Elucidating the Role of Subcortical Circuitry in Cue-Motivated Behaviors

by

Amanda Guadalupe Iglesias

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Doctoral Committee:

Professor Shelly B. Flagel, Chair
Professor Kent C. Berridge
Assistant Professor Ada D. Eban-Rothschild
Professor Carol Elias
Professor Emeritus Malcolm J. Low

Amanda Guadalupe Iglesias

agiglesi@umich.edu

ORCID iD: 0000-0003-2525-7796

Dedication

This dissertation is dedicated to Michelle Iglesias. Mom, you gave us your all so this is just as much your degree as it is mine.

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Abstract

Environmental cues can influence behavior in an adaptive manner by signaling the availability of valuable resources. For some individuals, however, such cues can attain inordinate control and promote maladaptive behavior. That is, when cues are attributed with incentive motivational properties, they become incentive stimuli that gain the power to evoke complex emotional states and subsequently influence behavioral responding. The propensity to attribute incentive motivational value to such cues is also an indicator of behavioral traits that can render one vulnerable to cue-influenced psychopathologies (e.g. substance abuse disorder). We can study these phenomena through an animal model, the sign-tracker (ST)/goal-tracker (GT) model, through a behavioral task termed Pavlovian conditioned approach (PavCA). In PavCA training, presentation of a neutral cue is followed by delivery of a food reward, and after repeated cue-reward pairings, distinct behavioral phenotypes emerge –sign-trackers (STs), goal-trackers (GTs), and intermediate responders (IRs). While both GTs and STs attribute predictive value to the cue, STs also attribute incentive motivational value to the cue, which leads to greater engagement with the cue. IRs show both sign-tracking and goal-tracking conditioned responses, with no clear predominance of either. These differential responses in the Pavlovian conditioned approach task have been associated with distinct neurobiological mechanisms. An investigation of the subcortical neural systems that subserve these behaviors will be the focus of this dissertation.

Dopamine has long been implicated in reward and motivated processes, and prior work suggests that the behavior of sign-trackers is dopamine-dependent. In Chapter 2, we addressed a gap in the literature regarding the temporal dynamics of dopamine and sign-tracking. Using optogenetics we disrupted cue-elicited dopamine neural activity in the ventral tegmental area. We found that sign-tracking does not develop without cue-paired dopamine activity due to disrupted incentive salience attribution, but that sign-tracking behavior subsequently emerges with intact dopamine signaling (i.e. when laser inhibition is terminated). Another subcortical system of interest is the paraventricular nucleus of the thalamus (PVT) to the lateral hypothalamus (LH) pathway. The PVT has emerged as a neural hub that mediates the characteristic differences in associative learning captured in the ST/GT model. Prior findings have led us to postulate that bottom-up projections from the lateral hypothalamus (LH) to the PVT relay the incentive value of reward-associated cues. In Chapter 3 we utilized chemogenetics to inhibit the LH-PVT pathway and found that this manipulation attenuated goal-directed behavior and that this effect was apparent in IR rats, but not STs and GTs. In Chapter 4, we utilized a novel tissue multiplexing technology to label cue-elicited neuronal activity simultaneously with neuronal identity and projection specificity. We found that, in response to a Pavlovian food-cue, STs have more active orexinergic neurons that project from the LH to the PVT, relative to GTs.

Collectively, these studies illuminate the contributions of multiple subcortical systems in the acquisition and expression of behavioral responses that emerge through cue-reward learning. Regarding the dopaminergic system, we add to a body of literature suggesting that incentive value attribution, and by proxy sign-tracking behavior, is dopamine-dependent. For the LH-PVT pathway and orexinergic system, we open a line of questions to further investigate what role

these subcortical targets play in individual differences in cue-reward learning. In all, this research teased apart some of the neurobiological factors that contribute to divergent cue-influenced behaviors, which is of relevance to understanding multiple neuropsychiatric disorders.

Chapter 1
General Introduction

Note: Some of the text and figures within the Introduction have previously appeared in print (Iglesias & Flagel, 2021, Frontiers in Integrative Neuroscience).

Our modern world is organized to inundate us with constant stimuli that grasp our attention and influence our choices. The stimuli, or cues, that have the most control over our behavior are generally ones that we associate with memorable past experiences. This process (i.e. associative learning), by which we attribute predictive value to environmental cues, is an invaluable mechanism that can optimize behavioral responding. For instance, imagine eagerly hiking on a trail you have never been on. At some point you get turned around and become acutely aware of your surroundings to try and get back on trail. Remembering unique rock formations or trail features could be the difference between getting out of that scenario safely or needing rescue. Similar associative learning mechanisms are utilized to guide decisions, such as choosing water, food, or mates, in which adaptive choices encourage survivability.

It is through Pavlovian conditioning (Rescorla, 1988) wherein a relationship between events emerges. Successfully associating a discrete environmental stimulus with an outcome transforms a once neutral stimulus into a predictor that can also have powerful sway over behavior (Robinson & Berridge, 1993). When a stimulus is attributed with incentive salience, it becomes an incentive stimulus, capable of eliciting complex emotional and motivational responses. Incentive stimuli themselves become attractive and desirable (Berridge, 2001; Bindra, 1978; Bolles, 1972; Toates, 1986), and can trigger maladaptive behaviors characteristic of

psychiatric disorders (Robinson & Berridge, 1993). One such example is substance use disorder wherein the exposure to stimuli previously associated with the drug-taking experience can lead to detrimental patterns of behavior such as relapse to drug-taking despite the desire to remain abstinent and even in the face of adverse consequences (DeJong, 1994). In fact, incentive stimuli, are such powerful "motivational magnets" (Berridge et al., 2009) that mere exposure to drug-paired stimuli, or cues (e.g. people, places, and paraphernalia), can initiate this relapse (Robinson & Berridge, 1993; Tomie, 1996). These same drug-paired cues, however, are not "motivational magnets" for all individuals that consume illicit substances.

Similarly, there are a range of other cue-mediated psychopathological disorders that only impact a subset of the population (Albertella et al., 2019; María-Ríos & Morrow, 2020; Wang et al., 2021). Although the most common example of relapse is perhaps in relation to substance use disorder, there are other conditions that have similar features. For example, individuals with obsessive-compulsive disorder (OCD) might seek treatment but periodically slip back into old patterns of thinking and "relapse" to acting out compulsions. A similar sequence of events can happen for those with eating disorders, or those with post-traumatic stress disorder (PTSD) wherein cues associated with adverse life events are generalized and impact an individual's related encounters. Although these disorders (e.g., substance abuse disorder, OCD, eating disorders, PTSD) all manifest differently, they demonstrate that a relapse to adverse behaviors can be due to exposure to related cues. Further management of symptoms becomes particularly challenging in the face of cues with "motivational magnet" status, as was described for substance use disorder. Studying these cue-mediated relationships can therefore provide insight into vulnerabilities that foster psychopathology.

One such way to probe these psychopathological phenomena is to study individual differences in the attribution of incentive salience (Berridge, 2001; Robinson & Berridge, 1993) to reward-paired cues in animals (Flagel et al., 2009). These differences can be studied through Pavlovian learning paradigms (Pavlov, 1927). In Pavlov's paradigm dogs were presented with a tone-cue (conditioned stimulus, CS) that was followed by a food-reward (unconditioned stimulus, US) and the dogs subsequently developed conditioned responses (CRs). Pavlov demonstrated that a salivatory response in dogs could be elicited through such behavioral conditioning. However, the work of Karl Zener showed that diverse responses could emerge through these processes (Zener, 1937). Specifically, when dogs were unharnessed, it was reported that some dogs attended to the site of reward delivery, while others interacted with the CS itself (Zener, 1937). These divergent CRs were later coined goal- and sign-tracking behavior, respectively. The "goal-tracking" term emerged through studies with rats, wherein following cue (CS) presentation the rats would approach and interact with the site of reward delivery, the "goal" location (Boakes, 1977). Work in pigeons inspired the "sign-tracking" label (Hearst & Jenkins, 1974), in which the pigeons would approach and peck a key-light-cue, the "sign" that predicted a food reward (US; (Brown & Jenkins, 1968). Although the observations and nomenclature for sign- and goal tracking behavior were established many decades ago it was only more recently that the behaviors were studied together (Flagel et al., 2007). The sign-tracker/goal-tracker (ST/GT) animal model, captures individual differences in Pavlovian cue-reward learning (Flagel et al., 2007) and can be utilized to elucidate the neurobiological mechanisms that encode adaptive or maladaptive cue-driven behaviors. An expansion of this model and a review of the subcortical neural targets that can influence these individual differences is to follow.

1.1 The sign- and goal-tracker animal model

The ST/GT model was developed following the observation that rats exhibit individual differences in response to reward-paired cues in a Pavlovian conditioned approach (PavCA) paradigm (Flagel et al., 2007; Meyer et al., 2012; Robinson & Flagel, 2009). In PavCA an illuminated lever-CS is presented for 8 sec and is followed by a food reward-US delivered into an adjacent food-cup. Through repeated pairings, and via associative learning, the lever-CS becomes a predictor of the food reward-US and the conditioned responses (CRs) of sign- and goal tracking develop (Figure 1.1) (Robinson & Flagel, 2009). Sign-trackers (STs) exhibit more approach and engagement with the lever-CS when it is presented, often displaying consummatory behaviors towards the cue (e.g., grasping, licking, gnawing). GTs do not interact with the lever-CS and swiftly approach the location of reward delivery following lever-CS presentation. Another group, intermediate responders (IRs), vacillate between sign- and goal-tracking behavior, showing less rigidity in their response and no clear preference for either the lever-CS or reward location. The rate at which a behavioral phenotype is acquired is similar among STs, IRs, and GTs (Robinson & Flagel, 2009). Through extensive analyses using the ST/GT model, it has been shown that roughly 1/3 of animals (sampled from a population of 1598 outbred heterogenous stock rats) exhibit a sign-tracking CR, 1/3 a goal-tracking CR, and 1/3 are intermediate responders (Hughson et al., 2019; Meyer et al., 2012).

These differential responses during PavCA are due to the value ascribed to the lever-CS. As the lever-CS is reliably followed by a food reward-US, the lever-CS gains predictive value, but can also gain incentive value. All phenotypes, STs, IRs, and GTs, place predictive value on the lever-CS as they learn the Pavlovian association between the cue and the reward. For STs, however, the lever-CS can also acquire incentive motivational value. That is, the lever-CS gains

incentive salience and consequently becomes an incentive stimulus (Zhang et al., 2009). It is this process of incentive salience attribution (Berridge, 2001; Robinson & Berridge, 1993) that transforms a stimulus from being merely a predictor of reward to an object of desire. Incentive stimuli are characterized by three fundamental properties: (1) they can bias attention and elicit approach (Cardinal, Parkinson, Hall, et al., 2002; Harmer & Phillips, 1998; Peterson et al., 1972); (2) they themselves become "wanted", in that animals will learn new instrumental actions and work to get them (Wyvell & Berridge, 2000); and (3) they are able to evoke a conditioned motivational state that can promote ongoing instrumental actions (Di Ciano et al., 2007; Taylor & Horger, 1999).

The ability of an incentive stimulus to elicit approach can be captured through the PavCA paradigm described above, as enhanced approach toward the lever-CS is robustly displayed by STs. Further, for STs, the lever-CS becomes a conditioned reinforcer, such that they will work for presentation of the stimulus in the absence of a food reward. Specifically, in this test, the lever-CS is flanked by two nose ports and a poke into the "active" port results in lever-CS presentation whereas a poke into the "inactive" port has no consequence. Relative to GTs and IRs, STs exhibit more 1) nose pokes in the active port and 2) lever-CS deflections upon its presentation (Robinson & Flagel, 2009). A Pavlovian to instrumental transfer (PIT) paradigm is used to assess the third property of an incentive stimulus – the ability of the conditioned stimulus to promote ongoing instrumental actions (Estes, 1948). There are three components to PIT training, animals are first exposed to a Pavlovian cue-CS and food reward-US pairing and are then trained to perform an instrumental response (e.g., lever press) for receipt of a different reward (Estes, 1948). Lastly, in the absence of reward, presentation of the Pavlovian cue-CS results in enhanced instrumental responding, indicating that the cue-CS gained motivational

value. As STs would tend to show enhanced lever-CS regardless of the prior conditions, PIT training would be confounded (Robinson et al., 2014). However, although not examined in the traditional sense (i.e. PIT), STs are influenced by food- and drug-paired cues and thereby show food- and drug-seeking behavior, which demonstrates instrumental responding (Saunders & Robinson, 2011; Saunders et al., 2013; Yager & Robinson, 2010). Thus, it has been demonstrated that a Pavlovian conditioned stimulus exhibits the three fundamental properties of an incentive stimulus for STs to a greater extent than it does for GTs or IRs (Berridge, 2001; Cardinal, Parkinson, Lachenal, et al., 2002; Di Ciano et al., 2007; Flagel et al., 2009; Harmer & Phillips, 1998; Peterson et al., 1972; Robinson & Berridge, 1993; Taylor & Horger, 1999; Wyvell & Berridge, 2000; Zhang et al., 2009).

1.2 Behavioral correlates of sign- and goal-tracking behavior

Through the lens of the incentive-sensitization theory (Robinson & Berridge, 1993, 2001), propensity to attribute incentive salience to reward-paired cues renders one vulnerable to substance use and abuse. Indeed, relative to GTs, STs display a number of behaviors characteristic of substance use disorder (Robinson et al., 2014; Saunders et al., 2013). Sign-trackers are unduly attracted to both food- and cocaine-paired cues (Flagel et al., 2010; Uslaner et al., 2006; Yager et al., 2015; Yager & Robinson, 2013), acquire cocaine self-administration faster and are more motivated to respond for cocaine (Beckmann et al., 2011; Saunders & Robinson, 2011), are more likely to relapse to drug-seeking behavior upon exposure to drug-associated cues (Saunders & Robinson, 2010, 2011; Saunders et al., 2013), and show enhanced psychomotor sensitization following repeated cocaine exposure (Flagel et al., 2008). Of note, STs and GTs do not differ in opioid self-administration behavior (Chang et al., 2022) or in the motivation for cocaine following extended exposure (Kawa et al., 2016). Beyond drug-seeking

and taking sign-trackers exhibit a number of traits of relevance to substance use disorder and other psychiatric disorders. Sign-trackers have poor attentional control (Paolone et al., 2013), are more impulsive (Lovic et al., 2011), and show an exaggerated fear response to discrete aversive cues (Morrow et al., 2011; Morrow et al., 2015). Although not influenced by traditional discrete cues, goal-trackers are not resistant to influence by illicit substances and appear to be motivated by "higher order" discriminative cues (Rescorla, 1988). For instance, GTs have a higher propensity to relapse to drug-seeking in the presence of discriminative stimuli (i.e. occasion setters) (Pitchers, Phillips, et al., 2017), display drug-seeking following exposure to contextual cues (Saunders et al., 2014), and have an exaggerated fear response to contextual cues (Morrow et al., 2011). As such, both sign-trackers and goal-trackers display traits of relevance to psychopathology, but under different circumstances and likely via different psychological and neurobiological processes.

Further, there is evidence that sign- and goal-tracking traits are heritable. Rats selectively bred based on a "sensation-seeking" trait, or locomotor reactivity to a novel environment (Stead et al., 2006), exhibit predictable sign- and goal-tracking phenotypes. Bred high-responders (bHRs), or those that exhibit enhanced locomotor response to novelty, consistently engage with reward-paired cues in a PavCA paradigm, whereas bred low-responders (bLRs), or those that exhibit low levels of activity in a novel environment, reliably approach the location of reward delivery (Flagel et al., 2010). That is bHRs are almost always sign-trackers and bLRs goal-trackers. These findings suggest that the propensity to attribute incentive salience to reward-paired cues is heritable. We have also observed skewed populations in other transgenic rat colonies bred in-house (Iglesias et al., 2023) and in outbred rats from different vendors (Fitzpatrick et al., 2013; Khoo, 2022). This is likely due to some genetic variables, as our in-

house colonies experience the same environment, but different colonies are skewed in different directions (i.e. some have more sign-trackers, some more goal-trackers, and some are equally distributed).

1.3 Neurobiology underlying sign- and goal-tracking behavior

In Pavlov's early work he stated that a "chronic pathological state" likely occurred from "a conflict between excitation and inhibition which the cortex finds itself unable to resolve" or due to "the action of extremely powerful stimuli" (pg. 318) (Pavlov, 1927). Of relevance, distinct differences in neural circuit engagement between STs and GTs have been identified (Flagel, Cameron, et al., 2011; Haight et al., 2017), resulting in a "top-down" vs. "bottom-up" theory of goal- and sign-tracking behavior, respectively. Through studies of neuronal activity (via cFos), differential engagement of brain systems have been noted between sign- and goal-trackers in response to both food- and drug-associated cues (Flagel, Cameron, et al., 2011; Haight et al., 2017; Yager et al., 2015). During Pavlovian conditioning, GTs, are believed to utilize "top-down" cortico-thalamic-striatal circuits while STs are thought to employ "bottom-up" hypothalamic-thalamic-striatal circuits to guide their behavior (Flagel, Cameron, et al., 2011; Haight et al., 2017). Recent research has focused on "top-down" cortical mechanisms of these disparate behaviors, however, the "bottom-up" circuitry mediating incentive salience attribution have yet to be fully explored and will be the focus of this dissertation.

1.3.1 Cortical "top-down" involvement

The prefrontal cortex (PFC) is a neocortical structure with various subregions that can generally be divided into midcingulate cortex and anterior cingulate cortex, both with subdivisions of their own (Laubach et al., 2018; van Heukelum et al., 2020). The PFC promotes

executive functions such as cognitive control and emotion regulation, which are essential for selective attention (for review see: (Friedman & Robbins, 2022; Fuster, 2001)) and goal-directed behaviors (Duan et al., 2021; Mihindou et al., 2013). In behavioral tasks that demand sustained attention and response inhibition, GTs unsurprisingly display better performance than STs (King et al., 2016; Paolone et al., 2013). Further, GTs show enhanced impulse control on impulsive action tasks that require the ability to withhold responding in order to receive a reward (Flagel et al., 2010; Lovic et al., 2011). A proposed mechanism for these differing "cognitive-motivational styles" displayed by sign- and goal-trackers is cholinergic activity within the PFC (Sarter & Phillips, 2018). In support, GTs perform well in attentional control tasks and their performance is associated with acetylcholine (ACh) increases in the PFC (Paolone et al., 2013; Pitchers, Kane, et al., 2017). Alternatively, STs perform poorly on attentional control tasks and also have low task-related ACh levels that have been linked to inefficient choline transport in the PFC (Koshy Cherian et al., 2017; Paolone et al., 2013). Further, it has been observed that only GTs have increased ACh in response to a cocaine-cue whereas STs show increased dopamine (DA) (Paolone et al., 2013; Pitchers, Kane, et al., 2017). Cholinergic activity within the PFC, therefore, is thought to mediate "top-down" cognitive control resulting in traits related to and the manifestation of goal-tracking behavior, whereas a deficiency in this signaling increases the likelihood of sign-tracking behavior and related traits. Importantly, however, this line of thought is restricted to cortical ACh because systemic antagonism of ACh receptors reduce sign-tracking behavior (Gheidi et al., 2023), likely through action on other neural systems.

An alternative cortical modulator may be within the serotonergic system in the PFC. In support, following Pavlovian training serotonin (5-HT) levels and 5-HT receptor binding in the PFC increases (Tomie et al., 2003; Tomie et al., 2004). Further, lesions of serotonergic neurons

in the forebrain in rats (Winstanley et al., 2004) and depleting 5-HT in the PFC of mice (Campus et al., 2016) enhances sign-tracking behavior. Thus, the 5-HT system within the PFC appears to play a role in encoding the incentive motivational value of reward cues and, in turn, modulate sign-tracking behavior.

Beyond individual nodes, neuronal activity maps helped to identify neural pathways of relevance to sign- and goal-tracking behaviors. Using c-Fos mRNA as a marker of neuronal activity, it was discovered that only STs engage a number of brains regions in response to a food-cue, whereas GTs and unpaired rats were no different from one another (Flagel, Cameron, et al., 2011). From these data, correlative neural activity maps were developed and most notably showed that, both STs and GTs had correlated neural activity with the paraventricular nucleus of the thalamus (PVT) and other regions. For STs, a thalamo-striatal connection was present whereas for GTs the cortico-thalamic correlation was strongest. Here, the PVT became a target of interest in potentially mediating "top-down" versus "bottom-up" mechanisms involved in sign- and goal-tracking behavior. In support, chemogenetic potentiation of the prelimbic cortex (PrL)-PVT pathway decreases the incentive salience of the cue among STs and reduces dopamine in the nucleus accumbens shell (NAcSh) (Campus et al., 2019). Alternatively, chemogenetic inhibition of the PrL-PVT pathway increases the incentive salience of reward-cues among GTs, as well as dopamine transmission in the NAcSh (Campus et al., 2019). These data highlight a PVT-mediated "top-down" control mechanism with input from the PrL to the PVT encoding the predictive value of reward cues and thereby modulating output from the PVT-NAc to promote goal-directed behavior. When cortical systems are taken "offline", via chemogenetic inhibition, the subcortical mechanisms that guide sign-tracking behavior are able to tip the scales toward incentive salience encoding of reward-related cues (Campus et al., 2019).

1.3.2 Subcortical "bottom-up" involvement

Relative to GTs, STs showed greater c-Fos expression in subcortical regions such as the striatum, amygdala, hypothalamus and show correlated neural activity with the PVT (Flagel, Cameron, et al., 2011). As such, the PVT-mediated "bottom-up" neural targets are believed to guide incentive salience attribution and influence sign-tracking behavior. Outside of the correlative neural maps, numerous studies have indicated that sign-tracking behavior is dopamine-dependent (Flagel, Clark, et al., 2011; Flagel et al., 2007; Saunders & Robinson, 2012). The role of different subcortical systems in sign-tracking behavior are of focus here: 1) dopaminergic projections from the ventral tegmental area to the nucleus accumbens and 2) orexinergic projections from the LH to the PVT.

1.4 Dopamine

The motivating properties of both natural and drug-related rewards have been shown to be mediated by dopamine (Bassareo & Di Chiara, 1999; Berridge, 2007; Di Chiara & Imperato, 1988; Kelley & Berridge, 2002; Montague et al., 2004; Robbins & Everitt, 1999; Stuber et al., 2008; Volkow et al., 2011; R.A. Wise, 1980). One of the main dopaminergic (DAergic) projections exists within the mesocorticolimbic system that originates within the ventral tegmental area (VTA) and projects through the nucleus accumbens (NAc), amygdala, PFC, and forebrain (Fallon & Moore, 1978; Lindvall et al., 1974; Swanson, 1982). The VTA-NAc pathway represents a considerable projection (Swanson, 1982) and the NAc in particular has been widely studied and implicated in many aspects of reward-related behaviors such as mediating the reinforcing effects of rewards (Ikemoto et al., 1997; Kelley, 2004).

1.4.1 Neuroanatomical characteristics of the VTA

The VTA is a heterogeneous midbrain structure that was first identified and described through examinations of opossum and armadillo brains (Papez, 1932; Tsai, 1925). The VTA can be divided into subregions that can be assigned through an antero-posterior gradient. The anterior VTA projects mainly to lateral olfactory tubercle and the NAc core (NAcC) while the posterior VTA projects to the medial NAc shell (NAcSh) and medial olfactory tubercle (for review see: (Ikemoto, 2007; Sanchez-Catalan et al., 2014)). In the anterior portion of the VTA there are some DAergic cells, but the area mostly contains non-DAergic (i.e. glutamatergic and γ-aminobutyric acid (GABA)ergic) cells within the parafasciculusretroflexus area and ventral tegmental tail subdivisions (Morales & Root, 2014; Olson & Nestler, 2007; Olson et al., 2005). The majority of the dopamine neurons (tyrosine hydroxylase positive cells) exist in the posterior VTA in the paranigral nucleus and parabrachial pigmented area (Ikemoto, 2007). The neuronal architecture of the anterior and posterior areas of the VTA foreshadows their respective roles in behavior. Drugs that act on dopamine systems are not reinforcing in the anterior VTA, but are in the posterior VTA (Ikemoto et al., 2006; Rodd, Bell, Kuc, et al., 2005; Rodd, Bell, Zhang, et al., 2005; Zangen et al., 2006).

Early work showed that rats would lever press for microinjections of morphine into the VTA - implicating the region in drug reward (Bozarth & Wise, 1981). Continued research in this regard has revealed that rodents will readily self-administer opiates, cannabinoids, cocaine, and ethanol into the VTA (for review see: (Ikemoto & Bonci, 2014; McBride et al., 1999). Drug administration directly into the VTA increases dopamine activity (Bonci et al., 2003; Brodie et al., 1990), but can also influence dopamine release downstream in the NAc (Di Chiara & Imperato, 1988). As such, over the last few decades, an extensive body of work has developed to support a role for mesolimbic dopamine activity in the addictive properties of drugs of abuse and

other rewards (Adinoff, 2004; Berridge & Robinson, 2016; Koob & Volkow, 2010; Lüscher & Malenka, 2011; Lüscher & Ungless, 2006; Pierce & Kumaresan, 2006).

1.4.2 Mesolimbic dopamine

Mesolimbic dopamine projections target the NAc, olfactory tubercle, septum, amygdala, and hippocampus; however not all regions receive the same density of DAergic innervation from the VTA. The NAc is the primary target of mesolimbic dopamine (Albanese & Minciacchi, 1983; Ikemoto, 2007) and has received considerable attention for role in reward processing and as an interface between "motivation and action" (Berridge & Robinson, 1998; Ikemoto & Panksepp, 1999; Kelley, 2004; Mogenson et al., 1980; Pierce & Kumaresan, 2006). As such, dopamine levels in the NAc rise in response to reward presentation and consumption of food, sex, and various drugs of abuse (Bassareo & Di Chiara, 1999; Covey et al., 2016; Di Chiara & Imperato, 1988; Fiorino et al., 1997; Wise, 1996), as well as following intracranial self-stimulation (Cheer et al., 2007). Further, infusion of dopamine-stimulating drugs in the NAc are reinforcing (Hoebel et al., 1983; Rodd-Henricks et al., 2002) and hyperdopaminergic mice show enhanced incentive salience to a sucrose reward (Peciña et al., 2003) (see also: (Peciña & Berridge, 2013; Wyvell & Berridge, 2000).These fluctuations in neural activity based on environmental conditions puts the NAc in a position to guide motivated behavior. In support, NAc dopamine is essential for reward-seeking behavior (Berridge & Robinson, 1998; Cardinal, Parkinson, Hall, et al., 2002; Di Chiara, 2002; du Hoffmann & Nicola, 2014; Ikemoto & Panksepp, 1999). A historically extensive and growing library of literature beyond what can be covered here continues to expand the role of mesolimbic dopamine in reward processing and behavioral instigation.

1.4.3 Theoretical frameworks of dopamine in motivation

Dopamine plays a clear role in reward-related behaviors, and this is true for both natural rewards and drugs of abuse (Bassareo & Di Chiara, 1999; Covey et al., 2016; Di Chiara & Imperato, 1988; Fiorino et al., 1997; Wise, 1996). However, there is contention on precisely how this neuromodulator works and several theories have been developed to explain its role in motivated behavior (for review see: (Berridge, 2001; Berridge & Robinson, 1998; Wise, 2004). Responding to environmental rewards can encompass a number of complex events involving attention, motor control (i.e movement), and even effort. Similarly, anticipation and consumption of rewards elicits an affective response. The questions researchers grapple with is how dopamine activity fits into or guides any one of these systems. Concise descriptions of some of the most prominent theories of dopamine in motivation are presented below.

1.4.3.1 Hedonia

The *hedonia hypothesis* suggests that dopamine gates the subjective feelings of "pleasure" that accompanies rewards (R. A. Wise, 1980). In support, brain dopamine increases are noted in response to natural and drug rewards (Bassareo & Di Chiara, 1999; Covey et al., 2016; Di Chiara & Imperato, 1988; Fiorino et al., 1997; Wise, 1996), dopamine blockade via pimozide results in decreased responding for a food reward (Wise et al., 1978), and further dopamine loss reduces motivation for rewards (Wise, 1985). However, nearly complete dopamine depletion in rats (via 6-hydroxydopamine lesions) results in aphasia, but has no impact on affective orofacial reactions of "liking" to tastes (Berridge et al., 1989). This seminal study along with several others published in subsequent years (Peciña & Berridge, 2013; Peciña et al., 2003; Smith et al., 2011; Wyvell & Berridge, 2000) demonstrated that dopamine does not encode pleasure.

1.4.3.2 Reward learning

The *reward learning* hypothesis posits that dopamine is involved in associated learning related to rewards (Wise, 2004). As such, some believe that dopamine enhances stimulus-reward relationships and results in habitual and excessive drug taking (Everitt et al., 2001; Everitt & Wolf, 2002; Robbins & Everitt, 1999). Alternatively, if we embrace the reward prediction error (RPE) hypothesis (Montague et al., 2004; Schultz et al., 1997), dopamine is believed to encode the discrepancy between rewards "predicted" and those received, thereby acting as a learning signal in the brain. However, others have shown that learning is possible without dopamine activity. For instance, dopamine-deficient mice show impairments in motivation, but learn reward preferences at similar rates to controls (Cannon & Palmiter, 2003). Further, pretreatment with caffeine in dopamine-deficient mice encourages learning (i.e. learning through non-DAergic mechanisms) (Berridge, 2005; Hnasko et al., 2005; Robinson et al., 2005).

1.4.3.3 Incentive salience

The *incentive salience* hypothesis suggests that dopamine systems can imbue previously neutral stimuli with incentive salience, making them rewarding, and thereby, "wanted". This "wanting" manifests as approach, engagement, and effort put forth to interact with the incentive stimulus. Crucially, the hedonic impact "liking", incentive salience "wanting", and reward learning are all dissociable concepts under this framework, and only incentive learning has been consistently shown to be dopamine-dependent (Berridge, 2001; Berridge, 2018; Berridge & Kringelbach, 2015; Berridge & Robinson, 1998, 2003, 2016; Robinson & Berridge, 1993, 2000, 2001; Zhang et al., 2009). The 6-hydroxydopamine lesion study mentioned above initially showed that "liking" reactions are still possible in dopamine-depleted states, but that motivational "wanting" was diminished (Berridge et al., 1989). There are a number of studies

that show dopamine-dependent “wanting” exists without impacting “liking” (Evans et al., 2006; Leyton et al., 2002), and also the reverse (Peciña et al., 2003).

In support of the incentive salience hypothesis of dopamine, we revisit the ST/GT model, which parses predictive and incentive salience encoding. Of the most striking and important findings within the ST/GT model remains the differences in dopamine activity (Flagel, Cameron, et al., 2011; Flagel, Clark, et al., 2011; Flagel et al., 2007; Iglesias et al., 2023; Saunders & Robinson, 2012). Specifically, dopamine transmission within the NAc during Pavlovian learning is necessary for the acquisition and expression of sign-tracking behavior (i.e. attribution of incentive salience to reward-cues) (Flagel, Clark, et al., 2011; Saunders & Robinson, 2012). Several other lines of evidence have shown that only sign-tracking behavior is dopamine-dependent, notably in gene expression (Flagel et al., 2007) and phasic dopamine release in the NAc (Flagel, Clark, et al., 2011). Dopamine release patterns were captured through fast scan cyclic voltammetry in PavCA, and showed sign- and goal-trackers differ in this regard (Flagel, Clark, et al., 2011). As sign-trackers develop their conditioned response, dopamine peaks shift from reward consumption to cue-presentation, a “classic” reward prediction error signal. However, dopamine transmission in the NAc of goal-trackers does not shift from reward to cue presentation. Therefore, it was interpreted that this dopamine shift in sign-trackers encoded the incentive salience ascribed to the reward-cue, not a general learning signal (Flagel, Clark, et al., 2011). Thus, mesolimbic dopamine has been directly shown to play a role in encoding the incentive salience of reward-paired cues and is a critical mediator in the sign-tracking conditioned response that results through Pavlovian conditioning.

1.5 The paraventricular nucleus of the thalamus

Behavioral neuroscience research has long been focused on unveiling the brain mechanisms underlying motivated behavior. Olds and Milner (1954) were among the first to identify brain structures involved in appetitive motivation and reinforcement learning. Their pioneering experiments showed that rats would repeatedly press a lever for electrical stimulation of brain regions that we now consider part of the mesocorticolimbic "reward" system. Since these initial experiments, however, we have learned that ascribing terms such as reward or reinforcement learning to a specific brain mechanism or circuit is overly simplistic both in terms of the semantics and the underlying neural mechanisms (Milner, 1991). We now know that the classic "motive circuit" extends beyond those brain structures first identified by Olds and Milner (1954; see also Milner, 1991), and more closely resembles that put forth by Ann Kelley in the early 2000s (Kelley et al., 2005).

Among the brain regions included on Ann Kelley's maps of motivated behavior is the paraventricular nucleus of the thalamus (PVT). The PVT is a midline thalamic structure that is ideally placed to integrate information from arousal, limbic, cortical, and motor circuits in the brain (Figure 1.2). Not long after the initial studies by Olds and Milner (1954), Cooper & Taylor (1967) reported that rats with electrodes implanted within the PVT and surrounding areas demonstrated self-stimulation behavior. These findings were later supported by the work of Clavier and Gerfen (1982), who showed that levels of self-stimulation increased as electrodes became localized to midline thalamic structures, including the PVT. In the decade that followed, a number of studies demonstrated engagement of the PVT in response to drugs of abuse and associated stimuli (Deutch et al., 1998; Deutch et al., 1995; Pierce & Kalivas, 1997; Stephenson et al., 1999; Young & Deutch, 1998). Despite this earlier research identifying a potential role for the PVT in reward processing and reinforcement learning, and Ann Kelley's subsequent

recognition of this nucleus as a primary node of motivated behavior, only in recent years has the PVT gained increasing attention in behavioral neuroscience research (see reviews: (Barson et al., 2020; Curtis et al., 2021; Haight & Flagel, 2014; Hsu et al., 2014; James & Dayas, 2013; Kirouac, 2015; Martin-Fardon & Boutrel, 2012; Matzeu et al., 2018; Matzeu et al., 2014; McGinty & Otis, 2020; McNally, 2021; Millan et al., 2017; Zhou & Zhu, 2019).

1.5.1 Neuroanatomical and neurochemical characteristics of the PVT

Comprehensive tracing and anatomical studies have delineated the afferent and efferent connections of the PVT (see Figure 1.2), as well as the neurotransmitter and neuropeptide profiles within the region (for review see, (Barson et al., 2020; Curtis et al., 2021; Kirouac, 2015). The PVT is densely innervated by glutamatergic afferents from the medial prefrontal cortex, with the most abundant population coming from the prelimbic cortex (PrL) (Li & Kirouac, 2012). Although less dense relative to cortical inputs, the PVT also receives afferents from a range of subcortical regions including the hypothalamus, LH, hippocampus, and amygdala; and from brainstem structures including the periaqueductal grey and dorsal raphe (Hsu & Price, 2009; Li & Kirouac, 2012; Van der Werf et al., 2002; Vogt et al., 2008). The PVT has a number of reciprocal connections, with primarily glutamatergic efferents terminating in both cortical and subcortical structures including the PrL, hypothalamus, hippocampus, and amygdala (Li & Kirouac, 2008, 2012; Su & Bentivoglio, 1990; Vertes & Hoover, 2008). The PVT also sends glutamatergic efferents to the nucleus accumbens core (NAcC) and shell (NAcSh) (Dong et al., 2017; Parsons et al., 2006; Vertes & Hoover, 2008), making it ideally positioned to integrate information regarding internal states and the external environment and translate it into motivated actions (Kelley et al., 2005).

As its afferents and efferents are primarily glutamatergic, it is not surprising that glutamatergic markers, like vesicular glutamate transporter 2 (vGLUT2) mRNA, are highly expressed in the PVT (Barroso-Chinea et al., 2007). However, the neurochemical composition of the PVT extends beyond glutamate, and is complex and heterogeneous. Other neurotransmitter and neuropeptide systems that have been observed within this thalamic nucleus include dopamine (DA), gamma aminobutyric acid (GABA), opioids, cocaine-and amphetamine-regulated transcript (CART), and orexin (Koylu et al., 1998; Koylu et al., 1997; Lindvall et al., 1984; Peyron et al., 1998).

The PVT is often divided into anterior (aPVT) and posterior (pPVT) subregions based on anatomical boundaries that correspond with observational differences in cellular organization and function. The entire axis of the PVT is tightly linked to the limbic system via both afferent and efferent connections (Kirouac, 2015; Li & Kirouac, 2008, 2012), and some to a different degree in the aPVT versus pPVT. Compared to the pPVT, the aPVT receives more dense neuronal projections from areas including the hypothalamus, ventral hippocampal subiculum, and infralimbic cortex (Canteras & Swanson, 1992; Li & Kirouac, 2012); and has reciprocal connections with a number of brain regions, including the suprachiasmatic nucleus (Li & Kirouac, 2008; Moga & Moore, 1997; Novak et al., 2000; Vertes & Hoover, 2008). Both the aPVT and pPVT project to the nucleus accumbens (NAc), with the densest projections to the NAcSh (Li & Kirouac, 2008; Vertes & Hoover, 2008).

1.5.2 The PVT as a critical node of the hypothalamic-thalamic-striatal circuit

The PVT has been postulated to mediate motivated behavior via its placement in the hypothalamic-thalamic-striatal circuit (Kelley et al., 2005). Kelly and colleagues (2005)

recognized the PVT is a key node of this subcortical circuit, acting to encode information about energy and arousal states from the hypothalamus, while sending this information to the striatal complex to guide motivated behavior. Specifically, it was hypothesized that the PVT integrates orexinergic input from the LH and, in turn, sends information via glutamatergic projections to the nucleus accumbens to elicit actions (see Figure 1.2). These glutamatergic terminals from the PVT interface with dopamine neurons in the NAcSh (Pinto et al., 2003) and can alter dopamine release, independent of the VTA (Parsons et al., 2007). Despite these neuroanatomical and neurochemical findings, relatively little behavioral neuroscience research has focused on the LH-PVT-NAc circuit; but that which has supports its proposed role in motivated behaviors.

The LH, the primary source of orexin neurons in the brain, was originally studied for its role in feeding behaviors (Anand & Brobeck, 1951; Hoebel & Teitelbaum, 1962), and subsequently in other homeostatic functions, such as sleep regulation and circadian rhythms (Colavito et al., 2015; Kolaj et al., 2007). The two orexin peptides, orexin-A and orexin-B, are synthesized from the same mRNA transcript, prepro-orexin (de Lecea et al., 1998), found in the LH (Tsujino & Sakurai, 2013). Orexinergic neurons in the LH project to a wide variety of areas, including the cerebral cortex, hippocampus, and thalamus, with a large percentage terminating in the PVT (Kirouac et al., 2005; Peyron et al., 1998). The density of LH orexinergic input is stronger in the pPVT compared to aPVT (Goto & Swanson, 2004; Kirouac et al., 2005). It is known that orexin is excitatory (de Lecea et al., 1998), orexinergic neurons can co-release glutamate (Schöne et al., 2014; Torrealba et al., 2003), and that LH orexinergic inputs can have an excitatory effect on postsynaptic neurons in the PVT (Huang et al., 2006; Ishibashi et al., 2005).

The orexin peptides, and their two G-protein coupled receptors, orexin-1 (OX1) and orexin-2 (OX2) (Sakurai et al., 1998), are found throughout the PVT (Marcus et al., 2001; Parsons et al., 2006). Each receptor has a different binding affinity for each neuropeptide, with OX-1 having a higher affinity for orexin-A, and OX-2 having similar affinities for orexin-A and orexin-B (Marcus et al., 2001). Orexin signaling within the PVT has been implicated in behavioral responses to both natural rewards and drugs of abuse (Barson et al., 2015; Li et al., 2009; Stratford & Wirtshafter, 2013).

1.5.3 A proposed role for the LH-PVT-NAc circuit in incentive motivational processes

Many studies implicating the PVT in motivated behavior have relied on associative learning paradigms. In fact, Ann Kelley and colleagues (Schiltz et al., 2007) identified the PVT as a "hot spot" as indexed by immediate early gene expression in response to stimuli associated with food reward (Schiltz et al., 2007); and a number of subsequent studies demonstrated that reward-associated cues and contexts similarly engage the PVT (Choi et al., 2010; Hamlin et al., 2009; Igelstrom et al., 2010). Using the ST/GT model, we have identified the PVT as a critical node that mediates the propensity to attribute predictive or incentive motivational value to reward cues (Campus et al., 2019; Haight & Flagel, 2014; Haight et al., 2015; Haight et al., 2017; Kuhn, Campus, et al., 2018; Kuhn, Klumpner, et al., 2018). Further, our work suggests that goal-tracking behavior is facilitated via a cortico-thalamic-striatal pathway (see Figure 1.3) (Campus et al., 2019), whereas sign-tracking behavior is guided by hypothalamic-thalamic-striatal pathway (see Figure 1.3) (Flagel, Cameron, et al., 2011; Haight et al., 2017). We know from chemogenetic studies that manipulations of the PrL-PVT pathway impact dopamine activity in the striatum (i.e., the NAc); and we know that dopamine activity is critical for incentive learning (Flagel, Clark, et al., 2011). Focusing on subcortical systems, we most

recently demonstrated that orexin receptor antagonism in the PVT and lesions of the LH both attenuate sign-tracking behavior (Haight et al., 2020). Our combined findings led us to develop a neural model of sign-tracking (Figure 1.3); whereby orexinergic transmission from the LH-PVT encodes the incentive salience of cues, altering communication in the PVT-NAcSh pathway. Together, these findings point to the PVT as a critical node in the regulation of distinct cue-reward learning strategies, with the LH-PVT-NAc circuit playing a specific role in incentive motivational processes.

