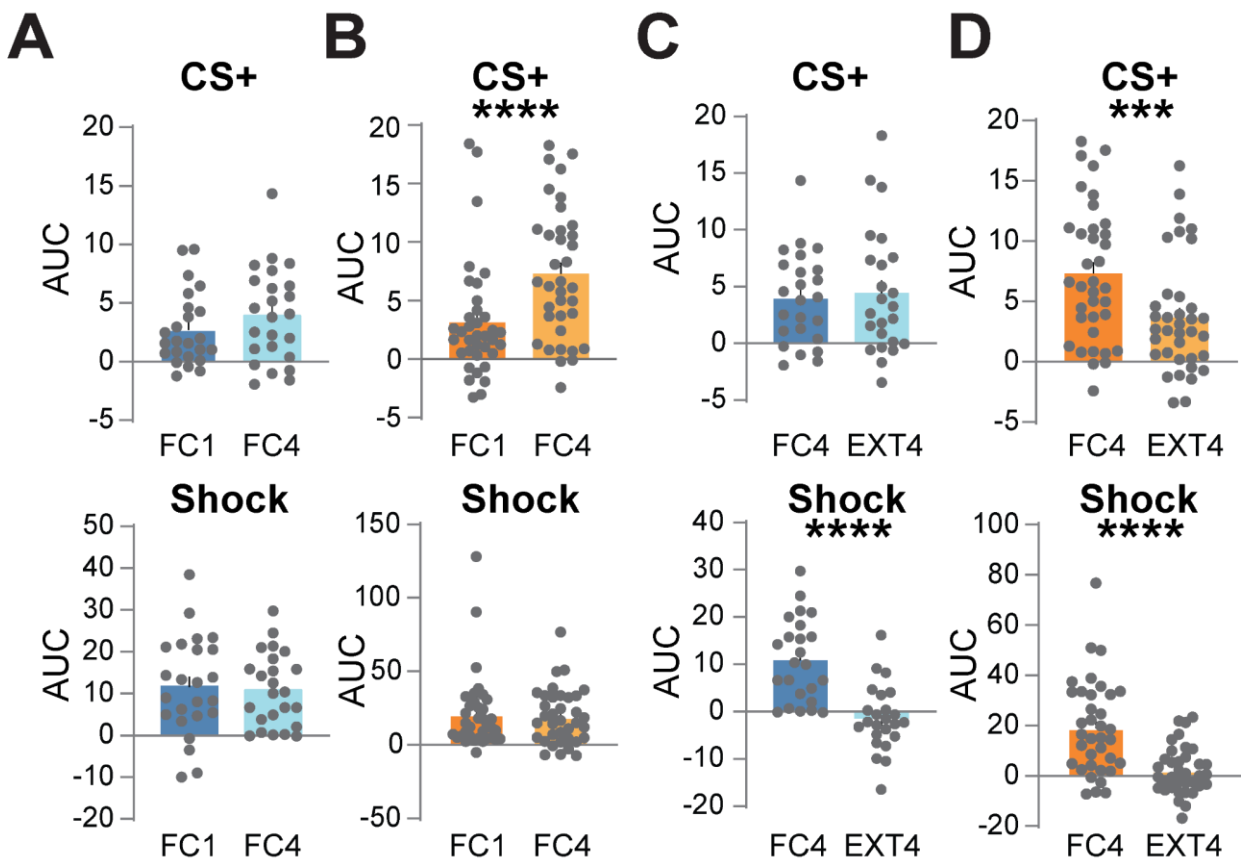

Fig. S5. D2 MSNs, and not D1 MSNs, show a learning-dependent response to a cue that predicts a shock.

(**A**) D1 MSNs show no difference in response to the CS+ (nested ANOVA $F_{(1,47)} = 1.94$, $p = 0.1713$, n = 4 mice) or the shock (nested ANOVA $F_{(1,47)} = 0.11$, $p = 0.7393$, n = 4 mice) following repeated fear conditioning sessions. (**B**) D2 MSNs show an increase in response to the CS+ following repeated fear conditioning sessions (nested ANOVA $F_{(1,71)} = 18.49$, $p < 0.0001$, n = 6 mice). D2 MSNs show no significant difference to the shock (nested ANOVA $F_{(1,71)} = 0.23$, $P = 0.6363$, n = 6 mice). (**C**) There was no significant change in the D1 MSN response to the CS+ following extinction (nested ANOVA $F_{(1,47)} = 0.13$, $p = 0.7197$, n = 4 mice). There was a significant difference in shock response between FC4 (shock presented) and EXT4 (no shock presented) (nested ANOVA $F_{(1,47)} = 44.81$, $p < 0.0001$, n = 4 mice). (**D**) There was a significant change in response to the CS+ following extinction (nested ANOVA $F_{(1,71)} = 15.09$, $P = 0.0003$, n = 6 mice). There was a significant difference in shock response between FC4 (shock presented) and EXT4 (no shock presented) (nested ANOVA $F_{(1,71)} = 44.43$, $p < 0.0001$, n = 6 mice). Data represented as mean ± S.E.M. *** $p < 0.001$, **** $p < 0.0001$. [fear conditioning session 1 [FC1]; fear conditioning session 4 [FC4]; extinction session 4 [EXT4]].

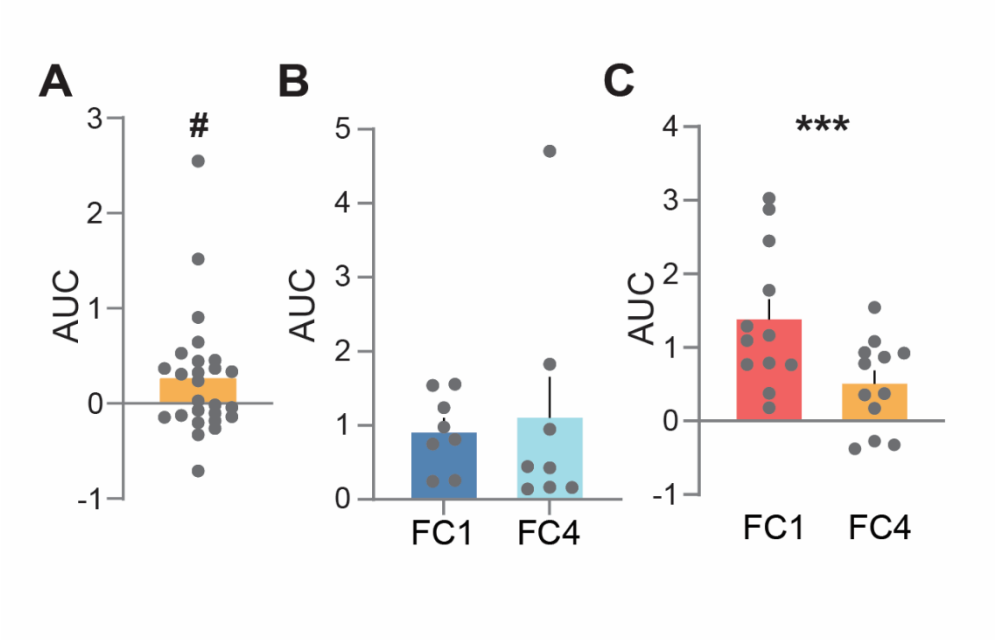

Fig. S6. D2 MSNs track the predictability of an outcome.

(**A**) The D2 MSN response goes up to the sucrose when the sucrose is not predicted (independent sample t-test, $t_{25} = 2.031$, $p = 0.0530$, n = 4 mice). (**B**) We did not see an effect on shock magnitude in D1 MSNs following repeated exposure to the cue-shock (nested ANOVA $F_{(1,15)} = 0.24$, $p = 0.6407$, n = 4 mice). (**C**) Repeated exposure to the cue-shock results in a smaller magnitude response to the shock in D2 MSNs (nested ANOVA $F_{(1,23)} = 14.31$, $p < 0.0026$, n = 6 mice). Data represented as mean ± S.E.M. *** $p < 0.001$. [fear conditioning session 1 [FC1]; fear conditioning session 4 [FC4].

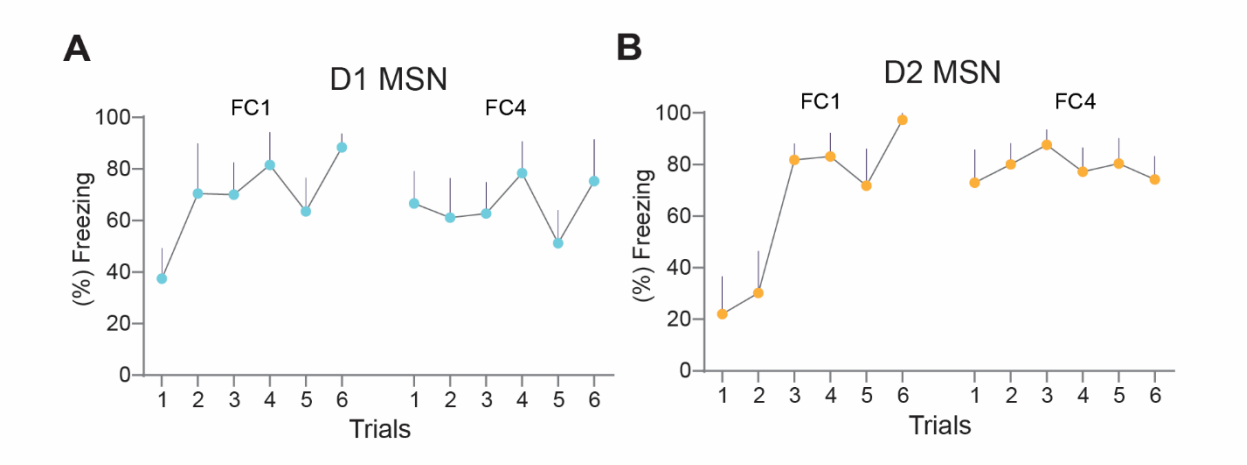

Fig. S7. Freezing data by genotype.

(A) Representative histology of calcium indicator (AAV5.CAG.FLEX.GCaMP6m.WPRE.SV40) expressed in the NAc core and a GRIN lens was implanted above the viral injection site to visualize activity patterns of single cells. (B and C) D1- and A2A-cre freezing response during fear conditioning session 1 (FC1) and fear conditioning session 4 (FC4).

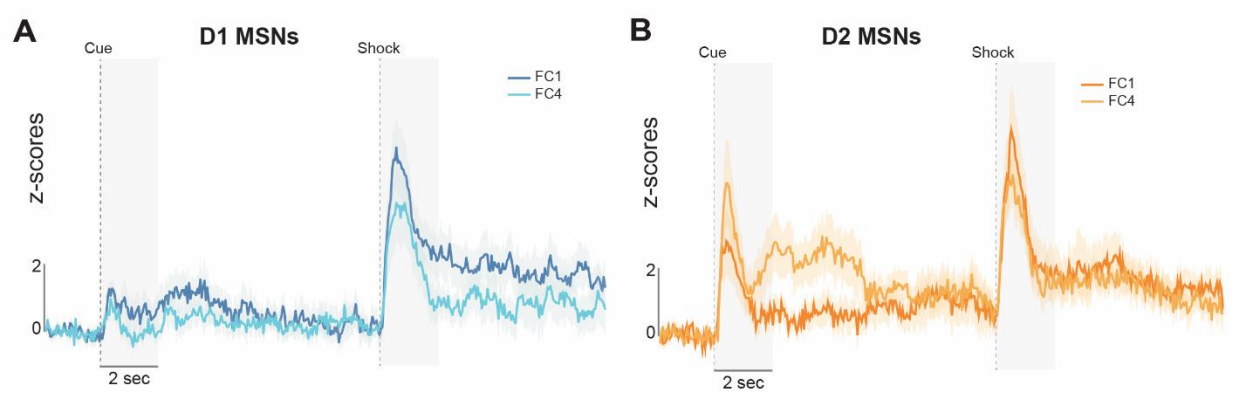

Fig. S8. Whole field traces of calcium signal from miniature endoscopes.

Selected the entire field of view (Region of Interest) for each recording and plotted the bulk calcium fluorescence for (**A**) D1 and (**B**) D2 MSNs during FC1 and FC4.

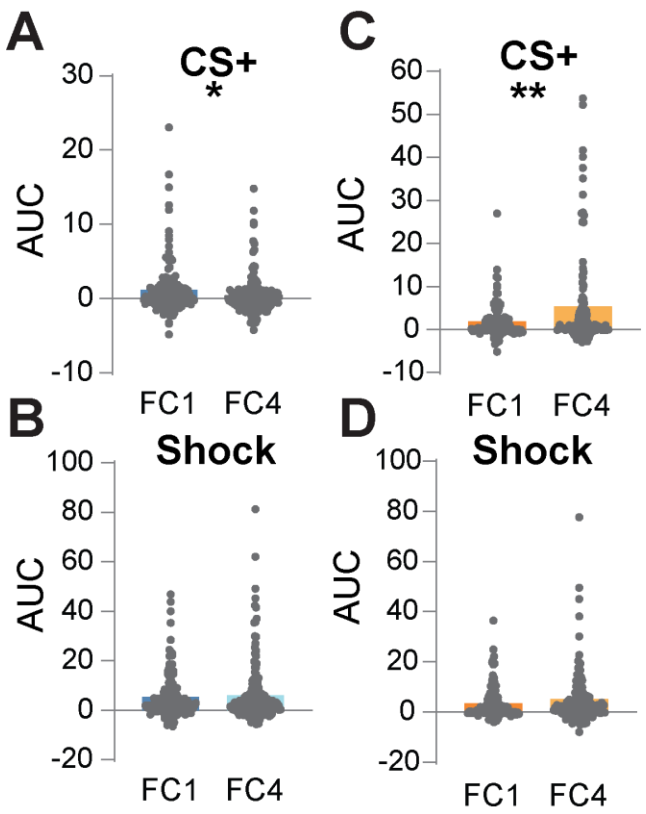

Fig. S9. D1 MSNs show a decrease to the predictive cue and D2 MSNs an increase following repeated fear conditioning sessions.

(**A**) There was a decrease in the peak response to the CS+ in FC4 (independent sample t-test, $t_{335} = 2.349$, $p = 0.0194$, n = 5 mice) and (**B**) no difference in the shock in D1 MSNs in FC1 verses FC4 (independent sample t-test, $t_{335} = 0.5603$, $p = 0.5756$, n = 5 mice). (**C**) There was a significant increase in response to the cue following repeated fear conditioning sessions (independent sample t-test, $t_{216} = 1.195$, $p = 0.2333$, n = 5 mice) and (**D**) no difference in the response to the shock in D2 MSNs in FC1 verses FC4 (independent sample t-test, $t_{216} = 0.7109$, $p = 0.4779$, n = 5 mice). Data represented as mean ± S.E.M. * $p < 0.05$, ** $p < 0.01$.

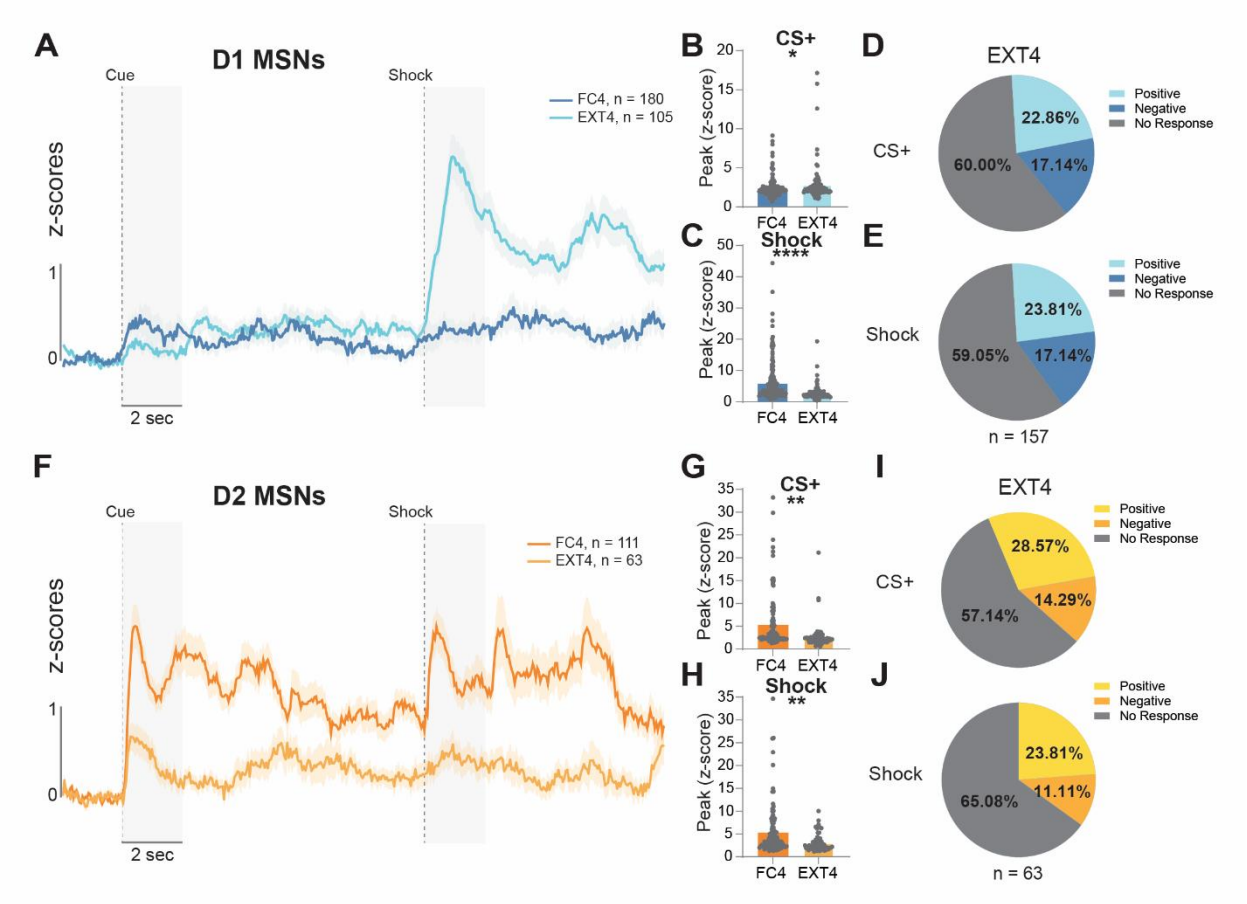

Fig. S10. The D2 MSN response to the cue decreases with repeated extinction sessions.

(**A**) D1 MSNs average response of 180 cells in FC4 (plotted in Figure 5D) as compared to 105 cells in EXT4. (**B**) There was a decrease to the cue in FC4 verses EXT4 (independent sample t-test, t_{283} = 2.027, p = 0.0436, n = 5 mice). (**C**) There was a difference in response to the shock (FC4) verses no shock (EXT4) (independent sample t-test, t_{283} = 5.009, p < 0.0001, n = 5 mice). Percentages of D1 MSNs that increased their response (positive), decreased their response (negative), or did not respond (no response) to the (**D**) CS+ and (**E**) shock in FC4 and EXT4. (**F**) D2 MSNs average response of 111 cells in FC4 (plotted in Figure 5I) as compared to 63 cells in EXT4. (**G**) There was a significant decrease in response to the cue following repeated fear extinction sessions (independent sample t-test, t_{172} = 3.251, p = 0.0014, n = 5 mice). (**H**) There was a difference in the response to the shock in D2 MSNs in FC4 (shock) verses EXT4 (no shock) (independent sample t-test, t_{172} = 3.161, p = 0.0019, n = 5 mice). Percentages of D2 MSNs that increased their response (positive), decreased their response (negative), or did not respond (no response) to the (**I**) CS+ and (**J**) shock in FC4 and EXT4. Data represented as mean ± S.E.M. * p < 0.01, ** p < 0.01, **** p < 0.0001.

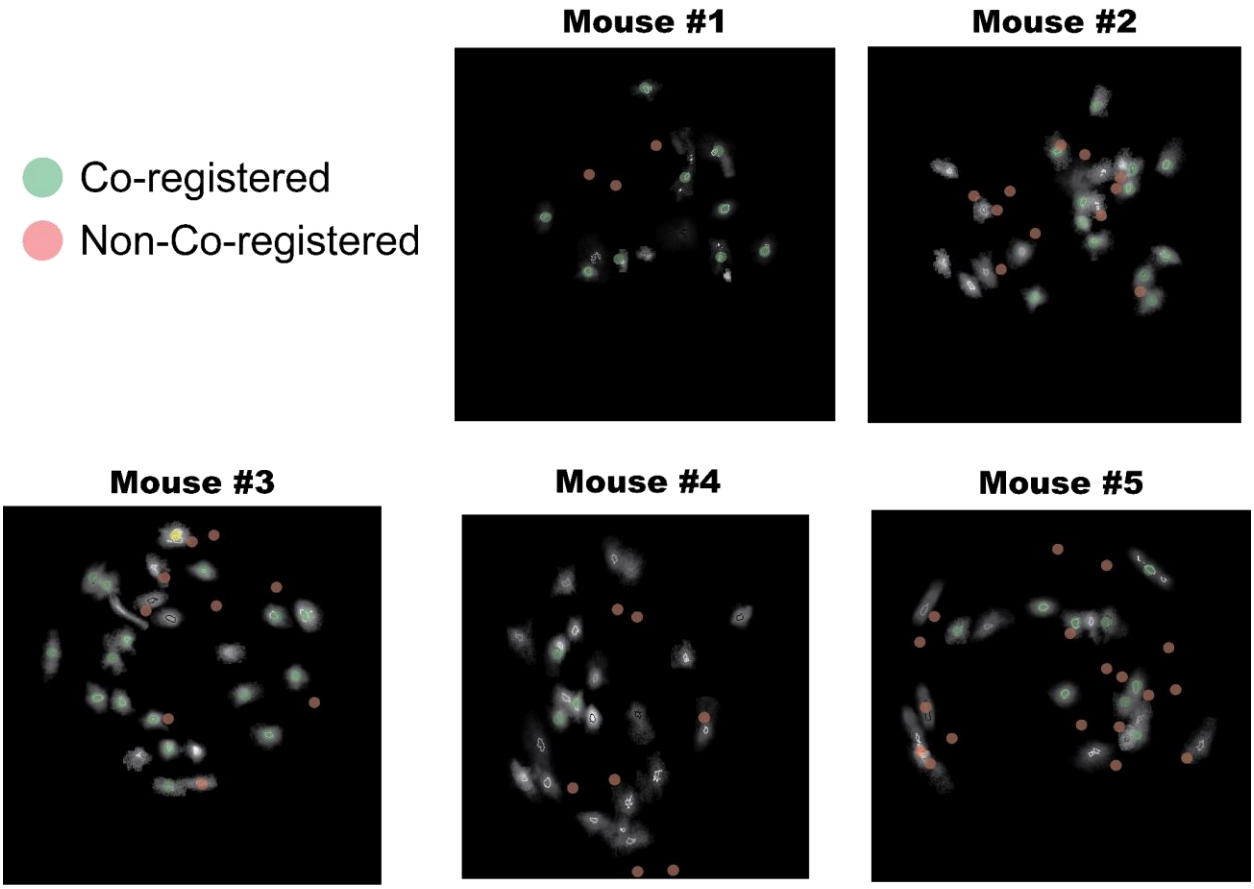

Fig. S11. Overlay of projected cell maps for A2A-cre animals showing co-registered cells.

