Elucidating the Role of the Paraventricular Nucleus of the Thalamus in Cue-Motivated Behavior

by

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A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
(Neuroscience)
in the University of Michigan
2016

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DEDICATION

This dissertation is dedicated to my wife, Emily, and my parents, Mark and Julie, for never letting me give up.

ACKNOWLEDGEMENTS

It would be impossible for me to acknowledge everyone who has had a significant impact on me, and my development as a person and scientist, these last five years. Nonetheless, I will try my best.

First off, I have to acknowledge the entire Flagel lab, starting with my mentor, Dr. Shelly Flagel. When I joined Dr. Flagel's lab in the Spring semester of 2012, I was a little worried about being 'the first graduate student'. It turns out it was the best decision I could have made for my graduate school career. I would not be where I am today without her never-ending support, in science and in life. She encouraged me to explore my own ideas, and think critically yet positively about my work. Dr. Flagel's contagious enthusiasm about science inspires me to this day, and will continue to inspire others for years to come. I know that, aside from a mentor, I have made a life-long friend.

To all the wonderful people I have gotten to work with in the Flagel Lab: thank you for making this journey such a wonderful one! Your companionship, as colleagues and as friends, has influenced me in such positive ways throughout the years. Our lab managers Rebeca Kelly, Katie Long, Julia Boese and Marin Klumpner, have all helped in various ways on the projects contained in this thesis, and dealt with my tendency to order necessary items at the last minute with patience. The post-docs that I have gotten to work with, Aram Parsegian, Nacho Covelo-Rivera, and Paolo Campus, have patiently answered all my questions and provided advice on my projects and career path. My fellow Flagel Lab graduate students, Brittany Kuhn and Sofia Lopez, have shared (and commiserated at times) all the ups and downs with me. And most importantly, this thesis would not exist without the tireless commitment of two undergraduates: Kurt Fraser and Zack Fuller, who worked by my side on many nights and weekends to make this work possible.

My dissertation committee also deserves special recognition. Drs. Kent Berridge, Terry Robinson, Robert Thompson, and Stanley Watson: thank you for your invaluable guidance and input on my thesis throughout the years, and for your patience with all the hiccups and

complications along the way. While Dr. Huda Akil was not an official member of my committee, she, along with Dr. Watson, took a special interest in my work. As Co-Directors of the Molecular and Behavioral Neuroscience Institute, they opened up countless lab resources to me, and gave me the opportunity to present my work at their joint lab meetings, allowing me to grow as a scientist. Dr. Watson and Dr. Robinson also read many drafts of manuscripts that have since been published, and their feedback on the work in progress was invaluable. Last, when I was in one of many panicked states during my qualification exams, Dr. Thompson loaned me a book with a title akin to "Genetics for Dummies". Without that book, I might never have passed those exams.

The Neuroscience Graduate Program deserves recognition as well. Thank you for admitting me to the Ph.D. program in the first place, and allowing me to pursue this career. It is certainly a privilege. Special thanks to our Director, Edward Stuenkel, and Assistant Director, Audrey Seasholtz, for upholding the integrity of higher education in Neuroscience. And to my cohort: while we may have drifted apart as the years went by, the friendships we built during those first two grueling years will stay with me forever.

Last, but certainly not least, I must acknowledge my family, for sticking with me over the last five years. To my parents, Julie and Mark: I would never have made it to graduate school in the first place, let alone completed my degree, without your endless love and encouragement. To my wife, Emily: you have seen me at my best, and also at my worst. Your strength and love have picked me up when times were rough, and inspired me to be the best husband and father that I could be. And to my son, Henry: even though you are young, and will not remember this time in Michigan, you have made *everything* worth it.

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ABSTRACT

Recently, there has been increased interest in the role of the paraventricular nucleus of the thalamus (PVT) in motivated behaviors, specifically in response to reward-paired cues. The precise role of the PVT in these behaviors has been difficult to identify since Pavlovian conditioned cues can act as both predictive and incentive stimuli. Using an animal model that captures individual variation in Pavlovian conditioned approach (PCA) behavior, these properties of stimulus-reward learning can be differentiated. When rats are exposed to a PCA paradigm, wherein presentation of a discrete cue predicts delivery of a food reward, some rats (goal-trackers; GTs) treat the cue exclusively as a reward predictor and approach the location of impending reward delivery upon cue presentation. Other rats (sign-trackers; STs) attribute both predictive and incentive value to the cue, and approach and engage the reward-paired cue upon presentation. STs will also work to a greater extent than GTs for cue presentation in the absence of reward, another measure of incentive salience attribution. Using this model, work from our laboratory has indicated that the PVT may play an important role in mediating the propensity to attribute incentive salience to reward cues. Here, I confirmed a role for the PVT in sign- and goal-tracking behavior through the use of pharmacological lesions. Next, I identified distinct populations of PVT efferents and afferents that are engaged following presentation of a predictive stimulus (i.e. for GTs) or one that is also attributed with incentive value (i.e. for STs). My work revealed that presentation of a predictive stimulus activates PVT afferents from the prelimbic cortex, while an incentive stimulus engages subcortical structures communicating with the PVT, including afferents from the lateral hypothalamus and efferents to the nucleus accumbens. Last, I used local pharmacology to demonstrate that blocking orexin-receptor 2 in the PVT attenuates sign-tracking behavior, indicating that this pathway is involved in the attribution of incentive salience to reward-paired cues. Taken together, this work has yielded a model in which the PVT is a central node between top-down processing, mediating goal-tracking behavior, and a sub-cortical drive that can override this top-down control, mediating sign-tracking behavior.

Chapter 1

Introduction

Note: Much of the text, as well as the majority of the figures, contained in the Introduction have appeared previously in print (Haight & Flagel, 2014, Frontiers in Behavioral Neuroscience).

Over the past few decades a large quantity of research has focused on elucidating the neurobiological mechanisms that contribute to addiction and related behaviors (for review see: (Grace, 2000; Kelley & Berridge, 2002; Lüscher & Malenka, 2011; Everitt & Robbins, 2013; Nestler, 2014). The majority of this work has focused on the classic mesocorticolimbic reward circuitry, but the field is beginning to recognize the importance of structures outside of this system (e.g. (Ikemoto, 2010). One such structure is the paraventricular nucleus of the thalamus (PVT), which has recently gained attention for its role in mediating cue-driven behaviors, especially as they relate to drug-seeking behavior and addiction (Martin-Fardon & Boutrel, 2012; James & Dayas, 2013; Browning *et al.*, 2014). Although there is now sufficient evidence implicating the PVT in mediating responses to both food- and drug-associated cues, its exact role in these processes has yet to be discovered.

The PVT is a midline thalamic nucleus located at the interface between the limbic, cortical and motor circuits. The PVT receives a complex set of sub-cortical afferents from areas known to be involved in motivated behavior, including but not limited to the hypothalamus, hippocampus, amygdala, locus coeruleus, periaqueductal grey, and dorsal raphe (Van der Werf *et al.*, 2002; Vogt *et al.*, 2008; Hsu & Price, 2009; Li & Kirouac, 2012). In addition to these sub-cortical elements, the PVT receives dense innervation from the medial prefrontal cortex, including the prelimbic (PrL), infralimbic (IL), cingulate and dorsal peduncular cortices (Li & Kirouac, 2012). The most abundant set of afferents to the PVT appears to be from the prelimbic cortex (Li & Kirouac, 2012), an area shown to be a critical mediator of drug- and cue-motivated behaviors in recent years (Di Pietro *et al.*, 2006; Di Ciano *et al.*, 2007; Rocha & Kalivas, 2010).

The efferents from the PVT are primarily glutamatergic, targeting both cortical and subcortical structures including the PrL, IL, nucleus accumbens (NAc) shell and core, amygdala, hippocampus, and hypothalamus (Jones *et al.*, 1989; Su & Bentivoglio, 1990; Van der Werf *et al.*, 2002; Pinto *et al.*, 2003; Li & Kirouac, 2008; Vertes & Hoover, 2008). Thus, the neuroanatomical positioning of the PVT is ideal for integrating information regarding environmental stimuli and internal states, and translating it into motivated actions.

The first study to implicate the PVT as a potential mediator of motivated behavior surfaced almost 50 years ago when it was demonstrated that rats will self-stimulate intracranial electrodes placed in or near the PVT (Cooper & Taylor, 1967). These findings were later supported by Clavier and Gerfen (1982), who confirmed that the most consistent patterns of thalamic self-stimulation occurred when electrode placements were close to, or within the midline nuclei, which included the PVT. Since then, numerous studies have supported a role for the PVT in motivated behavior, specifically in response to discrete and contextual cues that have previously been paired with food and drug rewards. Following is a review of the behavioral, pharmacological, and anatomical evidence supporting a role for the PVT in cue-motivated behaviors. In addition, an animal model that captures individual variation in Pavlovian conditioned approach (PCA) behavior to parse the incentive from the predictive qualities of reward paired cues is discussed; and, based on recent data, a hypothetical model for PVT function is proposed, implicating this structure in mediating specific aspects of cue-reward learning and PCA behaviors.

A role for the paraventricular nucleus of the thalamus in reward processing and cue-motivated behaviors

More than a decade following the intracranial self-stimulation studies (Cooper & Taylor, 1967; Clavier & Gerfen, 1982), the PVT was shown to play a role in psychoactive drug effects. Systemic administration of amphetamine and MDMA elicited an increase in neuronal activity in the PVT, as measured by c-fos (Deutch *et al.*, 1995; Deutch *et al.*, 1998; Stephenson *et al.*, 1999). Around this same time, a series of lesion studies sought to examine the role of the PVT in cocaine-induced behavioral sensitization. It was found that lesions of the PVT before a contextually conditioned regimen of repeated cocaine treatment (Young & Deutch, 1998), but not after repeated cocaine administration (Pierce *et al.*, 1997), attenuates the development of

behavioral sensitization. These studies were the first to suggest that the PVT was important for the acquisition of the relationship between drugs and conditioned stimuli.

By this time it had been well established that motivated behaviors, such as the behavioral sensitization to cocaine described above, are regulated by a complex set of cortical, striatal, thalamic and limbic brain areas, known as the 'motive circuit' (for review see (Pierce & Kalivas, 1997). However, it wasn't until later that work by Ann Kelley and colleagues highlighted the PVT as an important component of this circuitry (Kelley *et al.*, 2005a). In Kelley's model, the PVT is a critical interface between the limbic and motor circuitry, relaying information regarding arousal, environmental cues, energy needs, reward, and circadian rhythms from the hypothalamus to the striatum, including the nucleus accumbens (NAc). Once in the striatum, this information is incorporated with information from the ventral tegmental area (VTA) and prefrontal cortex, among other areas, and integrated with basal-ganglia motor output pathways to influence motivated behaviors. In support of this model, Kelley and colleagues demonstrated that exposure to a context previously paired with a highly palatable reward (chocolate Ensure) can induce robust cellular activation throughout many areas of the motive circuitry, including prefrontal cortical areas, the amygdala, NAc, and the PVT (Schiltz *et al.*, 2005a; Schiltz *et al.*, 2007). Interestingly, exposure to a context previously paired with nicotine administration also induces robust cellular activation in these areas (Schiltz *et al.*, 2005b). This similar pattern of neuronal activation in response to both food and drug cues led Kelley and colleagues to postulate that "addictive drugs induce neuroadaptations in brain circuits normally subserving learning and memory for motivationally salient stimuli" (pg. 12, (Kelley *et al.*, 2005b), and the PVT appears to be a critical locus of these circuits.

Other behavioral studies have built upon the initial studies by Kelley and colleagues (Kelley *et al.*, 2005b; Schiltz *et al.*, 2005a; Schiltz *et al.*, 2005b; Schiltz *et al.*, 2007), further supporting the notion that the PVT is an important mediator of contextual cue-reward associations and addiction-related behaviors (Martin-Fardon & Boutrel, 2012; James & Dayas, 2013). It has been previously reported that exposure to a context previously paired with experimenter-administered cocaine injections increases levels of c-fos in the PVT (Brown *et al.*, 1992; Johnson *et al.*, 2010). Moreover, lesions or chemical inactivation of the PVT prevent reinstatement of "beer-seeking" behavior following exposure to the previously alcohol-paired context (Hamlin *et al.*, 2009; Marchant *et al.*, 2010). Additionally, the expression of cocaine-

induced conditioned place preference is attenuated following inactivation of the PVT (Browning *et al.*, 2014), further confirming a role for the PVT in contextual cue-reward processes. One possible pathway in which the PVT can influence context-related behaviors is through its interactions with the ventral pallidum (VP), as context-induced reinstatement of alcohol seeking engages PVT projections to the VP (Perry & McNally, 2013).

The PVT has also been implicated in the response to discrete reward-paired cues. For example, repeated Pavlovian pairings of a discrete cue light with a water reward results in increased c-fos expression in the PVT relative to unpaired controls (Igelstrom *et al.*, 2010). Likewise, exposure to an odor cue previously associated with ethanol availability in a Pavlovian manner (Dayas *et al.*, 2008), or exposure to a discrete cue associated with cocaine availability (Matzeu *et al.*, 2015a), increased c-fos expression in the PVT. c-fos is also elevated in the PVT following reinstatement of drug-seeking behaviors after exposure to ethanol- (Wedzony *et al.*, 2003) or cocaine-associated (James *et al.*, 2011) cues. Furthermore, drug-seeking behavior can be disrupted by inactivation of the PVT. James and colleagues (2010) demonstrated that direct infusion of tetrodotoxin (a voltage-gated sodium channel antagonist) or the inhibitory peptide cocaine- and amphetamine-regulated transcript (CART) into the PVT is able to attenuate cocaine-primed reinstatement (James *et al.*, 2010). In addition, inactivation of the PVT with GABA receptor agonists prevents cue-induced reinstatement of cocaine-seeking behavior (Matzeu *et al.*, 2015b). Taken together, these findings demonstrate a role for the PVT in the conditioned-effects of both discrete and contextual reward-associated cues, and drug-seeking behavior.

Interactions between the paraventricular nucleus of the thalamus and the ventral striatum, with a focus on dopamine transmission

A series of elegant studies have been published that further support a role for the PVT in motivated behaviors via its interactions with the NAc, including dopamine transmission. Mesocorticolimbic dopamine transmission has long been known to play a role in cue- and drug-motivated behaviors. Exposure to food or drug rewards, as well as reward-paired cues, elicit robust dopamine transmission in the NAc (for review see (Baik, 2013). The PVT sends projections to the NAc core and, to a greater extent, the shell (Berendse & Groenewegen, 1990; Su & Bentivoglio, 1990; Moga *et al.*, 1995; Van der Werf *et al.*, 2002; Pinto *et al.*, 2003; Li &

Kirouac, 2008; Vertes & Hoover, 2008), and many of the these neurons are found in close proximity to tyrosine-hydroxylase positive (i.e. dopaminergic) axons (Pinto *et al.*, 2003). An early study showed that chemical excitation of PVT neurons increased the ratio of dopamine metabolites in the NAc (Jones *et al.*, 1989), but the exact mechanism of this increase was unknown. More recent work by Parsons and colleagues (2007) has pointed to presynaptic dopamine terminal regulation as the main mechanism of PVT-elicited dopamine release in the NAc. It was shown that electrical excitation of the PVT elicits dopamine efflux independent of the VTA, and that these PVT-evoked responses were attenuated following infusion of a glutamate receptor antagonist into the shell of the NAc (Parsons et al., 2007). It has also been postulated that hypothalamic orexin neurons that project to the PVT are part of the sub-cortical system that drives dopamine levels in the ventral striatum (Kelley *et al.*, 2005a). In support, *in vivo* administration of orexin-A peptide directly into the PVT has been shown to increase dopamine levels in the NAc, and knockdown of orexin-1 receptors in the PVT reduces 'hedonic' feeding (i.e. consumption of high-fat pellet consumption in sated rats) (Choi *et al.*, 2012), which is controlled in part by the NAc shell (Reynolds & Berridge, 2002).