1.6 Summary

Individual differences in associative learning strategies can be captured through the sign-tracker/goal-tracker model (Figure 1.1). These divergent behavioral responses reflect neurobiological differences that can confer vulnerabilities to psychopathological disorders such as substance use disorder, OCD, PTSD, or even eating disorders. An imbalance between cortical-control and subcortical drive are believed to influence the propensity to attribute incentive salience to reward cues. Thus, there is a "top-down" versus "bottom-up" view of sign- and goal-tracking behaviors, their associated traits, and their respective "cognitive-motivational" styles. It is believed that the "bottom-up" systems gate incentive salience attribution and therefore, sign-tracking behavior (Figure 1.3). Notably, sign-tracking, but not goal-tracking, behavior is dopamine-dependent, and cue-elicited dopamine in the nucleus accumbens (NAc) is thought to encode the value of reward cues. Further, prior findings have led us to postulate that orexinergic projections from the lateral hypothalamus (LH) to the paraventricular nucleus of the thalamus (PVT) relay the incentive salience of reward-associated cues (Figure 1.2). The role of these subcortical targets in sign- and goal tracking behavior will be the focus of this dissertation in which we employed: optogenetic inhibition of cue-elicited dopamine in the ventral tegmental

area (Chapter 2), chemogenetic inhibition of the LH-PVT pathway during the expression of a conditioned response (Chapter 3), and immunohistochemical analysis of the overlap between orexinergic neurons that project from the LH-PVT and are activated in response to a reward-cue.

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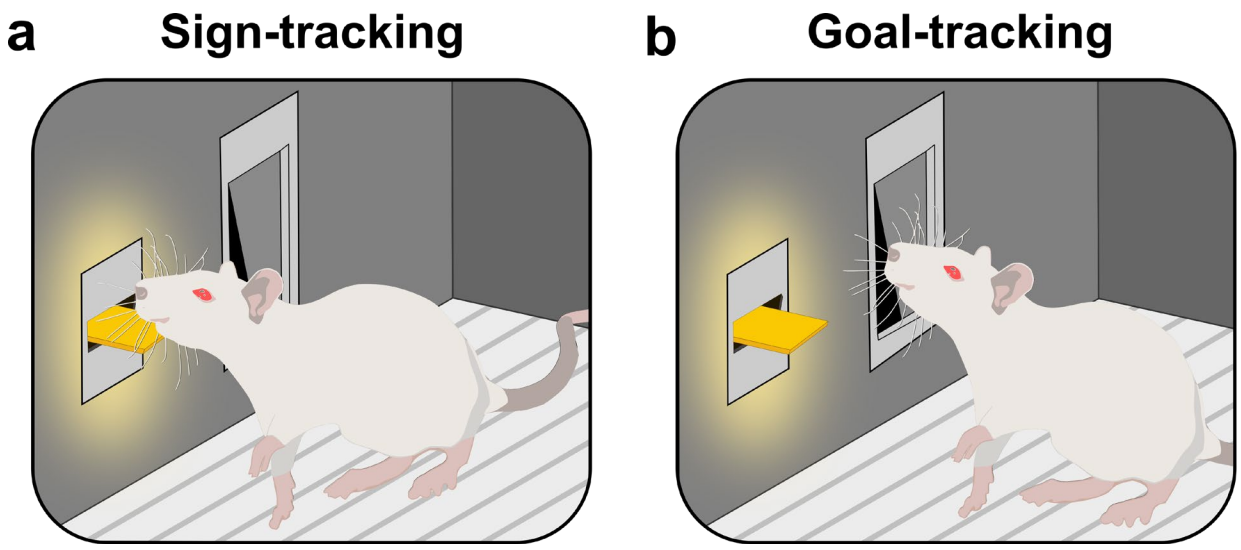

Figure 1-1 **Schematic representation of sign- and goal-tracking behaviors.**

(a,b) Behavioral chambers outfitted with a lever-cue on the left and a food cup (i.e. location of reward delivery) in the center. Examples of (a) sign-tracking and (b) goal-tracking behaviors in response to lever-cue presentation during a Pavlovian conditioning session. (a) Sign-trackers approach the lever-cue upon its presentation while (b) goal-trackers approach the food cup upon lever-cue presentation.

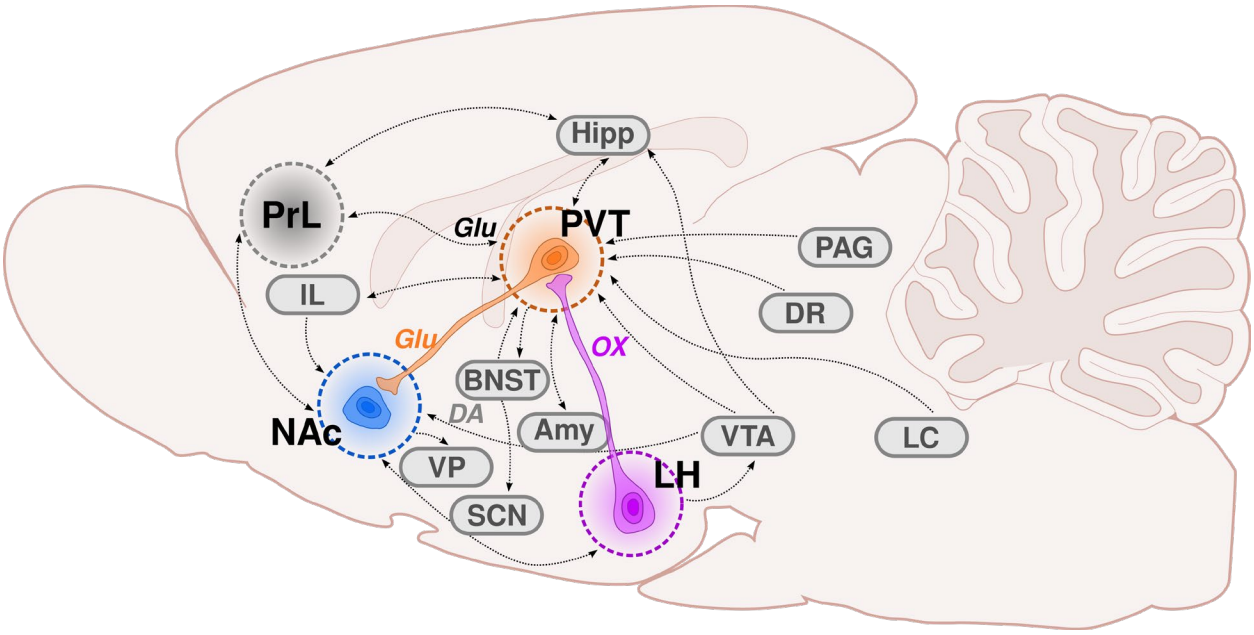

Figure 1-2 **The PVT as a critical node of the hypothalamic-thalamic-striatal circuit.**

This graphic illustrates the afferents and efferents associated with the paraventricular nucleus of the thalamus (PVT), with an emphasis on the hypothalamic-thalamic-striatal circuit. The purple neuron represents orexinergic (OX) innervation from the lateral hypothalamus (LH)-PVT. The orange neuron is illustrating glutamatergic (Glu) innervation from the PVT-nucleus accumbens (NAc). The blue circle depicts the NAc, which receives substantial innervation from the PVT. The gray circle represents the anatomical location of the prelimbic cortex (PrL), which sends dense glutamatergic innervation to the PVT. The dotted black lines throughout the schematic depict neuronal connections, some of which are reciprocal (double arrow). Amy, amygdala; BNST, bed nucleus of the stria terminalis; DR, dorsal raphe; Hipp, hippocampus; IL, infralimbic cortex; LC, locus coeruleus; LH, lateral hypothalamus; NAc, nucleus accumbens; PAG, periaqueductal gray; PrL, prelimbic cortex; PVT, paraventricular thalamic nucleus; SCN, suprachiasmatic nucleus; VP, ventral pallidum; VTA, ventral tegmental area; DA, dopamine; Glu, glutamate; OX, orexin.

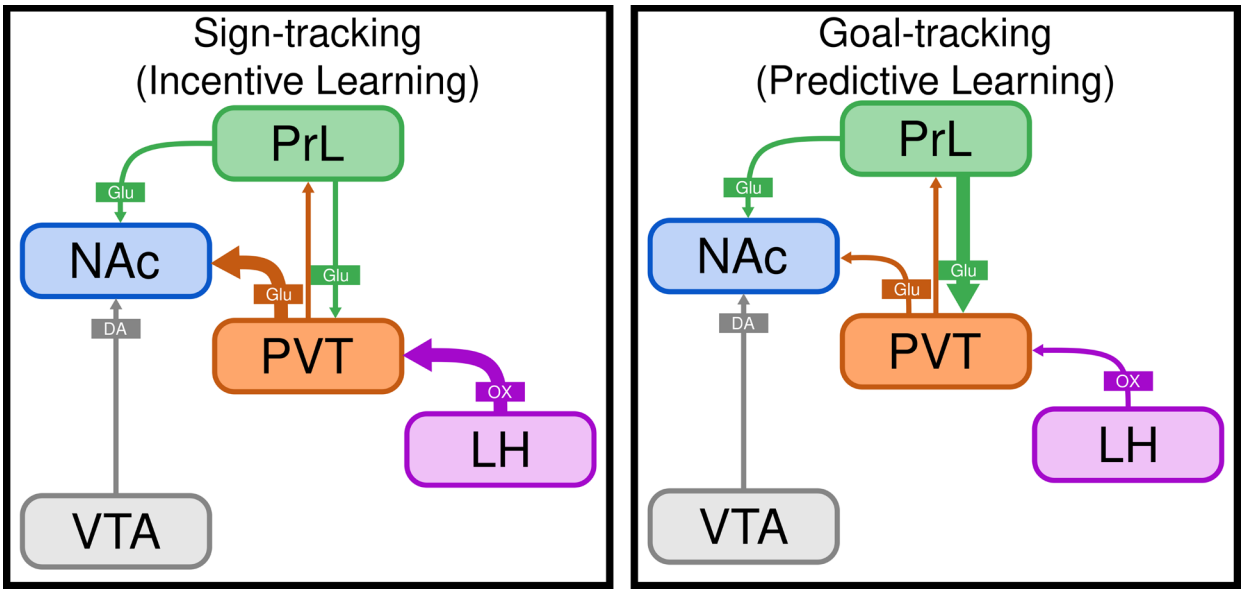

Figure 1-3 **The PVT differentially mediates sign- and goal-tracking behavior.**

Schematic illustrating the paraventricular nucleus of the thalamus (PVT) as a central locus that acts to differentially regulate sign-tracking and goal-tracking behavior. Sign-tracking is a result of incentive value (i.e. incentive salience) placed on reward-cues, whereas goal-tracking is the result of predictive value placed on reward cues. We hypothesize that the incentive salience of reward cues is encoded in the LH-PVT-NAc circuit (as indicated by thick purple and brown arrows), which is engaged to a greater degree in sign-trackers. In contrast, goal-trackers rely on top-down cortical control mechanisms (as indicated by thick green arrow) to encode the predictive value of reward cues and inhibit incentive motivational processes. LH, lateral hypothalamus; NAc, nucleus accumbens; PrL, prelimbic cortex; PVT, paraventricular thalamic nucleus; VTA, ventral tegmental area; DA, dopamine; Glu, glutamate; OX, orexin.

Chapter 2
Inhibition of Dopamine Neurons Prevents Incentive Value Encoding of a Reward Cue: With Revelations from Deep Phenotyping

Note: Much of the text and figures within Chapter 2 have appeared in a manuscript (Iglesias AG, Chiu AS, Wong J, Campus P, Li F, Liu ZN, Bhatti JK, Patel SA, Deisseroth K, Akil H, Burgess CR, Flagel SB. (2023) Inhibition of dopamine neurons prevents incentive value encoding of a reward cue: With revelations from deep phenotyping. J Neurosci., JN-RM-0848-23. doi: 10.1523/JNEUROSCI.0848-23.2023. Epub ahead of print. PMID: 37709540.) and is reproduced here with permission from the authors.

2.1 Abstract

The survival of an organism is dependent on its ability to respond to cues in the environment. Such cues can attain control over behavior as a function of the value ascribed to them. Some individuals have an inherent tendency to attribute reward-paired cues with incentive motivational value, or incentive salience. For these individuals, termed sign-trackers, a discrete cue that precedes reward delivery becomes attractive and desirable in its own right. Prior work suggests that the behavior of sign-trackers is dopamine-dependent, and cue-elicited dopamine in the nucleus accumbens is believed to encode the incentive value of reward cues. Here we exploited the temporal resolution of optogenetics to determine whether selective inhibition of ventral tegmental area (VTA) dopamine neurons during cue presentation attenuates the propensity to sign-track. Using male tyrosine hydroxylase (TH)-Cre Long Evans rats it was

found that, under baseline conditions, ~84% of TH-Cre rats tend to sign-track. Laser-induced inhibition of VTA dopamine neurons during cue presentation prevented the development of sign-tracking behavior, without affecting goal-tracking behavior. When laser inhibition was terminated, these same rats developed a sign-tracking response. Video analysis using DeepLabCut revealed that, relative to rats that received laser inhibition, rats in the control group spent more time near the location of the reward cue even when it was not present and were more likely to orient towards and approach the cue during its presentation. These findings demonstrate that cue-elicited dopamine release is critical for the attribution of incentive salience to reward cues.

2.2 Introduction

Associative learning strategies are utilized daily by humans and animals alike to make situational decisions. Such strategies often rely on cues, or stimuli, in the environment to guide behavior and can directly impact the survival of an organism. In rodents, individual differences in cue-motivated behaviors can be captured using a Pavlovian conditioned approach (PavCa) paradigm, wherein presentation of a discrete cue (conditioned stimulus, CS) is followed by delivery of a food reward (unconditioned stimulus, US) (Flagel et al., 2009). Following PavCa training, two distinct phenotypes emerge – goal-trackers (GT) and sign-trackers (ST) (Boakes, 1977; Hearst, 1974; Robinson & Flagel, 2009). While both GTs and STs attribute predictive value to the reward cue, STs also attribute incentive value to the cue. The attribution of incentive motivational value, or incentive salience, transforms the cue itself into an attractive and desirable stimulus (Berridge & Robinson, 2003). For STs, both food- and drug-associated cues gain appreciable incentive value and thereby the ability to elicit maladaptive behaviors (Saunders & Robinson, 2010, 2011; Yager et al., 2015; Yager & Robinson, 2013). The ST/GT model,

therefore, can be harnessed to elucidate the neurobiological mechanisms that encode the predictive versus incentive value of reward cues. Further, this model can help us better understand the neural processes that contribute to shared symptomatology between psychiatric disorders, as an increased propensity to attribute incentive salience to reward cues (i.e. to sign-track) has been associated with externalizing behaviors and deficits in executive control in both rodents and humans (Colaizzi et al., 2023; Flagel et al., 2010; Phillips & Sarter, 2020).

Dopamine has been implicated in a number of psychiatric disorders, predominantly via its role in learning, attention, and motivation (Grace, 2016; Howes & Kapur, 2009; Nestler & Carlezon, 2006; Volkow et al., 2017). However, the precise role of dopamine remains a subject of debate, especially as it pertains to reward processing and learning about stimuli in the environment (Berke, 2018; Berridge, 2007; T. N. Lerner et al., 2021; Schultz et al., 2017). While dopamine has long been considered a prediction error signal (Schultz et al., 1997), used to update the predictive value of reward-cues during associative learning, deficiencies in this theory have been recognized (Jeong et al., 2022; Kutlu et al., 2021; Saunders et al., 2018; Sharpe et al., 2020). Of particular relevance, the sign-tracker/goal-tracker model previously revealed that intact dopamine signaling is necessary for the attribution of incentive salience to reward cues, or what we refer to here as Pavlovian "incentive learning", and not the encoding of predictive value alone, or "predictive learning" (Flagel et al., 2011; Saunders & Robinson, 2012; Yager et al., 2015). Both systemic and local (nucleus accumbens core) blockade of dopamine receptors prevents the acquisition and expression of a sign-tracking conditioned response with no effect on goal-tracking (Flagel et al., 2011; Saunders & Robinson, 2012). Additionally, using fast-scan cyclic voltammetry, it was shown that dopamine-encoded "prediction error" signals are present in the nucleus accumbens core of sign-trackers, but not goal-trackers (Flagel et al., 2011).

Together, these findings led to the conclusion that dopamine encodes the incentive value of Pavlovian reward cues. However, it was not clear from these studies whether dopamine neuron activity precisely at the time of cue presentation is necessary for incentive value encoding and the acquisition of sign-tracking behavior. To address this question, we exploited the temporal resolution of optogenetics. Specifically, we utilized tyrosine hydroxylase (TH)-Cre rats to selectively inhibit dopamine neurons in the ventral tegmental area (VTA) during cue presentation early in Pavlovian training. We found that male TH-Cre Long Evans rats have an inherent tendency to sign-track, and that optogenetic inhibition of VTA dopamine neurons during cue presentation blocks this tendency, without affecting goal-tracking behavior. An in-depth analysis of behavior using DeepLabCut (Mathis et al., 2018) revealed that the effects of this manipulation were time-locked and specific to cue-elicited incentive motivation.

2.3 Materials and Methods

2.3.1 General Methods

2.3.1.1 Subjects

In total, one hundred twenty-eight male Long Evans rats were received from a breeding colony maintained by the Akil laboratory (University of Michigan, Ann Arbor, MI). Only male rats were used for these studies, which are a direct follow-up to prior studies that had been conducted with male rats (Flagel et al., 2011; Saunders & Robinson, 2012). The breeding colony originated in 2013 with two TH-Cre male Long Evans rats from the Deisseroth laboratory (Stanford University, Stanford, CA). These transgenic TH-Cre rats were developed utilizing BAC constructs wherein the Cre gene was inserted before the ATG (autophagy-related) sequence of the mouse TH gene (Witten et al., 2011). Rats were initially characterized for fidelity of the

transgene through confirmatory electrophysiological and optogenetics experiments described by Witten and colleagues. The colony has since been maintained by breeding TH-Cre Long Evans male rats with wild type (WT) Long Evans female rats. Rats were bred and weaned in either the Biological Science Research Building or the Molecular and Behavioral Neuroscience Institute building (University of Michigan, Ann Arbor, MI) and transferred to the Flagel laboratory in the Molecular and Behavioral Neuroscience Institute building around postnatal day (PND) 46. Rats were housed under a 12-hour light-dark cycle (lights on at 6 or 7 AM depending on daylight saving time) with climate-controlled conditions (22 ± 2ºC) and had ad-libitum access to food and water throughout the study. They were pair- or triple-housed prior to surgery, and single-housed following surgery and for the duration of the experiment. Rats were acclimated to the housing room for a minimum of one week before experimenter handling began. Behavioral testing took place during the light-phase between 10 AM and 4 PM. All procedures followed The Guide for the Care and Use of Laboratory Rats: Eighth Edition (2011, National Academy of Sciences) and were approved by the University of Michigan Institutional Animal Care and Use Committee.

2.3.1.2 Pavlovian conditioned approach (PavCA)

Prior to assessing Pavlovian conditioned approach (PavCA) behavior, rats were briefly handled by the experimenters for 2-4 days. As described below, a single pretraining session preceded PavCA sessions. For the two days prior to pre-training rats received ~25 banana-flavored grain pellets (each pellet 45-mg; Bio-Serv, Flemington, NJ, USA) in their home cage to acquaint them with the reward.

PavCA testing occurred inside Med Associates chambers (St. Albans, VT, USA; 24.1 × 21 x 30.5 cm) located in sound-attenuating boxes equipped with a fan to reduce background noise (Figure 2c). The chambers contained a magazine that was connected to a pellet dispenser

and placed in the center of one wall 3 cm above the chamber floor. An illuminated retractable lever (i.e. lever-cue) was located either to the left or right of the magazine (Med Associates, ENV-200R2M-6.0), 6 cm above the chamber floor. The magazines used were taller (2 x 6 in) than the standard (2 x 2 in) to allow rats to access food pellets without interference from their headcap and tethers. A white house light was placed at the top of the chamber on the wall opposite to the magazine and lever-cue and remained on for the duration of the session. Magazine entries were recorded by a break of the photo beam inside the cup. Lever-cue contacts were recorded following a minimum force of 10 g.

Prior to each session the rats were transferred to the testing room in their home cage. They were left in the room for a minimum of 30 min to allow them to acclimate. Rats were initially placed into the Med Associates chambers for a single pretraining session. At the start of the pretraining session, the food magazine was baited with two food pellets to direct the rats' attention to the location of reward delivery. During pretraining the lever-cue remained retracted, and rats received a food pellet in the food magazine on a variable 30 s (range 0-60 s) schedule. There was a total of 25 trials and the pretraining session lasted approximately 12.5 min wherein head entries were recorded, and food pellet consumption confirmed. Following pretraining rats had a single session of PavCA each day for either 5 (colony characterization) or 6 (optogenetic inhibition) consecutive days. The start of each PavCA session began with a 5-min waiting period followed by the house light turning on which signified the start of the session. As previously described (Campus et al., 2019; Meyer et al., 2012), during PavCA, the illuminated lever-cue (conditioned stimulus, CS) entered the chamber for 8 s and upon retraction a food pellet (unconditioned stimulus, US) was immediately delivered into the adjacent food magazine. PavCA sessions consisted of 25 lever-cue (CS)/ food-US trials on a variable 90-s schedule (range

30–150 s). Each session lasted approximately 40 min. It was confirmed that all food pellets had been consumed following each session.

Med Associates software recorded the following behaviors during PavCA sessions: 1) number of food magazine contacts made during the 8-s lever-cue presentation, (2) latency to contact the food magazine during lever-cue presentation, (3) number of lever-cue contacts, (4) latency to lever-cue contact, and (5) the number of food magazine entries made during the intertrial interval (i.e., food magazine contacts made in between lever-cue presentations). Contact and latency data were used to calculate the PavCA Index. As previously described (Meyer et al., 2012), the PavCA Index is a composite measure calculated using the following formula: [Probability Difference Score + Response Bias Score + (-Latency Difference Score)/3]. PavCA Index scores range from −1 to 1, with a score of −1 representing individuals with a conditioned response (CR) focused solely on the food magazine during lever-cue presentation (i.e., a "pure" goal-tracker) and a score of +1 representing individuals with a CR focused solely on the lever-cue upon its presentation (i.e., a "pure" sign-tracker) (Figure 1a). For colony characterization, the PavCA Index from sessions 4 and 5 were averaged to assess the frequency distribution of sign-trackers (range of 0.5 - 1) and goal-trackers (range of -0.5 - -1.0), as shown in Figure 1b.

2.3.2 Behavioral characterization of the Long Evans colony

2.3.2.1 Subjects

To determine the inherent behavioral phenotypes of the transgenic rats, a subset of male Long Evans (n=57) and TH-Cre (n=38) rats underwent "baseline" characterization of PavCA behavior (Figure 1). The WT rats were from 2 generations and 15 litters, and the TH-Cre rats were from 2 generations and 7 litters. Just over half of the WT rats (n=31) had sham surgery

prior to “baseline” characterization, whereas the remaining WT (n=26) and TH-Cre rats were left undisturbed prior to “baseline” characterization.

2.3.2.2 Sham surgery

Rats were around PND 90 at the time of surgery. All rats had anesthetic induction with 5% isoflurane (Vet One, Boise, ID) delivered via an induction chamber and were given an injection of carprofen (5 mg/kg, subcutaneous (s.c.)) for analgesia during surgery. Sham surgery consisted of levelling rats in the stereotaxic frame, shaving and cleaning the scalp, and drilling two holes directly above the VTA (bilaterally from bregma, AP -5.76; ML ± 2.98). A 1 μL Hamilton Neuros Syringe was lowered into the holes (from bregma, DV -8.4) and pulled up after 10 min. The surgical site was closed with clips (Stoelting, Wood Dale, IL) which were removed 7-10 days following surgery. After the clips were removed experimenter handling and behavioral testing procedures began.

2.3.2.3 Statistical analyses

Statistical analyses were conducted with the Statistical Package for the Social Sciences (SPSS) program version 27.0 (IBM, Armonk, NY, USA). To compare differences in the PavCA Index among the colony (Figure 1a), a linear mixed-effects model (LMM) with restricted maximum likelihood estimation was used. This analysis applies multiple covariance structures to the data set and the structure with the lowest Akaike information criterion (AIC) was selected as best fit (Duricki et al., 2016; Verbeke, 1997). LMM was conducted to compare genotypes and groups across sessions 1-5. Session was used as the repeated variable and genotype/group as the between-subjects variable. For one analysis the WT group was split into rats that were Naïve (WT Naïve) and those that received Sham surgery (WT Sham), and for another analysis, the WT groups were collapsed and compared to TH-Cre rats. To assess the differences in the relative

proportions of phenotypes between the genotypes in the Long Evans colony, a Fisher's Exact Test was conducted on the PavCA Index (Figure 1b). For all analyses, statistical significance was set at $p < 0.05$, and Bonferroni post hoc comparisons were made when significant main effects or interactions were detected.

2.3.3 Optogenetic inhibition of the VTA

2.3.3.1 Subjects

To determine the effects of optogenetic inhibition of dopamine neurons during Pavlovian cue-reward learning (Figure 2), 33 male TH-Cre rats from 3 generations and 11 litters were used. Some rats were excluded for not consuming pellets during pretraining (n = 4), poor virus expression/probe placement (n = 8), or head caps coming off prematurely (n = 4). Due to technical issues, session 4 data was lost for one rat in the halorhodopsin group and session 5 data was lost for one rat in the control group. Data from these two rats are included in the analyses for other sessions. In total, 17 out of 33 rats are included in the behavioral analyses assessing the effects of optogenetic inhibition, with 10 in the halorhodopsin group and 7 in the control group. For DeepLabCut analyses, three of these rats were excluded due to technical issues with video capturing, resulting in 8 in the halorhodopsin group and 6 in the control group.

2.3.3.2 Viral vectors

A Cre-dependent inhibitory optogenetic construct halorhodopsin (eNpHR, AAV5- Ef1a-DIO eNpHR 3.0-EYFP at titer $\geq 1\times10^{13}$ vg/mL, Addgene plasmid # 26966) or an empty vector (control, AAV5- Ef1a-DIO EYFP at titer $\geq 1\times10^{13}$ vg/mL, Addgene plasmid # 27056) were utilized. Both plasmids were provided by Dr. Karl Deisseroth and obtained from Addgene.

2.3.3.3 Virus and optogenetics probe implant surgery

Rats were around PND 90 at the time of surgery. All rats had anesthetic induction with 5% isoflurane (Vet One, Boise, ID) delivered via an induction chamber and were given an injection of carprofen (5 mg/kg, subcutaneous (s.c.)) for analgesia during surgery. Rats were placed into a stereotaxic frame and the scalp was shaved and cleaned. Two holes were drilled directly above the VTA (bilaterally from bregma, AP -5.76; ML ± 2.98). Four additional holes were drilled ± 2 mm ML from bregma. 2.4 mm stainless-steel screws (Plastics One, Roanoke County, VA) were secured into the four holes. A Hamilton Syringe (5 µL Model 85 RN, Small Removable Needle, 26s gauge, 2 in, point style 2) was placed into a pump (Harvard Apparatus Pump 11 Elite, Holliston, MA) and then connected to P50 tubing and a guide cannula (Plastics One, Roanoke County, VA) that screwed onto a 10 mm injector (Plastics One, Roanoke County, VA). A Cre-dependent inhibitory optogenetic construct (halorhodopsin, eNpHR) or Cre-dependent control virus (EYFP) was bilaterally injected into the VTA at a 10º angle (from bregma, AP -5.76; ML ± 2.98; DV -8.4) at a rate of 100 nL per min over a 10-min period (1 µL total) (Figure 2a). The injector remained in place for an additional 10 min. After diffusing, fiber optic implants were inserted 0.3 mm above the injection site at a 10º angle (from bregma, AP -5.76; ML ± 2.98; DV -8.1, Figure 2a). Fiber optic implants were made in house and consisted of 200 µm-diameter optic fibers (Thor Labs, Newton, NJ) inserted into 10.5-mm-long ferrules (Thor Labs, Newton, NJ). Only fibers above 85% laser emittance were used for surgery. The fiber optic implants were secured with acrylic cement (Bosworth New Truliner, Keystone Industries, Gibbstown, NJ). The plastic screw from a guide cannula (Plastics One, Roanoke County, Virginia) was placed at the most anterior portion of the headcap as the acrylic cement was drying (later used for securing the rats headcap during behavior). A 3-4-week period for

virus incubation followed surgery (Figure 2d). Prior to testing, the acrylic headcap was covered in black pet safe nail polish (Warren London Pawdicure Dog Nail Polish Pen) to occlude the laser light from illuminating the behavioral chamber.

2.3.3.4 PavCA sessions and laser parameters

Rats received ~25 banana-flavored grain pellets (each pellet 45 mg; Bio-Serv, Flemington, NJ, USA) in their home cage for the two days prior to testing. Before each session, the plastic screw at the front of the headcap was connected to a reinforced cannula spring (Plastics One, Roanoke, VA) and the optogenetic probes cleaned with ethanol and bilaterally connected to individual optogenetic cables that were secured with a ceramic mating sleeve (Thor Labs, Newton, NJ).

Rats had 1 pretraining session followed by 6 PavCA sessions. For the first 3 PavCA sessions (trials 1-75), rats received photoinhibition of the VTA continuously during the 8 s lever-cue presentation via a 593.5 nm Yellow DPSS Laser (Shanghai Laser & Optics Century CO., Ltd., Shanghai, China) (Figure 2b-d). Parameters known to be effective for inhibiting dopamine neurons were used (Gradinaru et al., 2010; McCutcheon et al., 2014). Laser power was calibrated to ~10 mW/mm2 from the tip of the optogenetic cables before each session. Cables were also tested after each session to ensure laser power was consistent throughout. For sessions 4-6 (trials 76-150), rats were connected to the reinforced cannula spring and optogenetic cables as described above, but the laser was turned off (i.e., no photoinhibition occurred on sessions 4-6). Each rat had 3 PavCA sessions of "laser on" followed by 3 PavCA sessions of "laser off", across 6 consecutive days.

2.3.3.5 Video analysis

Videos were obtained from session 3 of PavCA and additional behavioral metrics were obtained from experimenter observation and using DeepLabCut, as described below. DeepLabCut allowed us to verify experimenter observations and behavioral data obtained from Med Associates, and enhanced the granularity with which we can assess the effects of optogenetic inhibition on individual rats.

PavCA orienting response. An experimenter assessed whether a rat oriented towards the lever-cue or food magazine for each trial of session 3. An orienting response was defined as a head movement directed towards the lever-cue or food magazine at any point during the 8.2 sec lever-cue presentation. The probability of approaching either the lever-cue or food magazine was then calculated as the number of trials with an orienting response (to either the lever-cue or food magazine)/25. In addition, the percentage of trials on which an orienting response was directed towards the lever-cue, food magazine, or both was determined.

DeepLabCut. Session 3 videos were further analyzed using DeepLabCut (DLC) and custom MATLAB (R2021b) scripts. Raw videos were processed in Adobe Premiere Pro to increase contrast and enhance brightness within the behavioral chambers. Videos in this dataset on average contained 35,000 frames of which 75 frames were extracted for training. Training videos were from session 1. Labeling of all videos for training was completed by two experimenters who were blind to experimental groups. Each video was manually labeled with the following markers on the rats' body which were used for the planned analyses: tether, nose, left-shoulder, right-shoulder, and tail base (see Figure 7a). The food magazine in the chamber was also labeled. Training was conducted via DeepLabCut 2.1.10.4 downloaded from GitHub (https://github.com/DeepLabCut/DeepLabCut) and installed onto University of Michigan, Great

Lakes Computing Cluster. Following training of the network, locations of each marker for images analyzed on separate videos were extracted with a p-cutoff-parameter of 0.8 (see representative video, Multimedia File 1). Videos for analysis that failed to meet criteria (>10 outlier frames) were reanalyzed following relabeling of outlier frames and retraining of the network. Three videos were excluded from analyses due to camera angle (one control rat and one halorhodopsin rat) or an occluded camera (one halorhodopsin rat).

Data extracted from session 3 videos were post-processed in MATLAB and aligned with Med Associates data. Linear and spline interpolation was applied in addition to DLC filtering to further smooth pose estimation coordinates for frames during which body markers were not visible due to lighting or occlusion. Timepoints of each lever-cue presentation per trial were extracted from analyzed videos to validate alignment to lever-cue presentation based on Med Associates data. The following behaviors were extracted and analyzed in MATLAB: time in zone (food magazine or lever-cue), head orientation to food magazine or lever-cue, approach behavior (approach bouts) towards food magazine or lever-cue, locomotion, and latency to approach the food magazine. The length of the time bins for illustration and analyses were different across outcome measures to better capture behaviors that were time-locked to cue-presentation or retraction, and/or those that occurred during the intertrial intervals. Further, the reference body part differs for some of the measures as described below. For time in zone, body position was tracked by following the rats' headcap tether throughout the chamber. The tether was chosen as the reference point due to the robustness of this marker tracking, prior to interpolation.

For heatmap analyses, 8.2-s time bins were chosen to capture each rat's location immediately before lever-cue presentation (Figure 7b), during lever-cue presentation (Figure 7c),

immediately after lever-cue presentation, (Figure 7d), and during the intertrial interval (Figure 7e). This time bin reflects the duration of each lever-cue presentation; the lever is out for 8 s, and it takes 0.1 sec for the lever to present and 0.1 sec for the lever to fully retract. Each heatmap (time in zone, Figure 7b-e) represents the average location of rats across all trials on session 3, integrated over 123 video frames (8.2-s time bins) for each period of interest. A 2D Gaussian smoothing kernel with a standard deviation of 3 was applied to smooth the heatmap images (Figure 7b-e).

Head orientation (Figure 8a) was tracked by angle changes between two vectors: 1) from the points between the rats' nose to headcap tether and 2) from the rats' headcap tether to the food magazine. For head orientation, the center of the food magazine was the reference point, and the videos were assessed across 4-s time bins during different phases of the session: 4-s before lever-cue presentation (Figure 8b), the first 4-s during lever-cue presentation (Figure 8c), 4-s before pellet delivery (Figure 8d), the 4-s immediately after pellet delivery (Figure 8e), and 4 s during the ITI (Figure 8f). These data are illustrated as the average head direction for each individual rat and as group means (Figure 8).

For approach behavior (Figure 9), body position was tracked by following the rats' nose, the reference body part for this metric, throughout the chamber for the entire session. An approach bout was counted when the rats nose remained within 75 pixels (1 cm) of the lever-cue (Figure 9a-b) or food magazine (Figure 9c-d) for more than 15 frames. An approach bout ended when the interbout interval exceeded 15 frames (1 s). Approach directed towards the lever-cue or food magazine was quantified during the 8.2-s lever-cue presentation (Figure 9a,c), and during the intertrial interval (i.e., after lever-cue retraction) (Figure 9).

Locomotion was captured as the velocity (cm/s) and distance travelled (cm) based on tracking the rats' center of mass, the point between the rat's right and left shoulders. Velocity was quantified for the 4-s period immediately before and during lever-cue presentation (Figure 10a). The distance from the lever-cue during the 2-s-period preceding lever-cue presentation and the first 2-s during lever-cue presentation was also assessed (Figure 10b). Upon lever-cue retraction, the latency to approach the food magazine (Figure 10c) was analyzed as an index of activity when dopamine neurons were no longer inhibited.

2.3.3.6 Code Accessibility

DLC settings, desktop parameters, and code for performing post-processing reconstructions and analysis are made publicly available on GitHub: https://github.com/alvchiu/THCREOpto-dlc.

2.3.3.7 Perfusion and tissue processing

Rats were perfused within 5 days following the experiment. Rats were first anesthetized with ketamine (90 mg/kg, intraperitoneal (i.p.)) and xylazine (10 mg/kg, i.p.) and then transcardially perfused with 0.9% saline and 4% formaldehyde (pH = 7.4). Following brain extraction, the tissue was post-fixed in 4% formaldehyde for 24 hours at 4ºC and then placed in 30% sucrose at 4ºC (sucrose in 0.1M PBS, pH = 7.4) for 3 days. The brains were frozen using dry ice and coated in a Tissue-Plus Optimal Cutting Temperature compound (Fisher HealthCare, Houston, TX). Coronal brain slices were taken at 40 µm using a cryostat at -20ºC (Leica Biosystems Inc, Buffalo Grove, IL). The whole brain was collected, and slices were placed into well plates containing cryoprotectant and then stored at −20°C. Slices with the VTA were isolated, mounted onto SuperFrost Plus microscope slides (Fisher Scientific), and cover slipped with DAPI as a counterstain (diluted 1:5000 in 90% glycerol). Images were captured using a

Zeiss AxioImager M2 motorized fluorescent microscope (Carl Zeiss, Sweden). Fluorescent images of endogenous virus expression and optogenetic probe placement were evaluated by two experimenters blind to the experimental groups (Figure 2d, representative image). Virus expression was evaluated based on distinct localized cell body expression of enhanced yellow fluorescent protein (EYFP, virus tag) within the VTA (e.g., Figure 2d) and probe placements were confirmed if they were visualized bilaterally within the VTA (Figure 3b, c).

2.3.3.8 Histology

Tyrosine hydroxylase (TH) was utilized as a proxy for Cre expression in the VTA was evaluated following immunofluorescent staining of TH. Immunohistochemical procedures took place at room temperature and each step was followed by 3 washes of 0.1M phosphate buffered saline (PBS) for 5 min each. Sections were blocked with 2.5% normal donkey serum (NDS; Jackson ImmunoResearch) + 0.4% Triton X-100 (TX) + 0.1M PBS for 1 hour, then incubated overnight in the primary antibody solution (rabbit anti-TH, Abcam, ab16453) diluted 1:500 in 0.1M PBS + 0.4% TX + 1% NDS. The next day, sections were incubated in the secondary antibody solution containing biotinylated donkey anti-rabbit antibody (Jackson ImmunoResearch, 711-065-152), diluted 1:500 in 0.1M PBS + 0.4% TX + 1% NDS, for 2 hours. Sections were incubated with Streptavidin, Alexa Fluor 594 conjugated (Thermo Fisher Scientific, S11227), diluted 1:1000 in 0.1M PBS for 1 hour. Slides were mounted and cover slipped as described above. Z-stack and mosaic images were captured using a FV3000 confocal microscope and the FV31S-SW Viewer software (OLYMPUS Microscopes, Center Valley, PA, USA). Single channel and triple labelled fluorescent images of EYFP (green), TH (red), and 4',6-diamidino-2-phenylindole (DAPI, cobalt) to stain nuclear DNA are represented in Figure 3a.

2.3.3.9 Statistical analyses

A linear mixed-effects model (LMM) with restricted maximum likelihood estimation was used to assess PavCA behavioral outcome measures across sessions. When two sessions were directly compared a two-way ANOVA or t-test was performed, as described below. A simple linear regression analysis was used to test if the Acquisition Index (Δ PavCA Index between sessions 3 and 1) predicted the Final Index (the PavCA Index on session 6). For all analyses, statistical significance was set at $p < 0.05$, and Bonferroni post hoc comparisons were made when significant main effects or interactions were detected. Effect size (Cohen's d, (Cohen, 1988) was calculated for pairwise comparisons. Effect sizes were considered with respect to the following indices: 0.2, small; between 0.5 – 0.8, medium; between 1.2 – 2.0, large (Cohen, 1988; Sawilowsky, 2009).

LMM was conducted to compare experimental groups across sessions 1-3 ("laser on") or sessions 4-6 ("laser off"). That is, sessions 1-3 or sessions 4-6 were used as the repeated variable and experimental group (control vs. halorhodopsin) was used as the between-subjects variable. For lever-directed behaviors, a LMM was also conducted to compare control sessions 1-3 ("laser on") to halorhodopsin sessions 4-6 ("laser off"). A two-way ANOVA was conducted when session 3 ("laser on") was directly compared to session 6 ("laser off"), with session (3 and 6) as the within subject independent variable and experimental group (control or halorhodopsin) as the between subject independent variable. Differences in the number of lever-cue contacts (dependent variable) between sessions 1 and 4 were analyzed using an unpaired t-test (control session 1 vs halorhodopsin session 4) or a paired t-test (control session 1 vs control session 4 or halorhodopsin session 1 vs halorhodopsin session 4). For both the LMM and ANOVA, lever-directed behaviors (number of lever-cue contacts, probability to approach the lever-cue, latency

to approach the lever-cue), food magazine-directed behaviors (food magazine contacts during lever-cue presentation, probability to approach the food magazine during lever-cue presentation, latency to approach the food magazine during lever-cue presentation), and food magazine entries during the intertrial interval (ITI, non-CS food magazine head entries) were used as dependent variables.

Behavioral output from video analyses were also statistically analyzed. A two-way ANOVA was conducted to assess orienting responses directed towards the lever-cue or food magazine on session 3, with experimental group (control or halorhodopsin) and location (lever-cue or food magazine) as the independent variables. For the data generated by DLC, a Kolmogorov-Smirnov (KS) two-sample test compared the distributions for head direction responses (dependent variable) between experimental groups (control or halorhodopsin) at the following 4-s periods: 1) immediately before lever-cue presentation, 2) the first 4 s of lever-cue presentation, 3) the last 4 s of lever-cue presentation), 4) immediately after pellet delivery, and 5) during the intertrial interval. Differences in approach bouts (each bout $\geq$ 1 s, dependent variable) between experimental groups (control or halorhodopsin) towards the lever-cue or food magazine 1) during lever-cue presentation and 2) after lever-cue retraction were analyzed using unpaired t-tests. Differences in locomotor activity and latency to approach the food magazine between experimental groups (control or halorhodopsin) were analyzed using unpaired t-tests. A two-way repeated measures ANOVA was conducted to compare the velocity of movement during the 4-s period before lever-cue presentation and the first 4-s during lever-cue presentation, with time as the within subject independent variable and experimental group (control or halorhodopsin) as the between subject independent variable. A two-way repeated measures ANOVA was also conducted to compare distance from the lever-cue during the 2-s period immediately before

lever-cue presentation and the first 2 s during lever-cue presentation, with time as the within subject independent variable and experimental group (control or halorhodopsin) as the between subject independent variable.