Cell maps from the last fear conditioning session (FC4) with overlays (dots) from cells identified in the first fear conditioning session (FC1) for each A2A-Cre animal included in the study. The FC1 cells were represented as the co-registered (green) and non-co-registered (red) dots. Cells that are identifiable, but do not have dots are cells that were not identified in FC1 but were active in FC4.

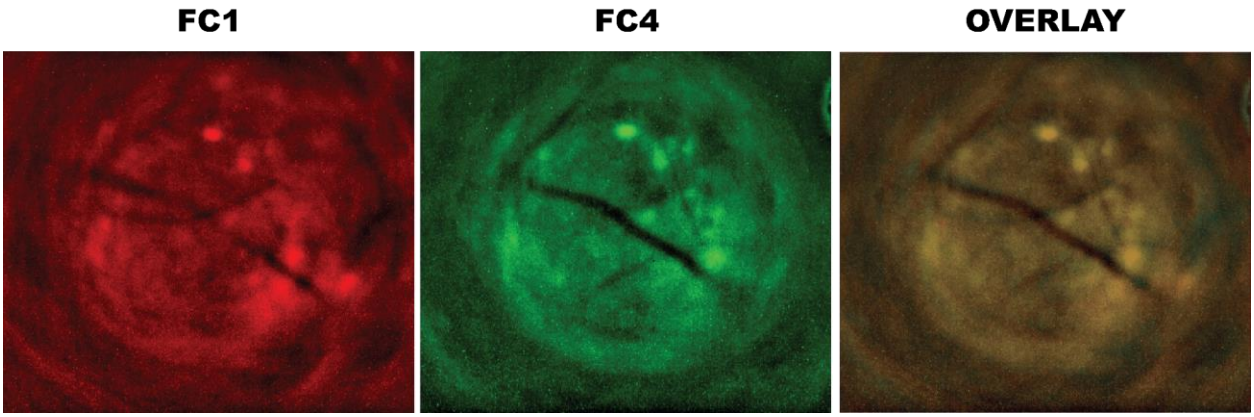

Fig. S12. Overlay of raw cell maps for A2A-cre animals showing close alignment of field of view and landmarks.

Representative projection images from single cell recordings during FC1 (red) and FC4 (green) as well as the overlay between FC1 and FC4 projection images.

CHAPTER 3

Dopamine signaling in the nucleus accumbens core causally mediates latent inhibition

Adapted from: Kutlu, Zachry*, Melugin* et al., 2022 Nature Neuroscience*

3.1 Summary

Systems neuroscience studies often focus on defining the neural mechanisms by which associations between cues and predicted outcomes control behavior. These studies regularly use associative learning frameworks to understand the neural control of behavior. While powerful, these frameworks do not always account for the full range of effects of novelty on behavior and future associative learning. Here we show that dopamine in the nucleus accumbens (NAc) core is evoked by novel, neutral stimuli in isolation, and that these responses causally influence future learning for valenced stimuli. We used optical approaches to record and manipulate dopamine signals in the NAc core of awake and behaving mice during exposure to neutral stimuli and defined their influence on future learned behavior. Dopamine was evoked by novel neutral stimuli and the trajectory of this response over time tracked habituation. Habituation to novel cues prior to associative learning reduced future associative learning, a psychological construct termed latent inhibition. Critically, trial-by-trial dopamine response patterns tracked this phenomenon. Finally, optically stimulating or inhibiting dopamine responses to the cue during the habituation period bidirectionally influenced future aversive and appetitive associative learning. Our findings

highlight the causal role of dopamine signaling in the NAc core in novelty-based learning in a way that cannot be predicted based on purely associative factors.

3.2 Introduction

Systems neuroscience studies have focused on the neural mechanisms of associative learning with a goal of defining how circuits in the brain encode associations between cues and predicted outcomes to control behavior. However, other experience-dependent factors, such as novelty, play an important and causal role in determining the trajectory of associative learning (Harris, 1943b; Lubow & Moore, 1959). For example, both valenced and neutral stimuli exert the highest influence on behavior when they are novel (Kamprath & Wotjak, 2004; Lubow & Moore, 1959). Habituation, in which stimulus responses are reduced over repeated presentations, is a critical form of novelty-based learning that guides organisms to ignore irrelevant stimuli in their environment (Harris, 1943a). Further, novelty also potently influences associative learning and conditioned behavioral responses (Lubow & Moore, 1959; R. A. Rescorla, 1973). Novel stimuli can alter conditioned responses to previously learned cues (i.e. external inhibition) – even when no errors in prediction are present (Kutlu et al., 2021; Pavlov, 1927). Additionally, unconditioned stimuli form stronger associations with neutral cues when the cues are novel while familiar cues impede this process – a psychological phenomenon termed latent inhibition (Lubow, 1973a). While parameters such as salience and novelty are accounted for in various respects in virtually all influential associative learning models, these frameworks still do not always account for the full range of effects of novelty on behavior (Mackintosh, 1975; Pearce & Hall, 1980; R. Rescorla & Wagner, 1972; Schmajuk et al., 1996). Thus, defining the neural underpinnings of interactions between stimulus novelty/habituation and future learning – and whether this is best explained by associative or non-associative factors – is critical to understanding fundamental neurobehavioral processes.

While dopamine is often studied in associative learning contexts (Oleson et al., 2012; Saunders et al., 2018), work has shown that dopamine in striatal regions, such as the nucleus accumbens (NAc) core, is modulated by novelty. Extracellular dopamine levels are influenced by novelty and habituation, and basal dopamine levels correlate with attention (Joseph et al., 1993; Kutlu et al., 2021; A. Young et al., 1993). While previous studies have focused on calcium imaging in ventral tegmental area (VTA) cell bodies and have suggested that dopamine neurons are critically involved in novelty detection (Morrens et al., 2020), the majority of work on how novelty alters the dopamine signal at its projection targets has been relegated to slow sampling techniques that only allow for the assessment of dopamine levels over long periods of time. Importantly, habituation occurs rapidly and understanding its neural correlates requires the ability to assess dopamine responses on a trial-by-trial basis. In sum, while prior work suggests that dopamine is influenced by novelty, technical limitations have prevented our ability to systematically define 1) if and how this occurs in a temporally specific fashion and the behavioral factors that influence these signals over experience in the NAc core and 2) whether the dopamine signal in the NAc core is causal to novelty effects on learning via non-associative factors and whether this affects future associative learning. The development of genetically encoded fluorescent dopamine sensors allows for direct, optical assessment of dopamine transients *in vivo* with a high signal-to-noise ratio. We can thus assess dopamine responses across single trials, across sessions, and across behavioral tasks within the same animals. To this end, we observed and manipulated dopamine responses during repeated presentations of neutral stimuli to understand how dopamine responses track novelty and habituation to influence future associative learning.

We show that neutral auditory and visual stimuli evoke a positive dopamine response in the NAc core in the absence of any valence-based predictions. Further, the magnitude of the dopamine response tracks the novelty of neutral stimuli whereby a response is reliably evoked during initial exposure and dissipates as a function of habituation to the stimulus. Moreover, with

repeated presentations, dopamine responses to neutral cues decreased to baseline as animals habituated to the stimulus. We subsequently employed a latent inhibition paradigm to define whether these signals were causal to future associative learning. Using optogenetics to increase or decrease dopamine responses during habituation, we showed that dopamine responses during the habituation period are causal to future learning and cannot be explained solely by associative or prediction-based accounts of dopamine coding in learning and memory (Hall & Channell, 1986; S. Killcross & Balleine, 1996; Westbrook et al., 2000). Our results show that dopamine in the NAc core is causal to latent inhibition. Further, we demonstrate a causal link between dopamine and novelty that influences current and future behavior that is best explained via non-associative mechanisms.

3.3 Materials and Methods

Animals. Male (N=35) and female (N=48) 6- to 8-week-old C57BL/6J mice obtained from Jackson Laboratories (Bar Harbor, ME; SN: 000664) were kept 5 per cage and maintained on a 12-hour reverse light/dark cycle, with all behavioral testing took place during the light cycle. Animals were given *ad libitum* access to food and water. All experiments were conducted in accordance with the guidelines of the Institutional Animal Care and Use Committee (IACUC) at Vanderbilt University School of Medicine. The order of testing was counterbalanced, and experimenters were blind to experimental groups throughout behavioral experiments. Male (N=10) and female (N=7) TH-Cre rats at around 12-16 weeks old at the beginning of the experiments were used to test the effect of photostimulation of dopamine neurons in the VTA on latent inhibition in an appetitive procedure using food rewards (**fig. S25**). These rats were bred at NIDA-IRP after the founders were obtained from the rat resource and research center (RRRC, University of Missouri). Rats were singly housed and maintained on a 12-hour light/dark cycle, with all behavioral testing took place during the light cycle. Rats were on *ad libitum* access to food and water unless undergoing the behavioral experiment, during which they received either 8g or 12g of chow- for females and

males, respectively, daily in their home cage following training sessions. Rats were monitored to ensure they did not drop below 85% of their initial body weight across the course of the experiment. All experimental procedures were conducted in accordance with the NIDA-IRP Institutional Animal Care and Use Committee of the US National Institutes of Health (NIH) guidelines.

Apparatus. For all mouse fiber photometry and optogenetic experiments, animals were trained and tested daily in individual operant conditioning chambers (Med Associates Inc., St. Albans, Vermont) fitted with visual and auditory stimuli including a standard house light, a white noise generator, and a 16-tone generator capable of outputting frequencies between 1 and 20 KHz (85 dB). For the optogenetic photostimulation experiments in rats, training was conducted in eight standard behavioral chambers (Coulbourn Instruments; Allentown, PA), which were individually housed in light and sound-attenuating boxes (Jim Garmon, JHU Psychology Machine Shop). Each chamber was equipped with a pellet dispenser that delivered 45-mg pellets into a recessed food port when activated. Access to the food port was detected by means of infrared detectors mounted across the opening of the recess. The chambers contained a speaker connected to an Arduino that was capable of generating many auditory sounds. A computer equipped with GS3 software (Coulbourn Instruments, Allentown, PA) controlled the equipment and recorded the responses.

Surgical Procedure. At least 1 hour prior to surgery, mice were administered Ketoprofen (5 mg/kg) via subcutaneous injection. Animals were anesthetized using isoflurane (5% for induction and 2% for maintenance) and placed on a stereotaxic frame (David Kopf Instruments). Ophthalmic ointment was continuously applied to the eyes throughout surgical procedures. A midline incision was then made down the scalp and a craniotomy was performed with a dental drill using aseptic technique. Using a .10-mL NanoFil syringe (WPI) with a 34-gauge needle,

AAV5.CAG.dLight1.1 (UC Irvine;[28]) was unilaterally infused into the NAc (bregma coordinates: anterior/posterior, + 1.4 mm; medial/lateral, + 1.5 mm; dorsal/ventral, −4.3 mm; 10° angle) at a rate of 50 nL/min for a total volume of 500 nL. Following infusion, the needle was kept at the injection site for 7 minutes before being slowly withdrawn. Fiber-optic cannulas (400 µm core diameter; .48 NA; Doric) were then implanted in the NAc and positioned immediately dorsal to the viral injection site (bregma coordinates: anterior/posterior, + 1.4 mm; medial/lateral, + 1.5 mm; dorsal/ventral, −4.2 mm; 10° angle) before being permanently fixed to the skull using adhesive cement (C&B Metabond; Parkell). Follow-up care was performed according to IACUC/OAWA and DAC standard protocol. Animals were allowed a minimum of 6 weeks to recover in order to ensure efficient viral expression before commencing experiments.

The surgical procedures for the optogenetic photostimulation of the dopamine neurons in the VTA have been described previously[55]. Briefly, rats received bilateral infusions of 1.2µl if AAV5-EF1α-DIO-ChR2-eYFP (n=8) or AAV5-EF1α-DIO-eYFP (n=9) into the VTA at the following coordinates relative to bregma: AP -5.3mm; ML ±0.7mm; DV -6.5mm and -7.7mm (females) or -7.0mm and -8.2mm (males). Virus was obtained from the University of North Carolina at Chapel Hill (UNC Vector Core). During this surgery, optic fibers were implanted bilaterally (200µl diameter, Thorlabs) at the following coordinates relative to bregma: AP -5.3mm; ML ± 2.61; DV -7.05mm (females) and -7.55mm (males) at an angle of 15° pointed towards the midline. All procedures were conducted in accordance with the Institutional Animal Care Use Committee of the US Institutes of Health (approved protocol: 18-CNRB-108).

For the mouse optogenetic experiments, we used a viral approach to target dopaminergic cells in the VTA in combination with a terminal specific stimulation strategy, which ensures added specificity on top of the viral approach, to achieve dopamine release manipulations. AAV5.Ef1a.DIO.hchR2.eYFP (ChR2; UNC vector core), Chrimson.FLEX: AAV5-Syn-FLEX-rc[ChrimsonR-tdTomato] (Chrimson; Addgene) or AAV5-Ef1a-DIO.eNpHR.3.0-eYFP (NpHR; Addgene) and AAV9.rTH.PI.Cre.SV40 (Addgene;[56]) were injected into the VTA (unilaterally for

ChR2 and Chrimson and bilaterally for NpHR; bregma coordinates: anterior/posterior, -3.16 mm; medial/lateral, + 0.5 mm; dorsal/ventral, −4.8 mm) of C57BL/6J mice. Unilateral (for ChR2 and Chrimson) or bilateral (for NpHR) 200um fiber optic implants were placed into the NAc core (bregma coordinates: anterior/posterior, +/- 1.4 mm; medial/lateral, + 1.5 mm; dorsal/ventral, −4.3 mm; 10° angle; at a rate of 50 nL/min for a total volume of 500 nL). This allowed for the photostimulation or photoinhibition of dopamine response only in dopamine terminals that project from the VTA and synapse in the NAc core. Control animals received AAV5.Ef1a.DIO.eYFP injections into the VTA instead of ChR2 or NpHR. Controls for the Chrimson group only received the Cre-dependent Chrimson but not the cre-inducing virus.

Histology: Mice were deeply anaesthetized with an intraperitoneal injection of a ketamine/xylazine mixture (100 mg/kg;10 mg/kg) before being transcardially perfused with 10 mL of 1x PBS solution followed by 10 mL of cold 4% PFA in 1x PBS. Animals were subsequently decapitated, and the brain was extracted and postfixed in the 4% PFA solution stored at 4 °C for at least 48 hours before being dehydrated in a 30% sucrose in 1x PBS solution stored at 4 °C. After sinking, tissue was sectioned (35 µm slices) on a freezing sliding microtome (Leica SM2010R) and then placed in a cryoprotectant solution (7.5% sucrose + 15% ethylene glycol in 0.1 M PB) stored at - 20 °C until immunohistochemical processing. For optogenetic experiments using AAV9.rTH.PI.Cre.SV40 and AAV5-Ef1a-DIO.eNpHR.3.0-eYFP/AAV5-EF1α-DIO-ChR2-eYFP/ AAV5-EF1α-DIO-eYFP, we also validated the targeting of TH+ cells in the VTA via an anti-tyrosine hydroxylase (TH) antibody (mouse anti-TH; Millipore #MAB318, 1:100) and an anti-GFP antibody (chicken anti-GFP (Abcam #AB13970). Sections were then incubated with secondary antibodies [gfp: goat anti-chicken AlexaFluor 488 (Life Technologies #A-11039), 1:1000 and TH: donkey anti-mouse AlexaFluor 594 (Life Technologies # A-21203), 1:1000] for 2 h at room temperature. After washing, sections were incubated for 5 min with DAPI (NucBlue, Invitrogen) to achieve counterstaining of nuclei before mounting in Prolong Gold (Invitrogen). Sections were

mounted on glass microscope slides with ProLong Gold antifade reagent. Fluorescent imaging was conducted using a BZ-X700 inverted fluorescence microscope (Keyence) under a dry 20x objective (Nikon). Injection site locations and optical fiber placements were determined with serial images in all experimental animals. For the TH-Cre rat optogenetic photostimulation experiments, all rats were euthanized with an overdose of carbon dioxide and perfused with phosphate buffered saline (PBS) followed by 4% Paraformaldehyde (Sigma-Aldrich Inc, NJ). Fixed brains were cut in 40µm sections to examine fiber tip position under a fluorescence microscope (Olympus Microscopy, Japan). Images of these brain slices were acquired by a fluorescence Virtual Slide microscope (Olympus America, NY) and later analyzed in Adobe Photoshop. Subjects were eliminated if viral expression was detected outside of the defined borders of the NAc core and VTA and/or the tip of the implants were identified outside of the NAc core borders based on the mouse brain atlas (Franklin & Paxinos, 2007). We excluded a total of 1 animal due to inaccurate placement of optic fiber in the NpHR group.

Fiber Photometry. The fiber photometry system used two light-emitting diodes (490nm and 405nm; Thorlabs) controlled by an LED driver (Thorlabs). The 490nm light source was filtered with a 470nm (the excitation peak of dLight1.1) bandpass filter and the 405nm light source was used as an isosbestic control [28]. Light was passed through an optical fiber (400 µm, .48 NA; Doric) that was coupled to a chronically implanted fiber optic cannula in each mouse. LEDs were controlled via a real-time signal processor (RZ5P; Tucker-Davis Technologies) and emission signals from each LED were determined by multiplexing. Synapse software (Tucker-Davis Technologies) was used to control the timing and intensity of the LEDs and to record the emitted fluorescent signals upon detection by a photoreceiver (Newport Visible Femtowatt Photoreceiver Module; Doric). LED power (125 µW) was measured daily and maintained across trials and experiments. For each event of interest (e.g., cue presentation, footshock), transistor-transistor logic (TTL) signals were used to timestamp onset times from Med-PC V software (Med Associates

Inc.) and were detected via the RZ5P in the Synapse software (see below). A built-in low-pass filter on the Synapse software was set to 10 Hz to eliminate noise in the fiber photometry raw data.

Behavioral Experiments:

***Latent Inhibition*.** Mice received 4 consecutive pre-exposure sessions (days 1 - 4) wherein animals were presented with either a tone (85 dB, 2.5 khz frequency) or light stimulus (total of 33 stimulus presentations per session). The pre-exposure stimuli were presented for a 10 second duration with a variable inter-trial interval (35 - 55 seconds). No footshocks were paired with these stimulus presentations. During 2 consecutive sessions (sessions 5 - 6), mice were then given pseudo-random presentations of house light and tone with a 10 second stimulus duration (6 trials of each) and a variable inter-stimulus interval (60 - 100 seconds). Animals received a footshock (1 mA, .5 second duration) immediately following both house light and tone. Pre-exposure to either house light or tone across all 4 sessions was counterbalanced between animals. Therefore, for half of the animals the tone was the pre-exposed CS+ and light was the non-pre-exposed CS+ and for the other half of the animals, these stimulus roles were reversed. In a subset of animals, we employed a modified pre-exposure paradigm where they received 2 cue presentations instead of 33 and remained in the context for the remainder of the session.

Repeated stimulus exposure. Intermixed with the CS+ presentations during the last 2 days of the latent inhibition experiment, mice were also presented with an auditory stimulus (white noise, 85 dB) a total of 12 times (6 presentations per session), for 10 seconds with a variable inter-trial interval (35 - 55 seconds) in the absence of the footshock. This was to test the dopamine response patterns during stimulus pre-exposure.

Test of conditioned inhibition following pre-exposure. In order to test the potential inhibitory properties of cues that are pre-exposed, we ran a "summation test". Mice received pre-exposure to a light or a tone cue (pre-exposed cue; counterbalanced) as explained above. Then received a session where they received 10 trials of tone- or light-shock pairings (excitor). Finally, for the summation test, mice received 3 trials of each test presentations: excitor alone, excitor and pre-exposed cue together, and excitor and a novel cue (white noise; external inhibition) together and freezing response to each test stimuli was scored.

Optogenetic photostimulation during cue pre-exposure. TH-Cre rats had previously undergone appetitive training with procedures described elsewhere, utilizing four auditory and two visual cues, which were generated by Coulbourn equipment. For latent inhibition, we used two auditory stimuli generated by an Arduino to produce two very distinct sounds that would be distinguishable from cues used previously (chime and warp). All trials consisted of 10-s presentations of the chime or warp. Training began with 2 days of pre-exposure to the pre-exposed cue (warp or chime, counterbalanced). On each day, rats received 12 presentations of the pre-exposed cue. During pre-exposure, we delivered light into the brain (470nm, 1s, 20Hz) at the onset of the cue. We have previously used a greater number of pre-exposure trials to generate successful latent inhibition[58]. We used less pre-exposure here as we wanted to give an opportunity to see either an enhancement or reduction in latent inhibition in our experimental group. Following 2 days of pre-exposure, rats received a single critical conditioning session in which the pre-exposed cue and another novel stimulus (chime or warp, counterbalanced) were presented 6 times each followed immediately by delivery of two 45-mg sucrose pellets (5TUT; Test Diet, MO).

In a group of C57BL/6J mice, AAV5.Ef1a.DIO.hChR2.eYFP (ChR2; UNC vector core) and AAV9.rTH.PI.Cre.SV40 (Addgene;) were injected into the VTA and a 200um fiber optic implant was placed into the NAc core. This allowed for photostimulation of dopamine response only in

dopamine terminals that project from the VTA and synapse in the NAc core. Control animals received AAV5.Ef1a.DIO.eYFP injections into the VTA instead of ChR2. For these experiments, mice were trained utilizing auditory and visual cues generated by MedPC equipment (MED Associates, Inc). For latent inhibition experiments, all trials consisted of 10-s presentations of the tone (5 kHz at 85 dB) or house light. Training began with 4 days of pre-exposure to the pre-exposed cue (tone or house light, counterbalanced). On each day, mice received 30 presentations of the pre-exposed cue. During pre-exposure, we delivered blue laser stimulation (470nm, 1s, 20Hz, 8mW) into the NAc core at the onset of the cue for 1s. Following 4 days of pre-exposure, mice underwent two fear conditioning sessions where the pre-exposed and novel stimuli (tone or house light, counterbalanced) were paired 6 times each with a shock (1mA, 0.5 sec).

