

**Role of dopamine signaling in the lateral amygdala in mediating associative learning with
relevance to substance use disorders**

by

Dana M. Smith

Bachelor of Arts, Colgate University, 2017

Submitted to the Graduate Faculty of the
School of Medicine in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy

University of Pittsburgh

2023

UNIVERSITY OF PITTSBURGH
SCHOOL OF MEDICINE

This dissertation was presented

by

Dana M. Smith

It was defended on

March 21, 2023

and approved by

Committee Chair: Susan Sesack, PhD, Professor, Department of Neuroscience

Yan Dong, PhD, Professor, Department of Neuroscience

Yanhua Huang, PhD, Associate Professor, Department of Psychiatry

Colleen McClung, PhD, Professor, Department of Psychiatry

Outside Examiner: Ryan LaLumiere, PhD, Associate Professor, Department of Psychological
and Brain Sciences, University of Iowa

Dissertation Advisor: Mary Torregrossa, PhD, Associate Professor, Department of Psychiatry

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Dana M. Smith, PhD

University of Pittsburgh, 2023

In classical conditioning, a form of associative learning, repeatedly pairing a neutral stimulus with a biologically significant unconditioned stimulus (US) results in an association between the two stimuli. The neutral stimulus becomes a conditioned stimulus (CS) and presentation of it alone elicits a conditioned response (CR) that mirrors the unconditioned response. Associative learning can promote maladaptive behaviors including drug abuse. Environmental cues paired with repeated drug use acquire strong incentive value such that exposure to them alone can trigger craving and relapse.

Initially weak afferents carrying CS information and strong afferents carrying US information converge in the lateral amygdala (LA) and through plasticity mechanisms, there is enhancement at the excitatory synapses carrying CS information. Evidence suggests that the ventral tegmental area (VTA) dopamine projection to the LA participates in encoding reinforcing effects that act as a US in conditioned cue reward-seeking as dopamine released in the amygdala is important for emotional and behavioral functions. In the context of drugs of abuse, dopamine in the LA has been shown to be necessary for maintenance of drug-cue associations, regulation of drug-seeking behavior, and reinstatement of drug-seeking.

In this dissertation, through a combination of fiber photometry, chemogenetics, and behavioral techniques, we found that dopamine activity in the LA is necessary for cue-mediated reward-

seeking. During conditioning, as learning occurs, dopamine activity develops responses to reward-predictive cues. Dopamine activity in the LA is associated with cue-related events as release is unchanged by reward or instrumental action. We also found that inhibiting dopaminergic input to the LA during cocaine self-administration slowed acquisition and weakened the ability of the previously cocaine-paired cue to elicit cocaine-seeking. Conversely, exciting the projection during self-administration boosted the salience of the cocaine-paired cue as indicated by enhanced responding during cue-induced reinstatement. Importantly, interfering with dopamine input to the LA has no impact on the ability of cocaine to elicit a place preference or induce reinstatement in response to a priming cocaine injection. Together, this work indicates that manipulation of projections underlying dopamine signaling in the LA may be useful for developing therapeutic interventions for substance use disorders (SUD).

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List of Common Abbreviations

| | |
|---------------|---|
| BLA | Basolateral amygdala |
| CeA | Central amygdala |
| CNO | Clozapine-N-Oxide |
| CPP | Conditioned place preference |
| CR | Conditioned response |
| CS | Conditioned stimulus |
| DREADD | Designer Receptors Exclusively Activated by Designer Drugs |
| DRN | Dorsal raphe nucleus |
| FR1 | Fixed ratio 1 |
| ITI | Intertrial interval |
| LA | Lateral amygdala |
| LTD | Long-term depression |
| LTP | Long-term potentiation |
| MGN | Medial geniculate nucleus |
| NAc | Nucleus accumbens |
| OFC | Orbitofrontal cortex |
| pDMS | Posterior dorsomedial striatum |
| PIT | Pavlovian to Instrumental Transfer |
| RPE | Reward prediction error |

| | |
|-------------|------------------------|
| RR10 | Random ratio 10 |
| RR5 | Random ratio 5 |
| SUD | Substance use disorder |
| US | Unconditioned stimulus |
| VEH | Vehicle |
| VTA | Ventral tegmental area |

1.0 INTRODUCTION

The amygdala is a relatively small region comprised of interconnected nuclei including the basolateral complex of the amygdala (BLA) made up of the lateral (LA), basal (BA), and basomedial (BM) cell groups and the central nucleus (CeA) with lateral (CeL) and medial (CeM) subdivisions (Janak & Tye, 2015). Two key functions of the structure include assigning valence to both fearful and rewarding environmental stimuli and mediating cue-related learning processes.

1.1 VALENCE ENCODING

The amygdala and its subdivisions contribute to circuits that encode valence. Imaging studies have highlighted the importance of the amygdala in valence circuitry by identifying a “valence-general affective space” consisting of cells in the anterior insula, rostral and dorsal anterior cingulate cortex, ventral striatum, thalamus, occipitotemporal cortex, and amygdala (Barrett & Bliss-Moreau, 2009). Amygdala neurons concurrently encode multiple dimensions such as the sensory properties of conditioned cues, the behaviors they elicit, and their valence (Kyriazi, Headley, & Pare, 2018). Approximately 37% of neurons in the amygdala are selectively responsive to motivationally relevant stimuli (Fuster & Uyeda, 1971). In addition, some of these neurons modulate their firing rate following reversal of the expected outcome value, further suggesting that neurons in the amygdala can track valence (Nishijo, Ono, & Nishino, 1988).

Specifically, in the BLA, about a fifth of the neurons track valence (Belova, Paton, Morrison, & Salzman, 2007; Namburi, Al-Hasani, Calhoun, Bruchas, & Tye, 2016; Nishijo et al.,

1988; Schoenbaum, Chiba, & Gallagher, 1999). There is evidence for inhibitory relationships between the positive and negative encoding neurons such that presentation of a positive valence stimulus results in increased firing rate in positive encoding neurons and decreased firing rate in negative encoding neurons (Belova et al., 2007). Some research shows these positive and negative valence-encoding neurons are intermingled (Beyeler et al., 2018; Redondo et al., 2014). For example, in animals performing a trace-conditioning task, electrophysiological recordings showed that cues of each valence elicited higher levels of activity in separate but anatomically interspersed populations of neurons (Paton, Belova, Morrison, & Salzman, 2006). Additionally, activity-dependent labeling of BLA neurons using nicotine or an opposite sex conspecific as a positive reinforcer or a footshock as a negative reinforcer showed non-overlapping populations in the rat BLA (Gore et al., 2015; Redondo et al., 2014).

Recent advances have allowed for more precise identification of amygdala neuron populations based on their function, projection target, and genetic markers (Namburi et al. 2015; Felix-Ortiz and Tye, 2014; Felix-Ortiz et al. 2013; Senn et al., 2014; Stuber et al. 2011; Kim et al., 2016). Interestingly, a genetic strategy to transcriptionally profile neurons in the BLA revealed that negative and positive encoding neurons were spatially segregated into the anterior and posterior portions of the BLA, respectively (Kim et al., 2016), implying that negative and positive neurons may only be intermingled at the transition between the two areas. Regardless of anatomical location, it is possible that specific amygdala neuronal subpopulations mediate valence (Berridge., 2019), but it has also been posited that BLA neurons that respond to positive valence stimuli may also respond to negative stimuli under different conditions. Thus, affective valence encoding in the amygdala may be the result of neuronal ensembles, neurons that have dynamic states, or a combination of both (Berridge, 2019; Kyriazi et al., 2018).

Amygdala neurons' contributions to valence encoding are linked both to projection target and afferent input. For example, projections from the BLA to the nucleus accumbens (NAc) encode positive valence (Beyeler et al., 2016; Namburi et al., 2015), BLA projectors to the ventral hippocampus (vHPC) respond to valence-related cues (Beyeler et al., 2016), and projections from the BLA to portions of the CeA encode negative valence (Namburi et al., 2015; Tye, 2018). There are a number of projections to the BLA from regions including the basal forebrain, dorsal raphe nucleus (DRN), and ventral tegmental area (VTA) that play a role in valence processing (Correia & Goosens, 2016). The dense cholinergic input from the basal forebrain responds to both appetitive and aversive stimuli (Hangya, Ranade, Lorenc, & Kepecs, 2015). The DRN sends a projection to the BLA (Burghardt & Bauer, 2013; Vertes, 1991) and these serotonergic DRN neurons show phasic firing changes to punishment and reward predictive cues (Cohen, Amoroso, & Uchida, 2015). Likewise, VTA dopamine axons innervating the BLA were activated by both reward and punishment (Lutas et al., 2019). Another crucial pathway for valence processing is the connection between the BLA and the posterior dorsomedial striatum (pDMS) (Corbit, Leung, & Balleine, 2013). After lesioning the BLA and disconnecting it from the pDMS with muscimol, rats were unable to use recently acquired associations to direct new response-outcome associations (Corbit et al., 2013), implying that valence information from the BLA is essential for response-outcome learning mediated by pDMS. Overall, positive and negative detecting cells in the amygdala participate in valence encoding circuits that are critical for reinforcement, decision-making based on motivationally relevant outcomes, and cue-reward learning.

1.2 ASSOCIATIVE LEARNING PROCESSES

The study of associative learning began over a hundred years ago with the seminal work of Thorndike and Pavlov. Thorndike narrowed in on the connections formed between stimuli and responses whenever the response was followed by reward (Pearce & Bouton, 2001). On the other hand, Pavlovian conditioning focuses on the pairing of two stimuli. More specifically, a neutral environmental stimulus is repeatedly paired with a biologically significant unconditioned stimulus (US) resulting in an association between the two stimuli. The once neutral stimulus becomes a conditioned stimulus (CS) such that presentation of the stimulus alone elicits a conditioned response (CR) that mirrors the unconditioned response (Pearce & Bouton, 2001). In the most recognized example, Pavlov's dogs experienced pairing of a bell each time they were given food, which triggered salivation. Eventually, ringing the bell alone caused salivation in the dogs (Pavlov, 1927). As the field has progressed, focus has shifted to the neural substrates underlying associative learning with a particular emphasis on the amygdala.

1.2.1 FEAR CONDITIONING

Early evidence for the amygdala's involvement in associative learning came from fear conditioning studies. In fear conditioning, an auditory tone CS is repeatedly paired with an aversive footshock US. Electrophysiology experiments examining circuitry involved in fear conditioning revealed that the lateral amygdala (LA) mediates associative learning for auditory conditioned cues as it receives CS information from the auditory thalamus via thalamo-amygdala and thalamo-cortico-amygdala pathways (Kim et al., 2007; Kwon et al., 2014; LeDoux, Farb, & Ruggiero, 1990; Rogan, Staubli, & LeDoux, 1997; Rogan & LeDoux, 1995). At the same time,

negative effects of the footshock that serve as the US reach the LA from somatosensory regions (Shi & Davis, 1999). Information is then transmitted to the central amygdala (CeA) which projects to downstream areas, including the bed nucleus of the stria terminalis, lateral hypothalamic area, and midbrain central gray, that control expression of fear responses such as the release of stress hormones, changes in blood pressure, and freezing (Medina et al., 2002; LeDoux et al. 1988). Importantly, damage to or functional inactivation of the LA prevents acquisition of conditioned fear, thus confirming its ability to integrate and relay sensory information integral to fear conditioning (Muller et al., 1997; LeDoux et al., 1990; Amorapanth, LeDoux, & Nader, 2000; Campeau et al., 1995; Maren, Aharonov, & Fanselow., 1996).

On a mechanistic level, fear conditioning has been shown to induce long-term potentiation (LTP) both in the pathways transmitting auditory CS information and within the LA itself (Rogan & LeDoux, 1995; Rogan, Staubli, & LeDoux, 1997). LTP is an activity-dependent form of neural plasticity that contributes to associative memory formation of the conditioning experience (Malenka & Nicoll, 1993). Following fear conditioning, increases in cue-induced freezing are related to increased synaptic strength at these thalamo-amygdala and cortico-amygdala synapses (Kim et al., 2007; Hong et al., 2009). These alterations in synaptic transmission and neuronal activity in the LA are long-lasting (Maren, 2000; Rogan, Staubli, & LeDoux, 1997) and the enhanced synaptic strength of the CS inputs allows for subsequent CS presentations to activate the same LA neurons that are activated by the footshock. In turn, this activation initiates conditioned freezing responses (Schafe et al., 2001).

Optogenetic techniques have been used to examine further the involvement of specific projections to and from the amygdala during fear conditioning. In one series of experiments, pairing an auditory CS with photoactivation of LA principal neurons expressing the excitatory

opsin channelrhodopsin 2 (ChR2) was sufficient as a substitute for the footshock US (Johansen et al., 2010). A similar study replaced the CS tone with photoactivation of ChR2-expressing terminals from the medial geniculate nucleus (MGN) of the auditory thalamus and the primary auditory cortex. Temporally pairing this optical CS with a footshock US resulted in both conditioned freezing and synaptic potentiation in the LA. Furthermore, *in vivo* optical long-term depression (LTD) conditioning extinguished the fear-evoked response (Nabavi et al., 2014). Focusing on BLA efferent projections, optical stimulation of the BLA to ventral hippocampus pathway following footshock training but not context training in a contextual fear conditioning paradigm enhanced retention, while optical inhibition of the pathway impaired retention. As BLA to ventral hippocampus projections participated in memory consolidation for footshock, but not context, it seems BLA projections modulate memory consolidation depending on the type of learning involved (Huff, Emmons, Narayanan, & LaLumiere, 2016). Collectively, the amygdala is an important node in circuits mediating learning and memory during fear conditioning.

1.2.2 APPETITIVE CONDITIONING

The amygdala has been implicated in many reward-related behaviors such as conditioned place preference (CPP) (Cador, Robbins, & Everitt, 1989; Hiroi & White, 1991; Everitt et al. 1991) and appetitive classical conditioning (Gaffan & Harrison, 1987; Gallagher, Graham, & Holland, 1990; McDonald & White, 2013). In human imaging studies, amygdala activation is seen in response to reward-related cues (Childress et al., 1999; Garavan et al., 2000; Jasinska, Stein, Kaiser, Naumer, & Yalachkov, 2014). Likewise, during electrophysiological recordings, amygdala neurons fire in response to reward-predictive cues and show activation during reward-seeking (Schoenbaum, Chiba, & Gallagher, 1999; See, 2002). A similar pattern of amygdala activity is

seen following training. When animals are trained to associate sensory stimuli with positive reinforcement, the number of sensory-responsive amygdala neurons increases (Ono, Nishijo, & Uwano, 1995). Furthermore, during appetitive Pavlovian conditioning, BLA principal neurons are activated at the time of stimulus-outcome pairings (Crouse et al., 2020; Sias et al., 2021).

Interestingly, these neurons show precision in their encoding. Rats that were trained to respond to sucrose paired with a reward-predictive cue had a higher proportion of phasically responsive neurons during reinstatement than rats that were trained with a randomly presented cue. Two separate populations of BLA neurons responded to cues in trials when the cue was used as an incentive stimulus or as a reinforcing stimulus, indicating that specific populations of BLA cue responsive neurons encode the motivating properties or reinforcing properties of a reward-related cue (Tye & Janak, 2007). This type of cue encoding points to the amygdala as part of a system in which cues acquire positive incentive value.

A large body of work highlights that the amygdala is not responsible for all behaviors elicited by reward-predictive cues, but rather underlies the ability to respond to cues in the face of changing reward value (Balleine & Killcross, 2006; Hatfield, Han, Conley, Gallagher, & Holland, 1996; Málková, Gaffan, & Murray, 1997; Murray, 2007). In second-order conditioning experiments, rats first received light-food pairings. The acquired reinforcing ability of the light CS was assessed by determining its ability to serve as a reinforcer in second-order conditioning of a tone when tone-light pairings were delivered without food reward. Acquisition of this second-order conditioning was impaired in rats with BLA lesions, highlighting BLA involvement in CSs' acquisition of incentive value (Hatfield et al., 1996). In monkeys, amygdala lesions had no effect on visual learning guided by a secondary reinforcer, but impaired reinforcer devaluation effects hinting that the amygdala is necessary for learning the association between stimuli and reward

value and not for maintaining value of reinforcers (Málková et al., 1997). In the BLA, NMDA glutamate receptors (Malvaez, Shieh, Murphy, Greenfield, & Wassum, 2019; Parkes & Balleine, 2013) and mu opioid receptors (Wassum et al. 2009; Wassum et al., 2011) support this type of incentive learning.

CSs can also acquire the ability to activate a motivational process through their association with reward. In this case, a CS can help to control the strength with which voluntary actions are performed (Bindra, 1974; Rescorla & Solomon, 1978). Initial studies of this process, known as Pavlovian to Instrumental Transfer (PIT), reported that stimuli paired with food were able to augment instrumental actions directed towards food (Estes & Skinner, 1941; Estes, 1948). In this paradigm, Pavlovian cues and instrumental actions are trained in separate phases. In the Pavlovian phase, one or more stimuli are paired with the delivery of reward such as food pellets or sucrose. During instrumental training, a contingency is established between lever pressing and one or more outcomes, typically food delivery. Then, in the test phase it is determined how CS presentation modulates instrumental responding. Generally, a CS paired with an appetitive outcome enhances responding (Cartoni, Balleine, & Baldassarre, 2016). Additionally, cues can enhance specific actions associated with the same outcome as the cue (specific transfer) or promote actions paired with different outcomes (general effects) (Corbit & Balleine, 2005; Corbit, Muir, & Balleine, 2001).

Lesion studies examining the neural underpinnings of these transfer effects reported that the nucleus accumbens (Corbit et al., 2001; De Borchgrave, Rawlins, Dickinson, & Balleine, 2002; Hall, Parkinson, Connor, Dickinson, & Everitt, 2001) and amygdala (Blundell, Hall, & Killcross, 2001; Hall et al., 2001; Holland & Gallagher, 2003) are necessary for PIT. Thus, many studies were conducted to characterize the transfer process in the amygdala and its subdivisions. In the

same animal, using two cues that predicted the outcomes earned by the actions in training and a third cue that predicted an outcome that was not earned by those actions dissociated general motivational and specific excitatory effects of the reward-related cue (Corbit & Balleine, 2005). Presentation of the first two cues resulted in outcome-selective PIT, while the third cue more generally increased the performance of both actions. BLA lesions in these animals abolished outcome specific effects of the cue while sparing its general motivational effects, whereas CeA lesions eliminated the general motivational effects of the cues, but not the specific effects (Corbit & Balleine, 2005). Examining conditioned reward-approach responses has revealed that the BLA plays a more precise role in representing the affective value of the conditioned stimuli and this information is used to support the translation of conditioned associations into instrumental action (Burns, Robbins, & Everitt, 1993; Cador et al., 1989; Malvaez et al., 2015; Whitelaw, Markou, Robbins, & Everitt, 1996).

1.2.3 CIRCUITS MEDIATING ASSOCIATIVE LEARNING

The amygdala is well positioned to participate in appetitive conditioning as it can integrate afferent input from many brain areas such as the prefrontal cortex, sensory cortex, thalamus, and VTA (Kim & Cho, 2017; Ciccocioppo, Sanna, & Weiss, 2001; Do-Monte et al. 2015; Peter et al., 2012; Cho, Rendall, & Gray, 2017). The role of the amygdala in associative learning and memory is a result of these diverse inputs that allow it to receive sensory information about the CS and interoceptive effects that serve as a US. The LA receives input and sends it to the basal and basomedial regions of the BLA and lateral portion of the CeA which then project to the medial portion of the CeA that acts as main output nucleus of the amygdala (Janak & Tye, 2015). Reciprocal projections between the orbitofrontal cortex (OFC) and the BLA are critical for

associative encoding as these connections underlie the ability to represent information about an expected outcome (Saddoris, Gallagher, & Schoenbaum, 2005). More specifically, the BLA to lateral OFC pathway allows for cues to predict available rewards, while the BLA to medial OFC pathway enables value predictions (Wassum, 2022). Additionally, reciprocal connections between the BLA and ventral striatal dopamine system assign value to neutral stimuli as well as update reinforcer representations as value changes (Colwill & Motzkin, 1994; Holland, 1990; Roberts, 1996). Pavlovian approach and conditioned reinforcement are integrated within the ventral striatum through its interactions with the amygdala and mesolimbic dopamine system (Everitt et al., 1999), again highlighting the role of the amygdala in circuits that mediate associative learning.

On a smaller scale, it is important to examine how amygdala neurons develop and maintain enhanced excitatory responses to a conditioned stimulus (CS) paired with a reinforcing unconditioned stimulus (US). In the LA, through plasticity mechanisms, there is enhancement at the excitatory synapses carrying CS information (Maren 2000; Rogan, Staubli, & LeDoux, 1997; McKernan & Shinnick-Gallagher, 1997). Others have also shown that acquisition of cue-directed reward-seeking requires neuronal plasticity in the LA (Tye, Stuber, De Ridder, Bonci, & Janak, 2008). In these experiments, beam breaks at a nose-poke response port were reinforced with a cue and sucrose reward in about 50% of trials. For rats that acquired the task, half of the recorded neurons that did not respond to the cue before acquisition developed a phasic response to cue onset following acquisition. Cue encoding increased across sessions and this increase was predictive of behavior as greater proportions of neurons were recruited to encode the cue as performance improved. Furthermore, rats that learned the cue-reward association had larger AMPAR/NMDAR ratio at thalamo-amygdala synapses relative to non-learners, indicative of strengthened glutamatergic synapses. Thus, these findings demonstrated that during cue-reward learning, cue-

responsive neurons were rapidly recruited and thalamo-amygdalar synapses were selectively strengthened (Tye et al., 2008). Identifying projections such as the thalamo-amygdala pathway that develop and maintain responses to cues greatly improves our knowledge of associative learning in the region.

1.3 ROLE OF VENTRAL TEGMENTAL AREA (VTA) IN REWARD LEARNING

The ventral tegmental area (VTA) is a heterogeneous midbrain structure containing dopamine, glutamate, and GABA neurons (Hnasko, Hjelmstad, Fields, & Edwards, 2012; Pupe & Wallén-Mackenzie, 2015; Trudeau et al., 2014). Research into VTA function has focused heavily on the role of dopamine neurons as 50-70% of VTA neurons express dopamine and these dopaminergic neurons have diverse roles in reward-related behaviors (Dobi, Margolis, Wang, Harvey, & Morales, 2010).

1.3.1 VTA DOPAMINE AND LEARNING

Early characterization of the VTA and its dopaminergic projections determined that the region is critical for performance and positive reinforcement of appetitive behaviors (Lyness, Friedle, & Moore, 1979; Roberts, Koob, Klonoff, & Fibiger, 1980; Wise, 2005). Dopamine neurons in the VTA respond to unexpected natural reward with a burst of action potentials (Ljungberg, Apicella, & Schultz, 1992; Schultz, Apicella, & Ljungberg, 1993). These reward responses take place in synchronous phasic bursts that influence learning and motivation (Joshua et al., 2009). Tonic dopamine release also contributes to learning, but it is thought to regulate the

intensity of the phasic dopamine response (Grace, 1991; Grace et al. 2007; Schultz, 2007; Lapish et al., 2007).

Another well-studied function of this cell population is reward-prediction error (RPE) or the difference between the received and anticipated value of an outcome (Schultz, 1998). If a reward is larger than predicted, dopamine neurons are strongly excited (positive prediction error). Conversely, if a reward is smaller than predicted, dopamine neurons are phasically inhibited (negative prediction error) (Schultz, Dayan, & Montague, 1997). This same principle holds true for dopamine responses to sensory cues that provide information about future rewards. Therefore, RPE serves as an important form of learning the value of actions and environmental states (Houk & Wise, 1995; Montague, Dayan, & Sejnowski, 1996; Schultz, Dayan, & Montague, 1997). Another property of the RPE-encoding dopamine neurons is the shift in their response from reward delivery to cue onset that occurs as learning progresses (Waelti, Dickinson, & Schultz, 2001; Bayer & Glimcher, 2005; Schultz, Apicella, & Ljungberg, 1993; Pan et al., 2005).

Several groups have employed optogenetics to drive phasic activation of dopamine neurons to examine the role of the population in learning. For example, mice developed a place preference for a chamber associated with phasic optical stimulation of VTA dopamine neurons (Tsai et al., 2009). Additionally, this type of phasic activation enhanced reinforcing actions in a food-seeking operant task, but had no effect when reward was absent (Adamantidis et al., 2011). Optogenetic tools can also be used to mimic RPE signaling in VTA dopamine neurons. Manipulation via this artificial signal was able to both drive new cue-reward associative learning and modify previously learned associations (Steinberg et al., 2013). Other recent work has investigated the contribution of VTA dopamine neurons to Pavlovian learning and the ways in which these neurons confer predictive and motivational properties to cues. Researchers used a cue

conditioning procedure where brief phasic optogenetic excitation of VTA dopamine neurons substituted for natural reward delivery. The optical stimulation was paired with a discrete sensory cue which in turn was able to evoke dopamine neuron activity and elicit cue-locked conditioned behavior (Saunders, Richard, Margolis, & Janak, 2018). VTA dopamine neurons, particularly those projecting to the nucleus accumbens core and dorsal striatum, function in Pavlovian conditioning by creating conditioned stimuli from previously neutral cues (Saunders et al., 2018). Taken together, these studies demonstrate that dopamine neuron activity can change in response to reward-related cues to modulate future reward-seeking behavior.

1.3.2 VTA TO AMYGDALA CIRCUITRY

The mesocorticolimbic system is thought to underlie reward and this system is comprised of dopaminergic cell bodies in the VTA that project to prefrontal cortex areas, nucleus accumbens, anterior cingulate cortex, bed nucleus of the stria terminalis and the amygdala (Pierce & Kumaresan, 2006; Wise, 2004). Historically, the VTA to nucleus accumbens (NAc) projection has drawn a lot of attention for its role in reward-related behaviors. However, dopaminergic input from the VTA to the amygdala is also critical for numerous emotional and behavioral functions (Brinley-Reed & McDonald, 1999).

In *ex vivo* preparations, it has been shown that dopamine enhances BLA projection neuron excitability through dopamine D1 receptor activation effects on a slow inactivating potassium current (Kröner, Rosenkranz, Grace, & Barrionuevo, 2004). It is also thought that dopamine released in the BLA participates in postsynaptic modulation of interneuron excitability (Kröner, Rosenkranz, Grace, & Barrionuevo, 2004). These dopaminergic functions may modulate BLA

projection neuron firing to affect encoding of reward-related cues (Rosenkranz & Grace, 2001; Kröner, Rosenkranz, Grace, & Barrionuevo, 2004).

VTA to BLA connections play an important role in valence encoding that promotes reward-seeking. For example, two-photon imaging and photometry in behaving mice revealed that VTA dopamine axons innervating the basal portion of the amygdala were activated by both reward and punishment and that they acquired responses to cues predicting these outcomes (Lutas et al., 2019). Moreover, plasticity of BLA neurons is enhanced by dopamine released during emotionally valenced tasks (Muller, Mascagni, & McDonald, 2006; Bissière, Humeau, & Lüthi, 2003), thus suggesting that VTA inputs mediate valence encoding in the BLA and promote reward-related behaviors. It has been shown that stimulating dopamine neuron terminals in the BLA can enhance food intake during unconditioned feeding and that this stimulation also strengthens the motivational impact of previously learned reward-predictive cues (Mahler et al., 2019).

Other data further suggest this projection participates in associative learning. In electrophysiological recordings from anesthetized rats, dopamine-dependent effects on BLA neuronal responses were identified after just a few conditioning trials (Grace & Rosenkranz, 2002; Rosenkranz & Grace, 2002). Immunohistochemical analysis of dopaminergic activity revealed that dopamine neurons innervating the CeA and BLA show selective responses to a CS associated with a sucrose US during early training, while the response diminished once the CS-US association was well learned (Phillips, Setzu, Vugler, & Hitchcott, 2003). Likewise, perturbations to dopamine function in the BLA impaired acquisition of conditioned instrumental responses that were contingent on close temporal proximity of the CS and US (Blundell et al., 2001; Blundell, Hall, & Killcross, 2003; Hitchcott & Phillips, 1998). Pharmacological experiments also hint at the role of dopamine in the amygdala in associative learning. For one, intra-amygdala infusions of a dopamine

agonist with selectivity for the D3 receptor subtype prior to Pavlovian training diminished the ability of the CS to support lever pressing. When these infusions occurred prior to a conditioned reinforcement test, there was a robust reduction in lever pressing (Hitchcott & Phillips, 1998). Collectively, these experiments implicate mesoamygdaloid dopamine in the acquisition of associative learning.

1.4 DEVELOPMENT OF SUBSTANCE USE DISORDER

Substance use disorders (SUD) are highly prevalent as they affect nearly 20 million Americans (National Survey of Drug Use and Health (NSDUH), 2019). This prevalence represents a huge economic burden and drug use costs nearly 700 billion dollars annually (NSDUH, 2019). SUD is characterized by cycles of drug use, withdrawal, abstinence, and resumption of drug taking. During periods of abstinence, there is an increase in craving that makes individuals vulnerable to relapse (Gawin & Kleber, 1988; Kassani, Niazi, Hassanzadeh, & Menati, 2015). As a result, SUDs are both difficult to overcome and a challenge to treat. In fact, only 11.8% of Americans diagnosed with SUD receive treatment (NSDUH, 2019).

1.4.1 ENVIRONMENTAL CUES AND DRUGS OF ABUSE

Environmental cues can become associated with the reinforcing properties of drugs of abuse, gaining positive valence that strongly drives drug-seeking behaviors. When animals are trained with a drug-associated cue, removal of that cue markedly decreases responding, implying that presentation of stimuli associated with drugs contribute to maintenance of drug-seeking and

drug-taking (Caggiula et al., 2002; Schenk & Partridge, 2001). Moreover, environmental cues associated with repeated drug use increase craving and the likelihood of relapse even in the absence of the drug (Di Ciano and Everitt, 2004; See, 2005; Fuchs et al., 2008). In humans, exposure to these environmental cues such as drug paraphernalia elicit drug craving (See, 2005). Even when treatment in the clinical setting is successful, drug-seeking behavior tends to be renewed when patients return to the drug-paired environment where they are re-exposed to drug-associated stimuli (Crombag et al., 1999; Thewissen, Snijders, Havermans, van den Hout, & Jansen, 2006).

Animal models of drug-seeking behavior include the self-administration model in which animals learn to self-administer drugs of abuse by making an operant response (nose poke or lever press) that results in delivery of the drug (US) paired with a discrete cue (CS). Over the course of many trials, the drug-cue association is consolidated and stored in memory (Schafe & LeDoux, 2000; Wilensky, Schafe, & LeDoux, 1999). After acquisition of self-administration, the operant response can be extinguished by allowing animals to make responses that no longer result in reinforcer or CS presentation. Finally, the extinguished behavioral response can be reinstated by re-exposure to the CS, reinforcing substance, or a stressful stimulus, which serve as valid translation models for human relapse (See, 2002; Shaham et al., 2003; Crombag et al., 2008; Bossert et al., 2013).

1.4.2 ROLE OF THE AMYGDALA

As expected, the amygdala is critical for the cue learning that drives drug-seeking behavior (Everitt et al., 2009). The CeA exerts influence over ascending arousal systems that lead to increased salience, attractiveness, and motivational properties of drug-associated cues (Everitt et al., 2009). As part of the extended amygdala (bed nucleus stria terminalis, transition zone in medial

nucleus accumbens, and CeA), the CeA is critical for integrating changes in reward associated with drug dependence (Koob, 2003). Additional work focusing on the BLA has shown that it mediates the learning processes that allow drug paired stimuli to acquire incentive value and control over drug-seeking behavior (Everitt et al., 1999). For example, inhibition of BLA function via lesions or reversible inactivation impairs the acquisition of self-administration on a second order schedule of reinforcement (Whitelaw et al., 1996). Similarly, these types of manipulations attenuate reinstatement following exposure to cocaine-paired cues (Grimm & See, 2000; Kruzich & See, 2001; Meil & See, 1997). These studies highlight that while the BLA is not responsible for the primary reinforcing effects of drug, it is essential for a cue to elicit the affective representation of the drug reinforcer (Whitelaw et al., 1996). Likewise, functional integrity of the BLA has been shown to be necessary for the reinstatement of cocaine-seeking behavior elicited by cocaine cues, but not by cocaine itself (Fuchs & See, 2002; Fuchs, Feltenstein, & See, 2006).

During reinstatement tests, rats trained to associate stimuli with response contingent availability of cocaine show dopamine efflux in the NAc and amygdala (Katner, Magalong, & Weiss, 1999). Further investigation into the role of dopamine released in the amygdala found that infusion of dopamine but not glutamate receptor antagonists into the BLA prevent cue-induced reinstatement (See, Kruzich, & Grimm, 2001). Blockade of amygdala dopamine D1 receptors during acquisition of cocaine cue associations significantly reduced conditioned-cued cocaine-seeking behavior, whereas dopamine D2 receptor blockade within the BLA at the time of acquisition had dose-dependent effects on cue-induced reinstatement such that a high dose of the antagonist attenuated reinstatement and a low dose potentiated reinstatement (Berglind et al., 2006). These results demonstrate that dopaminergic activity in the BLA plays a key role in regulating cocaine cue learning.

As with non-drug reward, it is crucial to determine how neurons in the amygdala develop and maintain responses to drug-paired cues. Prior cue-reward learning experiments highlighted the contribution of thalamo-amygdala synapses and our lab has found similar findings in the context of drugs of abuse. Our lab has found that the strength of a drug cue association corresponds to the strength of synapses between the medial geniculate nucleus (MGN) of the thalamus and the LA (Rich et al., 2019). In these studies, rats were trained to self-administer cocaine or saline paired with an audiovisual cue daily for 14 days. Animals that self-administered cocaine displayed potentiation of excitatory postsynaptic currents at MGN-LA synapses relative to the saline self-administration group (Rich et al., 2019). These findings suggest that MGN-LA synapses serve to encode and pair the drug predictive cue with the cocaine experience, giving the cue positive valence. Conversely, cue extinction, or repeated presentation of the cue without any cocaine, reversed the potentiation of synaptic strength at MGN-LA synapses and reduced cue-induced reinstatement (Rich et al., 2019). Furthermore, optogenetic induction of long-term depression (LTD) of MGN-LA synapses, which recapitulated the reversal of cocaine-induced potentiation induced by extinction, was sufficient to reduce subsequent cue-induced relapse-like behavior (Rich et al., 2019), thus implying that manipulation of these synapses can alter the salience attributed to environmental stimuli.

1.4.3 TREATMENT INTERVENTIONS

Understanding the strengthening and weakening of synapses involved in drug-associated memory has implications for reducing the risk of relapse. A major strategy for preventing relapse has been to interfere with drug-associated memories. Few pharmacological options have been successful at preventing relapse for an extended period of time (Bossert et al., 2013; Conklin &

Tiffany, 2002). Likewise, behavioral strategies for suppressing drug-associated memories have had limited success. Cue exposure treatment, which involves repeated unreinforced exposure to stimuli previously associated with drug use to extinguish conditioned responses, has been used in the clinical setting for almost all drugs of abuse with only modest benefits (Drummond & Glautier, 1994; Ehrman et al., 1998; Raw & Russell, 1980). Some researchers have attempted to improve the efficacy of cue exposure treatment by combining it with cognitive behavior techniques such as cue replacement or coping strategies (Monti et al., 1993; Symes & Nicki, 1997). Despite these efforts, drug-seeking behavior tends to be renewed when the patient returns to the original drug-paired environment (Crombag et al., 2008; Thewissen et al., 2006). Combining both behavioral therapy strategies and treatment medication shows the highest success rates for reducing the strength of drug-associated memories that contribute to relapse (Cleva et al., 2011; Dunbar & Taylor, 2017; Torregrossa & Taylor, 2013). Thus, continuing to examine the circuits and mechanisms underlying the formation and maintenance of drug-associated memories is an important research direction for discovering successful therapeutic interventions for SUD.