2.4 Results

2.4.1 Behavioral characterization of the Long Evans transgenic rat colony

2.4.1.1 PavCA distribution

The tendency to sign- or goal-track (without optogenetic manipulation) was assessed in wild type (WT) and TH-Cre littermates from our in-house breeding colony. There were no significant differences in the PavCA Index across sessions 1-5 when comparing WT Naïve vs WT Sham vs TH-Cre (Table 1). Further, PavCA Index did not differ significantly across sessions between WT rats that did or did not receive sham surgery (Fisher's exact test, $p = 0.60$); thus, these groups were collapsed for visualization (Figure 1a, b) and further analyses. While there were not robust differences between WT and TH-Cre rats across sessions, there was a significant group x session interaction ($F_{(4,172.373)} = 3.175$, $p = 0.015$; Table 1) and post-hoc analyses revealed that TH-Cre rats had a higher PavCA Index on session 5 relative to WT rats ($p = 0.040$, Cohen's $d = 0.46$; Figure 1a). Out of the total population of rats that were screened (N = 95), ~84% were sign-trackers. Of the WT (n = 57 in total) rats ~81% were sign-trackers and of the TH-Cre (n = 38 in total) rats, ~89% were sign-trackers; but the population distribution between WT and TH-Cre rats was not significantly different (Fisher's exact test, $p = 0.40$; Figure 1b). These data suggest that this colony of Long Evans male rats are skewed towards sign-trackers, regardless of genotype. This skew in the population provides an opportunity to assess the impact

of neuronal manipulations on the attribution of incentive salience to reward cues and thereby the development of sign-tracking behavior.

2.4.2 Optogenetic inhibition of the VTA

2.4.2.1 Effects of optogenetic inhibition during lever-cue presentation in PavCA Discussion

To assess the role of dopamine in the attribution of incentive salience to a reward cue, we expressed an inhibitory opsin (eNpHR) in dopamine neurons in the VTA of TH-Cre rats (Figures 2 and 3). Disrupting cue-elicited dopamine through optogenetic inhibition of the VTA reduced lever-directed behaviors. During sessions 1-3, when the laser was turned on concurrently with lever-cue presentation, there was a significant effect of experimental group and/or a group x session interaction for all measures of lever-directed behavior (Table 2a). As shown in Figure 4a, while rats in the control group increased the number of lever-cue contacts across sessions 1-3, rats in the halorhodopsin group did not (group x session interaction: $F(2,15.773) = 4.119$, $p = 0.036$; effect of session for control group: $F(2,15.279) = 4.418$, $p = 0.031$). Post hoc comparisons revealed a significant reduction in the number of lever-cue contacts among halorhodopsin rats compared to control rats on session 3 ($p = 0.02$, Cohen's $d = 1.27$; Figure 4a). In agreement with this, the probability to approach the lever-cue was lower among halorhodopsin rats relative to control rats across sessions 1-3 (effect of group: $F(1,15.405) = 4.954$, $p = 0.041$) and only those in the control group showed a significant increase in the probability to approach the lever-cue across sessions (group x session interaction: $F(2,18.848) = 5.237$, $p = 0.016$; effect of session for control group: $F(2,24.073) = 5.730$, $p = 0.009$; Figure 4b). Post hoc analyses revealed that control rats had a significant increase in the probability to approach the lever-cue on session 3 relative to session 1 ($p = 0.008$, Cohen's $d = 0.95$). Consistent with the results described above, the latency to approach the lever-cue significantly decreased across sessions 1-3 in control rats,

but not those in the halorhodopsin group (effect of group: $F(1,15.396) = 4.620$, $p = 0.048$; group x session interaction: $F(2,15.436) = 4.513$, $p = 0.029$; effect of session for control group: $F(2,18.848) = 6.053$, $p = 0.009$; Figure 4c). Again, post hoc comparisons revealed that the group differences were most apparent on session 3, when halorhodopsin rats took more time to approach the lever-cue relative to control rats ($p = 0.008$, Cohen's $d = 1.34$; Figure 4c). Thus, for all these measures, the difference between groups was most apparent on session 3, after control rats began to exhibit sign-tracking conditioned response.

The impact of VTA dopamine neuron inhibition was specific to lever-directed behaviors and did not affect goal-directed behaviors during sessions 1-3. Figures 4d-f illustrate the lack of differences between experimental groups for the number of food magazine contacts during lever-cue presentation, the probability to approach the food magazine during lever-cue presentation, and the latency to approach the food magazine during lever-cue presentation during "laser on" sessions (see also Table 2c).

Consistent with the data above, experimenter observation of the videos from session 3 revealed that the probability to orient towards the lever-cue significantly differed between experimental groups, whereas the probability to orient towards the food magazine did not (group x location interaction: $F(1,28) = 4.788$, $p = 0.039$; effect of group for lever-cue: $F(1,24) = 5.538$, $p = 0.027$; Figure 5a). As shown in Figure 5b, rats in the control group oriented to both the lever-cue and food magazine on approximately 46% of trials, whereas those in the halorhodopsin group did so on approximately 36% of trials. Orientation to both the lever-cue and food magazine on a given trial suggests that neither rats in the control group nor the halorhodopsin group were extreme sign-trackers or goal-trackers by session 3, and this is consistent with the data shown in Figure 4b. These data might also suggest that the value of the lever-cue has not

been fully learned by session 3. Nonetheless, a conditioned orienting response is apparent for rats in both groups and inhibition of VTA dopamine neurons selectively affects the response directed towards the lever-cue.

Importantly, the effects of VTA dopamine neuron inhibition did not extend to behavior during the intertrial interval, as there were no significant differences in head entries into the food magazine in between trials during sessions 1-3 (Table 2e). Further, every rat consumed all of the food pellets that were delivered each session.

2.4.2.2 Effects of lifting optogenetic inhibition in later sessions

In the absence of VTA dopamine neuron inhibition and laser presentation (sessions 4-6), there were no significant differences in lever-directed behaviors between halorhodopsin and control rats. For lever-directed behaviors, there were no significant differences between experimental groups for lever-cue contacts, probability to approach the lever-cue or latency to approach the lever-cue on sessions 4-6 (Figures 4a-c, Table 2b). Relative to rats in the halorhodopsin group, however, control rats showed a trend towards a greater probability to approach the lever-cue (effect of group: $F(1,14.997) = 4.157$, $p = 0.059$) and a decreased latency to approach the lever-cue (effect of group: $F(1,15.095) = 4.074$, $p = 0.062$; Figure 4b,c). As shown in Figure 4a-c, without VTA dopamine neuron inhibition during lever-cue presentation, the halorhodopsin rats began to exhibit lever-directed behaviors comparable to control rats. In support, the "learning curve" for lever-directed behaviors did not differ between rats in the control group on sessions 1-3 and those in the halorhodopsin group on sessions 4-6 (Table 2g), when there was no laser inhibition. Further, there were no significant differences in lever-directed behaviors on session 1 for control rats relative to session 4 for halorhodopsin rats, and only rats in the control group had a significant increase in lever-cue contacts between session 1

and 4 ($t(6) = -2.62$, $p = 0.04$). Taken together, these data demonstrate that rats in the halorhodopsin group did not attribute incentive salience to the lever-cue when they were receiving cue-paired laser inhibition, but once laser inhibition was removed, they were fully capable of doing so. Thus, VTA dopamine neuron activity is necessary for encoding the incentive value of reward cues.

There were no significant group differences on sessions 4-6 in head entries, probability, or latency to approach the food magazine during lever-cue presentation (Figure 4d-f, Table 2d). While all rats tended to decrease the number of head entries into the food magazine during the intertrial interval across sessions 4-6 (effect of session: $F(2,30) = 3.593$, $p = 0.04$; Table 2f) those in the control group tended to do so less than those with prior VTA dopamine neuron inhibition (effect of group, $F(1,15) = 4.476$, $p = 0.05$, Table 2f). These data suggest that rats in the halorhodopsin group may be less efficient in retrieving food pellets, and this is consistent with delayed entry into the food magazine upon lever-cue retraction, as presented below (Figure 10c).

2.4.2.3 Comparing "laser on" vs. "laser off" sessions

To further assess differences in behavior as a function of VTA dopamine neuron inhibition, we directly compared session 3, the last PavCA session with laser inhibition, to session 6, the last PavCA session without laser inhibition. Rats in both the control group and halorhodopsin group showed an increase in lever-directed behaviors on session 6 relative to session 3 (Figure 4a-c, Table 3). Relative to rats in the halorhodopsin group, however, rats in the control group had a higher probability to approach the lever-cue (effect of group, $F(1,15) = 4.623$, $p = 0.048$; Figure 4b) and had a tendency to do so more quickly (effect of group, $F(1,15) = 3.917$, $p = 0.066$). There were no significant differences in food magazine-directed behaviors between session 3 vs. session 6 (Figure 4d-f).

We also assessed whether cue-paired inhibition of dopamine neuron activity limited the ability of behavior during sessions 1-3 to predict subsequent behavior (Figure 6). Indeed, we found that the change in behavior from session 1 to session 3 (i.e. Acquisition Index) predicted the PavCA Index on session 6 (i.e. Final Index) in control rats ($F(1,5) = 9.629$, $p = 0.027$), but not in halorhodopsin rats ($F(1,8) = .549$, $p = 0.480$). The Acquisition Index was an excellent predictor ($\beta = .811$, $p = 0.027$) and explained 65.8% (adjusted $r2 = 0.658$) of the variance in the Final PavCA Index among controls. However, inhibition of dopamine neuron activity during lever-cue presentation restrained the predictive power ($\beta = .253$, $p = 0.480$), as only 6.4% (adjusted $r2 = 0.064$) of the variance in Final Index could be explained by the Acquisition Index in halorhodopsin rats.

2.4.3 Deep phenotyping expands behavioral analysis

Behavioral video analysis with DeepLabCut confirms that perturbing cue-elicited dopamine reduces multiple facets of cue-directed behaviors. Video analyses from session 3, the final session of VTA dopamine neuron inhibition, were partitioned into different periods to assess location and time spent near (Figure 7), orientation to (Figure 8), and approach towards (Figure 9) the lever-cue or food magazine, as well as locomotor activity throughout the session (Figure 10).

2.4.4 Location in chamber

Time spent in locations of the behavior chamber were analyzed in 8.2-s time bins, reflective of the period from lever-cue presentation to retraction. As indicated by the heatmaps shown in Figure 7b, in the 8.2 s period before the lever-cue was presented, control and halorhodopsin rats were found throughout the chamber with a tendency to gather near the food

magazine. Once the lever-cue was presented rats in the control group appeared to spend more time near and around the lever-cue (Figure 7c), while those in the halorhodopsin group were around the food magazine or in other locations in the chamber. Immediately after lever retraction, rats in both groups spent more time at the location of food delivery (Figure 7d). A subtraction analysis further illustrates the difference in time spent near the lever-cue and food magazine for rats in the halorhodopsin group compared to those in the control group (Figure 7, right panel).

2.4.5 Head orientation

DeepLabCut analysis of head orientation revealed a trend toward a significant difference between groups in the 4-s preceding lever-cue presentation (KS test, $p = 0.065$), with rats in the control group showing a greater tendency to orient to the side of the chamber containing the lever-cue and food magazine (Figure 8b). During the first 4 s of lever-cue presentation, rats in the control group preferentially oriented towards the lever-cue relative to those in the halorhodopsin group (KS test, $p = 0.034$; Figure 8c). During the last 4 s of lever-cue presentation, there was a trend for a significant difference in head orientation between groups (KS test, $p = 0.087$; Figure 8d).There were no significant differences in head orientation to the lever-cue location or food magazine after pellet delivery (KS test, $p = 0.328$; Figure 8e) or during the intertrial interval (KS test, $p = 0.118$; Figure 8f). These data are consistent with those presented above and with the experimenter-scored videos (Figure 5). We demonstrate that inhibition of VTA dopamine neurons impacts orientation towards the lever-cue upon its presentation, without affecting orientation towards the food magazine.

2.4.6 Approach behavior

Approach towards the lever-cue and food magazine was assessed as an additional metric that is not captured by the Med Associates output, but one that is a hallmark of incentive salience attribution (Berridge, 2001; Cardinal et al., 2002). Approach behavior was counted when the rats' nose was within 1 cm of either the lever-cue or food magazine for more than 1-s. Relative to rats in the control group, those in the halorhodopsin group showed less approach behavior towards the lever-cue upon its presentation ($t(12) = 4.911$, $p < 0.001$; Figure 9a). Interestingly, the same was true during the intertrial intervals (i.e. after the lever had been retracted) ($t(12) = 3.753$, $p = 0.003$; Figure 9b), reflecting a general tendency for rats in the control group to spend more time by the lever-cue location (see also Figure 7d). There were no significant differences between groups for approach towards the food magazine at any time point (i.e., during lever-cue presentation ($t(12) = -0.079$, $p = 0.939$; Figure 9c) or after lever-cue retraction ($t(12) = -0.788$, $p = 0.446$; Figure 9d).

2.4.7 Locomotor activity

Importantly, the experimental groups did not display significant differences in non-specific locomotion during session 3 ($t(12) = 0.406$, $p = 0.692$; data not shown). Upon lever-cue presentation, however, control rats displayed a significant increase in the speed of movement relative to the time period prior to lever-cue presentation ($p < 0.001$, Cohen's $d = 1.73$; Figure 10a). This change in velocity was not apparent in halorhodopsin rats (effect of group: $F(1,24) = 20.202$, $p < 0.001$, effect of time: $F(1,24) = 13.509$, $p = 0.001$, group x time interaction: $F(1,24) = 7.444$, $p = 0.012$); and post hoc comparisons revealed that control rats move to the lever-cue faster than halorhodopsin rats ($p < 0.001$, Cohen's $d = 0.26$). When lever-cue-elicited locomotion was evaluated as the distance from the lever-cue 2 s before and 2 s during presentation there was

a significant effect of time ($F(1,836) = 115.680$, $p < 0.001$), but as shown in Figure 10b, the lever-cue-elicited locomotion was more apparent in rats in the control group relative to those in the halorhodopsin group (effect of group: $F(1,836) = 1135.491$, $p < 0.001$; group x time interaction: $F(1,836) = 47.958$, $p < 0.001$; Figure 10b). Post hoc comparisons revealed that rats in the control group moved closer to the lever-cue relative to those in the halorhodopsin group both before ($p < 0.001$, Cohen's $d = 0.01$) and after lever-cue presentation ($p < 0.001$, Cohen's $d = 0.045$). Interestingly, relative to those in the control group, rats in the halorhodopsin group were delayed in approaching the food magazine once the lever-cue had been retracted ($t(12) = -2.409$, $p = 0.03$; Figure 10c). This delay could potentially be due to reduced salience of the food magazine as a result of VTA dopamine neuron inhibition (DiFeliceantonio & Berridge, 2012; Mahler & Berridge, 2009). Nonetheless, all rats consumed all of the food pellets and there were no significant differences between groups in general locomotor activity. Thus, these data support the notion that cue-elicited dopamine neuron activity in the VTA plays a selective role in encoding the incentive value of reward cues.

2.5 Discussion

It is known that VTA dopamine neuron activity is involved in reward processing, however, the precise contributions of dopamine in terms of temporal specificity and value encoding remain a subject of debate (for review see: (Berke, 2018; Berridge, 2007; Berridge, 2012; Stauffer, 2018; Triche et al., 2022; Zhang et al., 2009). Here we capitalized on the temporal precision of optogenetics and utilized a transgenic rat colony with a tendency to sign-track to further explore the role of dopamine in reward learning. We demonstrate that inhibition of VTA dopamine neuron activity during presentation of a discrete cue that predicts reward delivery prevents incentive value encoding. Specifically, inhibition of VTA dopamine neurons

during lever-cue presentation precludes the attribution of incentive motivational value to the reward cue and thereby blocks the development of a sign-tracking conditioned response. Detailed analysis of behavior using DeepLabCut reinforced the specificity of these effects, revealing that locomotor activity was not affected by selective inhibition of VTA dopamine neuron activity, nor was orientation or approach directed towards the location of reward delivery. Further, when VTA dopamine neuron activity was restored, rats developed a sign-tracking conditioned response indicative of incentive salience attribution. These data are in agreement with prior studies demonstrating that dopamine is essential for incentive learning and the acquisition and expression of sign-tracking behavior (Saunders & Robinson, 2012; Yager et al., 2015).

The role of dopamine in reward processing has been presented within the context of multiple learning theories and frameworks, some of which are in direct opposition (Berridge, 2007; Berridge, 2012; Gershman & Uchida, 2019; Langdon et al., 2018; Talia N. Lerner et al., 2021; Schultz, 2019). The long-prevailing view that dopamine encodes the predictive value of reward cues and reflects a universal learning signal (Schultz et al., 1997) has been met with conflicting data. With the use of new technologies to further probe the role of dopamine in reward learning (for review see (de Jong et al., 2022), it has been elegantly demonstrated that dopamine promotes associative learning (Sharpe et al., 2020) and encodes perceived saliency independent of valence (Kutlu et al., 2021), even when conditions are ripe for prediction error signals. Further, using a novel computational approach, it was shown that dopamine conveys causal associations without reward prediction error (Jeong et al., 2022). Our prior work with the sign-tracker/goal-tracker animal model is consistent with these more recent findings. We demonstrated that dopamine in the core of the nucleus accumbens encodes the incentive

properties of reward cues, not the predictive (Flagel et al., 2011). That is, the classic prediction error shift in dopamine from the reward (unconditioned stimulus) to the reward cue (conditioned stimulus) occurs in sign-trackers, but not goal-trackers. If dopamine were merely a predictive learning signal, the shift in dopamine would have been evident in both sign-trackers and goal-trackers, as the reward-cue is a predictor and elicits a conditioned response for both. Moreover, blockade of dopamine signaling prevented the learning of a sign-tracking conditioned response, but not goal-tracking (Saunders & Robinson, 2012).

Here we demonstrate that inhibition of VTA dopamine neurons selectively during lever-cue presentation prevents the attribution of incentive salience to the lever-cue and thereby precludes the development of a sign-tracking response. Upon restoration of VTA dopamine neuron activity, the same rats attributed incentive value to the lever-cue in a manner that was indistinguishable from rats in the control group during their initial Pavlovian training sessions. Consistent with these findings, dopamine neurons in the VTA are activated to a much greater extent in sign-trackers relative to goal-trackers during lever-cue interaction (Ferguson et al., 2020) and VTA dopamine neuron stimulation can itself transform a predictive stimulus into an incentive stimulus (Saunders et al., 2018). Specifically, neurons projecting from the VTA to the core of the nucleus accumbens were found to be especially important for incentive value encoding (Saunders et al., 2018); but others have reported that dopamine within the nucleus accumbens shell encodes incentive salience (Saddoris et al., 2015). The current findings add to the growing body of literature that supports a role for dopamine neurons in the VTA in incentive value encoding.

Dissociating the effects of cue-paired inhibition of VTA dopamine neurons on encoding the predictive versus incentive value of reward cues is complex. To better assess the effects of

this manipulation on predictive learning we evaluated the conditioned orienting response, which is indicative that a stimulus-reward relationship has been learned (Buzsaki, 1982). Experimenter observation and DeepLabCut analyses revealed that rats in both groups exhibited a conditioned orienting response directed towards the lever-cue and/or food magazine, but only lever-cue oriented responses were affected by VTA dopamine neuron inhibition. These findings are seemingly in contrast to prior studies that have shown that the conditioned orienting response directed towards the lever-cue remains intact in sign-trackers following administration of a dopamine antagonist into the core of the nucleus accumbens, and that only approach and interaction with the lever-cue is attenuated (Saunders & Robinson, 2012; Yager et al., 2015). It is important to note, however, that the manner in which we assessed conditioned orienting differs from these studies. For example, rats in the current study were not habituated to the presentation of the lever-cue in the absence of reward delivery (as in Yager et al., 2015). Further, we assessed conditioned orienting behavior across all trials on session 3, presumably as the value of the lever-cue was still being learned, whereas other studies assessed it at the time an extreme sign-tracking response was evident (Saunders & Robinson, 2012; Yager et al., 2015) and only during the latter half of the session (Saunders & Robinson, 2012). It is also possible that, in the current study, the lever-directed conditioned orienting response is affected via inhibition of dopamine neuron activity in non-striatal regions, such as the prefrontal cortex (Swanson, 1982), which is known to play a role in "cognitive" or model-based learning (Dayan & Balleine, 2002; Dickinson & Balleine, 2002; Ioanas et al., 2022; Kuhn et al., 2018; Morrens et al., 2020; Swanson, 1982). Regardless, our findings are consistent with the conclusion that dopamine neuron activity is necessary for attributing incentive salience and transforming a predictive stimulus into a "motivational magnet", but not for learning the stimulus-reward relationship.

Based on the current findings and those previously published (Flagel et al., 2011; Saunders & Robinson, 2012), we would not expect cue-paired inhibition of VTA dopamine neurons to affect the prepotent conditioned response in goal-trackers. Importantly, however, we were not able to directly assess the effects of VTA dopamine neuron inhibition on the development of goal-tracking behavior as the rats used in this study have an inherent predisposition to sign-track. Indeed, the low levels of goal-tracking behavior might have precluded us from detecting any significant decreases in this behavior as a function of VTA dopamine neuron inhibition. It is not clear why the current population of Long Evans rats is skewed towards sign-trackers, but similar shifts in behavioral phenotypes have been observed in other rat populations bred in house (unpublished). Further, inherent tendencies to sign- or goal-track are known to differ between rats from different vendors (Fitzpatrick et al., 2013; Khoo, 2022) and the same is true for mice of different strains (Dickson et al., 2015). The results reported here are also limited by the fact that only male rats were studied. While this research was based on prior findings using male rats, it will be important to determine if the same neural mechanisms encode the incentive motivational value of reward cues in female rats. To-date, there is little evidence to support robust sex differences in the propensity to attribute incentive salience to a food cue (Pitchers et al., 2015), but female rats tend to be skewed more towards sign-trackers (Hughson et al., 2019) and the underlying neural mechanisms warrant further investigation.

Although locomotor activity did not appear to be generally affected by inhibition of VTA dopamine neuron activity in the current study, it is possible that general motivation was affected in real time. It is difficult to dissociate the effects of this manipulation on the motivation to approach the lever-cue from incentive value encoding. However, if cue-paired inhibition of VTA

dopamine neuron activity were merely affecting motivation, we would expect rats to exhibit higher levels of approach on session 4, when the inhibition was terminated. Instead, we observed a gradual increase in approach to the lever-cue between sessions 4-6, presumably as a function of incentive salience attribution. Further, rats that received cue-paired inhibition showed little change in locomotor activity during inhibition. Finally, the fact that a conditioned orienting response directed towards the food magazine did not differ between groups and that all of the rats consumed all of the food pellets that were delivered suggests that cue-paired inhibition of dopamine neuron activity in the VTA did not generally affect learning or motivation under the current conditions.

While the optogenetic parameters used here have been shown to be effective in silencing dopamine neurons (Gradinaru et al., 2010; McCutcheon et al., 2014), local network excitability in opsin-expressing neurons (Alfonsa et al., 2016; Raimondo et al., 2012) as well as reduced excitability in non-opsin expressing neurons (Parrish et al., 2023) has also been reported following continuous optogenetic inhibition. Further, rebound excitation can induce behavioral changes that are apparent immediately upon laser cessation (Arrenberg et al., 2009). It is therefore possible that the results reported here were affected by rebound excitation, but we do not think this is the case, as there were few differences in behavior between halorhodopsin rats and those in the control group immediately upon laser cessation. Of note, however, halorhodopsin rats were slower to approach the location of reward delivery upon cessation of laser inhibition. This delay in approaching the food magazine upon lever-cue retraction could suggest that the perceived meaning of lever-cue retraction was still being learned. In support, rats in the halorhodopsin group continued to show increased responding in the food magazine relative to those in the control group during the intertrial interval. Further, others have reported

that, relative to goal-trackers, sign-trackers show greater engagement of VTA dopamine neurons upon both the presentation and retraction of the lever-cue (Ferguson et al., 2020). It is also possible that inhibition of VTA dopamine neurons attenuated any incentive motivational value placed upon the food magazine (DiFeliceantonio & Berridge, 2012; Mahler & Berridge, 2009), which could explain the slight increase in goal-directed behaviors on non-laser sessions in halorhodopsin rats. Thus, while we recognize the potential impact of rebound excitation on the reported findings, we do not believe it to significantly affect our interpretation or conclusions.

The current findings expand and enhance the existing literature pertaining to the role of dopamine in reward learning. We clearly demonstrate that cue-paired inhibition of VTA dopamine neuron activity prevents the attribution of incentive motivational value to the reward cue and the development of sign-tracking behavior. Using DeepLabCut, we were able to thoroughly assess metrics of learning and incentive motivation and rule out non-specific effects of VTA dopamine neuron inhibition. This study was designed around the fact that the TH-Cre population we used was skewed towards sign-trackers; yet, this precluded us from assessing the effects of this manipulation in goal-trackers. The findings were interpreted with this individual variability or lack thereof in mind, and we consider this a valuable example for the field to consider. Further, the complexity of behavior and nuances therein are illustrated here and should be noted for those using cutting-edge techniques to decipher brain-behavior relationships. We will continue to capitalize on “deep phenotyping” approaches to assess the effects of similar manipulations in female rats and to better elucidate the neural substrates underlying reward learning and incentive value encoding. Based on the current findings, however, we conclude that cue-elicited dopamine is critical for the attribution of incentive salience to reward cues.

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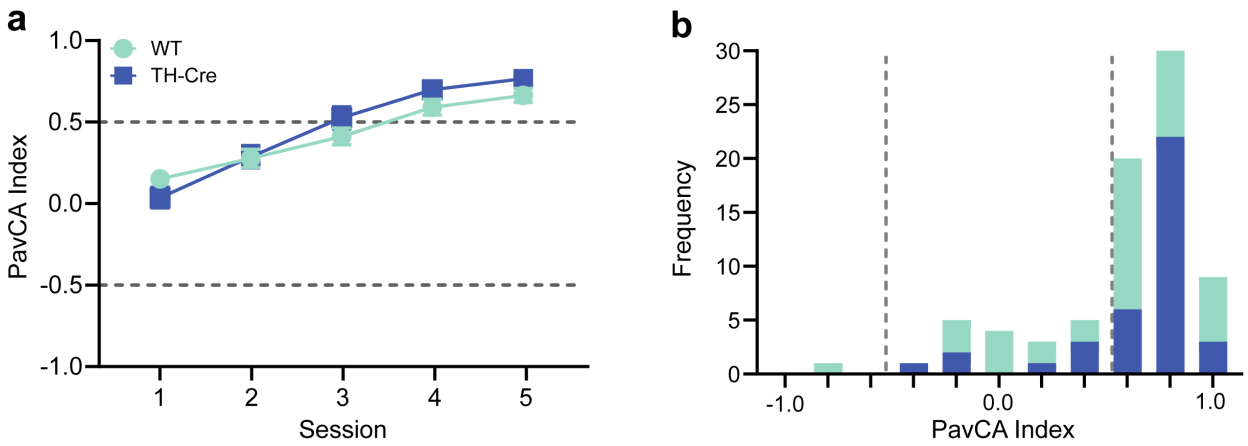

Figure 2-1 **The population of Long Evans male rats are skewed toward sign-trackers.**
(a) Pavlovian conditioned approach (PavCA) Index shown as mean ± SEM across 5 training sessions for WT rats (n = 57) and naïve TH-Cre rats (n = 38). (b) Frequency histogram illustrating the number of rats exhibiting a mean PavCA Index (averaged from sessions 4 and 5) between -1.0 and +1.0 for each of the groups depicted in panel b. 84% of the population (n = 95) was skewed toward sign-trackers (≥ 0.5, n = 46 WT and n = 34 TH-Cre), 14% of the population were intermediate responders (0.5 ≤ -0.5; n = 10 WT and n = 3 TH-Cre), and 2% goal-trackers (≤ -0.5, n = 1 WT and n = 1 TH-Cre).

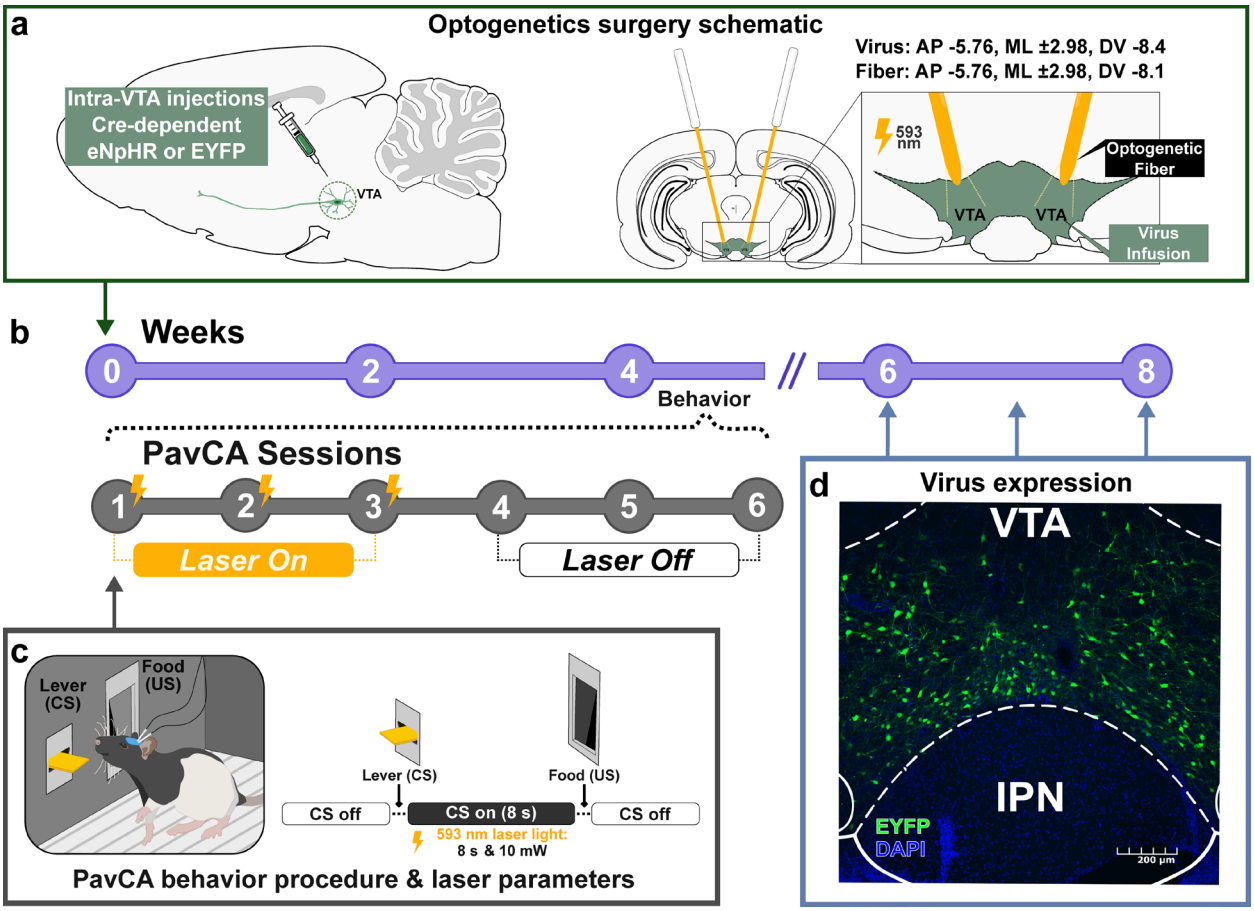

Figure 2-2 **Experimental timeline.**
(a) Schematic illustrating optogenetic viral infusion and fiber placement. 1.0 µl of either eNpHR (halorhodopsin) or EYFP (control) virus was infused and the optogenetic fiber was placed bilaterally in the ventral tegmental area (VTA). (b) Week of experimental timeline represented in purple. Week 4 of the experiment is expanded below in gray to show Pavlovian conditioned approach (PavCA) sessions. (c) Schematic of behavioral paradigm in which lever-cue (CS) presentation (8.2 sec) was paired with laser inhibition of dopamine neurons during PavCA sessions 1-3. The laser remained off for the last 3 PavCA sessions (4-6). (d) Representative image of virus expression in the VTA at 10x magnification depicting fluorescence of enhanced yellow fluorescent protein (EYFP, green) and 4',6-diamidino-2-phenylindole (DAPI, cobalt) to stain nuclear DNA.

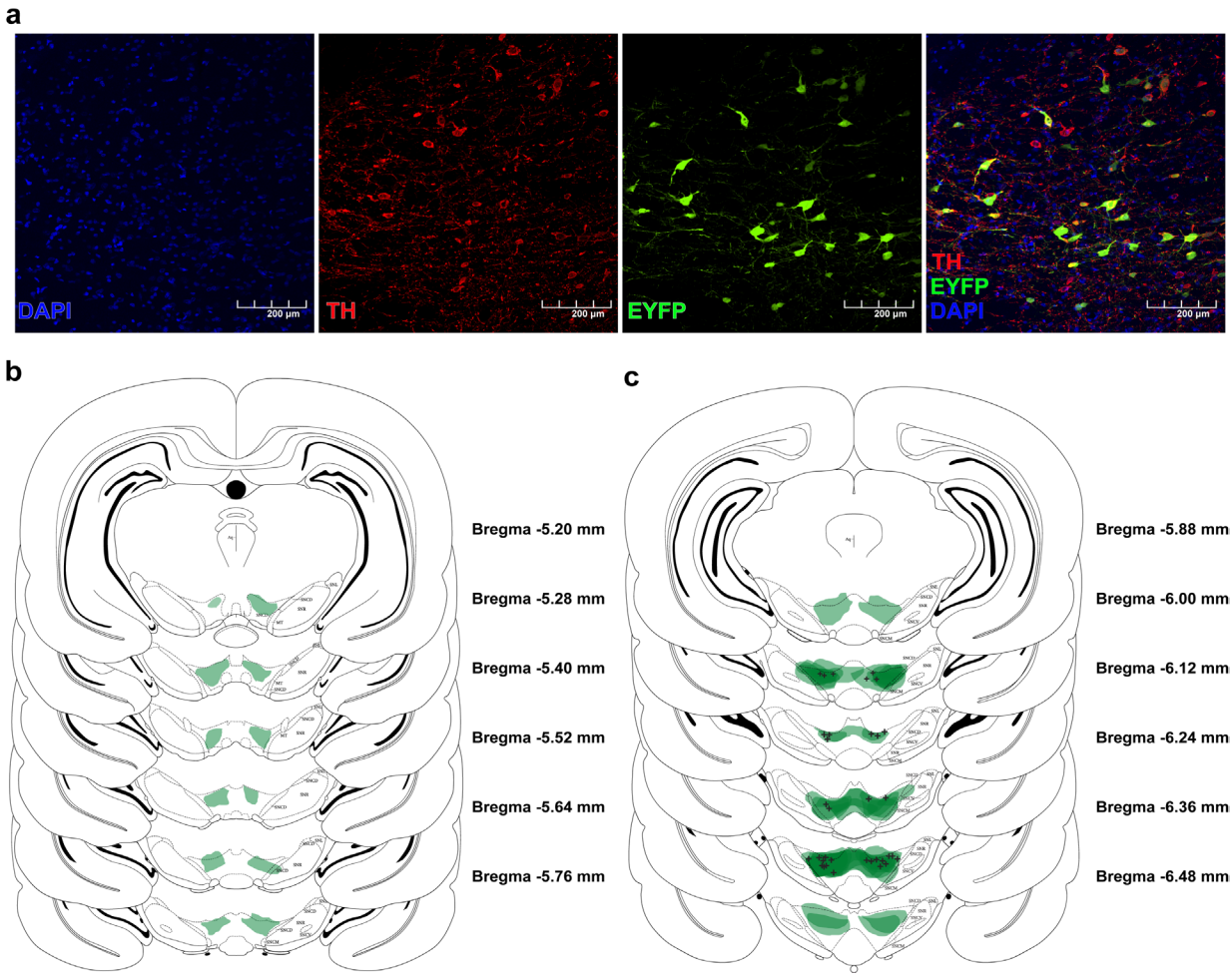

Figure 2-3 **Virus expression and optogenetic probe placement in VTA.**
(a) Representative images of virus expression in the VTA at 20x magnification depicting single channels of DAPI (cobalt), tyrosine hydroxylase (TH, red), and EYFP (green). A merged image of all channels depicts overlap of TH and EYFP in the VTA. Coronal atlas images showing (b) anterior to (c) posterior, relative to Bregma -5.20 to -6.48 are depicted with virus expression spread (in green) and probe placement markers (black + sign) for both experimental groups (EYFP and eNphR). The density of the virus expression is reflected by the different hues of green, with darker color reflecting greater density. The densest virus expression and successful probe placements were between Bregma -6.12 to -6.36.

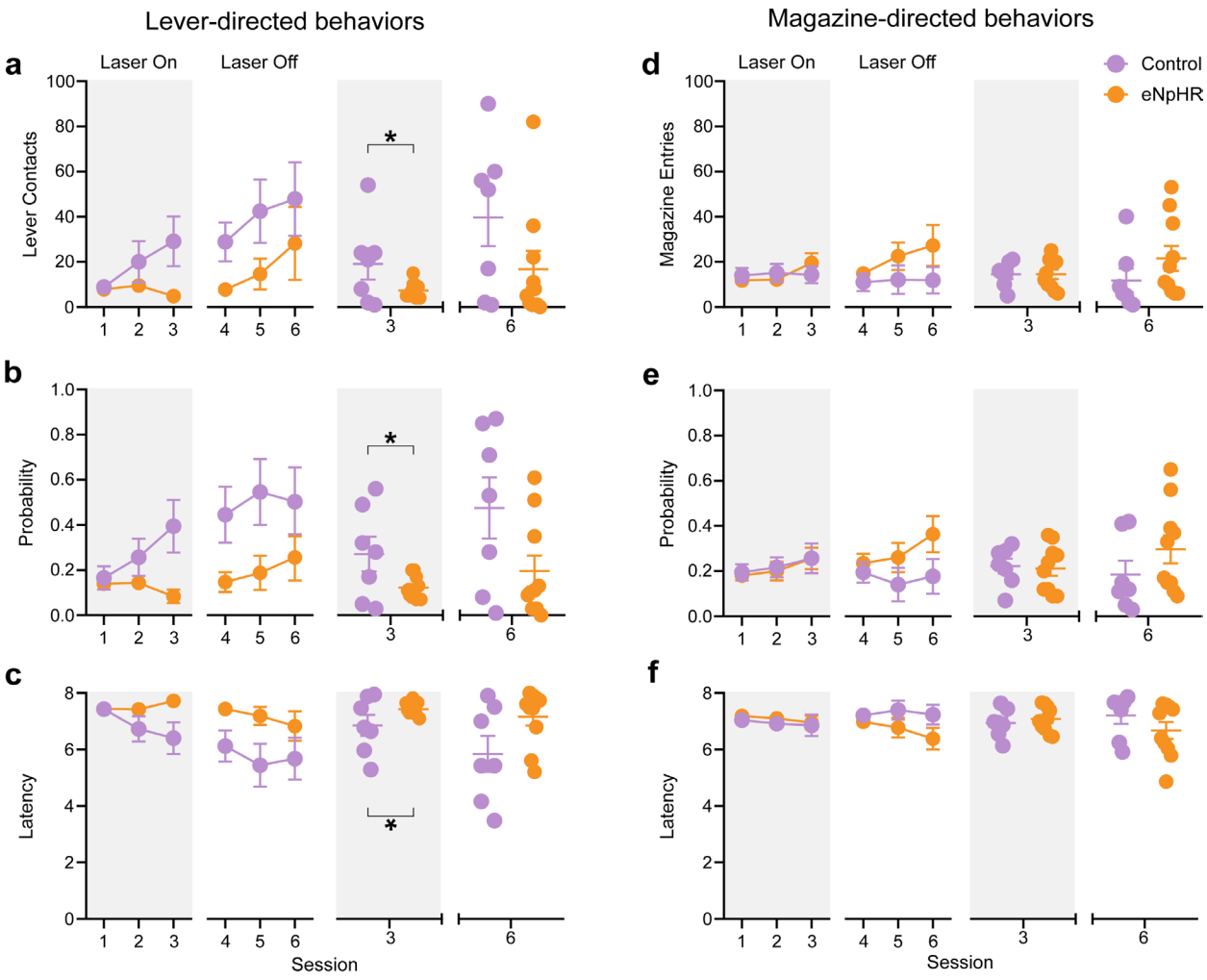

Figure 2-4 **Inhibition of dopamine in the VTA attenuates sign-tracking behavior.**
(a-c) Lever-directed and (d-f) magazine-directed behaviors for sessions 1-3 (laser on) and 4-6 (laser off). (a-f) A comparison of session 3 (laser on) to session 6 (laser off) is to the right of each panel. Data are shown as mean ± SEM for a,d) number of contacts or head entries, (b,e) probability, or (c,f) latency to approach the lever-cue (left) or food magazine (right). (a-c) Optogenetic inhibition of dopamine neurons in the VTA decreases lever-directed behaviors in eNphR rats (n = 10) compared to controls (n = 7) on sessions 1-3. Both groups had similar responding for lever-directed behaviors between sessions 4-6. (d-f) Optogenetic inhibition during sessions 1-3 had no effect on magazine-directed behaviors. Both groups had similar responding for magazine-directed behaviors between sessions 4-6. Bracket indicates significant difference between groups on session 3, $*p < 0.05$.

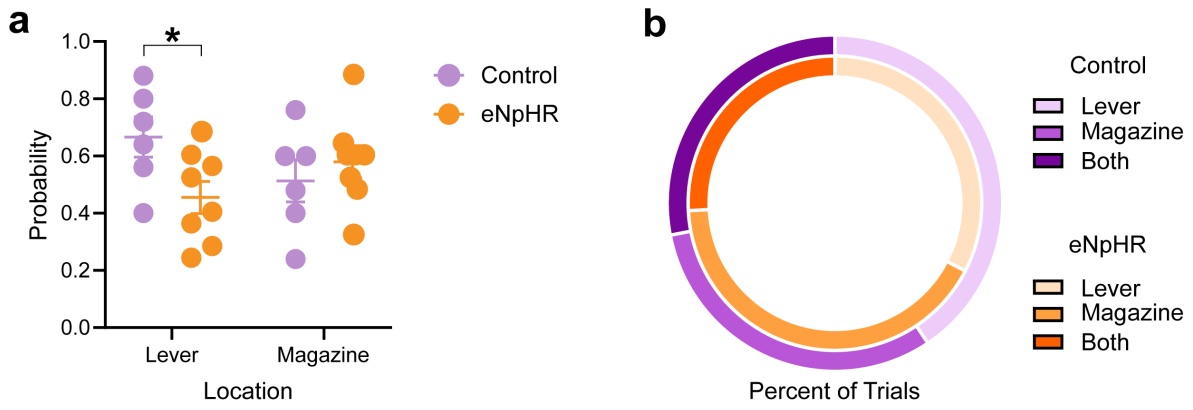

Figure 2-5 **Head orientation to the lever-cue and food magazine during lever-cue presentation.**
(a) Mean ± SEM for probability to orient to the lever-cue or food magazine during lever-cue presentation on session 3 (final day of VTA dopamine neuron inhibition). Rats in the control group oriented more towards the lever-cue than those in the eNpHR group that received VTA dopamine neuron inhibition. (b) Percent of trials that included orientation towards the lever-cue, food magazine, or both for control (purple) and eNpHR (orange) rats. Bracket indicates significant difference between groups on session 3, $*p < 0.05$.