In a separate group of C57BL/6J mice, AAV5-Ef1a-DIO.eNpHR.3.0-eYFP (NpHR; Addgene) and AAV9.rTH.PI.Cre.SV40 (Addgene;[56]) were injected into the VTA and a 200um fiber optic implant was placed into the NAc core. Control animals received AAV5.Ef1a.DIO.eYFP injections into the VTA instead of NpHR. For latent inhibition, all trials consisted of 10-s presentations of the tone (5 kHz at 85 dB) or house light as above. Training began with 4 days of pre-exposure to the pre-exposed cue (tone or house light, counterbalanced). On each day, mice received 30 presentations of the pre-exposed cue. During pre-exposure, we delivered yellow laser stimulation (590nm, 11s, constant, 8mW) into the NAc core at the onset of the cue for 11s. Following 4 days of pre-exposure, mice underwent two fear conditioning sessions where the pre-exposed and novel stimuli (tone or house light, counterbalanced) were paired 6 times each with a shock (1mA, 0.5 sec). Another group of mice received the photostimulation and inhibition of the NAc core dopamine terminals during each inter-trial interval during the pre-exposure when no other stimuli are presented. The photostimulation parameters were identical to those used for cue stimulation/inhibition experiment described above. Finally, we also ran another experiment in a different group of mice, where the animals received photostimulation or inhibition of NAc core terminals as described above but only for the first trial of the fear conditioning session on day 5.

Optogenetic photostimulation during pre-exposure combined with dopamine recording. In order to manipulate and record dopamine during pre-exposure and fear conditioning, respectively, in a separate group of C57BL/6J mice, Chrimson.FLEX: AAV5-Syn-FLEX-rc[ChrimsonR-tdTomato] (Chrimson; Addgene) and AAV9.rTH.PI.Cre.SV40 (Addgene) were injected into the VTA and AAV5.CAG.dLight1.1 (UC Irvine) was injected into the NAc core as described above. A 200um fiber optic implant was placed into the NAc core. Control animals received only Chrimson.FLEX: AAV5-Syn-FLEX-rc[ChrimsonR-tdTomato] injections into the VTA and AAV5.CAG.dLight1.1 into the NA core. This way, we were able to deliver a yellow laser stimulation (590nm, 11s continuous, 8mW) into the NAc core at the onset of the cue during pre-exposure and record dopamine at the same site using fiber photometry. This method has been validated previously[7].

Latent disinhibition via context switch. To test the effects of cue-context associations on latent inhibition, we ran a “latent disinhibition” experiment. For this experiment, mice were injected with AAV5.CAG.dLight1.1 (UC Irvine) into the NAc core. Following 4 days of pre-exposure to a cue in their regular context, mice received fear conditioning as described above in a novel context. For the novel context, we used MedPC boxes designed for rats with larger dimensions (11.625" L x 9.78" W x 7.35" H). Walls were added within these operant boxes made of cardboard boxes that contained spatial cues such as vertical stripes in addition to an olfactory cue (vanilla extract). All stimuli and experiment parameters were kept constant between the pre-exposure and fear conditioning contexts. NAc core dopamine responses to the cues were recorded in the new context.

Data Analysis and Statistics:

Behavioral Data Analysis. Statistical analyses were performed using GraphPad Prism (version 8; GraphPad Software, Inc, La Jolla, CA) and Matlab (Mathworks, Natick, MA). Freezing behavior, identified as the time of immobility except respiration during the stimulus duration, was calculated and converted into percent freezing ((freezing time * 100)/ stimulus duration)). Two blind reviewers scored all freezing behavior. For the statistical analyses of the freezing behavior during Session1 and Session2 of the latent inhibition experiments, we employed repeated measures ANOVA. For all other freezing data, we used a one-way ANOVA followed by Tukey's post-hoc analysis. We identified the mice that failed to show latent inhibition based on the second trial freezing response to the pre-exposed versus non-pre-exposed CS+. The mice included in the "No Latent Inhibition" group showed higher freezing response to the pre-exposed CS+ compared to the non-pre-exposed CS+. The data from these mice is included in **fig. S18.** Alpha was 0.05 for all statistical analysis. All data were depicted as group mean ± standard error of the mean (S.E.M.).

Fiber Photometry Analysis. The analysis of the fiber photometry data was conducted using a custom Matlab pipeline. Raw 470nm and isosbestic 405nm traces were used to compute ΔF/F values via polynomial curve fitting. For analysis, data was cropped around behavioral events using TTL pulses and for each experiment 2s of pre-TTL up to 20 seconds of post-TTL ΔF/F values were analyzed. In order to remove any movement and photobleaching artifacts, first, we used the isosbestic channel signal (405nm[28]) to calculate our ΔF/F (ΔF/F =F470-F405)/F405; see **fig. S13**). In addition, all fiber photometry data were converted to and reported as z-scores. We z-scored dopamine signals around the event of interests such as the CS+ using their own local baseline (2 seconds prior to the cue onset). Z-scores were calculated by taking the pre-TTL ΔF/F values as baseline (z-score = (TTLsignal - b_mean)/b_stdev, where TTL signal is the ΔF/F value for each post-TTL time point, b_mean is the baseline mean, and b_stdev is the baseline standard deviation). This allowed for the determination of dopamine events that occurred at the precise

moment of each significant behavioral event. For statistical analysis, we calculated the area under the curve (AUC), peak height, time to baseline, tau, and R^2 values for each individual dopamine event ([59]; see **fig. S13** for the visual description of these values). The AUCs were calculated via trapezoidal numerical integration on each of the z-scores across a fixed timescale. The peak height values were the maximum values after the TTL onset. The time to baseline was computed as the seconds to going back to the 0 z-score baseline and tau was the duration to the 2/3 of the peak height. For both of the measures where individual curves did not reach the baseline or tau the minimum value was taken into the statistical analysis. Finally, for slope analysis, we computed the R^2 values for the fitted curves (Linear polynomial curve) for a 15 sec duration. The duration of the data collection for the AUC, peak height, time to baseline, and tau values was determined by limiting the analysis to the z-scores between 0 time point (TTL signal onset) and the time where the dopamine peak of interest returns to baseline. Baseline dopamine responses were calculated as the z-scored dopamine values during the inter-trial interval 20 seconds prior to the CS+ presentations. Unpaired t-tests were employed to test the group differences for all fiber photometry-based dependent variables. We also calculated maximum z-scores for event fiber photometry traces and analyzed to see if these were significantly different from the critical z-score at p=0.05 level (1.645) using independent-t-tests.

3.4 Results

Neutral stimuli evoke dopamine responses that decrease as a direct function of habituation.

Our first goal was to determine if novel and neutral stimuli could evoke a dopamine response and how this changed with experience (i.e., following repeated exposure). To this end, we utilized optical methods for directly recording dopamine in awake and behaving animals. A majority of work overlaying dopaminergic activity with behavioral control has utilized electrophysiology (Hart et al., 2014) or calcium imaging (de Jong et al., 2019) to record action potentials at the soma of midbrain dopamine neurons (Schultz et al., 1997), or observed axonal calcium fluctuations as a proxy of dopamine release events (Menegas et al., 2017). Both approaches assume that dopamine itself follows the same pattern as these proxy measures. However, extracellular dopamine levels in the NAc results from both dopamine neuron firing patterns and rapid modulation of dopamine terminals by both homosynaptic mechanisms and heterosynaptic signaling via accumbal microcircuits (Nolan et al., 2020). These local modulatory mechanisms sculpt the timing and magnitude of dopamine transmission independent of dopamine cell body activity in the midbrain (Cragg et al., 1997). Indeed, recent work has shown that task-related VTA dopamine neuron spiking and dopamine release are dissociable *in vivo* (Mohebi et al., 2019) highlighting the need for direct assessment of dopamine response patterns in the NAc. To this end, we used the genetically encoded dopamine sensor, dLight1.1 (Patriarchi et al., 2018), to record *in vivo* dopamine dynamics at the level of its projection targets in the NAc core (**Fig. 7a;** see **fig. S13** for specific dopamine analyses conducted and representative dopamine traces)**.** Using this approach we recorded dopamine responses during the presentation of a neutral stimulus (white noise) presented at 85 dB for 6-7 presentations on a random-time schedule for two sessions on consecutive days (**Fig. 7a,b**).

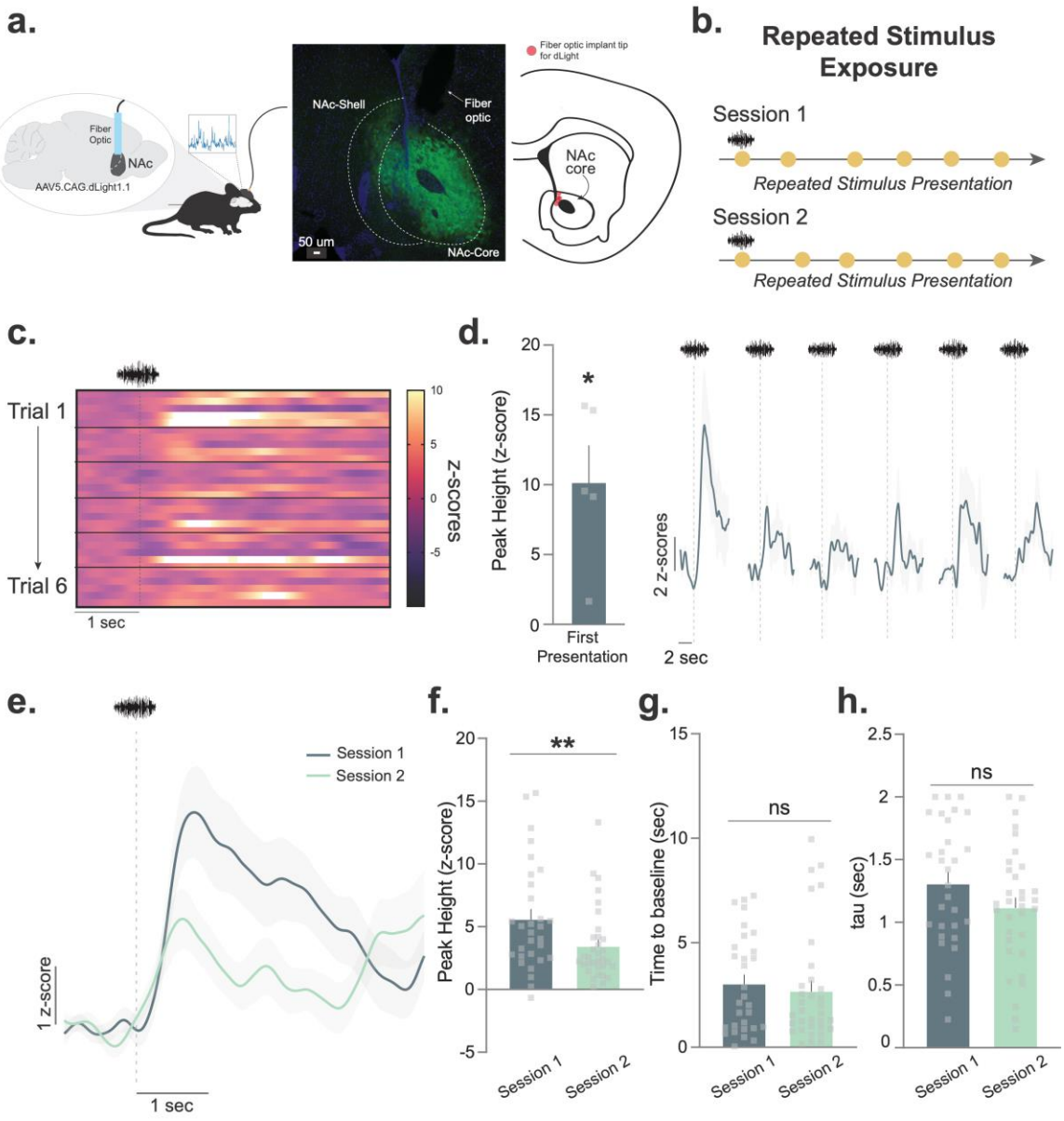

Fig. 7. Neutral stimuli elicit dopamine responses that decrease over repeated presentations.

(a) Mice (5 male, 2 female) received unilateral injections of the fluorescent dopamine sensor dLight1.1 in the nucleus accumbens (NAc). A fiber optic cannula was placed directly above the injection site in the NAc core. Representative histology showing viral expression (green) restricted to the NAc core and schematic showing fiber optic placements (red) in experimental animals. **(b)** Stimulus exposure paradigm. A white noise stimulus was pseudo-randomly presented at 85 dB for 6-7 presentations for two sessions. **(c)** Heatmap showing the trial-by-trial dopamine response (z-scores) to the neutral stimulus from each mouse (n=5 for each trial; 6 trials in total). **(d)** Session 1 dopamine signal to repeated white noise presentations (6-7 presentations per animal). The first presentation of the neutral stimulus evoked a significant positive dopamine response (Peak height for the first presentation; independent sample t-test, t_4=4.02, p=0.01, n=5 mice). **(e)** Averaged

dopamine responses to white noise presentations on session 1 versus session 2, showing that dopamine is reduced to neutral stimuli both within and across session. **(f)** Peak dopamine response evoked by the white noise decreased from session 1 to session 2 (Nested ANOVA $F_{(1,57)}$= 7.26, p=0.009, n=30-33 stimulus presentations). **(g)** The time for the dopamine signal to return to baseline in seconds did not significantly differ across sessions, suggesting that changes are driven by release, rather than clearance mechanisms (Nested ANOVA $F_{(1,57)}$= 0.40, p=0.5316, n=30-33 stimulus presentations). **(h)** Tau is another measure of dopamine clearance and is defined by the time in seconds for the signal to return to 2/3 of peak height. This measure did not differ across sessions (Nested ANOVA $F_{(1,57)}$= 2.65, p=0.1093, n=30-33 stimulus presentations). Data represented as mean ± S.E.M. * p<0.05, ** p<0.01.

First, we found that novel, neutral stimuli reliably evoked dopamine transients upon first exposure (**Fig. 7c,d**). Next, we found that dopamine responses to the same stimulus were progressively reduced over repeated exposure (**Fig. 7d;** see **fig. S14** for the second day of the exposure session). Specifically, we found that the peak of the dopamine response decreased both within (**Fig. 7d**) and across sessions (**Fig. 7e,f**). While the peak dopamine response tracked habituation to the neutral cue, there were no changes in dopamine clearance (**Fig. 7g**,**h**). These results show that in the absence of an outcome, NAc core dopamine responses track the novelty/familiarity of stimuli. That is, as the stimulus becomes more familiar during repeated exposure, the dopamine signal that the stimulus evokes diminishes.

Stimulus habituation decreases learning rates for subsequent conditioned associations.

Although we showed above that NAc core dopamine tracks the familiarity of a neutral stimulus, it is not known if this effect is consequential for the formation of future associations. To test this, we employed a latent inhibition paradigm (Lubow, 1973a). Latent inhibition is a novelty-based learning phenomenon whereby pre-exposure to a neutral stimulus before conditioning results in a reduced learning rate for that same stimulus in the future (Lubow, 1973a). This occurs because the novelty of the stimulus is reduced and thus attention to that stimulus is consequently reduced when an associative contingency is later imposed. Importantly, this is one of the main

challenges to prediction-based learning models, which have been used to explain the role of dopamine in learning and memory. Prediction-based models cannot account for the change in the conditioned response based on prior exposure to the stimulus (which influences novelty and attention (Pearce, 1987)).

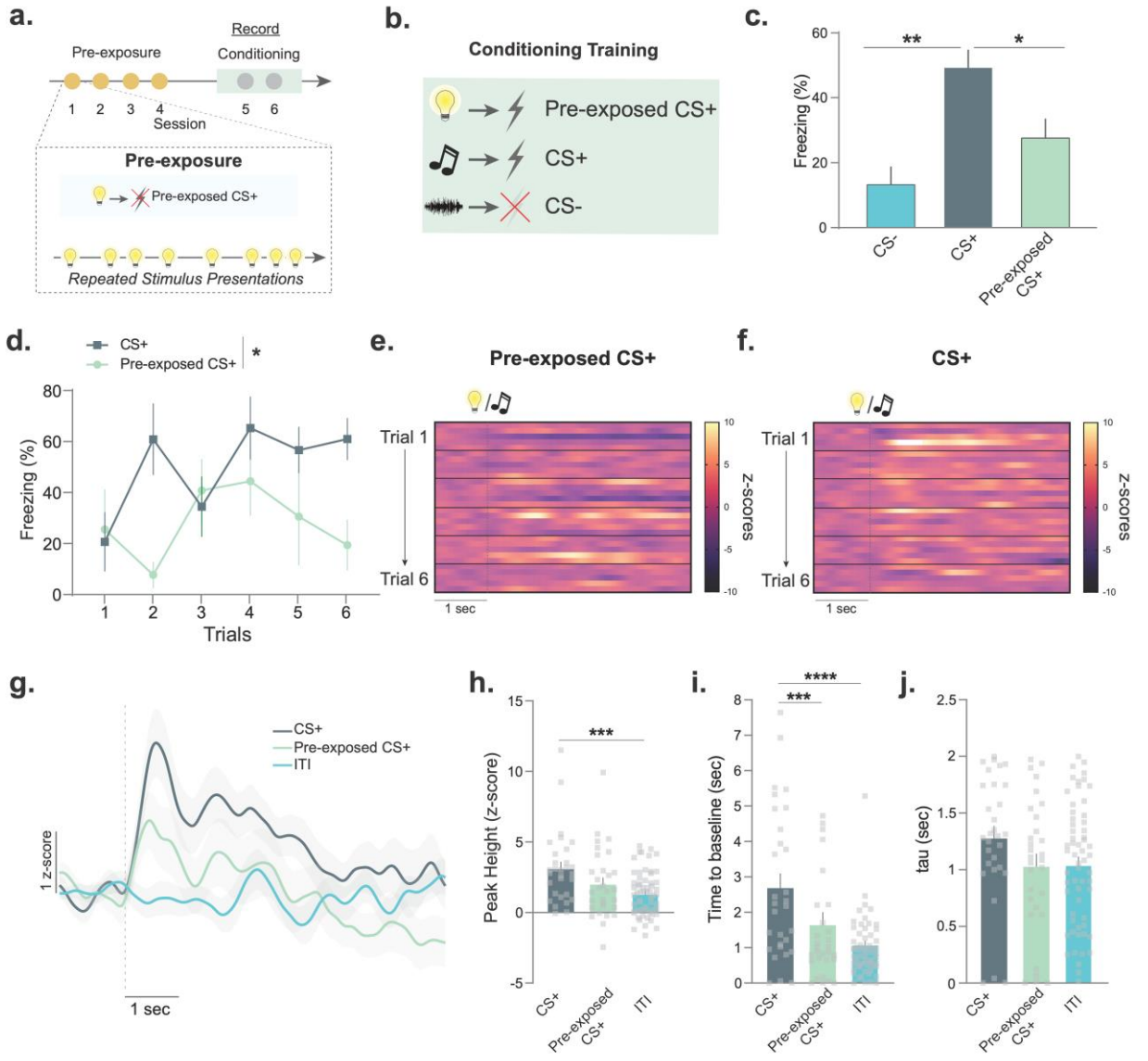

Fig. 8. Latent inhibition: Cue pre-exposure leads to decreased dopamine responses and learning rate during subsequent fear learning.

Latent inhibition training paradigm. Latent inhibition is a novelty-based learning phenomenon whereby pre-exposure to a neutral stimulus before conditioning results in a reduced learning rate for that stimulus. **(a)** In this paradigm, mice (5 male, 2 female) were pre-exposed to a stimulus (Pre-exposed CS+) for 4 sessions. **(b)** Next, the pre-exposed CS+ as well as a novel stimulus

(CS+) were paired with a footshock for 2 sessions. Another novel stimulus (CS-) was presented between each CS+ presentation and signaled the absence of the footshock. Cues were presented in the same session in an intermixed fashion. **(c)** Freezing responses to the pre-exposed CS+, CS+, and CS- were measured (session 1 is presented in this figure, for session 2 see **fig. S19**). There was a main effect of pre-exposure (RM ANOVA $F_{(2,12)}$= 11.50, p=0.001) and freezing was higher to the CS+ than the pre-exposed CS+ (Tukey post-hoc p=0.035). Freezing was increased to the CS+ as compared to the CS- (Tukey post-hoc p=0.001) **(d)** Percentage of time spent freezing across sessions 1. Freezing to the CS+ was greater than the pre-exposed CS+ (RM ANOVA main effect of pre-exposure $F_{(1, 8)}$= 9.76 p=0.014, n=5). **(e-f)** Heatmaps showing the trial-by-trial dopamine response (z-scores) to the CS+ and **(c)** the pre-exposed CS+ from each mouse (n=5 for each trial; 6 trials in total). **(g)** Averaged dopamine recordings over trials. ITI (inter-trial interval) values are the averaged dopamine responses during the inter-trial interval between CS+ presentations in the same session. **(h)** Peak dopamine response to the CS+ was higher than the ITI dopamine responses (Nested ANOVA $F_{(2,113)}$= 2.51, p=0.0006, Bonferroni post-hoc: CS+ vs. pre-exposed CS+ p=0.08; CS+ vs. ITI p=0.0002; n=30 trials); the pre-exposed CS+ did not differ from the ITI responses (Bonferroni post-hoc p=0.32). **(i)** Time in seconds for the dopamine response to return to baseline was significantly slower for the CS+ compared to the pre-exposed CS+ (Nested ANOVA $F_{(2,113)}$= 19.70, p=0.0001, Bonferroni post-hoc p<0.0001; n=30 trials) and the ITI dopamine response (Bonferroni post-hoc p<0.0001). **(j)** Tau is the time in seconds for the signal to return to 2/3 of peak height. Tau did not change between CS+ and pre-exposed CS+ (Nested ANOVA $F_{(2,113)}$= 2.13, p=0.123, Bonferroni post-hocs: p>0.05). Data represented as mean ± S.E.M., * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.

Mice were pre-exposed to a stimulus (either tone or light, counterbalanced) for 4 consecutive sessions, a total of 33 presentations for each session, to induce habituation (**Fig. 8a**). Following the pre-exposure period, animals underwent fear conditioning. During these sessions, animals were presented with a pre-exposed conditioned stimulus (pre-exposed CS+) or a non-pre-exposed conditioned stimulus (CS+) – both of which were immediately followed by a footshock. Further, there was a non-pre-exposed CS- (CS-) that signaled that no shock would occur (**Fig. 8b**). At the end of conditioning session 1, the CS+ yielded a stronger freezing response as compared to both the CS- and the pre-exposed CS+ (**Fig. 8c**). Importantly, there was no difference between the freezing to the CS+ and pre-exposed CS+ on the first trial before the first shock was presented (**Fig. 8d**); however, following conditioning, the CS+ yielded a stronger freezing response than the pre-exposed CS+. At the end of the second session, the difference

between the CS+ and pre-exposed CS+ disappeared, indicating that the observed differences were in the rate at which learning occurred (**fig. S15**). These results demonstrate a strong latent inhibition effect.

Dopamine responses track latent inhibition: response to the non-pre-exposed CS+ is greater than to the pre-exposed CS+.