While presynaptic regulation of DA terminals is one possible function of PVT neurons that project to the NAc, it has also been demonstrated that thalamic projections to the NAc can exert their influence through direct synaptic connections with NAc medium-spiny neurons (Christoffel *et al.*, 2015). A recent study examining PVT neurons that project to the NAc that specifically express the glucose transporter Glut2 found that activation of these neurons leads to glutamate-mediated excitation of post-synaptic NAc medium-spiny neurons, and also increased motivation to obtain a sucrose-reward in an operant-conditioning task (Labouebe *et al.*, 2016). Another study demonstrated that cocaine administration altered synaptic connections between the PVT and NAc, presumably on post-synaptic medium-spiny neurons, and that inactivation of this pathway led to decreased cocaine self-administration (Neumann *et al.*, 2016). Last, repeated morphine administration has been show to increase the synaptic strength of PVT projections onto D2-expressing medium-spiny neurons in the NAc, and inactivation of this pathway attenuates the symptoms of morphine withdrawal (Zhu *et al.*, 2016). Thus, there are multiple ways in which the PVT can influence activity in the nucleus accumbens, and in turn regulate motivated behaviors.

The PVT also receives direct sub-cortical input from dopaminergic neurons (Lindvall *et al.*, 1984; García-Cabezas *et al.*, 2009). Early biochemical evidence demonstrated that dopamine

innervation of the PVT was, at least in part, coming from the ventral tegmental area cell group in the ventromedial midbrain (Kizer *et al.*, 1976). This was later supported by a retrograde tracing study showing that dopaminergic cells (i.e. tyrosine-hydroxylase positive) in the VTA projected to the PVT (Takada *et al.*, 1990). However, it should be noted that tracing studies from other groups have not identified a circuit between the VTA and PVT (Cornwall & Phillipson, 1988; Chen & Su, 1990; Li & Kirouac, 2012). When we examined this circuit ourselves following retrograde tracer injection into the PVT, we found some retrograde labeling in the VTA, but this could be due to spread of the injection site to the medial portions of the mediodorsal thalamic nucleus or the ventral borders of the habenula. Alternatively, it has been suggested that dopaminergic innervation of the PVT arises from the A11, A13 and A14 cell groups residing in the hypothalamus and periaqueductal gray (Lindvall *et al.*, 1984). In support, it was more recently demonstrated in rats and monkeys that dopaminergic input to midline thalamic nuclei (PVT and centromedial nucleus) is coming from these cell groups, with the hypothalamus being the major source (Sánchez-González *et al.*, 2005; Li *et al.*, 2014). Interestingly, the PVT in humans, monkeys and rats is immunoreactive for DAT (García-Cabezas *et al.*, 2007; García-Cabezas *et al.*, 2009) and the majority of hypothalamic and lateral parabrachial dopamine axons do not express DAT(Sánchez-González *et al.*, 2005). Thus, further work is warranted to characterize the other sources of dopaminergic input to the rat PVT.

Dopamine in the PVT presumably acts on dopamine D3 receptors, the primary dopamine receptor in the PVT (Mansour & Watson, 1995). Here, the presence of D3 mRNA in the PVT has been confirmed using in situ hybridization, and remarkably, D3 expression is restricted to the PVT and not apparent in any of the surrounding thalamic nuclei (Figure 1.1). While the specific role of D3 activation in the PVT has yet to be examined, recent reports have demonstrated that systemic antagonism of D3 receptors can block both drug- and cue-induced reinstatement of drug-seeking behaviors (Xi *et al.*, 2006; Peng *et al.*, 2009; Khaled *et al.*, 2010; Higley *et al.*, 2011; Rice *et al.*, 2013). Interestingly, unpublished data from our own lab suggests that rats that are more susceptible to addiction-related behaviors (Flagel *et al.*, 2016), including both drug- and cue-induced reinstatement, have greater D3 mRNA expression in the PVT (Figure 1.1B).

Dopaminergic transmission in the thalamus has also been associated with addiction in humans. Work by Volkow and colleagues (2005) has shown that methylphenidate administration in cocaine abusers leads to increased dopamine levels in the thalamus (Volkow *et al.*, 2005).

Although the resolution in human imaging studies does not allow one to distinguish the PVT from other thalamic nuclei, these results are nonetheless interesting and relevant.

Taken together, the literature reviewed above supports the notion previously put forth by Kelley and colleagues (2005) that the PVT influences cue- and reward-motivated behaviors by integrating information from sub-cortical systems, such as the orexin and dopamine neurotransmitter systems, and relaying that information to the ventral striatum, where it can impact NAc activity.

Exploiting individual variation in Pavlovian conditioned responses to parse the incentive from the predictive properties of reward-paired cues

As summarized above, there is now sufficient evidence supporting the involvement of the PVT in motivated behaviors and the processing of reward-associated cues. However, it is difficult to draw conclusions about the *specific* role of the PVT in these processes, since many of these studies are confounded by the fact that Pavlovian conditioned reward cues can act not only as "predictors" of reward delivery, but can also come to act as "incentive" stimuli, capable of arousing complex emotional and motivational states (Stewart *et al.*, 1984; Childress *et al.*, 1993; Robinson & Berridge, 1993). It should be noted that here I am referring to incentive stimuli that have Pavlovian conditioned motivational properties, and not instrumental incentive value as described by Dickinson and colleagues (Balleine and Dickinson, 1998; Dickinson and Balleine, 2002). Pavlovian incentive stimuli have three fundamental properties: 1) they are attractive and elicit approach toward them, as in Pavlovian conditioned approach behavior; 2) they can reinforce the learning of new actions, acting as a conditioned reinforcer; and 3) they can energize ongoing instrumental actions, as in the Pavlovian instrumental transfer (PIT) effect (Estes, 1948; Lovibond, 1983; Berridge, 2001; Cardinal *et al.*, 2002a; Cardinal *et al.*, 2002b; Holmes *et al.*, 2010). Until recently, it was thought that the conditional relationship between a cue and reward was sufficient to confer incentive motivational value to the cue. That is, if a cue attained predictive value and was capable of eliciting a conditioned response, then it was assumed that it also had the ability to act as an incentive stimulus (de Wit & Stewart, 1981; Childress *et al.*, 1993). However, through the study of individual variation in PCA behavior, it has been demonstrated that this is not the case (Robinson & Flagel, 2009).

Some of the earliest work examining individual variation in PCA behavior was described by Zener (1937).Using a conditioning paradigm similar to Pavlov's, Zener observed that dogs developed a range of complex conditioned responses (CRs) following presentation of the conditioned stimulus (CS). Some dogs developed a CR directed towards the location of reward delivery, while some exhibited approach behavior directed towards the location of CS (Zener, 1937). This type of CS-directed behavior was later termed "sign-tracking," in reference to the 'sign', or CS that was approached, based originally on work in pigeons (Hearst & Jenkins, 1974). A few years later, in the late 1970's, this type of variation in CRs was described in rats (Boakes, 1977). Some rats, called "sign-trackers", approached the CS upon presentation. Others, which were termed "goal-trackers," approached the location of reward delivery upon CS presentation (Boakes, 1977).

As an extension of this early work, it has recently been shown that there is considerable variation in the conditioned response that emerges following Pavlovian conditioning, and this individual variation is thought to reflect differences in the propensity to attribute incentive salience to reward-paired cues (Flagel *et al.*, 2007; Robinson & Flagel, 2009; Meyer *et al.*, 2012). Briefly, when an illuminated lever (CS) is repeatedly paired with delivery of a food reward (unconditioned stimulus; US), rats will develop distinct and varied CRs. Rats termed goal-trackers (Boakes, 1977) attribute predictive value to the lever-CS, and promptly approach the location of reward delivery upon lever-CS presentation (Figure 1.2A). Rats termed sign-trackers (Hearst and Jenkins, 1974), not only attribute predictive value, but also attribute *incentive salience* to the lever-CS, and upon its presentation will approach and manipulate it (Figure 1.2B). Importantly, no interaction with the lever is required for food delivery, and all of the animals, regardless of their phenotype, retrieve and eat all of the food pellets that are delivered. Furthermore, if lever presentation is explicitly not paired with food delivery (i.e. unpaired conditions), neither conditioned response develops (Robinson & Flagel, 2009).

There is ample evidence supporting the notion that for sign-trackers, but not goal-trackers, the lever-CS is attributed with incentive salience (e.g. (Flagel *et al.*, 2009; Meyer *et al.*, 2012). For sign-trackers, the cue itself is attractive and elicits approach—indicative of the first property of an incentive stimulus (Flagel *et al.*, 2009). Further, the lever itself is desirable and acts as a more effective conditioned reinforcer for sign-trackers relative to goal-trackers. That is, sign-trackers will respond more than goal-trackers for lever-cue presentation in the absence of

food reward (Robinson & Flagel, 2009), demonstrating the second property of an incentive stimulus. Evidence demonstrating individual variation in the third fundamental property of an incentive stimulus, i.e. general PIT, is lacking, perhaps due to the complex nature of the paradigm. However, there is evidence suggesting that reward cues arouse a conditioned motivational state to a greater extent in sign-trackers than goal-trackers (Yager & Robinson, 2010; Saunders & Robinson, 2011; Saunders *et al.*, 2013b). In sum, the lever-CS is a predictor of reward delivery for both sign- and goal-trackers, as it elicits a conditioned response in both and the responses are learned at the same rate; but only for sign-trackers does the cue serve as an incentive stimulus.

Individual variation in the propensity to attribute incentive salience to reward cues and addiction vulnerability

In addition to advancing our knowledge regarding fundamental psychological processes underlying distinct forms of reward learning, there is general interest in understanding individual differences in incentive salience attribution, and elucidating the neural circuitry underlying this phenomenon, due to the theory that the attribution of incentive salience to reward cues underlies addiction (Robinson & Berridge, 1993; Robinson & Berridge, 2001). Utilizing the sign-tracker/goal-tracker model, evidence suggests that sign-trackers are more likely to exhibit addiction-related behaviors compared to goal-trackers (Saunders *et al.*, 2013a; Robinson *et al.*, 2014). Sign-trackers exhibit a greater propensity for psychomotor sensitization following repeated cocaine injections (Flagel *et al.*, 2008), a form of cocaine-induced plasticity that may contribute to the development of addiction. In addition, rats who sign-track to food-associated cues do the same for drug-associated cues (Flagel *et al.*, 2010; Yager & Robinson, 2013). Furthermore, cocaine-associated cues gain inordinate control over drug-taking behavior for sign-trackers, and these animals are more likely to exhibit reinstatement of drug-seeking behavior relative to goal-trackers, even in the face of adverse consequences (Saunders & Robinson, 2010; 2011; Saunders *et al.*, 2013b; Yager & Robinson, 2013). Sign-trackers have also been reported to acquire cocaine self-administration more rapidly than goal-trackers (Beckmann *et al.*, 2011), and sign-trackers are more impulsive than goal-trackers (Lovic *et al.*, 2011), another trait associated with addiction liability in both animal models and humans (Belin *et al.*, 2008; Ersche *et al.*, 2010). Thus, individual differences in the propensity to attribute incentive salience to discrete

food-paired cues may confer vulnerability to addiction-related behaviors. It should be noted, however, that recent work has called some of this evidence into question. In a study by Kawa and colleagues (2016), STs and GTs had long-term access to cocaine under an intermittent access schedule, and motivation to take cocaine was measured using a behavioral economics paradigm. As the previous work would predict, STs were more motivated that GTs to take cocaine early in the study, following limited drug exposure. These differences then disappeared as the study progressed, with STs and GTs displaying equal motivation to take cocaine by the end of the study (Kawa *et al.*, 2016). These findings suggest that STs and GTs might not differ in their long-term development of addiction-like behavior through differences in incentive-sensitization, as suggested earlier (Flagel *et al.*, 2008). Instead, STs might be more susceptible to continued drug use through a variety of other mechanisms, including sensitivity to discrete drug-paired cues (Saunders *et al.*, 2013b; Kawa *et al.*, 2016). Additionally, recent work suggests that goal-trackers may be more prone to attributing incentive motivational value to contextual stimuli (Saunders *et al.*, 2014), especially as they relate to drugs of abuse. This newly emerging finding provides further support for the notion that sign-trackers and goal-trackers process motivationally salient information in quite different ways (Flagel *et al.*, 2011a; Flagel *et al.*, 2011b; Robinson *et al.*, 2014); and the PVT may play a central role in the underlying processes as it has previously been implicated the conditioned effects of both discrete and contextual reward-associated cues (e.g. (Hamlin *et al.*, 2009; James *et al.*, 2011).

The neurobiology underlying differences in Pavlovian conditioned approach behavior

The role of dopamine in Pavlovian conditioned approach behavior

Important findings surrounding the neurobiological mechanisms of cue-motivated behaviors have emerged from the sign-tracker/goal-tracker animal model. Exploiting these individual differences in stimulus-reward learning, it has been demonstrated that systemically blocking dopamine transmission with the non-selective dopamine receptor antagonist flupenthixol attenuates the acquisition of a sign-, but not goal-tracking conditioned response (Flagel *et al.*, 2011b). In support, a more recent study showed that systemic flupenthixol administration biases individuals towards the development of a goal-tracking response during the acquisition of a Pavlovian CR (Scülfort *et al.*, 2016). In addition, flupenthixol administration specifically in the NAc core attenuates the expression of a sign-, but not goal-tracking behavior

and blocks cue-induced reinstatement of cocaine-seeking behavior specifically in STs (Saunders & Robinson, 2012; Saunders *et al.*, 2013b). From these studies, it was concluded that dopamine transmission is critical for the attribution of the incentive, but not the predictive, properties of reward cues. It should be noted, however, that a series of studies from our lab demonstrated that systemically manipulating dopamine transmission specifically at D_2/D_3 receptors attenuates the expression of *both* sign- and goal-tracking behavior (Fraser *et al.*, 2016). Due to the nature of systemic administration, it is unclear from the study by Fraser et al. (2016) if the effects observed are also mediated by dopamine blockade in the NAc, or different brain areas. In addition, the compound that was used in the previous studies (flupenthixol) also blocks dopamine transmission at D_1 receptors, while the compounds used by Fraser et al. (2016) did not. Thus, while dopamine in the core of the nucleus accumbens appears to be critical for the expression of a sign-tracking CR, activity at other dopamine receptors and in other brain regions may play a role in mediating the goal-tracking response.

The PVT and related circuitry underlying individual variation in Pavlovian conditioned approach behavior

To further delineate the neural circuitry underlying the attribution of incentive vs. predictive value to reward cues, cue-induced neuronal activity in areas outside of the classic mesocorticolimbic dopamine circuitry was examined (Flagel *et al.*, 2011a). Outbred rats were characterized as sign-trackers vs. goal-trackers based on Pavlovian training sessions consisting of lever-CS presentations paired with food reward. After rats had learned their respective conditioned responses, they were presented with the lever-CS in the absence of food reward to assess cue-induced expression of c-fos mRNA throughout the brain. Results showed that levels of c-fos mRNA were enhanced in the cortico-striatal-thalamic areas comprising the "motive circuit" (described by (Pierce & Kalivas, 1997; Kelley *et al.*, 2005a) in sign-trackers relative to goal-trackers and controls, who received an equal number of lever-cue and food presentations but in an unpaired fashion (Flagel *et al.*, 2011a). Thus, many parts of the motive circuit are engaged by the *incentive,* and not the predictive, properties of a discrete reward cue. Although sign-trackers exhibited enhanced cue-induced c-fos mRNA in all of the midline thalamic nuclei examined (i.e. central medial, intermediodorsal and PVT), the region with the most robust effect was the PVT (Flagel *et al.*, 2011a). In response to presentation of the food-paired cue, sign-

trackers exhibited almost twice as much c-fos expression in the PVT relative to goal-trackers. Importantly, goal-trackers did not significantly differ from the control group, suggesting that the PVT is highly engaged by cues attributed with incentive, but not predictive value. Similar findings have been reported following presentation of an opioid-paired cue (Yager *et al.*, 2014).