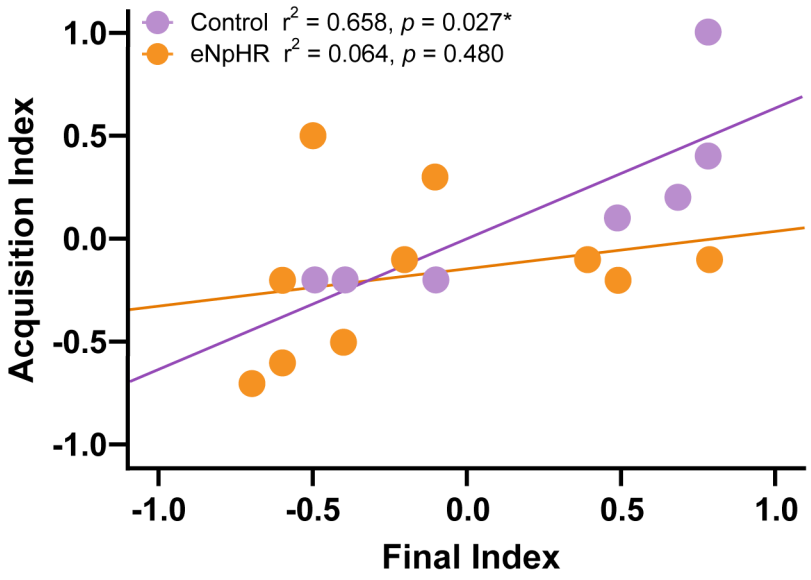

Figure 2-6 **The change in PavCA during Acquisition predicted the Final PavCA Index in control rats.** Individual data points are shown for control (purple, n= 7) and eNphR rats (orange, n = 10) with their respective regression lines. Acquisition Index reflects (Δ PavCA Index between sessions 3 and 1) and the Final Index reflects session 6 PavCA Index. The Acquisition Index significantly predicted the Final PavCA Index in controls, but not in eNphR rats. *$p < 0.05$.

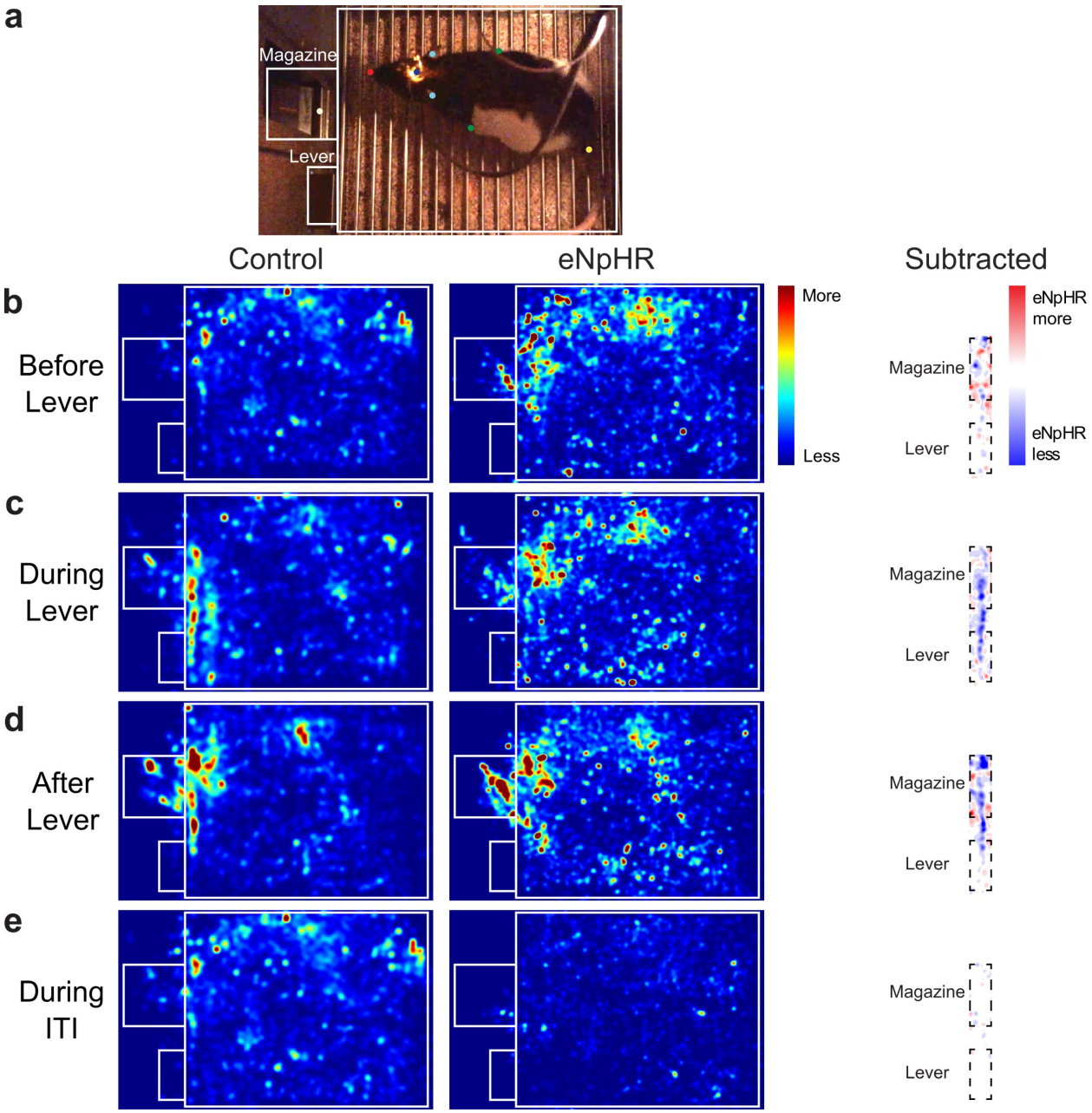

Figure 2-7 **Inhibition of dopamine in the VTA reduces time spent near the lever-cue.**
(a) Representative frame from a video with arena borders overlayed and magazine/lever labeled. Marker labeling from Deep Lab Cut: Nose (purple), tether (green), shoulders (orange/yellow), tail (blue), and magazine (red). (b-e) Each heatmap represents the rats' average location across all trials during session 3 of Pavlovian conditioned approach training (integrated over 123 video frames, 8.2-s time bins). Red indicates more time spent in a given location, blue indicates less. Location of the rats is shown for (b) the 8.2 s before lever-cue presentation, (c) 8.2 s during lever-cue presentation, (d) 8.2 s immediately after lever-cue retraction, and (e) 8.2 s during the inter-trial interval. Left: Control rats, Middle: eNpHR rats that received dopamine inhibition during lever-cue presentation, Right: Subtraction of eNpHR with control heat maps zoomed in on the magazine and lever. Red indicates that the eNpHR rats spent more time in a given location, blue indicates that control rats spent more time in a given location.

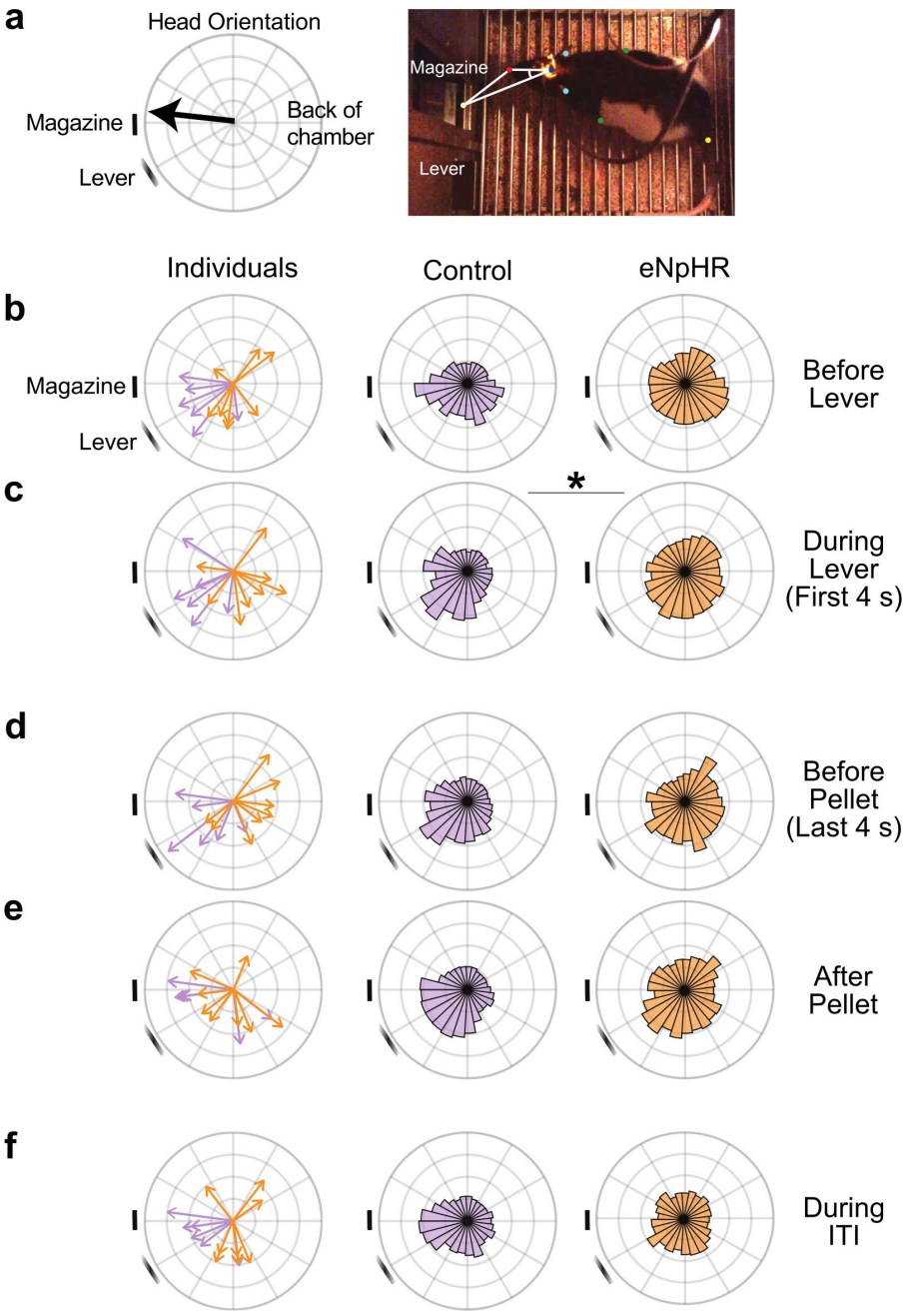

Figure 2-8 **Inhibition of VTA dopamine neurons blunts the orienting responses to the lever-cue.**
(a) Left: Head orientation was calculated from each video frame and is depicted as a unit vector on a polar histogram with the relative location of the magazine, lever, and back of chamber labeled. Right: Representative frame from a video with magazine and lever labeled. Marker labeling from Deep Lab Cut: Nose (purple), tether (green), shoulders (orange/yellow), tail (blue), and magazine (red). Head orientation was calculated from the angle between two vectors: 1) from the tether to the nose and 2) from the tether to the magazine. (b-f) Polar histograms showing individual rat average head direction (left) and group data head direction (center/right) (b) before lever-cue presentation (4 s), (c) during lever-cue presentation (4 s), (d) before pellet delivery (4 s), (e) after pellet delivery (4 s), and (f) during the intertrial interval (ITI, 4 s). (c) Compared to controls, eNphR rats that received inhibition of dopamine neurons in the VTA had more variability in their head direction during lever-cue presentation. *$p < 0.05$.

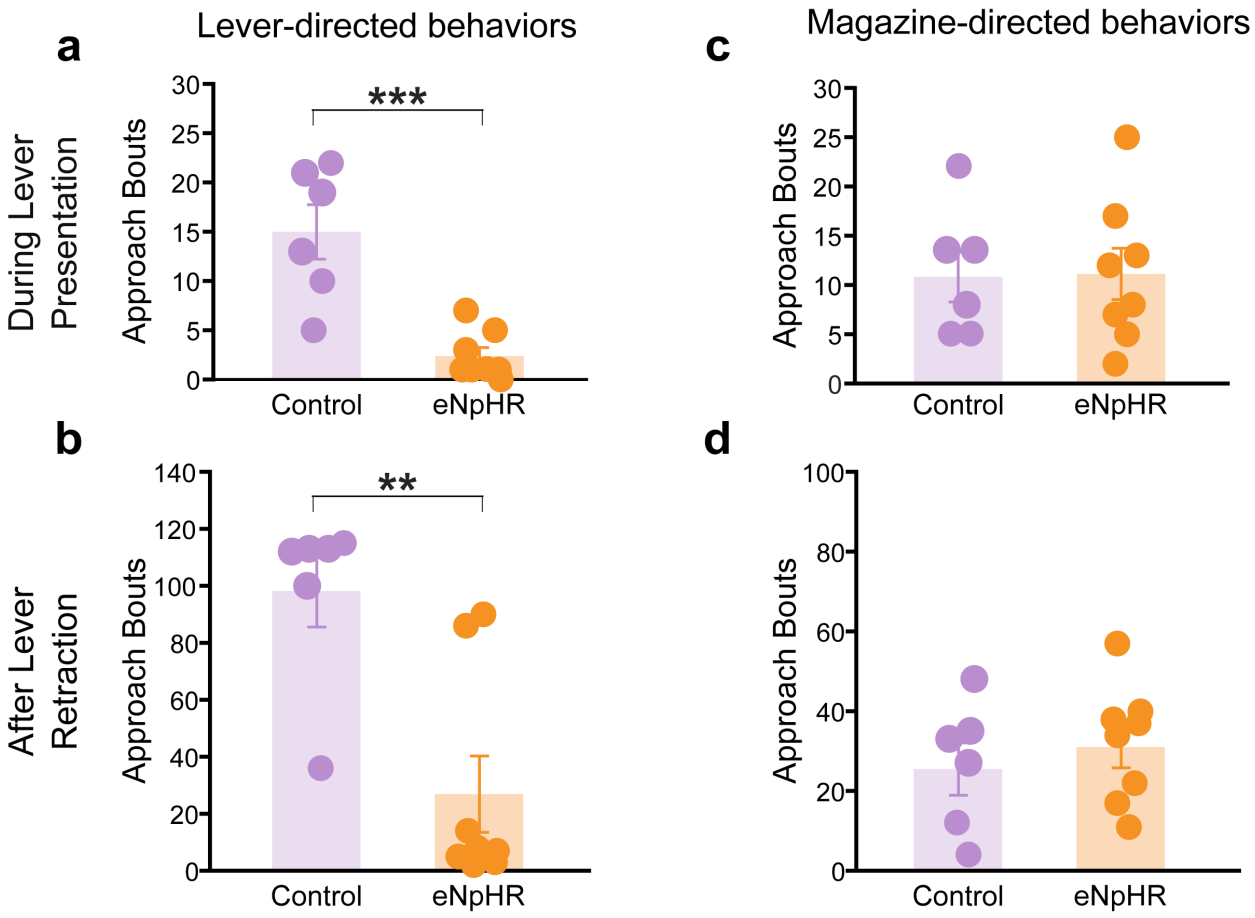

Figure 2-9 **DeepLabCut analysis confirms inhibition of VTA dopamine neurons suppresses sign-tracking behavior.**
Data are shown as mean ± SEM for approach behavior directed towards the lever-cue (left) or food magazine (right) during the 8.2 sec lever-cue presentation (top) or after lever retraction, during the intertrial intervals (bottom). An approach bout was counted when the rats nose remained within 75 pixels (1 cm) of the lever-cue or food magazine during more than 15 frames (1 s). A bout ended when the interbout interval exceeded 15 frames. Relative to control rats, eNphR rats exhibit less approach behavior towards the lever-cue, and this is true (a) during lever-cue presentation and (b) after lever-cue retraction (during the inter-trial intervals). Approach behavior directed towards the food magazine does not differ between control rats and those in the eNphR group. $^{**}p < 0.01$, and $^{***}p < 0.001$.

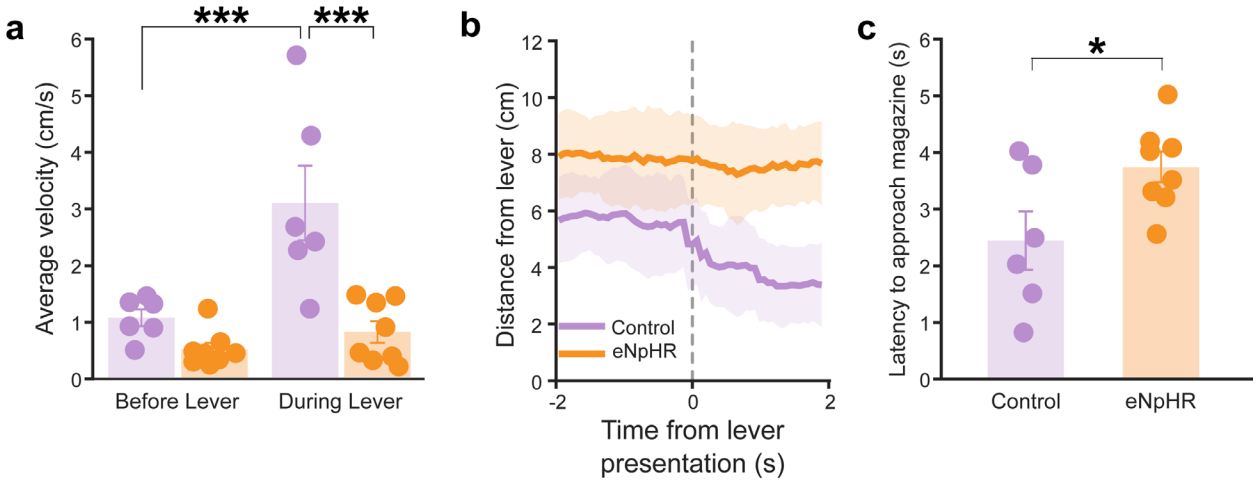

Figure 2-10 **Inhibition of dopamine neurons suppresses lever-cue elicited locomotion.**
Data are shown as mean ± SEM. (a) Average velocity (cm/s) of movement during the 4 s period immediately before and immediately upon lever-cue presentation, (b) distance (cm) from the lever-cue for the 2-s period immediately before and immediately upon lever-cue presentation, and (c) latency to approach the food magazine immediately following lever-cue retraction. (a) Control rats moved faster when the lever-cue was presented, relative to the period before lever-cue presentation. Control rats also moved quicker than eNpHR rats that received inhibition of dopamine neurons when the lever-cue was presented. (b) Control rats moved closer to the lever-cue when it was presented relative to eNpHR rats that received inhibition of dopamine neurons. (c) eNpHR rats that received lever-paired inhibition of dopamine neurons were delayed in approaching the food magazine upon lever-cue retraction. *$p < 0.05$ and ***$p < 0.001$.

Table 1

	Sessions 1-5: PavCA Index					
	WT Naïve vs WT Sham vs TH-Cre			WT vs TH-Cre		
	DF	*F*	*p*	DF	F	p
Group	1,94.710	0.534	0.588	1,94.469	0.642	0.425
Session	1,155.351	51.978	***<0.001	1,172.373	60.045	***<0.001
*Group*Session*	1,172.333	1.725	0.096	1,172.373	3.175	*0.015

Table 2-1 **Statistical analyses for PavCA Index across sessions 1-5.**
Data from linear mixed effects model analyses for PavCA Index on sessions 1-5. The left panel compares WT Naïve, WT Sham, and TH-Cre animals. The right panel compares WTs (WT Naïve and WT Sham collapsed) to TH-Cre animals. Significant effects and interactions are bolded (*$p < .05$ and ***$p < .001$).

Table 2

	a. Sessions 1-3 "Laser On": Lever-directed behaviors								
	Lever Contacts			Probability Lever			Latency Lever		
	DF	*F*	*p*	DF	*F*	*p*	DF	*F*	*p*
Group	1,15.060	4.166	**#0.059**	1,15.405	4.954	***0.041**	1,15.396	4.620	***0.048**
Session	2,15.773	1.774	0.202	2,18.848	2.186	0.140	2,15.436	3.528	**#0.055**
*Group*Session*	2,15.773	4.119	***0.036**	2,18.848	5.237	***0.016**	2,15.436	4.513	***0.029**
	b. Sessions 4-6 "Laser Off": Lever-directed behaviors								
	Lever Contacts			Probability Lever			Latency Lever		
	DF	*F*	*p*	DF	*F*	*p*	DF	*F*	*p*
Group	1,15.123	2.532	0.132	1,14.997	4.157	**#0.059**	1,15.095	4.074	**#0.062**
Session	2,16.918	2.912	0.082	2,20.648	1.149	0.336	2,17.219	1.828	0.191
*Group*Session*	2,16.918	0.787	0.471	2,20.594	0.239	0.789	2,17.219	0.322	0.729
	c. Sessions 1-3 "Laser On": Magazine-directed behaviors								
	Magazine Contacts			Probability Magazine			Latency Magazine		
	DF	*F*	*p*	DF	*F*	*p*	DF	*F*	*p*
Group	1,45	0.000	0.993	1,15.160	0.052	0.822	1,16.787	0.386	0.543
Session	2,45	0.764	0.472	2,20.026	1.641	0.219	2,19.534	0.468	0.633
*Group*Session*	2,45	0.866	0.427	2,20.026	0.020	0.980	2,19.534	0.022	0.979
	d. Sessions 4-6 "Laser Off": Magazine-directed behaviors								
	Magazine Contacts			Probability Magazine			Latency Magazine		
	DF	*F*	*p*	DF	*F*	*p*	DF	*F*	*p*
Group	1,15.015	1.544	0.233	1,14.925	1.407	0.245	1,14.793	1.301	0.272
Session	2,17.066	1.039	0.375	2,19.051	1.995	0.163	2,15.478	1.467	0.261
*Group*Session*	2,17.066	0.772	0.478	2,19.051	2.845	0.083	2,15.478	2.334	0.130
	e. Sessions 1-3 "Laser On": Magazine head entries during ITI						**f. Sessions 4-6 "Laser Off": Magazine head entries during ITI**		
	ITI Head Entries						ITI Head Entries		
	DF	*F*	*p*				DF	*F*	*p*
Group	1,15	0.320	0.580				1,15	4.476	**#0.052**
Session	2,30	1.027	0.370				2,30	3.593	***0.040**
*Group*Session*	2,30	0.366	0.696				2,30	1.461	0.248
	g. Control Sessions 1-3 "Laser On" vs Halorhodopsin Sessions 4-6 "Laser Off"								
	Lever Contacts			Probability Lever			Latency Lever		
	DF	*F*	*p*	DF	*F*	*p*	DF	*F*	*p*
Group	1,15.019	0.048	0.830	1,15.042	0.538	0.475	1,15.345	0.397	0.538
Session	2,15.963	2.148	0.149	2,18.668	4.870	***0.020**	2,17.539	3.983	***0.037**
*Group*Session*	2,15.963	0.350	0.710	2,18.668	0.551	0.586	2,17.539	1.073	0.363

Table 2-2 **Statistical analyses for lever- and magazine-directed behaviors.**
Data from linear mixed effects model analyses for "laser on" and "laser off" sessions. Contacts, probability, and latency are represented for lever-directed behaviors, magazine-directed behaviors, and head entries into the magazine during the intertrial interval. Significant effects and interactions are bolded (#trend for a significant effect and *$p < .05$)

Table 3

	a. Sessions 3 vs 6: Lever-directed behaviors								
	Lever Contacts			Probability Lever			Latency Lever		
	DF	*F*	*p*	DF	F	p	DF	F	p
Group	1,15	2.256	0.154	1,15	4.623	***0.048**	1,15	3.917	**#0.066**
Session	1,15	3.937	**#0.066**	1,15	4.752	***0.046**	1,15	5.984	***0.028**
*Group*Session*	1,15	0.047	0.831	1,15	0.243	0.629	1,15	0.063	0.805
	b. Sessions 3 vs 6: Magazine-directed behaviors								
	Magazine Contacts			Probability Magazine			Latency Magazine		
	DF	*F*	*p*	DF	F	p	DF	F	p
Group	1,15	1.792	0.201	1,15	1.306	0.271	1,15	0.906	0.356
Session	1,15	0.246	0.627	1,15	0.060	0.809	1,15	0.123	0.730
*Group*Session*	1,15	0.891	0.360	1,15	2.717	0.120	1,15	2.872	0.111
	c. Sessions 3 vs 6: Magazine head entries during ITI								
	ITI Head Entries								
	DF	*F*	*p*						
Group	1,15	1.927	0.185						
Session	1,15	4.158	**#0.059**						
*Group*Session*	1,15	1.270	0.277						

Table 2-3 **Statistical analyses comparing "laser on" vs "laser off" periods.**
Data from repeated measures ANOVA analyses comparing session 3 to session 6. Session 3 was the final session that animals received laser inhibition of dopamine neurons in the VTA and session 6 was the last session without laser inhibition. Statistical results for contacts, probability, and latency are represented for lever-directed behaviors and magazine-directed behaviors. Results for head entries during the intertrial interval are also shown. Significant effects are bolded (#trend for a significant effect, **p* < .05, ***p* < .01, and ****p* < .001).

Chapter 3

Chemogenetic Inhibition of Neuronal Projections from the Lateral Hypothalamus to the Paraventricular Nucleus of the Thalamus Attenuates Goal-tracking Behavior in Male Rats

3.1 Abstract

The paraventricular nucleus of the thalamus (PVT) has connections throughout the limbic system and has been implicated in motivated behaviors. The connections from the lateral hypothalamus (LH) to the PVT have specifically been shown to regulate arousal, feeding, and reward seeking. However, the involvement of the LH-PVT pathway in individual differences in cue-reward learning has yet to be explored and is the focus of the work presented here. During a Pavlovian conditioned approach paradigm, a reward is reliably preceded by a cue and rats subsequently develop different conditioned responses. Upon cue presentation we find that sign-trackers (STs) approach and interact with the cue, while goal-trackers (GTs) approach the reward delivery location. A subset of rats that exhibit approach directed towards the cue and/or reward location are termed intermediate responders (IRs). This animal model allows us to study individual differences in cue-reward learning. Here, we utilize a dual-vector surgical approach to selectively express inhibitory chemogenetic receptors in the LH-PVT pathway. We show that inhibition of the LH-PVT pathway selectively attenuates the expression of goal-tracking behavior. This effect seems to be driven primarily by IR rats, as inhibition of the LH-PVT blunts an increase in goal-tracking behavior in IR rats, without impacting the response of STs or GTs. Sign-tracking behavior was not affected by this manipulation in any of the phenotypes. We speculate that the flexibility of the IR rats (i.e. they do not exhibit solely sign- or goal-tracking

behavior) made them especially vulnerable to this manipulation. Together, these data support a role for LH-PVT pathway in goal-tracking behavior.

3.2 Introduction

Decades have been spent disentangling the neural circuits that contribute to motivated behavior and reward learning and much of these efforts have focused on the classic dopaminergic mesocorticolimbic system. However, the contributions of other brain regions, such as the paraventricular nucleus of the thalamus (PVT), have gained increasing attention over the years. The PVT is a midline thalamic structure intertwined in the limbic system as it receives projections from areas such as the prefrontal cortex, hypothalamus, and brainstem and projects to the nucleus accumbens shell, bed nucleus of the stria terminalis, and central amygdala (Hsu & Price, 2009; Li & Kirouac, 2008). Due to its anatomical position and connectivity, the PVT has been regarded as a relay center for arousal and feeding that thereby influences motivation to seek rewards (Kelley et al., 2005). The region has been shown to play a role in complex behavioral states such as arousal (Hsu et al., 2014; Kirouac, 2015), reward seeking (Kirouac, 2015; Matzeu et al., 2014), motivational conflict (Choi et al., 2019; Choi & McNally, 2017), and stress responsivity (Bhatnagar & Dallman, 1998; Bhatnagar & Dallman, 1999; Bhatnagar et al., 2003; Bhatnagar et al., 2002; Heydendael et al., 2011; Li et al., 2010b). Further, neurons within the PVT are known to respond to context and cue-associated stimuli (Choi et al., 2010; Igelstrom et al., 2010; Matzeu et al., 2017; Schiltz et al., 2007).

The lateral hypothalamus (LH) is one region that sends projections to the PVT and known to be involved in reward processing. The LH plays a critical role in appetitive and consummatory processes (Hoebel & Teitelbaum, 1962; Margules & Olds, 1962) and is regarded as a hub for feeding, due, in part, to early studies showing that electrical stimulation of the LH

results in intense feeding (Anand & Brobeck, 1951; Delgado & Anand, 1953). In more recent years, the cellular heterogeneity of the LH has prompted investigation of its involvement in processes outside of homeostatic regulation (for review see: (Bonnavion et al., 2016; Stuber & Wise, 2016). The orexinergic, glutamatergic, and GABAergic neurons in LH-PVT projections have been shown to promote feeding (Zhang & van den Pol, 2017), mediate arousal (Ren et al., 2018), and react to sensory stimuli (Otis et al., 2019). However, few have studied the LH-PVT pathway in relation to cue-reward learning, and even less research has been dedicated to individual differences in this regard.

In the current study, we examined the contributions of the LH-PVT pathway in cue-reward learning using an animal model that captures individual differences in cue-reward learning. With Pavlovian conditioned approach (PavCA) training in which a lever-cue precedes the delivery of a food reward, two distinct conditioned responses emerge. A sign-tracking response is directed towards the lever-cue upon its presentation and a goal-tracking response is directed towards the location of reward delivery. These conditioned responses result from the attribution of predictive and incentive value to the lever-cue in sign-trackers (STs) and the attribution of predictive value alone to the lever-cue in goal-trackers (GTs). It is the attribution of incentive motivational value, or incentive salience, that transforms the cue into an attractive and desirable stimulus that is able to control behavior (Berridge et al., 2009; Robinson & Berridge, 1993). For STs, both food- and drug-associated cues gain excessive incentive value and elicit maladaptive behaviors (Yager et al., 2015; Yager & Robinson, 2013). Another, less studied phenotype – intermediate responders (IR), vacillate between sign- and goal-tracking behavior.

Different neural circuits are recruited when predictive versus incentive value is ascribed to reward-cues, as noted with the ST/GT model. The model, therefore, can be utilized to

elucidate the neurobiological mechanisms that encode adaptive or maladaptive cue-driven behaviors. The PVT has been shown to play a role in mediating sign- and goal-tracking behaviors and is regarded as a neural hub for regulating these phenotypic responses (Campus et al., 2019; Flagel et al., 2011; Haight & Flagel, 2014; Haight et al., 2015; Haight et al., 2017; Kuhn et al., 2018). A "top-down" cortico-thalamic pathway is believed to encode the predictive value of reward cues and regulate the behavior of both phenotypes (Campus et al., 2019; Haight & Flagel, 2014). Prior findings have led us to postulate that "bottom-up" projections to the PVT relay the incentive value of reward-associated cues. Specifically, in STs, a subcortical hypothalamic-thalamic-striatal circuit is thought to encode the incentive value of reward cues and to override the top-down predictive value, with the LH, which sends dense orexinergic projections to the PVT (Kirouac et al., 2005; Peyron et al., 1998), acting as a critical neural node.

Prior work has indirectly implicated the LH-PVT pathway in mediating sign-tracking behavior as lesions of the LH attenuate the development of sign-tracking behavior and blockade of orexin signaling in the PVT reduces sign-tracking behavior and the conditioned reinforcing properties of the lever-cue. Thus, here we hypothesized that chemogenetic inhibition of LH-PVT projections would reduce OX-encoded incentive value and thereby decrease sign-tracking behavior. However, we found that LH-PVT inhibition had no effect on sign-tracking behavior but blunted the expression of goal-tracking. This effect was primarily carried by intermediate responders, as there was no effect of LH-PVT inhibition within the sign-tracker or goal-tracker populations.

3.3 Materials and Methods

In this experiment rats were run in two separate cohorts. Rats either had surgery to express DREADD (Designer Receptors Exclusively Activated by Designer Drugs) in the brain or

they had no surgery and were used as controls. Among cohort one, the rats were run in multiple rounds and there was either 30 or 40 rats run at one time per round. Cohort two was run in 1 round of 60 rats. Regardless of cohort, all rats had the same experiment. The rats had 7 consecutive sessions of Pavlovian conditioned approach (PavCA). On sessions 3-4 of PavCA rats were given vehicle (VEH) injections after behavior to acclimate them to receiving injections. On sessions 5-7 of PavCA rats were either given VEH or clozapine-*N*-oxide (CNO, DREADD ligand) 25 minutes before the session. On session 8 all rats had a conditioned reinforcement test (CRT) and no injections.

3.3.1 Subjects

Male Sprague Dawley rats (N=230), divided into two cohorts, were ordered from different barriers at either Charles River Laboratories or Taconic Biosciences. Rats from cohort one did receive surgery for DREADD (Designer Receptors Exclusively Activated by Designer Drugs). Rats from cohort two did not receive surgery for DREADD and were a control group. In the DREADD cohort (5 rounds, n=170), rats were from Charles River barrier R04 (n=41, Raleigh, NC), Charles River barrier R08 (n=64, Raleigh, NC), Taconic BU16 (n=20, Cambridge City, IN), or Taconic IBU (n=45, Germantown, NY). Some rats died before the experiment began (n=7). We had difficulty obtaining sign-tracker rats and had uneven phenotype distributions. Rats were excluded based on overrepresentation of a respective phenotype based on behavior from session 4 (i.e. before the DREADD ligand was administered) within each round (n=66). Rats were excluded for off-target virus expression or minimal expression (see methods) in either anterior PVT, posterior PVT, and/or bilaterally in the LH (n=43). Included rats from the DREADD cohort (n=54), were STs (n=13; 7 VEH/6 CNO), IRs (n=18; 7 VEH/11 CNO), or GTs (n=23; 15 VEH/8 CNO). In the no DREADD cohort (1 round, n=60), rats were

either from Charles River barrier R04 (n=30, Raleigh, NC) or Charles River barrier R08 (n=30, Raleigh, NC). We ordered from Charles River barriers for this cohort because, historically, these barriers yielded a more even distribution across phenotypes. Included rats from the no DREADD cohort (n=60), were STs (n=21; 11 VEH/10 CNO), IRs (n=23; 11 VEH/12 CNO), or GTs (n=16; 8 VEH/8 CNO).

Rats were approximately 9 weeks of age (~300-350g body weight) at time of arrival and were housed under a 12-hour light-dark cycle (lights on at 6 or 7 AM depending on daylight saving time) with climate-controlled conditions (22 ± 2ºC). They had ad-libitum access to food and water throughout the study. Rats were pair-housed for the duration of the experiment and were acclimated to the housing room for a minimum of one week before experimenter handling began. Behavioral testing took place during the light-phase between 10 AM and 4 PM. All procedures followed The Guide for the Care and Use of Laboratory Rats: Eighth Edition (2011, National Academy of Sciences) and were approved by the University of Michigan Institutional Animal Care and Use Committee.

3.3.2 Surgery

Surgeries were performed using a dual-vector approach, to selectively express hM4Di-DREADD receptors (G_i, inhibitory) in the LH-PVT pathway. Rats had anesthetic induction of 5% isoflurane (Vet One, Boise, ID) delivered via an induction chamber and were then placed into the stereotaxic frame (David Kopf instruments, Tujunga, CA or Stoelting, Wood Dale, IL) and given an injection of carprofen (5 mg/kg, subcutaneous (s.c.)) for analgesia. Surgeries were performed under aseptic conditions. All coordinates were relative to bregma (Paxinos & Watson, 2007). A Neuros Syringe (5 µL Model 33-gauge, point style 3, Hamilton Company) infused 0.75 µl of an inhibitory Cre-dependent Gi DREADD virus bilaterally into the LH (-2.7 mm AP

(anterior/posterior); ±1.6 mm ML (medial/lateral); -9 mm DV (dorsal/ventral)). The DREADD virus was infused over 8 min and the syringe was left in place for 10 more min. A Neuros Syringe (0.5 µL Model 33-gauge, point style 3, Hamilton Company) infused 0.10 µl of a retrograde Cre virus at a 10° angle into the anterior PVT (aPVT: -2.0 mm AP; -1 mm ML; -5.4 mm DV) and posterior PVT (pPVT: -3.0 mm AP; -1 mm ML; -5.5 mm DV) to target the entire rostral-caudal axis of the region. The Cre virus was infused over 2 min and the syringe was left in place for 5 more min. Following 3 - 5 weeks of virus incubation, all rats underwent Pavlovian training sessions.

3.3.3 Viral Vectors

Viruses were obtained from Addgene. The Cre-dependent G_i DREADD virus (AAV8-hSyn-DIO-hM4Di-mCherry, Addgene 44362-AAV8) was a titer of $\geq 1 \times 10^{13}$ vg/mL. The Cre virus (AAVrg-pmSyn1-EBFP-Cre, Addgene 51507-AAVrg) was a titer of $\geq 7 \times 10^{12}$ vg/mL.

3.3.4 DREADD ligand

We obtained clozapine-*N*-oxide (CNO) from the National Institute of Mental Health. DREADD receptor activation occurred via systemic administration of CNO which results in inhibition of the LH-PVT pathway through disruption of G-protein-dependent signaling, likely from induced hyperpolarization (Armbruster et al., 2007; Vardy et al., 2015) or inhibition of presynaptic release of neurotransmitters (Stachniak et al., 2014; Vardy et al., 2015). 5 mg/kg CNO was dissolved in 6% dimethyl sulfoxide (DMSO) and sterile H_2O and 5 mg/kg was injected intraperitoneally (i.p.) (Armbruster et al., 2007; Ferguson et al., 2011; Rogan & Roth, 2011; Stachniak et al., 2014). The control animals were administered vehicle i.p. (VEH; 6% DMSO and sterile H_2O).

3.3.5 Pavlovian conditioned approach (PavCA)

Pavlovian conditioned approach (PavCA) testing occurred inside Med Associates chambers (St. Albans, VT, USA; 24.1 × 21 x 30.5 cm) located in sound-attenuating boxes equipped with a fan to reduce background noise. The chambers contained a food cup that was connected to a pellet dispenser and placed in the center of one wall 3 cm above the chamber floor. An illuminated retractable lever was located either to the left or right of the food cup, 6 cm above the chamber floor. A white house light was placed at the top of the chamber on the wall opposite to the food cup and lever and remained on for the duration of the session. Food cup entries were recorded upon a photo-beam break inside the food cup and a lever contact was recorded following a minimum deflection force of 10 g.

All rats were handled by experimenters for two days before assessing PavCA behavior. For the two days prior to pre-training rats received ~25 banana-flavored grain pellets (each pellet 45-mg; Bio-Serv, Flemington, NJ, USA) in their home cage to familiarize them with the reward. Prior to each session the rats were transferred to the injection room 1 hour prior to behavior. Rats were initially placed into the Med Associates chambers for a single pretraining session. At the start of the pretraining session, the food cup was baited with two food pellets to direct the rats' attention to the location of reward delivery. During pretraining the lever-cue remained retracted, and rats received a food pellet in the food cup on a variable 30 s (range 0-60 s) schedule. There was a total of 25 trials and the pretraining session lasted approximately 12.5 min wherein head entries were recorded, and food pellet consumption confirmed. Following pretraining rats had a single session of PavCA each day for 7 consecutive days (Figure 1a,b). The acquisition phase of PavCA behavior was over 4 sessions. Following behavior on sessions 3-4 rats received i.p. VEH injections to acclimate them to receiving injections. Rats were assigned to phenotype and

treatment groups in a counterbalanced manner based on their behavior on session 4. On sessions 5-7 rats received i.p. CNO injections to activate the DREADDs 25 minutes before the start of the session each day. The start of each PavCA session began with a 5-min waiting period followed by the house light turning on which signified the start of the session. As previously described (Campus et al., 2019; Meyer et al., 2012), during PavCA, the illuminated lever (conditioned stimulus, CS) entered the chamber for 8 s and upon retraction a food pellet (unconditioned stimulus, US) is immediately delivered into the adjacent food cup. PavCA sessions consisted of 25 lever (CS)/ food-US trials on a variable 90-s schedule (range 30–150 s). Each session lasted approximately 40 min. It was confirmed that all food pellets had been consumed following each session.

Med Associates software recorded the following behaviors during PavCA sessions: 1) number of food cup contacts made during the 8-s lever presentation, (2) latency to contact the food cup during lever-cue presentation, (3) number of lever contacts, (4) latency to lever contact, and (5) the number of food cup entries made during the inter-trial interval (i.e., food cup contacts made in between lever presentations). Contact and latency data were used to calculate the PavCA Index to characterize rats into their behavioral phenotypes, as previously described (Meyer et al., 2012). The PavCA Index is a composite measure calculated using the following formula: [Probability Difference Score + Response Bias Score + (-Latency Difference Score)/3]. PavCA Index scores range from −1 to 1, with a score of −1 representing individuals with a conditioned response (CR) focused solely on the food cup during lever-cue presentation (i.e., a “pure” goal-tracker, GT) and a score of +1 representing individuals with a CR focused solely on the lever-cue upon its presentation (i.e., a “pure” sign-tracker, ST). The PavCA Index was generated from the

behavioral measures of session 4 to classify animals as STs (PavCA > 0.5), IRs (<0.5 PavCA >-0.5), or GTs (PavCA < -0.5).