1.5 PURPOSE OF STUDIES

This dissertation summarizes two major lines of inquiry concerning how dopamine release in the amygdala contributes to learning and memory. In **Chapter 2**, we use fiber photometry in the LA during a Pavlovian to Instrumental Transfer (PIT) task to test the hypothesis that dopamine and calcium activity increase in response to reward and reward-predictive cues to mediate learning and memory. To this end, we assess LA dopamine and calcium dynamics during multiple timepoints of Pavlovian training, instrumental training, and the transfer phase. Furthermore,

experiments in **Chapter 3** aim to examine the development of maladaptive behaviors by assessing the hypothesis that the VTA dopamine to the LA projection supports cocaine cue associative learning. Through chemogenetic techniques, we test if manipulation of VTA dopamine input to the LA affects acquisition of cocaine self-administration and/or reinstatement. In total, these studies suggest that dopamine in the amygdala is a key component of associative learning and that aberrant dopamine signaling in the region may confer vulnerability to drug use.

2.0 DOPAMINE ACTIVITY IN THE LATERAL AMYGDALA DRIVES CUE-REWARD LEARNING

2.1 INTRODUCTION

Associative learning is a process that allows organisms to acquire information about how events are related to one another. This type of learning promotes behavior modification based on the relationship between stimuli. In classical conditioning, a simple form of associative learning, repeatedly pairing a neutral stimulus with a biologically significant unconditioned stimulus (US) results in an association between the two stimuli. The once neutral stimulus becomes a conditioned stimulus (CS) and future presentation of it alone elicits a conditioned response (CR) that mirrors the unconditioned response (Pearce & Bouton, 2001). In the most recognized example, Pavlov's dogs were conditioned to salivate (CR) at the sound of a bell (CS) that was repeatedly paired with food delivery (US) (Pavlov, 1927).

The lateral amygdala (LA) is a brain region that is critical for associative learning. It mediates associative learning for auditory conditioned cues by receiving CS information from the auditory thalamus (Kim et al., 2007; Kwon et al., 2014; LeDoux, Farb, & Ruggiero, 1990; Rich, Huang, & Torregrossa, 2019; Rogan, Staubli, & LeDoux, 1997; Rogan & LeDoux, 1995). At the same time, input encoding the US, whether it be rewarding like sucrose or aversive like footshock, reaches the LA. At the synaptic level, acquisition of cue-directed reward-seeking depends on neuronal plasticity in the LA at glutamatergic thalamo-amygdala synapses (Tye, Stuber, De Ridder, Bonci, & Janak, 2008). Despite knowledge of these relevant circuits, the activity of LA neurons during the cue-reward memory formation process is not well characterized.

Evidence suggests that dopamine activity in the LA may contribute to the formation of cue-reward associations. Dopamine released in the basolateral amygdala (BLA), including the lateral portion (LA), is important for emotional and behavioral functions (Brinley-Reed & McDonald, 1999). This role likely results from dopamine's ability to enhance amygdala projection neuron excitability and to affect postsynaptic modulation of amygdala interneuron excitability (Kröner, Rosenkranz, Grace, & Barrionuevo, 2005). Additionally, ventral tegmental area (VTA) dopamine to BLA connections promote reward-seeking by responding to both reward and reward-predictive cues (Lutas et al., 2019) and stimulating dopamine neuron terminals in the BLA has been shown to strengthen the motivational impact of previously learned reward cues (Mahler et al., 2019). Yet, many questions about how LA dopamine activity and its timing contribute to associative learning remain.

Here, we took advantage of fiber photometry to determine how dopamine and calcium activity in the LA reflect cue learning and if this activity changed across training. We used a Pavlovian to Instrumental transfer (PIT) behavioral paradigm to disentangle the dopamine and calcium responses that may underlie cue-reward learning and/or instrumental learning. Our results demonstrate a specificity in dopamine response to reward predictive cues during Pavlovian conditioning that is absent in response to reward or during instrumental training. In general, we probed these cue learning mechanisms in the LA to better our understanding of the development of stimulus-outcome associations, which have implications not only for natural reward learning but also for conditions such as substance use disorder or PTSD where associative learning becomes maladaptive.

2.2 METHODS

2.2.1 Animals

Adult male and female Sprague Dawley rats (Envigo) were paired housed in auto-ventilated racks with automated watering in a temperature- and humidity-controlled room. They were kept on a 12 h light/dark cycle (lights on at 4:30) and given at least 5 days to acclimate to the facility before any procedures. Following optic fiber implantation, animals were single housed. Rats were food restricted 24 hours before habituation to the sipper bottle and were maintained at 95% of their free-feeding weight. All procedures were conducted in accordance with the National Institutes of Health's *Guide for Care and Use of Laboratory Animals* and were approved by the University of Pittsburgh's Institutional Animal Care and Use Committee.

2.2.2 Surgery

2.2.2.1 Anesthesia

Rats were fully anesthetized via intramuscular injections of ketamine (90-100 mg/kg, Henry Schein) and xylazine (5 mg/kg, Butler Schein). They were given subcutaneous injections of the analgesic Rimadyl (5 mg/kg, Henry Schein) and 5 ml of Lactated Ringer's solution. Surgical sites were shaved and treated with betadine (povidone iodine, 5%, Henry Schein) and 70% ethanol on all incisions as previously described (Rich et al., 2019).

2.2.2.2 Viral Constructs and delivery

For viral infusion surgery, rats were placed in a stereotaxic frame and injected with 0.2-0.3 ml of lidocaine (Henry Schein) to the scalp as a local anesthetic. The scalp was opened with a sterile scalpel and holes were drilled in the skull at the appropriate coordinates. A 26-gauge stainless steel injection cannula connected to a Hamilton syringe and pump (Harvard Apparatus) was used to inject the LA (males in mm from bregma, anterior and posterior (AP): -3.0; medial and lateral (ML): ± 5.0 ; dorsal and ventral (DV): -7.9 mm; females in mm from bregma, AP: -2.8, ML: ± 4.8 ; DV: -7.8 mm) bilaterally (1 μ l/hemisphere) with a 1:1 ratio mix of AAV5.hSyn.dLight1.2 (Addgene) and AAV1.hSyn.NES-jRCaMP1b.WPRE.SV40 (Addgene). In a separate experiment using the same setup, a 1:1 ratio mix of a custom made AAV2retro.PRSx8.HA-hM4D.SV40 (Penn Vector Core) and AAV5.hSyn.dLight1.2 was bilaterally infused into the LA (males in mm from bregma, anterior and posterior (AP): -3.0; medial and lateral (ML): ± 5.0 ; dorsal and ventral (DV): -7.9 mm; females in mm from bregma, AP: -2.8, ML: ± 4.8 ; DV: -7.8 mm). Injections occurred at a 0.1 μ l/s flow rate and injection cannula were left in place for 5 min after completion before being slowly withdrawn.

2.2.2.3 Intracranial fiber optic implantation

Five weeks after the viral infusion surgery, rats were again placed in a stereotaxic frame and treated with a small injection of lidocaine to the scalp (0.2-0.3 ml, Henry Schein) as an anesthetic. After opening the scalp with a sterile scalpel, two holes were drilled at the proper coordinates. Two fiber optics (02.5 mm SS Ferrule, 0400 μ m core, 0.39 NA, 10 mm length, ThorLabs) were implanted bilaterally, aimed to the lateral amygdala (males in mm from bregma, anterior and posterior (AP): -3.0; medial and lateral (ML): ± 5.0 ; dorsal and ventral (DV): -7.9 mm;

females in mm from bregma, AP: -2.8, ML: ± 4.8 ; DV: -7.8 mm). Optic fibers were secured to the skull with screws and OrthoJet dental cement (Lang Dental). Rats were given Rimadyl (5 mg/kg, Henry Schein) subcutaneously for 2 days after surgery.

2.2.3 Behavioral Procedures

2.2.3.1 Behavioral Apparatus

Experiments were conducted in a single standard operant conditioning chamber (MedAssociates) using MedPC software (MedAssociates). The chamber was equipped with a bar floor, a house light, two cue lights above two levers, a tone generator, a white noise generator, and 5 nose-poke apertures covered with a removable opaque plexiglass cover. The chamber had 2 plexiglass walls with one containing the levers, magazine and cues lights and the opposite wall containing the nose-poke apertures. A panel containing a bottle and sipper tube (MedAssociates) was inserted on the chamber wall between the levers. The chamber was contained in a sound-attenuating box with a fan for background noise and a camera (MedAssociates) was affixed to the door of the box.

2.2.3.2 Habituation

A sipper bottle filled with a 10% sucrose solution was placed in the animal's home cage for 1 hr to acclimate them to both the sipper and the solution. The following day, rats were habituated to the sipper entering the operant chamber. The 20-min session began with house light illumination and the sipper entered the chamber on a random interval schedule. Each sipper entry had a 5s duration. Sessions were video recorded to confirm rats were using the sipper before continuing to training.

2.2.3.3 Pavlovian conditioning

Rats underwent 20-min Pavlovian conditioning sessions for 12 days with the 10% sucrose solution reinforcer. Each session began with illumination of the house light. The session consisted of 5 2-min inter-trial intervals (ITI) and 5 2-min CS+ periods. For the 2-min ITI, no auditory cue played and no sipper entries occurred. During the 2-min CS+ period, an auditory cue played (tone or white noise) and the sipper (from bottle containing 10% sucrose solution) entered the chamber at a random interval for a 5s entry duration. Sessions were video recorded and scored. Time spent in the designated sipper area (rectangle drawn around the sipper entry hole) was measured for both the ITI and the CS+ intervals. The number of times the sipper entered the chamber was recorded as was the number of times the rat used the sipper. Performance criteria were set to determine if rats learned the task, but did not impact whether they continued in the experiment. Criteria included: using the sipper a minimum of 8 times during the session, using the sipper for $\geq 33\%$ of its total entries, time spent near the sipper during the CS+ at least twice that of time spent near the sipper during the ITI, and spending $\geq 33\%$ of time during CS+ near the sipper. Any rats that lost optic fibers during the Pavlovian training phase, continued in behavioral training but did not have any further recordings. One cohort of rats (n=4) underwent Pavlovian training before continuing into a different training protocol and as a result only Pavlovian conditioning data for these animals is reported here.

2.2.3.4 Instrumental training

Rats had 30-min instrumental training sessions that began with illumination of the house light and lever insertion. Training began on a fixed ratio 1 (FR1) schedule of reinforcement where a press on the active lever resulted in sipper entry for 5s while the levers retracted. Sessions were

video recorded and sipper use was scored. FR1 sessions continued for ~3 days or until rats met the criterion of ≥ 10 active presses and ≥ 8 sips. Rats progressed to a random ratio 5 (RR5) schedule of reinforcement where a random number of active presses between 1 and 5 resulted in sipper presentation and lever retraction. After at least 3 days of RR5, or until criterion was met, rats proceeded to a RR10 schedule for 5 days where a random number of active presses between 1 and 10 resulted in sipper entry. Across all schedules of reinforcement, the number of times the sipper entered the chamber was counted as were inactive lever presses that had no programmed consequence. Any rats that did not meet instrumental acquisition criterion did not continue in the experiment. Any rats that lost fibers during the instrumental training phase continued through behavioral training, but did not have any further recording sessions.

2.2.3.5 Pavlovian to Instrumental Transfer test

After the final instrumental training session, rats were tested for Pavlovian to Instrumental transfer under extinction conditions. This 30-min test began with illumination of the houselight and insertion of the levers. The session began with 2 min of the auditory cue that was paired with sucrose during Pavlovian training (CS+). This period was followed by a 2-min inter-trial interval (ITI) before a novel auditory cue was played for 2 min (CS-). The CS+, ITI, CS- presentation pattern continued until each cue type had been presented 4 times. The number of active and inactive lever presses made during each cue or during the ITI were recorded to determine if CS+ presentation modulated instrumental performance, but there were no consequences for active or inactive presses.

2.2.3.6 Chemogenetic manipulation during behavior

A separate cohort of rats was co-infused in the LA with a retrograde adeno-associated virus (rgAAV2) containing a sequence for the hM4Di (Gi-coupled) receptor under the synthetic dopamine beta hydroxylase PRSx8 promoter and the virus containing dLight1.2. These animals underwent habituation as described above, but had different training timelines for the rest of the experiment. These rats experienced 10 days of Pavlovian conditioning, 1 week of instrumental training at FR1, 3 days of RR5 instrumental training, and 3 days of RR10 training. Animals received i.p. injections of 1 mg/kg of Clozapine-N-oxide (CNO, graciously provided by NIDA Drug Supply Program, dissolved in 5% DMSO in 0.9% sterile saline) or DMSO vehicle on Pavlovian training days (Days 4, 6, 8, and 10) and Instrumental Training (RR5 days 1-2, RR10 days 1-2). The order of CNO and vehicle were randomized and counterbalanced between subjects. All animals received systemic CNO on the Pavlovian to Instrumental Transfer test day.

2.2.3.7 Behavior scoring

Recording sessions were videotaped using a MedPC camera attached to the cabinet door of the box containing the operant chamber. Each time a rat used the sipper during the session for Pavlovian conditioning and instrumental training was manually coded using CowLog software. Sip timestamps coded during scoring were aligned to Plexon timestamps and used in subsequent analysis.

2.2.4 Fiber photometry

2.2.4.1 Recording procedure

During habituation to the operant chamber and sucrose sipper bottle, rats were also habituated to the optical tether, but no light was delivered. Fiber photometry was used to image bulk calcium (RCaMP sensor) and dopamine activity (dLight sensor) in LA neurons during Pavlovian training (Days 1, 4, 8, and 12 or Days 4, 6, 8, and 10 in the PRSx8 DREADD cohort), instrumental training (RR5 days 1 and 3, RR10 days 1 and 4 or RR5 days 1-2 and RR10 days 1-2 in the PRSx8 DREADD cohort), and Pavlovian to Instrumental transfer (only the first group). dLight, RCaMP, and control fluorescence were simultaneously imaged using a commercial multi-wavelength fiber photometry system (Plexon). Three light-emitting LEDs (465 nm: dLight fluorescence, 560 nm: Ca²⁺-dependent RCaMP fluorescence, and 410 nm: isosbestic control) were phase cycled and coupled via a patch cord (2 branches: 400 μ m core and 440 μ m cladding, 0.37 NA, Doric) to the implanted optical fiber. The excitation light intensity was adjusted to 20-30 μ W at the tip of the patch cord. Laser light was passed through the patch cord for 30 min prior to recording sessions to minimize photobleaching of the cable during recordings. Fluorescence emission was passed through a bandpass filter and fluorescence data were collected at 30 frames per second using Plexon software. Behavioral events were aligned to fluorescence using TTL timestamp output from MedPC software. Photometry recordings were collected from both hemispheres.

2.2.4.2 Data processing and analysis

All fiber data were analyzed using Guided Photometry Analysis in Python (GuPPy), a free and open-source tool. The analysis pipeline is described previously (Sherathiya et al. 2021).

Briefly, Plexon data were extracted from stores containing data for each fiber in each signal channel and isosbestic control channel. A zero-phase moving average linear digital filter was applied backwards and forwards to the channels to reduce high frequency noise without time-shifting. The window for the moving filter was set at 10 data points based on the sampling rate of the recorded data. The initial second was removed from each recording and any artifacts within the recording were removed via manual selection. GuPPy computed $\Delta F/F$ for the entire recording trace by subtracting the fitted control channel from the signal channel and dividing by the fitted control. z-scores of each trace were computed as the deviation of the $\Delta F/F$ signal from its mean so that data could be combined across fibers and subjects. PSTHs were computed for each event timestamp based on a defined window. For all timestamps, windows were set at 1s before the event, 1s during the event, and 1s second after the event. The average of trials in the PSTH vector was calculated after baseline correction and the area under the curve and the peak of the PSTH for each trace were calculated. dLight and RCamP signal were analyzed separately. For the Pavlovian conditioning phase, animals' mean peak z-score for each event for each fiber were grouped by training day. For the instrumental training, animals' mean peak z-score for each event on days under the same reinforcement schedule were averaged to create a single schedule score for each fiber. For PIT, rats' mean peak z-score for each event for each fiber were grouped by stimulus type.

2.2.5 Histology

Rats were deeply anesthetized with sodium pentobarbital (Covetrus, 100 mg/kg i.p.) and then perfused through the aorta with 1X PBS for 5 min followed by 4% paraformaldehyde (Santa Cruz Biotechnology) in 1X PBS, pH 7.4 for 10 min. The brains were extracted, postfixed in 4%

paraformaldehyde for 24h before being transferred to a 30% sucrose solution. Brains were sectioned at 50 μm using a cryostat (Leica) with slices containing the lateral amygdala collected for histology. Slices were mounted on glass slides and coverslipped with Fluoroshield with DAPI mounting media (Sigma-Aldrich). Slides were imaged using an Olympus VS200 or Olympus VS120 slide-scanning microscope to verify virus expression and optic fiber placement. Rats lacking expression and those without properly positioned fibers were removed from the study.

In animals infused with the PRSx8 promoter Gi-DREADD, sections containing the lateral amygdala and locus coeruleus were also taken to examine immunoreactivity of the HA tag associated with the virus. These sections were washed with PBS containing 0.1% triton X (PBST+) and then incubated in a PBST+ and 5% donkey serum (Millipore Sigma) blocking buffer for 2 hr at room temperature. Sections were then placed in anti-HA primary antibody (1:1000 Cell Signaling C29F4) for 48 hours at 4°C. After incubation in primary antibody, sections were washed with PBST+ and moved to wells with second antibody in blocking buffer (1:500 Donkey anti-rabbit IgG Alexa Fluor 594, ThermoFisher) for 2 hr at room temperature. Sections were washed in PBS, mounted, and coverslipped with Fluoroshield with DAPI mounting medium (Sigma Aldrich). Slides were then scanned using an Olympus VS120 scanning microscope.

2.2.6 Statistical Analysis

Behavioral data were collected using MedPC software and all statistical analyses were performed using GraphPadPrism and SPSS Statistics Software. In all analyses significance was set at $p < 0.05$. For Pavlovian conditioning behavioral data, two-way rmANOVA with within-subjects factors of auditory stimulus (CS+ vs ITI) and training day were performed. For photometry data collected during Pavlovian conditioning, two-way rmANOVA with within-

subjects factors of event timing (pre-CS+ vs CS+ or pre-sip vs sip) and training day were performed. Two-way rmANOVA with within-subjects factors of lever (active vs inactive) and training day were used to analyze behavior during instrumental training. For instrumental training photometry data, two-way rmANOVA with two-way within-subjects factors of event timing (pre-press vs press or pre-sip vs sip) and schedule (RR5 vs RR10) were performed. Finally for PIT data, two-way rmANOVA with within-subjects factors of auditory stimulus (CS+ vs CS- vs ITI) and lever (active vs inactive) were performed, while two-way rmANOVA with within-subjects factors of auditory stimulus (CS+ vs CS- vs ITI) and event timing (pre-CS+ vs CS+, pre-CS- vs CS-, pre-press vs press) were performed for PIT photometry data. For data from the PRSx8 DREADD group, treatment (CNO vs veh) was introduced as an additional within-subjects factor.

2.3 RESULTS

2.3.1 Pavlovian conditioning

2.3.1.1 Rats spent more time near sipper during CS+ presentation.

During the Pavlovian training phase, animals experienced alternating 2-minute periods of CS+ (tone or white noise) presentation with a 2-minute inter-trial interval (ITI) (Figure 1C). During the CS+ periods, the sucrose bottle sipper entered the operant chamber at a random interval. For the entire conditioning session, the amount of time the rats' spent near the sipper area was assessed and there was a main effect of CS+ presentation ($F_{(1,12)}=29.53$, $p=0.0002$), a main effect of training day ($F_{(11,132)}=5.51$, $p<0.0001$), and a CS+ presentation x training day interaction ($F_{(11,$

$t_{108}=4.83, p<0.0001$). During the CS+ presentation, rats spent more time near the sipper than they did during the ITI period and this difference increased across training (Figure 1D).

2.3.1.2 Dopamine and calcium activity reflect cue reward learning.

During the Pavlovian training phase, fiber photometry recordings in the lateral amygdala (LA) were performed on days 1, 4, 8, and 12. These recordings measured bulk dopamine (dLight) and calcium (RCaMP) activity aligned to the CS+ presentation. Peak fluorescence was compared between the 1-second baseline period before CS+ presentation and the first second of the CS+ presentation on each recording day (5 CS+ presentations total per day). For dopamine activity in the LA, there was no main effect of training day ($F_{(3, 66)}=0.11, p=0.95$), but a trend for an effect of CS+ presentation ($F_{(1,22)}=4.02, p=0.058$), and training day x CS+ presentation interaction ($F_{(3,10)}=2.86, p=0.091$). Planned comparisons to determine changes in cue response within each session demonstrated that there was an increase in dopamine activity to the CS+ compared to the pre-CS+ baseline on day 12 ($p=0.041$, Figure 1E, G). Examining the calcium response in the LA yielded no main effect of training day ($F_{(2.88, 60.57)}=1.59, p=0.20$). There was a main effect of CS+ presentation ($F_{(1,21)}=5.88, p=0.024$) such that calcium activity was greater during the first second of the CS+ compared to the 1 second prior to its presentation (Figure 1F, H).

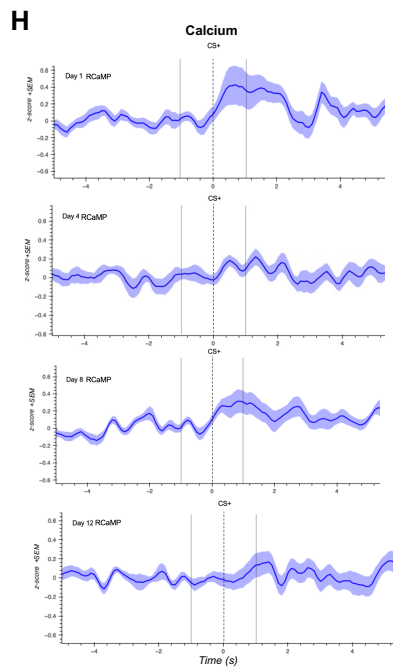
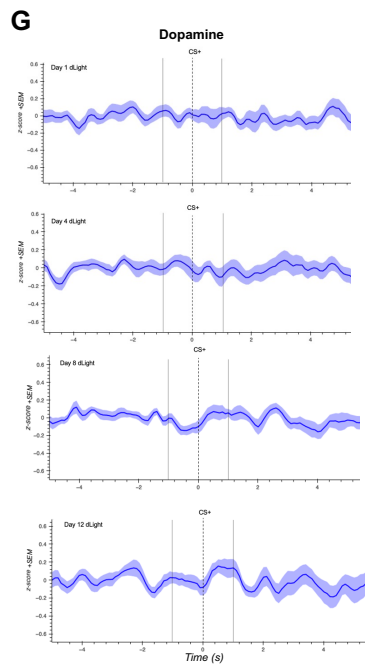
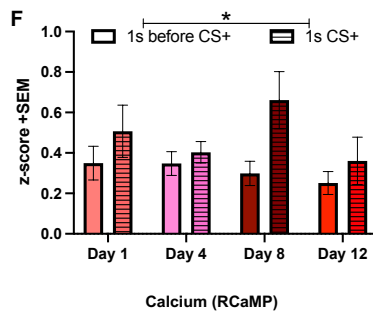
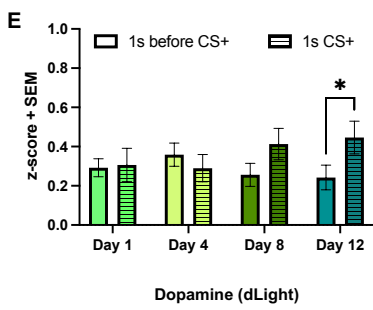
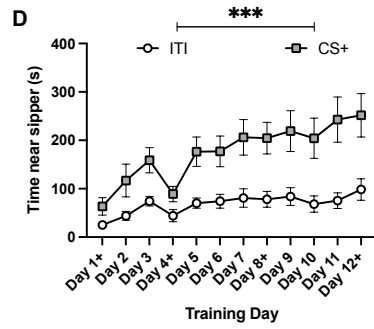
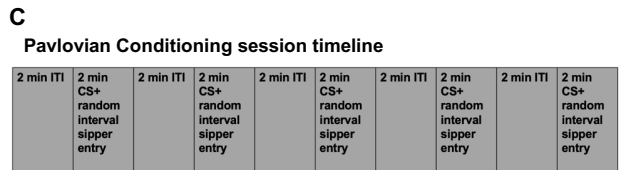
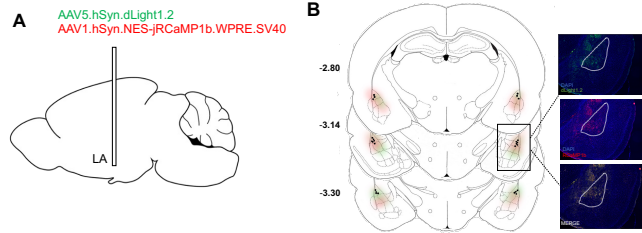


Figure 1. Dopamine and calcium activity reflect cue learning during Pavlovian conditioning.

Rats (n=13; recording: n= 9 [18 fibers]) underwent a Pavlovian conditioning task during which the time they spent near the sipper during both the CS+ and ITI was measured (C-D). During days 1, 4, 8 and 12 fiber photometry recordings measuring fluorescence linked to dopamine (dLight) and calcium (RCaMP) activity in the LA were performed (E-H). Schematic of viral injection of dLight1.2 (green) and RCaMP (red) in the LA (A). Schematic of viral injection and expression in the LA with representative images. Black circles mark optic fiber placements (B). Pavlovian training session timeline (C). Rats spent more time near the sipper during the CS+ than during the ITI and this difference increased across training (D). Dopamine activity tended to increase in response to CS+ presentation compared to the 1 second before CS+ baseline, particularly on day 12 (E). Calcium activity during the first second of the CS+ was greater than activity during the 1 second pre-CS+ at all timepoints of Pavlovian conditioning (F). Mean traces of dLight signal centered around CS+ presentation (top to bottom: Day 1, Day 4, Day 8, Day 12; G). Mean traces of RCaMP signal centered around CS+ presentation (top to bottom: Day 1, Day 4, Day 8, Day 12; H). Day+ on panel B represents recording days. *p<0.05, ***p<0.001

2.3.1.3 Animals that learn the cue-reward association show dopamine and calcium activity increases to the cue presentation.

As differences between the pre-CS+ baseline and the start of CS+ presentation emerged at later training timepoints, it is possible that these responses to the CS+ were related to acquisition of cue-reward associations. To examine if these effects were driven by learning, we set performance criteria to assess whether an animal learned the cue-reward association underlying the Pavlovian task (Figure 2A). To meet criteria, rats needed to use the sipper at least 8 times during the session. Additionally, they needed to use the sipper at least 33% of the time that it entered the operant chamber. To ensure that time spent near the sipper was driven by the auditory CS+ and not just a preference for that area of the chamber, rats had to spend twice as much time near the sipper during the CS+ period than they did during the ITI. Finally, when the CS+ was playing, they needed to spend at least 33% of that time near the sipper.

Animals were split into non-learners (did not meet criteria) and learners (met criteria) for each training day (Figure 2B). The dopamine and calcium peak fluorescence activity in LA in response to the CS+ on these days was examined with fiber photometry. No rats met criterion on day 1 so this timepoint was not included in analysis. For the non-learners, there was no main effect of training day ($F_{(2,22)}=0.13$, $p=0.88$) or CS+ presentation ($F_{(1,22)}=0.36$, $p=0.55$) on dopamine activity. For learners, there was no main effect of training day ($F_{(2,21)}=0.20$, $p=0.82$), but CS+ presentation impacted dopamine activity in the LA ($F_{(1,21)}=6.54$, $p=0.018$). Multiple comparisons revealed that on day 8, dopamine activity was on average greater during the first second of the CS+ than during the 1 second prior to the CS+ ($p=0.077$), while on day 12 activity during the CS+ was significantly greater than during the pre-CS+ period ($p=0.0038$, Figure 2C, E-F). These findings indicate that only rats that learned the task and the underlying cue-reward association had

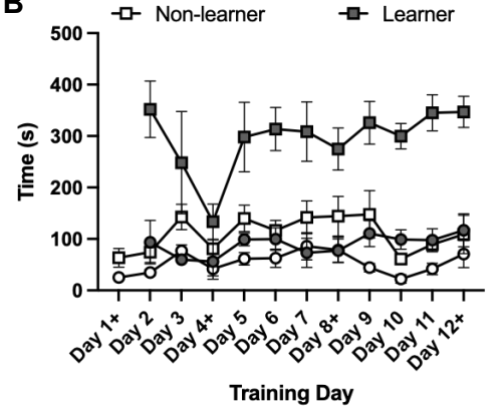
a change in the dopamine response to the CS+. When examining calcium activity in the LA of non-learners, there was a trend for an effect of training day ($F_{(2,20)}=3.40$, $p=0.053$). This effect was driven by calcium activity tending to be higher on day 8 relative to day 12 ($p=0.061$). However, there was no main effect of CS+ presentation on calcium activity ($F_{(1,20)}=1.27$, $p=0.27$), indicating that at no point during conditioning did non-learners display a change in calcium activity in response to the CS+ (Figure 2D, G-H). For learners, there was no main effect of training day ($F_{(2,20)}=0.12$, $p=0.89$) or CS+ presentation ($F_{(1,20)}=1.77$, $p=0.19$) on calcium activity, but there was a trend toward a training day x CS+ presentation interaction ($F_{(2,20)}=2.66$, $p=0.094$). Post hoc comparisons showed that on day 8, calcium activity was greater during the first second of the CS+ than during the 1 second baseline prior to CS+ onset ($p=0.034$, Figure 2D). Collectively, these data suggest that the CS+ is more salient for learners than non-learners.

A

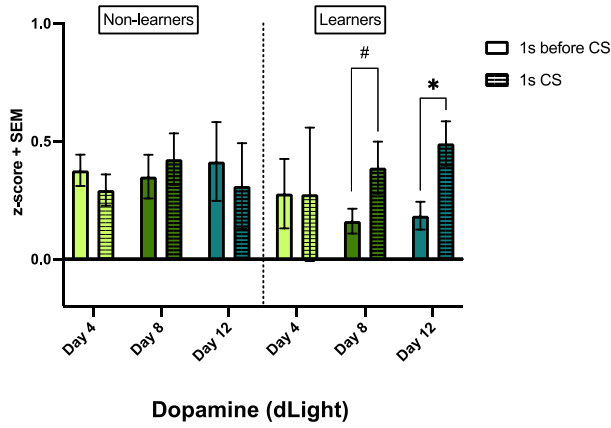
Performance criteria

| Sipper use | Sipper use per sipper entry | Sipper time CS vs ITI | Time near sipper during CS+ |
|------------------|-----------------------------|-----------------------|-----------------------------|
| ≥ 8 sipper bouts | ≥33% of entries | time CS ≥ 2X time ITI | ≥33% of time |

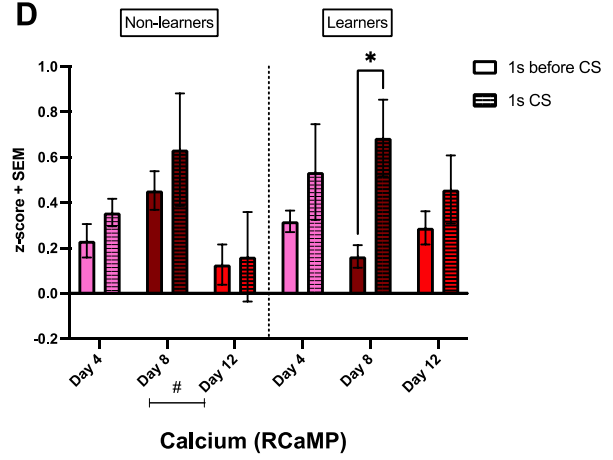
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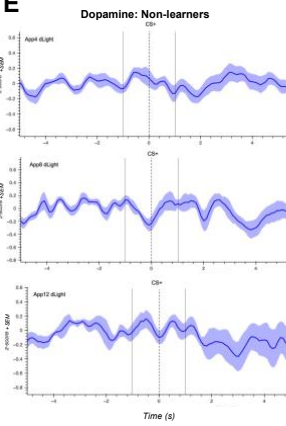
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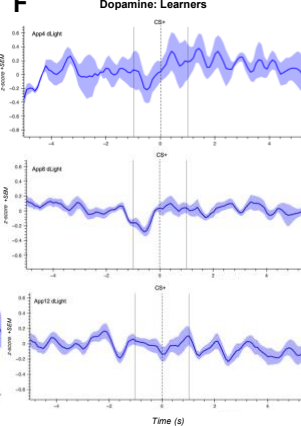
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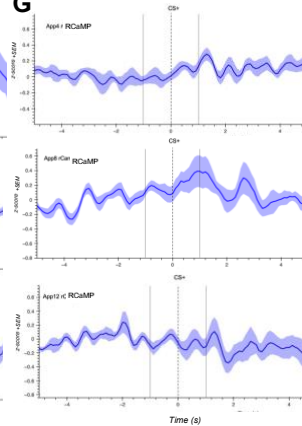
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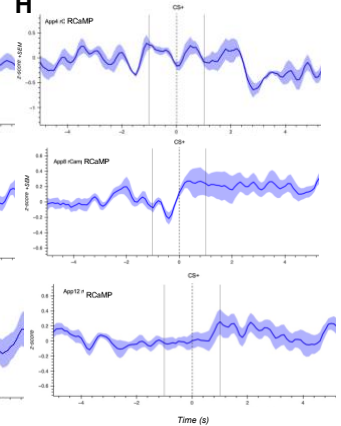


Figure 2. Acquisition of the cue-reward association is reflected in dopamine and calcium activity in response to CS+ presentation. Rats (n=13; recording: n=9 [18 fibers]) that underwent Pavlovian conditioning were split into non-learners and learners based on performance criteria for each training day (day 4: 2 learners, 11 non-learners; day 8: 5 learners, 8 non-learners; day 12: 10 learners; 3 non-learners; A-B). On all days, non-learners show no change in LA dopamine activity during the initial second of the CS+, while the difference between the pre-CS+ and CS+ emerged by days 8 and 12 for learners (C). Calcium activity in non-learners trended higher on day 8 relative to day 12, but there was no change in LA calcium activity in response to the CS+. For learners, on day 8, calcium activity in the LA during the first second of the CS+ was greater than activity in the 1 second period before CS+ onset (D). Mean traces of dLight signal centered around CS+ presentation for non-learners (top to bottom: Day 4, Day 8, Day 12; E). Mean traces of dLight signal centered around CS+ presentation for learners (top to bottom: Day 4, Day 8, Day 12; F). Mean traces of RCaMP signal centered around CS+ presentation for non-learners (top to bottom: Day 4, Day 8, Day 12; G). Mean traces of RCaMP signal centered CS+ presentation for learners (top to bottom: Day 4, Day 8, Day 12; H). Day+ on panel B represents recording days. *p<0.05

2.3.1.4 Dopamine responses to the CS+ develop on the day animals learn the cue-reward association.

To assess further the connection between associative learning and development of activity in response to CS+ presentation, we compared the first day of approach, the recording day closest to the day rats reached performance criteria, and the final day of approach for rats that never met criteria (Figure 3A). Dopamine and calcium peak activity during the 1 second prior to the CS+ and during the first second of CS+ presentation were examined. There were no main effects of day (day 1 vs day learned vs last day, $F_{(2,31)}=0.083$, $p=0.92$) or CS+ presentation ($F_{(1,31)}=0.46$, $p=0.50$) on dopamine activity, but there was a trend for a day x CS+ presentation interaction ($F_{(2,31)}=2.64$, $p=0.087$, Figure 3B, D). Planned comparisons at the different time points showed that on the day rats learned there was a significant increase in dopamine activity during the 1-second CS+ presentation compared to the 1-second baseline before the CS+ ($p=0.035$). As this response to the CS+ was absent during the first approach day and never developed for rats that never learned, it seems the activity reflects learning of the cue-reward association (Figure 3B). There were no main effects of day (day 1 vs day learned vs last day $F_{(2,25)}=2.33$, $p=0.12$) or CS+ presentation ($F_{(1,25)}=1.85$, $p=0.19$) on calcium activity in the LA (Figure 3C, E). The difference between the activity findings suggests that dopamine activity is specifically tied to cue-reward associative learning.