As shown in the initial repeated exposure experiment, NAc core dopamine responses decreased as novelty was reduced (i.e., with increasing familiarity of the stimulus). We hypothesized that if this effect had any impact on learning rate, the dopamine response to the CS+ and the pre-exposed CS+ would also differ. Supporting our hypothesis, we found that dopamine responses to the pre-exposed CS+ were weaker as compared to the CS+ (**Fig. 8e-j**; see **fig. S15-17** for additional analyses). The CS+ elicited a dopamine response which was larger than the baseline dopamine levels [during the inter-trial interval (ITI) when no stimuli were presented]; however, the dopamine response to the pre-exposed CS+ was not different from this baseline (**Fig. 8h**). In addition to changes in the dopamine response, there were also changes in some kinetic parameters. The time for the dopamine signal to return to baseline was increased following the presentation of the CS+ as compared to the pre-exposed CS+ (**Fig. 8i**,**j**). Supporting these results, we also found that in the mice that did not show latent inhibition, the dopamine response did not differ between the pre-exposed CS+ and CS+ (**fig. S18**). Thus, even though both cues were paired identically with an aversive stimulus, there were significant differences in dopamine responses, which tracked the novelty of the cue before it had acquired value.

Consistent with the behavioral data, on the second training day these behavioral and dopamine response differences disappeared (**fig. S19b**). At this time, there were no longer significant differences between the CS+ and pre-exposed CS+ for peak height (**fig. S19c,d**), or any of the kinetic parameters measured (**fig. S19e,f**). The work presented within the current manuscript is consistent with many previous studies showing that aversive stimuli increase

dopamine levels in the NAc (Budygin et al., 2012; A. M. J. Young, 2004). However, work has also suggested that dopamine encodes bi-directional valence where the dopamine response to the cues predicting aversive outcomes (e.g., fear cues) is negative (Oleson et al., 2012). These studies present fear conditioning responses as the average of many trials, rather than a trial-by-trial analysis during the early trials, as we present here. Replicating these results, we also showed that the dopamine response to the fear conditioning cues dipped below baseline with extensive training (**fig. S20**). However, these results cannot be explained by the cue coding for negative valence as animals have learned the aversive association – and thus freeze to the cue – before this signal becomes negative. Also, the positive dopamine response to the cue was correlated with learning rate for that cue during early training in the opposite direction of what would be predicted if this were a purely associative and valence-based signal, with larger dopamine responses predicting faster learning of the aversive association.

Overall, our data supports that, like the behavioral responses, the dopamine response to a CS+ decreases following pre-exposure and this effect disappears with additional training, highlighting that pre-exposure retards the learning rate for the neutral stimulus in the future.

Induction of latent disinhibition eliminates latent inhibition and associated dopamine responses.

It is well known that switching to a novel context following stimulus pre-exposure abolishes latent inhibition, an effect known as “latent disinhibition” (Lubow et al., 1976). We leveraged this behavioral manipulation as an additional mechanism to probe endogenous dopamine dynamics. We reasoned that if dopamine is encoding aspects of latent inhibition, eliminating the latent inhibition effect (via introducing a novel context) would abolish the differential dopamine response to pre-exposed versus novel cues. To this end, we conducted an experiment where the pre-exposure was done in a separate context than the subsequent fear conditioning session and conducted dopamine recordings within these animals at the time of fear conditioning following

pre-exposure (**Fig. 9a-c**). When the fear conditioning occurred in a different context than the cue pre-exposure, we found that the context switch indeed abolished the behavioral latent inhibition effect as expected (**Fig. 9**). The reduced dopamine response to the pre-exposed cue observed in previous experiments also disappeared when the pre-exposed cue was now presented in a novel context (**Fig. 9c**). Thus, behaviorally manipulating latent inhibition was able to alter the dopamine response in a predictable fashion, suggesting that dopamine may be causal to its expression.

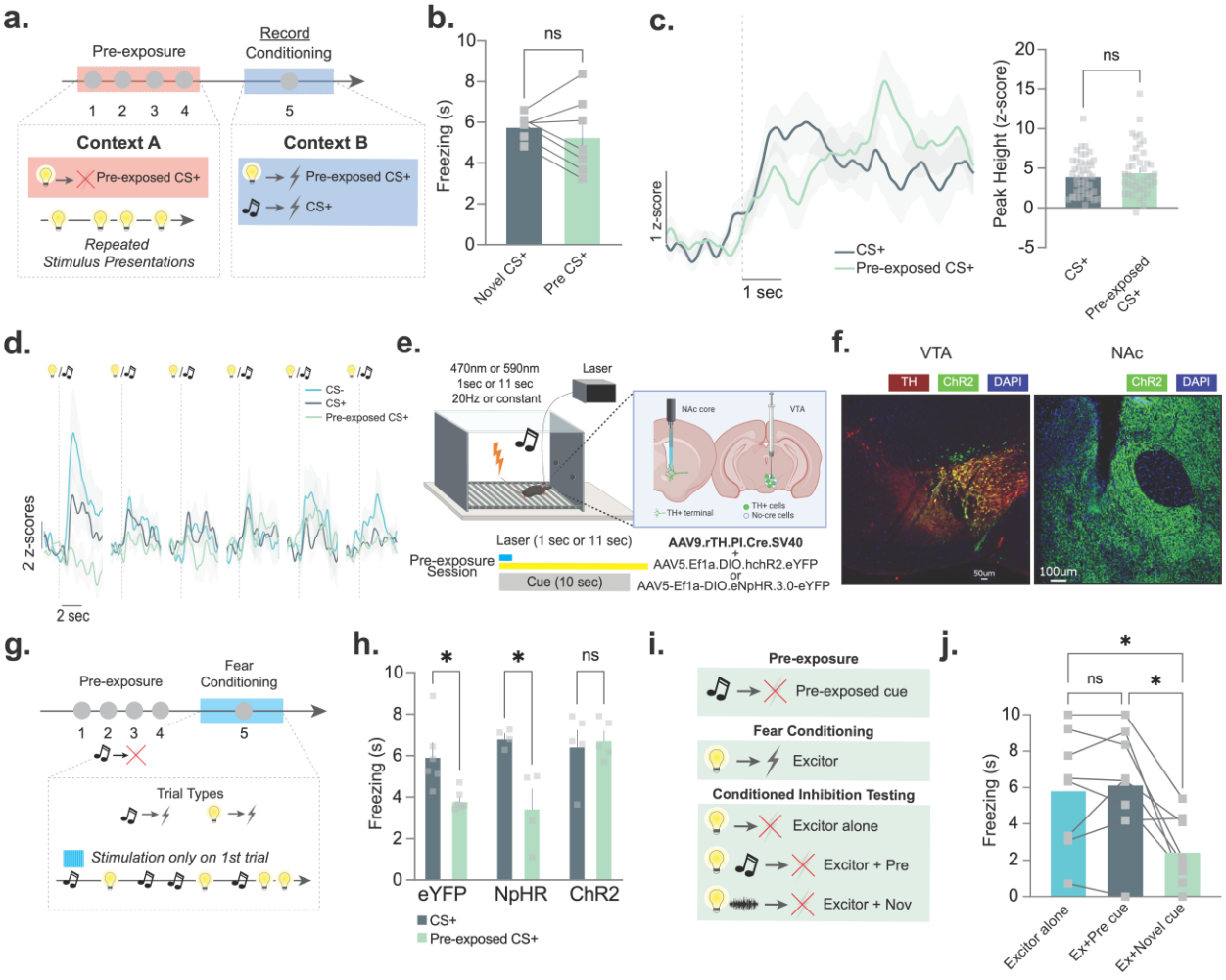

Fig. 9. Dopamine responses to pre-exposed cues are causal to future aversive learning; pre-exposed cues do not function as conditioned inhibitors.

(a) Mice (4 male, 3 female) underwent four sessions of pre-exposure in context A. Dopamine responses were recorded in two subsequent fear conditioning sessions in context B. **(b)** Switching the context disrupted the latent inhibition effect at the behavioral level. **(c)** Switching the context also eliminated differences in dopamine responses between the CS+ and pre-exposed CS+ (Nested ANOVA $F_{(1,76)}$= 0.77, p=0.3838). **(d)** Averaged fiber photometry traces showing dopamine

responses to the CS-, CS+, and pre-exposed CS+ during each trial during session 1 of fear conditioning training. **(e)** AAV5.Ef1a.DIO.eYFP (eYFP), AAV5-Ef1a-DIO.eNpHR.3.0-eYFP (NpHR), or AAV5.Ef1a.DIO.hchR2.eYFP (ChR2) were co-injected with AAV9.rTH.PI.Cre into the VTA to achieve dopamine-specific expression of excitatory or inhibitory opsins. **(f)** Representative histology showing expression of ChR2 and TH in the VTA and ChR2 in the NAc core dopamine terminals. **(g)** NAc core dopamine terminals were stimulated or inhibited at the time of the cue during the first cue-shock pairing of fear conditioning (7 male, 8 female mice). **(h)** Stimulating dopamine terminals at the time of the initial cue presentation disrupted the latent inhibition effect (2-way ANOVA cue x group interaction $F_{(2,12)}$=4.556 p=0.033; Bonferroni multiple comparisons: eYFP pre-exposed vs. non-pre-exposed p=0.049; ChR2 pre-exposed vs. non-pre-exposed p=0.999; NpHR pre-exposed vs. non-pre-exposed p=0.011), while inhibiting terminals had no effect. **(i)** In a pre-exposure session, mice (4 male, 4 female) were given repeated presentations of a cue. In a subsequent fear conditioning session, another cue (the excitor) was paired with a shock. In the conditioned inhibition testing session, three trial types were presented: excitor alone, excitor + the pre-exposed cue, and excitor + novel cue. **(j)** In the conditioned inhibition test session, the pre-exposed cue does not reduce freezing response to the cue that was paired with the shock outcome (RM ANOVA $F_{(1.37,9.60)}$= 10.21, p=0.0069; Bonferroni post-hocs Excitor alone vs. Excitor+Pre-exposed cue, p>0.05). A novel stimulus that was not presented before reduced freezing response to the excitor (Bonferroni post-hocs Excitor alone vs. Excitor+Novel Cue, p=0.026). Data represented as mean ± S.E.M., * p<0.05, ** p<0.01.

Dopaminergic responses to pre-exposed cues are causal to latent inhibition.

Next, we wanted to confirm if the reduced dopamine signal to the pre-exposed cue is indeed causal to latent inhibition. That is, the reduced dopamine response to the cue retards the learning rate for that cue when it is paired with another stimulus subsequently. The largest dopamine response was detected during the first trial of the fear conditioning session (**Fig. 9d**), suggesting the latent inhibition effect is determined during the initial trials of each session. Indeed, shorter cue exposures during pre-exposure were also able to induce latent inhibition (**fig. S21**). Next, we tested if stimulating or inhibiting dopamine terminals in the NAc core via optogenetics, only at the time of the cue during the first cue-shock pairing was able to alter the behavioral effect of the pre-exposure period (**Fig. 9e-h**). Concurrent with the pre-exposed stimulus presentation during the first fear conditioning trial, dopamine terminals that project from the VTA and synapse in the NAc core were either stimulated (channelrhodopsin-2, ChR2 group; 20 Hz 470nm photostimulation, 1s duration) or inhibited (halorhodopsin [eNpHR3.0], NpHR group; continuous

590nm photoinhibition, 11s duration; **Fig. 9e-g**; **fig. S22**). We found that stimulating dopamine terminals [via ChR2 expressed selectively in dopamine terminals in the NAc] during the cue was sufficient to block the latent inhibition effect and restore a normal learning trajectory for this cue (**Fig. 9h**). Inhibition of dopamine terminals [via NpHR 3.0 expressed selectively in dopamine terminals in the NAc] did not result in a larger latent inhibition effect (**Fig. 9h,** also see **fig. S23**), likely because of a floor effect. Overall, these results show that the diminishing dopamine response to the pre-exposed cue on the first day of conditioning (after the pre-exposure period) is causal to the behavioral effects observed.

Together, these data show that dopamine responses in the NAc core are 1) positively correlated with the novelty of a stimulus regardless of valence, conditioned value, or predictions, 2) increased to aversive stimuli (footshocks), elicited by appetitive (Day et al., 2007), novel, and neutral stimuli, 3) decreased with experience, 4) can be altered by altering latent inhibition, and 5) causal to its expression.

Dopaminergic control of latent inhibition cannot be explained by associative factors in isolation.

Most of the theoretical models and hypotheses for latent inhibition offer explanations that are hybrids of attentional-associative accounts (Schmajuk et al., 1996). However, some purely associative accounts have also been proposed (Bouton, 1993). Thus, there are two overarching hypotheses of how latent inhibition occurs on the behavioral level. The first is an attentional account. In this account, as novelty is reduced (via repeated exposure), the attention paid to these stimuli is also reduced as an animal is habituated to them. The core tenant of this hypothesis is that behavior in response to these cues is modulated by novelty in a non-associative fashion. The alternative hypothesis is the associative account. In this account, latent inhibition is the result of associative learning during the pre-exposure period (Hall & Channell, 1986; S. Killcross & Balleine, 1996; Westbrook et al., 2000). In this framework, associations are formed between the

neutral cue and the context during pre-exposure, and these associations compete with future cue-outcome associations to slow down the learning of new associations (Hall & Honey, 1989).

All of the previous studies presented could be explained by either an associative or attentional account of latent inhibition. Even the latent disinhibition experiment could be explained through either the introduction of general novelty – which alters attention to all stimuli in an environment and thus eliminates the effect of habituation (Schmajuk et al., 1996; Sokolov, 1960) – or through cue-context associations that were made during the pre-exposure period. We specifically designed the following series of studies to test these competing hypotheses and use these experiments to better define how dopamine's role in learning and memory is causally related to behavioral control.

Pre-exposed cues do not become conditioned inhibitors.

An associative account of latent inhibition suggests that associations formed either between cues and the absence of outcomes (i.e., cue-no outcome associations) or cues and contexts are responsible for the impaired learning during subsequent conditioning training. If cue-no outcome associations are responsible, then the pre-exposed cue might operate as a conditioned inhibitor during future learning, as it predicts that no outcome will occur. The pre-exposed cue (which would function as a conditioned inhibitor) would thus decrease the conditioned response to an excitor (a cue paired with an outcome) when presented together with that excitor in a summation test (**Fig. 9i**).

Our results support the attentional account since they show that the pre-exposed cue does not become an inhibitor as it does not reduce the freezing response to the excitor (the cue that was paired with a shock) (**Fig. 9j**). Additionally, the pre-exposed cue does not reduce the freezing response while a novel (distracting) stimulus alongside the excitor resulted in a marked reduction in the freezing response to the excitor, a purely attentional effect known as "external inhibition"

(Pavlov, 1927) (**Fig. 9j**). This suggests that the pre-exposed stimulus exerts no associative or attentional control over the conditioned response after an animal has been habituated to it.

Optogenetic photostimulation of dopamine terminals in the NAc during pre-exposure/habituation bidirectionally modulates subsequent associative learning.

Data presented thus far can be explained by novelty acting to alter attention to stimuli in a context. However, many of these findings can also be explained in part through associative learning about the relationship between the neutral cue and the context. Thus, we designed an optogenetic experiment to specifically parse these ideas from one another. In the accounts solely based on associative mechanisms, inhibiting dopamine responses during the pre-exposure period should prevent latent inhibition – as it would prevent the novel cue + context associations from forming -, conversely, increasing dopamine optogenetically should facilitate latent inhibition – as this would be necessary to facilitate the cue + context association that slows future learning. Alternatively, if dopamine responses reflect non-associative learning, as we predict, inhibiting dopamine responses during the cue pre-exposure period will facilitate latent inhibition and slow future learning - as this would signal that habituation occurred more rapidly -, whereas stimulating dopamine during pre-exposure will decrease the effect - by preventing habituation - and increase future learning.

We used optogenetics to increase or decrease the dopamine response to the cue during the pre-exposure period to determine how this influenced subsequent associative learning. Concurrent with stimulus presentation during the pre-exposure sessions, dopamine terminals that project from the VTA and synapse in the NAc core were either photo-stimulated or photo-inhibited **(Fig. 10a-e**; **fig. S23**). Following the pre-exposure sessions, mice underwent two fear conditioning sessions in which the pre-exposed cue (pre-exposed CS+) and a novel cue (CS+) were both paired with a shock over 6 trials. While the eYFP control group showed a significant reduction in freezing to the pre-exposed CS+ as compared to the CS+, in the ChR2 group freezing did not

differ between the CS+ and pre-exposed CS+ during fear conditioning (**Fig. 10c-e**). Therefore, stimulating dopamine to the pre-exposed stimulus disrupted the formation of a latent inhibition effect and enhanced subsequent associative learning. Conversely, inhibiting dopamine to the pre-exposed cue further impaired future associative learning, indicating a bidirectional effect of the dopamine signal on latent inhibition (**Fig. 10c-e**). The latent inhibition effect in the eYFP group disappeared with additional training during the second conditioning session, whereas in the NpHR group, the freezing response to the CS+ was still stronger compared to the freezing response to the pre-exposed CS+ (**fig. S24**). Therefore, the inhibition of NAc core dopamine during pre-exposure leads to a more persistent latent inhibition effect, where pre-exposed cues show a reduced ability to acquire novel associations in the future.

To verify that the optogenetic photostimulation of NAc core dopamine terminals at the time of the pre-exposure of stimuli did indeed augment subsequent dopamine response to the pre-exposed cue during learning sessions, we combined optogenetics (with a red-shifted opsin) with optical dopamine recordings within the same animal (**Fig. 10f-l**). Replicating our original optogenetics results, we showed that stimulating dopamine terminals in the NAc core at the time of the pre-exposure of cues [via expressing a red-shifted excitatory opsin in TH+ VTA cell bodies (Chrimson+rTH.PI.Cre virus)] resulted in disrupted latent inhibition compared to controls (rTH.PI.Cre virus only) (**Fig. 10h**). We also showed that this manipulation reversed the diminished dopamine response to the pre-exposed cue during fear conditioning while this effect was intact in the control animals (**Fig. 10i-l**), demonstrating a causal relationship between the dopamine response to cues during pre-exposure and future dopamine signatures that occur during subsequent associative learning.

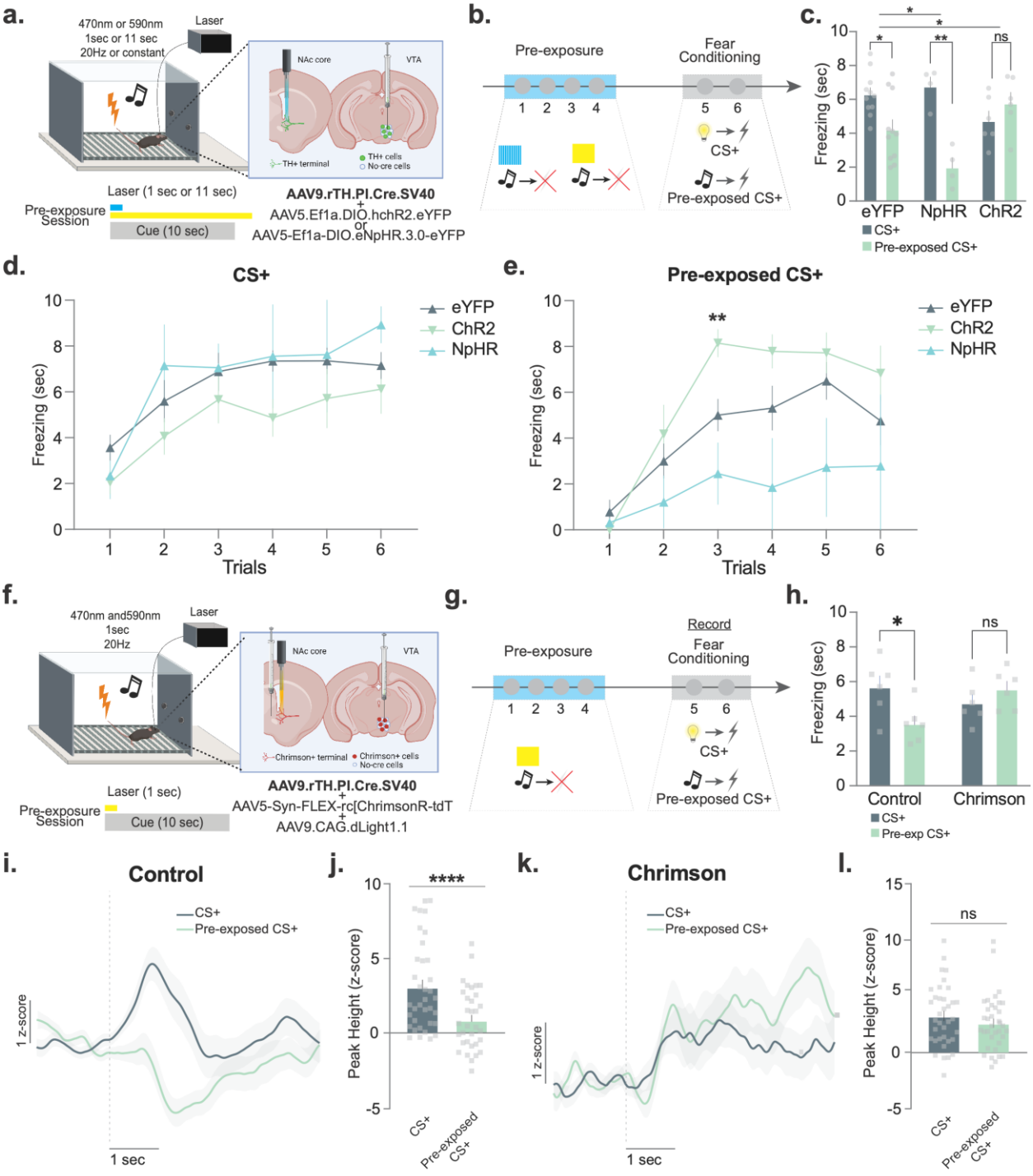

Fig. 10. Optogenetically evoking/inhibiting dopamine response during pre-exposure bidirectionally alters subsequent learning.