3.3.6 Conditioned reinforcement test (CRT)

Rats were exposed to a conditioned reinforcement test (CRT) the day following the final PavCA session (Figure 1a,c). This test required rats to perform an instrumental response for lever presentation, in the absence of reward (Hughson et al., 2019). That is, the lever that was previously the reward-cue now served as the reinforcer (Robinson & Flagel, 2009). Conditioning chambers were reconfigured, and the lever was placed in the center wall, flanked by nose ports. Nose pokes in to the "active" port resulted in presentation of the illuminated lever for 2 s, while nose pokes in the "inactive" port had no consequence. The active port was placed opposite the side of the lever location during PavCA sessions to minimize side bias. The conditioned reinforcement test lasted 40 minutes.

3.3.7 Perfusion and tissue processing

Rats that previously received surgery were perfused within 5 days following the experiment. Rats were anesthetized with a cocktail of ketamine (90 mg/kg, i.p.) and xylazine (10 mg/kg, i.p.). 10 min after the anesthetic injection rats were then transcardially perfused with 0.9% saline and 4% formaldehyde (pH = 7.4). Following brain extraction, the tissue was post-fixed in 4% formaldehyde for 24 hours at 4ºC and then placed in 30% sucrose at 4ºC (sucrose in 0.1M PBS, pH = 7.4) for 3 days. The brains were frozen using dry ice and coated in a Tissue-Plus Optimal Cutting Temperature (OTC) compound (Fisher HealthCare, Houston, TX). Coronal brain slices were taken at 40 µm using a cryostat at -20ºC (Leica Biosystems Inc, Buffalo Grove, IL).

3.3.8 Immunohistochemistry

DREADD expression between the LH-PVT pathway was evaluated following immunohistochemical staining of mCherry, the fluorescent tag on the G_i DREADD virus. Free-floating coronal sections from the LH and PVT were initially washed 6 times in 0.1M PBS (pH = 7.4) to remove Optimal Cutting Temperature (OTC) debris. Each step after this initial wash was followed by 3 washes in 0.1M PBS (pH = 7.4). Sections were incubated in 1% H_2O_2 for 10 min to block endogenous peroxidase activity. Sections were incubated in 0.1M PBS containing 0.4% Triton X-100 (TX) and 2.5% Normal Donkey Serum (NDS) (Jackson ImmunoResearch Laboratories, Inc, West Grove, PA) to block nonspecific binding of the secondary antibody. Then sections were incubated overnight at room temperature in primary antibody (rabbit anti-mCherry, Abcam, Cambridge, UK, diluted 1: 30,000) in 0.1M PBS + 0.4% TX + 1% NDS. The next day, sections incubated for 1 hr in a biotinylated donkey anti-rabbit secondary antibody (Jackson Immunoresearch, West Grove, PA, diluted 1: 500) in 0.1M PBS + 0.4% TX + 1% NDS. Sections incubated for 1 hour in Vectastain Elite ABC solution (1:1000 A and 1:1000 B, diluted in 0.1M PBS, mixed 30 minutes before use; Vector Laboratories). This stain was visualized by incubating the sections in 0.1M NaPB containing 0.02% 3,3'-diaminobenzidine tetrahydrochloride (Sigma-Aldrich) and 0.012% H_2O_2 for 10 minutes causing a brown precipitate to form at the location of mCherry detection. Sections were stored at 4°C until mounted, air dried and cover slipped with Permount (Thermo-Fisher Scientific, Waltham, MA). Bright-field images containing the LH, aPVT and pPVT were captured using a Leica DM1000 light microscope (Leica-Microsystems, Wetzlar, GER) and were analyzed by an experimenter blind to the experimental groups. The experimenter assigned a score of 0–3 to each image according to both the density and location of DREADD expression in the areas of interest

(Figure 2). A score of 0 was assigned to subjects that had no DREADD expression or off-target DREADD expression (e.g. expression outside regional boundaries) in either the LH or the aPVT and pPVT; a score of 1 was assigned to subjects that had adequate DREADD expression in both the LH and PVT; a score of 2 was assigned to subjects that had a strong DREADD expression in either the LH or PVT; a score of 3 was assigned to subjects that had a strong DREADD expression in both the LH and PVT. Representative images of what was considered adequate DREADD expression are shown in Figure 2. Rats that had a score of 0 (n = 43), meaning they had no expression in either the LH or PVT, were excluded from the statistical analysis.

To obtain representative images and visualize mCherry expression, the fluorescent tag on the Gi DREADD virus, a subset of brains were processed for immunofluorescence. Free-floating coronal sections from the LH and PVT were initially washed 6 times in 0.1M PBS (pH = 7.4) to remove OTC debris. Each step after this initial wash was followed by 3 washes in 0.1M PBS (pH = 7.4). Sections were incubated overnight at room temperature in primary antibody (rabbit anti-mCherry, Abcam, Cambridge, UK, diluted 1: 500) in 0.1M PBS + 0.4% TX + 2.5% NDS. The next day sections were incubated for 2 hr in a biotinylated donkey anti-rabbit secondary antibody (Jackson Immunoresearch, West Grove, PA, diluted 1: 500) in 0.1M PBS + 0.4% TX + 2.5% NDS. Sections were then incubated for 1 hr in Alexa Fluor 594-conjugated streptavidin (Thermo Fisher Scientific, Waltham, MA, diluted 1:1000) in 0.1M PBS + 0.4% TX then mounted onto slides and cover slipped with ProLong Gold Antifade Mountant (Thermo Fisher Scientific, Waltham, MA). Fluorescent images containing the LH, aPVT and pPVT were captured using a FV3000 confocal microscope and the FV31S-SW Viewer software (OLYMPUS Microscopes, Center Valley, PA, USA) (Figure 2).

3.3.9 Statistical analyses

Statistical analyses were conducted with the Statistical Package for the Social Sciences (SPSS) program version 27.0 (IBM, Armonk, NY, USA). To assess PavCA behavioral outcome measures across sessions, a linear mixed-effects model (LMM) with restricted maximum likelihood estimation was used to account for repeated measures. This analysis applies multiple covariance structures to the data set and the structure with the lowest Akaike information criterion (AIC) was selected as best fit (Duricki et al., 2016; Verbeke, 1997). When two variables were directly compared, ANOVAs were performed, as described below. For all analyses, statistical significance was set at $p < 0.05$, and Bonferroni post hoc comparisons were made when significant main effects or interactions were detected. Effect size (Cohen's d, Cohen, 1988) was calculated for pairwise comparisons. Effect sizes were considered with respect to the following indices: 0.2, small; between 0.5 – 0.8, medium; between 1.2 – 2.0, large (Cohen, 1988; Sawilowsky, 2009).

LMM was conducted to compare either treatment (VEH vs. CNO) or treatment (VEH vs. CNO) and phenotype (ST vs. IR vs. GT) across sessions 1-4 or sessions 5-7 in Pavlovian conditioned approach (PavCA). Either sessions 1-4 or sessions 5-7 were used as the repeated variable. Treatment (VEH vs. CNO) or treatment (VEH vs. CNO) and phenotype (ST vs. IR vs. GT) were used as the between-subjects variables. To ensure that subjects were counterbalanced between experimental groups after PavCA training, the average PavCA Index from session 4 was analyzed by using a two-way ANOVA with either treatment only (VEH vs. CNO) or treatment (VEH vs. CNO) and phenotype (ST vs. IR vs. GT) as independent variables. A two-way repeated measures ANOVA was conducted when session 4 ("pretest") was directly compared to the average of sessions 5-7 ("test"), with session ("pretest" vs. "test") as the within subject

independent variable and treatment (VEH vs. CNO) and phenotype (ST vs. IR vs. GT) as the between subject independent variables. For both the LMM and ANOVAs, PavCA Index, sign-tracking behaviors (number of lever-cue contacts, probability to approach the lever-cue, latency to approach the lever-cue), and goal-tracking behaviors (food cup contacts during lever-cue presentation, probability to approach the food cup during lever-cue presentation, latency to approach the food cup during lever-cue presentation) were used as dependent variables.

A mixed ANOVA was used to assess the effects of treatment (VEH vs. CNO) and port (active vs. inactive) or treatment (VEH vs. CNO), port (active vs. inactive), and phenotype (ST vs. IR vs. GT) on nose pokes during the conditioned reinforcement test (CRT). An ANOVA was used to assess the effects of effects of treatment (VEH vs. CNO) alone or treatment (VEH vs. CNO) and phenotype (ST vs. IR vs. GT) on lever presses during the CRT. For the ANOVAs, treatment or treatment and phenotype were the independent variables; nose pokes and lever presses were the dependent variables.

3.4 Results

3.4.1 Pavlovian conditioned approach index

The acquisition phase for Pavlovian conditioned approach (PavCA) occurred across sessions 1-4. The PavCA Index on session 4 was utilized to assign treatment groups. There were no differences between treatment groups during sessions 1-4 PavCA Index nor the session 4 PavCA Index alone when phenotypes were collapsed (Figure 3a, b; Table 1). When evaluated by assigned treatment groups (e.g. VEH vs CNO) the PavCA Index changed across sessions 1-4 (effect of session: $F_{(3,73.871)} = 3.164$, $p = 0.029$; Figure 3a), but a lack of treatment effect indicated that the groups were equally balanced. This was also evidenced by the session 4 (pretest) PavCA Index showing no significant differences between assigned treatment groups (Figure 3b).

When phenotype was considered, there were no significant effects of treatment, but there were significant differences between phenotypes, as would be expected (effect of session: $F_{(3,65.959)} = 3.614$, $p = 0.018$; effect of phenotype: $F_{(2,50.528)} = 42.631$, $p < 0.001$; phenotype x session interaction: $F_{(6,65.959)} = 20.242$, $p < 0.001$; Figure 3c). *Post hoc* comparisons revealed that STs, IRs, and GTs were all significantly different from one another by session 4 ($p < 0.001$). Importantly, however, the phenotypes were no different when compared by treatment on session 4 (e.g. VEH STs were similar to CNO STs etc.) (Figure 3d). Thus, treatment groups were adequately counterbalanced and any changes in behavior between treatment groups thereafter would presumably be due to LH-PVT inhibition.

3.4.2 Acquisition of PavCA Behavior

The treatment groups learned at similar rates across sessions 1-4, during the acquisition of PavCA behavior. Rats assigned to VEH and CNO treatment showed comparable learning for sign-tracking (Figure 4a, c, e; Table 2a) and goal-tracking (Figure 5a, c, e; Table 2d) behaviors as there was a significant effect of session for all measures. *Post hoc* comparisons revealed that engagement with the lever-cue and food cup significantly increased from session 1 to session 4 for all measures ($p < 0.001$). Similarly, the treatment groups within each respective phenotype had similar rates of sign-tracking (Figure 4g, i, k; Table 3a) and goal-tracking (Figure 5g, i, k; Table 4a) behaviors evidenced by significant effects of session and phenotype as well as a significant session x phenotype interaction for all sign- and goal-tracking measures, but no effect of treatment (Table 3a, 4a). *Post hoc* comparisons revealed that STs, IRs, and GTs were all significantly different from one another across all sign-tracking ($p < 0.001$) and goal-tracking measures ($p < 0.001$) by session 4.

3.4.3 Effects of inhibiting the LH-PVT pathway on PavCA behavior

3.4.3.1 Inhibition of the LH-PVT pathway has no impact on sign-tracking

Chemogenetic inhibition of the LH-PVT pathway via systemic CNO administration had no effect on sign-tracking behaviors (Table 2b, 3b). There were no significant differences in the number of lever-cue contacts (Figure 4a, g), probability to approach the lever-cue (Figure 4c, i), or latency to approach the lever-cue (Figure 4e, k) between VEH- and CNO-treated rats on sessions 5-7. This was true when comparing treatment groups collapsed across phenotypes (Figure 4a, c, e; Table 2b) and when considering phenotype as a variable (Figure 4g, i, k; Table 3b). As expected, sign-tracking behavior did change over the course of training and there was a significant effect of phenotype for each lever-directed behavior (Figure 4g, i, k; Table 3b). *Post hoc* comparisons revealed that, in general, STs had significantly more lever contacts ($p < 0.001$), a higher probability to approach the lever ($p < 0.001$), and approached the lever faster ($p < 0.001$) than IRs and GTs on sessions 5-7. Thus, LH-PVT pathway inhibition via DREADD activation had no effect on sign-tracking behavior.

To further assess differences in behavior as a function of LH-PVT pathway inhibition, we directly assessed the effect of treatment on session 4 (pretest) to the average of sessions 5-7 (test) for sign-tracking behaviors (Table 2c, 3c). There was a significant effect of session for all lever-directed behaviors (Figure 4b, d, f; Table 2c). *Post hoc* comparisons revealed that pretest sessions were significantly higher than test sessions for the number of lever-cue contacts ($p = 0.008$; Figure 4b), probability to approach the lever-cue ($p = 0.005$; Figure 4d), and latency to approach the lever-cue ($p = 0.002$; Figure 4f). This is to be expected with the additional training sessions. Consistent with the findings reported above, sign-tracking behavior was not altered due to LH-PVT inhibition when directly comparing “pre-“ vs. “post-“ test sessions. This was true

when collapsed across phenotype and when the effect of treatment was assessed specifically in IRs (Figure 4h, j, l; Table 3c). As such, IRs displayed no change in sign-tracking behavior as a result of LH-PVT inhibition.

3.4.3.2 Inhibition of the LH-PVT pathway decreases goal-tracking behavior

Chemogenetic inhibition of the LH-PVT pathway decreased goal-tracking behaviors and this reduction was phenotype-dependent. When collapsed across phenotype, there was an effect of treatment for goal-tracking behavior on sessions 5-7 (Table 2e). Relative to VEH-treated rats, CNO-treated rats had significantly decreased food cup contacts when the lever-cue was presented (effect of treatment: $F_{(1,52.137)} = 5.147$, $p = 0.027$; Figure 5a), a significantly decreased probability to approach the food cup (effect of treatment : $F_{(1,52.025)} = 5.119$, $p = 0.028$; Figure 5c), and a trend for an increased latency to approach the food cup (effect of treatment : $F_{(1,52.012)} = 3.655$, $p = 0.061$; Figure 5e). Thus, inhibition of the LH-PVT pathway decreased goal-tracking behavior.

Interestingly, when phenotype was considered as a variable, the differences in goal-tracking as a function of LH-PVT inhibition were apparent only in the IR group. As such, there was either a significant effect of treatment, effect of phenotype, and/or a treatment x phenotype interaction for all measures of goal-tracking behavior over sessions 5-7 (Table 4b). Rats treated with CNO had a significant reduction in food cup contacts (effect of treatment: $F_{(1,48.429)} = 4.349$, $p = 0.042$; effect of phenotype: $F_{(2,48.436)} = 32.846$, $p < 0.001$; Figure 5g) and an increase in latency to approach the food cup when the lever was out (trend for an effect of treatment for CNO-treated rats: $F_{(1,48.804)} = 3.945$, $p = 0.053$; effect of phenotype: $F_{(2,48.811)} = 56.982$, $p < 0.001$; Figure 5k) relative to VEH-treated rats. This CNO-mediated reduction in goal-tracking appears to be carried by the IRs and most apparent for probability to approach the food cup. As shown in

Figure 5i, while VEH-treated IR rats increased in probability to approach the food cup when the lever was out, CNO-treated IR rats did not (effect of treatment: $F_{(1,48.154)} = 7.561$, $p = 0.008$; effect of phenotype: $F_{(2,48.161)} = 57.539$, $p < 0.001$; treatment x phenotype interaction: $F_{(2,48.161)} = 4.886$, $p = 0.012$). *Post hoc* comparisons revealed that CNO-treated IRs had a significantly reduced probability to approach the food cup relative to VEH-treated IRs ($p < 0.001$, Cohen's $d = 1.53$). Further, VEH-treated IRs had a significantly higher probability to approach the food cup relative to VEH-treated STs ($p < 0.001$, Cohen's $d = 2.48$), but not VEH-treated GTs ($p = 0.372$). Along similar lines, CNO-treated IRs had a significantly lower probability to approach the food cup relative to CNO-treated GTs ($p < 0.001$, Cohen's $d = 2.65$), but not CNO-treated STs ($p = 0.063$). That is, VEH-treated IRs showed more goal-tracking behavior and were closer in responding to VEH-treated GTs, while inhibition of the LH-PVT attenuated goal-tracking behavior to similar levels as STs. So, inhibition of the LH-PVT pathway significantly decreased goal-tracking behavior only in IRs.

We also compared session 4 (pretest) to the average of sessions 5-7 (test) for goal-tracking behaviors in VEH- and CNO-treated rats (Table 2f, 4c). As shown in Figure 5d, there was a significant session x treatment interaction for probability to approach the food cup when the lever-cue was presented ($F_{(1,52)} = 10.532$, $p = 0.002$). *Post hoc* comparisons revealed that CNO-treated rats had a significantly higher probability to approach the food up on the pretest session relative to the test session ($p = 0.005$, Cohen's $d = 0.35$). Additionally, on the test session, CNO-treated rats had a significantly lower probability to approach the food cup relative to VEH-treated rats ($p = 0.028$, Cohen's $d = 0.15$). Accordingly, LH-PVT inhibition appears to blunt goal-tracking behavior between sessions.

When assessing the effect of treatment across pretest vs. test sessions specifically in IRs (Table 4c), the VEH-treated IRs had significantly more food cup contacts (effect of treatment: $F_{(1,16)} = 5.560$, $p = 0.031$; Figure 5h) and a significantly higher probability to approach the food cup (effect of treatment: $F_{(1,16)} = 5.149$, $p = 0.037$; Figure 5j) relative to CNO-treated IRs. Further, as shown in Figure 5j, there was a significant session x treatment interaction ($F_{(1,16)} = 8.311$, $p = 0.011$) for probability to approach the food cup. *Post hoc* comparisons revealed that CNO-treated IRs had a significantly higher probability to approach the food up on the pretest session relative to the test session ($p = 0.042$, Cohen's $d = 0.68$). Additionally, on the test session, CNO-treated rats had a significantly lower probability to approach the food cup relative to VEH-treated rats ($p = 0.006$, Cohen's $d = 0.36$). In all, it appears that LH-PVT inhibition has a specific impact on goal-tracking, and this is most apparent in IRs.

3.4.4 Prior LH-PVT inhibition has no influence on a test of conditioned reinforcement

Prior inhibition of the LH-PVT pathway had no effect on behavior during a conditioned reinforcement test (CRT). When collapsed across phenotypes, all rats responded more in the active port than the inactive port (effect of port: $F_{(1,52)} = 31.889$, $p < 0.001$; Figure 6a, Table 5). As expected, when phenotype was considered as a variable significant effects emerged as follows: (effect of port: $F_{(1,48)} = 44.721$, $p < 0.001$; effect of phenotype: $F_{(2,48)} = 9.221$, $p < 0.001$; port x phenotype interaction: $F_{(2,48)} = 8.537$, $p < 0.001$; Figure 6c). *Post hoc* analyses revealed that both STs ($p < 0.001$, Cohen's $d = 1.35$) and GTs ($p = 0.008$, Cohen's $d = 0.96$) had significantly more active than inactive nose pokes. However, STs had significantly more active nose pokes as compared to IRs ($p = 0.004$, Cohen's $d = 1.07$) and GTs ($p < 0.001$, Cohen's $d = 1.53$). Phenotypes also differed in the number of lever presses (effect of phenotype: $F_{(2,48)} = 11.430$, $p < 0.001$; Figure 6d); *post hoc* comparisons showed that STs had significantly more

lever presses relative to IRs ($p < 0.001$, Cohen's $d = 1.00$) and GTs ($p < 0.001$, Cohen's $d = 1.56$). These data support the notion that the lever-cue is a more effective conditioned reinforcer for STs relative to GTs and IRs; and suggest that LH-PVT inhibition during PavCA training do not affect the conditioned reinforcing properties of the lever-cue.

3.4.5 CNO without DREADD does not impact behavior

Our group previously showed that administration of 3 mg/kg CNO to non-DREADD rats had no off-target effects on behavior during PavCA or CRT (Campus et al., 2019). Here we assessed the effects of 5 mg/kg CNO in the absence of DREADD receptors in the brain. In this case, CNO-treated rats were no different from VEH-treated rats in sign-tracking (Figure 7a-c) or goal-tracking (Figure 7d-e) behaviors during PavCA sessions 1-4 or sessions 5-7 (Table 6). The same was true when phenotype was considered as a variable for sign-tracking (Figure 7a-c; Table 7) or goal-tracking (Figure 7d-e; Table 8) behaviors. That is, VEH and CNO-treated STs were no different from one another, and this was the case for IRs and GTs as well.

Consistent with the data presented above, rats exhibited more engagement with the active port relative to the inactive port when collapsed across phenotype (effect of port: $F_{(1,58)} = 22.034$, $p < 0.001$; Figure 7g) and when phenotype was considered as a variable (effect of port: $F_{(1,54)} = 21.243$, $p < 0.001$; effect of phenotype: $F_{(2,54)} = 3.648$, $p = 0.033$; Figure 7i). However, the behavioral differences in CRT were not due to treatment as the CNO-treated rats were no different from VEH-treated rats in number of nose pokes (Figure 7g, I; Table 8) or lever contacts (Figure 7h, j; Table 8). In all, we found that administration of 5 mg/kg CNO did not affect behavior on a conditioned reinforcement test in rats without DREADD expression.

3.5 Discussion

We examined the role of the LH-PVT pathway on the expression of conditioned responses in a Pavlovian conditioned approach paradigm. We observed that chemogenetic inhibition of this pathway decreased goal-tracking behavior, and this effect was apparent only in intermediate responder rats when phenotype was considered. LH-PVT inhibition had no impact on the behavior of sign- or goal-trackers. These findings are seemingly at odds with our previous reports that the LH and orexin signaling within the PVT are required for incentive value encoding and sign-tracking behavior (Haight et al., 2020). Nonetheless, these data suggest that the LH-PVT pathway regulates goal-tracking behavior without impacting reward consumption.

The fact that LH-PVT inhibition primarily affected the behavior of IRs is consistent with the fact that once a prepotent conditioned response is formed, it is difficult to disrupt, especially in the extremes of the population (Flagel et al., 2009; Flagel et al., 2008; Flagel et al., 2007). Indeed, the IR population displays flexibility in their responding, and it is therefore reasonable that a neural manipulation would have the greatest impact on the behavior of IRs. It should be noted that the IR rats were often overlooked, as the extreme phenotypes of sign-trackers and goal-trackers were largely the focus in prior work. As, such, we lack a map of the neural activity of IRs and this work has established pathway of interest for studying the behavior exhibited by this phenotype. This is important as the behavior of IRs and their flexibility in responding is likely more representative of the majority of the human population.

The current findings, that LH-PVT inhibition has no impact on sign-tracking behavior, is seemingly in opposition with the literature supporting a predominance of bottom-up processes in sign-trackers. While these data suggest that the LH-PVT pathway does not generally impact sign-tracking behavior, the orexin system specifically within this pathway may play a role

(Haight et al., 2020; Haight et al., 2017). Orexin is known to play a role in motivation, but little is known about its function in mediating individual variation in cue-motivated behaviors. It has been shown, however, that administration of orexin receptor antagonists into the PVT attenuates the incentive value of a food-paired cue in STs (Haight et al., 2020). Thus, there is evidence to suggest that orexinergic transmission in the LH-PVT pathway acts to encode the incentive value of reward cues, but further investigation is needed. We have found that LH-PVT projecting cells that are engaged by a reward-paired cue also tend to be orexinergic (Chapter 4), and these cells are activated to a greater extent in STs relative to GTs. Further, it has been shown that excitotoxic lesions of the LH resulted in a robust decrease in sign-tracking behavior (Haight et al., 2020). Importantly, these lesions were performed before the rats learned a conditioned response. Thus, it may be the case that the LH, and potentially its projections to the PVT, are utilized early in learning the cue-reward relationship and in the attribution of incentive salience to the reward-cue, but not necessarily in the expression of the resulting conditioned response. Elegant work by Zhu and colleagues demonstrated that the posterior PVT activity gates associative learning in the acquisition but not the expression of behavior. In head-fixed mice, the PVT was silenced via optogenetics either during cue presentation or reward consumption, and it was found that the strength of the associative CS-US relationship was diminished (via decreased anticipatory licking) during the acquisition of behavior, but not affected during the expression of behavior (Zhu et al., 2018). This notion of bottom-up processes, such as the LH-PVT pathway, differentially affecting the development versus the expression of sign-tracking behavior warrants further investigation.

While the current findings highlight a role for the LH-PVT pathway in goal-tracking behavior, it is important to note that the viral methods used likely affected multiple cellular

systems. That is, the DREADD virus was expressed in the entire LH-PVT pathway with no cellular specificity. The projections from the LH-PVT are heterogenous with the densest projections being orexinergic, GABAergic, and glutamatergic (Jennings et al., 2015; Kirouac et al., 2005; Li & Kirouac, 2012; Otis et al., 2019). In fact, multiple cellular systems play an integrative role in mediating reward properties and consumption. Orexinergic signaling within the PVT plays a predominant role in appetitive motivated behaviors (e.g.,(Barson et al., 2015; Li et al., 2009; Stratford & Wirtshafter, 2013), and stimulation of GABAergic neurons in the LH-PVT pathway elicits consummatory and reward-seeking behaviors (Wu et al., 2015; Zhang & van den Pol, 2017), consistent with the reported findings. Recently, it was shown that GABAergic projections from the LH-PVT are substantial (Otis et al., 2019) and optogenetic inhibition of LH GABA neurons following learning prevented the motivation to seek a reward but had no impact on reward consumption (Sharpe et al., 2017). Furthermore, LH GABAergic cells have been ascribed distinct functions such that one group of GABAergic cells is active in obtaining food rewards, while the other is active during food consumption (Jennings et al., 2015). This distinction is important because we show that goal-tracking is blunted with no impact on reward consumption within the task. Thus, further investigation is needed to determine exactly which cell types our viral manipulation targeted and to parse the role of orexinergic and GABAergic LH-PVT projection neurons in the development and expression of Pavlovian conditioned approach behavior.

Although we did not observe an effect of LH-PVT neuronal inhibition on food reward consumption during the PavCA task; we did not measure feeding behavior outside of this paradigm. Prior work has established that the LH is a critical region in modulating feeding responses (Hoebel & Teitelbaum, 1962; Margules & Olds, 1962). Further, the PVT is connected

to regions that regulate feeding such as the nucleus accumbens shell (Stratford, 2005; Stratford, 2007) and medial/lateral hypothalamus (Chen & Su, 1990; Moga et al., 1995), and has been shown to modulate food intake (Stratford & Wirtshafter, 2013). For instance, researchers measured food intake among sated rats through a test chamber that had food automatically dispensed without any effort needed from the rats. In this task, activation of $GABA_A$ receptors in the PVT dose-dependently increased food intake (Stratford & Wirtshafter, 2013). Methodological differences might underly these seemingly opposing results as systemic administration of the DREADD ligand CNO results in transient activation of the DREADD receptors, whereas application of the GABAergic drug acts sooner as its applied locally within the PVT. Additionally, our DREADD method was not cell specific whereas the approach by Stratford and colleagues was. We know that orexin receptor 1 (OX1R) expressing PVT neurons are activated in response to food-paired cues (Choi et al., 2010). Additionally, it has been shown that GABAergic LH-PVT inputs that project onto glutamatergic outputs to the nucleus accumbens mediate consummatory behavior (i.e. licking) (Otis et al., 2019). Additional work is needed to determine if LH-PVT pathway manipulations affect reward-seeking and consummatory behavior differentially, depending on the paradigm and neuronal cell types affected.

All the PVT-focused research within the ST/GT model has targeted the entire PVT (Campus et al., 2019; Haight et al., 2015; Haight et al., 2017; Kuhn et al., 2022; Kuhn et al., 2018). However, the anterior PVT (aPVT) and posterior PVT (pPVT) have distinct functions. Generally, the aPVT appears to regulate reward-seeking and arousal (Cheng et al., 2018; Do-Monte et al., 2017; Gao et al., 2020; Kolaj et al., 2012), whereas the pPVT in largely involved in stress and anxiety (Barson & Leibowitz, 2015; Bhatnagar & Dallman, 1999; Li et al., 2010a,

2010b). Here, we targeted the entire rostro-caudal gradient of the PVT, and it is therefore difficult to interpret to what extent these findings are mediated by either PVT subregion or how these data fit into the work of others who focus on one such subregion. Future work should focus on targeting either the aPVT or the pPVT. In relation to the ST/GT model, the orexinergic projections between the LH-aPVT pathway should be a target for further investigation studying the contributions of these signaling mechanisms in incentive salience encoding and attribution.

In the current study, we targeted afferents from the LH to the PVT to elucidate the role of this pathway in divergent cue-reward learning processes. Importantly, we demonstrate that CNO administration in non-DREADD rats has no off-target effects in PavCA or CRT. Through chemogenetic inhibition of the LH-PVT pathway during the expression of a learned conditioned response, we found that goal-tracking behavior in IR rats decreased, and that sign-tracking behavior remained intact. There was no effect of this manipulation on the conditioned reinforcing properties or incentive motivational value of the reward cue in IR rats. Further, the inhibition of the LH-PVT pathway had no impact on behavior in STs or GTs. Taken together, these data suggest that the LH-PVT pathway regulates goal-tracking behavior, but not sign-tracking behavior. Further, manipulation of this pathway after the cue-reward relationship has been learned and value attributed is not sufficient to affect the behavior of the extremes of the population. Continued work, however, should target specific cell systems within the LH, and also differentiate between the subregions of the PVT. In our approach of inhibiting of the entire LH-PVT pathway, we were unable to disentangle contributions of the orexin system or other neurotransmitter systems. In the future, targeting subregions of the PVT and specific cell types in the LH will hopefully lead to more clarity regarding the role of this subcortical pathway in cue-reward learning and individual differences in cue-motivated behaviors.

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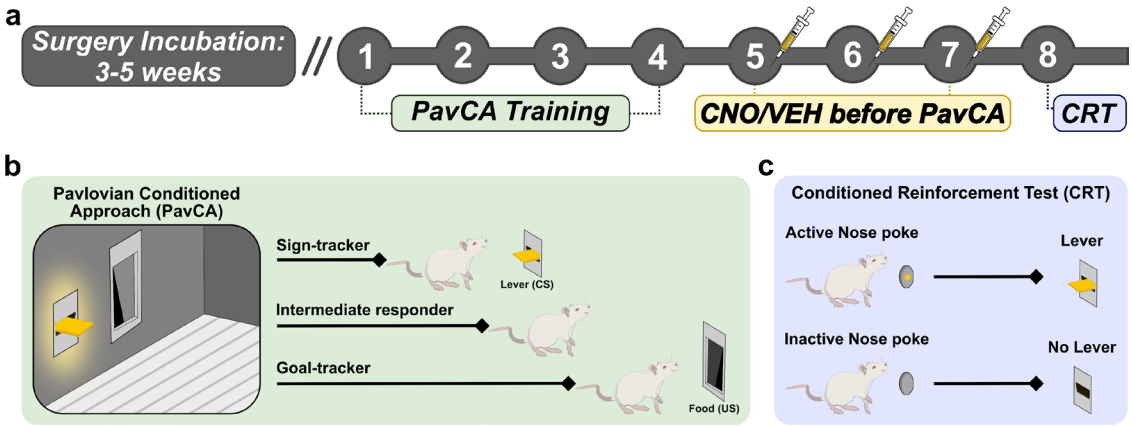

Figure 3-1 **Experimental timeline.**
(a) Schematic of the experimental timeline: A viral infusion surgery was performed to express inhibitory DREADD (Designer Receptors Exclusively Activated by Designer Drugs) virus in the lateral hypothalamus (LH) to the paraventricular nucleus of the thalamus (PVT) pathway. The virus incubated in the brain for 3-5 weeks and was followed by behavior. Pavlovian conditioned approach (PavCA) training occurred for 7 consecutive days. On days 5-7 of behavior, either clozapine-*N*-oxide (CNO; 5 mg/kg) or vehicle (VEH; 6% dimethyl sulfoxide) was injected intraperitoneally. On day 8 of behavior rats had a conditioned reinforcement test (CRT) with no injections. (b) PavCA Schematic illustrating the behavior box and responses of sign-tracker, intermediate responder, and goal-tracker. (c) CRT schematic wherein active nose pokes result in lever presentation and inactive nose pokes lead to no lever presentation.

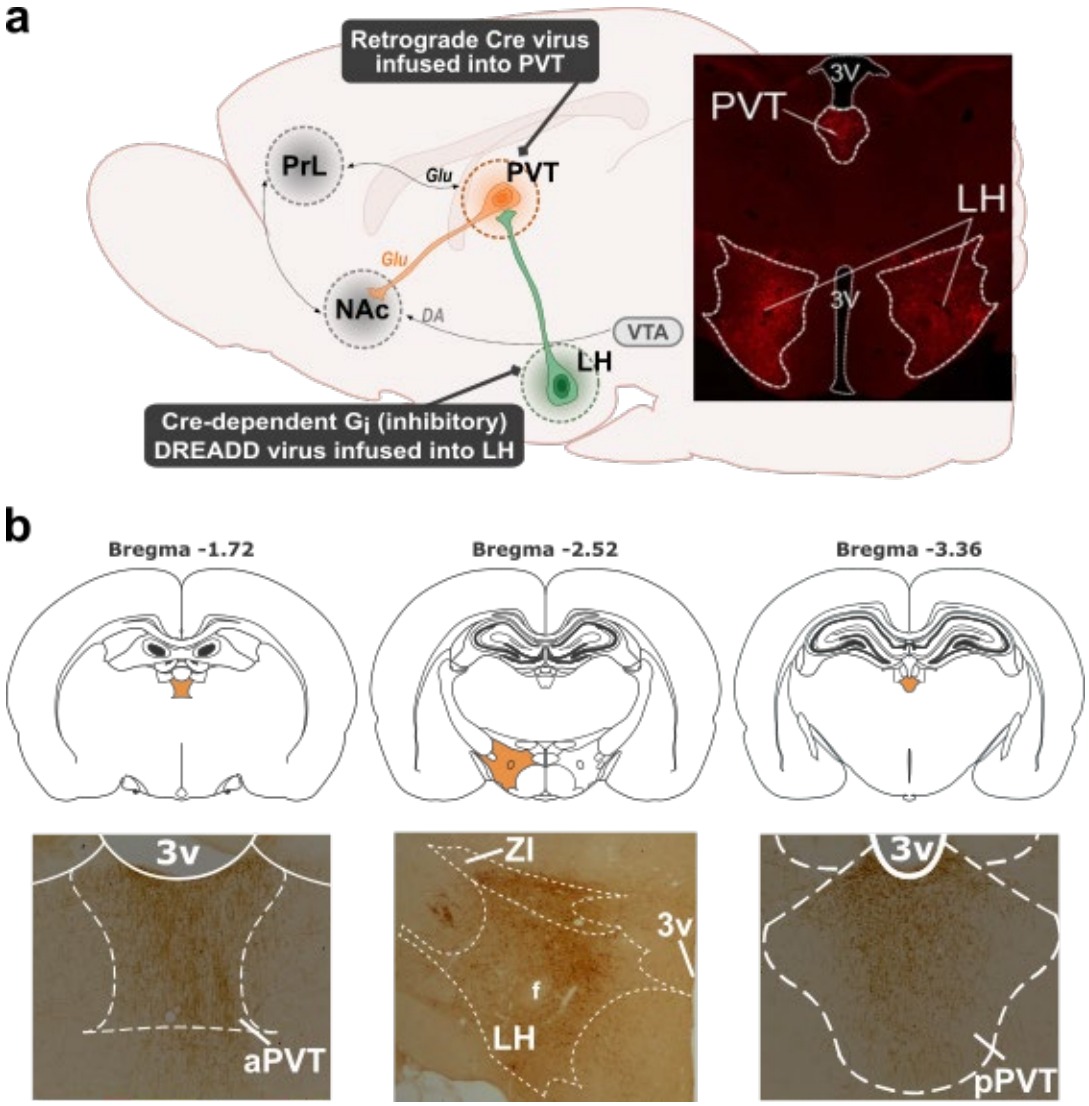

Figure 3-2 **Surgery schematic and virus expression.**
(a) Surgery schematic showing a dual-vector surgical approach to selectively express DREADD virus in the LH-PVT pathway. A retrograde Cre virus was infused into the anterior PVT (aPVT) and posterior PVT (pPVT). A Cre-dependent inhibitory DREADD virus bilaterally in the LH. A brain diagram highlighting brain regions and surgical infusion sights next to fluorescent virus expression in the LH-PVT pathway. (b) DREADD virus expression in the aPVT, representative section of the LH, and pPVT. PrL, prelimbic cortex; NAc, nucleus accumbens; VTA, ventral tegmental area; Glu, glutamate; DA, dopamine, 3v, third ventricle; ZI, zona incerta; f, fornix.

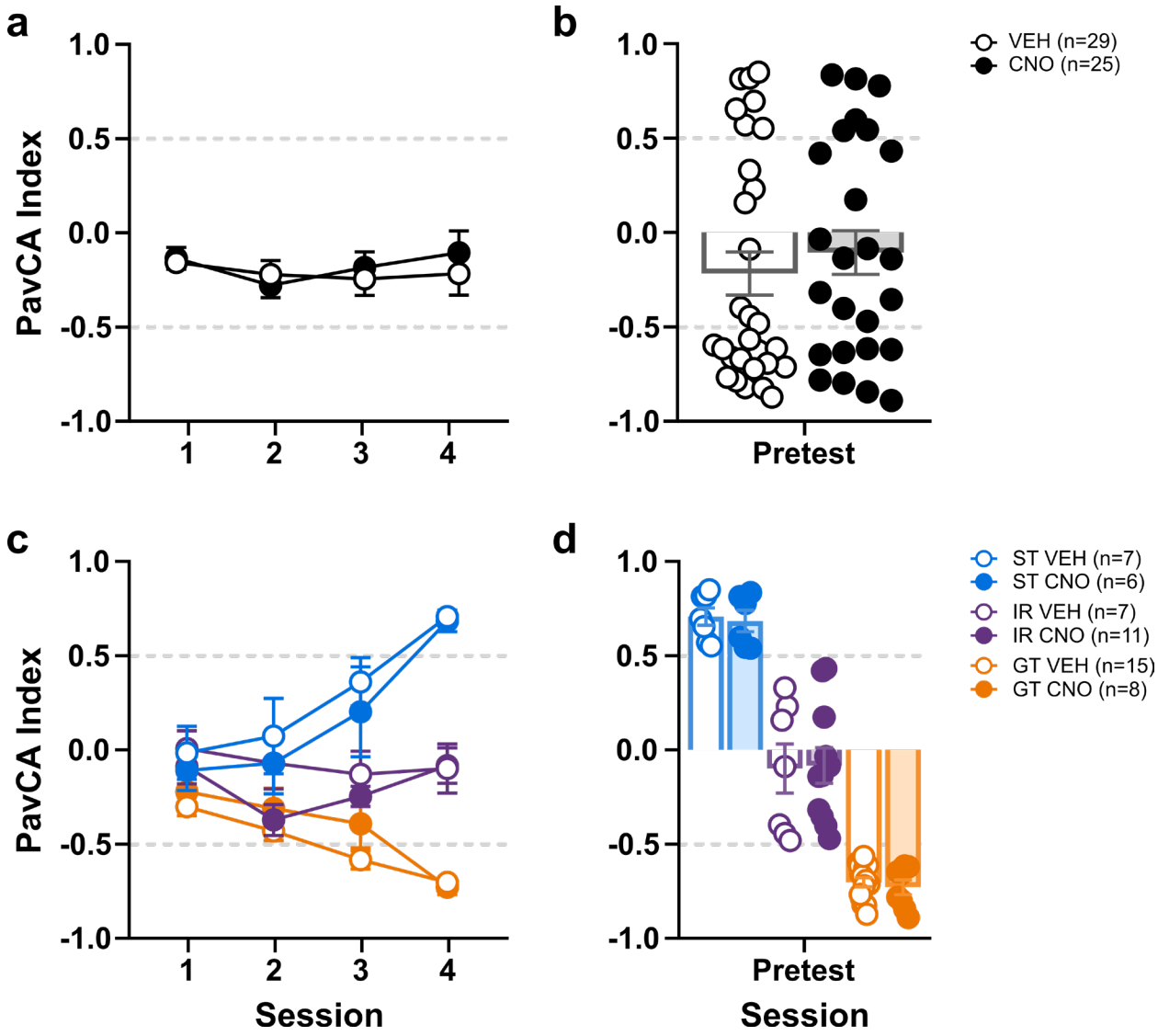

Figure 3-3 **Acquisition of Pavlovian Conditioned Approach (PavCA) prior to treatment.**
(a-b) Treatment (vehicle, VEH; clozapine-N-oxide, CNO) and (c-d) phenotype/treatment (sign-tracker, ST VEH/CNO; intermediate responder, IR VEH/CNO; goal-tracker, GT VEH/CNO) groups shown for PavCA Index. (a,c) PavCA Index over sessions 1-4 and (b,d) PavCA Index for session 4 (pretest). Data are expressed as mean ± SEM and show no differences between treatment or phenotype/treatment groups in PavCA Index (a,c) across sessions 1-4 or (b,d) in the pretest session.(c,d) There are differences between phenotypes for PavCA Index such that STs have a higher index than IRs and GTs.