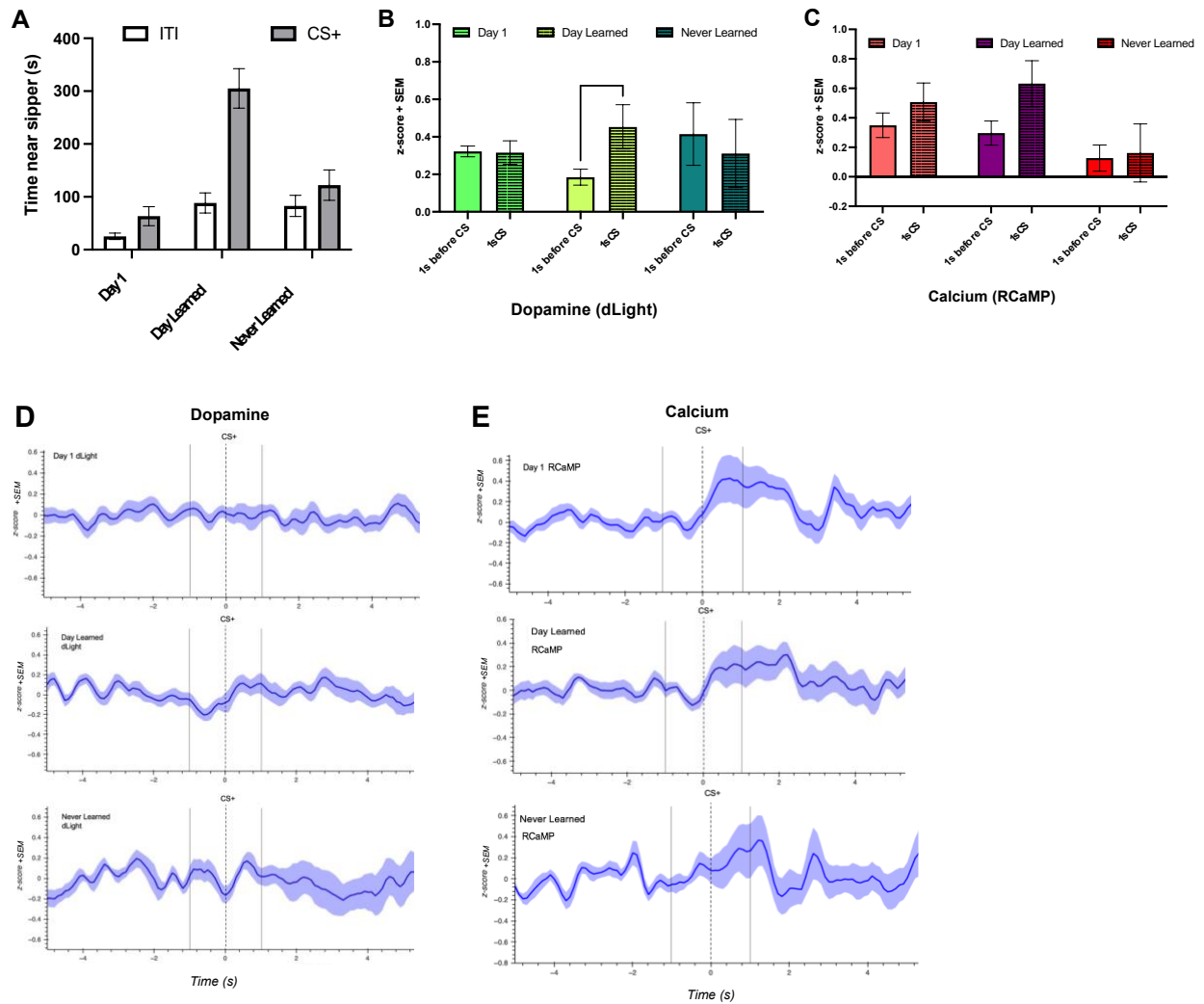


Figure 3. Increased dopamine activity to the CS+ emerged on the day rats learned the Pavlovian task. Rats ($n=13$; recording: $n=9$ [18 fibers]) were trained on a Pavlovian conditioning task and both behavioral and photometry data were split into the first day of training, the day learned, and the final day of training in the case where rats never learned the task (A-C). There was an increase in dopamine activity in response to the CS+ only on the day rats learned the task (B). Calcium activity did not significantly change in response to the CS+ or in response to task learning (C). Mean traces of dLight signal centered around CS+ (top to bottom: Day 1, Day Learned, Never Learned; D). Mean traces of RCaMP signal time-locked to CS+ (top to bottom: Day 1, Day Learned, Never Learned; E). $*p<0.05$

2.3.1.5 Activity changes in response to CS+ onset and not CS+ offset.

As our findings suggested that LA activity reflected salience of the CS+, we wanted to determine if there were changes in response to CS+ offset. We examined calcium and dopamine activity during the first second of the 2-minute CS+ presentation and the second the CS+ turned off. Analysis of dopamine activity yielded no main effect of training day ($F_{(3,66)}=0.44$, $p=0.72$). There was a main effect of CS+ timing on dopamine activity (onset vs offset, $F_{(1,22)}=6.51$, $p=0.018$) indicating that overall activity was greater in response to CS+ onset than CS+ offset and post hoc analysis revealed a trending difference between activity during onset and offset on day 12 ($p=0.095$, Figure 4A). In the case of the calcium response, we again found no main effect of training day on activity ($F_{(3,66)}=1.78$, $p=0.16$), but a main effect of CS+ timing (onset vs offset, $F_{(1,22)}=8.75$, $p=0.0073$). In general, calcium activity was increased in response to CS+ onset compared to CS+ offset (Figure 4B). Post hoc analysis showed that there was a significant difference in CS+ onset calcium activity relative to CS+ offset calcium activity on day 8 ($p=0.0066$, Figure 4B). These results suggest that CS+ onset, but not offset, is salient to rats, potentially even before they reach high levels of task performance.

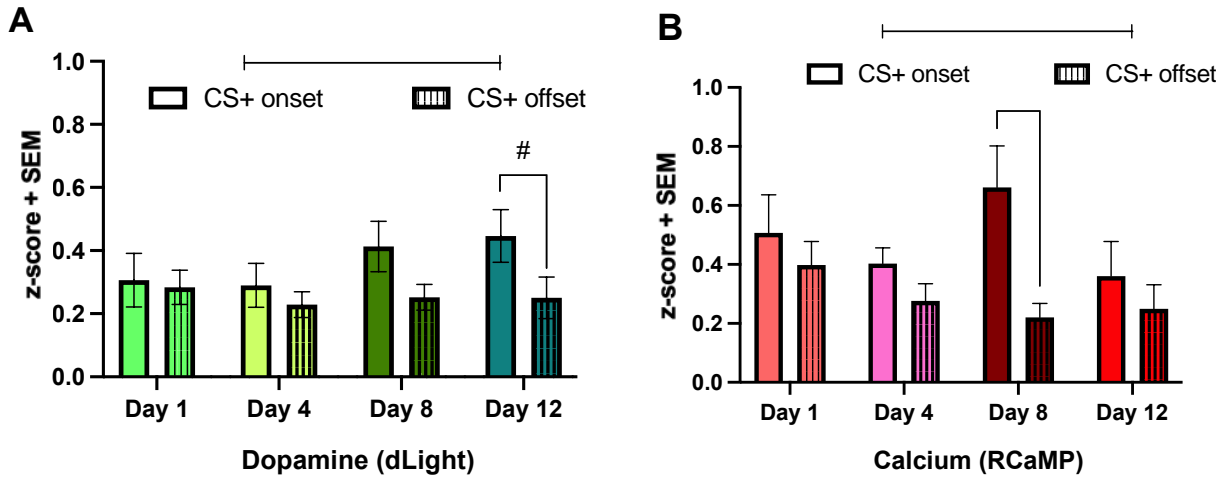


Figure 4. Dopamine and calcium activity changes in response to CS+ onset but not offset. During Pavlovian conditioning, rats (n=13; recording: n= 9 [18 fibers]) experienced a 2-minute CS+ presentation paired with sipper delivery. Dopamine activity was greater in response to CS+ onset than to CS+ offset (A). Calcium activity during CS+ onset was greater than activity during CS+ offset, especially on day 8 (B). #p<0.1, *p<0.05, **p<0.01

2.3.1.6 Sipper use increases across training.

During Pavlovian training, the CS+ presentation was paired with random interval sipper entry where rats could drink the 10% sucrose solution for 5 seconds (Figure 5A). We measured the number of times rats used the sipper each session across the 12 days of Pavlovian training. There was a main effect of training day on sipper use ($F_{(11,99)}=4.00$, $p<0.001$) such that animals increased their sipper use across training (Figure 5B). It is important to note that sipping behavior was low on day 4 when animals were connected to the optical tether even though rats had both been habituated and connected previously on day 1. Rats' behavior recovered by the following day and appeared unaffected by future connections.

2.3.1.7 Dopamine and calcium activity do not change in response to sipper use during Pavlovian training.

We measured dopamine and calcium peak fluorescence with fiber photometry to examine any dopamine and calcium activity changes in response to sipper use across Pavlovian training. Specifically, activity in the 1 second period before a sip was made was compared to the first second of the sip. There was not a main effect of either training day ($F_{(3,51)}=0.64$, $p=0.59$) or sip ($F_{(1,17)}=0.54$, $p=0.47$) on dopamine activity (Figure 5C, E). Likewise, there were no main effects of training day ($F_{(3,45)}=1.20$, $p=0.32$) or sip ($F_{(1,15)}=0.28$, $p=0.60$) on calcium activity (Figure 5D, F). These results indicate that activity in the LA remained stable from a pre-sip baseline to when rats took a sip from the sucrose sipper bottle.

A**Pavlovian Conditioning session timeline**

| | | | | | | | | | |
|-----------|--|-----------|--|-----------|--|-----------|--|-----------|--|
| 2 min ITI | 2 min CS+ random interval sipper entry | 2 min ITI | 2 min CS+ random interval sipper entry | 2 min ITI | 2 min CS+ random interval sipper entry | 2 min ITI | 2 min CS+ random interval sipper entry | 2 min ITI | 2 min CS+ random interval sipper entry |
|-----------|--|-----------|--|-----------|--|-----------|--|-----------|--|

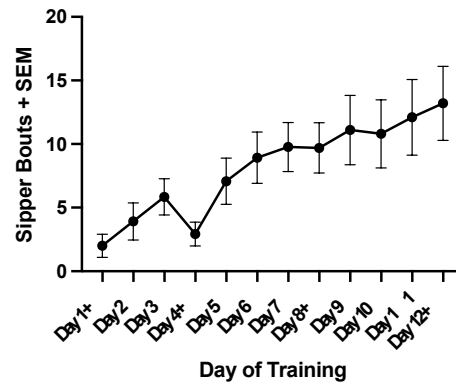
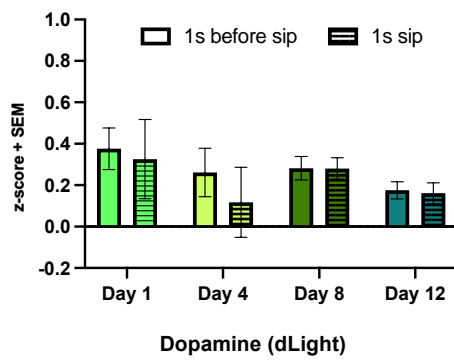
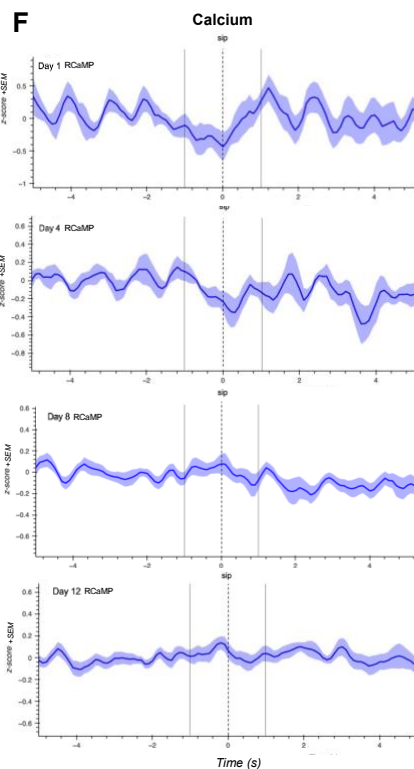
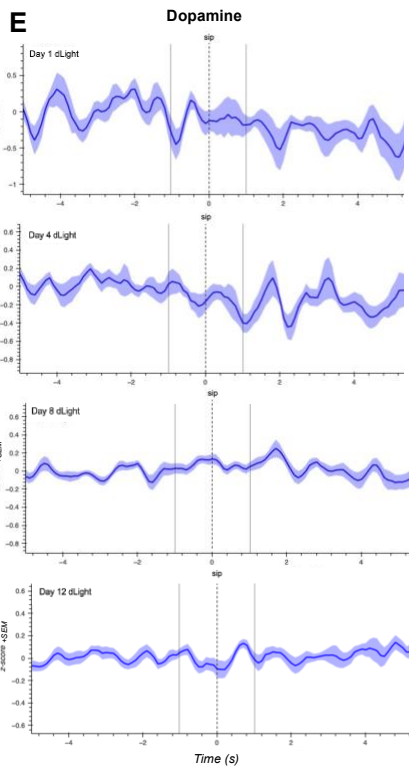
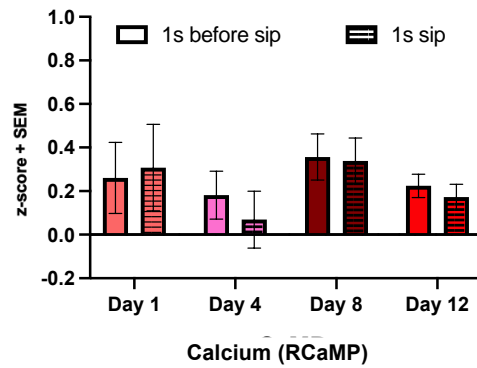
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Figure 5. Dopamine and calcium activity do not change in response to sipper use during Pavlovian conditioning. Rats (n=13; recording: n= 9 [18 fibers]) underwent 12 sessions of Pavlovian conditioning during which CS+ presentation was paired with sipper delivery that allowed them to drink a 10% sucrose solution (A). Rats increased their sipper use across training (B). Dopamine (dLight) and calcium activity (RCaMP) in the LA was assessed using fiber photometry (C-F). Dopamine activity did not change in response to rats taking a sip from the sipper and the activity before and during the sip remained similar across training days (C). Calcium activity as rats sipped from the sipper bottle was not different compared to the pre-sip baseline and this activity stayed similar across Pavlovian conditioning days (D) Mean traces of dLight signal centered around sip (top to bottom: Day 1, Day 4, Day 8, Day 12; E). Mean traces of RCaMP signal centered around sip (top to bottom: Day 1, Day 4, Day 8, Day 12; F). Day+ on panel B represents recording days.

2.3.1.8 Dopamine and calcium activity do not change in response to sipper use even as the Pavlovian task is learned.

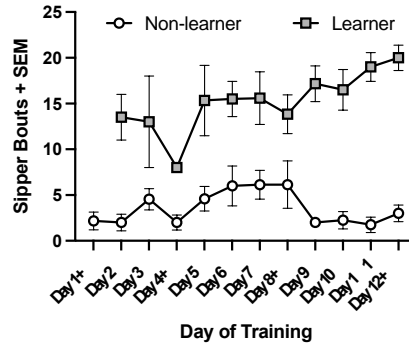
As with CS+ time-locked data, we wanted to see if there was a difference in the dopamine or calcium response to a sip that developed with better performance of the Pavlovian task. Animals were classified as non-learners or learners based on the previously described criteria (Figure 6A-B). There was no main effect of training day ($F_{(2,15)}=0.38$, $p=0.69$) or sip ($F_{(1,15)}=0.33$, $p=0.58$) on dopamine activity in non-learners (Figure 6C, E). Similarly, there was no main effect of training day ($F_{(2,22)}=0.39$, $p=0.68$) or sip ($F_{(1,22)}=0.88$, $p=0.36$) on dopamine activity in learners (Figure 6C, F), indicating that regardless of performance status, dopamine activity in the LA did not differ from a pre-sip baseline as the animals sipped. There was no main effect of training day ($F_{(2,15)}=0.19$, $p=0.83$) on calcium activity in the LA of non-learners (Figure 6D, G). There was a main effect of sip ($F_{(1,15)}=4.85$, $p=0.043$) on calcium activity where activity before the sip was higher than during the sip, which was related to a trend in the pre-sip to sip difference on day 4 (Figure 6D, G). As previously noted, sip behavior on day 4 may have been affected by being connected to the optical tether so this difference should be cautiously interpreted. Training day ($F_{(2, 19)}=0.79$, $p=0.47$) and sip ($F_{(1,19)}=0.0069$, $p=0.93$) had no effect on LA calcium activity for learners, further highlighting that activity in the LA does not meaningfully change when rats sip (Figure 6D, H).

A

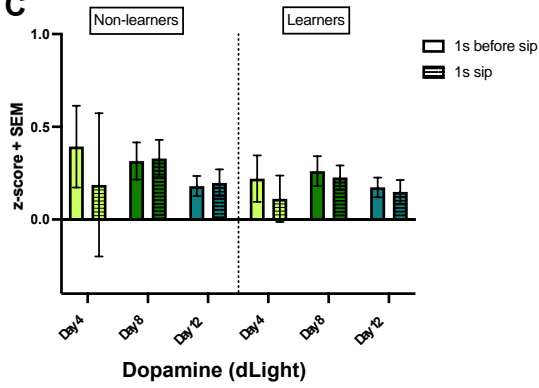
Performance criteria

| Sipper use | Sipper use per sipper entry | Sipper time CS vs ITI | Time near sipper during CS+ |
|------------------|-----------------------------|-----------------------|-----------------------------|
| ≥ 8 sipper bouts | ≥33% of entries | time CS ≥ 2X time ITI | ≥33% of time |

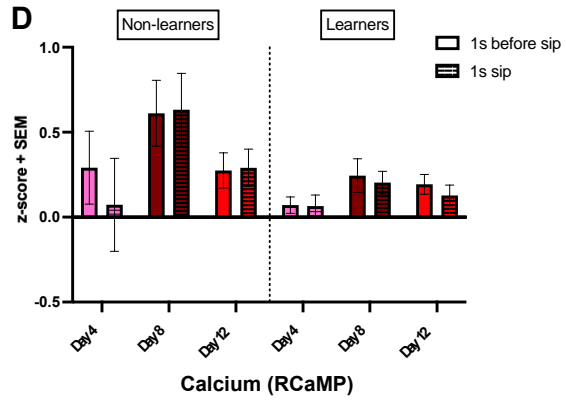
B



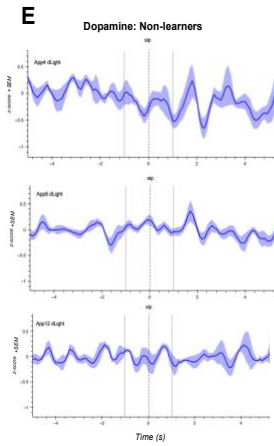
C



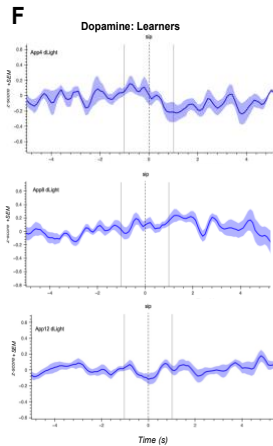
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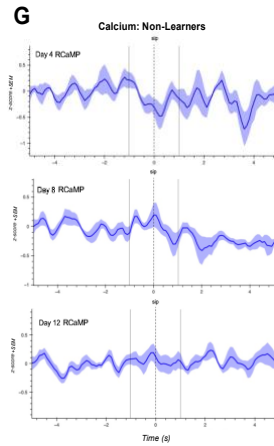
E



F



G



H

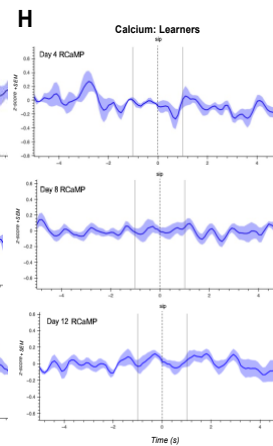


Figure 6. Dopamine and calcium activity do not change in response to sipper use even as performance improves. Rats (n=13; recording: n= 9 [18 fibers]) that underwent Pavlovian conditioning were split into non-learners and learners based on performance criteria (A-B). Both non-learners and learners show no change in dopamine activity from the pre-sip baseline to the sip at any point during conditioning (C). Non-learners displayed higher calcium activity during the pre-sip period relative to the sip period and this difference was reflected by the pre-sip activity tending to be greater than sip activity on day 4, a timepoint at which very few non-learners sipped. On all training days, learners showed no difference between calcium activity in the 1 second before sip and the activity during the first second of the sip (D). Mean traces of dLight signal centered around sip for non-learners (top to bottom: Day 4, Day 8, Day 12; E). Mean traces of dLight signal centered around sip for learners (top to bottom: Day 4, Day 8, Day 12; F). Mean traces of RCaMP signal centered around sip for non-learners (top to bottom: Day 4, Day 8, Day 12; G). Mean traces of RCaMP signal centered sip for learners (top to bottom Day 4, Day 8, Day 12; H). Day+ on panel B represents recording days.

2.3.1.9 Calcium and dopamine responses to the sucrose sip on the day animals acquire task

performance do not differ from responses at other timepoints in Pavlovian conditioning.

Lastly, we compared activity in response to a sip on the initial Pavlovian training day, the day animals reached performance criteria, and the final day of training in the event they never reached criteria (Figure 7A). There was no main effect of training day (day 1 vs day learned vs never learned) on dopamine activity ($F_{(2,23)}=0.96$, $p=0.39$). There was not a main effect of sip timing on dopamine activity either ($F_{(1,23)}=0.0099$, $p=0.92$), suggesting that no meaningful dopamine response to the sip was present on training day 1 nor did such a response develop as animals reached performance criteria (Figure 7B, D). For calcium activity in the LA, there was no main effect of training day ($F_{(2,22)}=0.67$, $p=0.52$) or sip timing ($F_{(1,22)}=0.02$, $p=0.89$), indicating that calcium activity did not reflect a response to the sip regardless if the Pavlovian task was learned or not (Figure 7C, E).

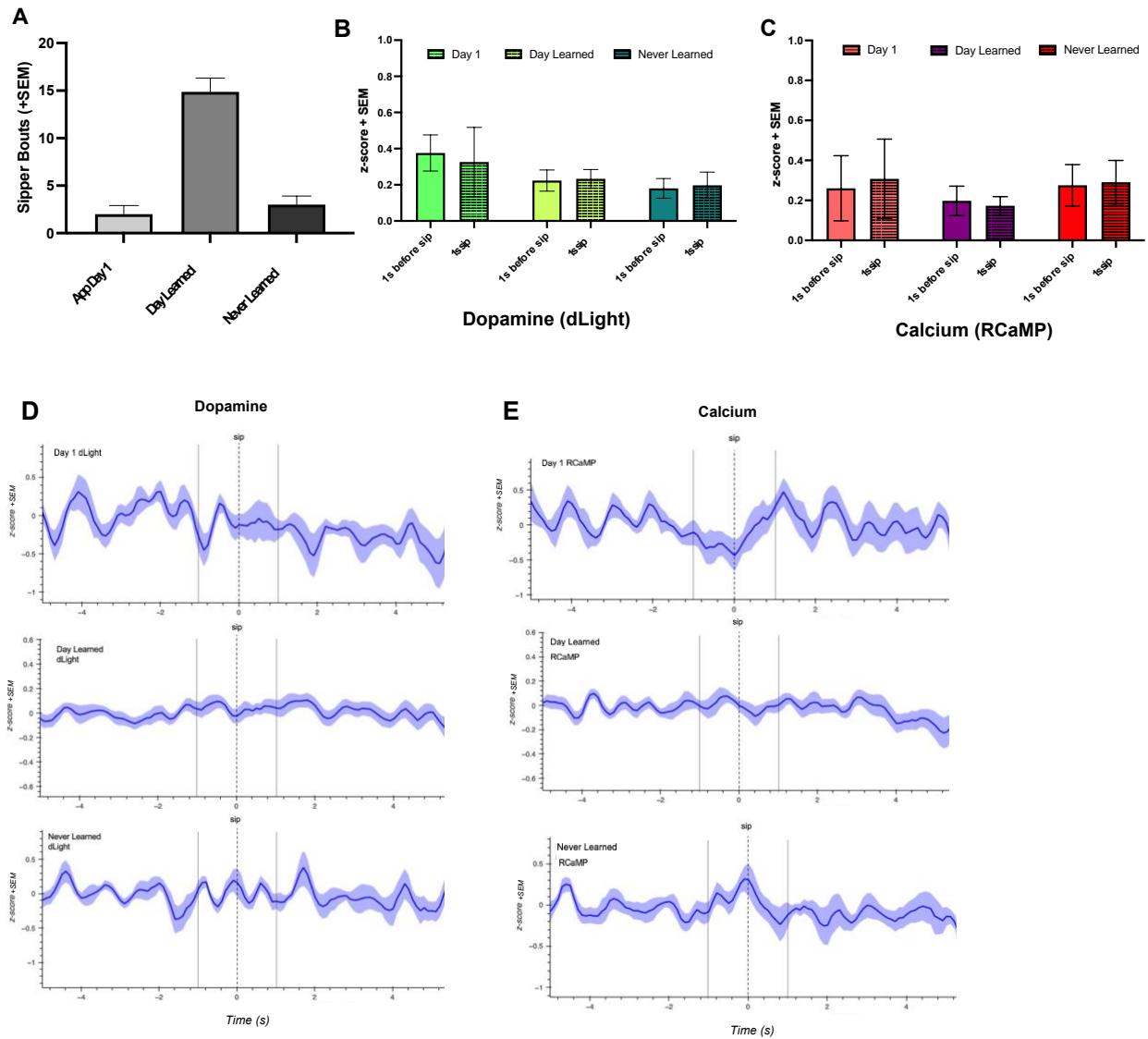


Figure 7. Dopamine and calcium responses to the sip on the day animals learned the Pavlovian task did not differ from responses on day 1 or responses for animals that never learned.

Rats (n=13; recording: n= 9 [18 fibers]) were trained on a Pavlovian conditioning task and both behavioral and photometry data were split into the first day of training, the day learned, and the final day of training for rats never learned the task (A-C). There was no change in dopamine activity in response to the sip on any day. (B). Calcium activity did not significantly change in response to the sip or as the task was learned (C). Mean traces of dLight signal centered around sip (top to bottom: Day 1, Day Learned, Never Learned; D). Mean traces of RCaMP signal centered to the sip (top to bottom: Day 1, Day Learned, Never Learned; E).

2.3.2 Instrumental Training

2.3.2.1 During instrumental training, rats discriminate between active and inactive levers.

After Pavlovian conditioning, animals progressed into the instrumental training phase of the PIT paradigm. Instrumental training began on a fixed ratio 1 (FR1) schedule of reinforcement where a press on the active lever resulted in sipper entry for 5 seconds with the levers retracted. FR1 sessions continued for at least 3 days or until rats made at least 10 presses followed by at least 8 sips. For FR1 training, there was a main lever effect ($F_{(1, 19)}=9.56$, $p=0.006$), but no effect of instrumental training day ($F_{(2.82, 30.47)}=0.99$, $p=0.40$) on pressing behavior. Across FR1 sessions, rats made more presses on the active lever than the inactive lever (Figure 8A). Rats continued to a random ratio 5 (RR5) schedule of reinforcement for 3 days during which there was a main effect of training day ($F_{(2,24)}=3.65$, $p=0.04$) and a main lever effect ($F_{(1,12)}=9.96$, $p=0.0083$). Overall, rats made more active presses than inactive presses and this was driven by significant differences between active presses and inactive presses on day 2 ($p=0.01$) and day 3 ($p=0.0027$) of training (Figure 8B). Lastly, rats had random ratio 10 (RR10) training for 5 days. During RR10, there was a trending effect of training day to impact pressing behavior ($F_{(1.67, 11.27)}=3.41$, $p=0.076$). There was a main lever effect ($F_{(1,12)}=17.66$, $p=0.0012$) and a trend for a lever x training day interaction ($F_{(4,27)}=2.57$, $p=0.061$, Figure 8C). Therefore, at all schedules rats were able to discriminate between the active sucrose-paired lever and the inactive lever.

2.3.2.2 Dopamine and calcium activity show no change in response to active presses made during instrumental training.

To determine dopamine and calcium dynamics in the LA during instrumental training, we used fiber photometry to examine peak fluorescence during a 1 second baseline before the active

lever press and during the first second of the active lever press. There was no difference in dopamine activity for either schedule (RR5 vs RR10, $F_{(1,7)}=1.38$, $p=0.28$), indicating that increased training did not affect dopamine activity in the LA (Figure 8D, F). Additionally, there was not a main effect of press ($F_{(1,7)}=0.45$, $p=0.52$) on dopamine activity (Figure 8D, F). As with dopamine, there was no main effect of schedule (RR5 vs RR10, $F_{(1,7)}=0.06$, $p=0.81$) or press ($F_{(1,7)}=0.49$, $p=0.51$) on calcium activity in the LA (Figure 8E, G). Thus, activity in the LA was not changed in response to active press on either reinforcement schedule.

When visually examining the mean traces, it appeared that variability began to grow around the active press event onset (Figure 8F-G). As a result, we used Levene's test for equality of variances to compare the variance during a 1-second period that occurred 5 seconds before the active lever press to the variance during 1-second of the active press. For the dopamine and calcium signal during RR5 training, variance was similar for the pre-press period 5 second before the press and during 1-second of the press ($F=0.33$, $p=0.58$; $F=1.50$, $p=0.24$, respectively). During RR10 training, the variance in the dopamine signal was greater during the press period compared to the timepoint 5 seconds earlier ($F=4.66$, $p=0.042$). Similarly, during RR10 training days, the variance in the calcium signal was greater during the press than it was 5 seconds before the press ($F=4.54$, $p=0.047$). While there was no change in dopamine or calcium activity in response to active presses, the trend for greater variance surrounding the press indicates that there may be meaningful individual differences in LA activity corresponding to the active press.

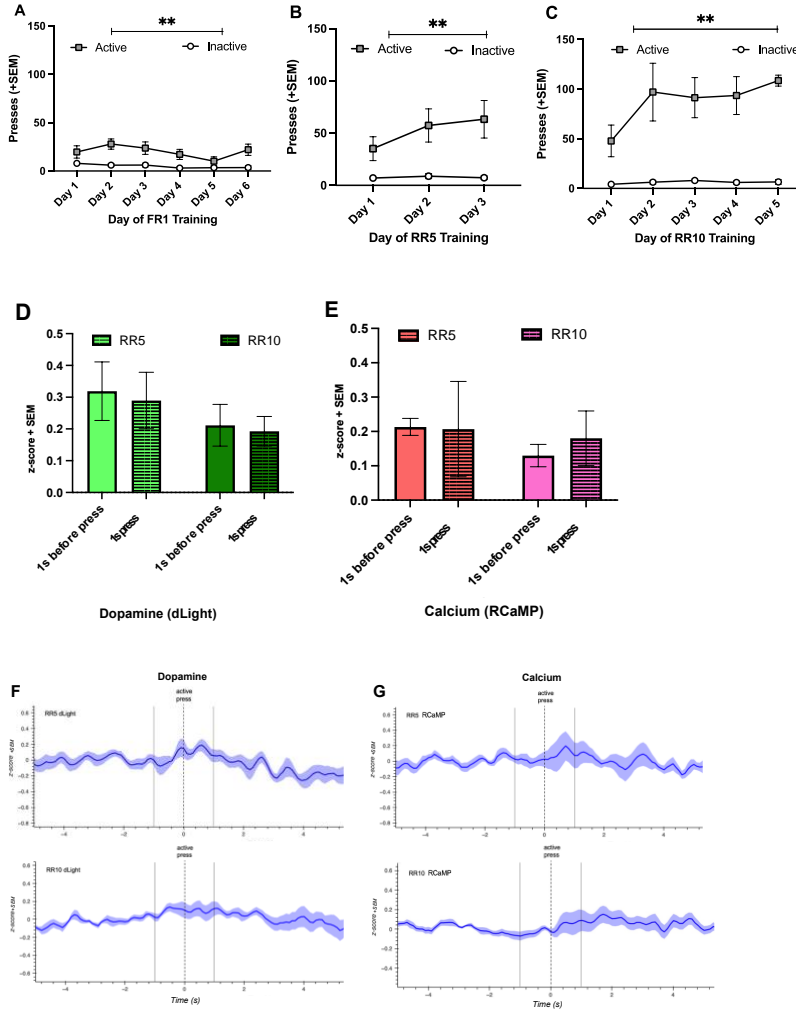


Figure 8. Rats learned to discriminate between levers, but show no change in activity in response to an active press.

Rats underwent instrumental training on FR1 (n=11), RR5 (n=7; recording n=4 [8 fibers]), and RR10 (n=6; recording n=4 [8 fibers]) schedules (A-C). Dopamine and calcium activity in the LA were measured during RR5 and RR10 sessions (D-G). Rats made significantly more active lever presses than inactive lever presses on FR1, RR5, and RR10 schedules (A-C). Dopamine activity was not different before the press and during the active press for either schedule (D). Calcium activity did not change in response to the active press for the RR5 or RR10 schedule (E). Mean traces of dLight signal centered around the active press (D). Mean traces of RCaMP signal aligned to the active press.

**p<0.01

2.3.2.3 Dopamine and calcium activity show no change in response to the first press or the last press in a series of presses

During RR5 and RR10 schedules, not every press results in presentation of the sipper so we wanted to examine calcium and dopamine activity in response to presses that may be more salient. First, we assessed activity during the first press in a series of presses to determine if initiating pressing behavior triggers a dopamine or calcium response. There was no main effect of schedule (RR5 vs RR10, $F_{(1,7)}=0.0013$, $p=0.91$) on dopamine activity prior to the first press or during the first press (Figure 9A, C). There was no main effect of the press ($F_{(1,7)}=0.18$, $p=0.68$) such that dopamine activity before the first press and during the first press was similar for both instrumental schedules (Figure 9A, C). Likewise, there was no main effect of schedule (RR5 vs RR10, $F_{(1,7)}=2.20$, $p=0.18$) or press ($F_{(1,7)}=0.087$, $p=0.78$) on calcium activity in the LA. Overall calcium activity was similar during both RR5 and RR10 instrumental training sessions and did not change from before the first press to during the first press (Figure 9B, D).

We also examined activity elicited by the last press of a series of presses. We compared calcium and dopamine activity during the 1 second before the end of the final press, during the last second of the final press where retraction begins, and during the first second of lever retraction. It is possible that lever retraction serves as a cue associated with sucrose reward so we also wanted to determine if activity differed from press to retraction. There was not a main effect of either schedule (RR5 vs RR10, $F_{(1,6)}=0.37$, $p=0.57$) or press (before end vs end vs retraction, $F_{(2,12)}=1.28$, $p=0.31$) on dopamine activity (Figure 9E, G). There were no main effects of schedule (RR5 vs RR10, $F_{(1,7)}=0.12$, $p=0.74$) or press (before end vs end vs retraction, $F_{(2,14)}=0.22$, $p=0.80$) on calcium activity (Figure 9F, H). Collectively, these findings indicated that although the last press

in a series is linked to sucrose reward and the retraction cue, dopamine and calcium activity in the LA did not show a response to the final press or its retraction.