(a) AAV5.Ef1a.DIO.eYFP (eYFP), AAV5-Ef1a-DIO.eNpHR.3.0-eYFP (NpHR), or AAV5.Ef1a.DIO.hchR2.eYFP (ChR2) were co-injected with AAV9.rTH.PI.Cre into the VTA to achieve dopamine-specific expression of excitatory or inhibitory opsins. **(b)** Dopamine terminals were optogenetically stimulated (via ChR2) or inhibited (via NpHR) at the time of the neutral cue

during pre-exposure. Mice received 4 sessions of stimulus pre-exposure followed by two sessions of fear conditioning. In the pre-exposure session, the pre-exposure cue (light or tone, counterbalanced) was presented in the absence of any outcome; in the conditioning sessions, both the pre-exposed (pre-exposed CS+) and non-pre-exposed (CS+) cues were followed by a footshock. **(c)** Freezing responses to the CS+ and pre-exposed CS+ for the eYFP, NpHR, and ChR2 groups throughout the 6 conditioning trials (2-way ANOVA cue x group interaction $F_{(2,36)}$=8.77, p = 0.0008; n=4-11 mice). Inhibition of dopamine response, which artificially reduced dopamine responses to the novel stimulus, during the pre-exposure reduced subsequent aversive conditioning. i.e. the NpHR group showed an enhanced latent inhibition effect compared to the eYFP controls (2-way ANOVA cue x group interaction $F_{(1,26)}$=4.34, p = 0.04; Multiple comparisons: NpHR pre-exposed CS+ vs. CS+ p=0.001; eYFP pre-exposed vs. non-pre-exposed p = 0.02). Conversely, enhancing dopamine signal, which artificially blocked the dopamine reductions we observed in **Figure 7** during the pre-exposure, enhanced subsequent aversive conditioning. i.e. the ChR2 group showed a reduced latent inhibition effect compared to the eYFP controls. (2-way ANOVA cue x group interaction $F_{(1,30)}$=7.22 p = 0.01; Bonferroni multiple comparisons: ChR2 pre-exposed vs. non-pre-exposed p = 0.85). **(d)** Trial-by-trial freezing responses to the non-pre-exposed cue in the NpHR, eYFP, and ChR2 groups (Repeated Measures ANOVA trial x group interaction $F_{(10,90)}$=0.62, p = 0.79; All multiple comparisons p>0.05). **(e)** Trial-by-trial freezing responses to the pre-exposed cue in the NpHR, eYFP, and ChR2 groups (Repeated Measures ANOVA trial x group interaction $F_{(10,90)}$=2.07, p = 0.03; Multiple comparison ChR2 vs. NpHR trial 2, p=0.007). **(f)** In mice (4 male, 8 female), AAV5-Syn-FLEX-rc[ChrimsonR-tdT] was co-injected with AAV9.rTH.PI.Cre into the VTA to achieve dopamine-specific expression of excitatory or inhibitory opsins. AAV9.CAG.dLight1.1 was injected in the NAc core. **(g)** Dopamine terminals were optogenetically photostimulated (via Chrimson) at the time of the neutral cue during pre-exposure in first four sessions. Dopamine was recorded in fear conditioning sessions via dLight1.1 in the same animals. **(h)** Photostimulation of dopamine terminals during the pre-exposure period disrupted the latent inhibition effect observed in the first fear conditioning session (2-way ANOVA $F_{(1,10)}$= 11.40, p=0.007; Bonferroni multiple comparisons: Controls pre-exposed vs. non-pre-exposed p=0.012; ChR2 pre-exposed vs. non-pre-exposed p>0.5). **(i)** Dopamine responses to the CS+ and pre-exposed CS+ in control animals. **(j)** Peak dopamine response to the CS+ was higher than to the pre-exposed CS+ (Nested ANOVA $F_{(1,65)}$= 19.02, p<0.0001). **(k)** Dopamine signal to the CS+ and pre-exposed CS+ in Chrimson+rTH.PI.Cre animals. **(l)** Peak dopamine response to the CS+ was not different as compared to pre-exposed CS+ (Nested ANOVA $F_{(1,65)}$= 1.23, p=0.2713). Data represented as mean ± S.E.M., * p < 0.05, **** p < 0.0001.

Finally, we showed that dopamine manipulations have comparable effects on future associative learning and latent inhibition, regardless of whether the subsequent associative learning is driven by aversive or appetitive stimuli (**fig. S25**). Overall, these results causally show that the dopamine signal in the NAc core is heavily influenced by novelty/familiarity and determines the associability of stimuli in future associative learning contexts. Together, these data

show the parameters under which dopamine controls novelty-driven learning. These data also rule out a purely associative explanation of dopamine in latent inhibition, while demonstrating that dopamine is a critical mediator of novelty-based effects on behavior in a fashion that can be explained, at least in part, via non-associative processes.

3.5 Discussion

Here we show that dopamine in the nucleus accumbens (NAc) core is evoked by novel, neutral stimuli in isolation, and that these responses causally influence future learning for valenced stimuli. Critically, trial-by-trial dopamine response patterns tracked both the habituation to novel neutral stimuli, as well as the dopamine response patterns that were observed to habituated and novel stimuli during future associative learning. Additionally, we demonstrated that these signals were causal to this process. Optogenetically evoking or inhibiting the dopamine response to neutral cues during a habituation period bidirectionally influenced the ability of these cues to form future associations with appetitive or aversive stimuli. Together, our results demonstrate a causal temporally specific link between the dopamine signal and the novelty of a stimulus (in a valence free fashion) that influences current and future behavior. Critically, our findings challenge theories of dopamine as a purely valence-based prediction signal and highlight the causal role of dopamine in the NAc core in novelty effects on current and future behavior.

While novelty effects on behavior, such as habituation, are important for animals to learn to ignore irrelevant stimuli in their environment, they can also influence associative forms of learning. As we show here, unconditioned stimuli form stronger associations with neutral cues when the cues are novel than when an animal has been habituated to them – a psychological phenomenon termed latent inhibition (Hall & Honey, 1989). We show here that dopamine patterns not only correlate with latent inhibition but are also causal to its development and expression. Our results demonstrate that NAc core dopamine responses evoked by a stimulus decreases as the stimulus becomes more familiar, or less novel, through repeated exposure. Further, this occurs

rapidly, within the first few trials of exposure. During subsequent associative learning for a cue-footshock pairing, the dopamine signal was weaker to the pre-exposed cue compared to a novel cue and resulted in a slower rate of learning of the association between the pre-exposed cue and footshock. Latent inhibition and conditioned attention theory were first proposed by Lubow and his colleagues, who described the pre-exposure effect as being an attentional deficit caused by repeated presentations (Lubow, 1973a; Schmajuk et al., 1996). Extant work has suggested that dopamine is influenced by these processes (Joseph et al., 1993; A. M. J. Young, 2004); however it was not clear if these responses were causal to its expression or the temporal dynamics by which these responses occurred. Here we temporally linked dopamine responses evoked by pre-exposed and non-pre-exposed stimuli to future associative learning and conditioned behavior.

Previous work has implicated certain populations of VTA dopamine neurons in the expression of latent inhibition (Jacob et al., 2021; Morrens et al., 2020). For example, Morrens et al. (2020) showed that dopamine cell bodies in the VTA respond to novel but not familiar odors and that activating these neurons or the dopamine terminals in the prefrontal cortex at the time of a familiar cue accelerated associative learning. However, these studies recorded VTA cell-bodies – not dopamine responses downstream – and the authors concluded that cortical, rather than striatal, projections were mediating these effects (Morrens et al., 2020). Here we show that dopamine is evoked by neutral auditory and visual stimuli and response patterns track habituation on a trial-by-trial basis. Moreover, our results concluded that preventing the habituation pattern influences not only the associative processes at that moment but also future learning as well. Further, the data contained within this manuscript contrast with conclusions drawn from earlier studies (Joseph et al., 1993; A. Young et al., 1993) which failed to show the involvement of VTA-striatum projections in detecting novel odors in the environment (Menegas et al., 2017). However, these previous studies examined NAc within a limited range of parameters and assessed the whole ventral striatum, rather than defined subregions which have been previously shown to have different response patterns during behavioral tasks (Corbit et al., 2001). Here we assessed

dopamine responses to neutral stimuli across sensory modalities as well as the influence of habituation on both appetitive and aversive learning in the NAc core. Further, using optogenetics we linked the dopamine response to novelty-driven effects on learning, ultimately supporting earlier studies showing dopamine levels are modulated by novelty and attention.

One particularly striking aspect of these findings is that habituation to neutral cues at the behavioral and neural level can occur very rapidly. We show that the latent inhibition effect can be manipulated at various points in the task with optogenetic dopamine manipulations on few – even just two – trials. Thus, this work highlights the transient nature of neural signals that track novelty. However, the speed at which this occurs is likely a result of the stimulus properties of these pre-exposed stimuli. Previous studies have demonstrated that stimulus duration (Westbrook et al., 1981), total pre-exposure time (Ayres et al., 1992), and stimulus intensity (Schnur & Lubow, 1976) employed during pre-exposure determine the size of the latent inhibition effect. Similarly, these stimulus properties also determine the habituation rate of a stimulus (Rankin et al., 2009). Therefore, depending on the stimulus characteristics, habituation may be achieved faster or slower suggesting a dynamic range in the habituation of behavioral and neural responses, such as the dopamine responses measured in this study. This also explains how only a single pre-exposure to a neutral stimulus is enough for latent inhibition in paradigms where the stimuli employed are less discrete (e.g., odor or flavor), such as conditioned taste approach/aversion in mammalian (Delacasa & Lubow, 1995) and non-mammalian species (Jacob et al., 2021). Overall, our data underscores the importance of stimulus properties determining not only the size of behavioral and neural responses but also the rate and shape of the progression of those signals. Further, and maybe more importantly, these data underscore the critical importance of trial-by-trial analysis when drawing conclusions about dopamine's involvement in novelty and novelty-related phenomenon.

While associative learning theories offer a powerful account of how animals learn relationships between stimuli, and include factors such as salience and novelty, they often fall

short in being able to fully capture the effects these factors on behavior. For example, while they can predict basic associative learning, they cannot model phenomena like external inhibition or latent inhibition that are primarily driven by the effects of novelty on behavior (Mackintosh, 1975; Pearce & Hall, 1980; Schmajuk et al., 1996). Supporting this hypothesis, we recently showed that when a novel, unpredicted stimulus was presented during external inhibition, the dopamine response to the cue increased and optogenetically stimulating dopamine during the presentation of a CS+ resulted in an external inhibition-like decrease in the freezing response (Kutlu et al., 2021). Thus, these results diverge from the canonical “dopamine as a prediction error” theory in several critical ways. First, prediction error for the footshock outcome is equal between the pre-exposed and non-pre-exposed cues throughout the training session as both stimuli were paired with the footshocks the same number of times. Second, we detected dopamine responses to the novel neutral cues before they acquired any predictive value. Therefore, it is not possible to attribute our results to differences in prediction error during these trials. These data show that dopamine is influenced by environmental factors that influence behavior and cannot be explained by traditional prediction-based models (Eshel et al., 2016; Schultz et al., 1997).

The data we present here shows that dopamine’s involvement in latent inhibition may also be influenced by associative factors. Our results showed that inhibiting dopamine responses to a novel neutral cue during pre-exposure enhanced latent inhibition, rather than preventing it as one would expect if the dopamine response during that period was critical for the encoding of the cue-context association that slows future learning through competition. Similarly, stimulating the dopamine response to the cue during pre-exposure did not enhance the latent inhibition effect, rather, it eliminated the effect. Although these results clearly show that associative mechanisms are in play in latent inhibition, they are also in line with a novelty-based account of dopaminergic control of latent inhibition. For example, novelty-based accounts of dopamine predict no effect of inhibition of dopamine during pre-exposure inter-trial intervals when novelty is already minimal in the absence of any stimuli, and the disrupted latent inhibition is a result of the increased novelty

in the environment due to the novel context or artificial photostimulation of dopamine. Nevertheless, novelty and associative terms such as associative strength, prediction, and prediction error are intrinsically linked to one another making it difficult to propose purely novelty-based or associative models of latent inhibition. For example, theoretical accounts like the one we proposed recently (Kutlu et al., 2021), assumes that stronger cue-context associations reduce novelty as the cue becomes more predicted in the environment, eventually resulting in decreased attention to the pre-exposed CS. Therefore, we acknowledge that our results do not completely rule out the involvement of any associative account in the pre-exposure process.

Furthermore, replicating earlier studies of latent inhibition (A. S. Killcross et al., 1995), we demonstrated that the pre-exposed cue does not become an inhibitor of conditioned behavioral responses, thereby ruling out the possibility that cue-no outcome associations are formed during pre-exposure and responsible for the impaired learning in the subsequent conditioning training via the suppression of a conditioned response. This potential explanation is also not supported by theoretical models that are solely based on associative terms, such as the Rescorla-Wagner model, as these models also assume that for cues to become inhibitory and predict the absence of an outcome, the presence of an outcome should be predicted by other cues or contexts that are present (R. Rescorla & Wagner, 1972). These results are in line with non-associative and novelty-based accounts of latent inhibition. Indeed, non-associative and non-reward prediction error-based accounts similar to our own framework here were previously put forward for dopaminergic encoding (Redgrave et al., 1999). Specifically, studies suggested that dopamine may be involved in novelty encoding in the form of novelty-induced exploration (Kakade & Dayan, 2002) and saliency detection (Horvitz, 2000; Redgrave et al., 1999). Here, in line with these accounts, we demonstrate that dopamine is involved in the non-associative processes by signaling novelty, while associative factors may be in play during the pre-exposure process.

Importantly, these data also explain a large body of human literature that has shown that dopamine deficits that characterize neurodegenerative diseases are concomitant with deficits in

non-associative learning. For example, habituation is a behavioral marker for many psychiatric diseases including Parkinsonism, which is marked by dopaminergic deficits (McDiarmid et al., 2017). Specifically, Parkinson's patients show decreased habituation to auditory stimuli (Teo et al., 1997) and these deficits are alleviated by drugs that enhance dopaminergic signaling (e.g., levodopa; (Rey et al., 1996)). Thus, understanding the neural mechanisms that underlie non-associative learning mechanisms, such as habituation, is important in understanding psychiatric disease symptomatology (McDiarmid et al., 2017) and is critical to our understanding of how dopamine deficits influence behavior in patient populations.

Overall, the results of the present study show that dopamine tracks the novelty of a given event regardless of the origin of novelty in the environment. Dopamine has been most widely studied under behavioral conditions of associative learning where a Pavlovian or discriminative cue acquires associative strength by predicting a significant outcome, such as a reward. Here, we critically show that dopamine is involved in non-associative types of learning, such as habituation of neutral stimuli. The novelty concept, as proposed by earlier theorists (e.g., (Kutlu & Schmajuk, 2012; Lubow, 1973a; Schmajuk et al., 1996)), is closely connected to prediction error as the source of novelty is ultimately the mismatch between prediction and actual occurrence of events. This is consistent with the literature suggesting that dopamine neurons in the VTA may compute sensory prediction errors (Morrens et al., 2020; Stalnaker et al., 2019) and even support formation of stimulus-stimulus associations (Sharpe et al., 2017). However, it is also possible that dopamine response patterns align more closely with non-associative terms, which are sensory adaptations rather than an associative process (Harris, 1943a), which the data within this manuscript support. Regardless, novelty and attention should be considered as principal components of the involvement of dopamine in associative learning and included in potential interpretation of data. Importantly, our results also suggest that more nuanced and updated understanding of predictions should be utilized when considering the role of mesolimbic dopamine in learning and memory.

3.6 Supplementary figures

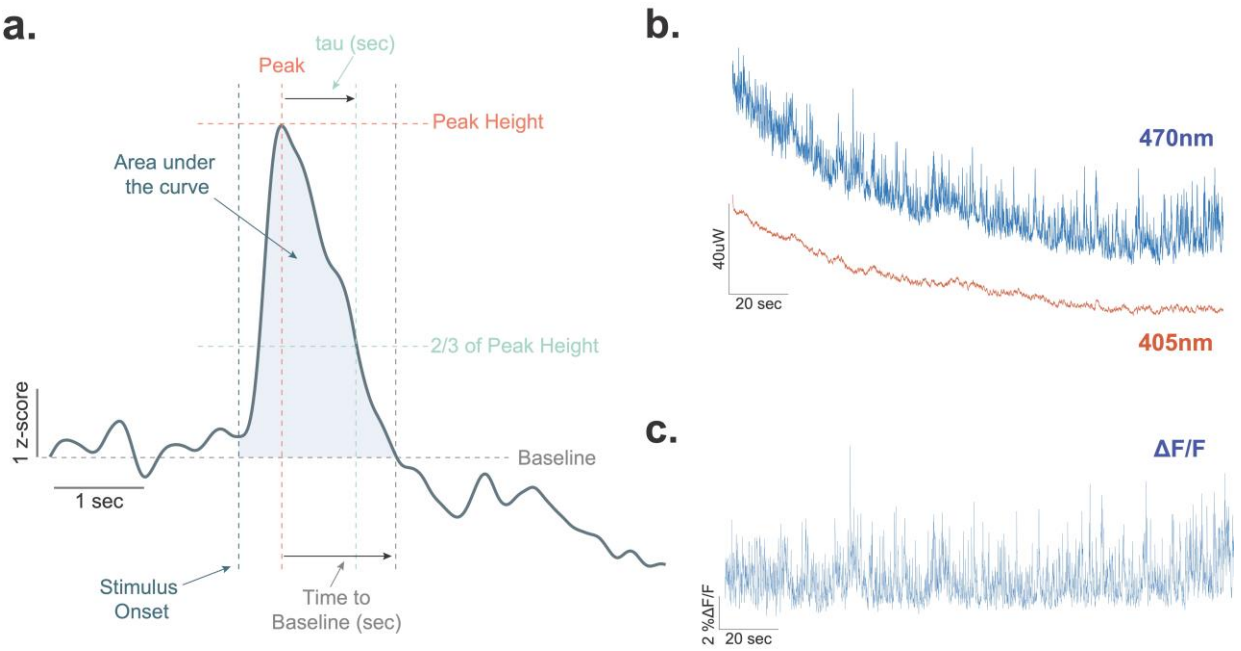

Fig. S13. Analysis of dopamine dynamics using fiber photometry.

(a) Diagram showing the methods used for calculating area under the curve, peak height, time to baseline, and tau. These analyses have been used extensively for defining the kinetics and dynamics of dopamine signals previously (Yorgason et al., 2012). Area under the curve (AUC) is the total area from stimulus onset to the return to baseline. Peak height is the maximal amount of dopamine that is evoked by the stimulus over the entire trace. Time to baseline is the time in seconds that it takes for the signal to return to baseline following the peak. Tau is the time it takes to return to 2/3 of peak height. **(b)** Representative traces for 470nm excitation (dLight) and 405nm excitation (isosbestic control) channels in an individual animal at baseline. **(c)** Representative ΔF/F trace showing dopamine transients in the nucleus accumbens core.

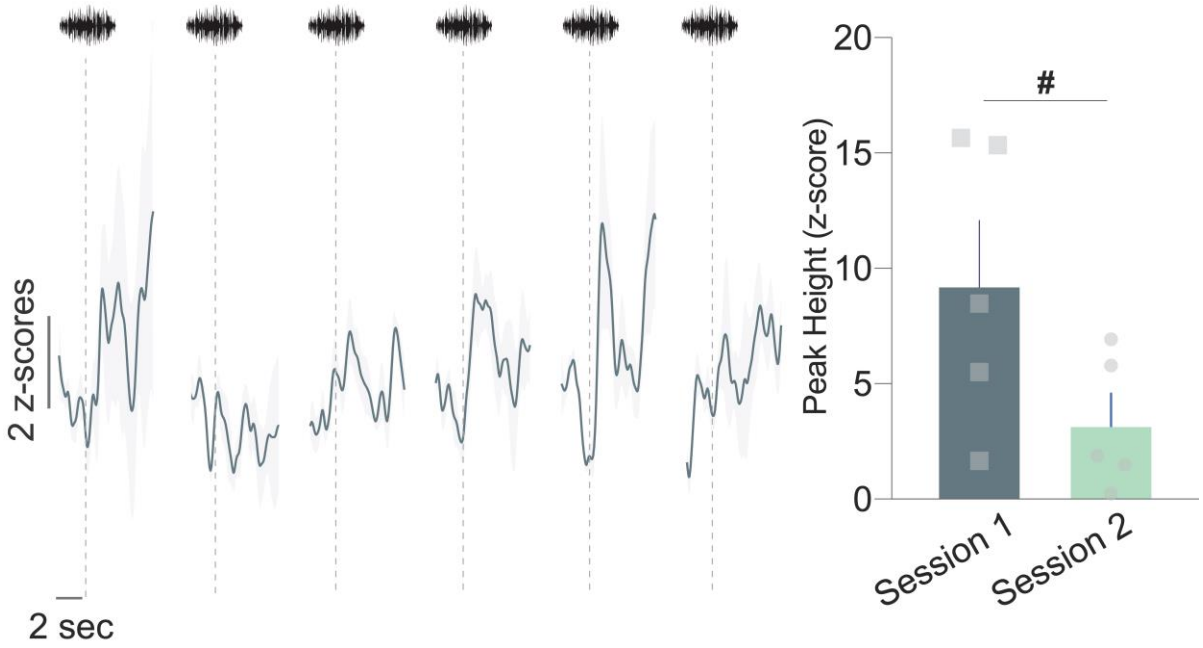

Fig. S14. Dopamine response to neutral cue during the second day of exposure.

Session 2 dopamine signal to repeated white noise presentations (6-7 presentations per animal). The first presentation of the neutral stimulus in Session 2 evoked a smaller dopamine response compared to the first presentation of the neural cue in the first session (Peak height for the first presentation of Session 1 vs. Session 2; paired t-test, t_4=2.429, p=0.07, n=5 mice). # p=0.0

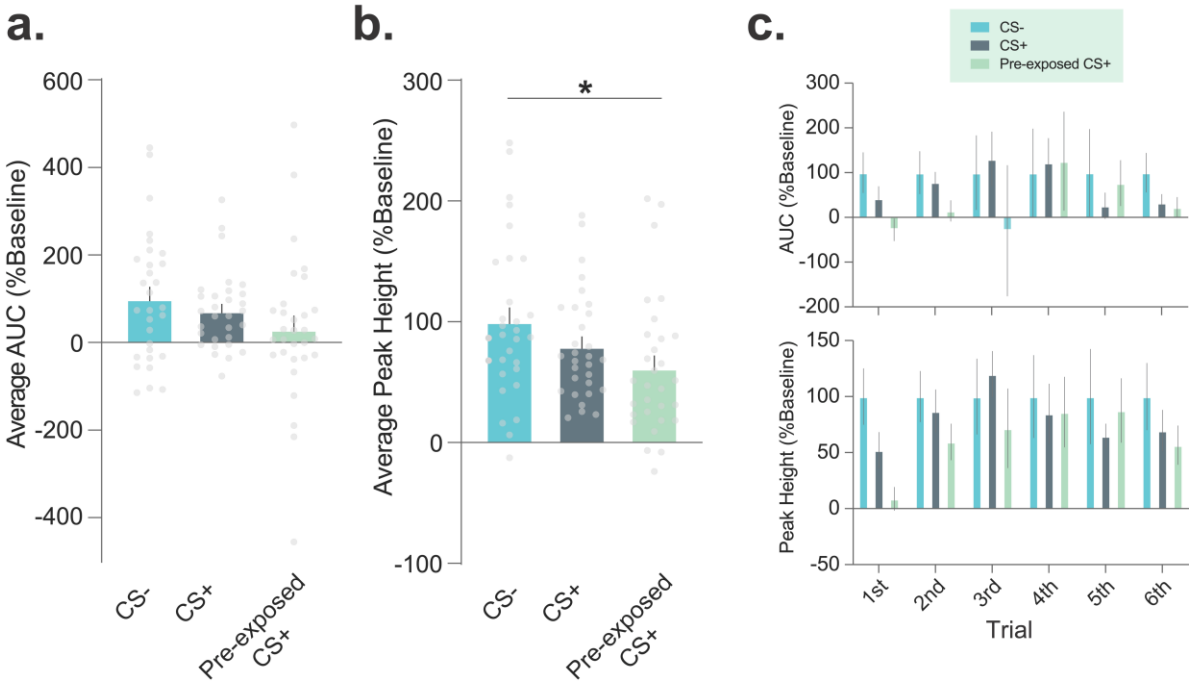

Fig. S15. Pre-exposure to stimuli decreases positive dopamine responses during subsequent fear conditioning.