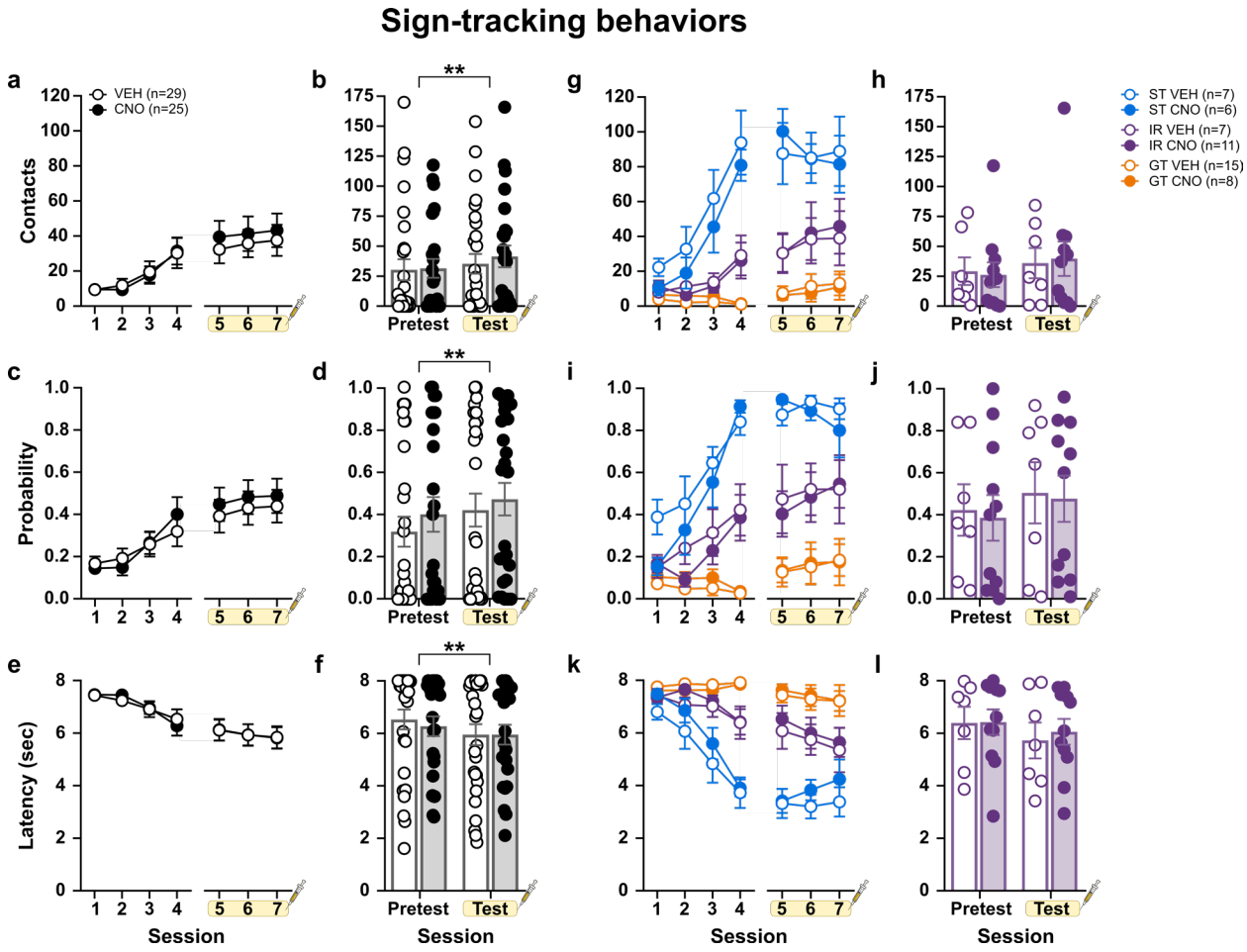

Figure 3-4 **Chemogenetic inhibition of the LH-PVT pathway has no impact on sign-tracking behavior.** (a-f) Treatment (VEH and CNO) or (g-l) phenotype/treatment (ST VEH/CNO, IR VEH/CNO, and GT VEH/CNO) groups shown for sign-tracking behaviors. Behaviors are shown (columns 1 and 3) across sessions 1-7 of PavCA and (columns 2 and 4) as pretest (session 4) vs. test (average of sessions 5-7) sessions. On sessions 5-7, either VEH or CNO were administered before behavior. Data are expressed as mean ± SEM for (top row) number of lever-cue contacts, (middle row) probability to approach the lever-cue, or (bottom row) latency to approach the lever-cue. (b,d,f) Graphs include individual data points for all rats, but (h, j, i) graphs include individual data points only for IR rats. There are no differences in sign-tracking based on (a,c,e) treatment or (g,i,k) treatment groups within each phenotype. There are (b) more lever-cue contacts (d) a higher probability to approach the lever-cue, and (f) a decreased latency to approach the lever-cue on the pretest sessions, relative to test sessions when treatment groups are collapsed. Brackets indicate significant differences between pretest and test sessions, $^{**}p < 0.01$.

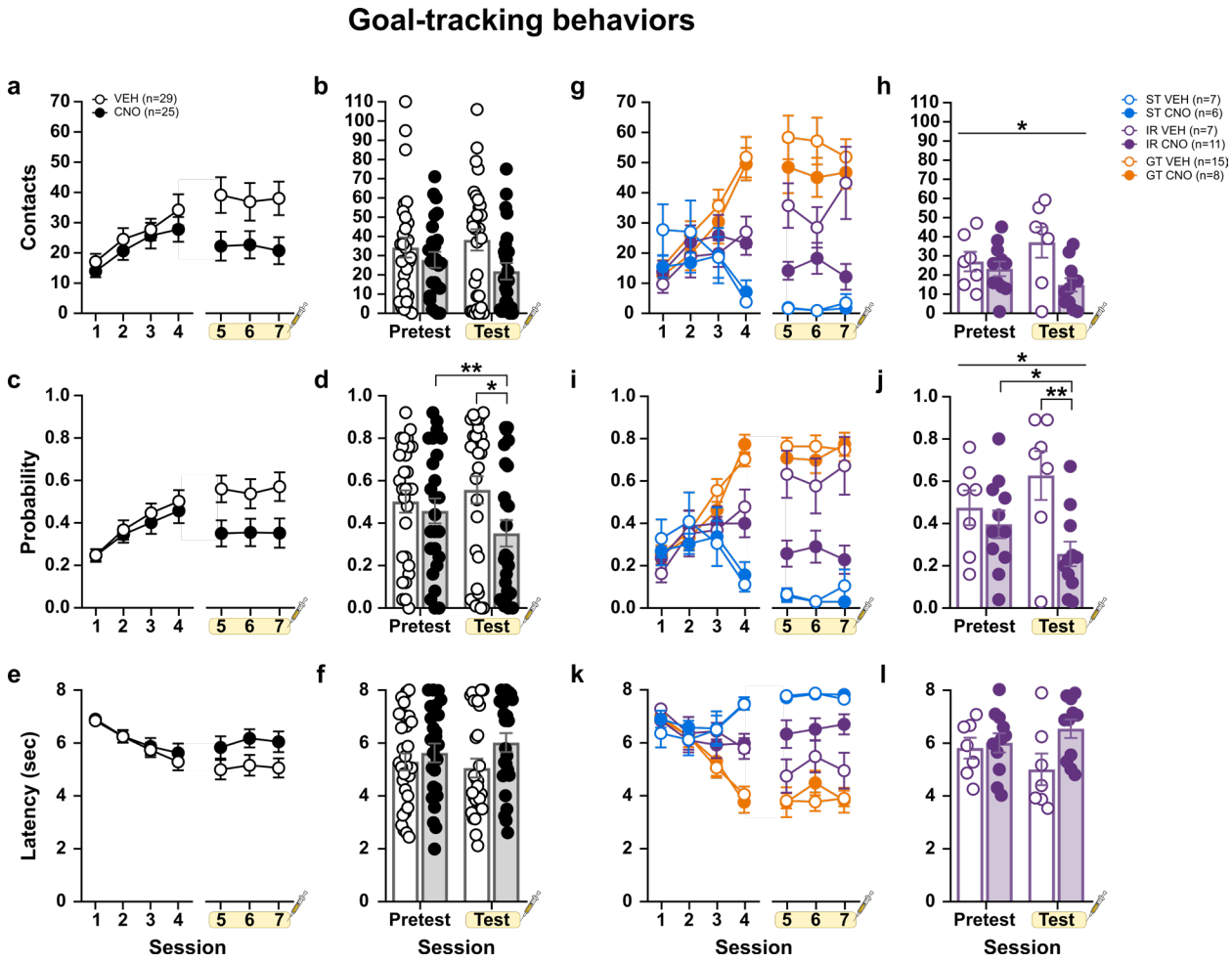

Figure 3-5 **Chemogenetic inhibition of the LH-PVT pathway attenuates goal-tracking behavior.** (a-f) Treatment (VEH and CNO) or (g-l) phenotype/treatment (ST VEH/CNO, IR VEH/CNO and GT VEH/CNO) groups shown for goal-tracking behaviors. Behaviors are shown (columns 1 and 3) across sessions 1-7 of PavCA and (columns 2 and 4) as pretest (session 4) vs. test (average of sessions 5-7) sessions. On sessions 5-7, either VEH or CNO were administered before behavior. Data are expressed as mean ± SEM for (top row) number of food cup contacts, (middle row) probability to approach the food cup, or (bottom row) latency to approach the food cup. (b,d,f) Graphs include individual data points for all rats, but (h, j, i) graphs include individual data points only for IR rats. CNO-treated rats have reduced (a,g,h) food cup contacts and (c,d,i,j) reduced probability to approach the food cup relative to VEH-treated rats. Straight line indicates a significant effect of treatment. Brackets indicate significant differences between or within pretest and test sessions, $*p < 0.05$ and $**p < 0.01$.

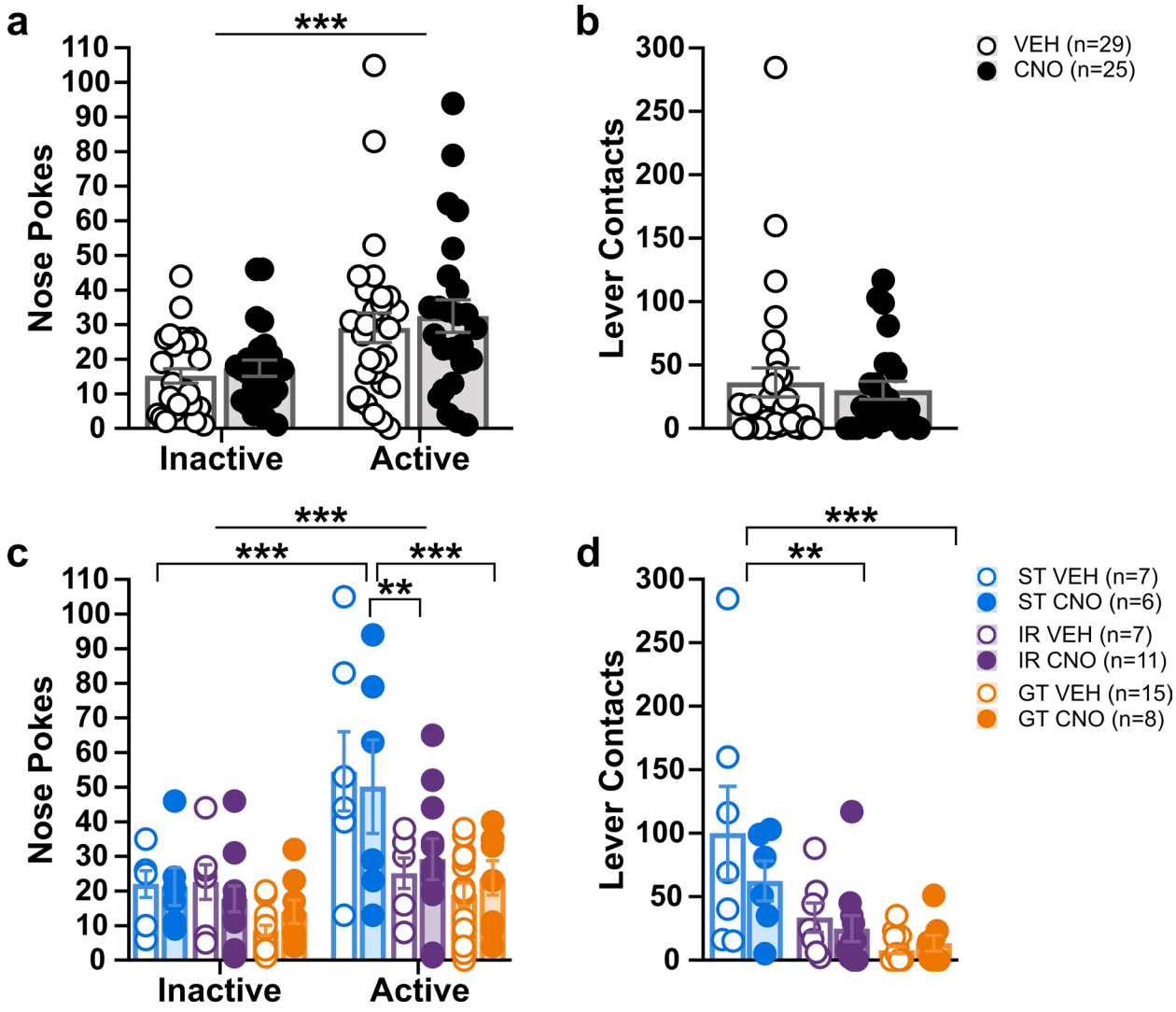

Figure 3-6 **Chemogenetic inhibition of the LH-PVT pathway does not impact the conditioned reinforcing properties of the lever-cue.**
(a-b) Treatment (VEH and CNO) or (c-d) phenotype/treatment (ST VEH/CNO, IR VEH/CNO and GT VEH/CNO) groups shown for behavior during the conditioned reinforcement test (CRT). Data are expressed as mean ± SEM for (a,c) nose pokes in the inactive and active ports and (b,d) lever contacts. There are no effects of treatment in the CRT. (a,c) Rats nose poke in the active port more than the inactive port. (c) STs have more active than inactive nose pokes; and they have more active nose pokes than IRs and GTs. (d) STs have more lever presses than IRs and GTs. Straight line indicates a significant effect of port. Brackets indicate significant differences between phenotypes or ports, $^{**}p < 0.01$ and $^{***}p < 0.001$.

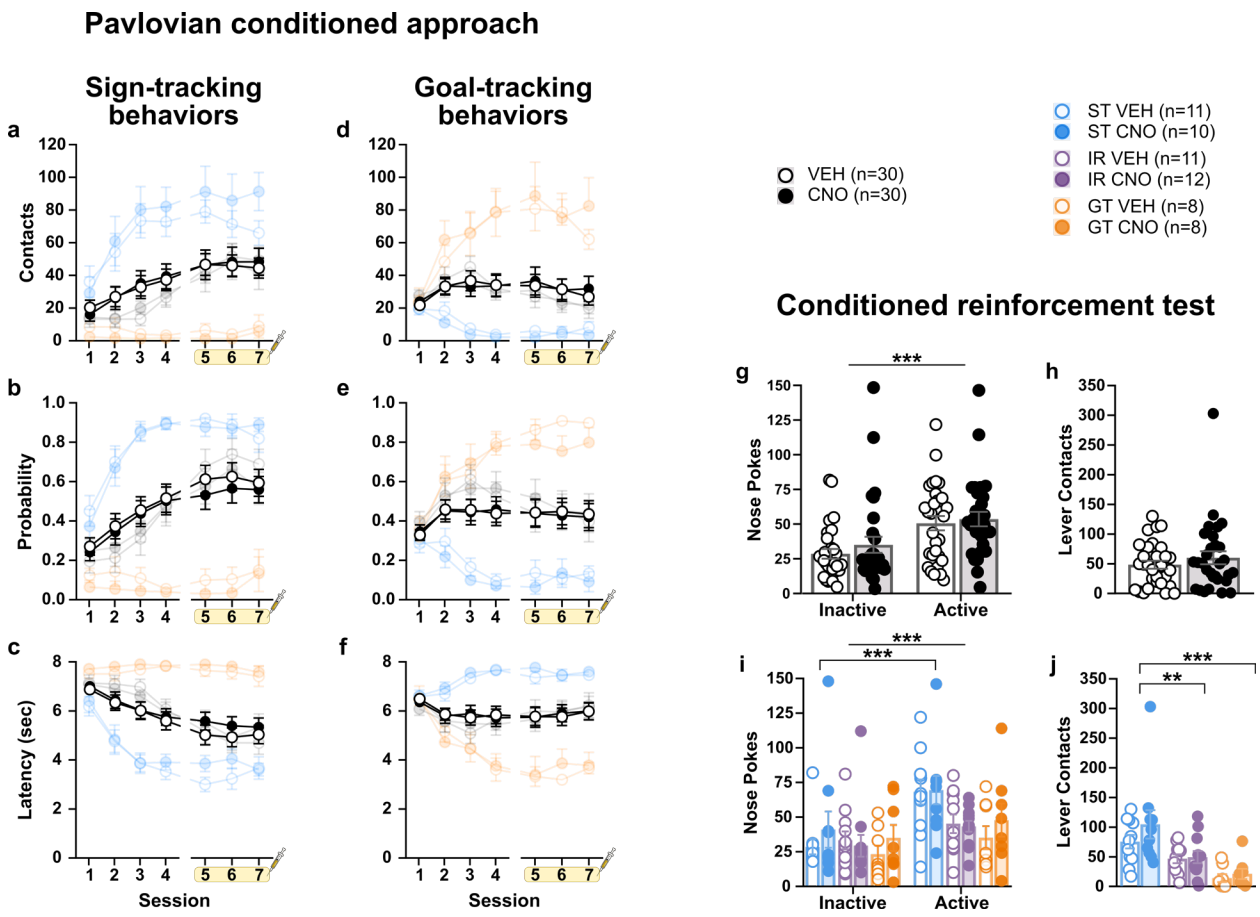

Figure 3-7 **Clozapine-N-oxide has no effects on Pavlovian Conditioned Approach (PavCA) behavior or the conditioned reinforcing properties of the lever in rats without DREADD receptors.**
Behavior is expressed as mean +/- SEM for rats that did not receive surgery for DREADD expression during (a-f) Pavlovian conditioned approach (PavCA) and g-j) a conditioned reinforcement test (CRT). (a-c) Sign-tracking and (d-f) goal-tracking behaviors across sessions 1-7 shown with treatment groups (black lines) collapsed and in the foreground and with phenotype/treatment groups expanded in background (muted color) (g-j). (a-c) Lever- and (d-f) food cup-directed behaviors for (a,d) number of contacts, (b,e) probability to approach, or (c,f) latency to approach. Treatment had no effect on sign- or goal-tracking behaviors in PavCA. Behavior during the CRT is shown as (g-h) treatment groups collapsed and (i-j) treatment and phenotype groups expanded. (g,i) Nose pokes in the inactive and active ports and (h,j) lever contacts. There were no significant effects of treatment on behavior during the CRT. (g,i) Rats nose poke in the active port more than the inactive port. (i) STs have more active than inactive nose pokes. (j) STs have more lever presses than IRs and GTs. Straight line indicates a significant effect of port. Brackets indicate significant differences between phenotypes or ports, $^{**}p < 0.01$ and $^{***}p < 0.001$.

Table 1

	Sessions 1-4: PavCA Index					
	Treatment			Treatment x Phenotype		
	DF	F	p	DF	*F*	*p*
Session	3,73.871	3.164	**.029**	3,65.959	3.614	**.018**
Treatment	1,54.052	.121	.729	1,50.528	.581	.450
Phenotype	-	-	-	2,50.528	42.631	**<.001**
*Session*Treatment*	3,73.871	1.009	.394	3,65.959	.508	.678
*Session*Phenotype*	-	-	-	6,65.959	20.242	**<.001**
Treatment Phenotype*	-	-	-	2,50.528	1.484	.236
*Session*Treatment*Phenotype*	-	-	-	6,65.959	1.057	.397

Table 3-1 **Statistical analyses for PavCA Index across sessions 1-4.**
Data from linear mixed effects model analyses for PavCA Index on sessions 1-4. The left panel compares rats by treatment, collapsed across phenotype. The right panel compares rats with treatment and phenotype as variables. Significant effects and interactions are bolded.

Table 2

	a. Sessions 1-4: Sign-tracking behaviors								
	Lever Contacts			Probability Lever			Latency Lever		
	DF	*F*	*p*	DF	*F*	*p*	DF	*F*	*p*
Session	3,62.664	7.647	**<.001**	3,68.418	7.703	**<.001**	3,62.467	8.544	**<.001**
Treatment	1,52.222	.014	.907	1,52.491	.008	.929	1,52.285	.000	.984
*Session*Treatment*	3,62.664	.358	.784	3,68.418	.972	.411	3,62.467	1.093	.359
	b. Sessions 5-7: Sign-tracking behaviors								
	Lever Contacts			Probability Lever			Latency Lever		
	DF	*F*	*p*	DF	*F*	*p*	DF	*F*	*p*
Session	2,95.247	.868	.423	2,59.794	1.634	.204	2,93.324	1.145	.323
Treatment	1,52.028	.252	.618	1,52.570	.228	.635	1,52.066	.000	1.000
*Session*Treatment*	2,95.247	.038	.963	59.794	.009	.991	2,93.324	.010	.990
	c. Pretest vs Test: Sign-tracking behaviors								
	Lever Contacts			Probability Lever			Latency Lever		
	DF	*F*	*p*	DF	*F*	*p*	DF	*F*	*p*
Session	1,52	7.506	**.008**	1,52	8.604	**.005**	1,52	10.417	**.002**
Treatment	1,52	.098	.756	1,52	.401	.530	1,52	.062	.805
*Session*Treatment*	1,52	.847	.362	1,52	.260	.612	1,52	.839	.364
	d. Sessions 1-4: Goal-tracking behaviors								
	Magazine Contacts			Probability Magazine			Latency Magazine		
	DF	*F*	*p*	DF	*F*	*p*	DF	*F*	*p*
Session	3,55.853	7.157	**<.001**	3,53.899	11.957	**<.001**	3,68.593	15.438	**<.001**
Treatment	1,57.616	.996	.322	1,58.158	.347	.558	1,56.378	.217	.643
*Session*Treatment*	3,55.853	.246	.864	3,53.899	.132	.941	3,68.593	.171	.916
	e. Sessions 5-7: Goal-tracking behaviors								
	Magazine Contacts			Probability Magazine			Latency Magazine		
	DF	*F*	*p*	DF	*F*	*p*	DF	*F*	*p*
Session	2,69.444	.159	.853	2,103.058	.225	.799	2,51.585	.928	.402
Treatment	1,52.137	5.147	**.027**	1,52.025	5.119	**.028**	1,52.012	3.655	.061
*Session*Treatment*	2,69.444	.253	.777	2,103.058	.362	.697	2,51.585	.216	.807
	f. Pretest vs Test: Goal-tracking behaviors								
	Magazine Contacts			Probability Magazine			Latency Magazine		
	DF	*F*	*p*	DF	*F*	*p*	DF	*F*	*p*
Session	1,52	.126	.724	1,52	1.044	.312	1,52	.249	.620
Treatment	1,52	3.072	.086	1,52	2.363	.130	1,52	1.970	.166
*Session*Treatment*	1,52	3.487	.067	1,52	10.532	**.002**	1,52	3.857	.055

Table 3-2 **Statistical analyses for sign- and goal-tracking behaviors by treatment.**
Data from linear mixed effects model analyses for (a,d) sessions 1-4 and (b,e) sessions 5-7, or a two-way repeated measures ANOVA (c,f) pretest vs test sessions comparing rats by treatment, collapsed across phenotype. Contacts, probability, and latency are represented for (a-c) sign-tracking behaviors and (d-f) goal-tracking behaviors. Significant effects and interactions are bolded.

Table 3

	a. Sessions 1-4: Sign-tracking behaviors								
	Lever Contacts			Probability Lever			Latency Lever		
	DF	*F*	*p*	DF	*F*	*p*	DF	*F*	*p*
Session	3,137.409	32.727	**<.001**	3,73.415	25.881	**<.001**	3,59.093	29.867	**<.001**
Treatment	1,50.676	.894	.349	1,50.507	.961	.331	1,48.867	1.043	.312
Phenotype	2,50.676	27.831	**<.001**	2,50.507	40.353	**<.001**	2,48.867	34.499	**<.001**
*Session*Treatment*	3,137.409	.152	.928	3,73.415	.774	.512	3,59.093	.813	.492
*Session*Phenotype*	6,137.409	18.003	**<.001**	6,73.415	13.343	**<.001**	6,59.093	16.300	**<.001**
Treatment Phenotype*	2,50.676	1.040	.361	2,50.507	.923	.404	2,48.867	1.127	.332
*Session*Treatment*Phenotype*	6,137.409	.237	.964	6,73.415	2.135	.059	6,59.093	1.053	.401
	b. Sessions 5-7: Sign-tracking behaviors								
	Lever Contacts			Probability Lever			Latency Lever		
	DF	*F*	*p*	DF	*F*	*p*	DF	*F*	*p*
Session	2,70.114	.364	.696	2,95.565	1.123	.329	2,72.946	.551	.579
Treatment	1,48.057	.009	.923	1,49.042	.036	.850	1,48.227	.686	.412
Phenotype	2,48.057	22.509	**<.001**	2,49.042	26.715	**<.001**	2,48.227	30.622	**<.001**
*Session*Treatment*	2,70.114	.254	.776	2,95.565	.173	.841	2,72.946	.058	.944
*Session*Phenotype*	4,70.114	1.636	.175	4,95.565	1.160	.333	4,72.946	1.710	.157
Treatment Phenotype*	2,48.057	.038	.962	2,49.042	.019	.981	2,48.227	.091	.914
*Session*Treatment*Phenotype*	4,70.114	.551	.699	4,95.565	.890	.473	4,72.946	.274	.894
	c. Pretest vs Test: Sign-tracking behaviors in intermediate responders								
	Lever Contacts			Probability Lever			Latency Lever		
	DF	*F*	*p*	DF	*F*	*p*	DF	*F*	*p*
Session	1,16	3.650	.074	1,16	2.767	.116	1,16	3.829	.068
Treatment	1,16	.000	.984	1,16	.038	.847	1,16	.056	.816
*Session*Treatment*	1,16	.400	.536	1,16	.008	.928	1,16	.321	.579

Table 3-3 **Statistical analyses for sign-tracking behaviors by treatment and phenotype.**
Data from linear mixed effects model analyses for (a) sessions 1-4 and (b) sessions 5-7, comparing rats by treatment and phenotype. A two-way repeated measures ANOVA assessing the effect of treatment within intermediate responders during pretest vs test sessions. Contacts, probability, and latency are represented for sign-tracking behaviors. Significant effects and interactions are bolded.

Table 4

	a. Sessions 1-4: Goal-tracking behaviors								
	Magazine Contacts			Probability Magazine			Latency Magazine		
	DF	*F*	*p*	DF	*F*	*p*	DF	*F*	*p*
Session	3,76.837	7.035	**<.001**	3,95.188	18.529	**<.001**	3,86.212	17.968	**<.001**
Treatment	1,51.163	.278	.601	1,50.286	.022	.883	1,51.460	.001	.972
Phenotype	2,51.163	4.666	**.014**	2,50.286	5.562	**.007**	2,51.460	7.078	**.002**
*Session*Treatment*	3,76.837	.284	.837	3,95.188	.260	.854	3,86.212	.068	.977
*Session*Phenotype*	6,76.837	11.583	**<.001**	6,95.188	19.421	**<.001**	6,86.212	17.423	**<.001**
Treatment Phenotype*	2,51.163	.409	.666	2,50.286	.056	.946	2,51.460	.303	.740
*Session*Treatment*Phenotype*	6,76.837	.665	.678	6,95.188	1.562	.167	6,86.212	.881	.512
	b. Sessions 5-7: Goal-tracking behaviors								
	Magazine Contacts			Probability Magazine			Latency Magazine		
	DF	*F*	*p*	DF	*F*	*p*	DF	*F*	*p*
Session	2,64.226	.203	.817	2,95.255	.812	.447	2,69.999	1.615	.206
Treatment	1,48.429	4.349	**.042**	1,48.154	7.561	**.008**	2,48.804	3.945	.053
Phenotype	2,48.436	32.846	**<.001**	2,48.161	57.539	**<.001**	2,48.811	56.982	**<.001**
*Session*Treatment*	2,64.226	.563	.572	2,95.255	.579	.563	2,69.999	.083	.920
*Session*Phenotype*	4,64.283	.451	.771	4,95.255	.097	.983	4,70.005	.234	.918
Treatment Phenotype*	2,48.436	1.426	.250	2,48.161	4.886	**.012**	2,48.811	2.655	.080
*Session*Treatment*Phenotype*	4,64.283	1.742	.152	4,95.255	1.752	.145	4,70.005	1.938	.114
	c. Pretest vs Test: Goal-tracking behaviors in intermediate responders								
	Magazine Contacts			Probability Magazine			Latency Magazine		
	DF	*F*	*p*	DF	*F*	*p*	DF	*F*	*p*
Session	1,16	.035	.853	1,16	.016	.901	1,16	.165	.690
Treatment	1,16	5.560	**.031**	1,16	5.149	**.037**	1,16	2.998	.103
*Session*Treatment*	1,16	4.448	.051	1,16	8.311	**.011**	1,16	4.105	.060

Table 3-4 **Statistical analyses for goal-tracking behaviors by treatment and phenotype.**
Data from linear mixed effects model analyses for (a) sessions 1-4 and (b) sessions 5-7, comparing rats by treatment and phenotype. A two-way repeated measures ANOVA assessing the effect of treatment within intermediate responders during pretest vs test sessions. Contacts, probability, and latency are represented for goal-tracking behaviors. Significant effects and interactions are bolded.

Table 5

	a. Session 8: Conditioned Reinforcement					
	Nose Pokes			Lever Contacts		
	DF	*F*	*p*	DF	*F*	*p*
Port	1,52	31.889	**<.001**	-	-	-
Treatment	1,52	.433	.514	1,52	.196	.660
*Port*Treatment*	1,52	.053	.819	-	-	-
	b. Session 8: Conditioned Reinforcement					
	Nose Pokes			Lever Contacts		
	DF	*F*	*p*	DF	*F*	*p*
Port	1,48	44.721	**<.001**	-	-	-
Treatment	1,48	.035	.853	1,48	1.282	.263
Phenotype	2,48	9.221	**<.001**	2,48	11.430	**<.001**
*Port*Treatment*	1,48	.096	.758	-	-	-
*Port*Phenotype*	2,48	8.537	**<.001**	-	-	-
*Treatment*Phenotype*	2,48	.365	.696	1,48	1.055	.356
*Port*Treatment*Phenotype*	2,48	.623	.541	-	-	-

Table 3-5 **Statistical analyses for behavior during the Conditioned Reinforcement Test (CRT).**
Data from a mixed ANOVA comparing (left column) nose pokes into each port and a two-way ANOVA comparing lever contacts during the conditioned reinforcement test. Analysis was conducted between (a) treatment groups and (b) with treatment and phenotype as variables. Significant effects and interactions are bolded.

Table 6

	a. Sessions 1-4: Sign-tracking behaviors								
	Lever Contacts			Probability Lever			Latency Lever		
	DF	*F*	*p*	DF	*F*	*p*	DF	*F*	*p*
Session	3,58	10.974	**<.001**	3,58	14.495	**<.001**	3,87.520	12.523	**<.001**
Treatment	1,58	0.000	0.987	1,58	0.078	0.781	1,58.355	0.065	0.799
*Session*Treatment*	3,58	0.316	0.814	3,58	0.020	0.996	3,87.520	0.170	0.916
	b. Sessions 5-7: Sign-tracking behaviors								
	Lever Contacts			Probability Lever			Latency Lever		
	DF	*F*	*p*	DF	*F*	*p*	DF	*F*	*p*
Session	2,72.906	0.063	0.939	2, 116	0.595	0.553	2, 116	0.733	0.483
Treatment	1,58.163	0.035	0.851	1,58	0.368	0.546	1,58	0.745	0.392
*Session*Treatment*	2,72.906	0.293	0.747	2, 116	0.502	0.607	2, 116	0.547	0.580
	d. Sessions 1-4: Goal-tracking behaviors								
	Magazine Contacts			Probability Magazine			Latency Magazine		
	DF	*F*	*p*	DF	*F*	*p*	DF	*F*	*p*
Session	3,58	4.241	**0.009**	3,80.815	5.666	**0.001**	3,58	5.175	**0.003**
Treatment	1,58	0.005	0.943	1,58.507	0.006	0.939	1,58	0.015	0.904
*Session*Treatment*	3,58	0.372	0.773	3,80.815	0.204	0.893	3,58	0.593	0.622
	e. Sessions 5-7: Goal-tracking behaviors								
	Magazine Contacts			Probability Magazine			Latency Magazine		
	DF	*F*	*p*	DF	*F*	*p*	DF	*F*	*p*
Session	2,113.482	3.402	**0.037**	2, 76.021	0.249	0.780	2,115.032	2.262	0.109
Treatment	1,58.003	0.067	0.797	1, 58.036	0.018	0.893	1,57.999	0.008	0.931
*Session*Treatment*	2,113.482	1.147	0.321	2, 76.721	0.102	0.903	2,115.032	0.358	0.700

Table 3-6 **Statistical analyses for sign- and goal-tracking behaviors by treatment in no-DREADD rats.** Data from linear mixed effects model analyses assessing the effect of treatment in rats that did not have DREADD surgery for (a,d) sessions 1-4 and (b,e) sessions 5-7. Contacts, probability, and latency are represented for (a-b) sign-tracking behaviors and (d-e) goal-tracking behaviors. Significant effects and interactions are bolded.

Table 7

	a. Sessions 1-4: Sign-tracking behaviors								
	Lever Contacts			Probability Lever			Latency Lever		
	DF	*F*	*p*	DF	F	p	DF	F	p
Session	3,54	19.784	**<.001**	3,144.296	18.010	**<.001**	3,95.656	23.674	**<.001**
Treatment	1,54	0.019	0.890	1,55.497	0.098	0.756	1,58.901	0.045	0.832
Phenotype	2,54	34.867	**<.001**	2,55.497	62.546	**<.001**	2,58.901	52.097	**<.001**
*Session*Treatment*	3,54	1.061	0.373	3,144.296	0.069	0.976	3,95.656	0.208	0.891
*Session*Phenotype*	6,54	16.765	**<.001**	61,44.296	10.977	**<.001**	6,95.656	15.878	**<.001**
Treatment Phenotype*	2,54	0.178	0.838	2,55.497	0.368	0.694	2,58.901	0.257	0.774
*Session*Treatment*Phenotype*	6,54	0.625	0.710	6,144.296	0.251	0.958	6,95.656	0.227	0.967
	b. Sessions 5-7: Sign-tracking behaviors								
	Lever Contacts			Probability Lever			Latency Lever		
	DF	*F*	*p*	DF	*F*	*p*	DF	*F*	*p*
Session	2,108.463	0.003	0.997	2,108	.404	.669	2,108	.713	.493
Treatment	1,56.702	0.162	0.689	1,54	.865	.356	1,54	1.449	.234
Phenotype	2,56.702	35.261	**<.001**	2,54	70.615	**<.001**	2,54	51.708	**<.001**
*Session*Treatment*	2,108.463	0.207	0.813	2,108	.555	.575	2,108	.534	.588
*Session*Phenotype*	4,108.463	2.672	**0.036**	4,108	2.422	.053	4,108	2.100	.086
*Treatment*Phenotype*	2,56.702	1.076	0.348	2,54	.349	.707	2,54	.079	.925
*Session*Treatment*Phenotype*	4,108.463	0.615	0.652	4,108	.589	.671	4,108	1.599	.180

Table 3-7 **Statistical analyses for sign-tracking behaviors by treatment and phenotype in no-DREADD rats.** Data from linear mixed effects model analyses in rats that did not have DREADD surgery for (a) sessions 1-4 and (b) sessions 5-7, comparing rats by treatment and phenotype. Contacts, probability, and latency are represented for sign-tracking behaviors. Significant effects and interactions are bolded.

Table 8

	a. Sessions 1-4: Goal-tracking behaviors								
	Magazine Contacts			Probability Magazine			Latency Magazine		
	DF	*F*	*p*	DF	*F*	*p*	DF	*F*	*p*
Session	3,95.753	14.598	**<.001**	3,131.599	14.093	**<.001**	3,107.097	14.808	**<.001**
Treatment	1,57.395	0.028	0.868	1,55.848	0.013	0.908	1,56.370	0.000	0.990
Phenotype	2,57.395	32.442	**<.001**	2,55.848	27.536	**<.001**	2,56.370	25.099	**<.001**
*Session*Treatment*	3,95.753	0.538	0.658	3,131.599	0.139	0.936	3,107.097	0.594	0.620
*Session*Phenotype*	6,95.753	19.507	**<.001**	6,131.599	17.863	**<.001**	6,107.097	18.449	**<.001**
*Treatment*Phenotype*	2,57.395	0.242	0.786	2,55.848	0.125	0.883	2,56.370	0.049	0.952
*Session*Treatment*Phenotype*	6,95.753	0.880	0.513	6,131.599	0.739	0.619	6,107.097	0.630	0.706
	b. Sessions 5-7: Goal-tracking behaviors								
	Magazine Contacts			Probability Magazine			Latency Magazine		
	DF	*F*	*p*	DF	*F*	*p*	DF	*F*	*p*
Session	2,110.272	4.158	**.018**	2,71.686	.129	.879	2,104.005	2.233	.112
Treatment	1,54.017	.148	.702	1,54.109	.294	.590	1,54.001	.146	.704
Phenotype	1,54.017	46.545	**<.001**	2,54.109	44.326	**<.001**	2,54.001	53.707	**<.001**
*Session*Treatment*	2,110.272	2.122	.125	2,71.686	.131	.878	2,104.005	.492	.613
*Session*Phenotype*	4,110.272	1.903	.115	4,71.686	.924	.455	4,104.005	.687	.602
*Treatment*Phenotype*	2,54.017	.238	.789	2,54.109	.493	.614	2,54.001	.159	.853
*Session*Treatment*Phenotype*	4,110.272	3.112	**.018**	4,71.686	1.667	.167	4,104.005	1.044	.388

Table 3-8 **Statistical analyses for goal-tracking behaviors by treatment and phenotype in no-DREADD rats.** Data from linear mixed effects model analyses in rats that did not have DREADD surgery for (a) sessions 1-4 and (b) sessions 5-7, comparing rats by treatment and phenotype. Contacts, probability, and latency are represented for goal-tracking behaviors. Significant effects and interactions are bolded.

Table 9

	a. Session 8: Conditioned Reinforcement					
	Nose Pokes			Lever Contacts		
	DF	*F*	*p*	DF	*F*	*p*
Port	1,58	22.034	**<.001**	-	-	-
Treatment	1,58	.538	.466	1,58	.832	.365
*Port*Treatment*	1,58	.163	.687	-	-	-
	b. Session 8: Conditioned Reinforcement					
	Nose Pokes			Lever Contacts		
	DF	*F*	*p*	DF	*F*	*p*
Port	1,54	21.243	**<.001**	-	-	-
Treatment	1,54	.990	.324	1,54	.369	.546
Phenotype	2,54	3.648	**.033**	2,54	10.304	**<.001**
*Port*Treatment*	1,54	.095	.759	-	-	-
*Port*Phenotype*	2,54	2.848	.067	-	-	-
*Treatment*Phenotype*	2,54	.677	.512	1,54	.062	.940
*Port*Treatment*Phenotype*	2,54	.255	.776	-	-	-

Table 3-9 **Statistical analyses for behavior during the Conditioned Reinforcement Test (CRT) in no-DREADD rats.**
Data from a mixed ANOVA comparing (left column) nose pokes into each port and a two-way ANOVA comparing lever contacts during the conditioned reinforcement test. Analysis was conducted between (a) treatment groups and (b) with treatment and phenotype as variables. Significant effects and interactions are bolded.

Chapter 4

Male Rats that Attribute Incentive Salience to Reward Cues Show Enhanced Engagement of Orexinergic Projections from the Lateral Hypothalamus to the Paraventricular Nucleus of the Thalamus

*Note: Some of the methodological details within Chapter 4 have appeared in a previous manuscript (*Haight JL, Fuller ZL, Fraser KM, Flagel SB. A food-predictive cue attributed with incentive salience engages subcortical afferents and efferents of the paraventricular nucleus of the thalamus. *Neuroscience*. 340, 135-152.*) and is reproduced here with permission from the authors.*

4.1 Abstract

Associative learning strategies can dictate survival. Cues in one's environment can gain motivational properties through the attribution of incentive salience and lead to maladaptive behaviors that are characteristic of psychopathologies including substance use disorders. Individuals vary in the extent to which they attribute incentive value to a reward-paired cue. These differences in cue-motivated behavior are captured through a Pavlovian conditioned approach (PavCA) paradigm, where two distinct phenotypes emerge. Sign-trackers (STs) develop a conditioned response towards reward-paired cues and attribute both predictive and incentive value to the cues, while goal-trackers (GTs) approach the reward location and attribute only predictive value to the Pavlovian cue. Differential engagement of "top-down" or "bottom-up" neural processes is believed to underly these two behavioral phenotypes, with the paraventricular nucleus of the thalamus (PVT) as a potential mediator in this regard. Prior work

has shown that the lateral hypothalamus (LH) plays a role in "bottom-up" processes, which are involved in attributing motivational properties to reward-associated cues. A subset of LH-PVT projecting neurons are orexinergic, and this peptide has been implicated in cue-motivated behaviors. To determine whether the LH-PVT projecting neurons that encode the incentive value of a reward cue in sign-trackers are indeed orexinergic, we used a novel combination of techniques: hairpin chain reaction *in situ* hybridization (HCR FISH) and immunofluorescence (IF). We demonstrate that STs show more engagement of the LH throughout its rostrocaudal axis, and that the LH-PVT projection neurons that show more activity in STs relative to GTs are indeed orexinergic.

4.2 Introduction

Cues in the environment can reliably predict biologically relevant stimuli; and recognition of these associations can aid in one's survivability. For some individuals, environmental cues signal valuable resources, but for others, those same cues can become distractors and bias attention. Parsing the neural mechanisms underlying differences in cue-reward learning strategies is relevant to a number of psychopathologies. For instance, mere exposure to drug-related cues in individuals who suffer from substance abuse disorder can elicit relapse (Robinson & Berridge, 1993; Tomie, 1996), and this presumably occurs via cue-induced activation of motivationally relevant neural circuitry (for review see: (Goodyear, 2019; Hill-Bowen et al., 2021; Michaelides et al., 2012; Tanabe et al., 2019; Volkow et al., 2004; Volkow et al., 1999). When cues gain motivational value through incentive salience attribution (Robinson & Berridge, 1993) the cues themselves can drive behavior and lead to adverse consequences in vulnerable populations (Berridge & Robinson, 2003; Frank et al., 2020; Hellberg et al., 2019; Mahler & de Wit, 2010; Versace et al., 2016).