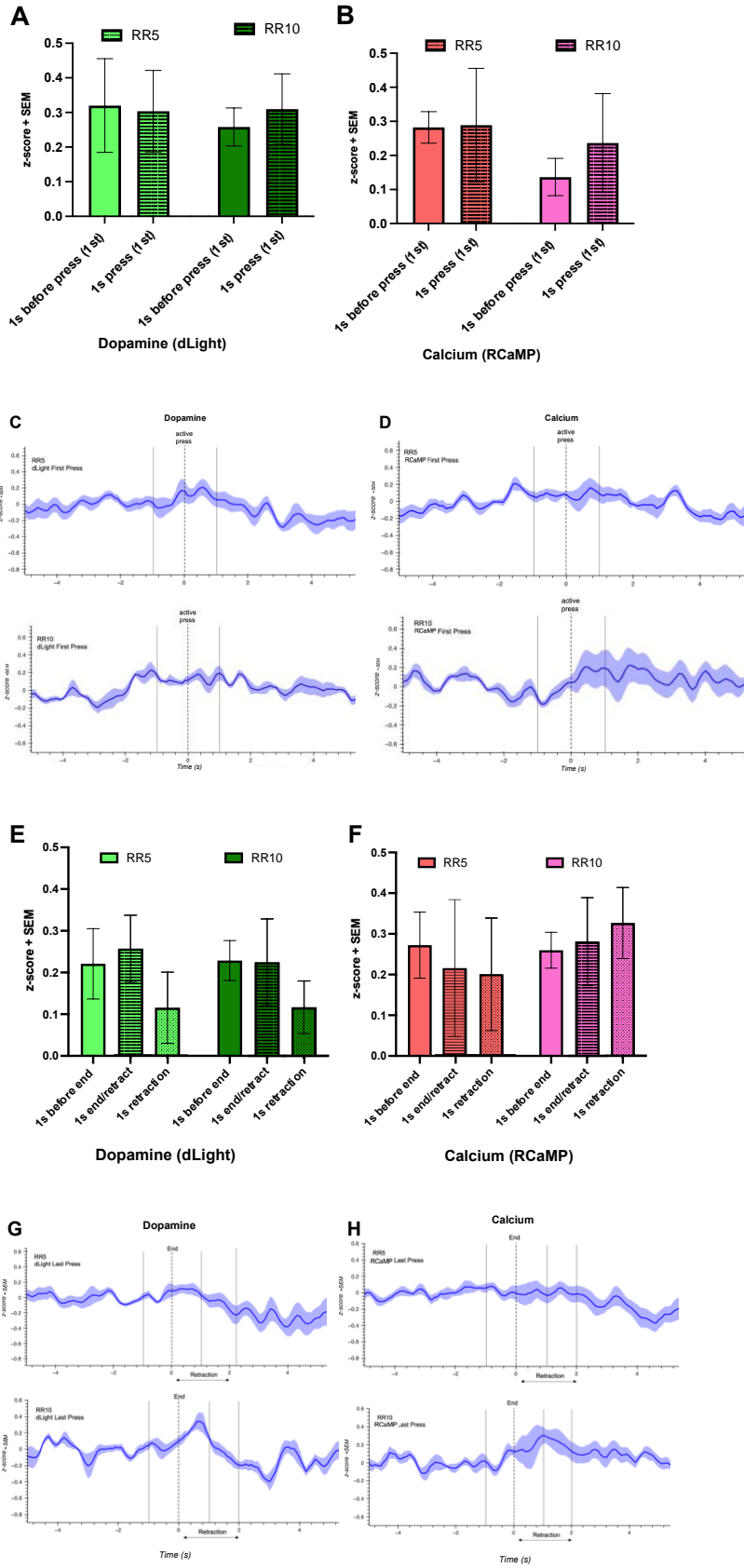


Figure 9. Dopamine and calcium activity show no change in response to the first active press or the last active press in a series of presses during instrumental training. Rats underwent instrumental training on both RR5 (n=7; recording: n=4 [8 fibers]) and RR10 schedules (n=6; recording: n=4 [8 fibers]). The first press in a series of presses is the initiation of seeking behavior and thus may be salient, so LA dopamine and calcium activity in response to the first press were examined (A-D). Dopamine activity was similar during both the 1-second pre-press timepoint and the first second of the first press (A). Calcium activity was similar before the first press and in response to the first press (B). Mean trace of dLight signal aligned to the first press (top: RR5, bottom: RR10; C). Mean trace of RCaMP signal aligned to the first press (top: RR5, bottom: RR10; D). The last press in a series during instrumental training was paired with sipper delivery and lever retraction, which could serve as reward-related stimuli for these animals. There was no change in dopamine activity from the period before the end of the press to the end of the press or to the first second of retraction (E). Calcium activity was similar before the end of the press, during the end of the press, and during initial retraction (F). Mean trace of dLight signal centered around the end of the last press (top: RR5, bottom: RR10; G). Mean trace of RCaMP signal centered around the end of the last press (top: RR5; bottom: RR10; H).

2.3.2.4 Dopamine and calcium activity are similar during both rewarded and unrewarded presses.

Finally, we were interested in examining if activity would differ between rewarded and unrewarded presses since under both RR5 and RR10 schedules not every lever press is followed by sipper presentation (Figure 10A-B). For presses made under the RR5 schedule, dopamine activity was compared between presses not followed by a sip (unrewarded) and presses followed by a sip (rewarded) at 1 second before the press, during the initial second of the press, and 1 second after the press. There was no effect of press (before press vs press vs after press) on dopamine activity ($F_{(2,10)}=0.96$, $p=0.41$), indicating that there was no change in dopamine response to making a press. There was no effect of sip reward ($F_{(1,5)}=0.029$, $p=0.87$) on dopamine activity. This result suggests that dopamine activity associated with rewarded presses is no different than that of unrewarded presses, even during the post-press period when rats would be initiating a sip (Figure 10C, G-H). Analyzing calcium activity during RR5 presses yielded similar results as there was no main effect of sip reward ($F_{(1,5)}=0.98$, $p=0.37$), implying that LA calcium activity does not differ between rewarded and unrewarded presses (Figure 10E, I-J). Additionally, there was no main effect of press on calcium activity (before press vs press vs after press, $F_{(1,30, 6.51)}=4.75$, $p=0.11$) meaning that calcium activity did not differ during the pre-press, press, or post-press periods.

Examining dopamine activity related to presses made under the RR10 schedule revealed no main effects of press (before press vs press vs after press, $F_{(2,6)}=0.27$, $p=0.77$) or sip ($F_{(1,3)}=0.067$, $p=0.81$, Figure 10D, G-H). Likewise, there was no main effects of press (before press vs press vs after press, $F_{(2,6)}=2.23$, $p=0.19$) on calcium activity in the LA. There was a trend for an effect of sip ($F_{(1,3)}=5.82$, $p=0.095$) on calcium activity during the RR10 phase. In general, activity during the rewarded presses was trending to be greater than activity during unrewarded presses.

However, this difference was present at the pre-press baseline suggesting the trending difference did not reflect a meaningful change in activity (Figure 10F, I-J). Overall, for both schedules of reinforcement, there was no change in activity during the press or in the post press period during which animals could be initiating a sip. Moreover, activity was similar for unrewarded and rewarded presses implying that the instrumental learning involved in pairing the press with reward is not reflected by LA dopamine and calcium activity.

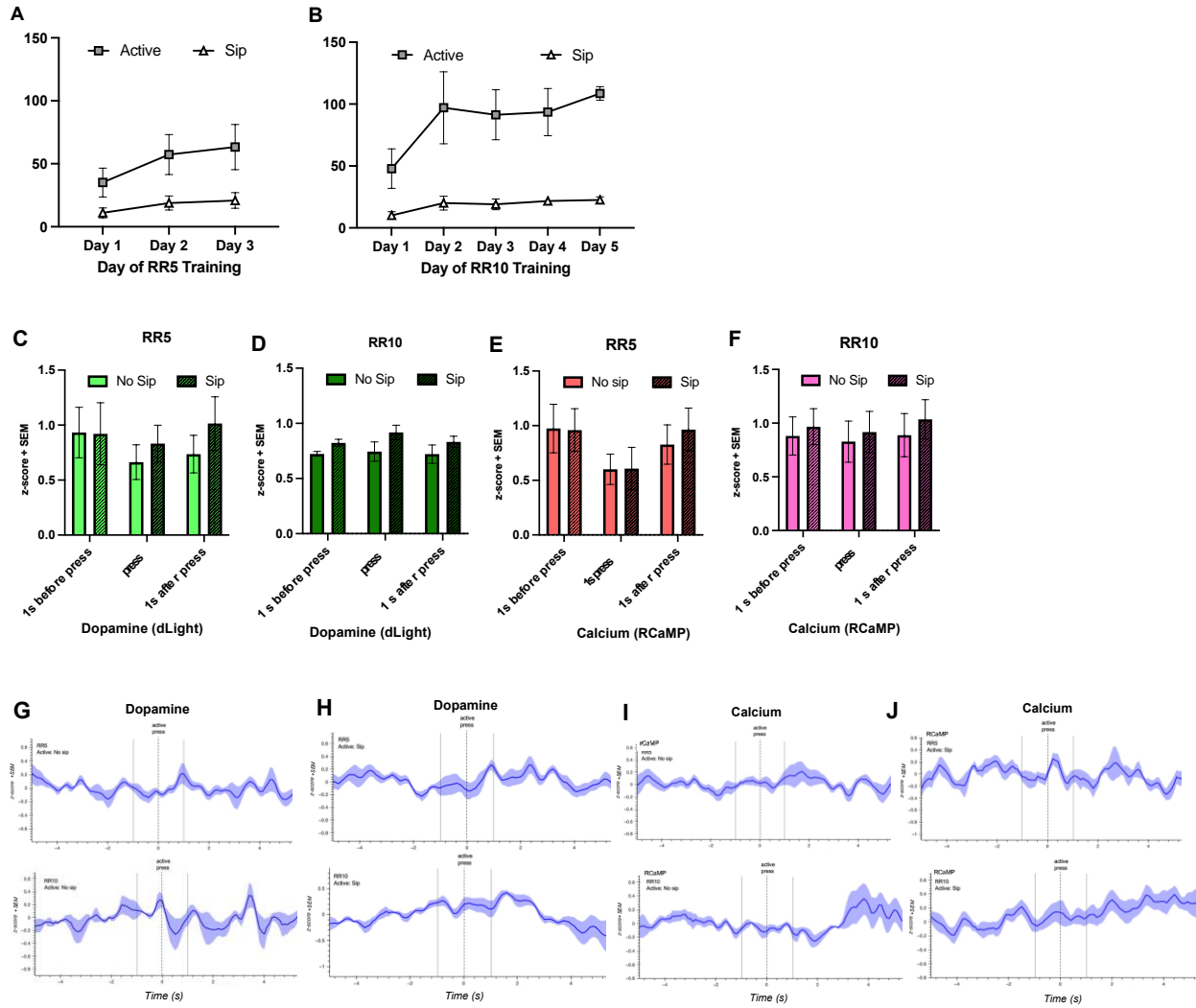


Figure 10. Dopamine and calcium activity is similar for both rewarded and unrewarded presses.

Rats underwent instrumental training under both RR5 (n=7; recording: n=4 [8 fibers]) and RR10 (n=6; recording: n=4 [8 fibers]) schedules (A-B). Their press behavior was split into active presses followed by no sip (unrewarded) and those followed by a sip (rewarded). Dopamine activity was measured during the pre-press period, the active press, and the post-press period and there were no differences in activity between rewarded and unrewarded presses at any timepoint for either the RR5 or RR10 schedule (C-D). Calcium activity was measured for both rewarded and unrewarded presses under both schedules and no differences between each press type were found before the press, during the press, or after the press (E-F). Mean traces for dLight signal centered to the rewarded press (top: RR5, bottom: RR10; G) and unrewarded press (top: RR5, bottom: RR10; H). Mean traces for RCaMP signal aligned to rewarded presses (top: RR5, bottom: RR10; I) and unrewarded presses (top: RR5, bottom: RR10; J).

2.3.2.5 Activity did not change in response to making inactive lever presses.

Behaviorally, animals discriminated between the active and inactive levers during instrumental training (Figure 8A-C). To determine if dopamine and calcium activity responded to inactive lever presses, activity at a 1-second pre-inactive press baseline was compared to activity during the first second of the inactive press. There was no main effect of schedule (RR5 vs RR10, $F_{(1,22)}=0.49$, $p=0.48$) on dopamine activity in the LA. There was a trend for inactive press (1s baseline vs 1s press) to impact dopamine activity ($F_{(1,22)}=3.51$, $p=0.074$), but multiple comparisons to examine the effect of inactive press within each schedule did not reach significance (Figure 11C, E). As inactive presses are not rewarded, it is possible that this trend for decreased dopamine activity represents a reward prediction error. There were no effects of schedule ($F_{(1,7)}=1.87$, $p=0.21$) or inactive press ($F_{(1,7)}=0.49$, $p=0.51$) on calcium activity (Figure 11D, F). Collectively, inactive presses made under the RR5 or RR10 schedule during instrumental training did not strongly modulate LA activity.

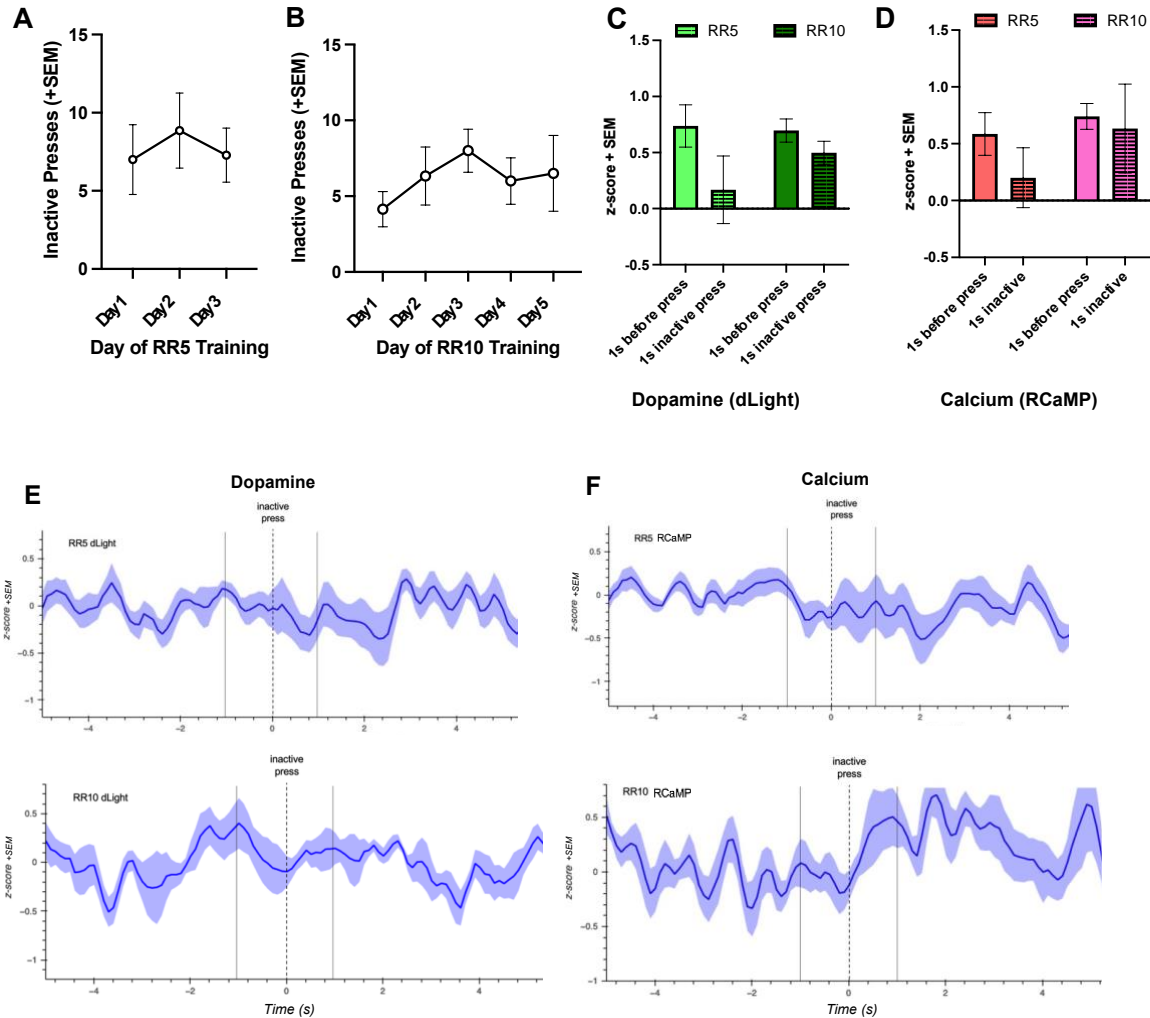


Figure 11. Activity did not change in response to inactive presses made during either schedule. Rats underwent instrumental training on an RR5 (n=7; recording: n=4 [8 fibers]) and an RR10 instrumental schedule (n=6; recording: n=4 [8 fibers]). Rats made few inactive lever presses during training (A-B). There was no significant change in dopamine response in the LA from before the inactive to during the inactive press under either schedule (C). The calcium activity in the LA was similar before the inactive press and during the inactive press (D). Mean traces for dLight signal aligned to the inactive press (top: RR5, bottom: RR10; E). Mean traces for RCaMP signal aligned to the inactive press (top: RR5; bottom: RR10; F).

2.3.2.6 Dopamine and calcium activity show no response to sipper use during instrumental training.

During instrumental training, presses on the active lever were paired with sips from the sucrose sipper bottle (Figure 12A-B). Using fiber photometry in the LA, we measured peak dopamine and calcium fluorescence to examine activity in a pre-sip baseline period 1 second before a sip and during the first second of the 5 second sip. There was no change in dopamine activity in response to the sip ($F_{(1,7)}=2.58$ $p=0.15$) for either instrumental schedule (RR5 vs RR10, $F_{(1,7)}=1.99$, $p=0.15$, Figure 12C, E). Additionally, there was no main effect of schedule (RR5 vs RR10, $F_{(1,7)}=3.30$, $p=0.11$) or sip ($F_{(1,7)}=0.29$, $p=0.61$) on LA calcium activity such that the pre-sip response and the sip response were similar under both schedules (Figure 12D, F).

As was observed with active presses during instrumental training, variation seemed to increase around the time of the sip (Figure 12E-F). We used Levene's test for equality of variances to evaluate variance 5 seconds prior to the sip and variance during the first second of the sip. For dopamine signal during RR5 training, the variance was not different for these two 1-second timepoints ($F=0.003$, $p=0.96$). During the RR10 schedule, the variance in dopamine signal during the sip was greater than the pre-sip baseline variance ($F=6.19$, $p=0.029$). Under both training schedules, calcium activity had more variance during the sip than before the sip ($F=6.77$, $p=0.026$; $F=3.55$, $p=0.078$; respectively). Taken together, these results indicate that although rats likely acquire the association between the operant response and sipper use, the sip itself does not strongly influence dopamine or calcium activity in the LA although there may be individual differences in the response (Figure 12).

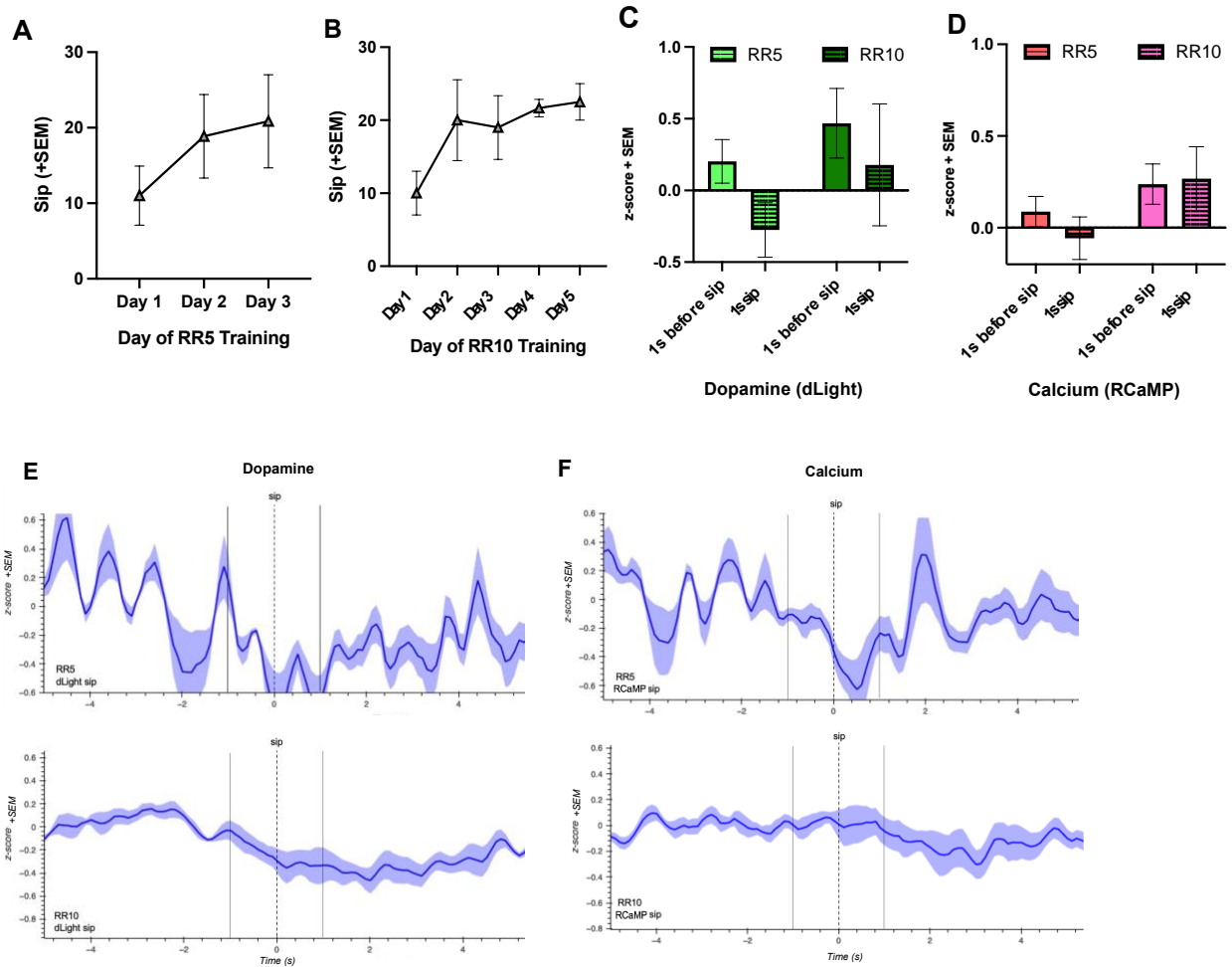


Figure 12. Dopamine and calcium activity show no response to sipper use during instrumental training. Rats underwent RR5 (n=7; recording: n=4 [8 fibers]) and RR10 (n=6; recording: n=4 [8 fibers]) instrumental training during which they could sip a 10% sucrose solution from a sipper that enter the chamber following a random number of active presses (1-5 for RR5 schedule, 1-10 for RR10 schedule) (A-B). Dopamine activity did not significantly change in response to the sip for either instrumental schedule (C). Calcium activity showed no response to the sip under either instrumental schedule (D). Mean traces for dLight signal aligned to the sip (top: RR5, bottom: RR10; E). Mean traces for RCaMP signal aligned to the sip (top: RR5, bottom: RR10; F)

2.3.3 Pavlovian to Instrumental transfer

2.3.3.1 Instrumental behavior did not show robust invigoration by auditory stimulus presentation.

Pavlovian to Instrumental transfer can determine how a cue that has been associated with a rewarding stimulus can alter the motivational salience of operant responding. In this 30-minute transfer test under extinction conditions, rats were presented with the CS+ they experienced during Pavlovian training for 2 minutes, then they experienced a 2-minute ITI with no cues followed by 2-minute presentation of a novel CS-, and finally a 2-minute ITI before the pattern repeated (Figure 13A). During each stimulus type and ITI, the number of lever presses per minute was measured. There was a main effect of lever ($F_{(1,3)}=12.64$, $p=0.038$) where the ratio of active presses was greater than that of inactive presses during the transfer test (Figure 13B). There was no main effect of stimulus type (CS+ vs CS- vs ITI, $F_{(1.16,3.48)}=3.005$, $p=0.17$), likely because we were underpowered at this phase of testing to detect a statistical difference. Comparing the ratio of presses made during the CS+ to the ratio during CS- did not result in a significant difference ($t(3)=1.43$, $p=0.25$), although there was a moderate effect size ($d=0.72$). Likewise, the ratio of presses made during the CS+ tended to be greater than the ratio during the ITI ($t(3)=2.74$, $p=0.071$) as suggested by a large effect size ($d=1.37$, Figure 13B).

2.3.3.2 Dopamine activity in the LA shows a greater response to CS+ presentation than CS- presentation.

Although animals' performance during PIT was not conclusively different between the type of CS presentations, we were interested in examining dopamine and calcium activity in the LA during the CS+, CS-, and ITI periods, particularly in response to the onset of each stimulus

type. There was no effect of stimulus onset ($F_{(1,7)}=0.012$, $p=0.92$) or stimulus type (CS+ vs CS- vs ITI, $F_{(1.39, 9.73)}=3.08$, $p=0.10$). However, planned multiple comparisons revealed that dopamine activity during the first second of stimulus presentation was significantly higher for the CS+ than the CS- ($p=0.021$, Figure 13C, E), suggesting that dopamine activity may be suppressed during presentation of the novel auditory cue. In the case of calcium activity, there were no main effects of stimulus onset ($F_{(1,6)}=2.27$, $p=0.18$) or stimulus type (CS+ vs CS- vs ITI, $F_{(1.12, 6.73)}=0.03$, $p=0.89$), though it appears that calcium activity may be related to detecting general effects of any stimulus turning on or off (e.g., first second of ITI) (Figure 13D, F). Therefore, it seems dopamine but not calcium activity may have a specific response to the stimulus paired with reward during Pavlovian conditioning.

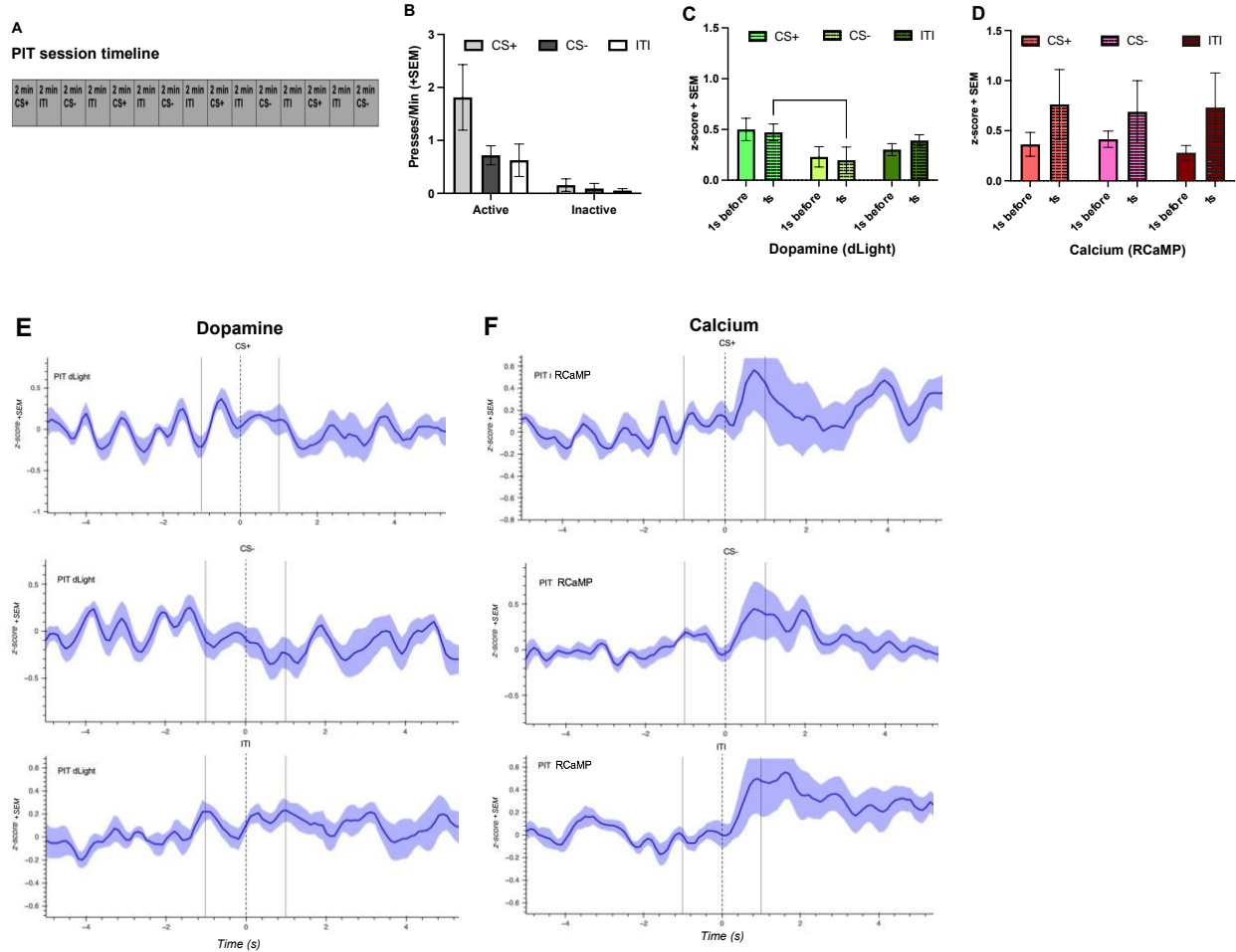


Figure 13. Dopamine activity reflects salience of Pavlovian conditioned cues. Rats ($n=4$; recording: $n=4$ [8 fibers]) underwent a test of Pavlovian to Instrumental transfer to examine if a conditioned stimulus from Pavlovian training modulated instrumental responding (B). Dopamine and calcium activity in response to each auditory stimulus and the ITI were measured (C-F). Timeline of PIT session (A). Instrumental behavior during each stimulus and ITI (B). In general, LA dopamine activity was not impacted by stimulus onset or stimulus type, but the dopamine response was greater during the first second of the CS+ than the first second of the CS- (C). Calcium activity in the LA did not change as a result of the stimulus type or its onset (D). Mean traces of dLight signal centered around stimulus or ITI start (top to bottom: CS+, CS-, ITI; E). Mean traces of RCaMP signal aligned to stimulus or ITI onset (top to bottom: CS+, CS-, ITI; F). * $p<0.05$

2.3.3.3 Dopamine and calcium activity did not change in response to presses made during the CS+, CS-, or ITI.

As rats made instrumental responses during the CS+, CS-, and ITI periods (Figure 14A, D), we wanted to determine if the dopamine and/or calcium response to an active press differed based on the stimulus type. There was no effect of stimulus type on press-related dopamine activity (CS+ vs CS- vs ITI, $F_{(1.39, 9.72)}=0.053$, $p=0.89$) and there was no effect of active press ($F_{(1,7)}=0.02$, $p=0.89$) such that dopamine activity did not change in response to making an active press during any stimulus (Figure 14B, G). Additionally, there were no effects of stimulus type (CS+ vs CS- vs ITI, $F_{(1.96, 13.71)}=1.13$, $p=0.35$) or active press initiation ($F_{(1,7)}=0.10$, $p=0.76$) on calcium activity, indicating that calcium activity did not change in response to active presses made during any stimulus period (CS+ vs CS- vs ITI, Figure 14C, H). Dopamine and calcium activity during inactive presses made during PIT were analyzed. There were no main effects of stimulus type (CS+ vs CS- vs ITI, $F_{(1.36,4.09)}=1.49$, $p=0.31$) or inactive press ($F_{(1,3)}=0.71$, $p=0.46$) on dopamine active (Figure 14E, I). Likewise, inactive press related calcium activity did not differ during any stimulus period (CS+ vs CS- vs ITI, $F_{(1.19, 8.35)}=0.65$, Figure 14F, J). There was no main effect of inactive press ($F_{(1,14)}=0.0039$, $p=0.95$) on calcium activity meaning that the calcium response in the 1 second before the inactive press was similar to the response during the 1-second press (Figure 14F, J).

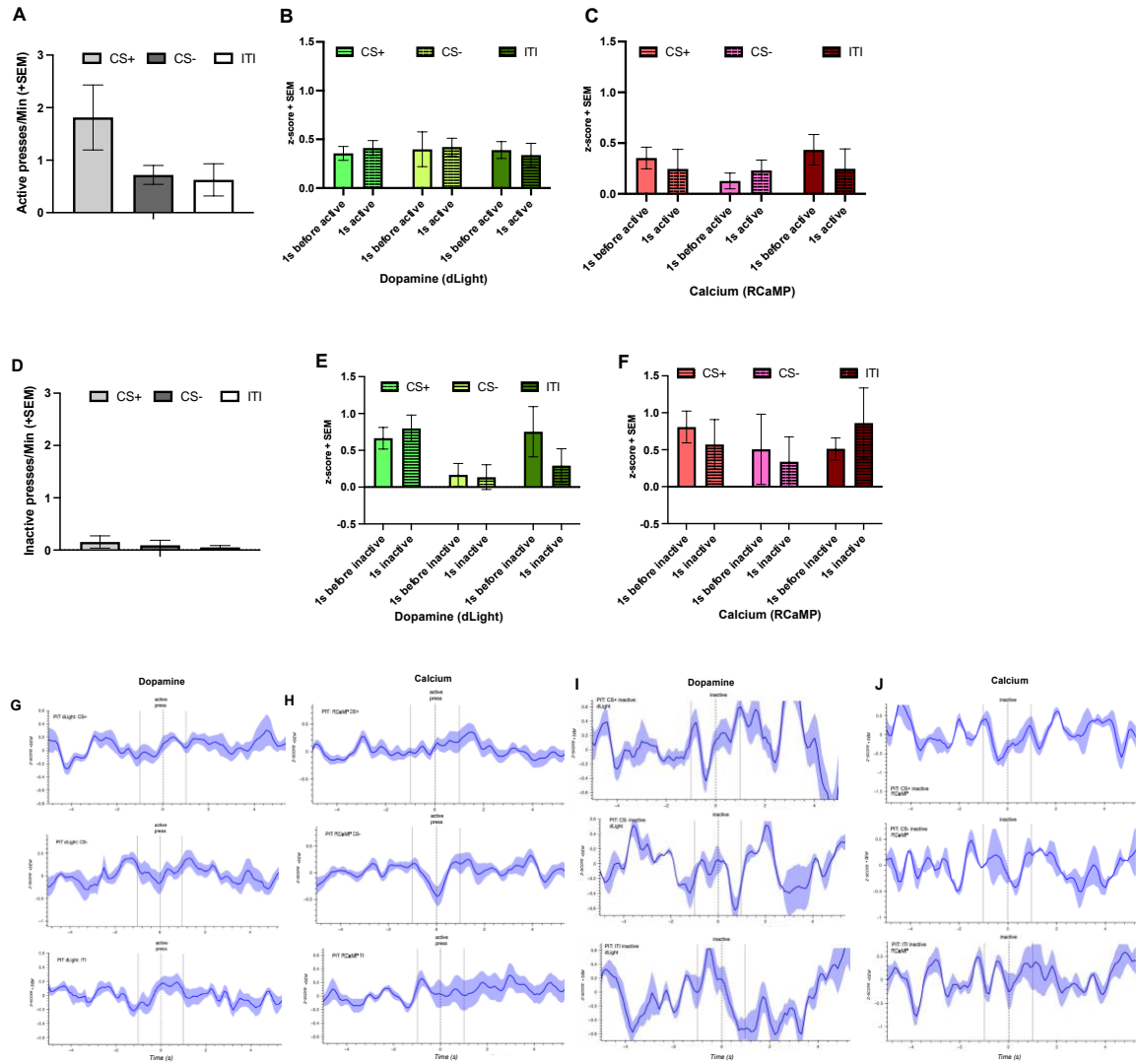


Figure 14. Dopamine and calcium activity does not change in responses to presses made during CS+, CS- or ITI. The number of active and inactive presses made by rats ($n=4$; recording: $n=4$ [8 fibers]) during PIT were reorded (A,D). The dopamine and calcium activity 1-second before the press and during the first second of the press were measured. There was no change in LA dopamine or calcium activity in response to active presses under any stimulus presentation (CS+, CS-, or ITI; B-C). There was no change in dopamine or calcium activity in response to inactive presses made during the CS+, CS-, or ITI (E-F). Mean traces for dLight signal aligned to active press (top: CS+, middle: CS-, bottom: IT; G). Mean traces for RCaMP signal aligned to active press (top: CS+, middle: CS-, bottom: IT; H). Mean traces for dLight signal aligned to inactive press (top: CS+, middle: CS-, bottom: IT; I). Mean traces for RCaMP signal aligned to inactive press (top: CS+, middle: CS-, bottom: IT; J).