When 'functional connectivity' was examined in sign-trackers vs. goal-trackers by identifying correlations in cue-induced c-fos mRNA between brain regions in a given phenotype, a different picture emerged (Figure 1.3). Originally, this analysis included only brain regions in which there was a significant difference in cue-induced c-fos mRNA between sign-trackers and goal-trackers (Flagel *et al.*, 2011a). Here, however, it has been expanded to include all of the brain areas examined in order to get a more complete picture of network activity in the motive circuit. In sign-trackers, cue induced c-fos mRNA expression was correlated between the thalamus and the NAc shell. Although this correlation was significant for multiple thalamic nuclei, the strongest was a negative correlation between the PVT and the NAc shell. It should be noted that, with this analysis, the direction of the correlation is uninformative, since the type of cell (e.g. inhibitory or excitatory) in which the c-fos is expressed remains unknown. Regardless, this finding further supports a role for the PVT in dopamine-dependent, sub-cortical processing underlying the sign-tracking response. For goal-trackers, cue-induced c-fos mRNA was correlated between the prefrontal cortex and the PVT. Of particular interest is the significant correlation between the PVT and the PrL, since the densest set of afferents to the PVT comes from the PrL (Li & Kirouac, 2012). Interestingly, there were no significant correlations between the PrL and other thalamic nuclei. There was also evidence of cortico-striatal communication in goal-trackers, which was not present in sign-trackers. Together, these data suggest that goal-trackers may utilize 'top-down' cortical processes to regulate their behavior (*see below*). The distinct patterns of connectivity between sign- and goal-trackers highlight the extent to which different neural systems are engaged when a cue is attributed with incentive vs. only predictive value and suggest a potential role for the PVT in these learning processes.

A potential role of the paraventricular nucleus of the thalamus in mediating sign- vs. goal-tracking behaviors

The discovery that the PVT is more engaged following exposure to a reward-associated cue in sign-trackers, compared to goal-trackers and unpaired control subjects, suggests a role for

the PVT in the attribution of incentive salience. Here it is hypothesized that the attribution of incentive salience is mediated by subcortical afferents to the PVT, coupled with the dense PVT efferents to the ventral striatum. The mesolimbic dopamine system has long been known to be active in response to reward cues, and the hypothalamus, which contains PVT-projecting orexin neurons, has recently been recognized for a similar role (Choi *et al.*, 2010; Petrovich *et al.*, 2012). It is possible therefore, that exposure to a reward-paired cue elicits robust activity in sub-cortical projections to the PVT, including orexin input from the hypothalamus, which could result in increased excitation in PVT neurons. This increased activity in the PVT could ultimately lead to an increase in NAc activity, including dopamine transmission, and may do so to a greater extent than VTA-NAc transmission alone. Presumably, activity in the pathways that mediate dopamine release in the NAc are enhanced to a greater extent in sign-trackers than goal-trackers in response to reward cues.

While previous work has demonstrated that sign-tracking behavior is dependent on dopamine transmission in the NAc core (Flagel *et al.*, 2011b; Saunders & Robinson, 2012); the role of dopamine in the NAc shell, where the PVT sends more dense projections, has yet to be investigated in this regard. Importantly, the NAc core and shell send direct projections to one another via medium spiny neurons and interneurons (van Dongen *et al.*, 2005).The NAc shell also sends projections directly to the VTA (Nauta *et al.*, 1978; Heimer *et al.*, 1991), and these projections heavily overlap with VTA cells that in turn project back to the NAc core (Haber *et al.*, 2000). Therefore, there are both direct and indirect pathways in which the NAc shell can influence activity in the NAc core. Further, while ample evidence supports a role for the NAc core in cue-reward processing, recent evidence has demonstrated a potentially similar role for the NAc shell (Blaiss & Janak, 2009; Grimm *et al.*, 2011; Peciña & Berridge, 2013). In relation, enhanced cue-induced c-fos activity has been observed in both the core and shell in sign-trackers relative to goal-trackers (Flagel *et al.*, 2011a). Thus, the specific involvement of the NAc core versus shell in cue-motivated behaviors is not yet entirely clear. We suspect, however, that PVT projections to the NAc affect activity in both the core and the shell and it is, at least in part, via this circuit that the PVT regulates sign-tracking behavior.

Based largely upon the 'functional connectivity' (i.e. c-fos mRNA correlational analysis) data described above, we postulated that the goal-tracking CR is mediated largely by cortical 'top-down' control. The correlational analyses revealed that PVT and PrL activity is correlated in

goal-trackers, but not sign-trackers, following cue presentation. The PrL is important for regulating goal-directed behavior (Balleine & Dickinson, 1998), and has recently been thought to represent a 'cognitive-control' mechanism capable of inhibiting conditioned responding to cues (Jonkman *et al.*, 2009; Kober *et al.*, 2010; Mihindou *et al.*, 2013). Indeed, we have shown that goal-trackers exhibit more self-control, as they are found to be less impulsive than sign-trackers (Flagel *et al.*, 2010; Lovic *et al.*, 2011), and perform better on a prefrontal-dependent sustained-attention task (Paolone *et al.*, 2013). Moreover, acquisition of a goal-tracking response appears to be dopamine-independent (Flagel *et al.*, 2011b; Scülfort *et al.*, 2016), and cognitively-mediated learning processes are also thought to be dopamine independent (Dickinson & Balleine, 2002). Together, these findings lead to the hypothesis that goal-trackers utilize the discrete reward cue as an informational stimulus which results in the attribution of predictive (but not incentive) value to the cue, via a 'top-down' (e.g. PrL-PVT) cognitive learning strategy. In consideration of the circuitry proposed above for sign-trackers, it is possible that for goal-trackers PrL input to the PVT is suppressing the subcortical (i.e. orexinergic/dopaminergic) signaling induced by the reward cue, preventing an increase in accumbens dopamine levels, and thereby preventing the attribution of incentive salience to the cue. For example, the PVT shows dense expression of group II metabotropic glutamate receptors, and agonism of these receptors leads to hyperpolarization of post-synaptic PVT neurons (Hermes & Renaud, 2011). PrL glutamatergic activity at these receptors could therefore result in the suppression of sub-cortical signaling (e.g. orexin and dopamine) at the level of the PVT.

Conclusions and Hypothesis

Based on the anatomical, pharmacological, and behavioral evidence reviewed above, the PVT appears to play an important role in mediating cue-motivated behaviors. More specifically, recent data from our laboratory and others suggests that the role of the PVT in motivated behavior lies in processing *both* the predictive and incentive properties of reward cues. It is hypothesized that the PVT is a critical regulator in biasing an individual towards either dopamine-dependent (sign-tracking) or dopamine–independent (goal-tracking) behaviors. In this model, sign-tracking behavior is mediated by a 'subcortical drive' involving input from multiple subcortical regions, including orexinergic input from the hypothalamus, and relaying this input to the NAc. In contrast, goal-tracking behavior is mediated by 'top-down' cortical control, in the

form of dense glutamatergic PrL innervation of the PVT. Thus, the PVT appears to represent a critical node wherein integration of sub-cortical and cortical inputs can influence the propensity to attribute incentive vs. predictive qualities to discrete reward cues (Figure 1.4). This hypothesis will be addressed in several steps, as outlined below, in this dissertation.

Chapter 1: Lesions of the paraventricular nucleus of the thalamus differentially affect sign- and goal-tracking conditioned responses.

Due to the preponderance of evidence identifying the PVT as a modulator of cue-motivated behaviors, and recent data from our lab supporting a role for this nucleus in individual variation in cue-motivated behaviors, the aim of the first chapter is to establish a causal link between the PVT and sign- and goal-tracking CRs. First, the role of the PVT in the *acquisition* of sign- and goal-tracking behavior will be examined following lesions of the PVT. This will be accomplished using bred high-responder (bHR) and bred low-responder (bLR) rats (Stead *et al.*, 2006). Briefly, these rats have been bred based on their locomotor response to a novel environment. During this breeding process, several other traits seem to have been co-selected, including sign- and goal-tracking behavior. That is, bHR rats will almost always develop a sign-tracking phenotype, and bLR rats a goal-tracking phenotype (Flagel *et al.*, 2010; Flagel *et al.*, 2011b; Flagel *et al.*, 2014). Knowing the phenotypes *a priori* allows us to assess the effects of PVT lesions on the *acquisition* of sign- and goal-tracking CRs. Second, the effects of PVT lesions on the *expression* of sign- and goal-tracking CRs will be assessed. This will be accomplished by training outbred Sprague-Dawley rats in a PCA paradigm and classifying them as STs or GTs using the PCA Index score (Meyer *et al.*, 2012). Following classification, PVT lesions will be performed, and animals will continue PCA training to evaluate changes in the previously acquired CR. These studies will allow us to form a causal link between the PVT and PCA behavior.

Chapter 2: A food predictive cue engages subcortical afferents and efferents of the paraventricular nucleus of the thalamus if it is attributed with incentive salience.

A key component of the model proposed in this Introduction revolves around the role of cortical and subcortical afferents to the PVT, as well as efferents from the PVT to the NAc. The aim of Chapter 2 is to directly assess the engagement of these afferent and efferent pathways in

response to presentation of reward-predictive and incentive stimuli. This will be accomplished in a series of immunohistochemical studies utilizing retrograde tracing and c-fos protein expression. Briefly, the retrograde tracer Fluorogold (FG) will be injected into either the PVT or NAc. Following FG injection, rats will be trained in a PCA paradigm, and classified as STs and GTs. These animals will then be presented with the lever-CS, which is predictive of reward delivery for both STs and GTs, but also serves as an incentive stimulus for STs. Following lever presentation, the levels of c-fos protein expression in afferent and efferent pathways labeled with FG will be examined, to assess differences in the engagement of PVT circuits between STs and GTs. This will further our understanding of the PVT and related circuitry in incentive salience attribution.

Chapter 3: Investigating the role of orexin receptor 2 in the paraventricular nucleus of the thalamus in Pavlovian conditioned approach behavior.

As described above, the role of orexin transmission in the PVT has been gaining attention in both cue-motivated and reward-seeking behaviors (Martin-Fardon & Boutrel, 2012; Matzeu *et al.*, 2014). Additionally, slice physiology studies have demonstrated that orexin administration in the PVT excites PVT cell bodies (Kolaj *et al.*, 2007), and it is believed that this effect is mediated by orexin receptor 2 (OX-2R) (Ishibashi *et al.*, 2005; Huang *et al.*, 2006). Last, orexin administration in the PVT leads to an efflux of dopamine in the NAc, potentially linking this circuit to motivated behavior, including sign-tracking. The aim of this chapter is to assess the role of orexin transmission at OX-2Rs in the PVT in sign- and goal-tracking behaviors. This will be accomplished in a series of experiments utilizing local administration of the OX-2R antagonist TCS OX2 29 directly into the PVT to assess the effects of this compound on the expression of sign- and goal-tracking behavior and on the conditioned reinforcing properties of the lever-CS. In addition, the role of orexin transmission at OX-2Rs in the PVT during a feeding test will be examined, since previous work has demonstrated that the PVT is important for feeding behavior (Stratford & Wirtshafter, 2013), and it is believed that this is in part mediated by orexin transmission in the PVT (Choi *et al.*, 2012).

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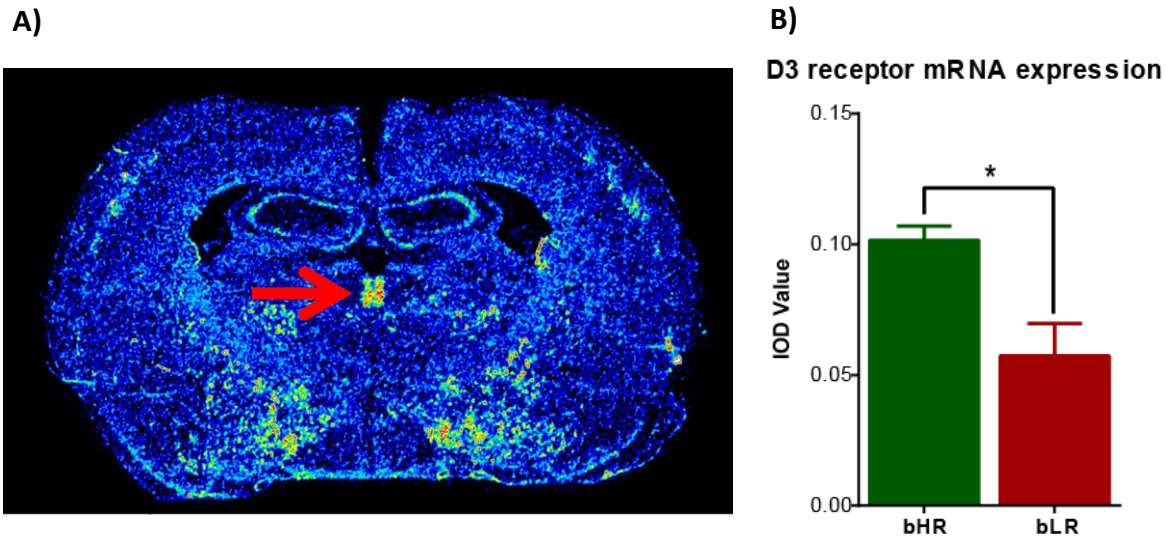

Figure 1.1. Dopamine D3 receptor mRNA expression in the PVT. A) Color-enhanced in situ hybridization image of D3 mRNA in the PVT (red arrow) in a coronal rat brain section. Warmer colors (red, followed by yellow, then green) represent areas of greater D3 mRNA expression compared to background (blue). Approximate bregma level is -2.28 (Paxinos & Watson, 2007). B) Levels of D3 mRNA expression in bred High-Responder (bHR; n = 7) or bred Low-Responder (bLR; n = 7) rats, measured as integrated optical density (IOD) value. bHR rats are predisposed developing a sign-tracking conditioned response ,and are susceptible to addiction-like behaviors, while bLR rats are predisposed to developing a goal-tracking phenotype. An unpaired t-test shows that bHRs have greater D3 receptor mRNA expression in the PVT than bLRs (*p = 0.007).

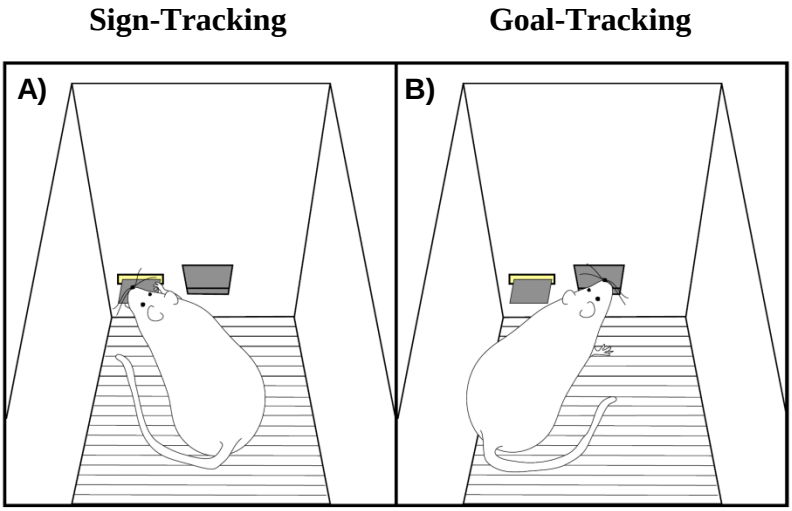

Figure 1.2. Cartoon representation of sign-tracking and goal-tracking behaviors. Examples of A) sign-tracking and B) goal-tracking behaviors in response to lever-cue presentation during a Pavlovian conditioning session. A) Sign-trackers approach the lever-cue during its presentation, even though no response is required for food delivery, while B) goal-trackers approach the food cup (i.e. location of reward delivery) upon lever-cue presentation.