The sign-tracker (ST)/goal-tracker (GT) animal model allows us to capture individual variation in response to reward cues (Robinson & Flagel 2009). Some individuals, sign-trackers (STs), attribute both predictive and incentive value to cues and other individuals, goal-trackers (GTs), primarily attribute predictive value to the cue (Flagel et al., 2009). These differences in cue-responsivity have been associated with other psychological traits. For example, rats with an inherent tendency to sign-track to reward-cues also exhibit more impulsive behavior (Lovic et al., 2011), inadequate attentional control (Flagel et al., 2010; Paolone et al., 2013), an exaggerated fear response (Morrow et al., 2011; Morrow et al., 2015), and enhanced responsivity drugs and drug-paired cues (Flagel et al., 2010; Saunders & Robinson, 2010, 2011; Saunders et al., 2013; Yager & Robinson, 2013). Conversely, rats that are inherently prone to goal-track are more influenced by contexts in drug-related (Saunders et al., 2014) and fear-conditioning paradigms (Morrow et al., 2011). Not surprisingly, different neural processes have been shown to contribute to these distinct "cognitive-motivational" styles exhibited by STs and GTs.

There is evidence to suggest that the behavior of GTs is dependent on "top-down" neural circuitry; whereas that of STs is driven by "bottom-up" pathways (Flagel et al., 2011; Haight et al., 2017). The paraventricular nucleus of the thalamus (PVT) has emerged as a key neural node that may subserve behavior in both GTs and STs (Campus et al., 2019; Flagel et al., 2011; Haight et al., 2015; Haight et al., 2017). The LH-PVT pathway has been identified as a potential key mediator of "bottom up" processes in reward learning (Kirouac et al., 2005; Peyron et al., 1998). Specifically, this pathway shows greater neuronal engagement (i.e., c-fos) in STs relative to GTs in response to a reward cue (Haight et al., 2014). Further, lesions of the LH and orexin (OX) receptor antagonism in the PVT both attenuate the propensity to sign-track, with no effect on goal-tracking behavior (Haight et al., 2020).

Here we utilized tissue from a prior fluorogold (FG) tracing experiment (Haight et al., 2017) to determine if LH-PVT projecting neurons engaged by a cue are also orexinergic. We hypothesized that STs engage more orexinergic LH-PVT projecting neurons than GTs in response to the reward cue. We utilized a novel combination of immunofluorescent multiplexing techniques (Choi et al., 2010; Choi et al., 2018; Kumar et al., 2021) and confirmed that STs show more engagement of the LH, as previously reported (Haight et al., 2017). In addition, we found that STs engage more orexinergic LH-PVT projection neurons than GTs in response to the reward cue.

4.3 Materials and Methods

The following methods, up until tissue processing, are adapted from Haight et al., 2017 (Figure 1a).

4.3.1 Subjects

Forty male Sprague-Dawley rats, at approximately eight weeks of age, were obtained from Envigo, weighing 230 to 300 grams upon arrival. Rodents were pair-housed in standard acrylic cages (46 x 24 x 22 cm) in a climate-controlled room. Rats were able to acclimate to the new environment for 10 days prior to any experimentation. In addition, subjects were kept on a 12-h light:dark cycle during the experiment (lights were on at 7:00h), and food and water were accessible *ad libitum*. All behavioral training was performed in the light cycle between 12:00 and 17:00 h.

4.3.2 Surgery

After the 10-day acclimation period, the retrograde tracer fluorogold (FG; Fluorochrome, Denver, CO, USA) was infused into the PVT via stereotaxic surgery. Aseptic conditions were

maintained throughout the surgery. First, a surgical plane of anesthesia was induced with inhalation of 5% isoflurane. The scalp was then shaved and sterilized with swabs of a 70% alcohol and Betadine solution (Betadine, Stamford, CT, USA). A small incision was subsequently made to reveal bregma and lambda coordinates, and the skull was leveled within ± 0.1mm. Small burr holes were drilled above the PVT, and a 0.5 μL Hamilton Neuros syringe (Hamilton Company, Reno, NV, USA) placed in a Kopf Model 5000 Microinjection Unit (David Kopf Instruments, Tujunga, CA, USA) was used to make two 50-nL injections of 2% FG solution diluted in 0.9% sterile saline into the PVT. This was the smallest volume that could be reliably injected. Some argue that damaged, and possibly undamaged, axons of passage could take up FG, resulting in erroneous neuronal labeling (Dado et al., 1990). To minimize this risk, FG injections were performed with a Hamilton Neuros syringe with a small 32-gauge injector tip, limiting damage to the FG injection site and thus uptake by damaged axons.

The injections were performed at the following coordinates relative to bregma: AP -2.0, ML -1.0, DV -5.4 and AP -3.0, ML -1.0, DV -5.5. The stereotaxic arm was angled at 10° toward the midline. Each injection lasted approximately 2 min, and the syringe was left in place for 5 min after the injection to minimize diffusion of the FG solution up the injection track. After, the syringe was slowly retracted, and the scalp was closed with wound clips. Immediately prior to surgery, and 24 h after surgery, subjects received subcutaneous injections of the nonsteroidal anti-inflammatory drug flunixin (2.5 mg/kg FlunixiJect diluted in 0.9% sterile saline; Butler Schein Animal Health, Dublin, OH, USA) for pain management. Rats were then allowed to recover for 8 to 9 days prior to any further experimentation.

4.3.3 Behavioral Testing

4.3.3.1 PavCA Training:

After surgical recovery, all rats underwent Pavlovian conditioned approach (PavCA) procedures like those previously described (Meyer et al., 2012). Standard behavioral test chambers (MED Associates, St. Albans, VT, USA) were enclosed in sound-attenuating boxes that were equipped with ventilation fans that provided constant air flow and background noise. All behavioral data were collected using MED PC software (Med Associates, St. Albans, VT, USA).

A food cup was connected to a pellet dispenser located in the middle of one of the walls in each chamber. Upon activation of the pellet dispenser, one 45-milligram banana-flavored grain pellet (Bio-Serve, Flemington, NJ, USA) was delivered. Each food cup was equipped with an infra-red photo beam and breaks of this beam were registered and recorded as head entries. Flanking the food cup to the left or right was a retractable, illuminated lever, positioned at equal height with the food cup. Chambers alternated in whether the lever was to the left or right of the food cup. All levers were calibrated such that approximately 10-15 grams of force would cause a deflection of the lever and would be registered as a lever contact. A white house light was placed on the upper middle portion of the wall directly across from the food cup and lever. This light was illuminated for the duration of each Pavlovian conditioning session.

Two days before behavioral training, rats were briefly handled by the experimenters in the housing room, and a small amount of banana-flavored grain pellets (approximately 25 pellets per rat) were placed in the home cage to familiarize the rats with the experimenters and novel food. Following these two days, all rats underwent one pretraining session in the test chambers as follows. Each food cup was baited with three banana-flavored pellets before the pre-training

session to direct the rats' attention to the location of the food reward delivery. At the start of the pre-training session, the house light remained off for 5 min to allow the rats to acclimate to the training chamber. After this acclimation period, the house light was illuminated, and 25 food pellets were delivered one at a time into the food cup on a variable interval 30-second schedule (range 0-60 s). The lever remained retracted for the entirety of the session, which lasted an average of 12.5 min. Following pretraining, rats underwent five sessions of PavCA training, one session occurring each day. Each session consisted of 25 trails in which the 8-second insertion of the illuminated lever (CS) into the test chamber was paired with delivery of one banana-flavored pellet (US) into the food cup. CS-US presentation occurred on a variable interval 90-second schedule (range 30-150 s). In addition, a small subset of rats from the experimental group (n=8) were unpaired as a control group. These animals received the same number of CS and US presentations, but the lever-CS and food-US were unpaired. Each PavCA and Unpaired Control session lasted approximately 40 min. The following data were recorded per trial during each session to quantify PavCA behaviors: (1) the number of food cup entries during the 8 s lever-CS period, (2) the latency to first food-cup entry upon lever-CS presentation, (3) the number of lever-CS contacts, (4) latency to first lever-CS contact, and (5) the number of food-cup entries during the inter-trial interval.

After session 5 of PavCA, rats in the "paired" group were classified as STs, GTs, or intermediate responders (INs) based on their average PavCA Index scores from sessions 4 and 5. The PavCA Index is a composite score that is used to assess the propensity of a rat to approach the lever-CS vs. the food cup (location of US delivery) upon CS presentation. This Index relies on three different metrics: response bias [(total lever contacts – total food cup contacts)/(sum of total contacts)], probability difference score [$prob_{(lever)} - prob_{(food\ cup)}$], and latency difference

score [– (lever contact latency – food cup entry latency/8]. These three measures are then averaged together to create the PavCA Index score, which ranges from -1.0 to 1.0, with -1.0 representing an individual whose behavior is solely directed toward the lever-CS.

4.3.3.2 CS re-exposure

After PavCA training, the test chambers were reconfigured such that the food cup and pellet dispenser were removed, the lever was placed in the center of the wall it was previously located on, and new metal grate flooring was inserted. To minimize the influence of contextual cues, rats classified as STs, GTs, and the unpaired control groups were placed into the reconfigured test chambers on three consecutive days. During these sessions, following an initial 5-min acclimation period, the house light was illuminated, and the animals remained in the chambers for another 30 min, with the lever retracted. On the fourth day (i.e., cue-test session), rats were placed into the chambers, and following the 5-min acclimation period, the house light was illuminated, and the illuminated lever-CS was inserted into the cage for 2 s, once a min, over a period of 10 min, for a total of 10 lever-CS presentations. Importantly, these presentations were not paired with pellet delivery, but lever contacts were recorded during the session. Following the 10th lever presentation, rats were placed back into their home cages and transferred to the housing room, where they were left undisturbed for 60 min. Following this 60-min period, the rats were deeply anesthetized with an intraperitoneal injection of a cocktail containing ketamine (90 mg/kg) and xylazine (10 mg/kg) and transcardially perfused with approximately 100 mL of room temperature 0.9% saline, followed by approximately 200 mL of room-temperature 4% formaldehyde (pH = 7.3–7.4, diluted in 0.1 M sodium phosphate buffer; Fisher Scientific, Hampton, NH, USA).

4.3.4 Tissue Processing

Following perfusion, brains were extracted and post-fixed overnight in 4% formaldehyde at 4° C. Brains were then cryoprotected over three nights in graduated sucrose solutions (10%, 20%, and 30%, dissolved in 0.1M sodium phosphate buffer, NaPB; pH =7.3-7.4) at 4° C. Next, brains were sectioned at 40µm on a frozen cryostat (Leica Biosystems Inc, Buffalo Grove, IL, USA). Starting with the anterior prelimbic cortex and continuing through the thalamus, brain sections were serially collected in 6-well plates. Each well contained a full brain series, with each section approximately 200 µm caudal from the previous section. Toward the hindbrain, where the ventral subiculum is located, sections were collected in 48-well plates, one section per well. All sections were stored in 0.1 M NaPB at 4° C. For long-term storage, tissue was stored in cryoprotectant at -20 °C.

4.3.4.1 Hairpin Chain Reaction Fluorescent In-Situ Hybridization (HCR FISH)

Brain tissue of 9 STs and 8 GTs were chosen from the aforementioned behavioral study based on amount of tissue available to process. The unpaired group was not quantified here due to unavailable tissue. The tissue underwent a free-floating hairpin chain reaction fluorescent *in situ* hybridization (HCR FISH) protocol in order to detect orexin mRNA (Figure 1a,b). Using FASTA sequence, a text-based format that represents nucleotide or peptide sequences, approximately 20 pairs of short oligonucleotide probes (~40 nucleotides) were designed to bind to prepro-orexin mRNA via complementary binding. Prepro-orexin mRNA was chosen as it is cleaved into orexin-A and orexin-B (de Lecea et al., 1998). The following prepro-orexin probe sequence was used:

1 ggctcggcgg cctcagactc cttgggtatt tggaccactg caccgaagat accatctctc

61 cggattgcct ctccctgagc tccagacacc atgaaccttc cttctacaaa ggttccctgg

121 gccgccgtga cgctgctgct gctgctactg ctgccgccgg cgctgctgtc gcttggggtg

181 gacgcgcagc ctctgcccga ctgctgtcgc cagaagacgt gttcctgccg tctctacgaa

241 ctgttgcacg gagctggcaa ccacgccgcg ggcatcctca ctctgggaaa gcggcgacct

301 ggacccccag gcctccaagg acggctgcag cgcctccttc aggccaacgg taaccacgca

361 gctggcatcc tgaccatggg ccgccgcgca ggcgcagagc tagagccata tccctgccct

421 ggtcgccgct gtccgactgc aaccgccacc gctttagcgc cccggggcgg atccagagtc

481 tgaacccgtc ttctatccct gtcctagtcc taactttccc ctctcctcgc cggtccctag

541 gcaataaaga cgtttctctg ctaaaaaaaa aaaaaaaaaa aaaaa

Pairs of these nucleotide segments, each separated by two nucleotides, were designed to also complement an initiator sequence that binds exclusively to these pairs. Finally, metastable DNA HCR hairpins that bind to these initiators amplify fluorescence by creating a chain of fluorescent polymers.

In preparation for HCR FISH, free-floating brain sections were first washed five times in 0.5 M saline sodium citrate (SSC) buffer and a mild detergent, Tween-20, for 5 min per wash. The tissue was then incubated for 10 min in 0.1 M triethanolamine (Sigma-aldrich, # 90279-500ML) buffer (pH 8.0) mixed with 0.25% vol/vol acetic anhydride. Sections were subsequently washed briefly in deionized water and delipidated in 1:1 acetone:methanol for 5 min. Slices were then washed again in 5x SSC-Tween-20 five times for 5 min each.

For the fluorescent *in situ* hybridization, brain tissue was first pre-hybridized in probe hybridization buffer (30% formamide + 5x SSC + 9mM citric acid (pH 6.0) + 0.1% Tween-20 + 1x Denhardt's solution + 10% low molecular weight dextran sulfate) for 30 min at 37 °C. After this 30-min incubation period, tissue was removed from the pre-hybridization solution and added to a solution containing probe hybridization buffer and 1pmol of each of the OX probes for

overnight incubation at 37 ℃. The next day, excess probe was removed via a series of washes. First, three 15-min washes in 30% probe buffer (30% formamide + 5x SSC + 9mM citric acid (pH 6.0) + 0.1% Tween-20) at 37 ℃. Then, two 15-min washes in 0.5M SSC+Tween-20 at room temperature. Next, sections were incubated in pre-amplification buffer (0.04 M TBS + 0.1% Tween-20 + 10% low molecular weight dextran sulfate) for 30 min at room temperature. During this time, H1 and H2 hairpin solutions for amplification were prepared by diluting each in 20x SSC to a 60nM concentration. This mixture was heated at 90℃ for 90 s and then cooled to room temperature in the dark for 30 min. The cooled final hairpin solution was then added to amplification buffer at room temperature. Sections were subsequently removed from the pre-amplification solution and incubated for 1-2 h in hairpin solution at room temperature in the dark. Then, unbound hairpins were removed by washing tissue two times with 0.5 M SSC + Tween-20 and then once in 0.1 M phosphate-buffered saline (PBS) to prepare the tissue for further immunohistochemical processing (Figure 2).

4.3.4.2 Immunofluorescence (IF)

Immediately following HCR FISH amplification, the same tissue was processed for detection of FG and c-Fos via free-floating immunofluorescence (IF, Figure 1a, c). All IF procedures took place at room temperature on a shaker providing gentle agitation. Free-floating sections were rinsed 3-4 times for 5 min each in 0.1 M PBS in between incubations. Tissue was blocked in 2.5% normal donkey serum (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA), 0.4% Triton-X (Acros Organics, Geel, Belgium), and diluted in 0.1 M PBS for 1 h. Sections were then incubated overnight in a double-antibody cocktail. The solution contained 1:500 mouse anti-c-Fos antibody (GR3454884-1, abcam) and 1:1000 rabbit anti-FG primary antibody (this antibody was a generous gift from Dr. Stanley Watson's Laboratory at the

University of Michigan and is commercially available from Fluorochrome, Denver, CO, USA) diluted in 1% normal donkey serum and 0.4% Triton X in 0.1 M PBS. Both primary antibodies were diluted in sterile water. The next day, sections were again rinsed three times in 0.1 M PBS. After this, sections were incubated in a secondary antibody solution containing 1:500 donkey anti-mouse antibody conjugated with Alexa Fluor 594 (lot 153991; Invitrogen, Thermo Fisher Scientific), with biotinylated donkey anti-rabbit antibody (lots 162263 and 159880; Invitrogen, Thermo Fisher Scientific). Sections were then rinsed with 0.1 M PBS. Finally, all sections were incubated in 1:1000 Streptavidin conjugated with Alexa Fluor 488 (lot 1802442; Invitrogen, Thermo Fisher Scientific), each diluted in 1% normal donkey serum, and 0.4% Triton X-100 in 0.1 M PBS, for 2 h in the dark. Sections were subsequently rinsed in 0.1 M PBS. Prior to mounting onto SuperFrost Plus microscope slides (Fisher Scientific, Hampton, NH, USA), sections were incubated with 4',6-diamidino-2-phenylindole (DAPI), a fluorescent DNA stain for cellular nuclei, diluted 1:2000 in PBS for 20 min then rinsed 3 times for 5 min each in 0.1 M PBS. The sections were then mounted and coverslipped with ProLong Gold Antifade Mountant (lot 2406594; Thermo Fisher Scientific), a hard mount that preserves fluorescent signal.

4.3.5 Imaging and Image Analysis

After the tissue processing protocols were completed, the tissue was imaged using a FV3000 confocal microscope with FV31S-SW Viewer software (OLYMPUS Microscopes, Center Valley, PA, USA). For each rat a minimum of 3 brain slices were processed and images were taken in each hemisphere. The following settings were used when imaging: 1.0% Gain, 600-700 HV, 2-8% Offset. Anterior LH was defined between dorsal/ventral coordinates -1.72 to -2.16 (relative to Bregma, (Paxinos & Watson, 2007)) and cell counting areas were chosen directly lateral to the fornix. Middle LH was defined between dorsal/ventral coordinates -2.28 to

-3.12 (relative to Bregma, (Paxinos & Watson, 2007)) and cell counting areas were chosen around the perifornical part of the LH. Posterior LH was defined between dorsal/ventral coordinates -3.24 to -3.48 (relative to Bregma, (Paxinos & Watson, 2007)) and cell counting areas were chosen around the perifornical part of the LH. The literature supports defined subregions for the anterior and posterior LH, however the middle portion of the LH is not as well defined. As such we chose the limits of the anterior and posterior portions of the LH to determine middle LH (Larsen et al., 1994; Perez-Leighton et al., 2017; Villalobos & Ferssiwi, 1987). Sample images are included in Figure 2.

Image processing and analysis were based upon the previously reported methods for HCR FISH (Bossert et al., 2023; Wei et al., 2022). Here, it was further modified to include the FG and cFos immunostaining signal and was automated using an ImageJ (Fiji, National Institutes of Health & Laboratory for Optical and Computational Instrumentation) macro. Briefly, open-source ImageJ/Fiji software (Schindelin et al., 2012; Schneider et al., 2012) was used for processing and quantitation. 3D image stacks were processed for background subtraction (rolling ball radius = 50 pixels), filtered using 'Non-local means denoising' (auto estimate sigma) and 'Median 3D filter' (radius = 3x3x3 pixels) tools before applying the 'Gaussian 3D Blur' (sigma radius = 2x2x2 pixels) to concentrate the signal towards the center of each cell. Image stacks were then Z-projected (standard deviation) and duplicated to be processed in parallel with '3D Maxima Finder' tool (min. = 20 radius xy=3, radius z=3, noise=0) of 3D Suite (Ollion et al., 2013) and segmentation tool 'Auto local threshold' algorithm (method=Phansalkar, radius=15, parameter_1=0, parameter_2=0). A '3D watershed split tool' (binary=segmented image, seeds=peaks, radius=2) was subsequently applied to separate individual neuronal cell bodies, and was then converted to a mask (MaxEntropy algorithm) suitable for automated cell courting

which was performed using ‘Analyze Particles’ tool ("size=25-500 circularity=0.60-1.00 show=Masks display exclude summarize). Finally, the image calculator tool ‘AND operator’ was used to quantify overlapping populations of neurons. Cell counting with ImageJ was done by experimenters naïve to the behavioral phenotypes of the subjects. All cell counts and image analyses were conducted on images representing a 824 × 824 μm square section of the LH. The total amounts of c-Fos+ nuclei, FG+ nuclei, and OX+ nuclei in a given LH section were quantified according to their anteroposterior region in the LH. A series of colocalization counts were then completed to determine the number of FG+/OX+, FG+/cFos+, and OX+/cFos+ cells in the anterior, middle, and posterior LH. The resulting intersection image containing common pixel data was then used with the ‘analyze particles’ tool (size=15-500 circularity=0.001.00 show=Masks display exclude summarize) to count the overlapping cells. Using a maximum thresholding value, an area of each ROI was calculated and then used to estimate the neuronal number per unit area (number density) and the triple-labeled cells were quantified as the number of cells expressing FG, cFos, and OX. The percentage of colocalized cells was then calculated. The percentage of active lateral hypothalamic cells that project to the PVT was assessed within each LH section as: (the total number of FG and c-Fos double labeled cells) ÷ (the total number of FG+ cells) × 100%. The percentage of neurons that project from the LH to the PVT and are also OXergic was measured as: (the total number of FG and OX double labeled cells) ÷ (the total number of FG+ cells) × 100% within each LH section. The percentage of neurons that project from the LH to PVT and are both cellularly active and OXergic was calculated as: (the number of triple-labeled cells expressing c-Fos, FG, and OX) ÷ (the total number of FG+ cells) × 100% within each LH section. Statistical analysis was performed on both raw cell counts and the percentage of colocalized cells.

4.3.6 Statistical Analysis

The Statistical Package for the Social Sciences (SPSS) program version 28.0 (IBM, Armok, NY, USA) was used to analyze behavioral outcome measures as well as the cell counts and percentages. A two-way ANOVA was performed, as described below. For all analyses, statistical significance was set at $p < 0.05$, and Bonferroni *post hoc* comparisons were made when significant main effects or interactions were detected. A two-way ANOVA was conducted when region ("anterior LH" vs "middle LH" vs "posterior LH") was directly compared to phenotype ("ST" vs "GT"), with region ("anterior LH" vs "middle LH" vs "posterior LH") as the within/between subject independent variable and phenotype ("ST" vs "GT") as the between subject independent variable. All statistical findings are reported in Table 1.

4.4 Results

Data reflecting the acquisition of sign-tracking and goal-tracking is available in the paper written by Haight and colleagues (2017).

The number of cells labeled for FG, cFos, and OX were assessed and compared between phenotypes for the anterior, middle, and posterior subregions. The number of FG+ cells did not significantly differ between phenotypes or subregions (i.e. anterior, middle, and posterior) (Figure 3a). The same was true for the number of OX+ cells (Figure 3b). Neither FG+ cells nor OX+ cells differed between subregions (i.e. anterior, middle, and posterior). Thus, the density of projections from the LH to the PVT and the number of orexinergic neurons appear to be similar between neuroanatomical subregions and between STs and GTs. As expected based on prior reports (Haight et al., 2017; Yager et al., 2015), STs expressed more cue-induced cFos+ cells in the LH relative to GTs (Figure 3c, effect of phenotype, $F_{(1,45)} = 22.434$, $p < 0.001$).

Upon analysis of colocalization, we found no significant differences in number of OX+ cells in LH-PVT projecting neurons (i.e. FG+ neurons) (Figure 4a). However, relative to GTs, STs exhibit a greater number of cue-induced cFos+ cells in LH-PVT projecting neurons, (Figure 4b, effect of phenotype, $F_{(1,45)} = 22.541$, $p < 0.001$), with no effect of subregion. Orexinergic cells are also more active in STs relative to GTs, as revealed by greater colocalization of OX and c-Fos across the subregions (i.e. anterior, middle, and posterior) (Figure 4c, effect of phenotype, $F_{(1,45)} = 48.905$, $p < 0.001$). When analyzing the triple labelled cells, we found that STs show more colocalization of FG+, cFos+, OX+ cells relative to GTs (Figure 4d, effect of phenotype, $F_{(1,45)} = 19.788$, $p < 0.001$), but entire rostro-caudal gradient was similar in STs (i.e., no effect of subregion).

When accounting for the total number of PVT-projecting cells labeled with FG+, there were no significant differences between phenotypes in the percentage of the of cells labeled with both FG+ and OX+ (Figure 5a). There was, however, a significant difference between phenotypes in percentage of FG+ cells that also expressed cFos (Figure 5b, effect of phenotype, $F_{(1,45)} = 27.835$, $p < 0.001$). Specifically, STs had a higher percentage of active LH-PVT-projecting cells than GTs. Lastly, the percentage of FG+ cells that were both OXergic and active in response to cue presentation also differed between phenotypes, with STs displaying a higher percentage than GTs (Figure 5c, effect of phenotype, $F_{(1,45)} = 20.231$, $p < 0.001$). Together, these data suggest that the incentive motivational value of a reward cue is encoded, at least in part, by orexinergic neurons projecting from the LH to the PVT.

4.5 Discussion

In this study, we examined differences in connectivity and engagement of orexin neurons in the LH-PVT pathway between STs and GTs. We were able to replicate previous findings from

the lab showing that STs have more cue-induced activity in LH-PVT projecting cells (Haight et al., 2017). Furthermore, we demonstrate that STs, have significantly greater engagement of orexinergic LH-PVT projection neurons than do GTs. Importantly, we show that STs and GTs do not differ in number of orexinergic LH-PVT neurons. Thus, these findings suggest that there are not inherent differences in connectivity between STs and GTs; rather, distinct neural processes are engaged in response to a cue that has incentive value (i.e. for STs) versus one that has only predictive value (i.e. for GTs). Specifically, these data are in line with our current framework regarding ST/GT neurobiology; that the incentive value of reward-paired cues is encoded by "bottom-up" subcortical circuitry.

The neurobiological correlates of sign- and goal-tracking behavior stemmed from "functional connectivity" maps reflecting cue-induced cFos activity throughout the brain (Flagel et al., 2011; Haight et al., 2017). This foundational work established the "top-down" versus "bottom-up" view of sign- and goal-tracking behavior, wherein the PVT lies at the center as a modulator between the competing neural systems (Haight & Flagel, 2014; Haight et al., 2015). Over the years, increasing evidence has emerged to support this theory. Through chemogenetic manipulations of a prelimbic cortex-PVT pathway, our group found that excitation of this "top-down" projection increases sign-tracking, while inhibiting the same pathway reduces goal-tracking (Campus et al., 2019). In targeting the "bottom-up" systems, we found that both lesions of the LH and OX receptor antagonism in the PVT attenuate sign-tracking behavior (Haight et al., 2020). Until now, however, we did not have direct evidence that STs were engaging OX neurons the LH-PVT differently than GTs. Through our novel combination of two immunofluorescent techniques, we showed that, relative to GTs, STs have more active (i.e. cFos+) orexin neurons that project from the LH-PVT. Further, we reproduced prior findings with

a different staining method than that which was previously used (Haight et al., 2017). Specifically, we used immunofluorescence to identify cFos+ and FG+ neuronal populations in the LH; whereas Haight and colleagues utilized 3,3'Diaminobenzidine (DAB) immunohistochemical staining to do so. These different histological methods yielded similar results, demonstrating that relative to GTs, STs show greater cue-induced activity in the LH-PVT pathway.

Of note are a few limitations to the current study. For one, the tissue we used is older and not the same quality as it was several years ago. As such, we were at times limited in the number of tissue slices that could be utilized as each rat had a varying number of slices to choose from. While the FG-tracing study this tissue originated from included an unpaired control group (i.e. during behavioral training the rats did not have paired reward-cues, the reward and cues were randomly presented) (Haight et al., 2017), we were not able to utilize this tissue in current study as sections of optimal quality were limited. Thus, we are lacking a potential control group in the study. Additionally, our image analysis was conducted on a subsection of the LH, limiting our ability to quantify the entire LH in a selected slice. As such, the total cell numbers and percentages reported may not reflect the density of expression in other areas above, below, or to the sides of our selected cell counting zone in the LH. Future work could quantify a larger area of the LH across its rostro-caudal axis. Finally, this tissue is only from male rats, and we thus lack the ability to make general statements about orexin activity in LH-PVT-projecting neurons. Sex differences in sign- and goal-tracking are still being evaluated, with some groups reporting minimal differences while others have observed that female rats generally sign-track more than males (Hughson et al., 2019; Pitchers et al., 2015). There is, however, evidence of sex differences and sexual dimorphism in the orexin system. Female rats show higher orexin-A and

prepro-orexin mRNA levels compared to males (Taheri et al., 1999); and orexin isoforms and receptor expression are both known to be influenced by male and female hormones (Dorsey et al., 2020; Gao & Horvath, 2022; Karteris et al., 2004; Silveyra et al., 2007). As such, it is imperative to add females for future comparison of the orexin system between sign-trackers and goal-trackers.

We did not observe differences in FG+, OX+, or cFos+ cellular expression between subregions of the LH. However, both the anterior and posterior subregions of the LH send and receive projections to and from different brain areas (Berk & Finkelstein, 1982; Barone et al., 1981). One study found that while anterior LH neurons terminate in regions including the lateral septum, only the posterior LH projects considerably to the nucleus accumbens, caudate, and putamen, which all play a role in the reward system (Villalobos & Ferssiwi, 1987). Additionally, relative to the anterior PVT, the posterior PVT has been shown to receive a higher density of projections from the LH (Goto & Swanson, 2004; Kirouac et al., 2005). It is possible that the lack of regional differences in the current study is a function of the approach used for retrograde labeling. There was robust expression of fluorogold throughout the entire LH because fluorogold infusions targeted the entire PVT, and thus it is presumably the case that the majority of orexinergic LH-PVT projections were labelled. As such, we, unfortunately, cannot make a distinction between where the orexinergic PVT-projecting neurons terminate. Knowing where these neurons in the LH-PVT pathway terminate is important, as recent findings have highlighted the molecular, genetic, cellular, and functional diversity of the PVT (Gao et al., 2023; Gao et al., 2020). Specifically, the anterior PVT has been implicated in reward-seeking and arousal (Cheng et al., 2018; Do-Monte et al., 2017); whereas the posterior PVT appears to mediate anxiety-related behaviors and stress-responsivity (Bhatnagar & Dallman, 1998; Bhatnagar & Dallman,

1999; Bhatnagar et al., 2003; Bhatnagar et al., 2002; Heydendael et al., 2011). To complicate this further, distinct subregions of the PVT project to distinct subregions of the nucleus accumbens, each of which differentially influence motivated behavior (Beas et al., 2023). The PVT literature is sometimes accompanied by opposing findings that are difficult to parse. However, these differences are likely a result of activating disparate subregions with specialized functions. As such, targeting specific cellular systems in specific areas of the PVT will hopefully lead to more interpretable discoveries. As it relates to our findings and the ST/GT model, we showed that despite a lack of regional specificity, the orexin system is differentially engaged by sign-trackers versus goal-trackers.

The total number of orexin neurons within the LH in the current study were low. This is consistent with the literature that indicates more conservative expression of orexin relative to other major neurotransmitter systems (Kirouac et al., 2005). However, as noted above only a small defined region near and around the fornix/perifornical area in the LH was counted here. Further orexin neurons are known for extensive arborization (de Lecea et al., 1998; Sakurai et al., 1998); thus, one orexin-expressing cell may widely influence activity in other brain regions. We also observed relatively low levels of colocalization between FG+ and OX+ cells, reaching a maximum of ~25% overlap. Although the OX projections from the LH-PVT are dense (Kirouac et al., 2005), recent reports suggest that the majority of projection neurons are γ-Aminobutyric acid (GABA)ergic (Otis et al., 2019). Although the percentages were minimal, we found that of the cFos+ cells that project from the LH-PVT (~9%), nearly half of them were also orexinergic (~5%) in STs. So, although orexinergic projections from the LH-PVT represent a small proportion relative to others, their influence is likely greater than expected based on numbers alone.

Taken together, these differences in orexinergic activation in the LH-PVT pathway between STs and GTs suggest that orexin is mediating some of the "bottom-up" processing in the behavior of sign-trackers, likely through encoding the incentive value of reward-paired cues. These findings are in agreement with others from the lab indicating a role for the orexin system and LH-PVT projection neurons in sign-tracking behavior (Haight et al., 2020; Haight et al., 2017). However, it is clear that there are other neurotransmitter and/or neuropeptide systems that are involved as we do not know the type of cells that represent the other ~5% of active LH-PVT projecting neurons. Further, the majority of PVT-projecting cells were not cFos+ and thereby, not "active" in response to cue presentation. We do not know the identity of the majority of PVT-projecting cells, and this represents a future direction for this work. Through our novel combination of tissue processing methods, we can begin to further characterize the neuronal ensembles that are 1) active in response to a reward-cue (i.e. encoding incentive value) and 2) project from the LH-PVT. We hope to extend the methodology detailed in this work to other cell types that have been shown to project to and from the PVT, including dopaminergic and glutamatergic projections, since other cell types such as these could enhance orexin signaling in the PVT (Choi et al., 2012; Lindvall et al., 1984; Parsons et al., 2007; Pinto et al., 2003). To reiterate, however, the findings here are notable as nearly half of the PVT-projecting cells that are active in response to a cue are also orexinergic in STs. The orexin system, therefore, likely plays a substantial role in sign-tracking behavior and warrants further investigation.

4.6 References

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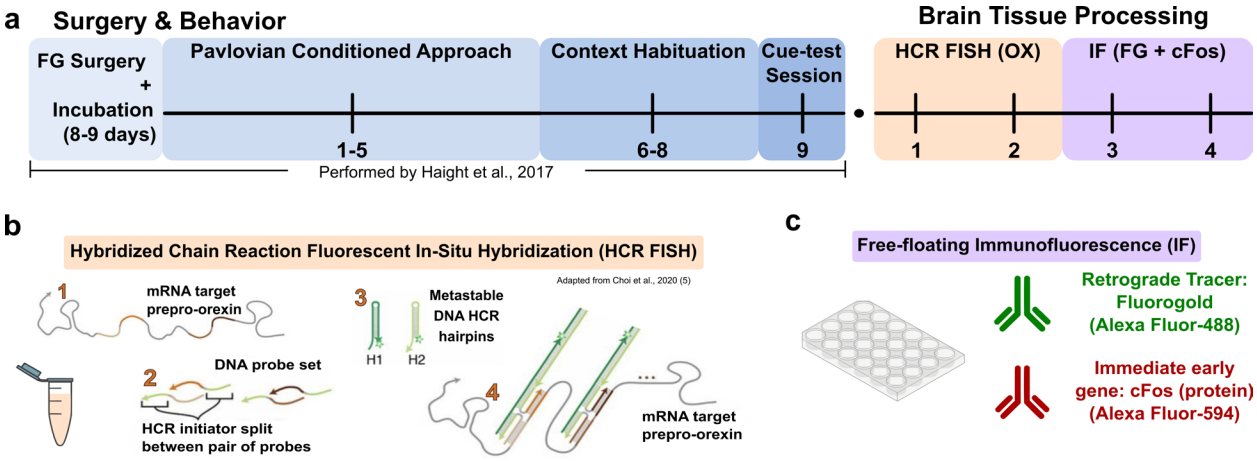

Figure 4-1 **Surgery, behavior, and experimental timeline.**
a) Surgery and behavior schematic detailing the procedural events performed by Haight et al., 2017 and the tissue processing methods in this experiment. b) Schematic of hybridized chain reaction (HCR) fluorescent in situ hybridization methods detailed as steps 1-4. 1- mRNA target prepro orexin, 2- HCR DNA hairpins hybridize to probes that bind to target mRNA present in the tissue. 3- HCR probes then bind to the target mRNA, 4- HCR DNA hairpins containing fluorophores hybridize to the probes bound to the target mRNA, creating a chain of fluorescent DNA hairpins that amplify the fluorescent signal emitted by the target mRNA. (Adapted from Choi et al., 2020). c) Schematic of free-floating immunofluorescence along with antibodies to label for fluorogold and cFos.

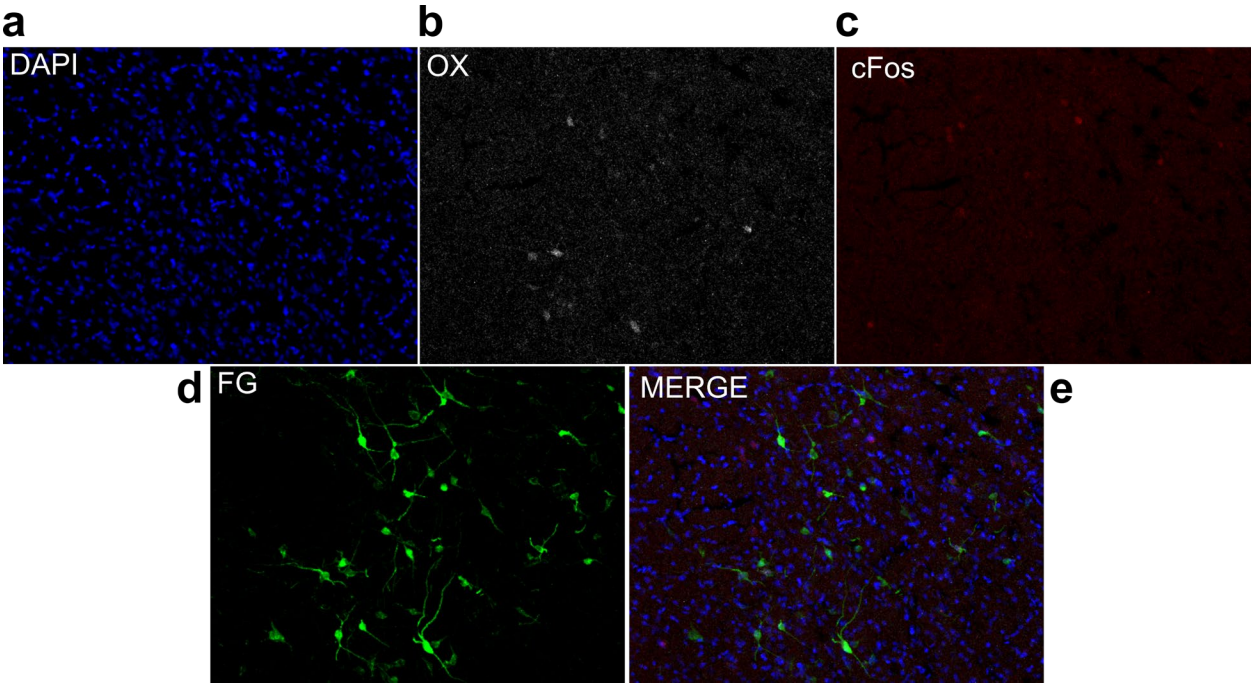

Figure 4-2 **Labeling orexin, cFos, and fluorogold neurons in the lateral hypothalamus using IF and HCR-FISH**
Confocal images at 20x magnification. (a) DAPI (blue) to stain cell nuclei, (b) prepro-orexin mRNA hybridization with HCR-FISH (OX, white), (c) cFos staining with IF (red), (d) fluorogold staining with IF (FG, green), and (e) merge of all three channels.

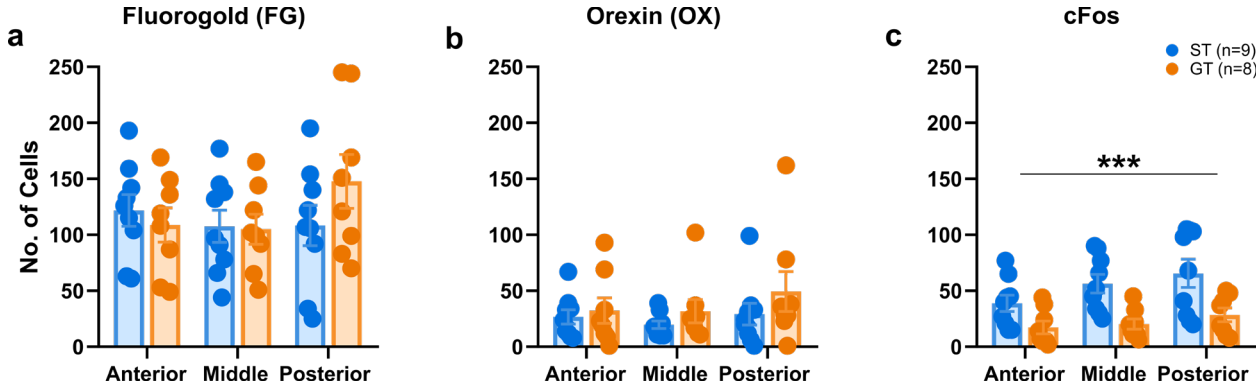

Figure 4-3 **Number of FG+, cFos+, and OX+ cells in the LH.**
Data are depicted as mean ± SEM total number of cells in the lateral hypothalamus (LH) that are a) fluorogold (FG)+, b) orexin (OX)+, and c) cFos+ between sign-trackers (ST) and goal-trackers (GT). a) No significant differences observed between subregions or phenotypes in the number of FG+ cells in the LH. b) No significant differences observed between subregions or phenotypes in the number of OX+ cells in the LH. c) STs have significantly more cFos across the entire LH as compared to GTs. (***$p < .001$).