2.3.4 Assessing norepinephrine contribution to dLight signal

2.3.4.1 Rats spend more time near the sipper, even when noradrenergic input to the LA is inhibited.

In order to determine if the dLight signals measured in the experiments above were truly driven by dopamine signaling or if there were contributions to the signal from the substantial norepinephrine inputs to the LA, rats were infused with a retrograde virus expressing the hM4di (Gi-DREADD) receptor under the PRSx8 promoter to enable inhibition of noradrenergic input to LA via systemic treatment of 1 mg/kg CNO (Figure 15A-B). Rats underwent 10 days of Pavlovian conditioning during which they experienced alternating 2-minute periods of CS+ (tone or white noise) presentation with a 2-minute ITI. During the CS+ presentation, the sucrose bottle sipper entered the operant chamber at a random interval (Figure 15C). On conditioning days where rats had photometry recordings in the LA, they received either 1 mg/kg CNO or vehicle injections prior to the behavior session. There was a main effect of CS+ presentation ($F_{(1,3)}=23.23$, $p=0.017$) such that rats spent more time near the sipper during the CS+ than during the ITI (Figure 15D). There was also a main effect of training day ($F_{(9,27)}=3.93$, $p=0.0027$) where animals' time spent near the sipper increased across training both during the CS+ and ITI (Figure 15D). However, post hoc analysis revealed that the difference between time spent near the sipper during the CS+ relative to during the ITI was significant on day 8 ($p=0.015$) and day 9 ($p=0.019$).

Animals were administered CNO or vehicle on the recording days of Pavlovian conditioning. To determine if administration of CNO, which inhibited noradrenergic input to the LA, affected behavior, we compared time near the sipper during the CS+ and ITI on these days. As the order of injections were counterbalanced, not all rats received the same injection type on the same day. Therefore, we collapsed across injection type for the early training timepoint (days

4 and 6) and the late training timepoint (days 8 and 10). For early training (days 4 and 6), there was no main effect of treatment (CNO vs veh, $F_{(1,3)}=5.00$, $p=0.11$) on time spent near the sipper, indicating that inhibition of the noradrenergic input did not alter behavior (Figure 15E). At this timepoint, there was no main effect of CS+ presentation ($F_{(1,3)}=3.28$, $p=0.17$) on time spent near the sipper suggesting that the Pavlovian task was not yet learned. However, post hoc analysis showed that in the CNO treatment group, time spent near the sipper was greater during the CS+ than during the ITI ($p=0.047$, Figure 15E). During late training (days 8 and 10), there was no main effect of treatment (CNO vs veh, $F_{(1,3)}=0.057$, $p=0.83$), again indicating that inhibition did not affect the amount of time rats spent near the sipper (Figure 15F). There was a main effect of CS+ presentation ($F_{(1,3)}=14.87$, $p=0.031$) on sipper time, meaning that animals were spending more time near the sipper during the CS+ presentation (Figure 15F). However, within each treatment group (CNO or veh), there was not a significant difference in sipper time during the CS+ or during the ITI. In general, approach behavior during Pavlovian conditioning is comparable whether the norepinephrine input is silenced or not.

2.3.4.2 Dopamine activity in the LA did not change in response to the CS+, but signal was unaffected by silencing of norepinephrine input.

Dopamine activity in the LA was examined with fiber photometry using the dopamine sensor dLight1.2. dLight1.2 is specific for dopamine release, but there is minimal sensitivity to norepinephrine (Patriarchi et al., 2018). As the LA receives noradrenergic innervation, it is possible the dLight signal has both dopaminergic and noradrenergic components. Therefore, rats were bilaterally infused in the LA with a retrograde inhibitory DREADD under the synthetic dopamine β hydroxylase promoter PRSx8 such that the receptor would be expressed in noradrenergic neurons projecting to the LA (Figure 15A). On recording days, rats received vehicle

and CNO (order counterbalanced) in order to compare the dopamine signal with and without this input. Early in conditioning (day 4/6), there was no main effect of treatment (CNO vs veh, $F_{(1,5)}=1.76$, $p=0.24$) on dopamine activity, suggesting that silencing norepinephrine input did not alter the dopamine signal (Figure 15G, I). There was also no main effect of CS+ presentation ($F_{(1,5)}=0.12$, $p=0.74$) on dopamine activity (Figure 15G, I). This result indicates that early in training, there is no specific dopamine response to the CS+. At a later conditioning timepoint (day 8/10), there were still no main effects of treatment (CNO vs veh, $F_{(1,5)}=2.48$, $p=0.18$) or CS+ ($F_{(1,5)}=0.11$, $p=0.76$) on dopamine activity in the LA (Figure 15H, J). These findings imply that while the dopamine signal does not change when norepinephrine input is inhibited, this inhibition has an effect on cue learning. As a result of the within-subjects design, rats receive both vehicle and CNO across training meaning that under vehicle conditions rats have already experienced conditioning days where noradrenergic input was silenced. It is likely that this repeated inhibition impacts their ability to form cue-reward associations. When we used the performance criteria from the prior experiment (Figure 2A), only one rat was classified as a learner by day 8 of conditioning. This effect on cue learning may explain why animals do not develop dopamine responses to the CS+ presentation much like the non-learners as previously described.

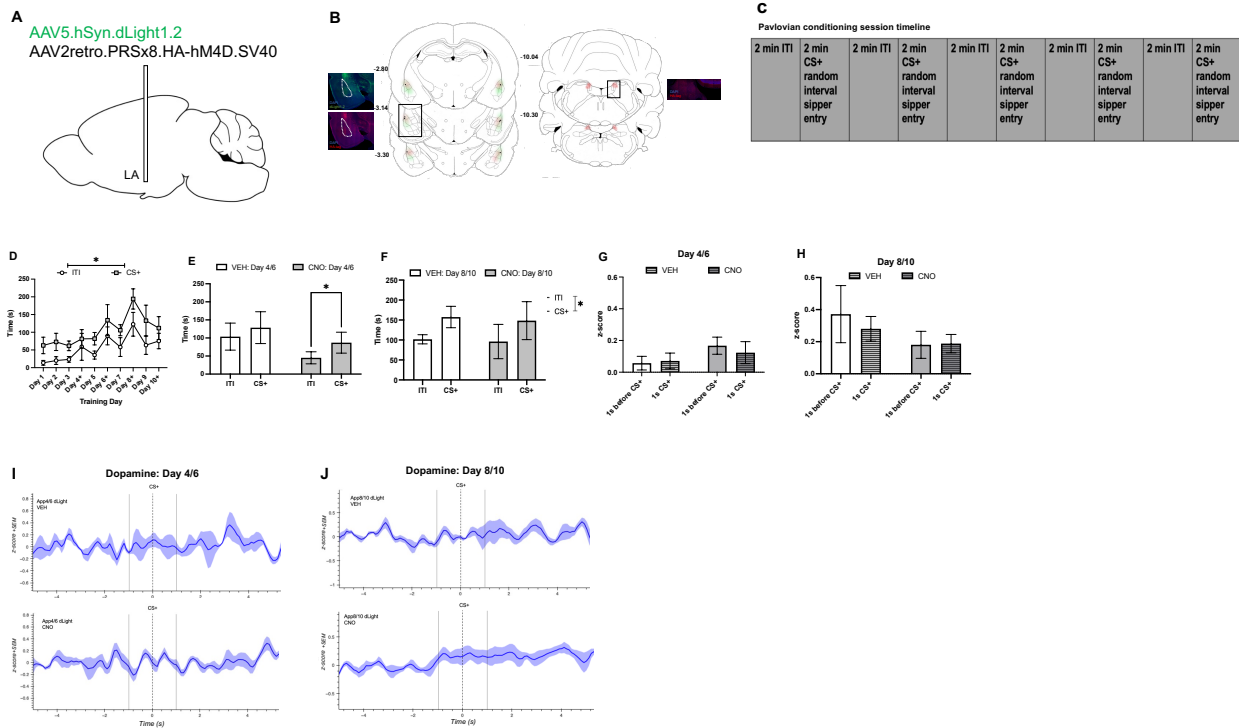


Figure 15. Rats spend more time near the sipper during the CS+, but do not show dopamine changes in response to CS+ presentation.

Rats (n=4; recording: n=4 [8 fibers]) underwent 10 sessions of Pavlovian conditioning during which both the time they spent near the sipper and LA dopamine activity were measured (C-D). On recording days, rats were administered CNO or vehicle in a counterbalanced order so behavior and dopamine activity were combined into CNO and vehicle groups on days 4/6 and day 8/10 (E-H). Schematic of viral injection of dLight1.2 and PRSx8 promoter Gi-DREADD in the LA (A). Schematic of viral injection and expression in the LA and expression in the LC with representative images. Black dots indicate optic fiber placement. (B). Timeline of conditioning session (C). Rats spent more time near the sipper during the CS+ than during the ITI (D). Early in training (days 4/6), CNO administration did not affect the amount of time spent near the sipper during CS+ or ITI. Following CNO treatment, rats spent more time near the sipper during the CS+ (E). Late in training (days 8/10), CNO treatment did not impact the amount of time spent near the sipper and following both vehicle and CNO rats spent more time near the sipper during the CS+ than during the ITI (F). On all recording days, there was no effect of CNO or CS+ presentation on dopamine activity (G-H). Mean traces of dLight signal aligned to CS+ on day 4/6 (top: veh, bottom: CNO; I). Mean traces of dLight signal aligned to CS+ on day 8/10 (top: veh, bottom: CNO; J). Day+ in panel B represents recording days. *p<0.05.

2.3.4.3 Sipper use stays low and stable across Pavlovian conditioning.

In Pavlovian training, the 2-minute CS+ presentations were paired with random interval sipper entry providing rats the opportunity to drink a 10% sucrose solution. The number of times the rats used the sipper each session was measured across the 10 training sessions. There was no main effect of training day ($F_{(9,27)}=0.551$, $p=0.82$) on sipper use. Overall, rats' sipper use was minimal and stayed stable across conditioning days (Figure 16A).

Animals were treated with systemic CNO or vehicle injections on recording days during training. We combined treatment type for the early timepoint (days 4 and 6) and the late timepoint (day 8 and 10). There was not a main effect of training (early vs late ($F_{(1,3)}=0.28$, $p=0.64$) on sipper use (Figure 16B). There was no main effect of treatment (CNO vs veh, $F_{(1,3)}=4.58$, $p=0.12$) on sipper use during training (Figure 16B), although it is important to note that not all animals made sips after CNO treatment. In general, inhibiting norepinephrine input to the LA did not alter how much the animals used the sipper.

2.3.4.4 Dopamine activity does not change in response to sipper use during Pavlovian training, but signal may be changed by altering norepinephrine input.

On recording days, animals were treated with CNO or vehicle and the dopamine response to sipper use on these days was assessed with fiber photometry. Initially in training (days 4 and 6), there was no main effect of sip ($F_{(1,3)}=0.072$, $p=0.81$), indicating that dopamine activity was the same during the pre-sip baseline and during the first second of the sip (Figure 16C, E). However, there was a trend for treatment to affect dopamine activity (CNO vs veh, $F_{(1,3)}=5.58$, $p=0.099$). This result suggested that norepinephrine had some contribution to the overall signal (Figure 16C, E). At the later training timepoint (days 8 and 10), there was no main effect of treatment (CNO vs veh, $F_{(1,5)}=2.13$, $p=0.20$) on dopamine activity, implying that norepinephrine did not contribute to

the measured signal (Figure 16D, F). Additionally, there was no effect of sip initiation ($F_{(1,5)}=2.25$, $p=0.19$) such that dopamine in the LA did not show a response to the sucrose sip (Figure 16D, F). Taken together, while sipper use does not influence dopamine activity, there appears to be a possible norepinephrine component of the dLight signal.

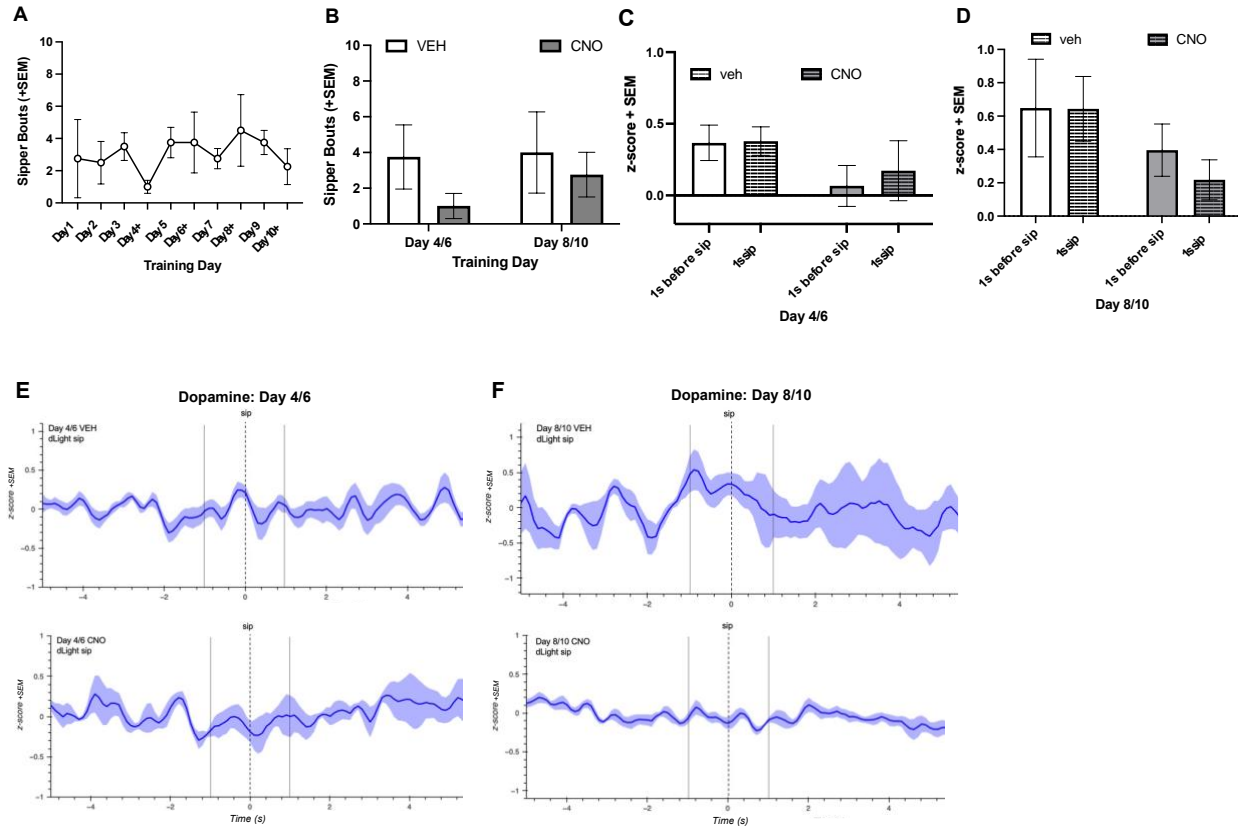


Figure 16. Rats have low and stable sipper use across Pavlovian training that does not impact dopamine activity.

Rats (n=4; recording: n=4 [8 fibers]) used the sipper as the CS+ played during Pavlovian training. Sipper use was relatively infrequent and did not increase with training (A). Sipper use during both early training (day 4/6) and late training (day 8/10) was similar following vehicle and CNO treatment (B). Dopamine activity in the LA did not change from the 1-second sip baseline to the 1-second start of the sip following vehicle or CNO treatment early in conditioning but the signal appeared dampened by inhibition (C). Late in conditioning, dopamine activity did not change following vehicle or CNO treatment and activity did not show a response to the initiation of a sip at the sucrose sipper bottle (D). Mean traces of dLight signal aligned to sip on day 4/6 (top: veh, bottom: CNO; E). Mean traces of dLight signal aligned to sip on day 8/10 (top: veh, bottom: CNO; F).

2.3.4.5 *During instrumental training, rats learn to discriminate between the active and inactive levers.*

During instrumental training, rats were trained first on a fixed ratio 1 (FR1) schedule of reinforcement for a week followed by a 3 days of a random ratio 5 (RR5) schedule and finally 3 days of an RR10 schedule. Under FR1, there was no main effect of training day ($F_{(2,30, 6.91)}=1.61$, $p=0.27$) on presses made, but there was a main lever effect ($F_{(1,3)}=26.24$, $p=0.014$) such that rats made more active presses than inactive presses (Figure 17A). Post hoc analysis found that on day 6, the number of active presses made was significantly greater than inactive presses ($p=0.030$). For presses made during the RR5 training, there were no main effects of training day ($F_{(2,6)}=1.85$, $p=0.24$) or lever ($F_{(1,3)}=2.17$, $p=0.24$) on behavior, but post hoc paired comparisons revealed that on days 1-3, rats made more active presses than inactive presses ($p=0.001$, $p=0.002$, $p=0.0005$, Figure 17B). For presses made during RR10, there were no main effects of training day ($F_{(2,6)}=0.73$, $p=0.52$) or lever ($F_{(1,3)}=1.71$, $p=0.28$) on pressing behavior, but post hoc analysis revealed that on days 1-3, rats made more active presses than inactive presses ($p=0.0007$, $p=0.0003$, $p=0.0005$, Figure 17C).

On instrumental recording days, animals were treated with CNO and vehicle to determine the contribution of norepinephrine to the dLight signal. There was no effect of treatment on pressing behavior (CNO vs veh, $F_{(1,3)}=0.009$, $p=0.93$) during RR5 training (Figure 17D). There was a trending effect of lever ($F_{(1,3)}=6.86$, $p=0.079$) where rats tended to make more active lever presses than inactive lever presses for the combined timepoint (Figure 17D). During the combined RR10 training day, there was no effect of treatment (CNO vs veh, $F_{(1,3)}=0.05$, $p=0.84$) or lever ($F_{(1,3)}=5.15$, $p=0.10$) on behavior such that animals made a similar number of lever presses even when norepinephrine activity was silenced (Figure 17E).

2.3.4.6 Dopamine activity did not change in response to presses made during instrumental training, but inhibition of norepinephrine input altered the recorded signal.

To determine dopamine dynamics in the LA during instrumental training, we examined activity during a 1-second baseline before an active press was made and during the first second of the active lever press. We assessed these timepoints when animals were administered CNO or vehicle both for the RR5 and RR10 schedule. For activity during RR5 training, there was no effect of press on dopamine activity ($F_{(1,3)}=1.39$, $p=0.32$) meaning rats showed no change in dopamine activity from pre-press to the active press. There was an effect of treatment (CNO vs vehicle, $F_{(1,3)}=10.89$, $p=0.046$) on dopamine activity indicating that during inhibition of noradrenergic input, the signal was diminished (Figure 17F, J). Analysis of the inactive presses made under the RR5 schedule yielded no main effect of treatment (CNO vs vehicle, $F_{(1,3)}=4.69$, $p=0.12$). There was a main effect of press ($F_{(1,3)}=20.48$, $p=0.020$) and a treatment x press interaction ($F_{(1,3)}=51.66$, $p=0.006$) such that when animals were treated with CNO, there was a reduction in dopamine activity during the press relative to before the press (Figure 17H, L). As animals underwent RR10 training, there was no effect of treatment (CNO vs vehicle, $F_{(1,3)}=3.59$, $p=0.15$) or active press ($F_{(1,3)}<0.0001$, $p=0.99$) on dopamine activity (Figure 17G, K). Additionally, there were no effects of treatment (CNO vs vehicle, $F_{(1,3)}=0.21$, $p=0.68$) or inactive press on dopamine activity measured during RR10 training ($F_{(1,3)}=0.24$, $p=0.66$, Figure 17I, M) Despite the activity reduction seen with inactive presses made following CNO treatment, the overall results pattern for pressing behavior indicates that dopamine activity does not robustly change in response to presses. Moreover, noradrenergic silencing impacted the recorded signal only during active presses made during RR5 training implying that norepinephrine may have contributed to the signal early in instrumental training.

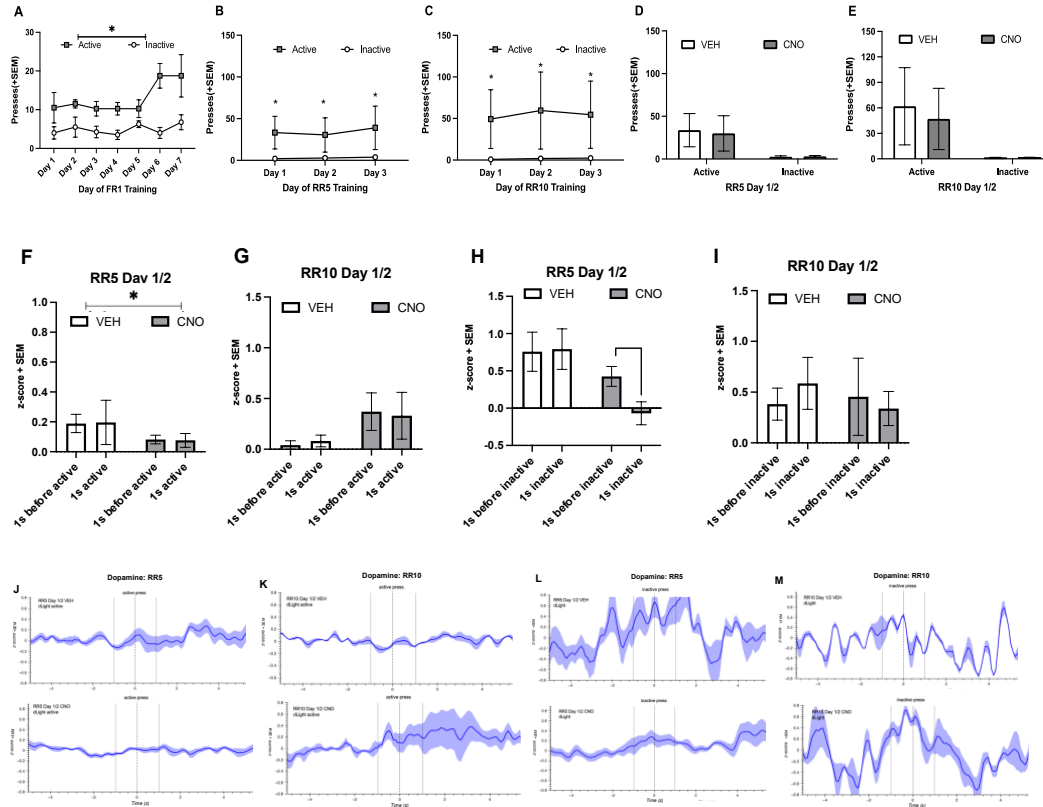


Figure 17. Animals discriminate between levers and pressing did not elicit a change in dopamine response. Rats ($n=4$; recording RR5: $n=4$ [8 fibers]; recording RR10: $n=2$ [4 fibers]) underwent instrumental training on FR1, RR5, and RR10 schedules of reinforcement where they earned access to the sucrose sipper. Animals learned to discriminate between the active sucrose-paired lever and inactive lever under FR1 (A), RR5 (B), and RR10 (C) schedules. Administration of CNO to silence norepinephrine input did not change pressing behavior relative to vehicle on either schedule (D-E). Dopamine activity did not change from a pre-press baseline as rats made an active press during both RR5 and RR10 training (F-G). During RR5 but not RR10, CNO administration meant to eliminate norepinephrine's role diminished the dLight signal compared to the signal recorded under vehicle conditions (F-G). Examining dopamine signal during the 1 second before an inactive press and during the first second of the press revealed a reduction in activity for inactive presses made during RR5 that disappeared with RR10 training (H-I). Mean traces of dLight signal aligned to the active press on RR5 days (top: veh, bottom: CNO; J). Mean traces of dLight signal centered to active presses made under RR10 (top: veh, bottom: CNO; K), Mean traces of dLight signal aligned to RR5 inactive presses (top: veh, bottom: CNO; L). Mean traces of dLight signal aligned to inactive presses made during RR10 training (top: veh, bottom: CNO). $*p<0.05$

2.3.4.7 Sipper use does not change across instrumental training.

Over the course of instrumental training, sipper use did not change across training day ($F_{(2,6)}=0.92$, $p=0.45$) or reinforcement schedule (RR5 vs RR10, $F_{(1,3)}=0.51$, $p=0.53$). Much like sipper use during Pavlovian training, the number of sips remained relatively low and stable across instrumental training (Figure 18A). On instrumental days during which animals were administered CNO or vehicle, there was no effect of treatment (CNO vs veh, $F_{(1,3)}=0.60$, $p=0.49$) or schedule (RR5 vs RR10, $F_{(1,3)}=0.038$, $p=0.86$) on sipper use during instrumental training (Figure 18B). Collectively, these data indicate that sipper use behavior remained stable across reinforcement schedule and when norepinephrine input was affected.

2.3.4.8 Dopamine activity does not change in response to sipper use following both vehicle and CNO treatment.

Rats were treated with CNO or vehicle on instrumental training days and the dopamine response to the sipper use associated with the lever presses under RR5 and RR10 training was measured. During RR5 training, there was no main effect of treatment (CNO vs veh, $F_{(1,3)}=2.32$, $p=0.23$) or sip ($F_{(1,3)}=0.81$, $p=0.43$) on dopamine activity (Figure 18C, E). For the RR10 schedule, neither treatment (CNO vs veh, $F_{(1,3)}=0.16$, $p=0.71$) nor sip ($F_{(1,3)}=1.74$, $p=0.28$) affected dopamine activity (Figure 18D, F). Therefore, dopamine activity does not display a response from the pre-sip period to the sip and this response is not further modulated by silencing norepinephrine projections to the LA.

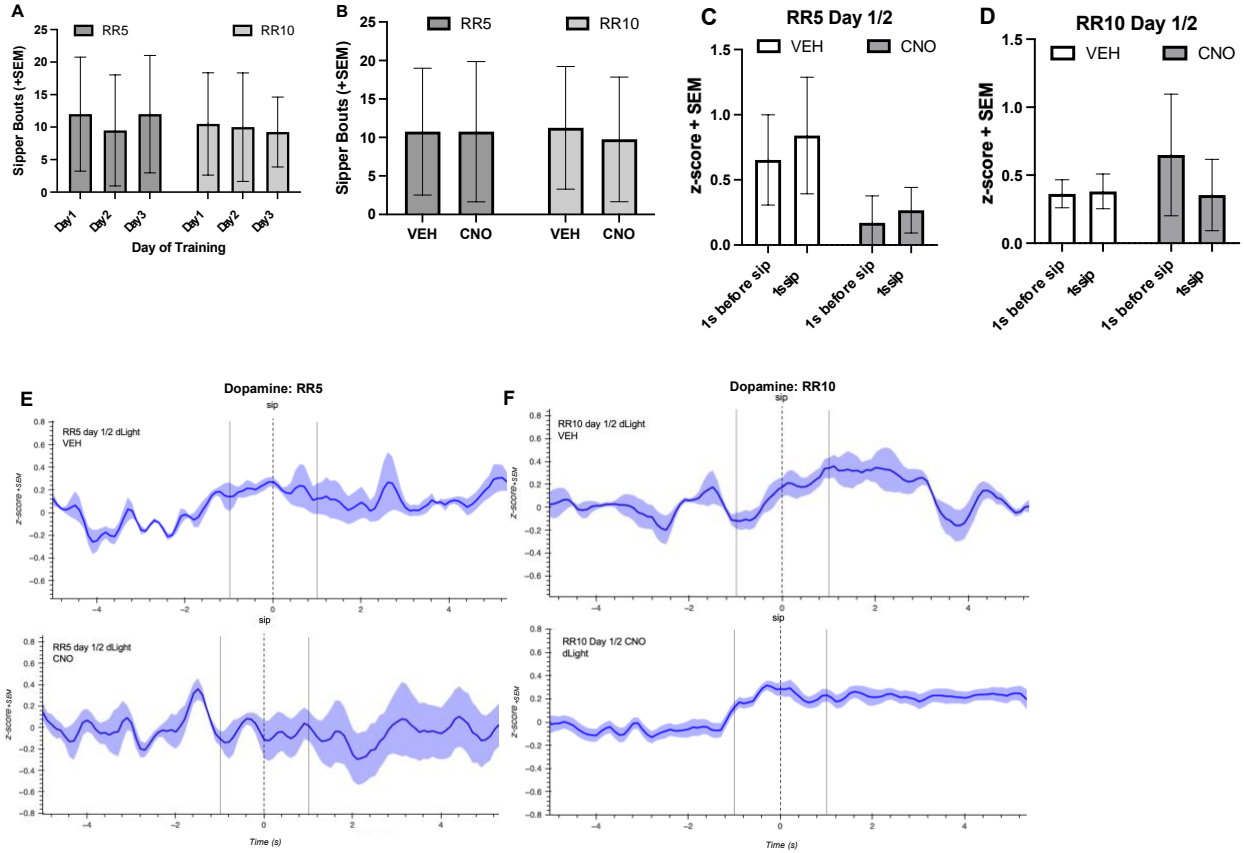


Figure 18. Steady sipper use across instrumental training is not associated with changes in dopamine activity. Rats ($n=4$; recording RR5: $n=4$ [8 fibers]; recording RR10: $n=2$ [4 fibers]) used the sipper following active presses made during instrumental training. Sipper use was low and steady across both training schedules (A). Rats made a similar number of sips after vehicle and CNO treatment during RR5 and RR10 training (B). During RR5 training, dopamine activity did not change as animals made a sip. The dopamine signal was not significantly different from the vehicle control as animals received CNO to inhibit norepinephrine release in the LA (C). At recording timepoints during RR10 training, there was no change in LA dopamine activity corresponding to a sip and activity did not differ based on vehicle or CNO treatment (D). Mean traces of dLight signal time-locked to a sip made under the RR5 schedule (top: veh, bottom: CNO; E). Mean traces of dLight signal centered around sip behavior during RR10 training (top: veh, bottom: CNO; F).

2.3.4.9 Inhibiting norepinephrine input to the LA may impair performance during the Pavlovian to Instrumental transfer test.

Following the Pavlovian conditioning phase and the instrumental phase, animals underwent a Pavlovian to Instrumental Transfer test where the original CS+ was played for 2 minutes followed by an ITI and then a novel CS- played for 2 minutes (Figure 19A). These presentations cycled through for the 30-minute session as rats could make unreinforced instrumental presses. Prior to the start of the PIT test, all rats were administered CNO to determine if inhibition of norepinephrine input to the LA would affect instrumental behavior. There was no main effect of stimulus presentation on pressing behavior (CS+ vs CS- vs ITI, $F_{(1,18, 3.55)}=1.61$, $p=0.29$, Figure 19B). There was no lever effect (active vs inactive, $F_{(1,3)}=0.86$, $p=0.42$, Figure 19B). Notably, there was a lot of variability in the active lever responses made during the PIT test as one animal made many more presses than the others. With a relatively small number of animals, it is difficult to determine if performance during the PIT test was impacted by norepinephrine inhibition. It is also possible that inhibition during training phases blunted both Pavlovian and instrumental learning which would affect subsequent expression of PIT.

A

PIT session timeline

| | | | | | | | | | | | | | | | |
|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|
| 2 min | 2 min | 2 min | 2 min | 2 min | 2 min | 2 min | 2 min | 2 min | 2 min | 2 min | 2 min | 2 min | 2 min | 2 min | 2 min |
| CS+ | ITI | CS- | ITI | CS+ | ITI | CS- | ITI | CS+ | ITI | CS- | ITI | CS+ | ITI | CS- | ITI |

B

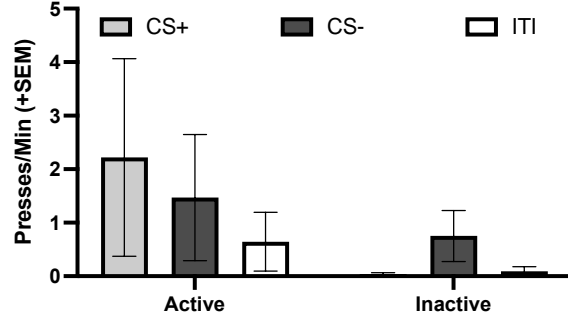


Figure 19. Inhibition of norepinephrine input to LA may weaken PIT.

Rats (n=4) underwent a Pavlovian to Instrumental Transfer test where they received CS+ and CS- presentations separated by an intertrial interval (ITI) to determine if the conditioned stimuli from Pavlovian training affected instrumental behavior. All rats received 1 mg/kg systemic CNO prior to the PIT test to minimize the role of norepinephrine in the LA. PIT session timeline (A). The number of active presses was similar during both stimulus presentations and the ITI (B).

2.4 DISCUSSION

Here, we determined how activity in the LA reflects the cue-reward associative learning process. Using a Pavlovian to Instrumental Transfer paradigm along with fiber photometry in the LA, we found that dopamine and calcium activity show responses to reward-predictive cues and that dopamine activity specifically reflects learning that occurs during Pavlovian conditioning. We also showed that activity in the LA does not change in response to sipper use at any timepoint in training, suggesting that calcium and dopamine dynamics in the region are not linked to primary reinforcement, but rather to the stimuli associated with reinforcement. Our results from instrumental training indicated that performing an instrumental action does not alter dopamine or calcium within the LA. Overall, results show that dopamine activity in the LA is precisely related to stimulus-outcome learning and memory formation.

Previous studies have shown that the LA is activated by reward-predictive stimuli (Ono, Nishijo, & Uwano, 1995; Schoenbaum, Setlow, Saddoris, & Gallagher, 2003; See, 2002). Our results are consistent with this observation as we found that dopamine and calcium activity in the LA increased in response to the beginning of the CS+ presentation. This dopamine activity represents both the time course and concentration of endogenous dopamine release (Patriarchi et al., 2018), while the calcium signal likely reflects calcium responses and spiking activity. However, calcium activity should be carefully interpreted as recent work detailing the photometry signal in the striatum suggested it may reflect non-somatic changes in calcium rather than somatic spiking-related changes (Legaria et al., 2022). It is not yet clear if these findings generalize to the LA or hold true for all calcium sensor variants, but we can conclude that the elevations seen in calcium activity following CS+ presentation represent activation in response to reward-related cues.

During the Pavlovian conditioning phase, rats spent more time near the sucrose sipper during the auditory CS+ presentation indicating that they acquired the association between the cue and sucrose reward. Importantly, neural responses to reward predictive cues may correspond to acquisition of cue-reward associations. For example, animals trained to respond for sucrose paired with a reward predictive cue show more phasically responsive amygdala neurons during reinstatement than animals trained with a randomly presented cue (Tye & Janak, 2007).

Changes in activity in the LA emerged as training progressed, which prompted us to probe the relationship between learning and neural responses by classifying rats' performance during each conditioning session. Rats that spent little time near the sucrose bottle sipper and infrequently used the sipper were grouped together as "non-learners". Conversely, animals that spent most of their time during the CS+ presentation near the sipper area and consistently drank from the sipper were grouped as "learners". Increases in dopamine and calcium activity from a pre-CS+ baseline to the CS+ were only found in the learner group, highlighting that the development of neural responses to the CS+ may require learning the association between the cue and sucrose sip.

Another way in which we assessed the connection between associative learning and LA activity was to compare responses to the CS+ on the initial day of training when no animals had acquired the cue-reward association, on the day animals reached performance criteria, and on the final day of training for the rats that never met performance criteria. Interestingly, only dopamine activity increased at CS+ presentation on the day that the task was learned. Although the elevated calcium response to the CS+ was strongest later in Pavlovian training, it appeared present, though not significantly, for early conditioning even in response to the stimulus on the first day of training. Moreover, the fact that the calcium response to the CS+ diminished by the end of training and was not evident on the day learned suggests that extensive experience with the CS+ reduces calcium

activity. Collectively, results from Pavlovian conditioning suggest that calcium activity in the LA has a general response to the novelty of the onset of CS+ presentation, whereas dopamine activity serves as a specific learning signal.

In other contexts, dopamine in the amygdala has been shown to contribute to associative learning and cue-dependent behaviors. Rats trained to associate stimuli with availability of cocaine show increased dopamine efflux in the amygdala in response to the drug-paired cue relative to the response elicited by a non-reward cue (Katner, Magalong, & Weiss, 1999). Additionally, dopamine receptor antagonism within the basolateral amygdala during acquisition of drug-cue associations attenuates later conditioned cue drug-seeking behavior (Berglund, Case, Parker, Fuchs, & See, 2006; See, Kruzich, & Grimm, 2001). Therefore, both previous evidence and our current results indicate that dopamine activity in the amygdala is a key component underlying associative learning.