(a) Fold change (in AUC) from average CS- values across 6 trials (Nested ANOVA, $F_{(2, 83)}$= 2.10 p=0.1287). **(b)** Percent change (in peak dopamine response) from CS- values across 6 trials (Nested ANOVA, $F_{(2, 83)}$= 3.91 p=0.0239). **(c)** Area under the curve (RM ANOVA Stimulus x Trial interaction $F_{(10, 72)}$= 0.42 p=0.92) and peak height values (RM ANOVA Stimulus x Trial interaction $F_{(10, 72)}$= 0.52 p=0.86; main effect of Stimulus $F_{(2, 72)}$= 3.26 p=0.04) for CS-, CS+, and pre-exposed CS+ computed as percent changes from CS- values. Data represented as mean ± S.E.M. * p<0.05.

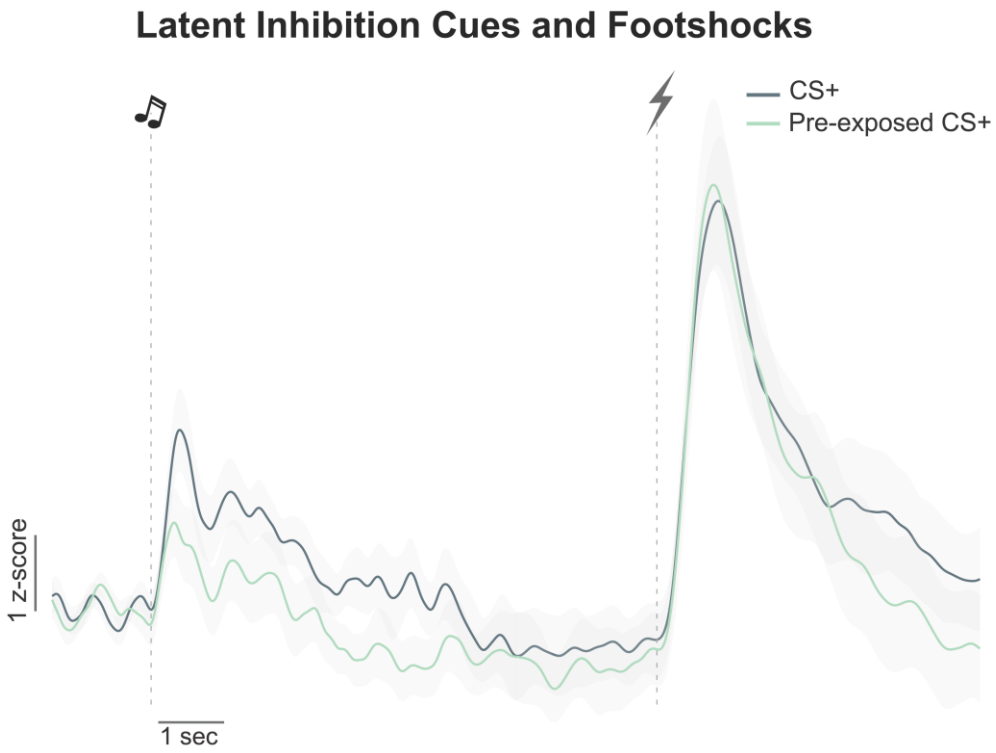

Fig. S16. Averaged dopamine responses during CS+ footshock and pre-exposed CS+ footshock trials during fear conditioning session 1.

Averaged dopamine response (z-scores) during the CS+ and pre-exposed CS+ cues and footshocks in the first fear conditioning session. The music note represents the cue onset and the lightning symbol denotes the footshock onset. Data is presented as mean ± S.E.M.

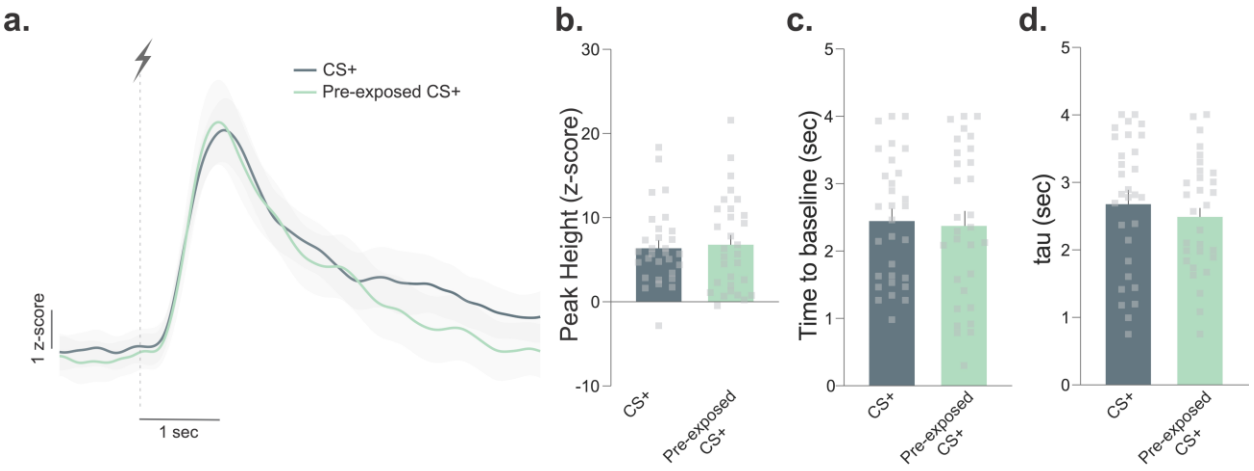

Fig. S17. Pre-exposure to the predictive cue does not affect dopamine response to the subsequent footshock.

(a) Averaged dopamine signal to footshocks following the CS+ and pre-exposed CS+ on fear conditioning session 1. **(b)** Peak dopamine response to the footshock following a pre-exposed or non-pre-exposed cue during session 1 (Nested ANOVA $F_{(1,54)}$= 0.13, p=0.3738), **(c)** time for the signal to return to baseline following peak evoked by the footshock across trial types did not differ (Nested ANOVA $F_{(1,54)}$= 0.10, p=0.7475), and **(d)** tau also did not differ between groups (Nested ANOVA $F_{(1,54)}$= 0.71, p=0.4040). Data represented as mean ± S.E.M.

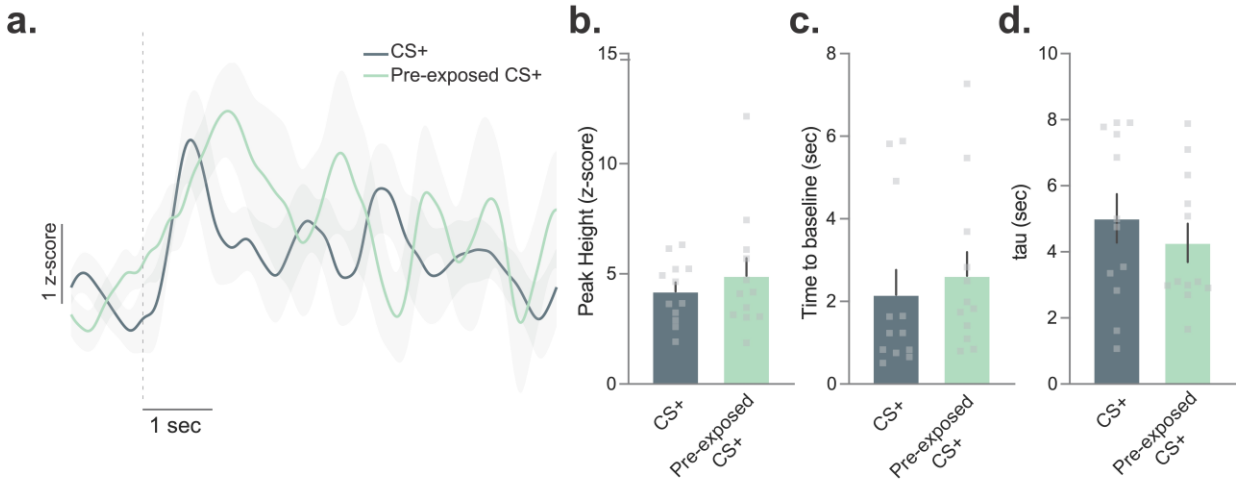

Fig. S18. Latent inhibition: In the absence of the latent inhibition effect, dopamine response to the pre-exposed and novel CS+ do not differ.

(a) Dopamine responses did not differ between the CS+ and pre-exposed CS+ for the animals that did not show latent inhibition. **(b)** The peak heights (Nested ANOVA, $F_{(1, 21)}$= 0.61 p=0.4449, n=30 presentations), **(c)** the time to return to baseline (Nested ANOVA, $F_{(1, 21)}$= 0.30 p=0.5888, n=30 presentations), and **(d)** tau were not different between the CS+ and pre-exposed CS+ (Nested ANOVA, $F_{(1, 21)}$= 0.60 p=0.4467, n=30 presentations). Data represented as mean ± S.E.M.

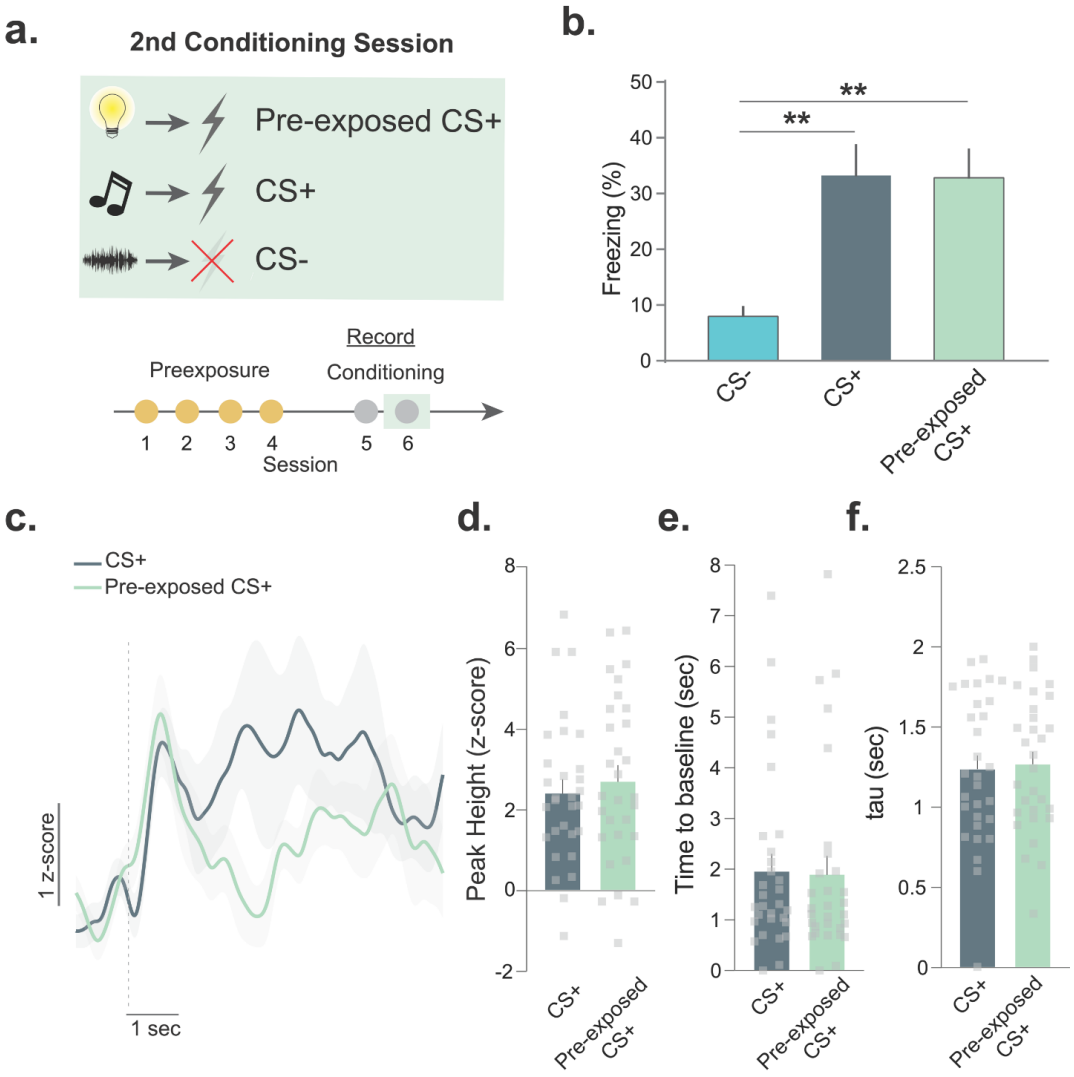

Fig. S19. Latent inhibition: Behavior and dopamine responses to non-pre-exposed and pre-exposed stimuli converge following extensive experience.

(a) On the second day of fear conditioning, the mice received the same training procedure as fear conditioning session 1. **(b)** Freezing responses to the pre-exposed CS+, non-pre-exposed CS+ (CS+), and non-pre-exposed CS- (CS-) were measured on session 2 of a two session fear conditioning paradigm (RM ANOVA pre-exposure main effect, $F_{(1.466,5.863)}$= 19.99, p=0.0032), the difference between the CS+ and pre-exposed CS+ disappeared on the second conditioning session (Tukey post-hoc, p=0.9979). Both the CS+ (Tukey post-hoc, p=0.0034) and the pre-exposed CS+ (Tukey post-hoc, p=0.0037) yielded a stronger freezing response compared to the CS-. **(c)** Averaged dopamine responses to the CS+ and pre-exposed CS+ during session 2 over all trials. **(d)** Dopamine responses did not differ between the CS+ and pre-exposed CS+ (Nested ANOVA, $F_{(1, 54)}$= 0.42 p=0.8901, n=30 presentations). **(e)** The time to return to baseline was not different (Nested ANOVA, $F_{(1, 54)}$= 0.07 p=0.7864, n=30 presentations). **(f)** Tau is another measure of dopamine clearance and is defined by the time in seconds for the signal to return to 2/3 of peak height. Tau was not different between the CS+ and pre-exposed CS+ (unpaired t-test, t_{58}=0.27, p=0.78, n=30 presentations). Data represented as mean ± S.E.M. ** p<0.01.

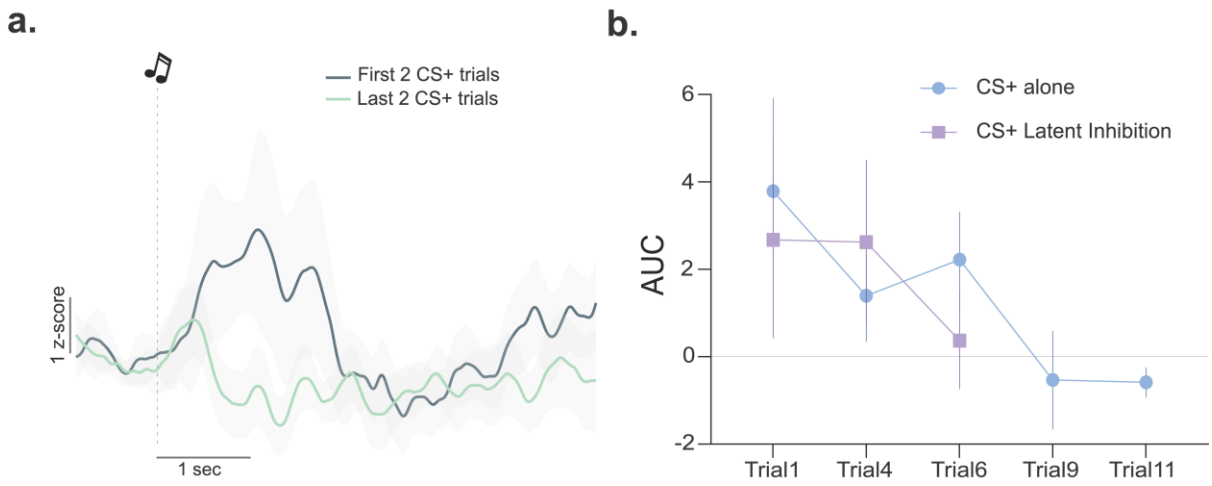

Fig. S20. Fear conditioning with additional trials yielded a negative dopamine response to the fear cues.

(a) Averaged dopamine signal to fear cues during the first two versus last two CS+ trials in a separate group of C57BL6/J mice (n=4). **(b)** Dopamine response to the CS+ (area under the curve, AUC) following 6 trials of the latent inhibition experiment compared to the dopamine response to the CS+ in an additional group with extensive fear conditioning trials did not differ for the first 6 trials (RM ANOVA Group x Trial interaction $F_{(2, 14)}$= 0.52 p=0.60; main effect of Group $F_{(1, 7)}$= 0.12 p=0.20) before becoming a negative response after the 9th trial. Data represented as mean ± S.E.M.

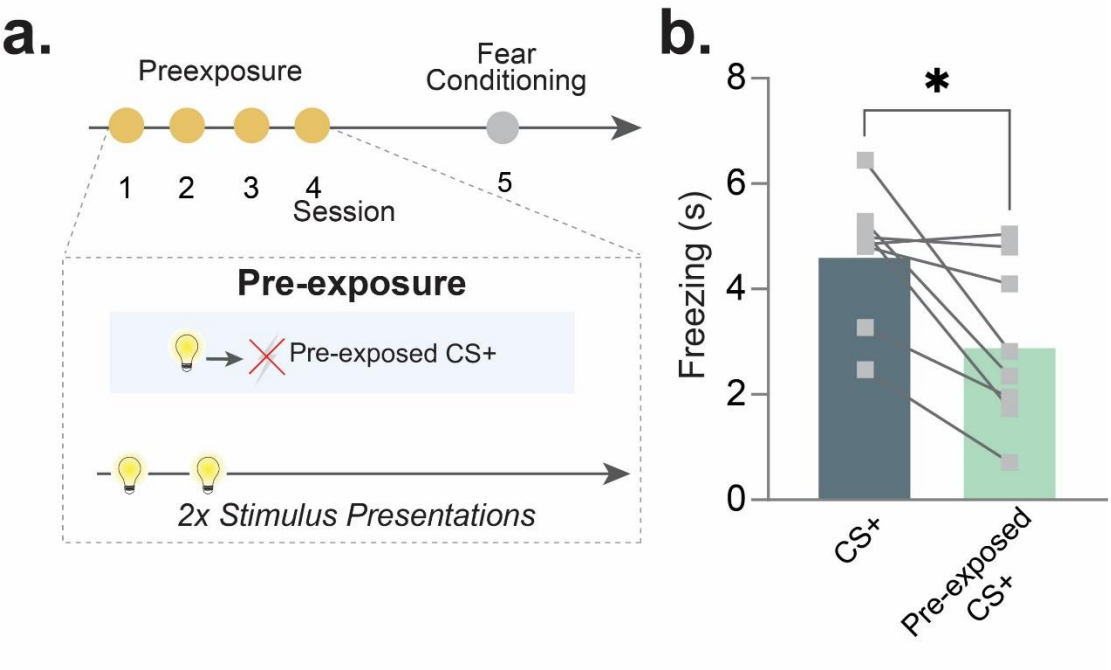

Fig. S21. Fewer pre-exposure presentations result in latent inhibition.

(a) Mice (4 male, 4 female) received two sessions of pre-exposure rather than four. **(b)** Fewer pre-exposure sessions still produced a latent inhibition effect (paired t-test t_7= 3.314, p=0.0129). Data represented as mean ± S.E.M., * p<0.05, ns = not significant.

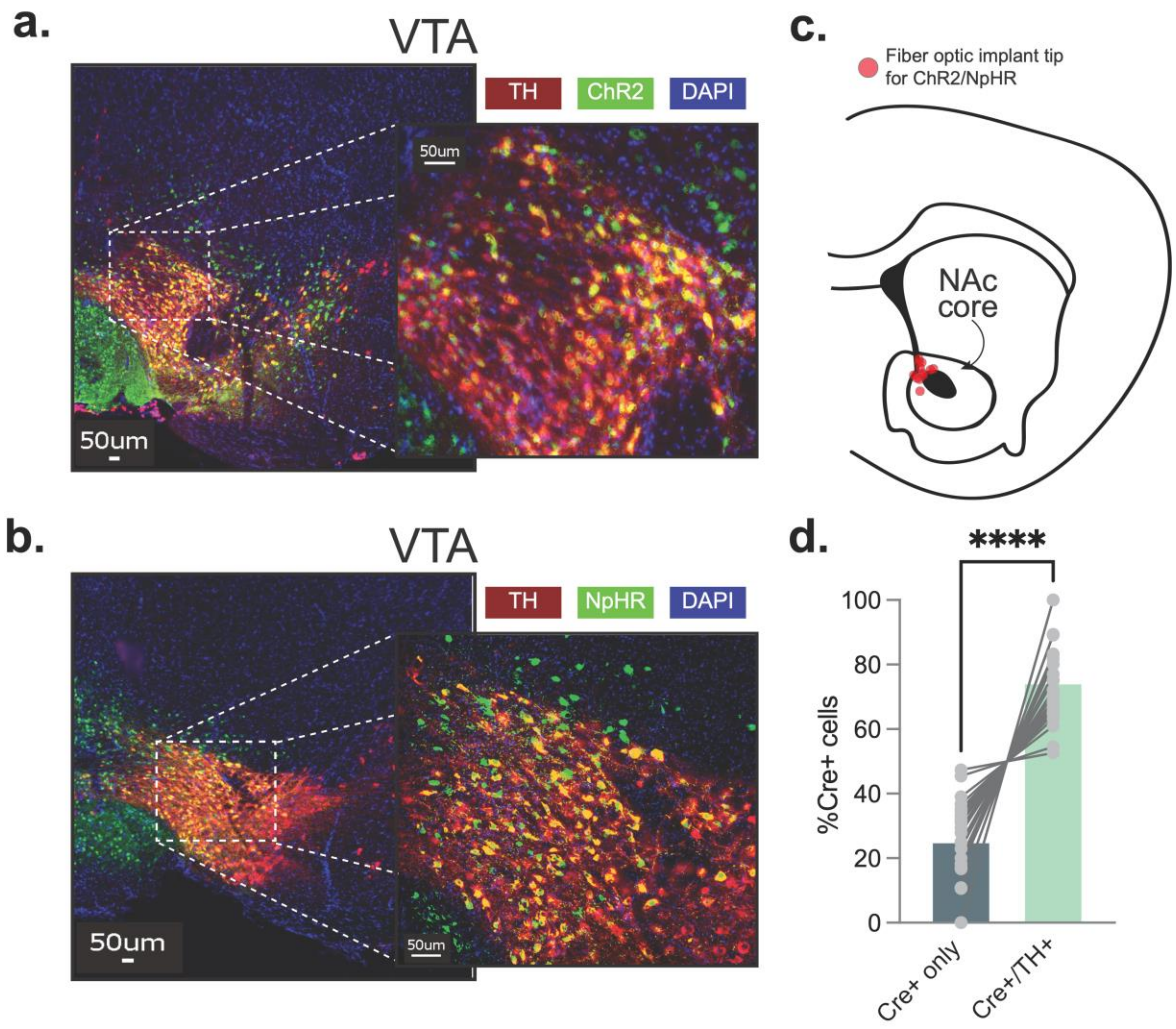

Fig. S22. Validation of TH+ cell-specific opsin expression.