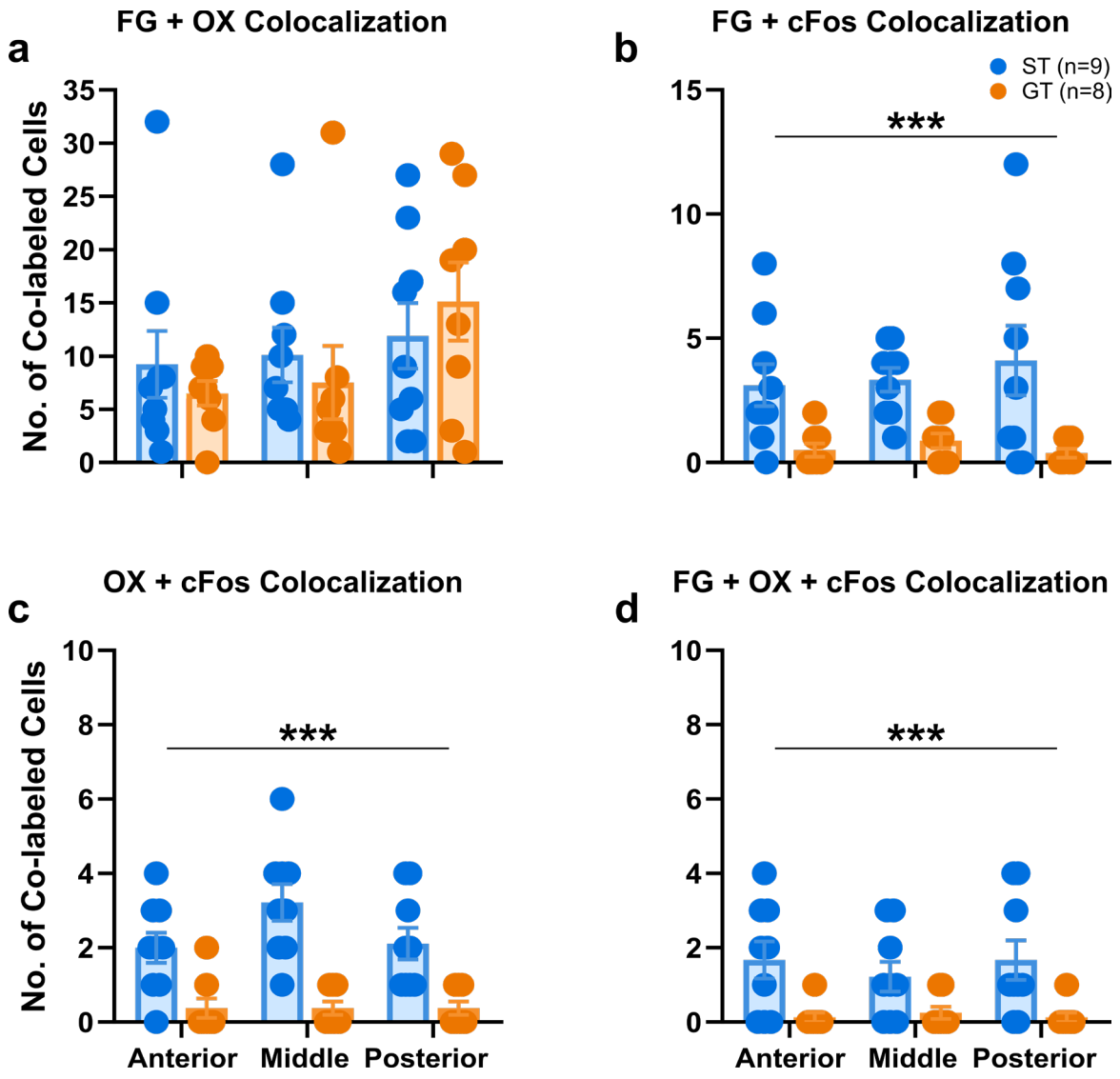

Figure 4-4 **Number of colocalized cells in the LH.**
Data are depicted as mean ± SEM total number of co-labeled cells in the lateral hypothalamus (LH) that are a) FG+ and OX+, b) FG+ and cFos+, c) OX+ and cFos+, and d) FG+, OX+, and cFos+ for STs and GTs. a) No significant differences were observed between subregions or phenotypes in the number of FG+/OX+ cells in the LH. b-d) STs have significantly more b) FG+/cFos+ cells, c) OX+/cFos+ cells, and d) FG+/OX+/cFos+ cells in the LH compared to GTs. (***$p < .001$).

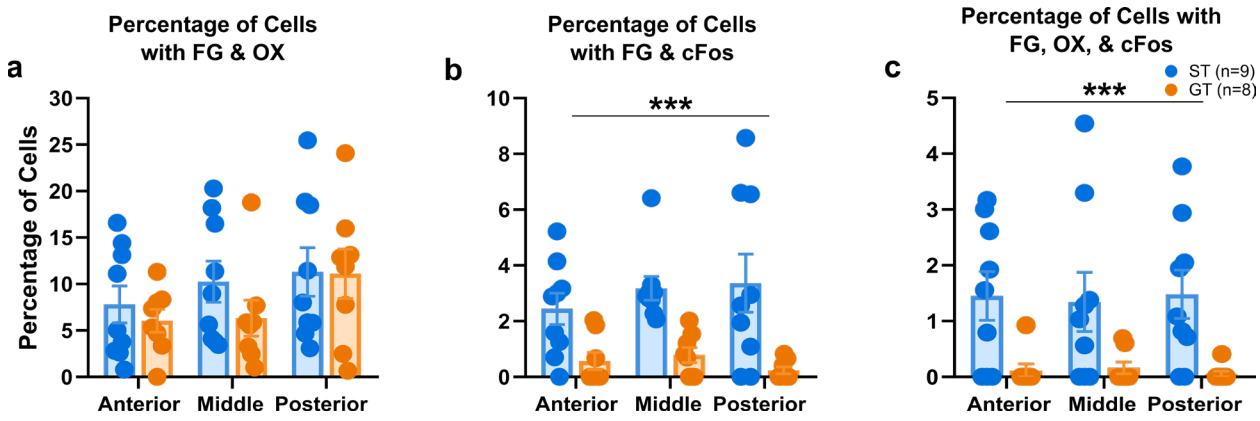

Figure 4-5 **Percentage of FG+ cells expressing cFos, OX, and cFos + OX.**
Data are depicted as mean ± SEM. Percentage of co-labeled cells in the lateral hypothalamus (LH) that are a) FG+ and OX+, b) FG+ and cFos+, and c) FG+, OX+, and cFos+ between ST and GTs. Percentage was calculated by ((number of colocalized cells/number of FG+ cells) x 100). a) No significant differences observed between subregions or phenotypes in the percentage of FG+/OX+ cells in the LH. b, c) STs have a significantly higher percentage of b) FG+/cFos+ cells, c) FG+/OX+/cFos+ cells in the entire LH as compared to GTs. (***$p < .001$).

	a. Number of Cells								
	Fluorogold			Orexin			cFos		
	DF	*F*	*p*	DF	F	p	DF	F	p
Phenotype	1,45	0.332	0.567	1,45	2.202	0.145	1,45	22.434	**<0.001**
Subregion	2,45	0.839	0.439	2,45	0.902	0.413	2,45	2.710	0.077
*Phenotype*Subregion*	2,45	1.350	0.269	2,45	0.240	0.787	2,45	0.579	0.565
	b1. Number of Co-labelled Cells								
	FG + OX Colocalization						FG + cFos Colocalization		
	DF	*F*	*p*				DF	*F*	p
Phenotype	1,45	0.083	0.775				1,45	22.541	**<0.001**
Subregion	2,45	2.072	0.138				2,45	0.174	0.841
*Phenotype*Subregion*	2,45	0.658	0.523				2,45	0.425	0.657
	b2. Number of Co-labelled Cells								
	OX + cFos Colocalization						FG + OX + cFos Colocalization		
	DF	*F*	*p*				DF	*F*	*p*
Phenotype	1,45	48.905	**<0.001**				1,45	19.788	**<0.001**
Subregion	2,45	1.739	0.187				2,45	0.123	0.885
*Phenotype*Subregion*	2,45	1.739	0.187				2,45	0.390	0.679
	c. Percentage of PVT-projecting Cells								
	Percent OX			Percent cFos			Percent OX & cFos		
	DF	*F*	*p*	DF	F	p	DF	F	p
Phenotype	1,45	1.210	0.277	1,45	27.835	**<0.001**	1,45	20.231	**<0.001**
Subregion	2,45	1.999	0.147	2,45	0.351	0.706	2,45	0.004	0.996
*Phenotype*Subregion*	2,45	0.371	0.692	2,45	0.608	0.549	2,45	0.061	0.941

Table 4-1 **Statistical analyses for cellular expression of fluorogold, orexin, and cFos within the lateral hypothalamus.**
Data from two-way ANOVA analyses for fluorogold (FG), orexin (OX), and cFos expression. (a) Total number of FG+, OX+, and cFos+ cells. (b1-2) Number of co-labelled cells for FG + OX, FG + cFos, OX + cFos, and FG + OX + cFos. (c) Percentage of PVT-projecting OX, cFos, and OX & cFos cells. Significant effects are bolded (***$p <$ .001).

Chapter 5
General Discussion

Note: Some of the text within the Discussion have previously appeared in print (Iglesias & Flagel, 2021, Frontiers in Integrative Neuroscience).

This dissertation aimed at examining the contributions of two subcortical systems in cue-reward learning. To do so we leveraged the sign-tracker (ST)/goal-tracker (GT) animal model which captures individual differences in associative learning styles. At the present a "top-down/bottom-up" theory of sign- and goal-tracking behavior posits that predictive value encoding is gated by cortical substrates, while incentive salience encoding is subserved by subcortical processes. Here, we were specifically interested in examining the subcortical drivers of incentive motivation.

Early work utilizing the sign-tracker/goal-tracker model demonstrated that sign-tracking behavior is dopamine-dependent and, in particular, that dopamine in the nucleus accumbens core is critical for incentive salience attribution (Flagel, Clark, et al., 2011; Flagel et al., 2007; Saunders & Robinson, 2012). As a follow-up to these findings, in Chapter 2 we utilized optogenetics to selectively manipulate ventral tegmental dopamine activity at the time of reward-cue presentation during the early learning phase of a Pavlovian conditioned approach task. We predicated that cue-elicited dopamine would be necessary for incentive value attribution and thereby, sign-tracking behavior to develop. Within the "top-down/bottom-up" framework, the paraventricular nucleus of the thalamus (PVT) has emerged as a critical node that may act as an arbitrator to gate predictive versus incentive value encoding. The lateral hypothalamus (LH)-

PVT pathway has been identified as a pathway of interest in this regard, as both the LH and PVT have been implicated in sign-tracking behavior as has a role of orexin signaling within the PVT (Haight et al., 2020; Haight et al., 2017). Thus, in Chapter 3, we used chemogenetics to selectively inhibit LH-PVT projection neurons and predicted that inhibition of this pathway would result in a decrease in sign-tracking behavior due to disrupted incentive salience encoding by orexin systems. As a continuation of our interest in this LH-PVT system, in Chapter 4, we utilized tissue multiplexing methods to identify orexin neurons that project in the LH-PVT pathway and are activated in response to a reward-cue (Haight et al., 2017). We predicted that STs would demonstrate greater engagement of orexinergic LH-PVT neurons than GTs. Below, a general discussion of the results from Chapters 2-4 will be followed by how these findings fit into the existing literature. Last some limitations and future directions are addressed.

5.1 Summary of findings and discussion

5.1.1 Dopamine and incentive salience attribution

The role of dopamine in reward processing and motivation continues to be up for debate. Some research supports dopamine as a learning signal under the reward prediction error theory (Montague et al., 2004; Schultz et al., 1997). As it relates to sign- and goal-tracking behavior, the initial studies investigating a role for dopamine occurred using fast scan cyclic voltammetry and support the incentive salience theory of dopamine in motivated behavior (Flagel, Clark, et al., 2011). In this experiment, dopamine activity in the nucleus accumbens core (NAcC) was captured during Pavlovian conditioned approach (PavCA) sessions. Over the course of training peak dopamine in the NAcC shifted from reward consumption to lever-cue presentation in sign-trackers, which represents a classic reward prediction error signal. However, goal-trackers learned the task perfectly well, but this phasic dopamine shift only occurred in sign-trackers,

indicating that dopamine did not subserve general associative learning. Rather, the dopamine shift encoded incentive salience ascribed to the lever-cue. These findings were further supported by pharmacological work employing systemic and local NAcC blockade of dopamine receptors which prevented the acquisition and expression of sign-tracking behavior, with no effect on goal-tracking ((Flagel, Clark, et al., 2011; Saunders & Robinson, 2012) see also: (Yager et al., 2015)). These findings demonstrated a critical role for dopamine in sign-tracking behavior; however, it remained unclear whether dopamine activity specifically during cue-presentation was necessary for attribution of incentive salience to reward-cues.

In Chapter 2, we addressed the timing query utilizing the temporal precision of optogenetics in a cohort of rats with a predisposition to sign-track. Dopamine neuron activity in the ventral tegmental area (VTA) was inhibited for the duration of lever-cue presentation and we found that this dopamine inhibition prevented sign-tracking behavior from developing, which supported our predictions. We contribute to these findings by exploring nuanced behavioral changes through DeepLabCut video analysis, which has not been included in other assessments with our model. There are a number of measures that are not quantified through our behavioral monitoring system, such as approach, movement initiation, and head orientation to task relevant objects, all of which signal interest and attention during behavioral tasks. Our work demonstrates VTA dopamine inhibition robustly and specifically impacts sign-tracking behavior, which supported our predictions. The prior work that laid the foundation for this experiment focused on dopamine signaling within the NAcC (Flagel, Clark, et al., 2011; Saunders & Robinson, 2012), which can be influenced through numerous means. Various presynaptic mechanisms alter signaling within this node, such as cholinergic interneuron modulation (Threlfell et al., 2012) or excitatory afferents from cortical (Suska et al., 2013), thalamic (Chisholm et al., 2021),

hippocampal (LeGates et al., 2018), or amygdala (Wang et al., 2020) regions, all of which mediate motivation in some facet. Our focus on VTA dopamine, being upstream from the NAc core, gleans further insight into the additional circuitry that might mediate incentive value encoding.

Due to our experimental design, the optogenetic virus was expressed in tyrosine hydroxylase expressing (TH+) cells, which could project to regions other than the NAc because the virus was infused directly into the VTA. A few alternative approaches could have been utilized to achieve VTA-NAc pathway specific manipulation (Tye & Deisseroth, 2012). A projection specific approach could have been to infuse a retrograde Cre-dependent optogenetic virus in the NAc. Alternatively, we could have utilized the current approach wherein we infuse the Cre-dependent virus in the VTA, but placed the optogenetic probes above the NAc and inhibit the terminal projections of VTA dopamine ((Attachaipanich et al., 2023) for review see: (Rost et al., 2022)). This approach would help avoid inhibiting collateral VTA dopamine projections that terminate in other regions such as the prefrontal cortex (Beier et al., 2015). However, as our findings were complementary to those that did target the NAc DA system (Flagel, Clark, et al., 2011; Saunders & Robinson, 2012; Yager et al., 2015), we have reason to believe the effects observed were primarily due to VTA-NAc dopamine signaling disruption.

5.1.1.1 VTA-NAc heterogeneity

Another potential caveat of the work presented in Chapter 2 is the potential impact the optogenetic manipulation had on the NAc downstream of the VTA. Both the core and shell of the NAc have been implicated in different aspects of reward and disparate research findings are observed when targeting one region versus the other. For instance, researchers were interested in the role of VTA dopamine in impulse control and attention and examined this using a five-choice

serial reaction time task (5-CSRTT). Inhibition of the VTA-NAcSh pathway during the intertrial interval before cue presentation resulted in enhanced impulsive action without impacting attention or motivation (Flores-Dourojeanni et al., 2023). Interestingly, work from the same group showed that not distinguishing between the NAc subregions had alternative effects. Chemogenetic stimulation of the VTA-NAc had no impact on impulsivity, but impaired attention (Boekhoudt et al., 2017). Although both findings are valuable, they are not complimentary, likely due to targeting either the NAcSh specifically or the NAcSh and NAcC combined, which underscores the importance of subregional differences. Other work indicates that the effort and vigor with which an animal initiates lever pressing in an operant task is signaled by the NAcC, and not the NAcSh (Ko & Wanat, 2016). In addition, it has been shown that NAcC dopamine codes for action initiation when movement is correctly initiated in a go-no go task (Syed et al., 2016). While we suspect that the effects of cue-paired VTA dopamine inhibition reported in Chapter 2 are a function of downstream effects in the NAcC, further investigation is warranted to parse the role of ventral striatal subregions in incentive motivational processes.

5.1.2 The PVT as a critical node of the hypothalamic-thalamic-striatal circuit

The PVT was appropriately interwoven within the classic reward circuitry by Kelley and colleagues almost 20 years ago (Kelley et al., 2005). Various anatomical, pharmacological, and behavioral studies have since implicated the PVT in emotional valence, appetitive and aversive motivation, and behavioral regulation. Work from our lab had identified a role for the PVT in mediating individual differences in incentive salience attribution (Haight & Flagel, 2014). Relative to GTs, STs show greater neuronal activity (c-Fos) in the PVT in response to both food- and drug-associated cues (Flagel, Cameron, et al., 2011; Yager & Robinson, 2013). Cue-induced c-Fos mRNA levels in the PVT are correlated with those in a number of subcortical regions for

STs, and cortical regions for GTs (Flagel, Cameron, et al., 2011; Haight & Flagel, 2014). Further, there is greater cue-induced neuronal activity (c-Fos) specifically in LH-PVT and PVT-NAc projection neurons in STs relative to GTs (Haight et al., 2017). To further investigate the role of the LH-PVT pathway in incentive learning, our group examined the effects of lesioning the LH and blocking orexinergic activity in the PVT. They found that LH lesions block the development of sign-tracking behavior, and administration of OX-1 or OX-2 antagonists into the PVT attenuate the expression of sign-tracking behavior (Haight et al., 2020). These findings established a basis for the LH-PVT pathway and orexin signaling in incentive salience attribution and sign-tracking behavior.

The purpose of Chapters 3 and 4 were to establish a role for the LH-PVT pathway and the orexin system in mediating individual differences in cue-reward learning. In Chapter 3, we utilized a chemogenetic approach to inhibit the LH-PVT pathway once a conditioned response had been learned. Specifically, rats performed in PavCA and were then characterized as STs, intermediate responders (IRs), or GTs. On the subsequent sessions the LH-PVT pathway was inhibited which resulted in the decreased expression of goal-tracking behavior. Upon further inspection, this decrease in goal-tracking was only evident in the IR rats. Following PavCA, rats performed in a conditioned reinforcement test wherein prior LH-PVT inhibition had no impact on responding. From these data is appears that the LH-PVT pathway mediates goal-tracking behavior, not sign-tracking. In Chapter 4, we utilized tissue that had expression of a retrograde tracer within the LH-PVT pathway, and examined expression of prepro-orexin mRNA in cells that project within this pathway and were also active in response to a food cue (c-Fos). We confirmed prior findings that STs engage more cells in the LH than GTs (Haight et al., 2017).

Additionally, we found that STs have more active orexinergic cells that project from the LH-PVT pathway.

The results from Chapter 3 were contrary to our prediction, as we expected for the LH-PVT manipulation to decrease sign-tracking behavior. However, these findings do align with what is observed in studies wherein they focus on the GABAergic LH neurons (Otis et al., 2019; Sharpe et al., 2017; Wu et al., 2015; Zhang & van den Pol, 2017). The LH has historically been a difficult area to study due to its heterogeneity not only in cell type, but also in connectivity to other regions (Bonnavion et al., 2016). Nonetheless, many functional studies have considered the LH as one single area without accounting for the heterogeneity that could lie within (Diaz et al., 2023). The overall density of LH-PVT projecting neurons has also been unclear as previous reports suggest that the projections are mainly orexinergic (Kirouac et al., 2005; Li & Kirouac, 2012). However, a recent report suggests that LH-PVT neurons are primarily GABAergic (Otis et al., 2019). Given the characteristics and temporal dynamics of GABA- versus orexinergic-signaling, the influence of these systems on PVT function and motivated behaviors likely occurs via distinct mechanisms. The Otis and colleagues finding is based largely on electrophysiological recordings, and possibly due to the relatively low numbers of PVT-projecting orexinergic neurons (Kirouac et al., 2005), which would preclude the ability to detect an electrophysiological signature of these neurons. Indeed, there is likely a disconnect between electrophysiological evidence and function, as there are several studies demonstrating that orexinergic signaling within the PVT plays a predominant role in appetitive motivated behaviors (e.g.,(Barson et al., 2015; Li et al., 2009; Stratford & Wirtshafter, 2013). Notably, stimulation of GABAergic neurons in the LH-PVT pathway also elicits consummatory and reward-seeking behaviors (Wu et al., 2015; Zhang & van den Pol, 2017). Although we lack clarity regarding the identity of

neurons targeted between the LH-PVT pathway in Chapter 3, the literature suggests we may have targeted primarily GABAergic neurons or that the signaling mechanisms of GABAergic neurons override those that are orexinergic. Further work is needed to map the relative contributions of different cell types involved in cue-reward learning within this pathway. A starting point to explore this would be to employ the immunohistochemical multiplexing methods introduced in Chapter 4 for projects moving forward and to expand our targets beyond prepro-orexin mRNA. Regardless, of the cell populations we targeted, it is clear the LH-PVT pathway remains a pathway of interest for studying the neural underpinnings of sign- and goal-tracking as well as intermediate responding.

The PVT sends dense glutamatergic projections to the NAc (Barroso-Chinea et al., 2007) and is believed to represent a final common pathway integrating signals from other regions projecting to the PVT as noted in our previous work (Campus et al., 2019). Recently, Otis and colleagues elegantly exploited new technologies to elucidate the circuits that facilitate cue-reactivity in the PVT (Otis et al., 2019). Their findings suggest that cue-reward information is transmitted via inhibitory responses in the PVT-NAc pathway, and that this information is differentially encoded by input to the PVT from the prefrontal cortex (PFC) and LH. Specifically, they report that information about the cue-reward association is carried by glutamatergic axons from the PFC, whereas consummatory (i.e. licking) information is carried by GABAergic input from the LH (Otis et al., 2019). Of note, the animals in these studies were head-fixed animals (Otis et al., 2019), making it is difficult to parse the value attributed to the cue, but these findings are, nonetheless, in line with what we observed (Chapter 3). Additionally, this work underscores the value of capturing the downstream effects of neuronal manipulations.

Although the findings presented in Chapter 3 suggest that the LH-PVT pathway plays a role in goal-tracking, and not sign-tracking behavior, other work from our lab has demonstrated that the LH and orexin signaling within the PVT mediate sign-tracking behavior (Haight et al., 2020; Haight et al., 2017). A role for the LH in reward-related behaviors dates back decades, as initial experiments showed that animals will work to receive stimulation of the LH (Hoebel & Teitelbaum, 1962; Margules & Olds, 1962). Further, orexinergic projections to the PVT have specifically been implicated in motivated behavior (Barson et al., 2015; Li et al., 2009). Support for a role for orexin neurons in cue-motivated behavior comes from studies showing that exogenous stimulation of LH orexin neurons led to the reinstatement of drug-seeking behavior (Harris et al., 2007) and that antagonism of orexin A in the LH decreased morphine preference (Harris et al., 2005). Further, orexin cells in the LH also are responsive to stimuli associated with rewards including drugs of abuse as well as discrete and contextual food cues (Mahler et al., 2012). Manipulations of the orexin system within the PVT are largely aligned with the results reported above. For example, microinjections of orexin-A into the PVT elicits food- and drug-seeking behavior (Barson et al., 2015; Matzeu et al., 2018); and antagonism of OX-1 in the PVT decreases cue-induced reward-seeking behavior (Cole et al., 2015). Other reports have demonstrated the involvement of OX-2 (Matzeu et al., 2016), but not OX-1 (James et al., 2011), receptors in the PVT in cocaine-seeking behavior. Work from our lab showed that antagonism of OX-1r or OX-2r selectively in the PVT attenuates sign-tracking behavior and the conditioned reinforcing properties of reward cues (Haight et al., 2020). Regardless of the mechanism, these findings highlight comparable roles for both the LH-PVT pathway and orexin signaling within the PVT in motivated behaviors (Barson et al., 2015; Matzeu et al., 2018; Matzeu et al., 2016).

Orexin, therefore, appears to have multiple roles in the PVT, with different receptor subtypes mediating distinct aspects of stimulus-reward processing.

As future work will undoubtedly investigate the role orexin plays in cue-reward learning it will be imperative moving forward to be deliberate in the population of orexin neurons that are targeted. Our prior work coupled c-Fos counts in the LH and perifornical area (Haight et al., 2017), however, different populations of orexin neurons appear to be involved in either reward-related or stress-related behavior. In a conditioned place preference test animals had contexts paired with food, morphine, or cocaine. Researchers found that orexin neurons in the LH were active if the animal had previously shown a place preference, whereas animals that showed no preference did not have active orexin neurons in the LH (Harris et al., 2005). These same neuronal activation patterns were not observed in adjacent hypothalamic regions as neither the perifornical area nor the dorsomedial hypothalamus showed activated orexin neurons. However, these hypothalamic subregions appear to be more involved with stress systems as the orexin neurons in the perifornical area/dorsomedial hypothalamus respond to foot shock, while the orexin neurons in the LH did not (Harris et al., 2005; Harris et al., 2007). Further, in sleep studies, orexin neuron activation in the perifornical area correlated with wakefulness (Estabrooke et al., 2001). However, it should be noted that LH orexin neurons are, in general, known to control wakefulness (Alam et al., 2008; Gerashchenko & Shiromani, 2004), and the perifornical area is not the sole subregion involved in wakefulness. As such it appears that the lateral hypothalamus orexin neurons mediate behaviors more on the appetitive reward-seeking spectrum, while those in the perifornical area mediate stress and arousal systems. Nonetheless a population of orexin neurons does mediate reward-related behaviors and there is evidence to

suggest that the PVT-projecting orexin neurons are especially tuned to influence reward functionality.

Manipulations of the orexin system within the PVT are known to impact DA transmission in the NAc, a critical component of reward processing. For example, in vivo administration of orexin-a into the PVT significantly increases dopamine levels in the NAc (Choi et al., 2012). Further, it was recently shown that infusion of orexin-A into the pPVT facilitates cue-elicited behavioral responses for a food reward and concurrent neuronal activity in the NAcC in sated rats (Meffre et al., 2019). In the same report, it was shown that optogenetic stimulation of the pPVT elicits similar behavioral and neuronal results (Meffre et al., 2019). Others have reported that targeted optogenetic stimulation of the aPVT-NAcSh pathway also enhances motivation to consume food in hungry mice (Cheng et al., 2018), presumably via dopamine release in the NAcSh. These findings are consistent with those of Choudhary et al., (2018), who reported that food-seeking behavior in sated rats was impacted by CART signaling in LH-PVT projection neurons and, in turn, by glutamatergic PVT-NAcSh neurons. Similarly, activation of neurons in the PVT that express glucose transporter 2 and project to the NAc results in increased sucrose-seeking behavior, highlighting a potential role for this neuronal pathway in maintaining balance between homoeostatic and hedonic control of food intake (Labouèbe et al., 2016). More recently, it was shown that optogenetic stimulation of the PVT-NAcSh pathway is itself reinforcing; yet inhibition of the same pathway elicits responding for food reward even when it is not available (Lafferty et al., 2020). Thus, the PVT-NAcSh pathway appears to promote efficient reward seeking behavior and should be a target for future works. Taken together, these findings support the notion that the PVT acts to integrate information about intrinsic motivational states and the external environment, and, in turn, communicate with the

NAc to guide consummatory and reward-seeking behaviors in an adaptive manner. In our case, inhibition of the LH-PVT pathway hindered goal-tracking behavior (Chapter 3) presumably through altering downstream PVT-NAc signaling.

5.1.3 PVT connectivity

Beyond the complexities of the LH and orexin signaling, the PVT has its own complexities which might alter our findings. The afferents and efferents of the PVT are primarily glutamatergic (Barroso-Chinea et al., 2007). However, other neurotransmitter and neuropeptide systems that have been observed within this thalamic nucleus include dopamine (DA), gamma aminobutyric acid (GABA), opioids, cocaine-and amphetamine-regulated transcript (CART), and orexin (Lindvall et al., 1984; Koylu et al., 1997, 1998; Peyron et al., 1998). Through retrograde labeling of tyrosine hydroxylase (TH) neurons, it was shown that the majority of DA fibers in the PVT originate in the hypothalamus and periaqueductal grey (Li et al., 2014). Although the majority of dopamine cells in the hypothalamus are not in the lateral hypothalamus, rather they are in the A10, A11, A13, and A15 cell groups (Li et al., 2014). Of the dopamine receptors, the D3 receptor, known to play a role in drug-seeking behavior (Higley et al., 2011; Khaled et al., 2010; Peng et al., 2009; Rice et al., 2013; Xi et al., 2006), appears to be the most abundant in the PVT (Haight & Flagel, 2014; Mansour & Watson, 1995). GABAergic neurons seem to be lacking within the PVT (Feldblum et al., 1993; Ottersen & Storm-Mathisen, 1984); see also (Alamilla & Aguilar-Roblero, 2010), but the PVT receives dense GABAergic innervation from several brain regions, including the reticular nucleus of the thalamus, the brainstem and a number of hypothalamic nuclei (Chen & Su, 1990; Cornwall & Phillipson, 1988; Krout et al., 2002; Li & Kirouac, 2012; Zhang et al., 2006). Projections arising from the LH (Otis et al., 2019) and the zona incerta (Zhang & van den Pol, 2017) seem to be particularly

important for motivated behaviors. Several neuropeptide systems are also abundant in the PVT. From the opioid family, expression of dynorphins and enkephalins are apparent, as are the kappa, mu, and delta-opioid receptors (Curtis et al., 2021; Marchant et al., 2010). CART containing neuron fibers are also found throughout the PVT (Kirouac et al., 2006; Koylu et al., 1998), and have been implicated in drug-seeking behavior (Choudhary et al., 2018; Dayas et al., 2008; James et al., 2010). Similarly, as noted above, orexin signaling within the PVT has been shown to play a role in drug-seeking and other reward-related behaviors (Choi et al., 2010; Choi et al., 2012; Matzeu et al., 2016; Matzeu & Martin-Fardon, 2020, 2021; Meffre et al., 2019), with both isoforms of orexin and the associated orexin receptors apparent throughout the nucleus (Marcus et al., 2001).

5.1.4 Anatomical and functional distinctions of the PVT

The PVTs connections (outlined in Chapter 1) support the proposed role for the aPVT in reward-seeking behavior (Cheng et al., 2018; Do-Monte et al., 2017) and arousal (Gao et al., 2020; Kolaj et al., 2012). The neuroanatomical connections of the pPVT support a proposed role for the subregion in stress-responsivity (Bhatnagar & Dallman, 1998; Bhatnagar & Dallman, 1999; Bhatnagar et al., 2003; Bhatnagar et al., 2002; Heydendael et al., 2011) and anxiety-like behaviors (Barson & Leibowitz, 2015; Li et al., 2010a, 2010b). Further, neuropeptides involved in arousal (e.g., galanin and proenkephalin) are apparent in the aPVT, while those involved in depressive- and anxiety-like behaviors (e.g., tachykinin 2, cholecystokinin, corticotropin-releasing hormone) are apparent in the pPVT (fore review see (Curtis et al., 2021)). Neuropeptides associated with reward related behaviors (e.g., tachykinin 2 and CART) are most abundant in the middle portion of the PVT (for review see Curtis, 2021). While much of the existing literature focuses on aPVT versus pPVT, new technologies are pushing many in the field

to study this nucleus based on molecular and cellular indicators. For example, recent work in mice identified two major classes of PVT neurons based on genetic markers (Gao et al., 2020). These Type I (Drd2) and Type II (Gal) PVT neurons were shown to differ in anatomy and function, representing a novel approach to characterizing the PVT along antero-dorsal and postero-ventral gradients (Gao et al., 2020). Anatomical, cellular, and functional characterization of the PVT is complex, often ambiguous, and sometimes conflicting; yet, there is clearly a role for this nucleus in motivated behaviors. If one were to assign functions based on anatomical boundaries and connectivity, the aPVT appears to play a predominant role in positive emotional valence and appetitive motivation (Barson et al., 2015; Cheng et al., 2018; Do-Monte et al., 2017), whereas the pPVT may be more involved in negative emotional valence and aversive motivation (Barson & Leibowitz, 2015; Bhatnagar & Dallman, 1998; Bhatnagar & Dallman, 1999; Bhatnagar et al., 2003; Bhatnagar et al., 2002; Heydendael et al., 2011; Li et al., 2010a, 2010b). Additionally, the aPVT projects more heavily to the dorsomedial NAcSh, and the pPVT to the ventromedial NAcSh (Dong et al., 2017). In line with the presumed functional distinctions between the aPVT and pPVT, the dorsomedial NAcSh has been implicated in positive emotional valence (e.g. appetitive and reward-related behaviors) (Peciña et al., 2006; Reed et al., 2015), whereas the ventromedial NAcSh has been implicated in encoding negative emotional valence (e.g., defensive behaviors) (Berridge & Kringelbach, 2015; Reynolds & Berridge, 2008). Due to the lack of regional specificity employed in targeting the LH-PVT in Chapters 3 and 4, it is unclear what contributions the aPVT versus pPVT had in our findings. However, in future work, the aPVT appears to be a better target. More specifically, targeting the aPVT to dorsomedial NAcSh could be a future approach in capturing specific subsystems of the PVT projections that can influence the ventral striatum.

5.1.5 PVT and stress responsivity

Beyond a specified role in reward-seeking behaviors, a number of reports suggest a more general role for the hypothalamic-thalamic-striatal circuit, and the PVT in particular, in mediating adaptive responses to both appetitive and aversive stimuli. In support, neurons in the PVT were found to respond similarly to stimuli associated with reward or punishment, with the neural response dependent on the intensity of the stimulus rather than the valence (Zhu et al., 2018). Further, PVT activity during Pavlovian cue presentation was shown to be required for both associative reward and aversive learning, as inhibition of PVT neurons decreases behavioral indices of a learned response to both positive and negative stimuli (Zhu et al., 2018). The seemingly indiscriminate encoding of value of appetitive and aversive stimuli within the PVT supports the proposed role for this nucleus as a mediator of motivational conflict (McNally, 2021). McNally and colleagues have demonstrated that the PVT is necessary for enabling adaptive responding under conflicting behavioral tendencies toward danger and reward (Choi et al., 2019; Choi & McNally, 2017). Specifically, chemogenetic inhibition across the anterior-posterior axis of the PVT disrupts cue-elicited appetitive behavior and increases cue-elicited aversive behavior under conditions of motivational conflict (Choi et al., 2019). More recently, it was shown that a subpopulation of neurons in the aPVT that express corticotrophin releasing factor (CRF) are particularly important for regulating responses during motivational conflict (Engelke et al., 2021). Inactivation of these neurons biases an animal's response toward food, whereas activation enables suppression of food-seeking behavior during conflict. Further, it was determined that input from the ventromedial hypothalamus to aPVT CRF neurons, and output from aPVT CRF neurons to the NAc, are critical components of the circuit that modulates reward-seeking behavior under competing demands of avoiding threats (Engelke et al., 2021).

These studies collectively support a role for the PVT as an arbitrator that encodes the value of external stimuli and internal states and, in turn, facilitates adaptive behavior. Additionally, these findings add to the complexities of targeting specific subpopulations of the PVT, as the optimal target is sometimes unclear. However, as researchers continue to outline the relative contributions the subsystems of the PVT, we will hopefully begin to understand its role in complex behaviors.

5.1.6 Limitations

5.1.6.1 Potential sex differences

The work reported here is limited by the fact that only male rats were studied. This research was cut short by the COVID-19 pandemic, and we were unable to conduct parallel studies in female rats. However, there is evidence for sex differences in the dopaminergic and orexinergic signaling systems. Dopamine activity can be modulated by hormones (Yoest et al., 2018), providing a basis for sex differences in the dopamine system (for review see (Zachry et al., 2021)) and an important next step to investigate. Additionally, sex differences in studying the orexin system have also been noted (Dorsey et al., 2020; Gao & Horvath, 2022; Karteris et al., 2004; Taheri et al., 1999). To-date, however, there is little evidence to support robust sex differences in the propensity to attribute incentive salience to a food cue (Pitchers et al., 2015), but female rats tend to be skewed more towards sign-trackers (Hughson et al., 2019) and the underlying neural mechanisms warrant further investigation.

5.1.7 Future directions

The immediate future directions should focus on examining the above findings in female rats to explore any potential sex differences in how subcortical neural systems guide behavior.

Additionally, moving forward it is important to utilize approaches that achieve neuronal and neuroanatomical specificity.

5.1.7.1 Cue-elicited dopamine

There are a few follow up studies that would nicely compliment the observations in Chapter 2. For one, we do not know the signaling changes downstream in the NAc that were caused by VTA dopamine inhibition. One approach would be do conduct a similar experiment in which we optogenetically inhibit the VTA and capture neural activity downstream in the NAc. Fast scan cyclic voltammetry has the best temporal resolution for capturing cue-paired dopamine signaling. In addition, it would be important to examine cue-elicited dopamine signaling during different phases of learning and once optogenetic inhibition has been terminated. Does the cue-paired dopamine signal, like the behavior, resemble patterns suggesting that learning has not yet taken place, as the behavior does? The experiment reported in Chapter 2 was conducted during the acquisition phase of behavior before the animals had learned. However, we do not know how the same manipulation would impact behavior after learning has occurred. Prior work has shown that DA in the NAcC is required for the acquisition and expression of sign-tracking (Flagel, Clark, et al., 2011; Saunders & Robinson, 2012). With an optogenetics approach, however, we could cycle inhibition at different time periods. For instance, we could allow the animal to acquire behavior, and then move forward with an inhibition protocol. Alternatively, we could employ a within session approach wherein animals perform, for example, 50 cue-reward pairings and on the last 25 cue-reward pairings we could optogenetically inhibit the pathway. Experiments such as these would allow us to determine the necessity of cue-elicited dopamine signaling for sign-tracking behavior under different circumstances. Additionally, experiments could be pushed out to study when sign-tracking behavior becomes dopamine independent

(Clark et al., 2013) and what neural mechanisms might drive behavior. This would be particularly relevant to psychopathology as neural mechanisms driving behavior change over time through plasticity. Work in the ST/GT model has been conducted within a relatively short period of time once an animal has learned the task. Lastly, it would be interesting to know if cue-elicited excitation of DA in the VTA-NAc would be sufficient to push a rat toward sign-tracking behavior before or after a conditioned response has developed. There are many avenues for next steps as it relates to the work completed in Chapter 2.

5.1.7.2 Orexin in the LH-aPVT-NAc pathway

Although the results from Chapter 3 provide an interesting avenue of research to investigate IR rats and goal-tracking behavior, there is still incongruency between these findings and those presented in Chapter 4 and other work from our lab. As such, a follow up experiment should employ tools to allow for more specificity in targeting the LH. Using, orexin-Cre rats, we could utilize a dual-vector approach to specifically express an optogenetic virus in orexinergic cells originating in the LH that synapse in the aPVT and project to the dorsomedial NaCSh. Relative to the pPVT, the aPVT appears to be involved in reward-related behaviors rather than motivational conflict and stress-responsivity (Cheng et al., 2018; Do-Monte et al., 2017; Gao et al., 2020; Kolaj et al., 2007). Further, older work showed that STs had greater c-fos mRNA expression than the other two groups in the aPVT compared to GTs and rats in an unpaired group (Flagel, Cameron, et al., 2011). As optogenetics affords more temporal specificity, it is a useful tool in examining cue-motivated behaviors. However, some troubleshooting might be needed as the signaling speed of orexin differs from classic neurotransmitter systems in which many optogenetics parameters were established (Gradinaru et al., 2010).

5.1.7.3 Orexin in the LH-VTA pathway

Future areas of study could also determine the role of orexin in reward processing by studying other lateral hypothalamic projections to reward-related brain areas. Orexin projections from the LH to the VTA dopamine neurons play an important role in reward-seeking behaviors (Thomas et al., 2022). Early work suggested that orexin activates the dopamine system resulting in increased dopamine release in the PFC (Vittoz and Berridge, 2006) and NAcSh (Narita et al., 2006). However, the NAcC is not activated in response to orexin administration (Narita et al., 2006). In fact, this dopamine system pattern of activation (i.e. PFC and NAcSh, but not the NAcC) can be observed following a stress response (Berridge et al., 1999; Dunn, 1988). Orexin activation in the VTA is limited to the caudomedial VTA and the effects of orexin activation within this region impacted VTA-PFC and VTA-NAcSh projections, but had no influence on NAcC projections (Vittoz et al., 2008). However, orexin in the VTA continues to be studied in relation to reward systems. The VTA receives orexin projections from the LH and expresses OX1r (Marcus et al., 2001; Trivedi et al., 1998), and we know OX1 receptor signaling is necessary for cue-driven cocaine -seeking behavior (Pantazis et al., 2022). It has also been found that administration of a selective OX receptor antagonist in the VTA reduced the rewarding properties of morphine, suggesting that OX projections to the VTA are important in encoding the rewarding aspects of drug cues (Narita et al., 2006). Further, recent literature has implicated a role of VTA dopaminergic neurons in encoding both predictive and incentive value of reward cues, highlighting its relevance to the study of cue-motivated behavior (Ferguson et al., 2020). Because it has been reported that either OX1r or OX2r receptor antagonism in the PVT attenuates sign-tracking behavior (Haight et al., 2020), it would be interesting to determine whether OX receptor antagonism in the VTA would also yield similar behavioral outcomes.

5.2 Concluding remarks

Collectively, the work presented in this dissertation serves as evidence for a role of subcortical neural systems in individual differences in cue-reward learning. First, we confirmed a role for cue-elicited dopamine signaling in mediating incentive salience attribution and sign-tracking behavior. This work confirms prior expectations as it relates to sign-tracking behavior and opens additional avenues for studying dopamine and individual differences. Beyond the dopamine system, we focused on the LH-PVT pathway and showed that it mediates goal-tracking behavior. More specifically we found that LH-PVT inhibition decreased goal-tracking behavior only in the intermediate responder rats. This finding provides some initial evidence for subcortical systems that might mediate goal-tracking behavior as well as a neural basis for studying intermediate responder rats, which have largely been overlooked to-date. Additionally, we demonstrate that sign-trackers engage the orexin system between the LH-PVT more so than goal-tracker rats. With that we confirm prior findings that the orexin system may mediate some of the aspects of sign-tracking behavior and provide additional evidence for studying the orexin system in this regard. The work in this dissertation illuminated the contributions of multiple subcortical systems in the acquisition and expression of behavioral responses that emerge through cue-reward learning.

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