Studies examining reward responses in the amygdala point to the idea that the amygdala is not necessary for primary reinforcement, but rather mediates responding to cues in the face of changing reward value (Balleine & Killcross, 2006; Hatfield, Han, Conley, Gallagher, & Holland, 1996; Málková, Gaffan, & Murray, 1997; Murray, 2007). For this PIT paradigm, sips from the sucrose sipper bottle were the primary reinforcer paired with an auditory cue. We found no change in dopamine or calcium activity in response to sips made across both Pavlovian conditioning and instrumental training, thus supporting the idea that the LA is more responsive to reward-related cues than to reward itself.

Likewise, operant responses made during instrumental training failed to elicit changes to dopamine and calcium signaling in the LA. Prior studies have found that the amygdala plays a modest role in instrumental behavior. For one, basolateral amygdala (BLA) lesions do not impair

acquisition of instrumental conditioning or performance. These lesion studies found that an intact BLA is crucial for encoding post-training changes in the incentive value of the instrumental outcome, implying that during instrumental learning the BLA establishes the reward-related properties of the outcome (Balleine, Killcross, & Dickinson, 2003). While excitatory neurotransmission in the BLA has been shown to be involved in instrumental learning, it may be that the BLA supports instrumental performance by underlying the Pavlovian associations between contextual stimuli and the reinforcer that influence acquisition of instrumental actions (Baldwin, Holahan, Sadeghian, & Kelley, 2000). Other evidence has shown the dopamine receptor activation in the amygdala is required for initial instrumental learning but not performance (Andrzejewski, Spencer, & Kelley, 2005). As we assessed LA calcium and dopamine dynamics after animals completed early training on a FR1 schedule, it is not surprising that we do not find LA neural responses as the animals make a press during the later RR5 and RR10 training schedules. Other regions that participate in instrumental learning including the striatum (Faure, Haberland, Condé, & El Massioui, 2005; Hernandez, Sadeghian, & Kelley, 2002; Reynolds, Hyland, & Wickens, 2001), medial prefrontal cortex (Matsumoto, Suzuki, & Tanaka, 2003), and orbitofrontal cortex (Schoenbaum & Roesch, 2005; Schoenbaum, Setlow, Saddoris, & Gallagher, 2003; Thorpe, Rolls, & Maddison, 1983) may be more likely to show robust calcium or dopamine activity changes in response to instrumental actions.

Pavlovian to Instrumental transfer determines how conditioned stimuli influence instrumental responding. Here, we found that the auditory CS+ from the Pavlovian phase did not significantly modulate the animals' instrumental behavior, although it appeared that more presses were made during the CS+ relative to the CS-. Despite the lack of significant behavioral differences, dopamine release during the first second of the CS+ presentation was significantly greater than release during

the first second of the CS-. This finding suggests that dopamine activity may reflect the incentive value of the cue. Many researchers have found that the amygdala plays an important role in representing the affective and incentive value of cues and in turn the representation supports the translation of conditioned associations to instrumental action (Burns, Robbins, & Everitt, 1993; Cador, Robbins, & Everitt, 1989; Malvaez et al., 2015; Whitelaw, Markou, Robbins, & Everitt, 1996). Increased dopamine activity during the CS+ likely contributes to encoding of the incentive value of the cue even when it does not influence behavior.

In all experiments, the dopamine signal in the LA was recorded with fiber photometry using the dopamine sensor dLight1.2. This sensor is specific for dopamine release, but there is also limited sensitivity to norepinephrine (Patriarchi et al., 2018). As the LA receives dense noradrenergic innervation from the locus coeruleus (Asan, 1998; Van Bockstaele, Bajic, Proudfit, & Valentino, 2001), there is a potential for the dLight signal to have both dopaminergic and noradrenergic components. We delivered a retrograde virus expressing the inhibitory DREADD receptor under the synthetic dopamine β hydroxylase promoter PRSx8 to allow for inhibition of norepinephrine input to the LA when CNO was administered. During all training phases, rats received both vehicle and CNO treatment to evaluate the dopamine signal as noradrenergic input was and was not silenced. This inhibition seemingly impacted conditioning behavior as these animals did not reach the performance levels of animals from the first cohort during the Pavlovian phase. Moreover, their pressing behavior and sipper use during instrumental training was lower than animals that never experienced inhibition of noradrenergic projections. There was likely an effect of inhibition on learning and there also appeared to be a general dampening of dLight signal resulting from this inhibition. This effect was not task specific and was not consistent across all measures, although it tended to be present at early timepoints for both Pavlovian and instrumental

training. Therefore, some of the signal we observed from the dLight sensor may reflect norepinephrine in addition to dopamine release.

The inhibition timepoints we used may be a limitation associated with determining the contribution of norepinephrine to the dLight signal with our PIT paradigm. Based on the behavioral data during conditioning, inhibition affected the rats' ability to acquire the cue-reward association as only one rat reached criteria to be considered a "learner". Additionally, no animals learned the task prior to the inhibition that occurred on day 4/6 so it is challenging to disentangle inhibition effects on signal from learning effects at that timepoint. We did not take any earlier recordings to examine the baseline dopamine signal and while a baseline recording could help identify underlying norepinephrine contributions, it would not capture a timepoint at which there would be a learning-related response to CS+ presentation. Waiting to impact norepinephrine signaling until after animals learned the task also poses an issue in that the dLight response to the CS+ may diminish after the cue-reward association is well learned. It may be beneficial to use an excitatory DREADD to increase noradrenergic signaling and compare any excitation-related changes to behavior and signal to those seen under inhibition. Future experiments designed with this caveat in mind may better address how norepinephrine input to the LA influences learning and whether norepinephrine release is a learning signal captured by the dLight sensor.

Another important consideration is the individual differences present in both associative learning and LA activity. Rats did not learn identically during Pavlovian conditioning as evidenced by some reaching performance criteria early in training while others never adequately performed the task. Differences were also seen during instrumental training when some animals took longer to learn the operant task before progressing to a more demanding schedule. Anecdotally, during the instrumental phase, rats used unique lever pressing strategies that ranged from pressing with

one paw to biting at the lever from underneath. At times, particularly in response to event-related stimuli, variability in the dopamine or calcium signal was substantial. While our studies are not designed to thoroughly examine individual differences, understanding how they may contribute to variability provides valuable insight into our findings and presents an opportunity for future investigation

Overall, these results indicate activity in the LA drives associative learning and memory. Calcium activity appears to serve as a general cue-related signal that diminishes with experience, whereas dopamine (and possibly norepinephrine) release underlies associative learning of reward-predictive cues. Fitting with the amygdala's known role in assigning and updating incentive value rather than underlying primary reinforcement, only conditioned stimuli elicited changes in dopamine and calcium activity. Identifying these responses to conditioned stimuli not only improves our understanding of stimulus-outcome learning and memory, but also has implications for when associative learning promotes maladaptive behavior modification.

3.0 VTA DOPAMINE TO LATERAL AMYGDALA PROJECTION SUPPORTS COCAINE CUE ASSOCIATIVE LEARNING

3.1 INTRODUCTION

Learning and memory mechanisms are critically involved in drug craving and relapse (Torregrossa & Taylor, 2013). As environmental cues become paired with repeated drug use, these cues acquire strong incentive and motivational value such that exposure to them alone can trigger craving and relapse. The amygdala is a key region underlying cue-related learning processes that assign valence to environmental stimuli including drug-paired cues (Kyriazi, Headley, & Pare, 2018). Many studies concerning cue associative learning in the amygdala have focused on threat processing and fear conditioning. In this work, an auditory tone conditioned stimulus (CS) is paired with an aversive footshock unconditioned stimulus (US), leading to threat appropriate responses, such as freezing, occurring in response to the CS alone. Electrophysiology experiments revealed that the lateral amygdala (LA) mediates this type of associative learning via integration of sensory inputs from auditory thalamus and cortex (Kim et al., 2007; Kwon et al., 2014; LeDoux, Farb, & Ruggiero, 1990; Rich, Huang, & Torregrossa, 2019; Rogan, Staubli, & LeDoux, 1997; Rogan & LeDoux, 1995) with the negative effects of the footshock via projections from somatosensory regions (Shi & Davis, 1999).

More recent work has examined how neurons in the LA develop and maintain neural responses to a CS that has been paired with positive reinforcing stimuli. The traditional model for this process is that an initially weak afferent pathway carrying sensory information about the CS and strong afferents carrying US information converge in the LA. Through plasticity mechanisms,

there is enhancement at the excitatory synapses carrying CS information (Maren, 2000; McKernan & Shinnick-Gallagher, 1997; Rogan, Staubli, & LeDoux, 1997). In the context of drugs of abuse, it has been shown that drug-cue associations develop through enhancement of CS inputs from auditory thalamus onto principal neurons in the LA (Rich et al., 2019). Yet, the regions that provide inputs to the LA to encode the reinforcing effects of cocaine that serve as a US are not well characterized.

Many lines of evidence lend support to the hypothesis that the ventral tegmental area (VTA) projection to the LA participates in encoding the reinforcing effects of drugs that act as a US in conditioned cue drug-seeking. Dopamine in the LA has been shown to be necessary for the maintenance of drug-cue associations, the regulation of drug-seeking behavior, and the conditioned reinstatement of drug-seeking (Berglind, Case, Parker, Fuchs, & See, 2006; Kruzich & See, 2001; Liu et al., 2010; Mashhoon, Tsikitas, & Kantak, 2009). Much of the dopamine released in the LA originates in the VTA (Brinley-Reed & McDonald, 1999). These VTA to LA projections have been shown to contribute to aversive and appetitive stimulus-outcome learning (Lutas et al., 2019; Tang, Kochubey, Kintscher, & Schneggenburger, 2020). Here, we used various chemogenetic approaches to manipulate the VTA to LA projections during cocaine cue associative learning and subsequent reinstatement to determine their role in cocaine cue associative learning. We then used tracing techniques in combination with RNAscope in the VTA to examine the neuronal populations that may underlie our behavioral findings. Our results extend the current knowledge of dopamine's role in the LA to show an important contribution of these VTA dopaminergic projections in associative learning and point to a pathway for further investigation into the development of substance use disorder.

3.2 METHODS

3.2.1 Animals

Adult male and female Sprague Dawley rats (Envigo) were paired housed in auto-ventilated racks with automated watering in a temperature- and humidity-controlled room and maintained on a 12 h light/dark cycle (lights on at 4:30 am). Rats were given ≥ 5 days to acclimate to the facility before any surgical procedures. Following catheter implantation, animals were single housed. Rats were food restricted 24 hours before the start of training and were maintained at 95% of their free-feeding weight. In experiments using TH-Cre rats, males and females were bred in house from Tyrosine Hydroxylase TH-Cre knockin (HsdSage:SD-TH^{em1(IRES-CRE)_{Sage}}) dams and wild-type males or the offspring of these breeding pairs. Genotypes were determined by Transnetyx® genotyping and only heterozygous TH-Cre animals were used. All procedures were conducted in accordance with the National Institutes of Health's *Guide for Care and Use of Laboratory Animals* and were approved by the University of Pittsburgh's Institutional Animal Care and Use Committee.

3.2.2 Drugs

Cocaine hydrochloride (graciously provided by NIDA) was dissolved at 2 mg/mL (intravenous cocaine self-administration) or 5 mg/mL (intraperitoneal injections) in 0.9% sterile saline (ThermoFisher) and filter-sterilized. Clozapine-N-oxide (CNO, Tocris Bioscience or graciously provided by NIDA Drug Supply Program) was dissolved in 5% DMSO in 0.9% sterile saline (1 or 3 mg/ml) and injected intraperitoneally 30 min before behavioral tests. For intracranial

injection, CNO was dissolved in 0.5% DMSO in artificial CSF (ACSF, Fisher Scientific; 1 mM/0.3 ul; total dose, ~100 ng/hemisphere) and microinjected 5 min before the start of behavior.

3.2.3 Viral or toxin constructs and delivery

Rats were placed in a stereotaxic frame and given a small injection (0.2-0.3 ml) of lidocaine (Henry Schein) to the scalp as a local anesthetic. For chemogenetic manipulations in TH-Cre rats, a 26-gauge stainless steel injection cannula connected to a Hamilton syringe was used to inject the LA (males in mm from bregma, anterior and posterior (AP): -3.0; medial and lateral (ML): ± 5.0 ; dorsal and ventral (DV): -7.9 mm; females in mm from bregma, AP: -2.8, ML: ± 4.8 ; DV: -7.8 mm) bilaterally (1 μ l/hemisphere) through a pump (Harvard Apparatus) with retrograde adeno-associated virus (rgAAV) containing a double-floxed, inverted open reading frame (DIO) sequence for the mCherry-tagged hM4D(Gi-coupled) or mCherry control (hSyn-DIO-hM4D(Gi)-mCherry, hSyn-DIO-mCherry, Addgene). In a separate experiment using the same setup, a custom made AAV2retro.PRSx8.HA-hM4D.SV40 (Penn Vector Core) was bilaterally infused into the LA (males in mm from bregma, anterior and posterior (AP): -3.0; medial and lateral (ML): ± 5.0 ; dorsal and ventral (DV): -7.9 mm; females in mm from bregma, AP: -2.8, ML: ± 4.8 ; DV: -7.8 mm). In all other chemogenetic manipulation experiments, the same injection setup was used to inject the VTA (males and females: in mm from bregma AP: -5.5, ML: ± 0.9 ; DV: -8.20) bilaterally with 1 μ l/hemisphere adeno-associated virus 2 (AAV2) containing the mCherry-tagged hM4Di (Gi-coupled) or hM3Dq (Gq-coupled) DREADDs or EGFP control (CaMKII α -hM4D(Gi)-mCherry, CaMKII α -hM3D(Gq)-mCherry, CaMKII α -EGFP, Addgene). For experiments involving retrograde tracing, injection cannula connected to the Hamilton syringe were used to bilaterally

inject the LA (males in mm from bregma, anterior and posterior (AP): -3.0; medial and lateral (ML): ± 5.0 ; dorsal and ventral (DV): -7.9 mm; females in mm from bregma, AP: -2.8, ML: ± 4.8 ; DV: -7.8 mm) with 1 μ l/hemisphere with cholera toxin subunit B (recombinant) Alexa Fluor-555 Conjugate (ThermoFisher). All injections were at a 0.1 ml/min flow rate and injection cannula were left in place for 5 min after completion before being slowly withdrawn. More than 5 weeks was allowed between virus injection and training.

3.2.4 Surgery

3.2.4.1 Anesthesia

Rats were fully anesthetized via intramuscular injections of ketamine (90-100 mg/kg, Henry Schein) and xylazine (5 mg/kg, Butler Schein) and then were given subcutaneous injections of the analgesic Rimadyl (5 mg/kg, Henry Schein) and 5 ml of Lactated Ringer's solution. Surgical sites were shaved and treated with betadine (povidone iodine, 5%, Henry Schein) and 70% ethanol on all incisions as previously described (Rich et al., 2019).

3.2.4.2 Catheter implantation

All rats were implanted with a chronic indwelling intravenous catheter into the right jugular vein and fed subcutaneously to exit the midscapular region where a bent cannula (PlasticsOne) exited through a round incision, as previously described (Torregrossa & Kalivas, 2008). Catheters were capped to prevent blockage.

3.2.4.3 Intracranial cannulation

Following jugular vein catheterization, rats used in experiments involving intra-LA microinfusions were placed in a stereotaxic frame. A small injection of lidocaine (0.2-0.3 ml, Henry Schein) was used as a local anesthetic. Two 22-gauge guide cannula (cut 11 mm below an 8 mm pedestal, PlasticsOne) were implanted bilaterally, aimed 2 mm dorsal to the lateral amygdala (males in mm from bregma, anterior and posterior (AP): -3.0; medial and lateral (ML): ± 5.0 ; dorsal and ventral (DV): -7.9 mm; females in mm from bregma, AP: -2.8, ML: ± 4.8 ; DV: -7.8 mm). Cannula were secured to the skull with screws and OrthoJet dental cement (Lang Dental). Once cured, dummy cannula (C313DC, PlasticsOne) the length of the guide cannula were inserted to prevent obstruction.

3.2.4.4 Post-operative care

For two days following surgery, rats were given Rimadyl (5 mg/kg, Henry Schein) subcutaneously. Catheter patency was maintained by daily infusion of 0.1 ml of a .09% sterile saline solution containing Gentamicin (3 mg/ml, Henry Schein) and heparin (30 USP/ml; Henry Schein).

3.2.5 Behavioral Procedures

3.2.5.1 Behavioral Apparatuses

Experiments were conducted in 24 standard operant conditioning chambers (MedAssociates) using MedPC software (MedAssociates). Each animal underwent all training and testing in the same chamber. Each chamber was equipped with bar floors, a house light, two cue lights above two levers, a tone generator, a head-entry magazine, a sucrose pellet dispenser, and a

syringe pump connected to a swiveled leash. All chambers had 2 plexiglass walls with one containing the levers, magazine and cues lights and the opposite wall containing nose-poke apertures. Half of the chambers were equipped with 2 nose poke apertures, while the others were equipped with 5 nose-poke apertures with a removable opaque plexiglass cover. Chambers were contained in sound-attenuating boxes with fans for background noise. Conditioned place preference experiments were conducted in a custom made 60 cm X 30 cm X 30 cm apparatus. The apparatus consisted of 2 25 cm X 30 cm X 30 cm chambers connected by a 10 cm X 30 cm X 30 cm pathway containing 15 cm X 15 cm entryways. During conditioning and testing, chambers differed from one another in odor (vanilla or almond), floor texture (Plexiglass or rough plastic covering), and wall pattern (vertical stripes or horizontal stripes). During conditioning, a 30 cm X 30 cm X 2 cm divider was inserted over the entryway to restrict the animal to a single chamber.

3.2.5.2 Self-Administration

Food Training: In the case where rats were restricted to 5 days of cocaine-self administration, they were initially trained to self-administer sucrose pellets in a single 6 hr session on a fixed ratio (FR1) schedule of reinforcement. The session started with illumination of the houselight and the insertion of the active and inactive levers (counterbalanced between animals). A press on the active lever resulted in the dispense of a sucrose pellet. Inactive lever presses were recording, but had no programmed consequence.

Cocaine or saline self-administration: For all experiments, rats were trained to self-administer cocaine (2 mg/mL) in 1 hr daily sessions on FR1 schedule with a 10s timeout. For the tracing experiment, half of the animals were trained to self-administer saline (0.9%) instead of cocaine. Each self-administration session began with house light illumination and lever insertion.

A press on the active lever (counterbalanced between animals) produced an infusion paired with a 10s audiovisual cue consisting of a tone and the illumination of a cue light above the active lever. Pump durations were adjusted daily according to body weight to deliver 1 mg/kg/infusion. Inactive lever presses were recorded, but had no programmed consequence. Sessions terminated after 1 hr or delivery of 30 infusions to prevent overdose. In experiments with systemic CNO delivery, animals received intraperitoneal CNO (1 or 3 mg/kg) or vehicle injection 30 min prior to the start of the session, while in experiments with local microinfusions, the sessions began 5 min after microinfusion of CNO or ACSF into the LA.

3.2.5.3 Instrumental Lever Extinction

After self-administration, rats underwent instrumental lever extinction for 7-14 days. During these daily 1-hour sessions, responses on the active and inactive levers were recorded but had no programmed consequences. There were no cue presentations or cocaine infusions during the extinction phase. Extinction continued until rats made an average of <25 lever presses on the last two days of extinction and any animals that did not reach criterion after 14 days were excluded.

3.2.5.4 Cue-induced Reinstatement

Cue-induced reinstatement was assessed during a 1-hr session during which a lever press on the active lever resulted in a 10s presentation of the cocaine associated cue on an FR1 schedule. Lever presses on the inactive lever were recording but had no programmed consequences. When rats had repeated cue reinstatement tests, the sessions were separated by ~2 days of instrumental re-extinction. In these sessions, rats received intraperitoneal injections of 1 mg/kg CNO, 3 mg/kg CNO, or vehicle with the order counterbalanced between animals. Any animals that did not reach criterion after 5 days of re-extinction were excluded.

3.2.5.5 Cocaine primed reinstatement

Cocaine prime reinstatement was examined during a 1-hr session immediately prior to which animals received a 10 mg/kg cocaine intraperitoneal injection. During the session, rats had access to both active and inactive levers. Presses were recorded, but neither active nor inactive presses had any programmed consequences. Rats that underwent 3 sessions of cocaine prime reinstatement separated by ~2 days of re-extinction received intraperitoneal injections of vehicle, 1 mg/kg CNO, or 3 mg/kg CNO with the order counterbalanced between rats. Any animals that did not reach criterion after 5 days of re-extinction were excluded.

3.2.5.6 Conditioned Place Preference

Rats were tested with the conditioned place preference (CPP) paradigm in a custom CPP chamber as described above. At baseline, rats were placed in the center neutral area of the chamber and were allowed to freely move between both chambers for 15 minutes. Time spent in each of the chambers was scored and analyzed. An animal was considered to be in a compartment if all four paws were located in the compartment, while two paws out of the compartment was considered an exit. Following baseline, rats had 6 days of conditioning. On alternate days, rats were intraperitoneally (i.p.) injected with cocaine and restricted to a randomly assigned (paired) compartment for 20 min or injected with saline and restricted to the unpaired compartment for 20 min. Males received a 20 mg/kg injection of cocaine (Russo et al., 2003) and females received a 5 mg/kg injection of cocaine, as these doses have been shown to produce equivalent levels of CPP between the sexes (Russo et al., 2003). Any i.p. injection of 1 mg/kg CNO or vehicle occurred 30 minutes prior to the start of the conditioning session, while local LA microinfusions of CNO occurred 5 minutes before the start of the conditioning session. The test session was conducted 24 h after the final conditioning session. On test day, the door separating the two chambers was

removed and each animal was placed in the center neutral area to begin the 15-minute session. Time spent in each of the chambers was scored and analyzed.

3.2.6 Staining, fluorescence, and imaging

Rats were perfused by first deeply anesthetizing them with sodium pentobarbital (Coventrus, 100 mg/kg i.p.) and then perfusing through the aorta 1X PBS for 5 min followed by 4% paraformaldehyde (Santa Cruz Biotechnology) in 1X PBS, pH 7.4 for 10 min. The brains were extracted, postfixed in 4% paraformaldehyde for 24h before being transferred to a 30% sucrose solution. Brains were sectioned at 50 μ m using a cryostat (Leica). Slices containing the LA or VTA were collected for histology or immunohistochemistry. In the case of histology, slices were mounted on glass slides and coverslipped with Fluoroshield with DAPI mounting media (Sigma-Aldrich). Slides were imaged using an Olympus VS120 slide-scanning microscope to verify virus expression and/or cannula placement. Rats lacking expression of AAV in LA or VTA and those with improperly positioned cannula were removed from the study.

3.2.6.1 Immunohistochemistry

c-Fos immunoreactivity was used as an indicator of activity to determine the efficacy of CNO in DREADD expressing animals. These animals were sacrificed via transcardial perfusion 90 minutes following a final cocaine self-administration session. To verify the effects of CNO in TH-Cre rats, animals were sacrificed 90 minutes following a 10 mg/kg cocaine i.p. injection that was preceded by an i.p. injection of CNO. To confirm CNO effects following a local microinfusion, rats received a CNO or vehicle microinfusion prior to a final cue reinstatement session and were euthanized via cardiac perfusion 90 minutes after the session. Brains were

harvested and sectioned at 50 μm using a cryostat (Leica). Sections were washed with PBS with 0.1% triton (PBST+) and then left in a PBST+ and 5% donkey serum (Millipore Sigma) blocking buffer for 2 hours at room temperature. Sections were then incubated in anti c-Fos primary antibody (1:2000, Abcam ABE457) for 48 hours at 4°C. After primary incubation, sections were again washed with PBST+ and then placed in wells with secondary antibody in blocking buffer (1:500, Donkey anti-rabbit IgG Alexa Fluor 488, Invitrogen Antibodies) for two hours at room temperature. Sections were then washed in PBS, mounted, and coverslipped with Fluoroshield with DAPI mounting medium (Sigma Aldrich). Slides were scanned using an Olympus VS120 scanning microscope with the DAPI, RFP, and GFP channels. c-Fos+ cells were counted with Fiji (ImageJ) software. Three sections per region per animal were counted and averaged to give a cell count per animal.

3.2.6.2 RNAscope

Animals were euthanized by decapitation 30 minutes from the middle of the final self-administration session. Brains were harvested and flash frozen using isopentane (Fisher Scientific). Brains were sectioned at 12 μm using a cryostat (Leica) and directly mounted onto glass slides. Slices were stored in airtight slide boxes at -80°C. The RNAscope Multiplex Fluorescent V2 Assay (ACD Bio now BioTechne) was performed according to the manual instructions. Briefly, the sections were fixed in chilled buffered formalin (Fisher Scientific) then dehydrated in series of 5-minute ethanol washes of increasing concentration from 50% to 100%. After a hydrophobic barrier was drawn around the sections, they were treated with RNAscope Hydrogen Peroxide, RNAscope Protease IV, and a probe mix consisting of RNAscope Probe RN-Fos, RNAscope Probe RN-Th-C2, and RNAscope Probe RN-Slc17a6-C3. A series of amplifications in the HybEZ Oven were performed to develop signals for each probe channel. These fluorescent detection channels were

paired with fluorophores from Akoya Biosciences. Finally, slides were counterstained with DAPI and mounted with ProLong Gold Antifade Mountant (ThermoFisher Scientific). Slides were scanned using an Olympus VS200 scanning microscope with the DAPI, Cy3, Cy5, Cy7, and FITC channels. Section images were cropped using OlyVia software and RNAscope puncta present on cells were counted with QuPath software. Three sections per region per animal were counted and averaged to give a cell count per animal.

3.2.7 Statistical Analysis

Behavioral data were collected using MedPC software and whenever possible experimenters were blinded to the rats' treatment conditions. Statistical analyses were performed using GraphPad Prism and SPSS Statistics Software. For all analyses, significance was set at $p < 0.05$.

3.3 RESULTS

3.3.1 Chemogenetic inhibition of VTA dopamine input to LA slows acquisition of cocaine self-administration.

To determine the contribution of VTA dopamine input to LA to cocaine cue associative learning, we chemogenetically inhibited the projection as animals learned cocaine self-administration. Prior to self-administration, TH-Cre rats received bilateral injections into the LA of a Cre-dependent retrograde AAV expressing hM4Di (Gi-DREADD) or a Cre-dependent

retrograde virus expressing mCherry (control) (Figure 20A-B). Rats were then trained to self-administer cocaine (1mg/kg/infusion) for 14 days while receiving injections (i.p) of either 1 mg/kg CNO or DMSO vehicle before each session (Figure 20C). Males and females were used for all experiments, but there was no effect of sex on infusions ($F_{(1,12)}=0.47$, $p=0.51$), active presses ($F_{(1,12)}=0.22$, $p=0.65$), or inactive presses ($F_{(1,12)}=0.004$, $p=0.95$) so data were collapsed across sex for subsequent analysis. During self-administration training, there were main effects of both treatment ($F_{(2,15)}=8.39$, $p=0.004$) and training day ($F_{(13,195)}=10.73$, $p<0.001$) and a treatment x training day interaction ($F_{(26,195)}=2.89$, $p<0.001$) on the number of infusions earned. CNO administration in Gi-DREADD rats decreased the number of infusions earned across training relative to Gi-DREADD rats treated with vehicle ($p=0.004$) and mCherry controls treated with either vehicle or CNO (combined control group) ($p=0.025$, Figure 20D). Analysis of the interaction indicated that Gi-DREADD rats that received CNO earned fewer infusions than the control group on days 6-9 and 11-14, whereas they earned fewer infusions than the Gi-DREADD vehicle group on days 2, 4, 6-7, and 9-12 (Figure 20D). Analysis of active lever presses revealed a main effect of treatment ($F_{(2,15)}=6.36$, $p=0.01$), training day ($F_{(13,195)}=5.46$, $p<0.001$), and treatment x training day interaction ($F_{(26,195)}=1.94$, $p=0.006$). Post hoc analysis showed Gi-DREADD rats that received CNO made fewer active lever presses compared to those that received vehicle ($p=0.010$, Figure 20E) and were trending to make fewer active presses compared to controls ($p=0.071$, Figure 20E). On a day-by-day basis, Gi-DREADD rats given CNO made fewer presses than control rats on days 7-9 and 11-12, while they made fewer presses than the Gi-DREADD rats given vehicle on days 2, 4, 6-7, and 9. No main effect of treatment on inactive lever presses emerged ($F_{(2,15)}=0.88$, $p=0.44$), but training day did affect inactive presses ($F_{(13,195)}=2.42$, $p=0.05$) as all groups decreased inactive lever pressing across training (Figure 20F).

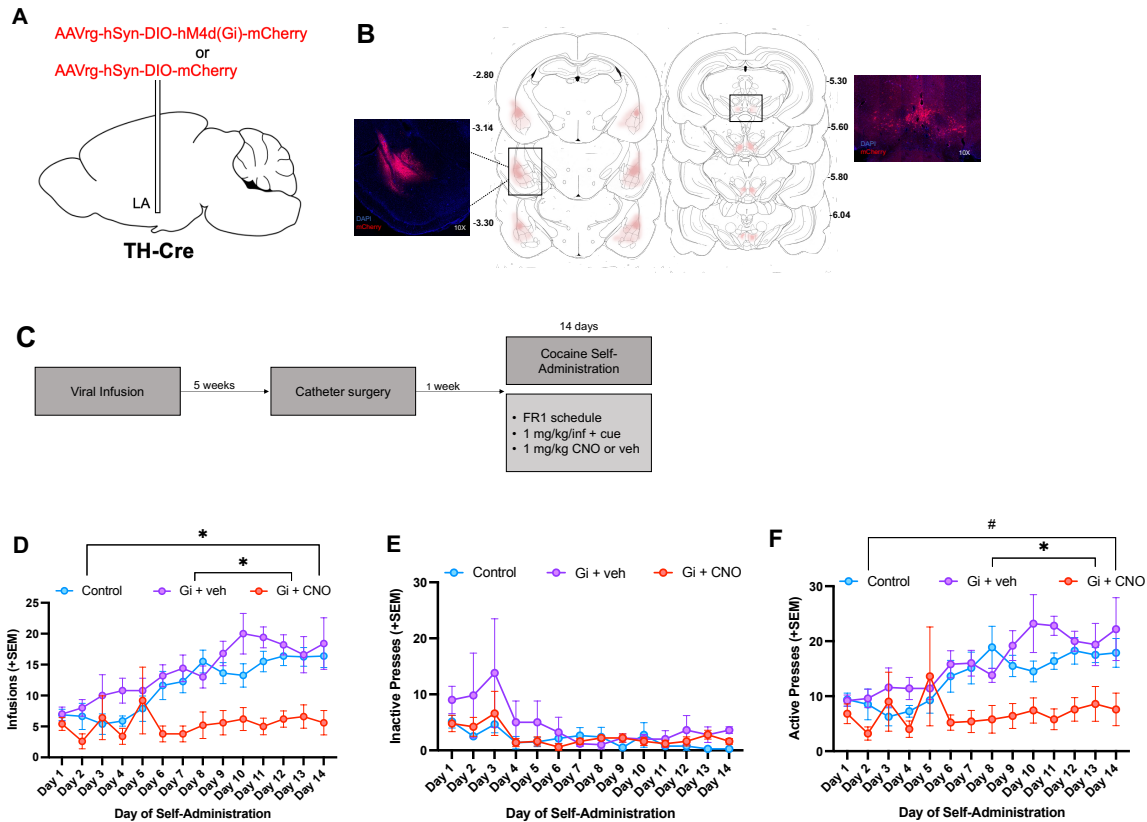


Figure 20. Inhibition of VTA dopamine input to LA slows acquisition of cocaine self-administration.

Rats (n=18, Control=4, Gi-DREADD + veh=9, Gi-DREADD + CNO=5) were trained to self-administer cocaine on FR1 schedule of reinforcement and received i.p. injections of either 1 mg/kg CNO or vehicle prior to each session (F-G). Schematic of virus surgery (A). Schematic showing injection of virus in LA and spread throughout the VTA. Representative images of LA and VTA expression (B). Experimental timeline (C). Gi-DREADD + CNO rats earned fewer cocaine infusions than Gi-DREADD + veh and mCherry control rats (D). Gi-DREADD + CNO rats made fewer active presses than Gi-DREADD + veh rats and tended to make fewer active presses than control rats (E). During self-administration, there was a main effect of training day such that all animals decreased inactive pressing across training, but did not differ in the number of inactive presses made (F). Graphs show group means \pm SEM. # $p < 0.1$, * $p < 0.05$

3.3.2 Prior chemogenetic inhibition of VTA dopamine input to LA affects early but not late instrumental extinction.

Self-administration was followed by instrumental extinction in order to extinguish lever pressing behavior before evaluating the effects of inhibition on the ability of the cocaine-paired cue or cocaine to reinstate drug-seeking behavior (Figure 21A). There was a main effect of treatment on active presses made during extinction ($F_{(2,15)}=4.77$, $p=0.025$) and a main effect of extinction day on presses ($F_{(6,90)}=12.61$, $p<0.001$, Figure 21B). These effects were driven by rats in the mCherry control group that made significantly more active presses than Gi-DREADD rats that received CNO ($p=0.028$) and made more presses than Gi-DREADD rats that were given vehicle ($p=0.074$). Multiple comparisons showed that the control group made more active presses than the inhibition group on extinction days 1 and 4. However, all groups equally extinguished their pressing behavior by the final extinction day (Figure 21B).

3.3.3 Chemogenetic inhibition alters reinstatement of cocaine-seeking behavior.

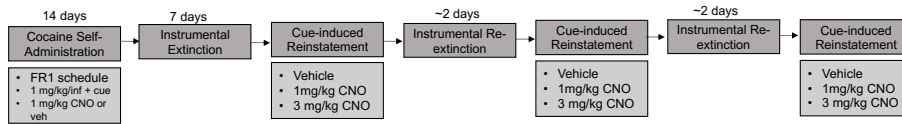
Reinstatement can serve as an indicator of relapse-like behavior and cue-induced reinstatement probes the ability of the conditioned stimulus (CS) to elicit drug-seeking. Rats experienced cue-induced reinstatement sessions after injection (i.p.) of vehicle, 1 mg/kg CNO, and 3 mg/kg CNO. Both male and female rats underwent cue-induced reinstatement, but there was no effect of sex on presses made during cue reinstatement ($F_{(1,13)}=1.32$, $p=0.27$) so all data were collapsed across sex for further analysis. Analysis of active lever pressing yielded a main effect of treatment during training ($F_{(2,15)}=7.44$, $p=0.006$), but no effect of in-session treatment ($F_{(1,15)}=0.009$, $p=0.93$). Across all sessions, previous administration of CNO in Gi-DREADD rats

attenuated active lever pressing during reinstatement compared to Gi-DREADD rats that received vehicle during training ($p=0.034$) and control rats ($p=0.010$). After vehicle injection, Gi-DREADD rats that received CNO during self-administration made fewer active lever presses than controls ($p=0.010$, Figure 21C). Following 1 mg/kg CNO treatment, Gi-DREADD rats that had CNO during training made fewer active lever presses relative to both Gi-DREADD rats that received vehicle during training ($p=0.038$, Figure 21C) and controls ($p=0.019$, Figure 21C). After an injection of 3 mg/kg CNO, Gi-DREADD rats that received CNO during training made fewer active presses compared to the Gi-DREADD rats that received vehicle during training ($p=0.0045$). These results paired with the lack of effect of in-session CNO, suggest that inhibition during self-administration and not during the session drives a reduction in relapse-like behavior elicited by cue re-exposure (Figure 21C).