Optogenetics studies were designed to test whether the latent inhibition effect is controlled by the NAc core dopamine response to the pre-exposed fear cue. **(a)** Representative images showing the expression of ChR2 and TH in the VTA dopamine cell bodies. AAV9.rTH.PI.Cre.SV40 and AAV5.Ef1a.DIO.hchR2.eYFP or AAV5.Ef1a.DIO.eYFP was injected into the VTA to achieve specific expression of Chr2 in dopamine neurons. Specifically, AAV9.rTH.PI.Cre.SV40 injections resulted in Cre expression in all Tyrosine Hydroxylase (TH) positive cells within the VTA. By placing a fiberoptic above the NAc core, we were able to stimulate dopamine release from VTA projecting dopamine terminals in the NAc core. **(b)** Representative images showing the expression of NpHR and TH in the VTA dopamine cell bodies using the same approach as described. AAV9.rTH.PI.Cre.SV40 and AAV5.hSyn.eNpHR.3.0.eYFP or AAV5.Ef1a.DIO.eYFP were injected into the VTA and a fiberoptic was placed in the NAc core. **(c)** Schematic showing histologically verified fiber optic placements for all mice (9 male, 12 female). **(d)** Cell counts were completed within the VTA from the experiments using the TH-specific excitatory/inhibitory opsin strategy. About 75% of the Cre+ cells in the VTA were also TH+ suggesting a significant portion of the ChR2 and NpHR cells were dopaminergic (paired t-test t_{22}= 8.96, p<0.0001). Data represented as mean ± S.E.M., **** $p < 0.0001$.

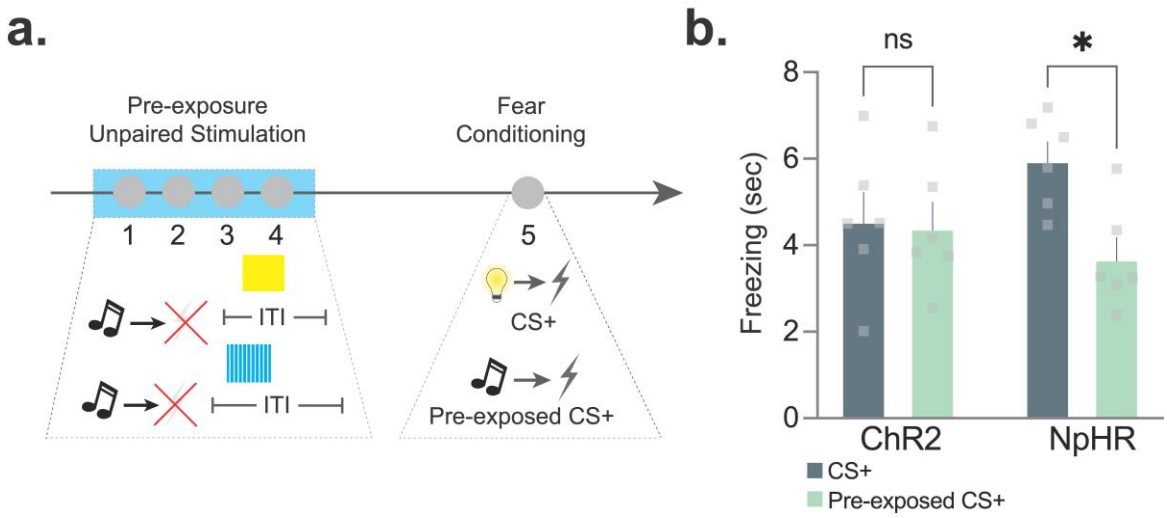

Fig. S23. Optogenetic stimulation, but not inhibition, of dopaminergic terminals during inter-trial interval abolishes latent inhibition.

(a) Mice (5 male, 7 female) underwent four sessions of pre-exposure where they received unpaired stimulations (ChR2) or inhibitions (NpHR) during inter-trial interval windows. **(b)** Unpaired stimulation of the NAc core dopamine response abolished latent inhibition (2-way ANOVA cue x group interaction $F_{(1,10)}$=4.078 p = 0.071; Bonferroni multiple comparisons: ChR2 pre-exposed vs. non-pre-exposed p=0.973; NpHR pre-exposed vs. non-pre-exposed p = 0.023) while inhibition of the terminals resulted in a latent inhibition effect. Data represented as mean ± S.E.M., * p<0.05, ns = not significant.

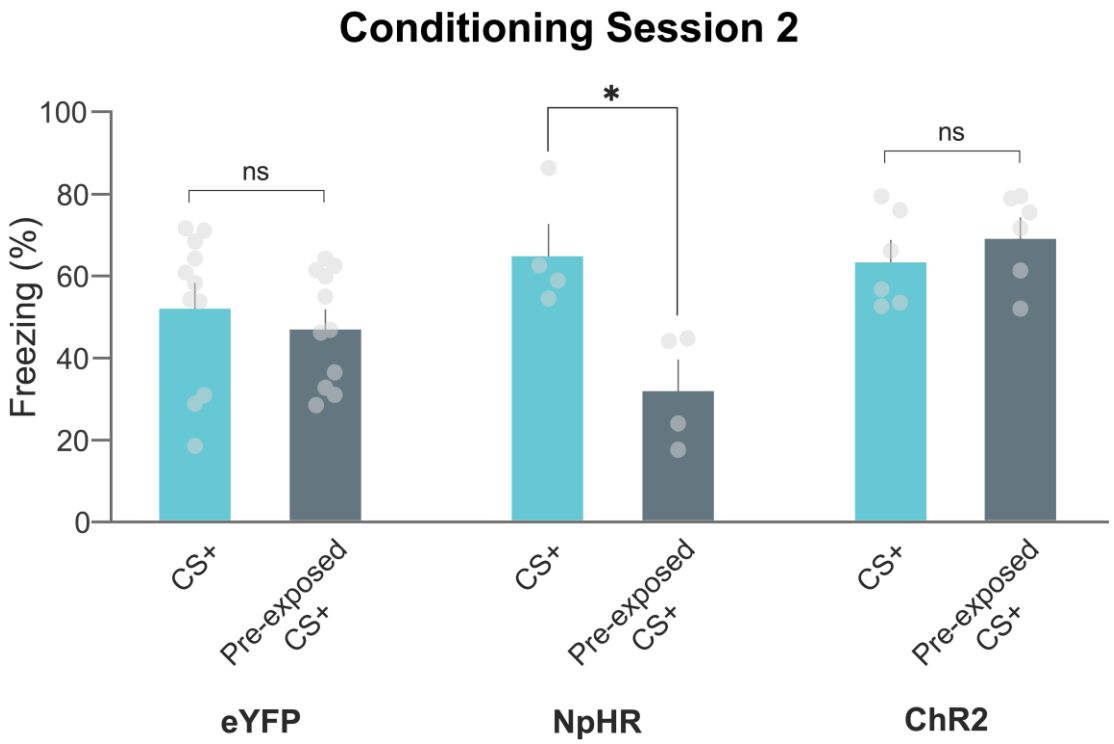

Fig. S24. The effect of the optogenetic inhibition and excitation of dopaminergic terminals disappears with additional fear conditioning training.

Freezing response to the CS+ and pre-exposed CS+ did not differ in the eYFP or ChR2 groups on the second session of fear conditioning (Multiple comparison *p*s >0.05). Freezing to the CS+ was still greater than the freezing response to the pre-exposed CS+ at the end of the session 2 (2-way ANOVA cue x group interaction $F_{(2,36)}$=4.31, p = 0.02; Multiple comparisons: NpHR pre-exposed CS+ vs. CS+ p=0.04). This suggest that the freezing response to all cues (pre-exposed and non-pre-exposed) reached the asymptotic level with additional training but the enhancing effect of dopamine inhibition during pre-exposure on latent inhibition persisted beyond the initial fear conditioning session. Data represented as mean ± S.E.M., * p<0.05, ns = not significant.

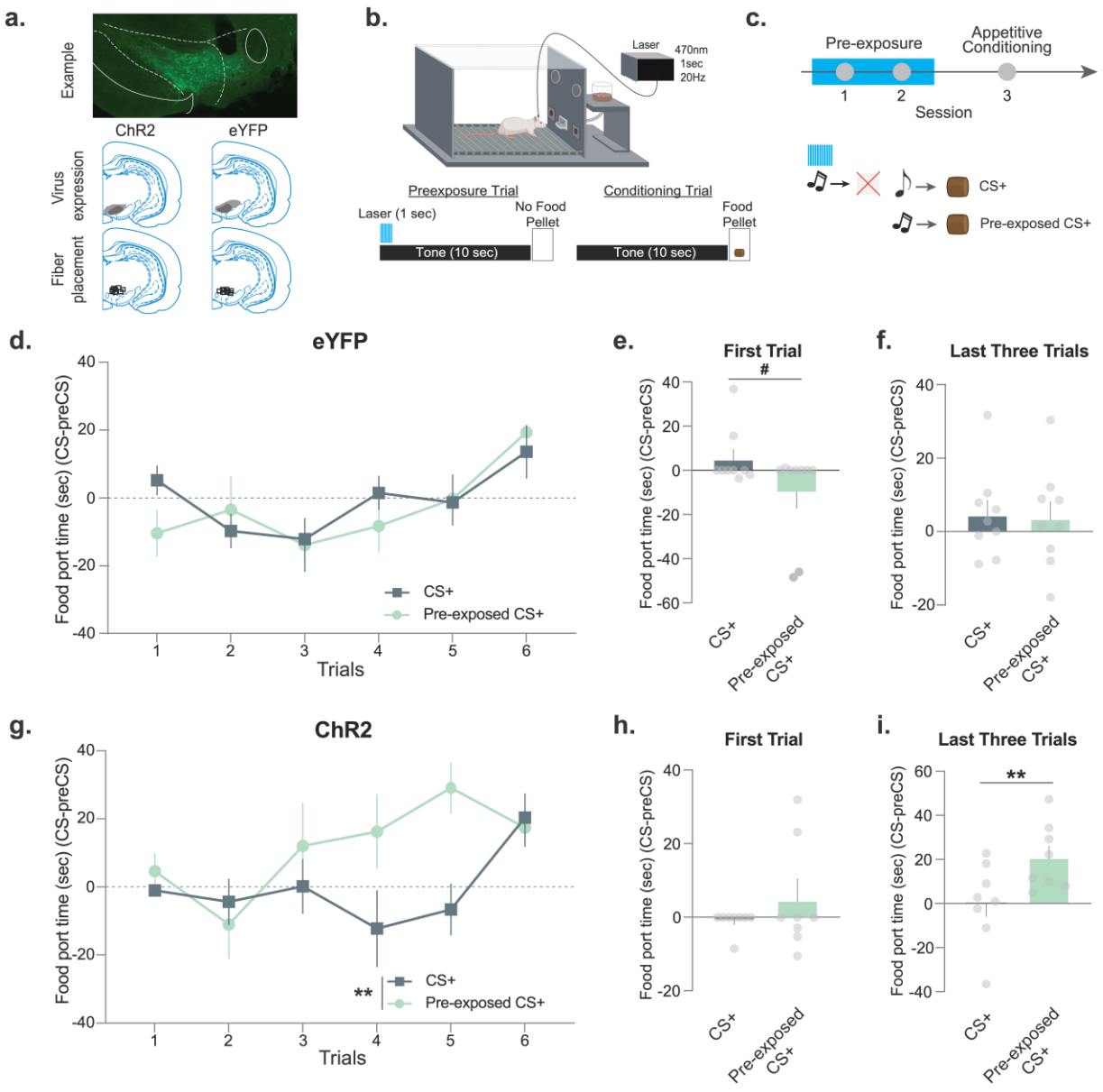

Fig. S25. Optogenetically stimulating VTA dopamine cell bodies during cue pre-exposure enhances subsequent associative learning for that stimulus.

(a) Representative histology showing ChR2 expression in the VTA dopamine cells in the TH-Cre rats. Histology maps showing ChR2 and eYFP expression and fiber placements in the VTA. **(b)** These experiments were designed to look at the effects of dopamine stimulations during the pre-exposure period when the cues are novel and have not yet acquired value. Ventral tegmental area (VTA) dopamine neurons were stimulated using a blue laser at the time of the cue presentation during pre-exposure sessions. **(c)** Rats received 2 sessions of stimulus pre-exposure followed by a single session of appetitive conditioning without any stimulation. In the pre-exposure session, the auditory cue was presented in the absence of an outcome whereas in the conditioning sessions, both the pre-exposed and non-pre-exposed cues were followed by the delivery of a food pellet. **(d)** Averaged responses (Appetitive response = CS response - preCS

response) for the eYFP group throughout the 6 conditioning trials (Repeated Measures session x group interaction ANOVA $F_{(5,80)}$=0.78 p = 0.56). **(e)** The difference between the first trial responses to the pre-exposed and non-pre-exposed cues trended towards significance in the eYFP group (paired t-test, t_8=2.13, p =0.06, n=9 rats). **(f)** There was no difference between pre-exposed versus non-pre-exposed cue responses during the last 3 trials of the conditioning session in the eYFP group (paired t-test, t_8=0.25, p =0.80, n=9 rats). (**g)** Averaged responses for the ChR2 group throughout the 6 conditioning trials (Repeated Measures session x group interaction ANOVA $F_{(5,70)}$=2.42, p = 0.04). **(h)** The difference between the first trial responses to the pre-exposed and non-pre-exposed cues did not differ in the ChR2 group (paired t-test, t_7=1.11, p =0.30, n=8 rats). **(i)** The pre-exposed cue responses were significantly higher compared to the non-pre-exposed cue responses during the last 3 trials of the conditioning session in the ChR2 group (paired t-test, t_7=0.008, p =0.02, n=8 rats). This demonstrates that stimulation of the VTA dopamine cell body response to stimuli during pre-exposure enhances the learning of cue-reward associations in the subsequent appetitive conditioning training. Data represented as mean ± S.E.M., # p=0.056, ** p<0.01.

CHAPTER 4

Discussion

5.1 D1 and D2 MSNs encode learning parameters necessary for associative learning

One of the primary aims of my PhD work was to define the information encoded in D1 and D2 MSNs – first, by showing that they do not have opposing roles (in action or valence) in the NAc core and second, by identifying whether there are stable and dynamic properties of learning represented by these subpopulations. We showed that D1 MSNs respond to stimulus saliency and presence and that D2 MSNs respond to predictions and prediction errors.

As noted, we showed that D1 and D2 MSNs do not encode valence, as they both support reinforcement, respond to aversive stimuli, and respond in reward and aversive learning tasks (**Chapter 2; Fig. 1-2**). We next showed D2 MSNs respond to predictions and prediction errors, as the signal to the cue in D2 MSNs supports the association between the cue and the outcome (**Chapter 2; Fig. 2-3**). Finally, we showed that the D2 MSN response to the cue is necessary for the development of a cue-outcome association in fear learning (**Chapter 2; Fig. 6**).

One of the primary papers I noted that showed D2 MSNs do not encode opposing valence demonstrated that optogenetic stimulation of D2 MSNs to a cue in a progressive ratio task increased the number of cumulative presses and the breakpoint (Soares-Cunha et al., 2016). This fit nicely with our data, although it is interesting to note that in our task design mice first acquired the task and that stimulation occurred after mice met criteria. They additionally demonstrated that

D2 activation during the inter-trial interval did not change the breakpoint, and furthermore, that if you instead inhibited these neurons during cue exposure (15mW constant light 10s), it reduced the number of cumulative presses and the breakpoint (Soares-Cunha et al., 2016).

In their 2022 paper, Soares-Cunha et al. probed the timing of optical activation and inhibition in a progressive ratio task in a specific projection population. Stimulating D2 MSN-VP projections during the reward-predictive cue and during the reward delivery had opposing effects on behavior, whereby activation to the cue enhanced breakpoint and activation to the reward decreased the breakpoint. The converse was true with inhibition (Soares-Cunha et al., 2022). This data fits well with D2 MSNs encoding a prediction/prediction error. Stimulating to the cue reinforces the strength of the association, while stimulating to the reward induces an error signal.

We thus concluded the timing of this signal in D2 MSNs is key to the behavioral outcome. This may explain disparate outcomes in studies where D2 MSNs are inhibited in discrete windows or for entire behavioral sessions. Nishioka et al. (2021) trained mice in a visual discrimination-based cue-guided attendance learning task and an avoidance learning task to probe the neural mechanisms underlying the strategy to choose a good option and the strategy to avoid a bad option – as these may represent separate processes even if resulting in the same choice behavior. They expressed an inhibitory designer receptor exclusively activated by a designer drug (iDREADD (hM4Di-mCherry)) in D1 and D2 MSNs of the NAc core during two tasks. In the first, a response during the correct cue (visual cue A) resulted in a reward and a response to an incorrect cue (a random image) resulted in a time-out (VD-attend). The second task was the inverse (VD-Avoid), in which the random image represented the correct cue and the mouse must withhold responding during the incorrect cue (visual cue B). Essentially, to perform on the VD-avoid task, mice do not directly learn the correct cue but get the reward by avoiding the incorrect cue (visual cue B). Nishioka et al. (2021) found that chemogenetic inhibition of D1 MSNs during of the session, after mice were well-trained, decreased their performance on both the VD-Attend and VD-Avoid tasks. However, chemogenetic suppression of D2 MSNs decreased performance

only on the VD-Avoid task, suggesting that in fact choosing a good option and avoiding a bad option are independent processes and that disruption in the D2 MSNs selectively affects the avoidance of a bad option (Nishioka et al., 2021).

However, as our data indicates, the timing of this signal is critical, especially as it relates to encoding learning parameters. Chemogenetic inhibition, while providing support for the importance of D1 and D2 MSNs to a task that requires associative learning, is nonselective in the timing of its inhibition. Secondly, the authors are looking at the execution of behavior, not the acquisition of it. They are suppressing activity at a time when mice have already learned the task parameters. Given the nature of the role we think D2 MSNs play in prediction-based signaling, the authors may have seen a delay in learning of the VD-Attend task contingency had they suppressed activity during the learning phase (Nishioka et al., 2021).

When the authors probed this effect further and inhibited (via archaerhodopsin (ArchT) just during the outcome period on an error trial in the VD-Avoid task, they found a decrease in performance on the subsequent trial. However, this effect was only significant on the trials that followed an error. This makes sense given a met prediction would not result in a response in the D2 MSNs during the outcome. However, on an error trial, inhibition would ablate the signal produced to an unmet prediction, thus altering the mouse's performance on the subsequent trial (Nishioka et al., 2021).

We next performed cellular resolution calcium imaging of D1 and D2 MSNs via miniature endoscopes (**Chapter 2; Fig. 5**). We identified a heterogeneity among responses in D1 and D2 MSNs also identified by Nishioka et al. It is important to note that Nishioka et al. utilized a D2-Cre mouse line. As other populations in the NAc express D2 receptors (cholinergic interneurons), it is possible that some of the cells recorded were not in fact D2 MSNs (Gallo et al., 2018). They classified cells as Type I (sustained activation after making an error choice in the VD-Avoid task) and Type II (activated in a correct trial). 60% of the D2 MSNs were categorized at Type I or responding to the error. Conversely, the majority of D1 MSNs (56%) were activated by the correct

choice (Type II). The authors did show that D2 MSNs predominantly encoded the error and D1 MSNs predominantly encoded the correct choice (the sucrose reward) (Nishioka et al., 2021). This would fit with our data indicating that D1 MSNs respond to stimulus presence. They are not necessarily encoding the outcome but encoding the presence of the reward on a correct trial.

One of the interesting potential risks associated with D2/D3 agonists used in treatment for patients with Parkinson's disease is gambling (Dodd et al., 2005), an effect that makes considerable sense given the role we identified of D2 MSNs in prediction error. Risk preference is modulated by prior outcomes and a hallmark feature of both rat and human behavior is that a win promotes a "stay" strategy whereas a loss promotes a "switch" strategy (Hayden et al., 2009; Niv et al., 2012).

Zalocusky et al., 2016 probed the effect of D2 MSNs in a model of risk-preference in rats. Rats were presented with both a 'safe' lever (same volume of sucrose every trial) and a 'risky' lever (75% of trials small reward; 25% large reward). They first isolated the contribution of D2Rs in rats, showing that a D2R agonist increased risk-seeking behavior. Trials were initiated with what the authors termed a 'decision-period', a 1s nosepoke hold preceding the lever press. From this task, the authors classified rats as risk-seeking or risk-averse, although both groups were inclined to choose risk after a gain as compared to a loss (Zalocusky et al., 2016).

If the rat, on the prior trial, had experienced a loss, the D2 MSN response to the decision period on the subsequent trial would be larger than had the rat experienced a win or had chosen the safe outcome. However, the signal was also higher if the rat was about to make a safe choice over a risky one. The authors also looked at forced choice trials, where the rat was only given the option of one lever in a trial. There appeared to be a loss signal continually represented during the decision period on the forced choice risky lever trials. This leads to a somewhat confusing result that the decision period can both represent a recent loss and also whether the rat will make a safe choice on the subsequent trial (Zalocusky et al., 2016). The decision period technically represents a cue, so the magnitude of the response could reflect the prediction of the stimulus or

the outcome (prediction error) on a previous trial. However, the nature of this task design makes it difficult to determine what the decision period signal means. The decision period requires an action that cannot be dissociated from the trial onset and the signal increases prior to the nosepoke. Instead, if a trial were initiated with an auditory cue, we could better dissociate the trial onset from motivated action.

But perhaps more interestingly, the authors only probed the D2 signal during the decision period, and not on the outcome. I would be curious to see the D2 MSN calcium signal on the trials in which the reward was larger than expected (error signal) or on trials in which the reward is the anticipated amount (no error signal). I am inclined to think that you would identify similar trends in D2 MSNs as we see in our own data (**Chapter 2; Fig. 4**) if the authors were to probe their data around the reward retrieval.