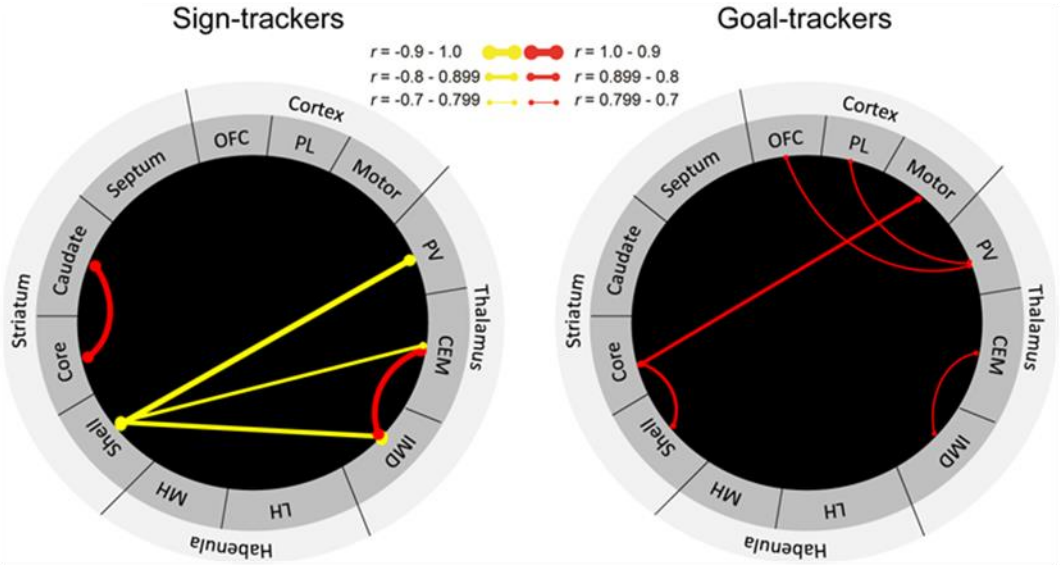

Figure 1.3. 'Functional connectivity' in sign-trackers and goal-trackers. Illustration of significantly correlated levels of c-fos mRNA expression between brain regions for sign-trackers and goal-trackers. Red lines are indicative of a significant positive correlations and yellow lines represent negative correlations. The thicker the line, the stronger the correlation. Abbreviations: OFC, orbitofrontal cortex; PL, prelimbic cortex, PV, paraventricular nucleus of the thalamus; CEM, centromedial nucleus of the thalamus; IMD, intermediodorsal nucleus of the thalamus; LH, lateral habenula; MH, medial habenula. Adapted from (Flagel et al., 2011a).

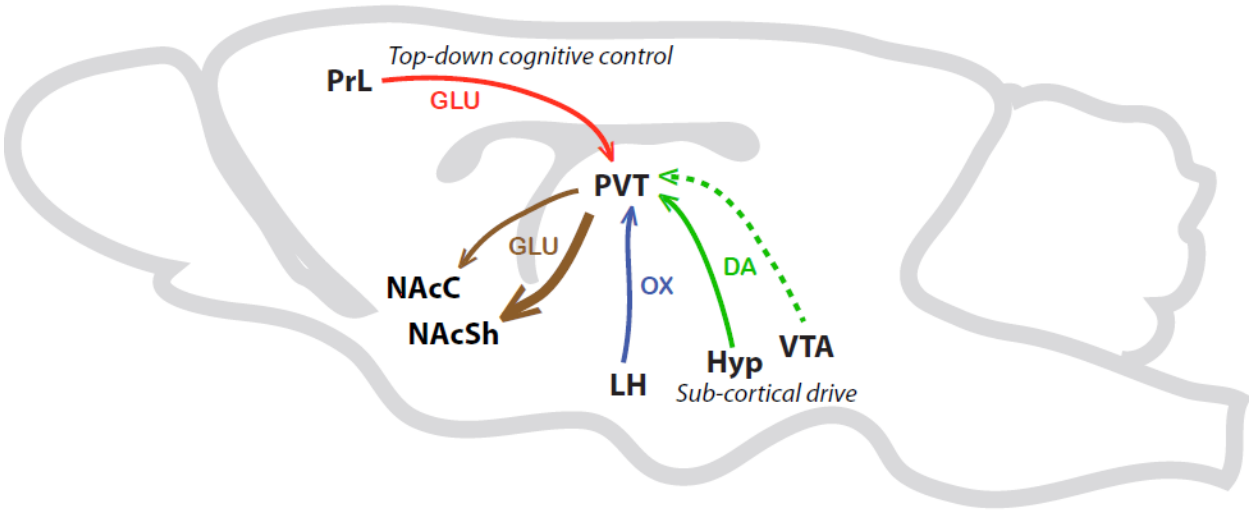

Figure 1.4. Schematic illustrating afferents and efferents of interest in the PVT. This simplified schematic illustrates afferents and efferents of the paraventricular nucleus of the thalamus (PVT) that are potentially involved in Pavlovian conditioned approach behavior. The solid green arrow represents sub-cortical dopamine inputs from the hypothalamus (Hyp). The dashed green line represents a minor dopaminergic input from the ventral tegmental area (VTA). The blue arrow represents orexin (OX) input from the lateral hypothalamus (LH) and the red arrow represents glutamatergic (GLU) projections from the prelimbic cortex (PrL) to the PVT. Efferent pathways from the PVT to the nucleus accumbens shell (NAcSh), and to a lesser extent the nucleus accumbens core (NAcC), are represented with brown arrows.

Chapter 2

Lesions of the paraventricular nucleus of the thalamus differentially affect sign- and goal-tracking conditioned responses

Note: This text, as well as the figures, have appeared previously in print (Haight JL et al., 2015, European Journal of Neuroscience), and are reproduced here with permission from the publisher, John Wiley and Sons.

Introduction

It is well established that environmental stimuli that are repeatedly paired with rewards can acquire motivational control over behavior, and do so through complex cortico-striatal-thalamic brain networks (Pierce & Kalivas, 1997; Kelley *et al.*, 2005). Within the past 10 years, evidence has emerged suggesting that the paraventricular nucleus of the thalamus (PVT) is a critical component of this circuitry (Martin-Fardon & Boutrel, 2012; James & Dayas, 2013), and its anatomical location supports a role for modulating cue-motivated behaviors (Vertes & Hoover, 2008; Li & Kirouac, 2012). In agreement, it has been shown that discrete cues associated with both natural rewards and drugs of abuse elicit robust activity in the PVT. For example, repeated pairings of a cue light with a water reward in a Pavlovian manner elicit enhanced c-fos expression in the PVT relative to controls exposed to random cue-reward presentations (Igelstrom *et al.*, 2010). In addition, elevated c-fos levels are found in the PVT following cue-induced reinstatement of drug-seeking behaviors (Wedzony *et al.*, 2003; Dayas *et al.*, 2008; James *et al.*, 2011). Taken together, these data suggest that the PVT may be involved in mediating cue-motivated behaviors, including Pavlovian conditioned responses (CRs), but its specific role in these processes is unknown.

Importantly, Pavlovian conditioned reward cues can act as both predictive and incentive stimuli, and individuals differ in the extent to which they attribute reward cues with motivational properties (Flagel *et al.*, 2009; Robinson & Flagel, 2009). When rats are exposed to a Pavlovian conditioned approach (PCA) paradigm, wherein a discrete cue (conditioned stimulus; CS)

predicts a food reward (unconditioned stimulus), some rats, termed sign-trackers (STs), will develop a conditioned response directed towards the cue. For these individuals, the cue itself becomes attractive, eliciting approach; and desired, such that STs will work to obtain it in the absence of a food reward (Robinson & Flagel, 2009). For others, termed goal-trackers (GTs), the cue elicits a CR directed towards the site of reward delivery. Thus, the cue is a predictive stimulus for both STs and GTs, and is effective at evoking a CR in both groups of animals; but only for the STs is the cue imbued with incentive salience, rendering it attractive and desired (Robinson & Flagel, 2009).

Using the sign-tracker/goal-tracker animal model, it has been shown that presentation of an incentive stimulus previously paired with a food or drug reward can elicit robust c-fos expression in the PVT (Flagel *et al.*, 2011a; Yager *et al.*, 2014). In addition, STs and GTs show different patterns of 'functional connectivity' (correlated levels of c-fos mRNA) between the PVT and other brain areas following cue presentation, suggesting the PVT might differentially regulate these conditioned responses (Flagel *et al.*, 2011a; Haight & Flagel, 2014). While these data further support the notion that the PVT is involved in cue-motivated behaviors, a causal link between the PVT and PCA behavior has yet to be established. In addition, it is unknown whether the PVT is necessary for the acquisition of PCA behavior, or whether it is also critical for the ongoing expression of Pavlovian CRs after they have been acquired. Here we used excitotoxic lesions to specifically investigate the role of the PVT in the acquisition and expression of sign- and goal-tracking CRs. Based on our previous findings (Flagel *et al.*, 2011a; Haight & Flagel, 2014; Yager *et al.*, 2014), we hypothesized that the PVT is an integral part of the neural circuitry underlying the attribution of incentive salience to reward cues, and that lesions of the PVT would disrupt both the acquisition and expression of sign- and goal-tracking behaviors.

Materials and methods

All experiments followed the Guide for the Care and Use of Laboratory Animals: Eighth Edition, revised in 2011, published by the National Academy of Sciences, and all procedures were approved by the University of Michigan Institutional Animal Care and Use Committee.

Experiment 1: The effects of PVT lesions on the acquisition of sign- and goal-tracking conditioned responses

Subjects

Subjects were 52 adult male Sprague-Dawley rats from generations F38 and F40 of selectively bred high-responder (bHR) and low-responder (bLR) rat lines (Stead *et al.*, 2006). These rats have been bred based on their locomotor response to a novel environment. bHR rats show increased locomotor response to novelty relative to bLRs, who show relatively low levels of activity in a novel environment. Importantly, a number of other traits seem to have been co-selected in these rat lines, including sign- and goal-tracking. It has previously been shown that bHR rats are primarily sign-trackers, and bLRs are goal-trackers (Flagel *et al.*, 2010; Flagel *et al.*, 2011b; Flagel *et al.*, 2014). That is, we can predict with 90-100% certainty whether these rats will develop a sign-tracking or a goal-tracking CR based on their breeding history. Knowing this information *a priori* therefore allows us to examine the effects of experimental manipulations on the *acquisition* of these CRs.

Rats were drawn from 7-11 litters per phenotype within each generation. No more than 4 pups from any given litter were used and littermates were balanced across treatment groups within a phenotype. Thus, a maximum of 2 rats from the same litter were assigned to a single treatment group. Rats were approximately 65 days of age at the start of the study. Prior to surgery, rats were pair housed with the same phenotype in acrylic cages (46 x 24 x 22 cm) in a temperature controlled room and maintained on a 12-12 hour light-dark cycle, with lights on at 06:00 hours. Food and water were available *ad libitum.* Following surgery, all rats were single housed under the same conditions.

Surgery

Prior to behavioral training, all subjects underwent lesion or sham surgery (for Experimental Timeline, see Figure 2.1A). All surgery was performed under aseptic conditions. Rats were anaesthetized with isoflurane and placed in a stereotaxic device. The scalp was shaved, sanitized with 70% alcohol followed by Betadine (Stamford, CT) solution and incised to expose the cranium. The skull was leveled, and small holes were drilled above the PVT. A 33-gauge injector (PlasticsOne, Roanoke, VA), connected to a 1µl Hamilton syringe (Hamilton Company, Reno, NV) via P50 tubing, was then lowered into two sites in the PVT at the

following coordinates measured from bregma (based on Hamlin *et al.*, 2009): A-P -2.6, M-L 0.2, D-V -5.5; and AP -3.6, M-L 0.3, D-V -5.6. To produce a lesion, 200 nl of 0.06M ibotenic acid (Abcam, Cambridge, MA) dissolved in sterile-filtered 0.1M phosphate-buffered saline (pH = 7.3-7.4) was injected at a rate of 100 nl per minute using a syringe pump (Harvard Apparatus, Holliston, MA). Control rats received infusions of vehicle (phosphate-buffered saline) only. Following infusion, the injector was left in place for 5 minutes to minimize diffusion up the injection track upon removal. Immediately prior to surgery, as well as 24 hours later, rats received sub-cutaneous injections of 2.5 mg/kg flunixin for pain management. Rats were allowed to recover in their home cages for 5-7 days prior to any behavioral testing.

Pavlovian conditioning procedures

The equipment and procedures used for Pavlovian conditioned approach (PCA) training have been described previously in detail (Flagel *et al.*, 2007; Flagel *et al.*, 2008). Sixteen standard MED Associates test chambers (MED Associates, St. Albans, VT) were used. Each chamber was equipped with a pellet dispenser and food cup located in the middle of the front wall. An illuminated, retractable lever was located to either the left or right (counter-balanced) of the food cup at equal height. All levers were set so that 10 grams of force caused a deflection of the lever and would result in a "lever contact" being recorded. A white house light was located at the top of the wall opposite the food cup and lever, and was illuminated for the duration of the training sessions. Operation of the pellet dispenser resulted in the delivery of one 45-mg banana flavored food pellet (Bio-Serv, Flemington, NJ) into the food cup. Head entries into the food cup were detected by an infrared photo beam. Each chamber was housed in a sound-attenuated box equipped with a ventilation fan that generated background noise.

All training was conducted between 13:00 and 17:00 hours. For 2 days prior to training, rats were briefly handled by the experimenter, and banana flavored food pellets (approximately 25-30 pellets per rat) were delivered into the rats' home cages to familiarize them with the reward to be used in training. Two pre-training sessions were then conducted prior to Pavlovian conditioning. During these sessions the house light was illuminated and 50 food pellets were delivered one at a time into the food cup on a variable interval 30-second schedule, and the lever remained retracted for the duration of the session. Each pre-training session lasted approximately 25 minutes, and by the end of the second session rats were reliably retrieving the majority of

their food pellets. Following pre-training, rats underwent 12 Pavlovian conditioning sessions, one session per day. Rats were trained for 7 consecutive days, given a 2 day break, and then trained for 5 more consecutive days, for a total of 12 sessions. Each training session consisted of 25 trials in which an illuminated lever (conditioned stimulus; CS) was inserted into the test chamber for 8 seconds, and then immediately upon its retraction a food pellet (unconditioned stimulus) was delivered into the food cup. The lever-CS was presented on a variable interval 90 second schedule (range 30-150 s), and the session lasted approximately 40 minutes.

The following events were recorded by MED Associates software: (1) the number of lever contacts, (2) the latency to first lever contact, (3) the number of head entries into the food cup during the 8 second lever presentation, (4) the latency to first food cup entry upon lever presentation, and (5) the number of head entries into the food cup during the inter-trial interval (ITI). These measures allowed us to quantify sign-tracking (lever-CS directed) and goal-tracking (food-cup directed) behavior, as well as activity during the ITI.

Experiment 2: The effects of PVT lesions on the expression of sign- and goal-tracking conditioned responses

Subjects

Subjects were 120 adult male Sprague-Dawley rats obtained from two commercial vendors (Charles River Laboratories, Portage, MI; Harlan Laboratories, Indianapolis, IN and Haslett, MI). Rats were ordered from two different vendors in order to get an adequate number of sign- and goal-trackers (Fitzpatrick *et al.*, 2013). Rats were approximately 60 days of age at the time of arrival and allowed to acclimate for 10-14 days prior to any handling or behavioral testing. Rats were pair housed in acrylic cages (46 x 24 x 22 cm) in a temperature controlled room and maintained on a 12-12 hour light-dark cycle, with lights on at 06:00 hours. Food and water were available *ad libitum.* All training was conducted between 13:00 and 17:00 hours.