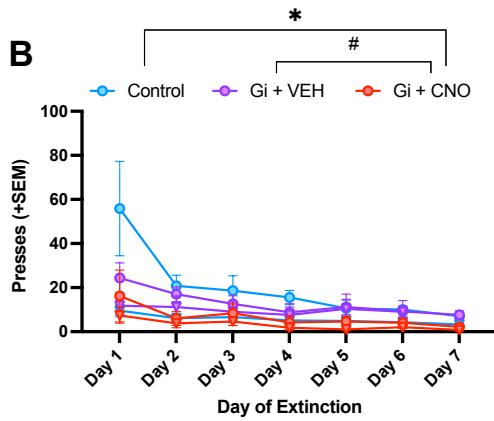
Cocaine primed reinstatement examines the ability of the cocaine US to elicit drug-seeking independently of the CS. As with cue-induced reinstatement, there was no effect of sex on active presses made during cocaine primed reinstatement ($F_{(1,11)}=0.29$, $p=0.60$) so data were combined. There was a trend for treatment during self-administration to impact active presses made during reinstatement ($F_{(2,13)}=3.13$, $p=0.078$), but no effect of in-session treatment ($F_{(2,13)}=0.022$, $p=0.98$). There was a treatment during training x in-session treatment interaction ($F_{(2,13)}=3.92$, $p=0.039$) on active lever presses where after vehicle, Gi-DREADD rats that were given vehicle during self-administration made more active presses than Gi-DREADD rats that received CNO during training ($p=0.042$, Figure 21D). Although, there was no main effect of in-session treatment, the Gi-DREADD group that received vehicle during self-administration did not maintain this heightened level of pressing. For these Gi-DREADD rats that received vehicle during training, 1 mg/kg CNO during reinstatement blunted active lever responding relative to the in-session vehicle ($p=0.051$,

Figure 21D), implying that inhibition of VTA dopamine input during cocaine primed reinstatement may decrease seeking behavior in Gi-DREADD animals that did not experience inhibition during training.

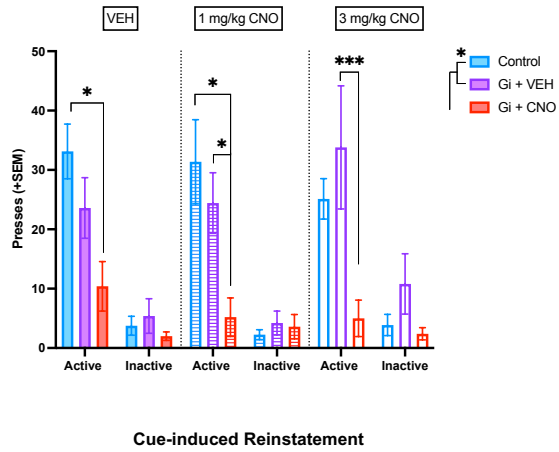
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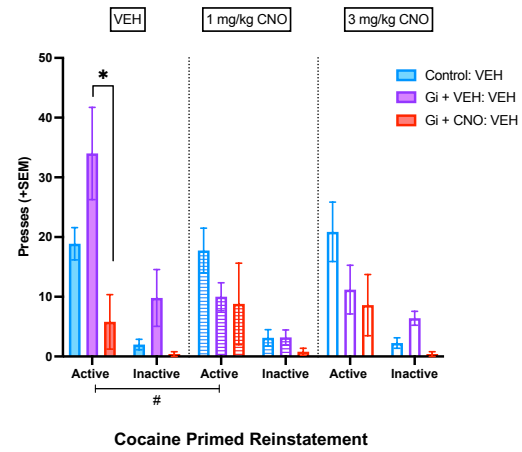


Figure 21. Chemogenetic inhibition alters reinstatement of cocaine-seeking. Rats (n=18, Control=4, Gi + veh=9, Gi + CNO=5) underwent instrumental extinction followed by 3 cue-induced reinstatement sessions in which they were administered injections of vehicle, 1 mg/kg CNO, or 3 mg/kg CNO (order counterbalanced). Cue-induced reinstatement was followed by cocaine primed reinstatement during which they received either vehicle, 1 mg/kg CNO, or 3 mg/kg CNO (order counterbalanced) (C-D). Experimental timeline (A). Treatment during self-administration affected the number of active lever presses made during instrumental extinction. Control rats made more active presses than Gi-DREADD rats that received CNO and trended to make more active presses than the Gi-DREADD rats that received vehicle. There was a main effect of extinction day on pressing indicating that all animals decreased their pressing across training and reached a low level of pressing by the final day (B). There was a main effect of treatment during self-administration on active lever presses made during cue-induced reinstatement where Gi + CNO rats made fewer presses. Control rats made more active presses than Gi + CNO rats following vehicle and 1 mg/kg CNO. Gi + veh rats made more active presses than Gi + CNO rats following both CNO doses. Within each group, in-session CNO treatment did not change reinstatement behavior (C). For cocaine-primed reinstatement, there was a treatment during training x in session treatment interaction where following vehicle, Gi + CNO rats made fewer active presses than Gi + veh rats. Despite no main effect of in-session treatment, Gi + veh rats trended to make fewer presses following 1 mg/kg CNO relative to when they received vehicle (D). Graphs show group means \pm SEM. Circle symbols indicate active presses and triangle symbols indicate inactive presses. #p<0.1, *p<0.05, ***p<0.0001

3.3.4 Chemogenetic inhibition does not affect rewarding effects of cocaine.

The previous results indicate that inhibition during training alters cocaine cue associative learning to weaken acquisition of self-administration and dampen reinstatement of drug-seeking, particularly in response to the formerly drug-paired cue. However, these experiments do not rule out that these effects on acquisition and reinstatement may result from inhibition impacting the rewarding effects of cocaine. To examine any effects of inhibition of dopamine inputs to the LA on the rewarding properties of cocaine, we used a similar chemogenetic approach as above to determine effects on acquisition of a conditioned place preference (CPP, Figure 22A). Analysis of time spent in the conditioning chamber revealed no main effect of CNO treatment during conditioning on time spent in each chamber ($F_{(2,26)}=0.65$, $p=0.53$). After conditioning, control rats, Gi-DREADD rats that received vehicle, and Gi-DREADD rats that received CNO spent more time in the cocaine paired chamber than the saline paired chamber ($F_{(1,26)}=14.03$, $p=0.009$, Figure 22B), suggesting that inhibiting VTA dopamine input to the LA does not interfere with the rewarding properties of cocaine or ability to learn a contextual association with cocaine.

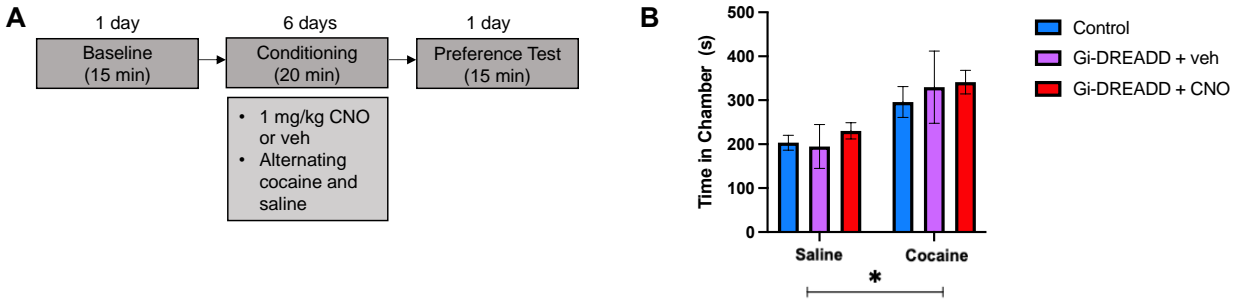


Figure 22. Inhibition does not affect rewarding effects of cocaine. Rats (n=16, Control = 8, Gi + veh =3, Gi + CNO =5) underwent Conditioned Place Preference (CPP) during which they received systemic vehicle or 1 mg/kg CNO during conditioning (A-B). Experimental timeline (A). Following conditioning, control rats, Gi + veh rats, and Gi + CNO rats spent more time in the cocaine paired chamber compared to the saline paired chamber. Experiencing inhibition of the VTA dopamine input to the LA did not affect time spent in the chambers (B). Graph shows group means \pm SEM. *p<0.05

3.3.5 Administration of systemic CNO in Gi-DREADD expressing TH-Cre rats impacts c-Fos immunoreactivity following a cocaine prime.

Binding of CNO to the Gi-DREADD receptor triggers hyperpolarization of the cell, inhibition of neurotransmitter release, and neuronal silencing (Roth, 2016). As a confirmation that systemic CNO administration reduced overall neuronal activity in the LA of Gi-DREADD expressing animals, we examined c-Fos immunoreactivity following a 1 mg/kg CNO injection followed by a 10 mg/kg cocaine prime. The number of c-Fos+ cells in the LA was lower in the Gi-DREADD group that received CNO compared to those expressing the control virus that received CNO ($t(6)=2.47$, $p=0.049$), indicating that CNO in Gi-DREADD rats reduced the number of active cells following cocaine exposure (Figure 23A).

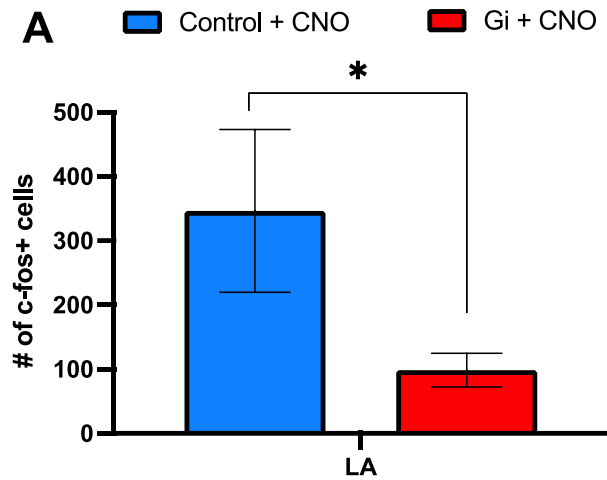


Figure 23. CNO administration in Gi-DREADD rats impacts c-Fos immunoreactivity. Rats (n=8, Control + CNO=4, Gi + CNO=4) expressing the control virus and rats expressing the Gi-DREADD virus were given i.p. injections of 1 mg/kg CNO before a 10 mg/kg cocaine prime injection. c-Fos immunoreactivity in the LA was examined and the number of c-Fos+ cells in LA was lower in Gi-DREADD animals relative to controls. Graphs shows group means \pm SEM. *p<0.05.

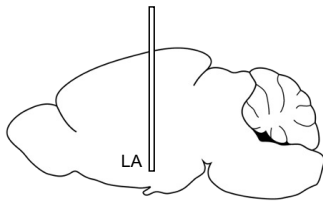
3.3.6 Cocaine self-administration and cue-induced reinstatement are not affected by inhibition of noradrenergic input to the LA.

Tyrosine hydroxylase (TH) is the rate-limiting enzyme in the synthesis of catecholamines including dopamine and norepinephrine (Molinoff & Axelrod, 1971). While the goal of our Cre-dependent viral approach in TH-Cre rats is to target dopaminergic VTA neurons that project to the LA, our strategy does not rule out the contribution of other TH+ cell populations such as noradrenergic input to the LA from the locus coeruleus (Asan, 1998; Robertson, Plummer, & Jensen, 2016). To determine if this population contributed to the inhibition effects on cocaine self-administration and cue-induced reinstatement, we infused a retrograde virus expressing the hM4Di inhibitory DREADD under the synthetic dopamine beta hydroxylase PRSx8 promoter into the LA of male and female rats (Figure 24A-B). These rats received CNO injections (i.p.) 30 minutes prior to the start of the session for 10 days of cocaine self-administration (Figure 24C). We compared the effect of CNO in the PRSx8 promoter Gi-DREADD rats to the inhibition and control groups from the previous Gi-DREADD experiments in TH-Cre rats. There was a main effect of treatment group ($F_{(2,17)}=6.20$, $p=0.0095$), a main effect of training day ($F_{(2.42, 41.17)}=8.76$, $p=0.0003$), and treatment group x training day interaction ($F_{(18,153)}=2.85$, $p=0.0003$) on the number of infusions earned (Figure 24D). Similarly, there was a main effect of treatment group ($F_{(2,17)}=3.66$, $p=0.048$), a main effect training day ($F_{(3.06, 52.03)}=4.23$, $p=0.0091$), and a treatment group x training day interaction ($F_{(18, 153)}=2.09$, $p=0.0087$) on active lever presses (Figure 24E). Finally, there were no main effects of treatment group ($F_{(2,17)}=0.45$, $p=0.64$) or training day ($F_{(1.60, 27.20)}=3.08$, $p=0.072$) on inactive lever presses made during self-administration, though inactive pressing behavior trended to decrease across training (Figure 24F). Despite having noradrenergic input silenced, these animals performed similarly to the TH-Cre vehicle-treated control group across self-

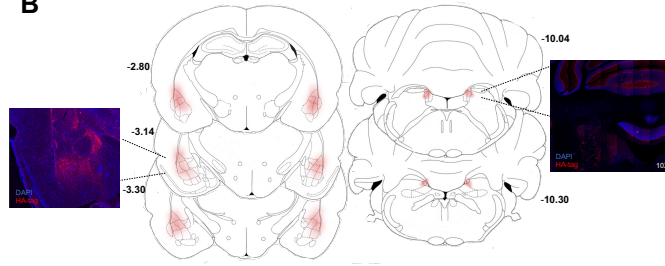
administration training and earned more infusions ($p=0.008$) and made more active presses ($p=0.10$) than the TH-Cre inhibition animals.

The PRSx8 promoter Gi-DREADD rats also underwent 2 counterbalanced cue-induced reinstatement sessions with vehicle and 1 mg/kg CNO (Figure 24C). There was no effect of in-session treatment ($F_{(1,17)}=8.43e-33$, $p>0.99$) for any group, but there was a main effect of treatment group during training ($F_{(2,17)}=8.07$, $p=0.0034$) on the number of active presses made during reinstatement (Figure 24G). Following both vehicle and CNO treatment, PRSx8 DREADD animals made significant more presses than the TH-Cre animals that had received CNO during training ($p=0.031$, $p=0.002$, respectively). There was no main effect of treatment group during training ($F_{(2,17)}=0.88$, $p=0.43$) or in-session treatment ($F_{(1,17)}=0.13$, $p=0.72$) on inactive presses. Again, the behavior of the PRSx8 group was similar to that of vehicle-treated TH-Cre control animals even though their norepinephrine input to the LA was altered during training and during the reinstatement session. (Figure 24G).

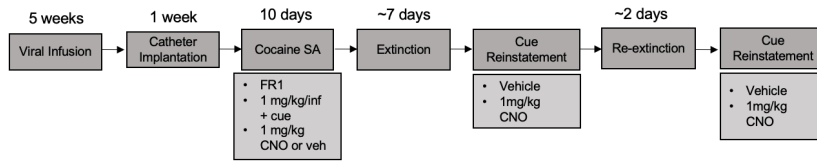
A AAV2retro.PRSx8.HA-hM4D.SV40



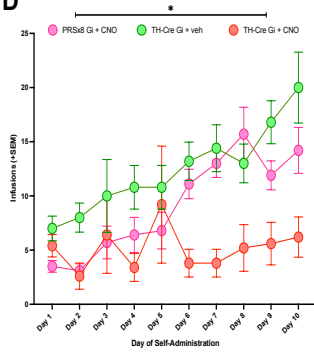
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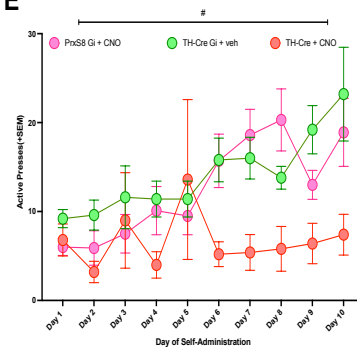
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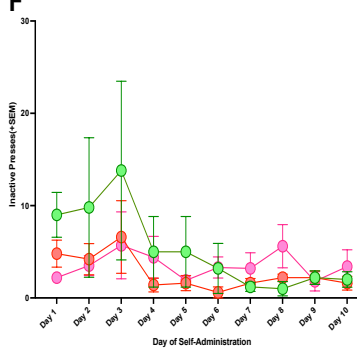
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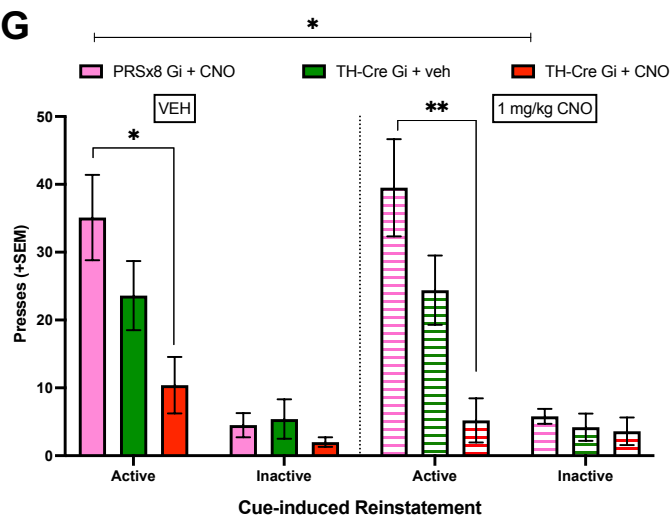


Figure 24. Inhibition of norepinephrine input to the LA does not affect cocaine self-administration or cue-induced reinstatement. Rats (n=10) were bilaterally infused in the LA with a retrograde AAV expressing the h4MDi inhibitory DREADD receptor under the PRSx8 promoter to infect noradrenergic cells projecting to the region. Schematic of virus surgery (A). Schematic showing injection of virus in the LA and expression in both the LA and the LC. Representative images of LA and LC expression (B). Experimental timeline (C). PRSx8 promoter Gi-DREADD animals were treated with daily i.p. injections of 1 mg/kg CNO to inhibit norepinephrine input during cocaine self-administration. Their behavior was compared to TH-Cre rats expressing the Gi-DREADD treated with either vehicle (n=5) or CNO (n=5) from the previous experiment. PRSx8 DREADD rats that were administered CNO earned significantly more cocaine infusions than TH-Cre DREADD animals that were administered CNO (D). PRSx8 DREADD + CNO rats tended to make more active lever presses than the TH-Cre DREADD + CNO rats (E). There were no group differences in the number of inactive lever presses made during cocaine self-administration (F). Following self-administration, rats underwent two counterbalanced cue-induced reinstatement sessions where they received vehicle or 1 mg/kg CNO prior to the session. Following both vehicle and CNO in-session treatment, PRSx8 DREADD + CNO animals made more active presses than the TH-Cre rats that also received CNO treatment during self-administration (G) Graphs show group means \pm SEM. #p<0.1, *p<0.05, ***p<0.0001

3.3.7 Chemogenetic inhibition of pathway from VTA to LA slows acquisition of cocaine self-administration

Our delivery of the Cre-dependent retrograde AAV virus containing hM4Di receptors results in expression in TH+ VTA cells that project to the LA, but this chemogenetic protein may also be taken up by other TH+ populations innervating the amygdala located in the substantia nigra, periaqueductal gray, or dorsal raphe (Poulin et al., 2018). Therefore, to probe further the VTA to LA input, we employed a DREADD strategy in which the virus was infused into the VTA and CNO was delivered to terminals in the LA by local microinfusion through cannula prior to the start of the cocaine self-administration session (Figure 25A-B). In this experiment, viruses expressing hM4Di (inhibitory) receptors, hM3Dq (excitatory) receptors, or an EGFP control were infused bilaterally into the VTA and infusion cannula were implanted over the LA (Figure 25A-B). CNO or ACSF vehicle was infused via the cannula to examine the effects of inhibition and excitation of the VTA to LA projection on acquisition of cocaine self-administration (Figure 25C). 48 hours before cocaine self-administration, animals began with an initial 6-hour food training session to learn the appropriate instrumental response to earn reinforcers before continuing into the next training phase. There were no group differences in the number of sucrose pellet reinforcers earned ($F_{(3,77)}=0.29$, $p=0.83$, Figure 25D), active presses ($F_{(3,77)}=0.32$, $p=0.81$, Figure 25E), or inactive presses ($F_{(3,77)}=1.13$, $p=0.34$, Figure 25E).

To avoid any excessive tissue damage caused by repeated microinfusions, the self-administration training was limited to 5 days. Male and female rats were tested, but there was no effect on sex on number of infusions earned ($F_{(1,60)}=0.70$, $p=0.41$), active lever presses made ($F_{(1,60)}=0.54$, $p=0.47$), or inactive lever presses made ($F_{(1,60)}=0.006$, $p=0.94$) so both sexes were combined for subsequent analysis. We also determined if there were group differences within our

controls. Post hoc analysis showed that animals expressing the control virus that received vehicle microinfusions did not differ from control-expressing animals that received CNO microinfusions in terms of infusions, active presses, or inactive presses so those animals were combined into one larger control group. Rats expressing the Gi-DREADD virus that received vehicle microinfusions had similar numbers of infusions, active press, and inactive presses as the rats expressing the Gq-DREADD virus that were given vehicle and those animals were combined into one larger DREADD control group to simplify data presentation. During the 5 days, there was a main effect of treatment ($F_{(3,68)}=4.79$, $p=0.004$) and a main effect of training day ($F_{(4,272)}=20.99$, $p<0.001$) on the number of cocaine infusions earned. Gi-DREADD rats that received CNO microinfusions earned significantly fewer infusions than the Gq-DREADD rats that received CNO ($p=0.003$). Gi-DREADD rats given CNO also showed a trend to earn fewer infusions than the control group ($p=0.063$) and the DREADD group ($p=0.10$, Figure 25F). Analysis of active presses revealed main effects of treatment ($F_{(3,68)}=4.31$, $p=0.008$) and training day ($F_{(4,272)}=9.09$, $p<0.001$). Post hoc tests of treatment found that the Gi-DREADD rats that received CNO made fewer presses than the Gq-DREADD rats that received CNO ($p=0.008$) and the controls ($p=0.046$). Gi-DREADD rats that received CNO were also trending to make fewer active presses than rats in the DREADD group ($p=0.08$, Figure 25G). There was no effect of treatment ($F_{(3,68)}=1.33$, $p=0.27$) or training day ($F_{(4,272)}=0.56$, $p=0.639$) on inactive lever pressing behavior (Figure 25H).

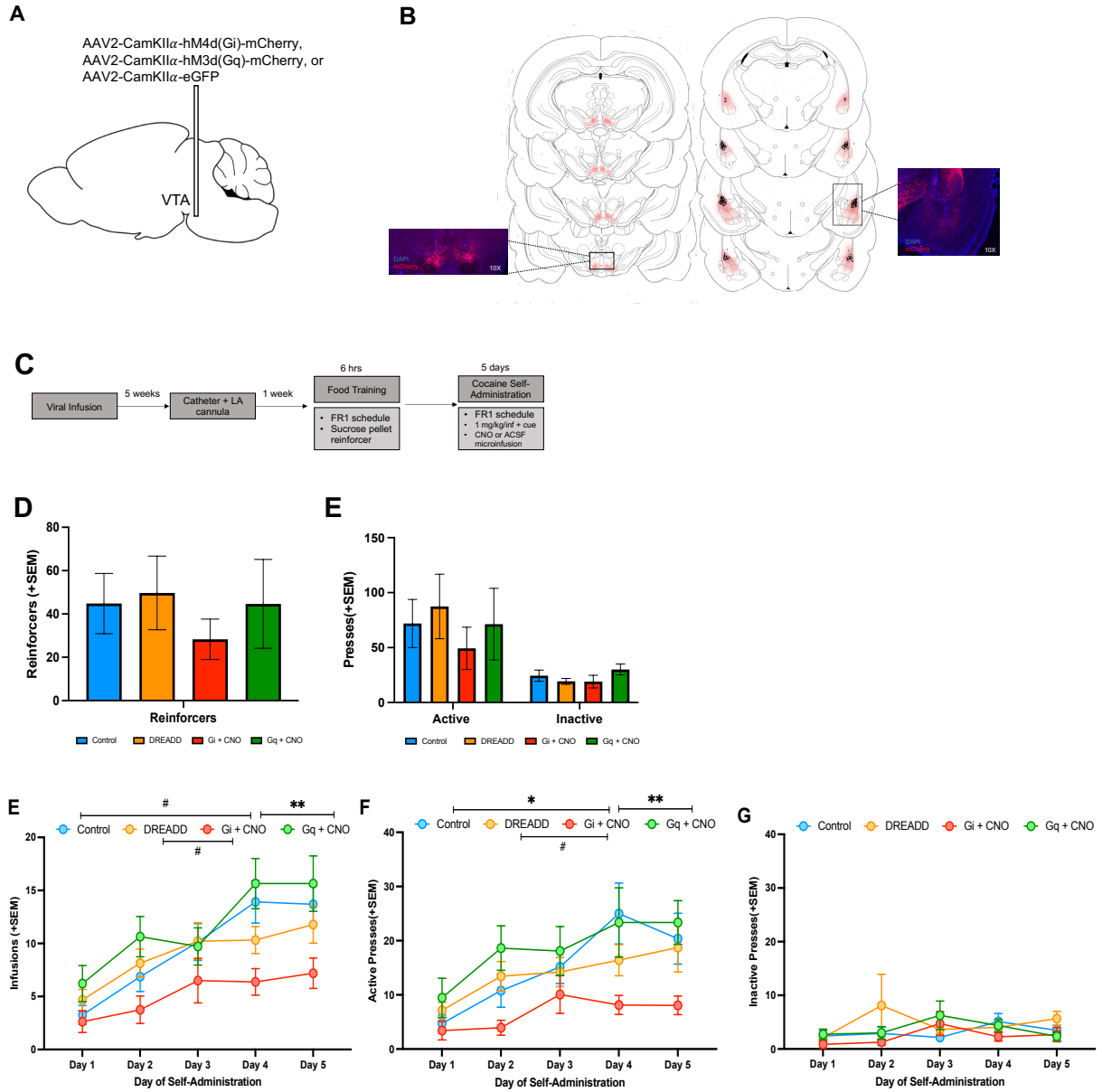


Figure 25. Inhibition of VTA to LA projection slows acquisition of cocaine self-administration.

Rats (n=72, Control=19, DREADD=27, Gi + CNO = 15, Gq + CNO = 11) were trained to self-administer cocaine on FR1 schedule of reinforcement and received LA microinfusions of either CNO or ACSF vehicle 5 min prior to each session (E-G). Schematic of viral surgery (A). Schematic showing injection of virus in VTA and representative image of VTA expression. Schematic showing expression throughout LA and cannula placements. Black circles show acceptable placement and black “X” indicate unsuccessful fiber placement. (B). Experimental timeline (C). Rats were trained to self-administer sucrose pellets during a 6-hour food training session before any microinfusions occurred. There were no significant group differences in reinforcers (D), active presses (E), or inactive presses (E). Gi-DREADD + CNO rats earned fewer cocaine infusions than Gq-DREADD + CNO rats and trended to earn fewer infusion than DREADD and control rats (E). Gi-DREADD + CNO rats made fewer active presses relative to Gq + CNO and control rats. There was also a trend for rats in the Gi + CNO inhibition group to make fewer active presses than those in DREADD group (F). Experimental groups did not differ in the number of inactive presses made across training (G). Graphs shows group means \pm SEM. *p<0.05, **p<0.01, #p<0.1

3.3.8 *Prior chemogenetic inhibition does not affect instrumental extinction.*

After cocaine self-administration, animals underwent instrumental extinction to extinguish their active lever pressing behavior before continuing into reinstatement testing. There was no effect of treatment ($F_{(3,68)}=1.13$, $p=0.34$) on the number of active presses made. There was a main effect of extinction day ($F_{(6,408)}=27.75$, $p<0.0001$) as lever pressing decreased across days of extinction (Figure 26A).

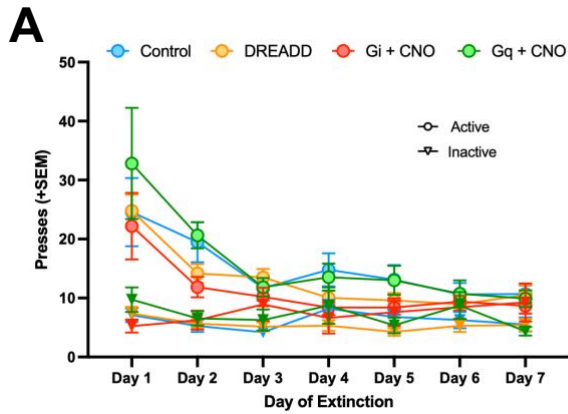


Figure 26. Prior inhibition of VTA to LA projection does not affect instrumental extinction.

Rats (n=72, Control=19, DREADD=27, Gi + CNO = 15, Gq + CNO = 11) underwent instrumental extinction following cocaine self-administration. Treatment during cocaine self-administration did not affect pressing behavior during extinction. There was a main effect of extinction day as all rats decreased their lever pressing across extinction days. Graphs shows group means \pm SEM.

3.3.9 Prior chemogenetic excitation of the VTA to LA pathway strengthens cue-induced reinstatement, but chemogenetic manipulation does not affect cocaine primed reinstatement.

To determine if manipulation of the VTA to LA pathway during self-administration affected relapse-like behavior, animals were tested on both cue-induced and cocaine-primed reinstatement (Figure 27A). Males and females were tested, but there was no effect of sex on active presses ($F_{(1,71)}=0.018$, $p=0.89$) or inactive presses ($F_{(1,71)}=3.14$, $p=0.08$) made during cue-induced reinstatement. The control animals that received vehicle and the control animals that received CNO during training were combined into one group as post hoc analysis revealed no significant difference between the groups. Both Gi-DREADD and Gq-DREADD groups that received vehicle during training were collapsed into a one group as post hoc analysis showed no significant differences in their behavior. During cue-induced reinstatement, analysis of active lever presses yielded a main effect of treatment during training ($F_{(3,68)}=1.46$, $p=0.016$, Figure 27B). Gq-DREADD animals that received CNO during training made significantly more active lever presses than animals in the DREADD group ($p=0.017$), and Gi-DREADD animals that received CNO during training ($p=0.042$, Figure 27B). A two-way ANOVA with the between subject factor of treatment during training and within subject factor of lever revealed a main effect of lever ($F_{(3,204)}=69.85$, $p<0.0001$) where the active lever presses made during reinstatement were greater than the number of inactive lever presses and the number of active presses made on the final day of instrumental extinction (Figure 27B).

Next, we wanted to examine if chemogenetic manipulation during training impacted cocaine-primed reinstatement. Again, males and females were used, but there was no effect of sex on the number of active presses ($F_{(1,71)}=2.52$, $p=0.12$). Post hoc analysis showed no group

difference between control animals that received vehicle or those that received CNO so they were pooled into one control group. Post hoc comparisons also revealed no group difference between Gi-DREADD animals that received vehicle and Gq-DREADD animals that received vehicle so the animals were combined into one DREADD-virus control group. There was no main effect of treatment during training on active presses during cocaine-primed reinstatement ($F_{(3,68)}=0.87$, $p=0.46$) (Figure 27C). There was a main effect of lever ($F_{(2,204)}=14.12$, $p<0.0001$) such that in general, during reinstatement, animals made more active presses than inactive presses and made more active presses relative to the final day of re-extinction (Figure 27C). However, planned comparisons showed that Gi-DREADD and Gq-DREADD animals that received CNO during training did not make significantly more active presses during reinstatement than they did on the final re-extinction day ($p>0.99$, $p=0.69$, respectively), indicating that responding to the cocaine prime was low (Figure 27C).

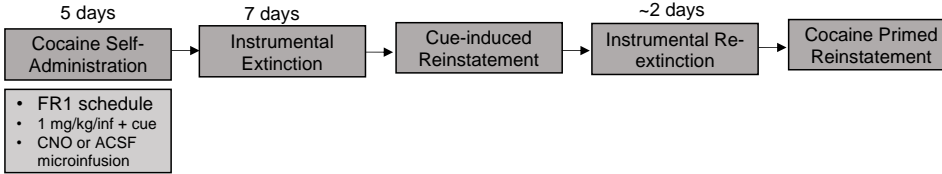
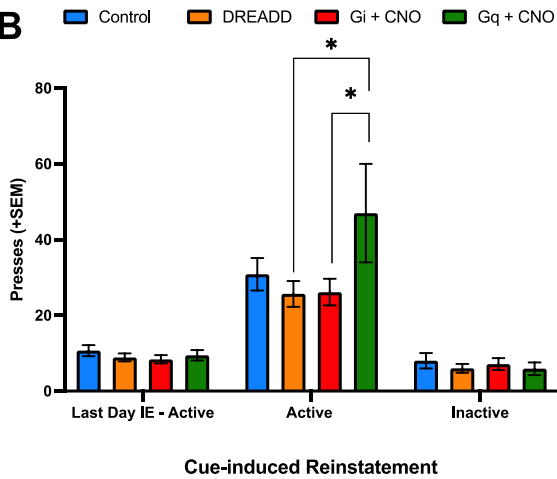
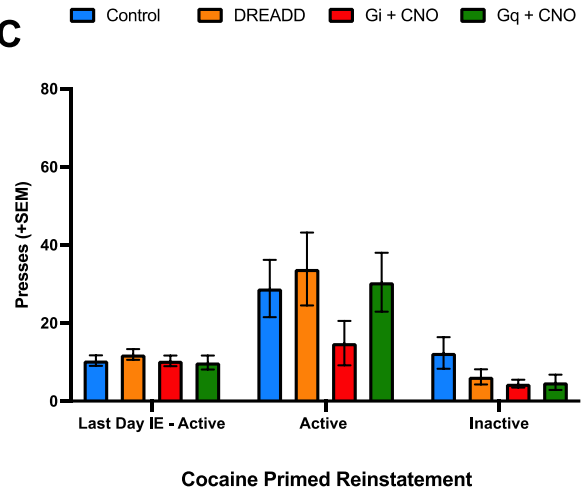
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Figure 27. Prior excitation of VTA to LA pathway strengthens cue-induced reinstatement, but manipulation does not affect cocaine-primed reinstatement.

Rats ($n=72$, Control=19, DREADD=27, Gi + CNO = 15, Gq + CNO = 11) underwent cue-induced and cocaine primed reinstatement (B-C). Experimental timeline (A). Gq-DREADD rats that received CNO during self-administration made more active presses than DREADD group rats and Gi-DREADD + CNO rats. During cue-induced reinstatement, rats made more active presses than inactive presses and pressed more in response to the formerly drug-paired cue than they did on the final day of extinction (B). There was no effect of treatment during self-administration on reinstatement to a cocaine prime. Rats made active presses than inactive presses during cocaine primed reinstatement and the degree of active lever pressing was greater in response to the drug prime than on the last day of re-extinction (C). Graphs shows group means \pm SEM. * $p<0.05$

3.3.10 Chemogenetic manipulation of the VTA to LA projection does not impact the rewarding effects of cocaine.

The results from self-administration and reinstatement tests suggest chemogenetic manipulation of the VTA to LA projection affects cue learning, but as we did not find strong reinstatement to a cocaine prime, we wanted to ensure that the chemogenetic manipulation was not altering the rewarding effects of cocaine. To determine the effects of the projection manipulation on the rewarding properties of cocaine, we assayed rats using conditioned place preference (CPP, Figure 28A). There was no effect of treatment during conditioning on time spent in each chamber ($F_{(2,38)}=0.38$, $p=0.69$, Figure 28B). Following conditioning, DREADD controls, Gi-DREADD rats that received CNO, and Gq-DREADD rats that received CNO spent more time in the cocaine paired chamber than the saline paired chamber ($F_{(1,38)}=20.88$, $p<0.001$, Figure 28B). Preference score was calculated as the time spent in the cocaine paired chamber divided by the time spent in both chambers. Again, there was no main effect of treatment during conditioning on preference score ($F_{(2,38)}=0.75$, $p=0.48$), but there was a main effect of conditioning such that preference score was greater following conditioning for all animals tested ($F_{(1,40)}=11.16$, $p=0.0002$, Figure 28C).

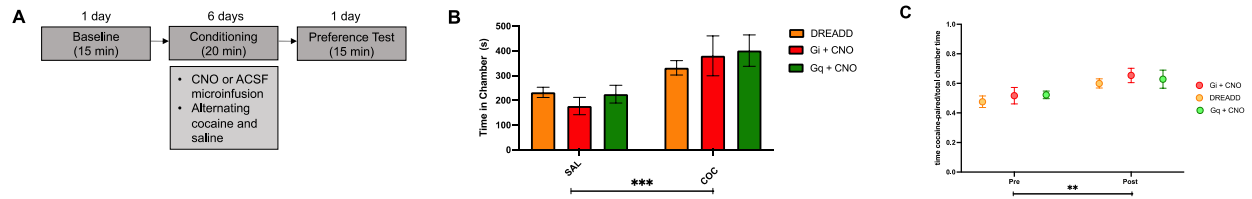


Figure 28. Manipulation of VTA to LA projection does not affect the rewarding effects of cocaine.