Our results have also caused us to reconsider data in a new light. For example, Calipari et al., (2016) identified a differential effect of D1 and D2 MSN activity in entering a chamber that was paired with cocaine. D1 MSN activity increased prior to entry into the drug-paired chamber and D2 MSN activity decreased following paired side entry (Calipari et al., 2016). Prolonged drug use can have an interesting effect on behavior, whereby the stimuli that predict drugs can become reinforcing themselves. For example, chronic cocaine induces a state where contexts associated with it engage the same neuronal mechanisms as acute experience. This is critical when thought of in relation to relapse – this circuitry is adapting in a way that supports strengthened cue–drug associations that ultimately drive relapse (Grimm et al., 2001). If entering the cocaine-paired side of the chamber becomes a reinforcing outcome, and not just a cue, then perhaps D2 MSNs encode an error prediction signal. The entry into the drug-paired chamber is then a predicted outcome, and so we see a moderate decrease in D2 MSN activity (Calipari et al., 2016).

We showed that D2 MSNs are necessary for the development of cue-outcome associations. However, this does not negate the role that other brain regions have in supplying information critical to cue-outcome associations. Our data indicates that the NAc serves as an

integrator, thus disruption of signals in a brain region that compiles and relays **information to motor nuclei output** would likely result in the blockade of a cue-outcome association. Furthermore, not all of the learning components necessary for associative learning (we have not recorded from other populations like interneurons in the NAc core), are represented in the NAc. Memory is likely not stored in the NAc (likely in the hippocampus) nor do we know if novelty is represented elsewhere. Lastly, we did not test the sufficiency of D2 MSNs to the cue-outcome association. To test this, we would have an unpaired cue and shock and stimulate (channelrhodopsin – ChR2) D2 MSNs to the cue. The cue and shock would then be associated (measured by increased freezing to the cue) only in the ChR2 group but not in EYFP control.

D1 and D2 MSN activity in other ventral striatal structures show some similarity to ventral activity.

Work from other ventral striatal brain regions has indicated roles for D1 and D2 MSNs in encoding learning parameters, suggesting there may in fact be conservation of MSN roles across other subregions. As noted above, D2 MSNs in the ventrolateral striatum responded to both a cue (presentation of two levers) and a food pellet reward (Natsubori et al., 2017). The authors did not look at how this signal evolved over learning. They did, however, show that optogenetic inhibition of both D1 and D2 MSNs with the trial start reduced the break point. While we did not see this effect with optogenetic inhibition of D1 MSNs on fear conditioning (**Chapter 2; Fig. 6**), I would suggest that collateral transmission between D1 and D2 MSNs may actually explain the importance of both of these populations to the enhanced motivation – a notion I will cover in the following section. Interestingly, optogenetic inhibition during the lever press selectively reduced the breakpoint only in D1-cre mice.

Martiros et al. identified representations of cue-outcome associations in the D1 and D2 neurons in the olfactory tubercle of the ventral striatum. This brain region receives dopaminergic input from the VTA and is also known to be involved in reward processing (Gadziola & Wesson, 2016; Ikemoto, 2003, 2007; Wesson & Wilson, 2011; Zhang et al., 2017). Martiros et al. conducted

two-photon calcium imaging with a GRIN lense of D1 and D2 receptor expressing neurons during odor-outcome associations. Five separate odors (cues) were paired with rewarding, aversive, or neutral outcomes (odors 1-2 paired with aversive airpuffs at two intensities; odor 3 with neutral stimulus; odors 4-5 with reward water drops at two intensities) (Martiros et al., 2022).

They showed D1- and D2-type neurons both responded to the odorant cues that predicted rewarding or aversive stimuli. They identified a subset of D1-type neurons that responded to the valence of the odorant cue-outcome association, a possibility that may be true in the NAc as well. Additionally, D1-type neurons did not respond to the saliency of the odorant cue that predicted differing intensities of the same stimulus (very few D2-type neurons responded to saliency either), although they did not look the at the D1-type neuron response to the outcome stimulus itself (Martiros et al., 2022). Interestingly, D2-type neurons responded to odorant identity and showed a training-dependent increase in identity scores. This suggests learning-related plasticity in the olfactory tubercle. This data, while not replicating what we see in the NAc core (and as would not be necessarily expected) does indicate a role of D1- and D2-type neurons for encoding parameters important for associative learning in another ventral striatal structure.

5.1.1 MSN activation patterns matter

There are important caveats to note in studies I referenced in the introduction that deserve re-evaluation in light of the work presented here and by others. Cole et al. showed that both D1 and D2 MSNs support reinforcement, as we identified (**Chapter 2; Fig. 1I-K**). Their data also paralleled with trends we saw in our own – including higher levels of reinforcement for D1 MSNs than for D2 MSNs (**Chapter 2; Fig. 1I-K**). However, while D2-ChR2 mice self-stimulated in a spout-touch task, they did not show positive self-stimulation in a location task, suggesting that D2 MSNs support reinforcement under some, but not all, contingencies. These findings also support

the notion that the activation pattern and context are key for the resulting behavioral outcome of D1 and D2 MSN activity.

In the Bock et al., (2013) paper, the bidirectional effects on behavior of D1 and D2 MSNs on progressive ratio following cocaine self-administration were dependent on prolonged drug use and/or schedule. Inhibition of D2 MSNs (hM4D1-DREADD) on an FR1 schedule of cocaine administration did not alter daily cocaine intake. Additionally, systemic administration of the D2-like agonist quinelorane did not change the breakpoint value for cocaine during progressive ratio sessions (Bock et al., 2013b). This may indicate that the bidirectional effects of D1 and D2 MSNs on behavior are the result of molecular, transcriptional, and physiological changes in D1 and D2 MSNs following prolonged drug use that alters normal/adaptive signaling in these circuits.

Furthermore, as an extension of the point that activation patterns of neuronal populations are key to the resulting behavior, optogenetic stimulation (ON, ChR2, 10ms pulses, 16.6Hz, 10 min OFF, 5 min ON throughout session) of D2 MSNs in the NAc core suppressed cocaine self-administration when normalized to the OFF period. Again, these are extremely long stimulation paradigms. While the authors do show downstream inhibition of ventral pallidum neurons with laser stimulation in ex vivo slices, this is brief stimulation (0.5 ms duration pulse at 16.6Hz), drastically shorter than for the in vivo experiments (Bock et al., 2013b). As we've shown (**Chapter 2; Fig. 2-6**), the temporal nature of the D2 MSN signal is key to its affect. Prolonged activation, which could result in rundown, could thus be disturbing important signals related to the execution of the learned cue-response-outcome association.

Regarding the hypothesis that stable, plastic changes in D1 and D2 MSNs could result in bidirectional roles of D1 and D2 MSNs, this was also observed in a model of chronic social defeat stress (CSDS). The resilience effect seen upon stimulating D1 MSNs following CSDS was only observed in the group identified as susceptible to social defeat stress, indicating innate potential differences in circuit signaling (Francis et al., 2015)

5.1.2 Multiple mechanisms permit direct and indirect communication between D1 and D2 MSNs that can be updated over time.

As we have noted, the temporal nature of the D2 MSN signal is key. This led us to the hypothesis that perhaps there is coordinated activity or population synchrony between D1 and D2 MSNs, suggestive of ensembles. Perhaps activity in one population alters activity in another in a way that is consequential for the development of associations between a cue and outcome. Essentially, D1 MSNs prime the system to say “this is an important stimulus, we should facilitate associations with cues and contexts that predict this stimulus”. Lateral inhibition, a previously underappreciated relationship that results from collateral transmission between D1 and D2 MSNs, offers a potential explanation for this information transfer.

Lateral inhibition results from the inhibitory synapses striatal MSNs make with nearby striatal neurons through axon collaterals. The extensive collateral plexus within the striatum has until recently been an under-appreciated aspect of MSNs (H. T. Chang & Kitai, 1985; Pennartz et al., 1991), largely due to what was considered a sparse amount of connectivity (34%, (Taverna et al., 2004)) between MSNs, emphasis on fast spiking GABAergic interneurons as the primary source of inhibition (Tepper et al., 2010; Tepper & Bolam, 2004), and a strong focus on MSN projection targets. These are unidirectional connections on distal dendrites of MSNs rather than on the soma as seen with FSI inputs (Wilson & Groves, 1980). Additionally, the majority of these synapses are D2 onto D1 MSNs, D2 onto D2 MSN, and D1 onto D1 MSN (Planert et al., 2010; Taverna et al., 2008).

For the supposed sparse amount of connectivity, an individual MSN receives approximately 1,200 to 1,800 contacts from other MSNs (Wilson, 2007). Thus, MSN collateral transmission, through co-activation of multiple MSNs, is poised to modulate striatal output through inhibitory actions on synaptic partners. Bock et al., (2013) showed that brief stimulation of indirect pathways D2 MSNs resulted in IPSCs in neighboring striatal MSNs (putative D1 MSNs) (Bock et al., 2013b). This has also been shown in slice, where excitation of D2 MSNs via channelrhodopsin

inhibited D1 MSN firing (Dobbs et al., 2016). Interestingly, pharmacological inhibitors of D2 receptors suppress this lateral inhibition, resulting in D1 MSN disinhibition (Dobbs et al., 2016). Dobbs et al., (2016) also showed that cocaine impacts the D1 → D2 MSN collateral transmission, mediated by D2 receptors, and resulting in greater excitability of D1 MSNs.

One of the questions we did not fully address in the NAc core D1-D2 MSN story was whether D1 MSNs may in fact function as a perceived saliency signal, rather than just as a saliency detector. Essentially, does heightened activity in D1 MSNs to a stimulus correlate to either a heightened likelihood for the development of a cue-outcome association or to the strength of a developed association? Cocaine, as well as the stimuli and contexts that predict it, are salient stimuli, which may be reflected in D1 activity and the ultimate strength of a cue-outcome association that depends on D2 MSN activity.

Lateral inhibition and collateral transmission primarily reflect a D2 to D1 MSN relationship, but there is evidence that activity in D1 MSNs can shape D2 MSNs through cholinergic interneurons. High-frequency excitation of D1 MSNs induces substance P release (an excitatory peptide selectively expressed in D1 MSNs), exciting cholinergic interneurons, and which (through GluR2-lacking AMPAR insertion), in concert with glutamatergic excitation, results in long-lasting, post-synaptic enhancement of glutamatergic drive on D2 MSNs (Francis et al., 2019). The excitatory activity in D1 MSNs is thus conserved and transmitted to D2 MSNs. This is particularly exciting when you consider what circumstances biologically would result in substance P release to drive this mechanism. Drugs of abuse and stressful events have both been shown to (Commons, 2010; Sandweiss & Vanderah, 2015). As a consideration, D1 to D2 MSN information transfer may not be necessary to form weak or neutral associations, but this may be a threshold mechanism by which very salient stimuli (or subjective perception of such) form fast and strong associations.

Collateral transmission as well as excitatory potentiation through interneurons suggests an ongoing relationship between D1 and D2 MSNs, in which the information encoded in one can

alter or update information in the other. We may not know the specific dynamics of this relationship, but the existence of these connections suggests that perhaps D1 MSNs provide salient information about a stimuli that can then inform how likely D2 MSNs are to form an association with a cue that predicts that stimulus. Soares-Cunhas et al., (2018) showed that enhanced motivation in a progressive ratio task, mediated by optogenetic activation of D2 MSNs, required both D1 and D2 receptor activation (Soares-Cunha et al., 2018). Thus, there is a strong case for local coordinated activity of MSNs being consequential to the ultimate behavioral output of an animal.

As a final note, one of the interesting consequences of the projection of both D1 and D2 MSNs to the ventral pallidum (Kupchik et al., 2015) (the canonically indirect pathway thought to be involved in action inhibition or aversion), is that the D1 and D2 MSNs could both inhibit or disinhibit the thalamus. Natsubori et al., (2017) proposed a model for this, although it is important to note they conducted these studies in the ventrolateral striatum. They concluded that D1 and D2 MSN projections to the VP were involved in action initiation (as they both responded to the trial start in a progressive ratio task) and D1 MSN direct projections to the VTA (which responded to the lever press) were involved in sustaining the action (Natsubori et al., 2017). Their model thus supports the notion that ventral striatum D1 and D2 MSNs are not encoding discrete behaviors based on output pathways related to activation or inhibition of movement (through their ultimate thalamic outputs).

5.2 The dopamine-MSN connection

The question of what information is transmitted by dopamine to MSNs and under what conditions has long been a critical question in the field. As dopamine is a neuromodulator, it operates on a slower timescale than glutamatergic activation of MSNs. Yet it does have the potential to shape the output of MSNs over time through D1 and D2 receptors and the microcircuit through which D1 and D2 MSN activity outputs to the VTA (Kupchik et al., 2015).

Soares-Cunha et al., (2018) proposed a model based on parsing the microcircuit underlying the role of D2 MSNs in increased motivation (Soares-Cunha et al., 2018). Once again, the direct pathway projections go to the VTA and indirect pathway to the VTA via the VP (Kupchik et al., 2015). As shown before (Soares-Cunha et al., 2016), D2 MSN activation increases the breakpoint on a progressive ratio task. The authors thus parsed the contribution of different elements in this circuit via pharmacological tools. They isolated the contribution of nicotinic acetylcholine receptors (nAChR), antagonism of which abolished the increased motivation in response to D2 MSN activation and which demonstrated the necessity of cholinergic activation of VTA terminals for this D2 MSN-dependent effect. This is important as cholinergic interneurons both regulate MSN activity and control dopamine release from VTA terminals (Cachope et al., 2012).

The D2 MSN-mediated enhancement of motivation also relied on activation of both D1 and D2 receptors, as pharmacological inhibition of either resulted in a reduction in breakpoint at baseline (without D2 MSN stimulation) and blocked the enhanced motivation with D2 MSN stimulation. This points to a dopamine-dependent effect. The dopamine signal is key to the enhanced motivation following D2 MSN activation. Furthermore, this work provides substantial evidence that the indirect pathway cannot encode aversion – as the D2 MSN projection to the VP results in enhanced motivation. This data indicates a microcircuit whereby output from D1 and D2 MSNs can be modulated via indirect projections to the VTA that alter dopamine release via cholinergic signaling (Soares-Cunha et al., 2018).

As to the nature of information transferred between dopamine and MSNs and given the evidence suggesting dopamine is critical for enhanced motivation (Soares-Cunha et al., 2018), I hypothesize that dopamine transmits a perceived saliency signal to D1 MSNs. They prime D1 MSNs to respond robustly to stimuli, that given the context and situation, should be attended to. This supports the development of associations that facilitate adaptive behavior and weaken those that do not support adaptive behavior or do not provide relevant information worth attending to.

D1 MSNs, in serving as a signal for stimulus presence and saliency, provide indirect information to the D2 MSNs that informs the development of associations between a cue and outcome.

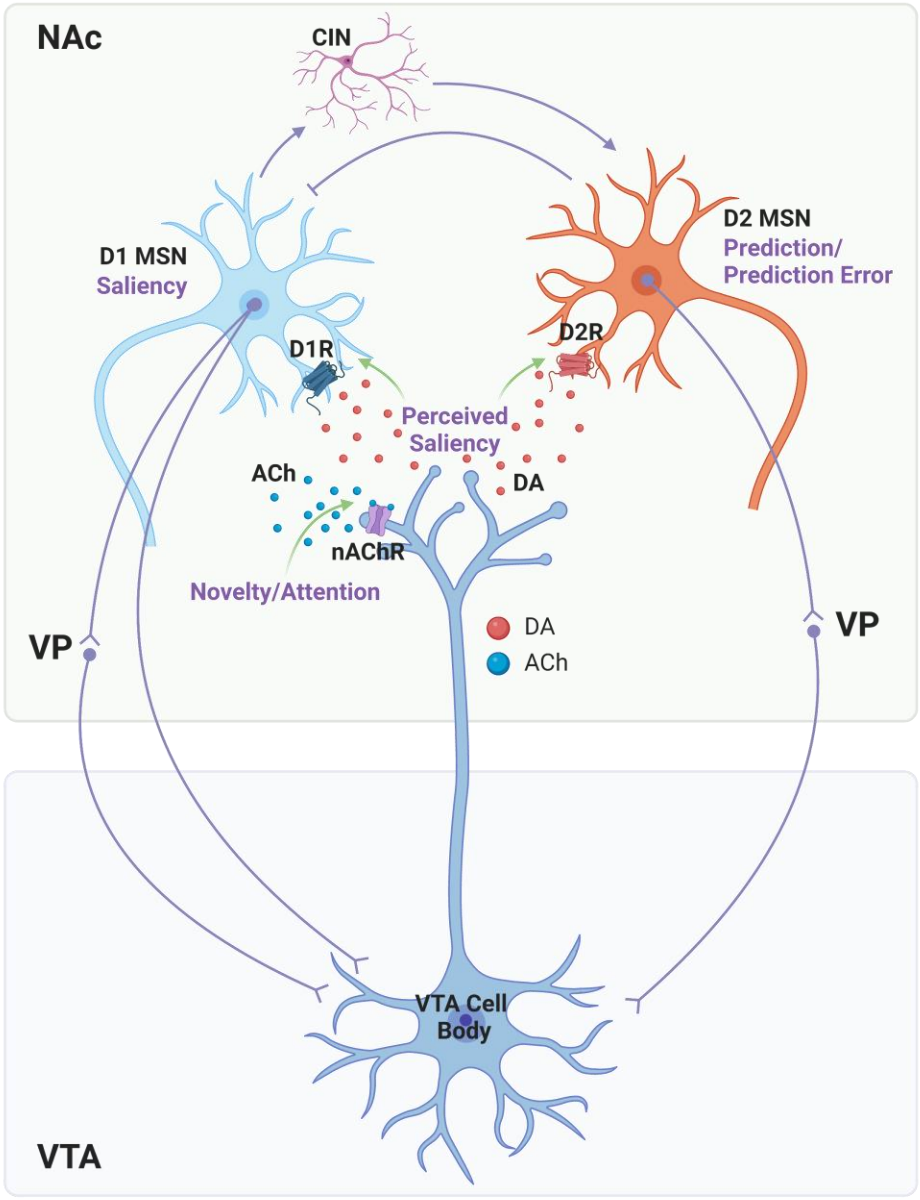

Fig. 11. Diagram of relationship between dopamine and D1 and D2 MSNs and their proposed function.

Dopamine-1 medium spiny neurons (D1 MSNs) express D1-receptors (D1R) and Dopamine-2 MSNs express D2Rs. D1- and D2-Rs are g-protein couple receptors that respond to dopamine released at dopaminergic terminals in the NAc core. MSN output feeds back to the ventral tegmental area (VTA) through direct and indirect connections through the ventral pallidum (VP). D1 MSNs can, through cholinergic interneurons (CIN), generate long-lasting, post-synaptic enhancement of glutamatergic drive on D2 MSNs. D2 MSNs, through lateral inhibition, can inhibit

neighboring D1 MSNs. Dopamine encodes a perceived saliency signal, D1 MSNs encode a saliency signal, and D2 MSNs encode a prediction/prediction error signal.

5.3 Conclusions and future directions

D1 and D2 MSNs encode distinct but complementary information that underlies the ability to associate cues and outcome in a valence-independent fashion to guide adaptive behavior. D1 MSNs track stimulus saliency and D2 MSNs respond to predictions/prediction errors. Furthermore, we show that D2 MSNs are necessary for the development of cue-outcome associations. The timing and coordination of D1 and D2 MSN activity is critical to the development of associations. Within this framework, we can reevaluate many psychopathologies as disorders of associative learning where the physiological, morphological, transcriptional, and in vivo activity changes that result from disease states (or lead to them) alter the balance in D1 and D2 MSNs and dopamine signaling to support maladaptive behavior.

There are a number of directions in which I would like to see this work expanded. I've characterized here how D1 and D2 MSNs respond across a number of contingencies in the NAc core. I would like to repeat some of the same studies in the NAc shell to determine whether any information is conserved across subtype in the core and shell and what information is differentially encoded. The NAc core is thought to subserve learning and action while the shell is involved in motivational value (Carelli, 2004; Castro et al., 2015; Kelley, 2004). These distinct functional outputs could be explained by anatomical organization. There are some differences in afferent and efferent projections. Subregions of the medial PFC selectively project to core and shell. The same is true of the BLA and the hippocampus (Kelley, 2004). The core projects primarily to the ventral pallidum and subthalamic nucleus while the shell projects to the lateral hypothalamus, bed nucleus stria terminalis, amygdala, ventral pallidum (West et al., 2018). Both brain regions are involved in reward processing and respond to reward-predictive cues yet show distinct responses, especially as they relate to the reinforcing effects of drugs of abuse (Ito et al., 2004).

I would next propose recording and manipulating specific inputs to the NAc, specifically, the dorsal hippocampus and the basolateral amygdala. It is likely that learning parameters identified in the nucleus accumbens are represented in other brain regions as well. We could thus determine whether information is encoded in the accumbens or simply transmitted and represented there. One of the questions we can address in the future is whether the D2 MSNs in the core that respond to the cue in fear conditioning session 1 are the same ones that respond on later fear conditioning sessions, indicating information is stored there over time. If no such ensemble exists, I would anticipate these memory cells to be present in the hippocampus and that disrupting information flow to the accumbens after acquisition of fear conditioning would reduce freezing.

Now that we have the tools to record and manipulate cells in awake and behaving animals, I would record in vivo activity of D1 and D2 MSNs during acquisition of cocaine self-administration. I would hypothesize that at first the cue-outcome association response in D2 MSNs would develop in similar fashion to sucrose positive reinforcement. However, as chronic cocaine administration alters morphological, physiological, and transcriptional properties of D1 and D2 MSNs, reshapes glutamatergic input, and downregulates dopamine receptors and production, I anticipate the timing and magnitude of D1 and D2 MSN activity – and the coordinated activity between the two -- to cues and outcomes would be altered (Volkow et al., 2010). Additionally, since there are known disruptions in motivation for non-drug related stimuli, I would expect to see this deficit represented in delayed acquisition and a smaller response to the predictive cue in D2 MSNs in a positive reinforcement task following chronic cocaine administration. It has also been well characterized that changes in the extended amygdala generate negative emotional states. This facilitates negative reinforcement, in which individuals take drug to alleviate the negative affect (Koob & Volkow, 2010). Thus, significant changes in upstream inputs to the ventral striatum could foreseeably alter activity in D1 and D2 MSNs to strengthen associations between drug and relief from negative emotional states.

Lastly, to tease apart the dopamine – D1/D2 MSN connection, I would record calcium activity in D1 and D2 MSNs while manipulating the dopamine signal in the NAc core. A long-standing question in the field that has historically been limited by the tools to study it is this: what information is transmitted between dopamine and medium spiny neurons? I have addressed some theories in section 5.2, but recording *in vivo* during reward and aversive learning would allow us to resolve the timing of the dopamine and MSN signals and the necessity and sufficiency of dopamine to associative learning parameters encoded in D1 and D2 MSNs.

Together, with novel tools and approaches we now have unprecedented access to neural circuits in the brain in awake and behaving animals. Using these tools in combination with behavior, as well as observing and manipulating these signals in models of maladaptive and disease states, will allow us to better understand exactly how the brain controls behavior and how dysfunction in these basic processes give rise to human disease.

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