Pavlovian conditioning procedures

Following the 10-14 day acclimation period, rats were handled and given banana pellets in their home cages for 2 days. Rats then underwent two pre-training sessions identical to those described in Experiment 1, to ensure rats were reliably consuming their food pellets. Following pre-training, rats underwent 7 sessions of Pavlovian conditioning (as described above; for an

Experimental Timeline, see Figure 2.1B). Importantly, unlike Experiment 1, this training occurred *before* surgery. Surgery was performed after 7 Pavlovian training sessions because previous studies have indicated that rats acquire the conditioned responses and approach asymptotic performance within the first week of training (Robinson & Flagel, 2009; Flagel *et al.*, 2011a).

Rats were characterized as sign-trackers, goal-trackers, and intermediate responders based on the average Pavlovian Conditioned Approach (PCA) Index scores (Meyer *et al.*, 2012) from sessions 6 and 7 of training. Briefly, the PCA Index Score is a composite measure used to quantify the degree to which an individual's behavior is directed towards the lever-CS or the food cup. The PCA Index score is based on three measures of Pavlovian approach behavior: the response bias for contacting the lever or food cup [(total lever-directed contacts – total food cup-directed contacts) ÷ (sum of total contacts)], the probability of lever or food cup contact [$Prob_{(lever)} - Prob_{(Mag)}$], and the latency to contact the lever or enter the food cup [(food cup entry latency – lever contact latency) ÷ 8]. These three values are then used to calculate the PCA Index score: [(response bias score + probability difference score + latency difference score) ÷ 3]. PCA Index scores range from +1.0 to -1.0, with +1.0 representing a rat whose behavior is exclusively directed towards the lever, and -1.0 representing a rat whose behavior is exclusively directed towards the food cup. In the current study, rats that were classified as STs had PCA Index scores ranging from 0.5 to 1.0, while rats classified at GTs had scores ranging from -0.5 to -1.0 (Meyer *et al.*, 2012).

Sign- and goal-trackers were then assigned to PVT lesion or sham treatment groups, which were balanced per phenotype based on both PCA Index Scores and Vendor (Harlan vs. Charles River). Importantly, although there were more sign-trackers from the Harlan population and more goal-trackers from Charles-River (as found in Fitzpatrick *et al.*, 2013), there were no behavioral differences based on Vendor within each phenotype. The average PCA index scores pre-lesion were similar across Vendor groups (ST, Charles River = 0.80; ST, Harlan = 0.84; GT, Charles River = -0.72; GT, Harlan = -0.68), such that behavior exhibited by sign-trackers from Harlan was indistinguishable from Charles-River sign-trackers, and the same was true when comparing goal-trackers from each vendor.

Surgery

The procedures for surgery were identical to those used in Experiment 1 above. However, for this experiment, surgical coordinates were altered in order to lesion a greater extent of the PVT. Ibotenic acid or vehicle was injected at the following coordinates from bregma, with the stereotaxic arm at a 10° angle toward the midline: A-P -2.0, M-L 1.0, D-V -5.4; A-P -3.0, M-L 1.0, D-V -5.5. Following a 5-7 day recovery period from surgery, rats underwent 14 additional sessions of PCA training to assess the effects of PVT lesions on the performance of sign- and goal-tracking conditioned responses.

Histological analysis of lesion sites (Experiments 1 and 2)

Following the completion of each experiment, rats were deeply anaesthetized with a cocktail of ketamine and xylazine (90 mg/kg ketamine; 10 mg/kg xylazine) and transcardially perfused with approximately 100 ml of 0.9% saline, followed by 200 ml of 4% paraformaldehyde in 0.1M phosphate-buffered saline (pH = 7.4). Brains were extracted from the skull and post-fixed overnight in 4% paraformaldehyde at 4^{0} C. Brains were then cryoprotected in graduated sucrose solutions (10%-30% in 0.1M sodium phosphate buffer, pH = 7.4) at 4° C over three days. Following cryoprotection, brains were mounted in Tissue-Plus O.C.T. compound (Thermo Fisher Scientific, Hampton, New Hampshire), frozen, and coronally sectioned on a cryostat at a thickness of 40 µm. Sections were mounted onto SuperFrost Plus slides (Thermo Fisher Scientific), stained with cresyl violet, dehydrated in graduated ethanol solutions followed by two xylenes washes, and coverslipped with Permount coverslipping medium (Thermo Fisher Scientific). To determine the presence of a PVT lesion, histological analysis was performed by an experimenter blind to treatment conditions. Lesions were identified by gliosis and a lack of cell bodies in the area of interest (Figure 2.2A-B).

Statistical methods (Experiments 1 and 2)

All statistical analyses were performed with the SPSS Statistics program, version 21 (IBM, Armonk, NY). Changes in Pavlovian conditioned approach behavior across sessions, measured by contacts, latency to contact, and probability of contact for either the lever or food cup were evaluated using linear mixed-effects models (Verbeke & Molenberghs, 2000), in which Session, Phenotype (bHR/bLR) and Treatment (lesion vs. sham) were treated as independent

variables. The covariance structure was explored and modeled appropriately for each dependent variable. A repeated measure ANOVA was used to further assess differences in sign- and goal-tracking behaviors pre- vs. post-lesion, with Treatment and Block (pre-lesion vs. post-lesion) as independent variables. For all analyses significance was set at $P \leq 0.05$ and Bonferroni post-hoc analyses were used to correct for multiple comparisons when significant main-effects or interactions were found.

Results

Experiment 1: The effects of PVT lesions on the acquisition of sign- and goal-tracking conditioned responses

Histology

Figure 2.2C shows a schematic representation of the lesion size and location for rats included in the study. In general, lesions spanned -2.3 mm to -3.8 mm posterior to bregma, encompassed the entire PVT, and only minimally damaged surrounding thalamic nuclei. Rats with small lesions that did not encompass the borders of the PVT, or lesions that resulted in extensive damage to neighboring nuclei or the hippocampus, were eliminated from the data analyses. Based on these criteria, 9 rats were excluded from the study and the following were included: bHR Lesion n= 9, bHR Sham n=12, bLR Lesion n=12, bLR Sham n=10.

Effects of PVT lesions in bLR rats

As indicated in Appendix A: Supporting Table 2.1, there was a significant Phenotype x Session and/or Phenotype x Session x Treatment interaction for all measures of sign- and goal-tracking behaviors in Experiment 1. Thus, bLRs and bHRs were analyzed separately, as described below.

For bLR rats, lesions of the PVT prior to acquisition of the conditioned response affected goal-tracking (Figure 2.3A-C), but not sign-tracking behaviors (Appendix B: Supporting Figure S2.1D-F; see also Appendix A: Supporting Information). Linear mixed-effects models revealed a significant effect of Treatment ($F_{(11,20)} = 4.48$, $P = 0.01$) and a Session x Treatment interaction ($F_{(11,20)} = 2.33$, $P = 0.05$) for food cup contacts (Figure 2.3A). There was a significant within-group effect of Session in bLR Sham rats ($F_{(11,20)} = 4.47$, $P = 0.002$), but not bLR Lesion rats, demonstrating that only bLR Sham rats learned a goal-tracking CR over the course of training. In

support, there was a trend towards significance for an effect of Treatment on measures of probability of food cup entry ($F_{(11,20)} = 3.77$, $P = 0.07$; Figure 2.3B) and latency to food cup entry ($F_{(11,20)} = 4.00$, $P = 0.06$; Figure 2.3C), but these effects did not reach statistical significance.

To determine whether PVT lesions were affecting general levels of activity in bLR rats when the CS was not present, we examined food cup responding during the inter-trial interval. While there was not a significant effect of Treatment on this measure, there was a significant Session x Treatment interaction ($F_{(11,20)} = 3.49$, $P = 0.01$; Appendix B: Supporting Figure S2.2). However, further analyses revealed a significant difference between bLR Sham and bLR Lesion groups only on Session 8 ($F_{(1,20)} = 7.22$, $P = 0.01$), which happened to be the first session of training after a 2 day break. Thus, although bLR Lesion rats had a tendency to enter the food cup with less frequency than bLR Sham rats during the inter-trial intervals, these differences were not as pronounced as those on measures of goal-tracking (Figure 2.3A-C; Appendix B: Supporting Figure S2.2).

It should also be noted that a small subset of bLRs did not consume all of their pellets during training, and three had to be excluded from the study for consistently leaving the majority of their pellets behind. Importantly, bLR Lesion and bLR Sham groups did not differ in their pellet consumption, and just a few rats (n = ~3 or 4) from both groups left an average of 5-7 pellets behind on any given training day. The number of omissions – or trials in which no sign- or goal-tracking response was occurred – was also analyzed. Although rats in the bLR Lesion group had a tendency to make more omissions relative to the bLR Sham group, this effect was not statistically significant (Effect of Treatment: $F_{(1,20)} = 3.83$, $P = 0.06$). Likewise, there was not a significant Treatment x Session interaction ($F_{(11,20)} = 1.78$, $P = 0.13$). Thus, taken together, we do not believe that the PVT lesions generally affected motivation to consume the food pellets or general locomotor activity in these subjects. These findings, therefore, demonstrate that PVT lesions selectively affect the development of a goal-tracking response in bLR rats that are inherently predisposed towards this behavior.

Effects of PVT lesions in bHR rats

For bHRs, lesions of the PVT affected the acquisition of sign-tracking behaviors (Figure 2.3D-F), but only during the latter phases of training. Linear mixed effects analysis revealed a significant Session x Treatment interaction for the number of lever contacts ($F_{(11,107)} = 2.04$, $P =$

0.03; Figure 2.3D) and the probability of lever contact ($F_{(11,107)} = 3.12$, $P = 0.01$; Figure 2.3E). For lever contacts, there was a significant effect of Session for both bHR Lesion ($F_{(11,107)} = 3.75$, $P = 0.001$) and bHR Sham ($F_{(11,107)} = 2.14$, $P = 0.02$) groups. However, when comparing the first training session to each subsequent session, lever contacts during the latter phases of training (sessions 9-12) were significantly different from early training (session 1) only for bHR Lesion rats ($P < 0.02$, Figure 2.3D). For probability of lever contact, a similar pattern was evident with a significant effect of Session for both bHR Lesion ($F_{(11,19)} = 7.50$, $P = 0.001$) and bHR Sham groups ($F_{(11,19)} = 7.99$, $P = 0.001$). The behavior of bHR Lesion rats during the latter phases of training (sessions 10-12) significantly differed from the first session ($P < 0.05$); whereas bHR Sham rats only significantly differed between session 12 and session 1 ($P = 0.03$; Figure 2.3E). For latency to contact the lever (Figure 2.3F), there was a significant effect of Session ($F_{(11,115)} = 5.85$, $P = 0.001$), but no effect of Treatment and no Session x Treatment interaction. These data suggest that both bHR sham and bHR lesion rats learned a sign-tracking conditioned response, as both approached the lever with decreasing latency over time. It should also be noted that there were no significant differences between sham-treated and lesioned rats on goal-tracking behavior (Appendix B: Supporting Figure S2.1A-C) or behavior during the ITI (data not shown). In sum, PVT lesions appeared to enhance sign-tracking behavior, as evident in the increased number of contacts and greater probability of contacting the lever in the latter phases of training. Although the effects of PVT lesions were less pronounced on sign-tracking behavior in bHRs compared to goal-tracking behavior in bLRs, these data suggest that the PVT is involved in regulating the development of both conditioned responses.

Experiment 2: The effects of PVT lesions on the expression of sign- and goal-tracking conditioned responses

Individual variation in PCA behavior

Similar to previous reports (Flagel *et al.*, 2009; Robinson & Flagel, 2009; Meyer *et al.*, 2012), considerable variation was seen in the form of the conditioned response acquired by individual rats following 7 sessions of Pavlovian training (Appendix B: Supporting Figure S2.3). Some rats came to preferentially direct their behavior towards the lever-cue, and were classified as sign-trackers (n = 37). Other rats directed their behavior towards the food cup upon lever-cue presentation, indicative of a goal-tracking CR (n = 33). The remaining rats showed a mixed

response in that they vacillated between the lever-cue and the food cup, and these rats were classified as intermediate responders (n = 50), and were not included in the study (data not shown).

Histology

All subjects were screened for the presence of a PVT lesion identical to the procedures described above for Experiment 1. Figure 2.4 shows a schematic representation of the lesion size and location for animals included in the study. In general, lesions spanned -1.6 mm to -3.4 mm posterior to bregma, encompassed the entire PVT, and only minimally damaged the mediodorsal, intermediodorsal, or centromedial thalamic nuclei. Based on the stated criteria, 13 ST and 11 GT lesioned animals were excluded from the study. One additional GT was excluded from the study because a lesion could not be verified. The final numbers of included animals were: ST Lesion n = 11, ST Sham n =13, GT Lesion n =9, GT Sham n =12 (see Appendix A: Supporting Information and Appendix B: Supporting Figure S2.4 for an analysis of the effects of missed lesions).

The effects of PVT lesions on the expression of sign- and goal-tracking conditioned responses

Given that these rats were classified as sign- and goal-trackers based on their PCA Index, and that the aim of the study was to compare the effects of PVT lesions on the previously acquired conditioned response, statistical comparisons were only made between treatment groups within a given phenotype. To assess the effects of PVT lesions on the expression of sign- and goal-tracking conditioned responses, we assessed longitudinal changes in post-lesion behavior across multiple sessions. To do this, all measures were normalized to pre-lesion baseline levels of responding for each individual rat by subtracting the average of sessions 5-7 (i.e. baseline) from the value for each of the post-lesion sessions, 8-21. Linear mixed-effects models were then used to assess changes in behavior across sessions with the normalized value as the dependent variable. For animals that were characterized as STs, there were no significant effects of PVT lesions on measures of goal- or sign-tracking behaviors or on behavior during the ITI (data not shown).

However, for rats characterized as GTs, PVT lesions after the acquisition of the conditioned response resulted in significant changes in both goal- and sign-tracking behaviors.

For goal-tracking measures, there was a significant effect of Treatment for contacts with the food cup ($F_{(13,119)} = 4.22$, $P = 0.05$; Figure 2.5A) and for latency to food cup entry ($F_{(13,20)} = 4.92$, $P = 0.04$; Figure 2.5C), but no significant interactions on these measures. There was, however, a significant Session x Treatment interaction for probability of food cup entries ($F_{(13,119)}=2.73$, $P = 0.02$; Figure 2.5B). GT Lesion rats decreased their probability of food cup entry with continued training (Effect of Session for GT Lesion group; $F_{(13,19)} = 3.91$, $P = 0.001$); whereas GT Sham animals did not show a significant decrease over time. In support, there were significant differences between the GT Lesion and GT Sham groups later in training (i.e. sessions 15, 18, 21; $P < 0.05$) for probability of food cup contact. These findings demonstrate that PVT lesions attenuate the expression of goal-tracking behavior in animals previously classified as GTs. Additionally, this reduction was not an immediate result of the PVT lesion, but was a learned effect over the course of post-lesion training. That is, probability to approach the magazine during the CS period diminished as a function of session. Importantly, this decrease in goal-tracking behavior is not the product of increased omissions (no Effect of Treatment, $F_{(1,20)} = 0.21$, $P = 0.65$; no Session x Treatment interaction, $F_{(20,195)} = 1.58$, $P = 0.06$).