Rats (n=24, DREADD=11, Gi + CNO= 4, Gq + CNO=6) underwent Conditioned Place Preference (CPP) during which they received LA microinfusions of CNO or ACSF vehicle (A-C). Experimental timeline (A). After conditioning, DREADD rats, Gi + CNO rats, and Gq + CNO rats spent more time in the cocaine paired chamber than the saline paired chamber. Inhibition or excitation of the VTA to LA projection during conditioning did not affect time spent in either chamber (B). Preference score for the cocaine paired chamber were higher following conditioning for all experimental groups (C). Graphs show group means \pm SEM. **p<0.01, ***p<0.001

3.3.11 Administration of CNO in LA of Gi-DREADD rats decreases c-Fos immunoreactivity after cue-induced reinstatement.

To confirm that CNO affected neuronal activity in the LA of DREADD expressing rats, we examined c-Fos immunoreactivity following a cue-induced reinstatement session since c-Fos expression serves as an indicator of activity. CNO or ACSF microinfusions were administered into the LA of Gi-DREADD expressing, Gq-DREADD expressing and control rats prior to the session. Analysis of c-Fos+ cells yielded a main effect of treatment ($F_{(2,13)}=5.78$, $p=0.016$). Gi-DREADD rats that received a CNO microinfusion had fewer c-Fos+ cells than DREADD controls ($p=0.039$) and Gq-DREADD animals that received CNO ($p=0.022$), indicating that CNO administration reduced the number of active LA cells following cue-induced reinstatement (Figure 29A). Behaviorally, there was no significant effect of treatment ($F_{(2,20)}=0.77$, $p=0.48$), but a trend for Gi-DREADD rats given CNO to make fewer active lever presses than Gq-DREADD rats given CNO ($p=0.09$, Figure 29B).

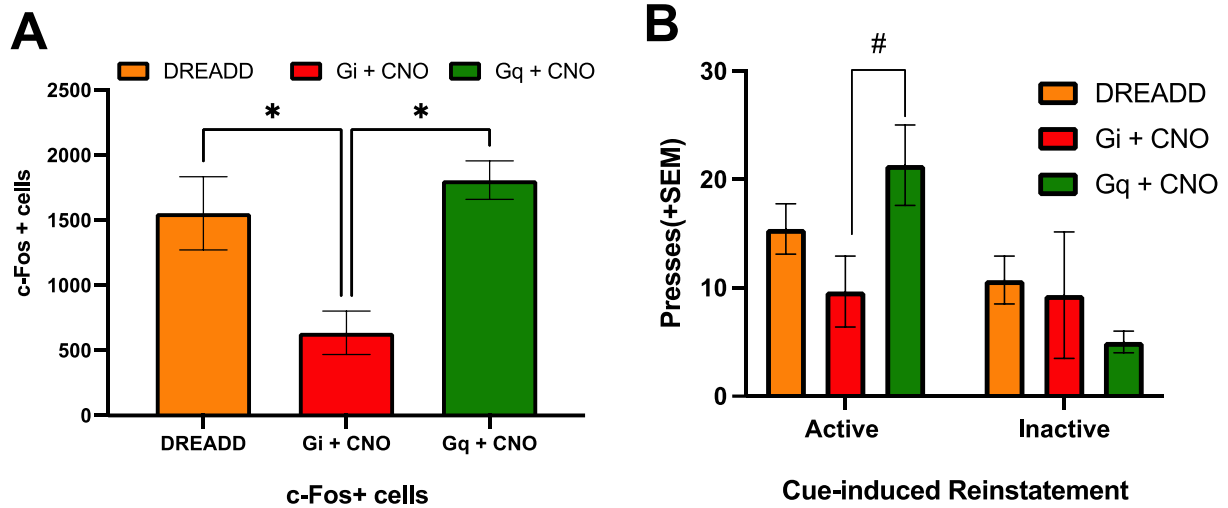


Figure 29. Administration of CNO in LA of Gi-DREADD rats diminishes c-Fos immunoreactivity after cue-induced reinstatement.

Rats (n=16, Control=7, Gi + CNO=5, Gq + CNO=4) underwent a cue-induced reinstatement session after LA microinfusion of CNO or ACSF. c-Fos immunoreactivity was then examined as an indicator of cell activity during the session (A-B). Gi-DREADD rats that received CNO microinfusions had fewer c-Fos+ cells compared to DREADD controls and Gq-DREADD rats that received CNO (A). Gq-DREADD expressing rats that received CNO were trending to make more active presses during cue-induced reinstatement than Gi-DREADD expressing rats that were treated with CNO (B). Graphs show group means \pm SEM. #p<0.1, *p<0.05

3.3.12 RNA expression patterns in the VTA differ following cocaine versus saline self-administration.

As we identified a role for the VTA to LA pathway in cocaine cue associative learning, we wanted to probe which cell types were projecting the LA and which were active following self-administration of cocaine or saline. Prior to behavior training, rats were infused with the retrograde tracer Ctb in the LA in order to label cells projecting to the region (Figure 30A). After self-administration, using RNAscope, we probed for Fos, TH, and VGlut2 RNA in the VTA (Figure 30A-B). Within the Ctb-expressing Fos+ population, we examined the proportion of cells that also expressed TH, VGlut2, or both. Although cocaine-trained rats had increased acquisition relative to saline-trained rats (Figure 30C-D), there was not a main effect of drug on RNA expression ($F_{(1,15)}=0.03$, $p=0.87$, Figure 30E), but the pattern of expression differed between saline-trained and cocaine-trained rats. Following saline self-administration, the proportion of Ctb-expressing Fos+ cells that were VGlut2+ was significantly higher than the proportion of cells that were both VGlut2+ and TH+ ($p=0.03$). Conversely, after cocaine self-administration, the TH+, VGlut2+, and double-labelled proportions were the same (Figure 30E). This expression pattern may highlight a function of VGlut2+ dopaminergic cells in the VTA for cocaine reward-responsiveness.

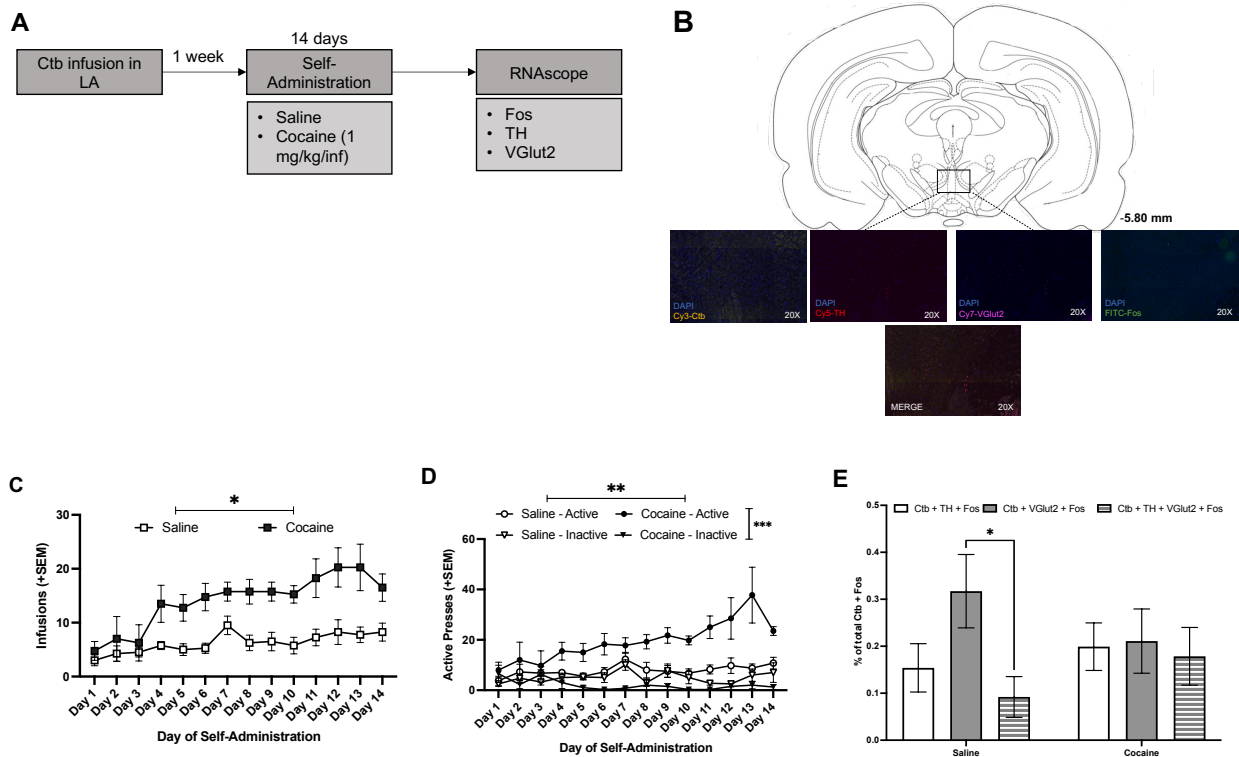


Figure 30. RNA expression pattern in the VTA following saline and cocaine self-administration.

Rats (n=8) were trained to self-administer saline (n=4) or cocaine (n=4) for 14 days following Ctb retrograde tracer infusion into the LA. Experimental timeline (A). Representative images of labeled probes in the VTA (B). Cocaine-trained rats had increased acquisition of self-administration compared to saline-trained rats (C-D). Within the ctb-expressing Fos+ population, saline-trained and cocaine-trained rats did not differ in RNA expression. The pattern of expression was different between saline-trained and cocaine-trained rats. After saline self-administration, the proportion of Ctb-expressing Fos+ cells that were VGlut2+ was significantly higher than the proportion of cells that were both VGlut2+ and TH+ (E). Graphs show group means \pm SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

3.4 DISCUSSION

Here, we thoroughly examined the effects of chemogenetic manipulation of the VTA dopamine projection to the LA during cocaine self-administration on acquisition and reinstatement. We showed that acquisition of cocaine self-administration is disrupted when animals have dopaminergic input from the VTA to LA inhibited. Our results indicated that when this projection is silenced during self-administration, animals later show a reduction in their reinstatement of cocaine-seeking elicited by the previously cocaine-paired cue. Conversely, excitation of the projection boosts subsequent cue-induced reinstatement. On the other hand, inhibiting this projection had no effect on the ability of cocaine to elicit a cocaine place preference or to induce reinstatement in response to a cocaine priming injection. Thus, these results suggest that the VTA dopamine projection to the LA plays a crucial role in cocaine cue associative learning/reinforcement of instrumental responding that is critical for acquisition of cocaine self-administration, but does not alter the acute rewarding effects of cocaine or spatial reward learning.

Previous research has demonstrated that cues facilitate acquisition of self-administration and that these cues do not change drug-reinforcing effects, but rather modulate drug-seeking behavior (Caggiula et al., 2002; Deroche-Gamonet, Piat, Le Moal, & Piazza, 2002; Schenk & Partridge, 2001). Our findings are consistent with these prior studies as our experiments showed that rats undergoing inhibition of the VTA to LA pathway still self-administered cocaine, but at much lower levels than controls. Given that the LA is critical for cocaine-cue associative learning, it appears that interfering with dopaminergic input to this region interfered with the ability of animals to associate the lever press response with the discrete cue and effects of cocaine, which weakened the ability to acquire cocaine self-administration.

Assessing cue-induced reinstatement helped determine how inhibition during self-administration affects the expression of cue learning. Cue-induced reinstatement is a commonly used model of relapse-like behavior that probes the strength of cue associations formed during self-administration (See, 2002; See, 2005; Shaham, Shalev, Lu, De Wit, & Stewart, 2003). Lesioning the basolateral amygdala, which contains the LA, has no effect on responding during cocaine self-administration, but does abolish cue-induced reinstatement (Meil & See, 1997; See, 2002), indicating that the region has a specific role in mediating cue-dependent behaviors. There was a similar specificity for rats that experienced excitation of the VTA to LA projection during the 5 days of training. These animals did not have increased responding during self-administration, but had the highest levels of cue-induced reinstatement, implying that excitation strengthened drug-cue associative learning.

In the first experiment, where rats received inhibition of dopamine projections to the LA for 14 days of self-administration training, we also found a reduction in cue-induced reinstatement, further highlighting the role of the projection in cue-dependent behavior. However, the low levels of self-administration in this group may have limited the amount of cue-induced reinstatement that could subsequently be observed. Indeed, when these same animals had additional cue-induced reinstatement sessions during which they received 1 or 3 mg/kg CNO, there was not a further reduction in reinstatement. Interestingly, in the DREADD expressing control group that acquired cocaine self-administration normally, acute inhibition of the dopamine projections to the LA did not disrupt cue-induced reinstatement. This was surprising given that others have found that the ability of cocaine-associated stimuli to elicit seeking behavior involves activation of dopamine transmission in the nucleus accumbens and amygdala (Weiss et al., 2000), and that dopamine receptor antagonism in the BLA can inhibit cued reinstatement (See, Kruzich, & Grimm, 2001).

Nevertheless, our results suggest that the dopamine projection to the LA is not needed for cue-motivated behavior once the behavior is already learned.

Comparing cocaine primed reinstatement with cue-induced reinstatement can disentangle the effects of cocaine itself from the cue's ability to trigger drug-seeking. In general, manipulation of the VTA dopamine projection to the LA during self-administration did not affect later cocaine primed reinstatement. One potential caveat was that there was a low level of responding during the reinstatement session. The amount of cocaine intake during self-administration and the magnitude of subsequent cocaine-induced reinstatement are directly related (Baker, Tran-Nguyen, Fuchs, & Neisewander, 2001; Deroche-Gamonet et al., 2002). Rats that have more access to cocaine whether it be via longer self-administration sessions or higher doses of cocaine are more susceptible to cocaine primed reinstatement (Knackstedt & Kalivas, 2007; Mantsch, Yuferov, Mathieu-Kia, Ho, & Kreek, 2004). This pattern may explain why in both our experiments reinstatement to the cocaine prime was not robust.

Despite low responding, we did observe a significant difference between TH-Cre animals expressing the Gi-DREADD that received vehicle during training and those that received CNO for the reinstatement session following vehicle treatment. This reflected the difference seen during self-administration where the Gi-DREADD animals treated with vehicle earned more infusions than the Gi-DREADD animals treated with CNO. Moreover, within the group of TH-Cre animals expressing the Gi-DREADD that received vehicle during training, 1 mg/kg CNO in-session treatment tended to blunt responding to a cocaine prime. Others have shown that chemogenetic inhibition of all VTA dopamine neurons attenuates cocaine primed reinstatement (Mahler et al., 2019). Thus, in the absence of inhibition-related plasticity changes during training, acutely altering the VTA to LA projection may contribute to weakening cocaine primed reinstatement.

As manipulating the VTA dopamine to LA projection during self-administration impacted acquisition and cue-induced reinstatement, but not cocaine primed reinstatement, it seems to be a pathway heavily involved in cocaine cue associative learning. Additionally, neither inhibition nor excitation of the VTA to LA projection during conditioned place preference (CPP) affected the animals' contextual learning or ability to develop a preference for the cocaine paired chamber. Amygdala lesions block cocaine-induced CPP (Brown & Fibiger, 1993), but silencing just the VTA dopamine projection to the LA left CPP intact, further highlighting its role in associative learning rather than contextual learning or reinforcement.

To examine the neuronal populations that underlie our behavioral results, we combined retrograde tracing with RNAscope labeling after rats had self-administered saline or cocaine. Our chemogenetic manipulations mainly targeted dopaminergic VTA neurons projecting to the LA, but did not exclude glutamatergic neurons and neurons that co-express dopamine and glutamate. Although cocaine-trained rats had increased acquisition relative to saline-trained rats, they did not differ in RNA expression within LA-projecting VTA cells that were active following self-administration. However, the pattern of expression differed between saline-trained and cocaine-trained rats. After saline self-administration, the proportion of cells that were VGlut2+ was higher than the proportion of cells that were both VGlut2+ and TH+, whereas TH+, VGlut2+, and co-expressing proportions were similar after cocaine self-administration. This finding suggests that dopamine and glutamate co-transmission may differ between the self-administration groups. Most of the published studies concerning dopamine and glutamate co-transmission have focused on projections from the VTA to nucleus accumbens (NAc) (Buck, Torregrossa, Logan, & Freyberg, 2021; Eskenazi et al., 2021). Disruption of glutamate release from these VTA dopamine neurons projecting to the NAc increases reward seeking and responsiveness (Alsiö et al., 2011; Wang et

al., 2017), but the contribution of dopamine-glutamate co-releasing fibers from the VTA to the LA remains unclear. Our expression pattern may hint at a role in reward processing that enhanced self-administration for cocaine compared to saline.

A potential limitation of this work is that we do not have a chemogenetic manipulation group that self-administers saline or undergoes cue-free self-administration. A saline self-administration group would help determine if the chemogenetic manipulation effects are drug-specific. Groups trained to self-administer cocaine without cues would provide insight into whether the deficit in acquisition was specific to associative learning related to the cue versus the lever pressing action. Although these additional experimental conditions could strengthen our conclusions, replicating our results using two different projection targeting strategies lends support to our findings. Additionally, the dissociable effects of chemogenetic manipulation on cue-dependent behaviors versus spatial reward-related learning provides strong evidence that the VTA dopamine to LA projection is not contributing to the rewarding effects of cocaine.

Examining the dopaminergic VTA to LA projection has implications for the ways in which therapeutic interventions may be targeted in the future. For one, cues paired with repeated drug use drive craving and relapse and here we demonstrated that weakening this input during acquisition of self-administration attenuated relapse-like behavior elicited by cues. Additionally, these experiments provide support for VTA dopamine as an input to the LA that encodes the reinforcing effects of cocaine that serve as a US in conditioned cue drug-seeking. Dealing with multiple drug-paired cues and contexts is a frequent obstacle for substance use disorder treatments such as cue exposure therapy, but preclinical work targeting reactivation of US memory to extinguish more than one CS highlights the promise of disrupting US encoding (Dunbar & Taylor,

2017). Our findings present a potential US-relevant pathway in the development of substance use disorder that warrants further investigation.

4.0 GENERAL DISCUSSION

The studies presented in this dissertation focus on pathways and mechanisms involved in associative learning. We investigated how activity in the lateral amygdala (LA), particularly related to calcium and dopamine release, was involved in the formation of an appetitive stimulus-outcome associative memory during Pavlovian conditioning. We also examined how conditioned stimuli could influence instrumental responses and whether this effect would be reflected by underlying calcium and/or dopamine activity patterns in the LA. Finally, with a focus on dopaminergic projections to the LA, we investigated whether the VTA projection to the LA supports the cocaine-cue associative learning required for acquisition of cocaine self-administration and subsequent reinstatement. Our main conclusion is that dopamine signaling in the LA encodes stimuli paired with both natural and drug reward. In this discussion, we will describe how our findings fit with current theories about associative learning in the amygdala. We will also detail how our results provide insight into learning and memory pathways involved in the development of substance use disorders (SUD) to inform potential therapeutic interventions.

4.1 UNDERSTANDING LA ACTIVITY IN RESPONSE TO REWARD-PREDICTIVE CUES

The results of this dissertation suggest that activity in the LA reflects the cue-reward associative learning process. Prior electrophysiological studies where recordings in the amygdala were conducted during cocaine self-administration revealed distinct neuron populations with firing

rates that increased before a response, increased during reinforcement, or decreased during reinforcement (Carelli, Williams, & Hollander, 2003). Only neurons that increased their firing rates during reinforcement showed activation to a cocaine-paired cue (Carelli et al., 2003), suggesting that changes to activity in the LA rely on the learned association between cue and drug reward. Moreover, rats trained to respond to sucrose paired with a reward-predictive cue had a greater proportion of phasically responsive BLA neurons during reinstatement than rats that were trained with a randomly presented cue (Tye & Janak, 2007). This result again indicates that amygdala neural activity reflects the acquisition of cue-reward associations. However, the timing and neurotransmitter dynamics of this activity have not been extensively studied.

In **Chapter 2**, using fiber photometry, we demonstrated that dopamine and calcium activity respond to reward-related cues during Pavlovian conditioning. As conditioning progressed, rats spent more time near the sucrose sipper during auditory CS+ presentations, which suggested that they acquired the cue-reward association. Over the course of training, dopamine activity changes developed in conjunction with cue-reward associative learning. Determining the degree to which animals successfully acquired the Pavlovian task further highlighted the link between dopamine activity and cue learning. Only in the group of animals whose behavior was indicative of learning the cue-reward association did we find activity changes in response to CS+ presentation. Focusing on the day that animals learned the value of the reward-predictive cue revealed that the dopamine response to the CS+ was not present on the first day of conditioning or for animals that never learned the Pavlovian task, but instead arose on the day animals learned the cue-reward association.

Calcium activity did not reflect learning in the same manner as dopamine activity. Calcium activity in response to the cue appeared present, though not significantly, on the first day of conditioning before animals would have learned the stimulus-outcome association. The calcium

response to the CS+ diminished by the end of training and was not evident on the day learned. Taken together, results from Pavlovian conditioning in **Chapter 2** suggest that calcium activity in the LA has a general response to the novelty of the onset of CS+ presentation, whereas dopamine activity serves as a specific learning signal.

It is not surprising that dopamine activity in the amygdala develops responses to reward-predictive cues as dopamine transmission in the basolateral portion of the amygdala is thought to be a crucial signal for initiating behavioral responses for reward. Rats trained to associate stimuli with availability of cocaine show greater dopamine efflux in the amygdala in response to the drug-paired cue than to a non-reward cue (Katner, Magalong, & Weiss, 1999; Weiss et al., 2000). Inhibition of dopamine signaling decreases discrete cue-primed reinstatement, while inhibition of glutamate signaling has no effect (See, Kruzich, and Grimm 2001), further indicating that dopamine activity in the amygdala contributes to encoding of reward-predictive cues.

After Pavlovian conditioning, rats underwent instrumental training followed by Pavlovian to Instrumental transfer (PIT), which assays the effects of cues on behavior. Generally during PIT, a Pavlovian CS+ paired with an appetitive outcome enhances instrumental responding (Cartoni, Balleine, & Baldassarre, 2016). In our case, the CS+ appeared to invigorate instrumental responding, though this was not statistically significant given the low number of animals. We also did not observe dopamine responses to the CS+ onset. On the other hand, calcium signaling during the PIT test acted as a change detector as the calcium response appeared to increase to the CS+ and CS- onset as well as to the start of the ITI when the preceding auditory cue ended. There was an overall suppression of dopamine activity during the CS- relative to the CS+, especially during the initial second of presentation. This decrease may reflect differences in salience between a previously sucrose-paired cue and a novel cue. This interpretation fits with a large body of work

showing that the amygdala encodes cue salience. Amygdala neurons are capable of encoding multiple task dimensions such as the sensory properties of conditioned cues, the behaviors they elicit, and their valence (Kyriazi, Headley, & Pare, 2018). By encoding the incentive value of cues, the amygdala supports the translation of conditioned associations to instrumental responses (Burns, Robbins, & Everitt, 1993; Cador, Robbins, & Everitt, 1989; Malvaez et al., 2015; Whitelaw, Markou, Robbins, & Everitt, 1996). Although we did not find robust transfer effects in behavior or activity, the dopamine and calcium activity patterns may hint at neural processes that contribute to translating conditioned associations into instrumental responses.

4.2 ROLE OF VTA DOPAMINE TO LA PATHWAY IN DRUG-CUE ASSOCIATIVE LEARNING

In **Chapter 3**, a major finding was that the VTA dopamine to LA projection is critical for assigning value to cocaine-paired cues during associative learning. Cues have been shown to modulate drug-seeking behavior and facilitate acquisition of self-administration (Caggiula et al., 2002; Deroche-Gamonet, Piat, Le Moal, & Piazza, 2002; Schenk & Partridge, 2001) as they acquire motivational value through association with the self-administered drug (Di Ciano & Everitt, 2003). In fact, if the drug-associated cue is not presented contingent on responding, drug-seeking is dramatically reduced (Arroyo, Markou, Robbins, & Everitt, 1998; de Wit & Stewart, 1981). Yet, projections involved in encoding the effects of drug that are paired with the cue have not been well characterized. The LA is critical for many cue-dependent processes including cocaine cue associative learning (Everitt, Morris, O'Brien, & Robbins, 1991; Everitt & Robbins, 2000; Kantak, Black, Valencia, Green-Jordan, & Eichenbaum, 2002; Whitelaw et al., 1996) and

dopamine signaling in the region contributes to this type of associative learning (Blundell, Hall, & Killcross, 2001, 2003; Hitchcott & Phillips, 1998). Moreover, evidence points to involvement of the VTA dopamine to LA projection in US encoding. Experiments conducted in **Chapter 3** found that when the VTA dopamine to LA projection was inhibited during cocaine self-administration, rats did not acquire self-administration as well as controls did. These findings suggest that inhibiting dopaminergic input to the LA interferes with the ability to associate the lever response with the cue and the effects of cocaine.

Cue-induced reinstatement is a commonly used model of relapse-like behavior that determines the strength of cue associations formed during self-administration (See 2002; See 2005; Shaham et al. 2003). As the basolateral amygdala (BLA), which contains the LA, plays an important role in mediating cue-induced reinstatement (Meil & See, 1997; See, 2002), we expected that altering the VTA dopamine projection to the LA during self-administration would impact the ability of the previously cocaine-paired cue to elicit cocaine-seeking behavior. Inhibition of the VTA dopamine to LA projection during cocaine self-administration led to a reduction in reinstatement of seeking elicited by the cue, while excitation of the projection during self-administration increased cue-induced reinstatement. Excitation during self-administration did not lead to elevated responding during self-administration, but the fact that these animals had the highest levels of cue-induced reinstatement suggests that excitation strengthens drug-cue associative learning.

When inhibitory DREADD expressing rats had multiple cue-induced reinstatement sessions during which they received 1 or 3 mg/kg CNO, there was not a further reduction in reinstatement with CNO administration. Interestingly, acute inhibition of the dopamine projections to the LA did not disrupt cue-induced reinstatement in DREADD expressing rats that acquired

cocaine self-administration normally. This was surprising given that others have found that the ability of cocaine-associated stimuli to elicit seeking behavior involves activation of dopamine transmission in the nucleus accumbens and amygdala (Weiss et al., 2000). Other evidence shows that dopamine in the BLA potentiates the effects of cocaine-paired cues during reinstatement since dopamine receptor antagonism in the BLA can inhibit cued reinstatement (See, Kruzich, & Grimm, 2001) and intra-BLA infusions of a nonspecific indirect dopamine agonist can increase responding during reinstatement (Ledford, Fuchs, & See, 2003). Nevertheless, our results encompass the effects of manipulations both during training and during reinstatement to indicate that the dopamine projection to the LA is not needed for cue-motivated behavior once the behavior is already learned, at least when using DREADD-based manipulations.

Broader circuits participate in cue-induced reinstatement so it is important to consider amygdala involvement as a node in these circuits. Both animal and human literature reveal a critical role of interactions between the amygdala and cortical regions in cue-primed reinstatement behavior (Kalivas & McFarland, 2003). The BLA provides input to the nucleus accumbens (NAc) to facilitate cue-induced cocaine seeking both directly and indirectly via prelimbic projections (Stefanik & Kalivas, 2013). Additionally, input from the lateral orbitofrontal cortex (IOFC) to the BLA promotes cue-induced cocaine seeking behavior (Arguello et al., 2017). Affecting the associative learning during acquisition by blocking VTA dopamine input to the LA may impact the subsequent interactions between the LA and other brain regions involved in cue-induced reinstatement.

With a focus on LA circuitry, in both **Chapters 2 and 3**, we wanted to make sure that the effects we found related to reward-predictive cue encoding were driven mainly by VTA dopamine input to the LA. In **Chapter 2**, we infused rats with a retrograde virus expressing the hM4di (Gi-

DREADD) receptor under the PRSx8 promoter to enable inhibition of noradrenergic input to LA via systemic treatment of CNO. This inhibition seemingly altered conditioned behavior as animals' approach performance and sipper use was indicative of having not learned the task. Furthermore, their lever pressing and sipper use during the instrumental phase was lower than animals that had never experienced inhibition of noradrenergic projections to the LA. There also seemed to be a general dampening of the dLight signal resulting from this inhibition, suggesting some of the observed signal was due to norepinephrine release, but the effect was not task specific or consistent across all measures. These experiments were underpowered and future experiments may better address how norepinephrine input to the LA influences learning and whether norepinephrine release is a meaningful learning signal captured by the dLight sensor. Interestingly, in **Chapter 3**, employing the same PRSx8 promoter Gi-DREADD strategy revealed that inhibiting noradrenergic input to the LA had no effect on animals' ability to acquire cocaine self-administration. Additionally, the previously cocaine paired cue was able to elicit cocaine-seeking normally even as these animals experienced inhibition during self-administration training. Compared to the training in the PIT paradigm, cocaine self-administration involves a compound stimulus (audiovisual cue) and more robust reinforcer (cocaine vs sucrose). While there may be a potential effect of inhibiting norepinephrine signaling in the LA on Pavlovian conditioning for sucrose reward, dopamine signaling on its own appears to drive cocaine cue associative learning.

A key implication of the studies in **Chapter 3** is that the VTA dopamine projections to the LA play a precise role in cue-dependent processes. Chemogenetic manipulation of the VTA dopamine to LA pathway had no effect on the ability of cocaine to induce reinstatement or elicit a cocaine place preference. Prior studies have found similar results showing that inactivation of the BLA does not inhibit cocaine-primed reinstatement (Grimm & See, 2000; McFarland & Kalivas,

2001). On the other hand, a prior study has found that amygdala lesions block cocaine-induced CPP (Brown & Fibiger, 1993), suggesting that silencing only the VTA dopamine projection to the LA is not sufficient to prevent CPP acquisition, and highlighted its role in cue-mediated associative learning rather than contextual learning or reinforcement.

4.3 IMPLICATIONS FOR SUBSTANCE USE DISORDER TREATMENT

Substance use disorder (SUD) affects nearly 20 million Americans and is a major societal and economic burden (National Survey of Drug Use and Health (NSDUH), 2019). SUDs are characterized by cycles of drug use, withdrawal, abstinence, and resumption of drug-taking. The focus of **Chapter 3** of this dissertation is on a model of cocaine use, which is related to a SUD category known as stimulant use disorder. In recent years, fatal overdoses when using stimulants have increased with more than half of these deaths related to illicit stimulant use involving fentanyl or other opioids (Hedegaard, Miniño, Spencer, & Warner, 2021; Hedegaard, Miniño, & Warner, 2021). To date, there are no medications specifically approved for stimulant use disorder and more generally, there are few pharmacological treatment options that have been successful at preventing relapse for extended periods of time (Bossert, Marchant, Calu, & Shaham, 2013; Conklin & Tiffany, 2002).

Drug-associated environmental stimuli are a major obstacle to the treatment of SUD as they elicit craving, support compulsive drug-seeking, and induce relapse (Childress et al., 1999; Robinson & Berridge, 1993; Stewart, de Wit, & Eikelboom, 1984). During abstinence, increases in craving driven by these stimuli can make individuals vulnerable to relapse (Gawin & Kleber, 1988; Kassani, Niazi, Hassanzadeh, & Menati, 2015). In the clinical context, drug-seeking

behavior is often renewed when the patient returns to the original drug-paired environment where they are re-exposed to cues and contexts associated with their drug use (Crombag, Bossert, Koya, & Shaham, 2008; Thewissen, Snijders, Havermans, van den Hout, & Jansen, 2006).

Behavioral strategies aimed at suppressing drug-associated memories have had limited success. Cue exposure treatment involves repeated unreinforced exposure to stimuli previously associated with drug use to extinguish drug-associated memories. This treatment has only shown modest benefits (Drummond & Glautier, 1994; Ehrman et al., 1998; Raw & Russell, 1980) and efforts to improve the efficacy of cue exposure treatment have involved combining the therapy with cognitive behavior techniques such as cue replacement or coping strategies (Monti et al., 1993; Symes & Nicki, 1997). Combining both behavioral therapy strategies and treatment medication shows the highest success rates for reducing the strength of drug-associated memories that contribute to relapse (Dunbar & Taylor, 2017; Torregrossa & Taylor, 2013; Cleva et al. 2011), but further understanding of the mechanisms underlying the formation and maintenance of drug-associated memories may improve development of therapeutic interventions.

Human imaging studies using techniques like positron emission tomography (PET) and functional magnetic resonance imaging (fMRI) show that the amygdala is activated in response to drug-associated cues and during reports of drug craving (Bonson et al., 2002; Breiter et al., 1997; Childress et al., 1999) These studies also emphasize the amygdala as a potential target for treatment. **Chapters 2 and 3** in this dissertation highlight the role of dopamine signaling in the LA in associative learning and point to VTA dopamine as an important input to the LA that participates in encoding the association between environmental cues and the reinforcing effects of cocaine. Human drug use is composed of many drug-paired cues and contexts that are an obstacle for current SUD treatment, but preclinical work targeting reactivation of cocaine (US) memory to

extinguish more than one CS highlights the promise of disrupting US encoding (Dunbar & Taylor, 2017).

Monfils and colleagues developed a memory retrieval extinction procedure where daily CS retrieval sessions followed by extinction attenuated CS-US memories (Monfils, Cowansage, Klann, & LeDoux, 2009). This procedure was adapted for rat models of drug relapse and for abstinent heroin users (Xue et al., 2012) and a more recent iteration of the procedure focused on US retrieval to inhibit drug-seeking and relapse in rodent models (Luo et al., 2015). In these studies, injecting rats non-contingently with the previously self-administered drug (cocaine) in their home cage 1-hour prior to daily extinction sessions showed decreased cocaine primed reinstatement, spontaneous recovery, and renewal of cocaine-seeking. In contrast to CS-based memory retrieval extinction, targeting US memories was able to diminish renewal and reinstatement of cocaine-seeking even in the presence of cocaine cues that were not present during extinction. Moreover, the US procedure was able to decrease cocaine-seeking after 28 days of abstinence when the CS manipulation showed no effect (Luo et al., 2015). Therefore, US-based interventions may be more effective and the work in this dissertation presents a relevant US pathway that could serve as a starting point.

Although pharmacotherapy and extinction procedures are potential avenues for interfering with drug-cue associative memories involved in SUD development, our chemogenetic experiments in **Chapter 3** suggest that DREADDs or related approaches may also serve a purpose for SUD treatment. This approach would require extensive preclinical evaluation and development before use in the clinic, but initial primate studies show efficacy and safety of DREADDs for modulating circuits and behavior (Eldridge et al., 2015; Grayson et al., 2016; Nagai et al., 2016; Raper & Galvan, 2022; Upright et al., 2018). Using chemogenetics in nonhuman primates is not

without challenges as it can be difficult to target specific cell subtypes, to achieve consistent levels of expression, and to overcome immune responses (Raper & Galvan, 2022). Despite the challenges, given the similarities of nonhuman primates to humans, continued development and application of chemogenetics is essential to eventually translate the approaches to treat human neuropsychiatric conditions including SUD.

4.4 SUMMARY AND CONCLUSIONS

In summary, this dissertation describes dopamine signaling in the LA as it relates to associative learning for both natural and drug reward. Collectively, we have shown dopamine activity in the LA is critical for encoding reward-predictive cues. For one, learned associations of an auditory cue with sucrose reward during Pavlovian conditioning led to increased dopamine release in the LA as the cue was presented, particularly as the association was learned. Additionally, inhibiting VTA dopamine input to the LA dampened acquisition of cocaine self-administration and blunted later reinstatement of seeking by a previous cocaine-paired cue. On the other hand, exciting VTA dopamine input to the LA during cocaine self-administration increased cue salience as indicated by greater responding to the cocaine-paired cue during reinstatement. Importantly, dopamine activity in the LA appears tuned to cue-related events as dopamine release is unchanged by primary reward or instrumental action and interfering with dopamine input to the LA leaves cocaine primed reinstatement and cocaine place preference intact. Taken together, these data indicate that manipulation of projections underlying dopamine input to LA that support CS-US encoding may be a useful starting point for developing therapeutic interventions to treat SUD.

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