There were also significant differences between GT Lesion and GT Sham rats on all measures of sign-tracking behavior. There was a significant Session x Treatment interaction for lever contacts ($F_{(13,19)} = 4.63$, $P = 0.001$; Figure 2.55D). For probability to contact the lever there was a significant effect of Treatment ($F_{(1,19)} = 5.22$, $P = 0.03$) and a Session x Treatment interaction ($F_{(13,19)} = 5.24$, $P = 0.001$; Figure 2.5E). There was also a significant Session x Treatment interaction for latency to contact the lever ($F_{(13,19)} = 2.71$, $P = 0.02$; Figure 2.5F). Post hoc analyses show that both GT Lesion (Effect of Session; $F_{(13,19)} = 4.56$, $P = 0.002$) and GT Sham (Effect of Session; $F_{(13,19)} = 2.75$, $P = 0.02$) rats showed an increase in lever contacts as training progressed, but GT Lesion rats appeared to do so to a greater extent (Figure 2.5D). The GT Lesion group also showed a robust increase over time for the probability to contact the lever (Effect of Session; $F_{(13,19)} = 5.12$, $P = 0.001$), and there was not a significant within-group effect of Session for the GT Sham group. A similar pattern was evident for the latency to contact the lever, with the GT Lesion group showing decreased latency (Effect of Session; $F_{(13,19)} = 2.52$, $P = 0.03$) to approach the lever over time; whereas sham controls exhibited relatively stable behavior on this measure. These results demonstrate that, for rats previously characterized as GTs, PVT lesions resulted in a significant increase in sign-tracking behavior. Further, for all

measures, the GT Lesion and GT Sham groups significantly differed no sooner than session 11, again indicating that the increase in lever-directed behavior in the GT Lesion group was not an immediate effect of the PVT lesion, but was a product of post-lesion Pavlovian training.

To examine whether PVT lesions had changed animals previously classified as GTs into STs, a repeated-measures ANOVA was used to compare pre-lesion (pre-lesion block; average of sessions 5-7) to post-lesion (post-lesion block; average of sessions 19-21) changes in response bias score (Figure 2.6A). Response bias is an index of an individual's bias towards the lever-cue vs. the food magazine during CS presentation, which is calculated using the following formula: [(total lever-directed contacts) – (total food cup-directed contacts)] ÷ (sum of total contacts). As a result, scores close to 1.0 represent behavior directed exclusively toward the lever (sign-tracking), while scores near -1.0 represent behavior directed exclusively towards the food cup (goal-tracking). Repeated-measures ANOVA showed a significant Block x Treatment interaction ($F_{(1,19)} = 6.02$, $P = 0.02$) for response bias score. Post-hoc analyses indicated a significant change in pre- vs. post-lesion behavior ($P < 0.001$) for the GT Lesion group, but not the GT Sham control group. In agreement, there was a significant difference between GT Lesion and GT Sham groups for the post-lesion block ($P = 0.05$). Prior to surgery, both GT Lesion and GT Sham groups showed response bias scores around -0.9, indicating that behavior was primarily directed towards the food cup. Following surgery, however, the response bias score of the lesion group moved to approximately -0.15, while the sham group stayed stable at approximately -0.75. A response bias score of -0.15 indicates that GTs with PVT lesions are not pure sign-trackers, but are showing an intermediate phenotype. Thus, their behavior is vacillating between the lever and the food cup; or, on a given trial, a rat may exhibit aspects of both a sign- and a goal-tracking response (e.g. Figure 2.6B).

Discussion

Here we assessed the effects of an excitotoxic lesion of the PVT on the acquisition and expression of sign- and goal-tracking CRs. Our results indicate that the PVT is required for the *acquisition* of a goal-tracking CR, but not a sign-tracking CR. In fact, lesioning the PVT prior to Pavlovian learning results in an exaggerated sign-tracking CR later in training. In addition, lesioning the PVT following the acquisition of sign- and goal-tracking CRs does not affect the behavior of STs, but leads to an overall shift towards sign-tracking behavior in animals

previously classified as GTs. These data suggest that the PVT is involved in mediating both sign- and goal-tracking behaviors, but in different ways.

A great advantage of utilizing the bHR/bLR rat lines is the ability to know *a priori* what CR the animals will develop, which was critical for examining the effects of PVT lesions on the *acquisition* of sign- and goal-tracking behaviors. Interestingly, locomotor response to novelty and the propensity to attribute incentive salience to reward cues (i.e. sign-tracking) are not highly correlated in outbred rats, as they are in the bred lines (Flagel *et al.*, 2010). In fact, the bHR/bLR rats differ on a number of genetic, neurobiological and behavioral traits that do not normally segregate with one another in outbred populations (for a detailed review, see Flagel *et al.*, 2014). Thus, any number of factors inherent to the bred lines could affect their initial tendency to sign- or goal-track, as well as any subsequent effects of manipulations, such as the lesion effects shown here. Nonetheless, any concerns due to the use of the selectively bred rats in Experiment 1 are mitigated by the fact that comparable results were found when commercially available outbred rats were used for Experiment 2. Further, additional analyses ruled out potential "general" effects on locomotor activity or motivational drive to obtain food in these studies, reinforcing the fact that findings from the *acquisition* study utilizing the bHR/bLR rats are indeed due to lesion effects on Pavlovian conditioned approach behavior.

In the *acquisition* study we found that the bHR Sham rats developed a sign-tracking CR consistent with previous findings (Flagel *et al*., 2010; Flagel *et al*., 2011b). The bLR Sham rats, however, did not develop as robust of a goal-tracking response as that seen in previous studies (Flagel *et al*., 2010; Flagel *et al*., 2011b). It is likely that the attenuated goal-tracking response in bLR rats in this study was due to extremely low levels of locomotor activity that have become characteristic of recent generations of bLR rats. Nonetheless, this does not detract from the current findings. In fact, had the bLR Sham group shown a more prominent goal-tracking response, the differences between the sham and lesioned animals would have been more pronounced. It is also important to note that there were not any significant differences in trial omissions between bLR Lesion and Sham groups; nor were there any differences in the number of reward pellets consumed. Thus, we believe the reduction in goal-tracking behavior seen in the bLR Lesion group is specific to the CR, and not due to a general loss of motivation or locomotor function in these animals.

Interestingly, when PVT lesions were performed following the acquisition of the CR in commercially available outbred rats, there were no effects on either sign- or goal-tracking behavior in rats characterized as STs. This was surprising since, in selectively bred HR rats, PVT lesions prior to CR acquisition led to increased asymptotic performance of a sign-tracking CR. It should be noted, however, that the levels of sign-tracking behavior exhibited by the outbred rats (Appendix B: Supporting Figure S2.3D-F) were greater than those exhibited by the bHR Sham rats (Figure 2.3D-F). Thus, the most likely explanation for these seemingly discrepant results is that PVT lesions could not further enhance the performance of STs in Experiment 2 because of a ceiling effect. To test this hypothesis, future studies might consider using rats that are intermediate responders to see if a PVT lesion could render these animals sign-trackers.

Perhaps the most interesting findings presented here are the effects of PVT lesions after outbred rats had acquired a goal-tracking CR. Goal-trackers with PVT lesions exhibited an attenuated goal-tracking CR relative to sham controls, which was consistent with the findings from the *acquisition* study using bLR rats. Concomitant with this reduction in goal-tracking behavior, however, was an increase in lever-directed behaviors, or sign-tracking. This behavioral shift was not apparent immediately following the PVT lesion, but developed over the course of post-lesion training. As a result, GT Lesion rats shifted to an intermediate phenotype by the end of training. That is, goal-tracking rats with a PVT lesion began to show increased interest in the lever upon its presentation, vacillating between it and the food cup. This is especially interesting since it is the first evidence to show that animals expressing a goal-tracking CR can be biased towards a sign-tracking CR via a neurobiological manipulation.

The fact that the behavioral shift towards sign-tracking for rats previously characterized as goal-trackers was not immediately apparent following the PVT lesion argues against the possibility that this effect could be attributed to an overall increase in locomotor activity. In agreement, others have assessed locomotor behavior following PVT lesions, and have found no differences between lesion and sham groups (Pierce *et al.,* 1997; Young & Deutch, 1998; Hamlin *et al.,* 2009). Further, we did not see an effect of PVT lesions when we assessed locomotor response to novelty in a subset of outbred animals from Experiment 2 (Appendix B: Supporting Figure S2.5; for detailed Methods and Results, see Appendix A: Supporting Information). Thus, we do not believe the increase in sign-tracking behavior reported here was due to an increase in general locomotor behavior.

In light of these results, it is interesting that an increased tendency to develop a sign-tracking CR following PVT lesion was not apparent in the bLR Lesion group in Experiment 1. However, as discussed above, this could be due to the overall lack of activity that is characteristic of the bLR lines. Thus, if the Experiment 1 was repeated with a population of outbred rats, it is possible that one would observe an overall population shift towards sign-tracking in PVT lesion animals. Unfortunately, it is difficult to interpret acquisition studies in outbred populations since it is impossible to know what CR rats will develop prior to training.

Another point to consider upon interpretation of Experiment 2 is that learning might have still been occurring at the time of the lesion. Although numerous studies have shown that rats acquire sign- and goal-tracking conditioned responses within the first week of training (Flagel *et al.*, 2009; Robinson & Flagel, 2009; Flagel *et al.*, 2011a; Flagel *et al.*, 2011b; Meyer *et al.*, 2012), we cannot rule out the possibility that ongoing learning processes are present even after rats have begun to exhibit their respective conditioned responses. As shown in Supplemental Figure S2.3, however, both STs and GTs appear to have reached stable levels of responding by Session 7, prior to the lesion. Thus, the apparent effects are likely specific to the ongoing performance of the CRs, rather than the learning process per se.

The current study was largely driven by previous findings showing that, relative to GTs, STs exhibit enhanced c-fos expression in the PVT in response to food- and drug-paired cues (Flagel *et al.*, 2011a; Yager *et al.*, 2014). Thus, it appears that only if a reward-cue is attributed with incentive salience will it robustly activate the PVT. In addition, correlations of cue-induced c-fos levels across brain regions within sign-trackers and goal-trackers reveal different patterns of 'functional connectivity' (Flagel *et al.*, 2011a; Haight & Flagel, 2014). Interestingly, one of the main points of divergence with this analysis was the PVT. For STs, cue-induced c-fos mRNA in the PVT was correlated with that in the shell of the nucleus accumbens (NAc); whereas GTs showed correlated levels of c-fos mRNA between the PVT and areas of the prefrontal cortex, particularly the prelimbic cortex (PrL). This suggests that, in response to cue presentation, STs and GTs might be utilizing different neural circuitry that converges on the PVT.

Our current data expand upon these recent findings, and affirm an important role for the PVT in both sign- and goal-tracking behavior. The findings stated above, namely correlated cue-induced c-fos mRNA between the PrL and PVT in GTs (Haight & Flagel, 2014), is consistent with the fact that the PVT receives dense cortical input from layer 6 of the PrL (Li & Kirouac,

2012). The PrL is known to be critical for goal-directed behavior (Balleine & Dickinson, 1998), but its role in these behaviors is complicated and not fully understood. For instance, inactivation of the PrL can facilitate or inhibit reinstatement of alcohol seeking in rats, depending on the context in which this behavior was extinguished (Willcocks & McNally, 2013). One view is that the PrL acts as a locus of 'cognitive-control,' capable of inhibiting responses to reward-paired cues (Jonkman *et al.*, 2009; Kober *et al.*, 2010; Mihindou *et al.*, 2013). In this regard, it is possible that STs and GTs differ in their degree of 'cognitive control' of their behavior, and PrL afferents to the PVT may be a critical component of this top-down circuitry. Specifically, these afferents might serve to suppress the motivational drive from subcortical areas, such as the hypothalamus, that are activated by presentation of reward-paired cues (Choi *et al.*, 2010; Mahler *et al.*, 2012). In the current study, it is therefore plausible that lesions of the PVT result in a loss of inhibitory control, presumably releasing the 'brake' on sign-tracking behavior. Thus, following PVT lesions, individuals who were previously goal-trackers appear to re-learn the cue-reward association using a different strategy—one that allows the cue to become attributed with incentive salience.

While these top-down cognitive processes might be stronger in GTs, it is also possible that sub-cortical motivational circuitry could be overriding this cortical control in STs. The PVT also receives input from subcortical brain areas known to be involved in motivated behavior, including dopamine and orexin projections from the hypothalamus (Kirouac *et al.*, 2005; Parsons *et al.*, 2006; Li *et al.*, 2014). Inside the PVT, these sub-cortical motivational signals are likely integrated with cognitive control signals from the PrL, to ultimately influence the activity of PVT neurons that project to the NAc. Importantly, these PVT efferents can modulate NAc dopamine release (Jones *et al.*, 1989; Parsons *et al.*, 2007), which is critical for sign-tracking behavior (Flagel *et al.*, 2011b; Saunders & Robinson, 2012). However, these signals would need to be strong enough to override the top-down control coming from the PrL to the PVT. Therefore, if the sub-cortical afferents to the PVT were specifically inhibited while leaving the PrL afferents intact, it is likely one would see an attenuation of sign-tracking behavior. Although this is not what we report in the current study, future studies will incorporate techniques capable of systematically targeting different components of this circuitry to determine what is driving sign-tracking behavior.

It has recently been shown that contextual, and not discrete, reward-paired cues can preferentially acquire motivational control over behavior in GTs compared to STs (Saunders *et al.*, 2014). Interestingly, previous reports have shown that exposure to contextual cues previously associated with administration of a highly palatable food (Schiltz *et al.*, 2005a; Schiltz *et al.*, 2007), nicotine (Schiltz *et al.*, 2005b), or cocaine (Johnson *et al.*, 2010) can elicit robust immediate early gene expression in the PVT, including c-fos expression. In addition, lesions or chemical inactivation of the PVT can attenuate contextual cue-induced reinstatement of alcohol-seeking behavior (Hamlin *et al.*, 2009; Marchant *et al.*, 2010); and the expression of cocaine-induced conditioned place preference is attenuated following inactivation of the PVT with GABA receptor agonists (Browning *et al.*, 2014). On a neuroanatomical level, these data are congruent with the fact that the PVT receives input from the ventral subiculum of the hippocampus (Li & Kirouac, 2012), an area critical for context-induced reinstatement of drug-seeking behavior (Sun & Rebec, 2003; Lasseter *et al.*, 2010). In addition, prelimbic afferents to the PVT might also be playing a role, since PrL function is needed to use contextual cues to guide goal-directed behavior (Marquis *et al.*, 2007). Given these recent findings (Hamlin *et al.*, 2009; Marchant *et al.*, 2010; Browning *et al.*, 2014; Saunders *et al.*, 2014), we postulate that the PVT might also be critically involved in the attribution of incentive motivational value to contextual cues. Thus, the attenuation of the goal-tracking response in lesioned GTs could have been due to a loss of motivational significance from contextual stimuli, resulting in behavior biased more towards the discrete cue (i.e. sign-tracking). Future studies will further assess the role of ventral subiculum and PrL afferents to the PVT, and whether these pathways play an important role in mediating the motivational significance of contextual cues.

In conclusion, we used an animal model that captures individual variation in response to discrete reward cues to assess the role of the PVT in different cue-reward learning processes. Using an excitotoxic lesion, we showed that PVT lesions attenuate the development of a goal-tracking CR, while increasing a sign-tracking CR. Taken together, these data support a role for the PVT in regulating individual differences in conditioned responding to discrete Pavlovian-conditioned reward cues. Specifically, the PVT may serve as a key node that regulates the attribution of incentive motivational values to reward-paired cues. Ongoing studies will further dissect the role of specific PVT efferents and afferents in incentive salience attribution to reward-paired cues.

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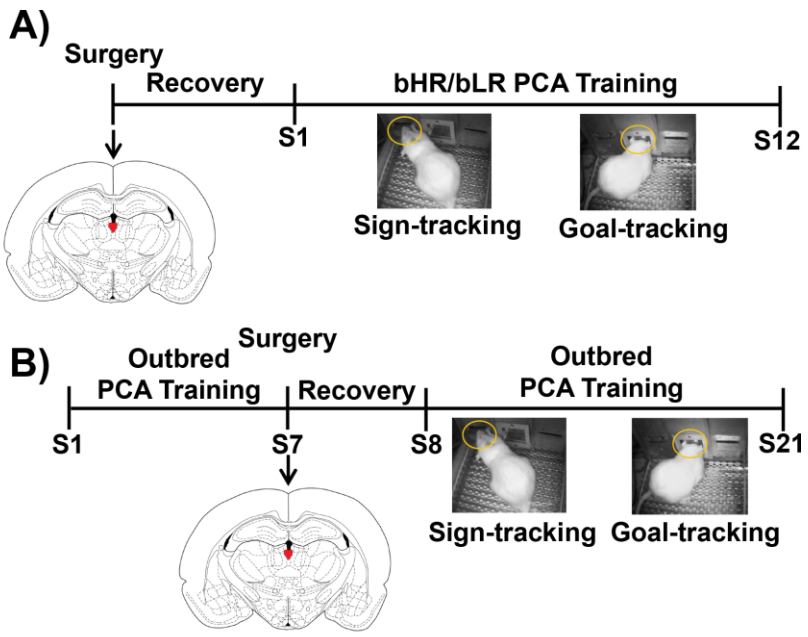


Figure 2.1. Experimental Timelines. Timeline for A) Experiment 1(Acquisition) and B) Experiment 2 (Expression) with representative photos capturing sign- and goal-tracking conditioned responses. Abbreviations: bHR, selectively bred high-responder; bLR, selectively bred low-responder; PCA, Pavlovian Conditioned Approach.

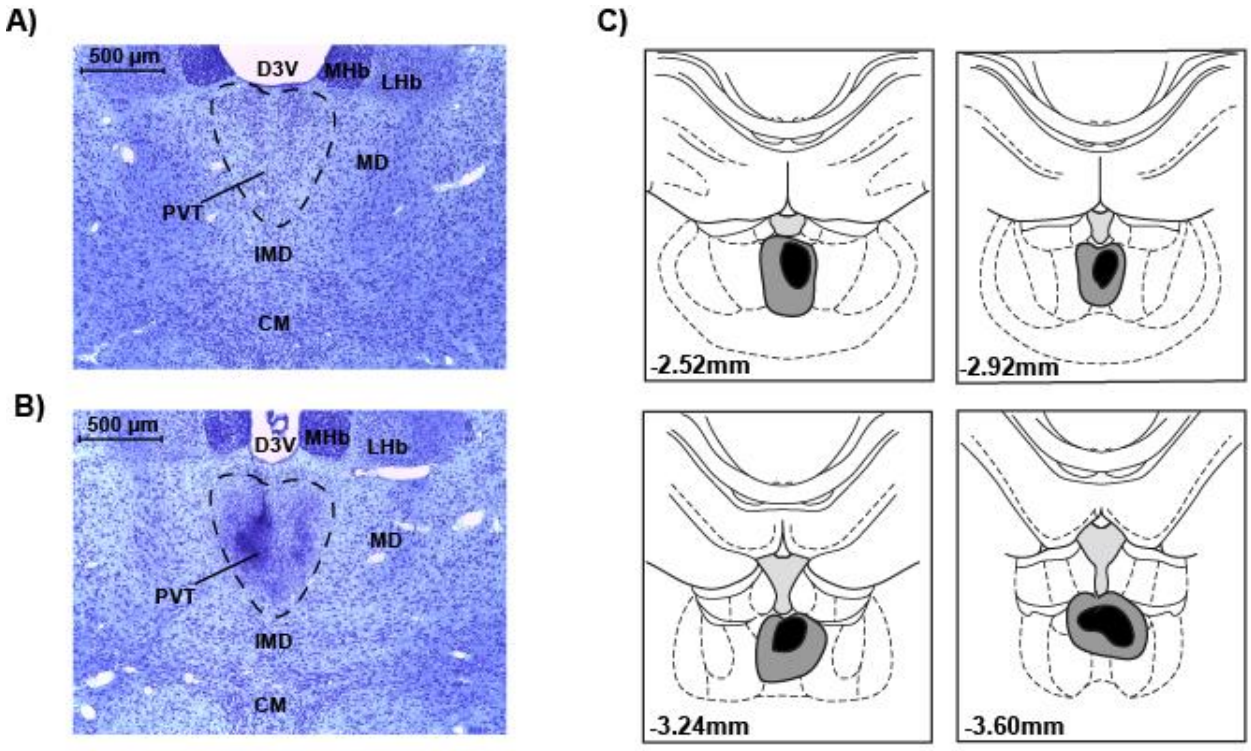


Figure 2.2. Histological analysis of lesion sites for Experiment 1. Photomicrograph showing representative cresyl-violet stained sections of A) an intact PVT and B) a PVT lesion; approximate bregma level = AP -3.00, scale bar = 500 µm. C) Illustration showing the largest (gray) and smallest (black) accepted excitotoxic lesions of the PVT for Experiment 1. Abbreviations: central medial thalamic nucleus, CM; dorsal 3rd ventricle, D3V; intermediodorsal thalamic nucleus, IMD; lateral habenular nucleus, LHb; mediodorsal thalamic nucleus, MD; medial habenular nucleus, MHb; paraventricular nucleus of the thalamus, PVT.

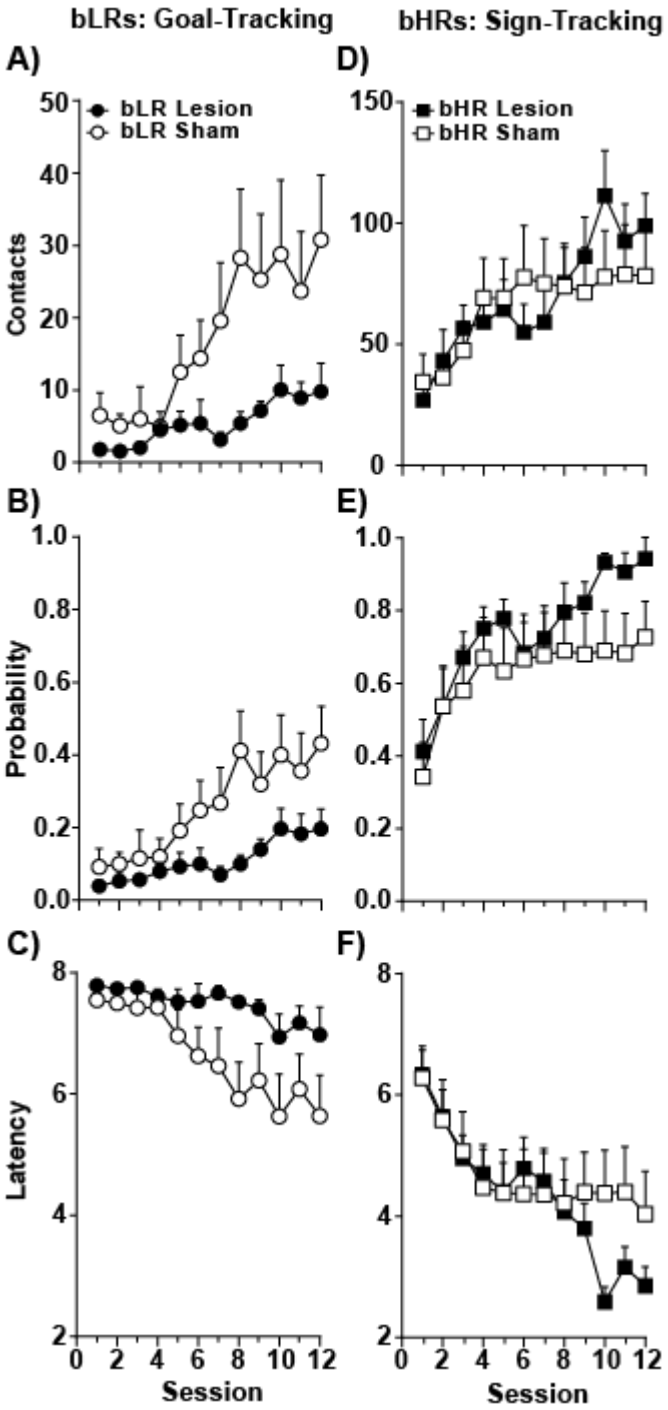


Figure 2.3. Effects of PVT lesion on the acquisition of (left) goal- and (right) sign-tracking conditioned responses. Mean + SEM of A) number of food cup contacts, B) probability of food cup contact, and C) latency to food cup contact for bLR animals (Lesion, n=12; Sham, n=10). For bHR animals (Lesion, n=9; Sham, n=12), D) number of lever contacts, E) probability of lever contact, and F) latency to lever contact.

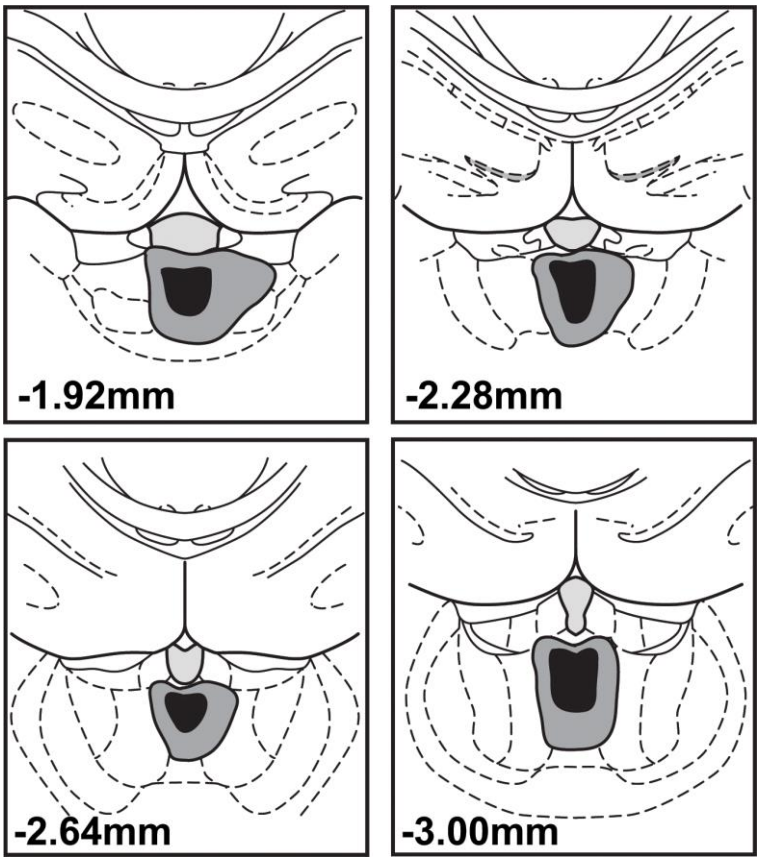


Figure 2.4. Histological analysis of lesion sites for Experiment 2. Representative atlas images showing the largest (gray) and smallest (black) excitotoxic lesions of the PVT for rats included in Experiment 2.

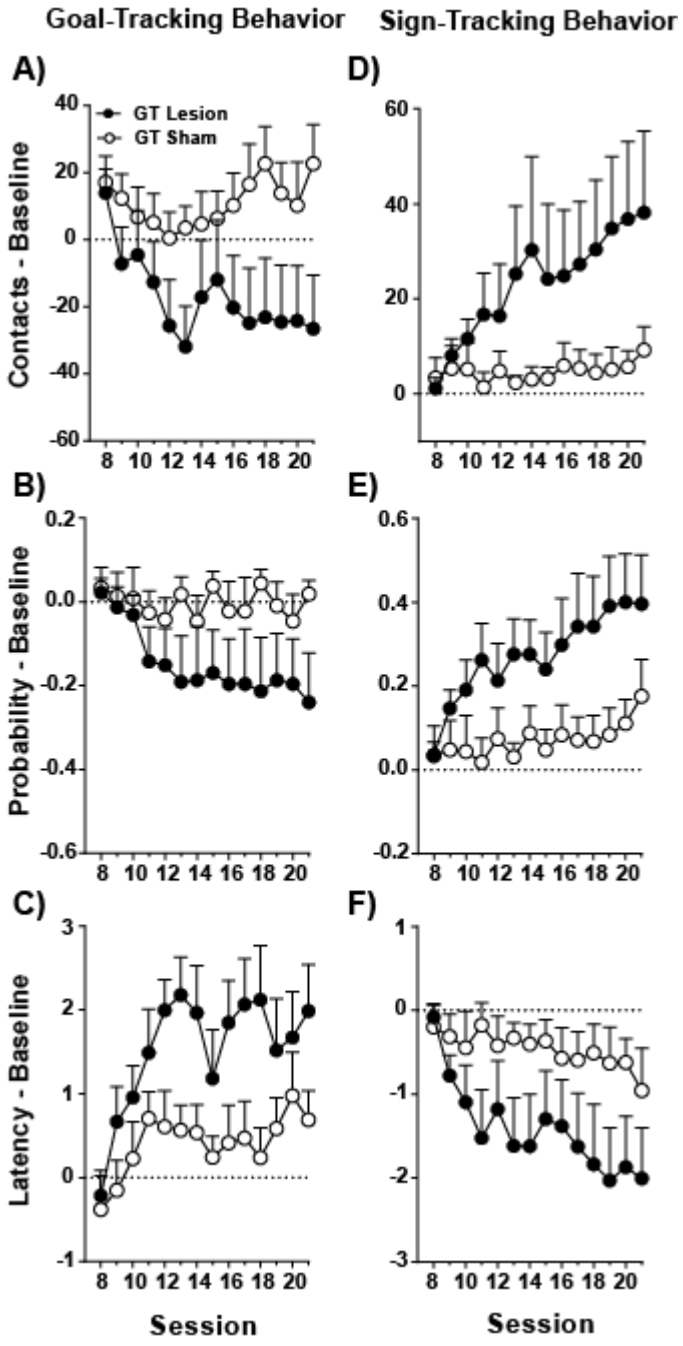


Figure 2.5. GTs learn to sign-track across sessions following PVT lesions. Mean + SEM of the subtraction of pre-lesion baseline behavior (average of sessions 5-7) from post-lesion data on sessions 8-21 for A) number of food cup contacts, B) probability of food cup contact, C) latency to food cup contact, D) number of lever contacts, E) probability of lever contact, and F) latency to lever contact (GT Lesion, n=9, GT Sham, n=12).

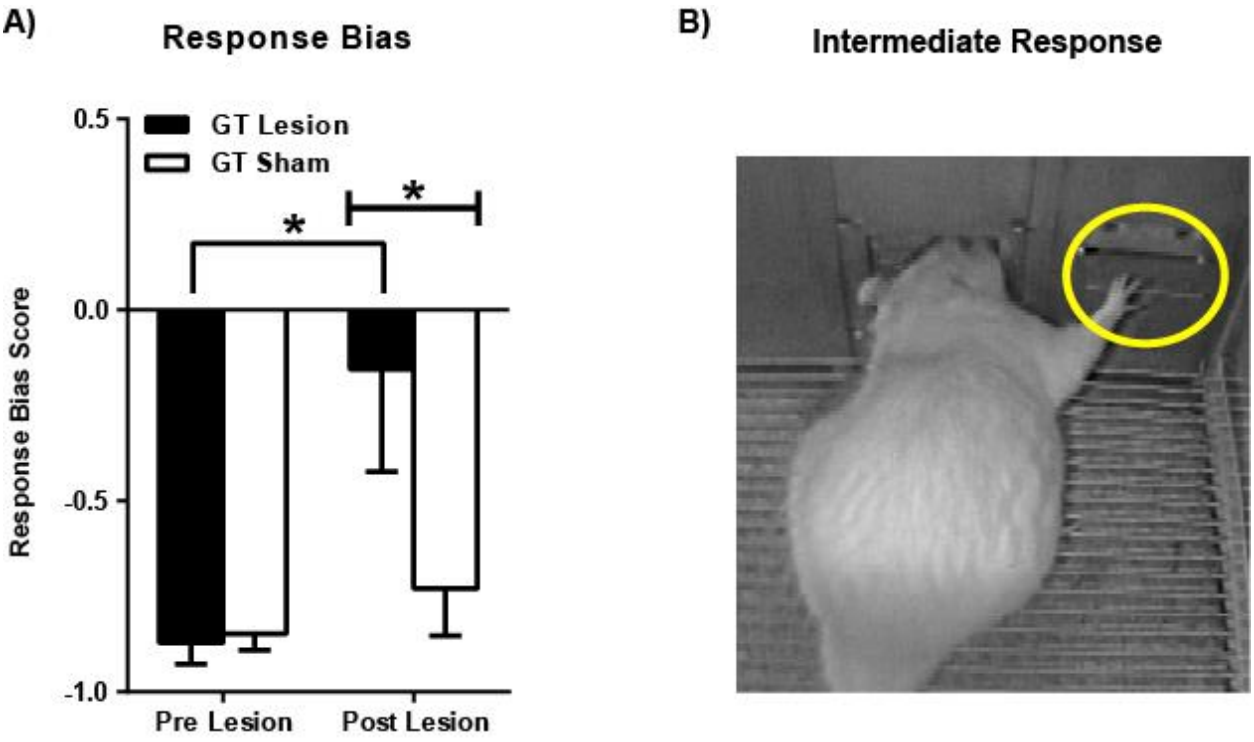


Figure 2.6. PVT lesions increase the tendency to sign-track in GTs. Mean + SEM of A) pre-lesion baseline behavior (average of sessions 5-7) and post-lesion behavior (average of sessions 19-21) for response bias score [(lever contacts - food cup contacts) ÷ (total contacts)]. The GT Lesion group (n=9) showed a significant increase in response bias score following PVT lesion ($P < 0.001$). The GT Lesion group also differed significantly from the sham control group (n=12) post-lesion ($P = 0.05$). B) A video image showing a rat performing an "intermediate" response, contacting both the lever-CS and the food cup simultaneously.

Appendix A

Supporting Information

Methods

Assessing the neuroanatomical specificity of the lesions

To further investigate the effects of damage to surrounding thalamic nuclei and neighboring brain structures, we analyzed data from GTs that were excluded from Experiment 2. GTs with missed lesions were classified into three groups: lesions that resulted in no PVT damage (PVT Miss; n=3), incomplete PVT lesions (PVT -, n=4), and lesions that encompassed the entire PVT, but also extensively damaged surrounding thalamic nuclei (PVT +, n=4).

Locomotor Response to Novelty

Following the completion of Pavlovian conditioned approach training in Experiment 2, a subset of outbred rats (GT Lesion, n=4; GT Sham, n=8; ST Lesion, n=5; ST Sham, n=8) were tested for locomotor response to a novel environment, as described previously (Stead *et al.*, 2006). Briefly, rats were taken into a novel room and placed individually into a standard acrylic cage (43 x 21.5 x 24.5 cm) with a novel floor. Locomotor activity was recorded by two rows of photocells to record both horizontal movement and rearing behavior. Photocell beam breaks resulted in activity counts that were recorded in 5 minute bins for 1 hour. Total locomotion scores were created by summing horizontal and rearing activity.

Results

Assessing the effects of PVT lesion on both sign- and goal-tracking behaviors in bLR and bHR animals

Prior to separating the bLR and bHR animals by Phenotype for analysis, linear mixed model analysis was performed for each sign- and goal-tracking measure (number of contacts, probability of contact, and latency to contact) with both Phenotype (bHR vs. bLR) and Treatment (lesion vs. sham) included as the between-subject factors, and Session as the repeated variable. The results from these analyses are summarized in Supporting Table 1. Importantly, for all sign- and goal-tracking measures a significant Phenotype x Session and/or Phenotype x Session x Treatment interaction was found, justifying the separation of the bLR and bHR animals for additional analyses.

Assessing the effects of PVT lesions on "off target" behavior in bHR and bLR animals

Linear mixed model analyses were also performed to ensure there were no changes in "off-target" behavior, i.e. sign-tracking behavior for bLRs and goal-tracking behavior for bHRs. As shown in Supporting Figure 1A-C, there was no effect of PVT lesion on goal-tracking behavior for bHRs. Specifically, for magazine contacts during the CS period, there was no effect of Treatment ($F_{(1,19)} = 0.000$, $P = 0.986$) or Treatment x Session interaction ($F_{(11,19)} = 0.90$, $P = 0.559$). Similarly, for probability of magazine contact there was no effect of Treatment ($F_{(1,19)} = 0.04$, $P = 0.850$) and no Treatment x Session interaction ($F_{(11,54)} = 0.39$, $P = 0.955$), and the same was true for latency to magazine contact (Effect of Treatment, $F_{(1,20)} = 0.01$, $P = 0.937$; Treatment x Session interaction, $F_{(11,46)} = 0.93$, $P = 0.525$). Thus, PVT lesions did not affect goal-tracking behavior in bHR rats with a predisposition to sign-track.

For bLRs, there was no effect of PVT lesions on sign-tracking behavior (Supporting Figure 1D-F). Specifically, for lever contacts there was no effect of Treatment ($F_{(1,19)} = 19.45$, $P = 0.659$) or Treatment x Session interaction ($F_{(11,109)} = 1.15$, $P = 0.328$). Likewise, no effects were seen for probability of lever contact (Effect of Treatment, $F_{(1,9)} = 0.23$, $P = 0.639$; Treatment x Session interaction, $F_{(11,9)} = 1.43$, $P = 0.301$) and latency to lever contact (Effect of Treatment, $F_{(1,20)} = 0.05$, $P = 0.826$; Treatment x Session interaction, $F_{(11,220)} = 1.27$, $P = 0.245$). These results indicate that PVT lesion did not cause a change in sign-tracking behavior in bLRs.

The extent of PVT damage is related to the shift from goal- to sign-tracking behavior

To assess whether the lesion effects that we saw were specifically due to the extent of PVT damage, we analyzed data from GT rats that were excluded from Experiment 2 (Supporting Figure 4). There were no significant differences revealed when a one-way ANOVA was conducted to compare post-lesion response bias score across the groups that were categorized based on the extent of the lesion. Given the small sample size and variance within the missed lesion groups, this is not surprising. Nonetheless, we believe the apparent trends evident in this dataset are meaningful. GTs in the PVT Miss group, who primarily had unilateral damage to the habenula or dorsal hippocampal damage, show a post-lesion response bias score identical to that of GT Sham controls at the conclusion of the experiment. Likewise, GTs in the PVT + group show a response bias score similar to GT Lesion rats that were included in the study. Further, GTs in the PVT - group show a response bias score between GT Sham and GT Lesion groups.

Thus, it appears, at least for GTs, that the size and extent of a PVT lesion corresponds with the change in response bias. Despite the lack of statistical significance, these data suggest that it is the PVT itself, and not the surrounding nuclei, that is important for sign- and goal-tracking behaviors.

The effects of PVT lesions on Locomotor Response to Novelty

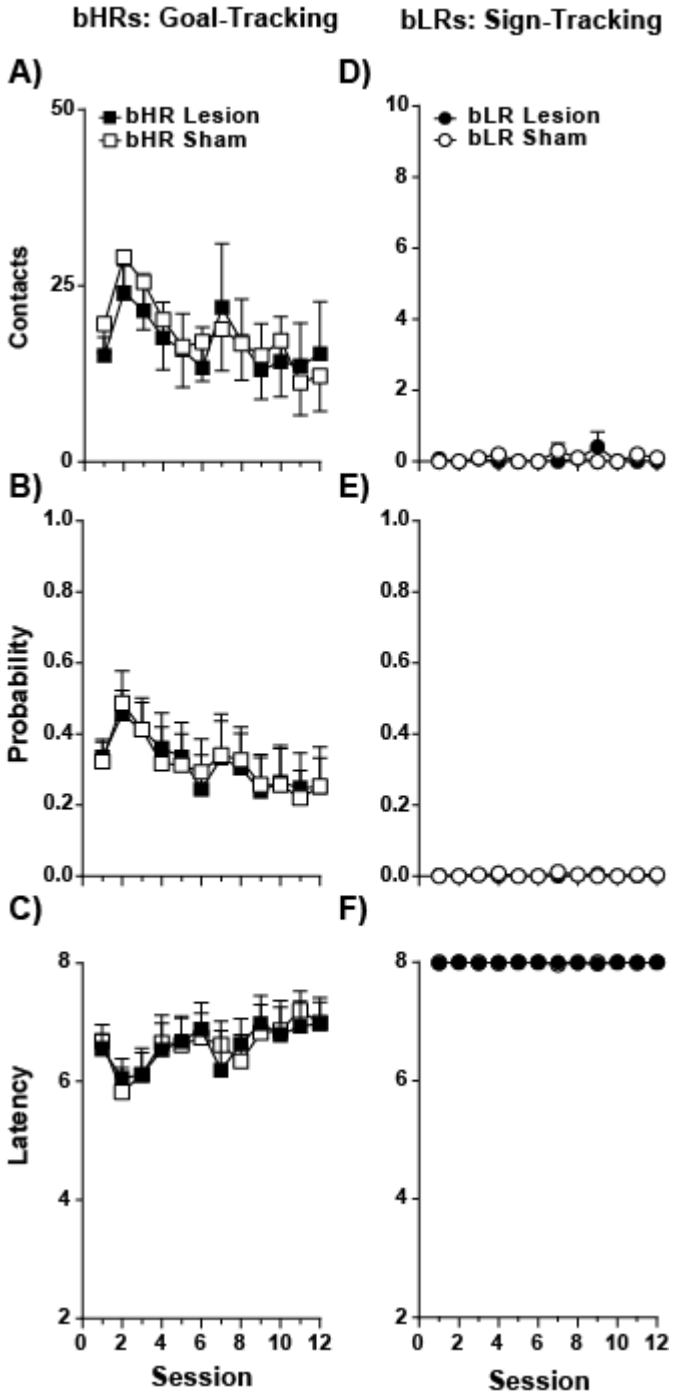
Linear mixed-effects models show no significant effect of Treatment, and no significant Treatment x Phenotype or Treatment x Phenotype x Interval interactions for locomotor response to novelty (Supporting Figure 5). These results indicate that PVT lesions did not affect novelty-induced locomotor behavior.

Supporting Table 2.1 – Statistical Results for Experiment 1

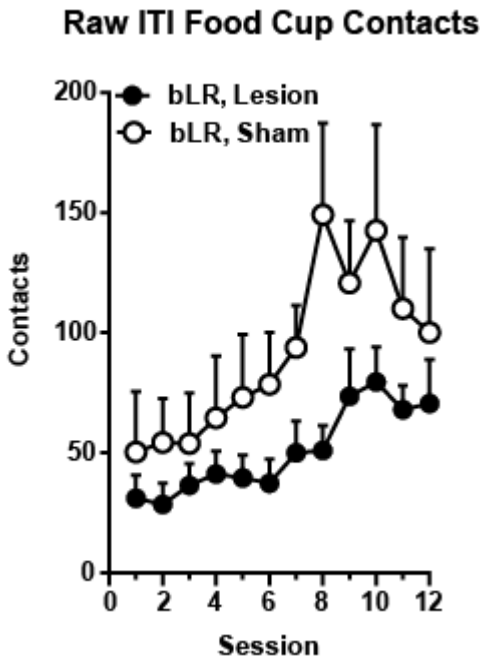
Measure	**Session**	**Treatment**	**Phenotype**	**Session x Treatment**	**Session x Phenotype**	**Treatment x Phenotype**	**Session x Treatment x Phenotype**
Lever Contacts	<0.001*	0.862	<0.001*	<0.001*	<0.001*	0.859	<0.001*
Lever Probability	<0.001*	0.353	<0.001*	0.002*	<0.001*	0.340	0.003*
Lever Latency	<0.001*	0.654	<0.001*	0.001*	<0.001*	0.652	0.001*
Magazine Contacts	0.026*	0.180	0.120	0.315	<0.001*	0.189	0.431
Magazine Probability	0.011*	0.320	0.024*	0.478	0.001*	0.187	0.847
Magazine Latency	0.005*	0.181	0.168	0.583	0.001*	0.144	0.757

* = statistically significant ($p < 0.05$)

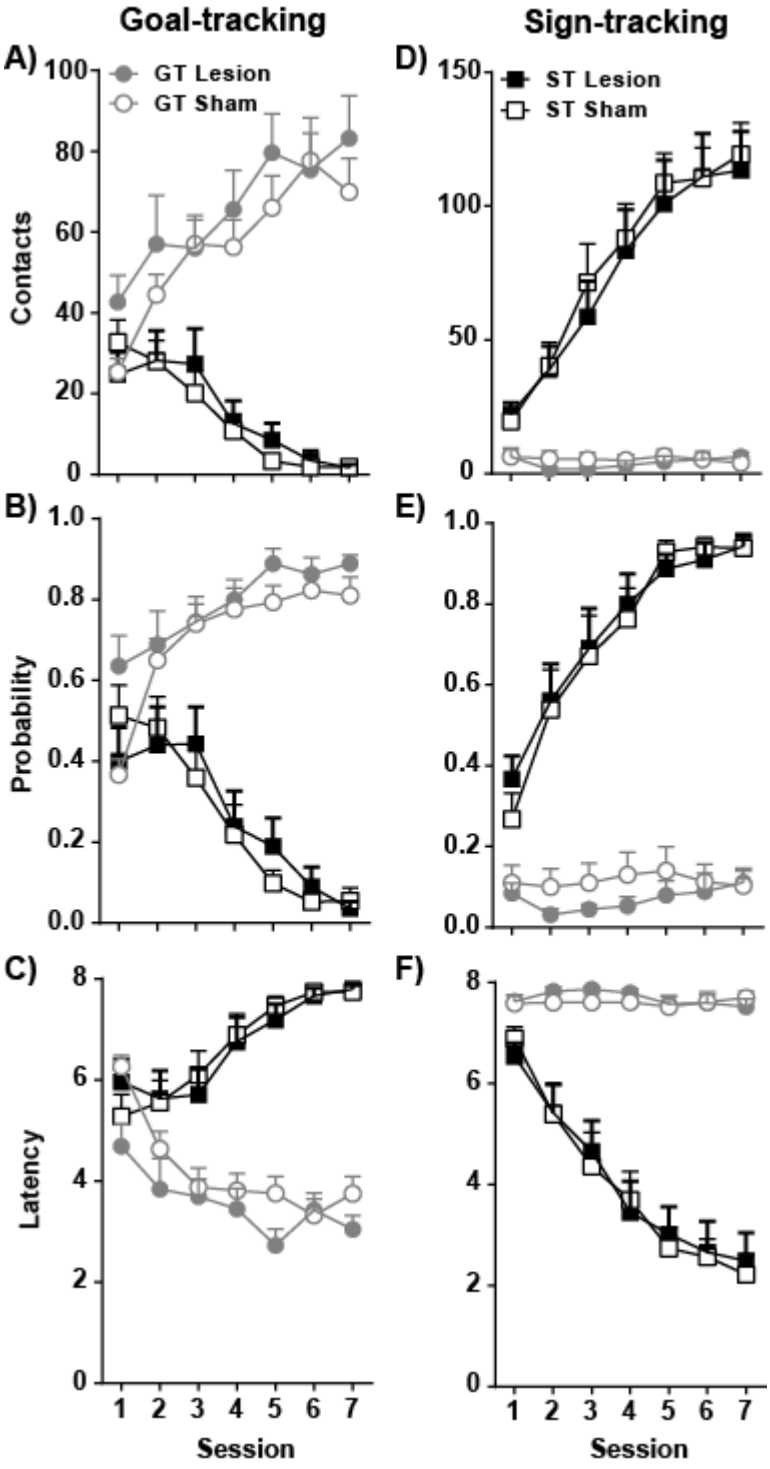
Appendix B



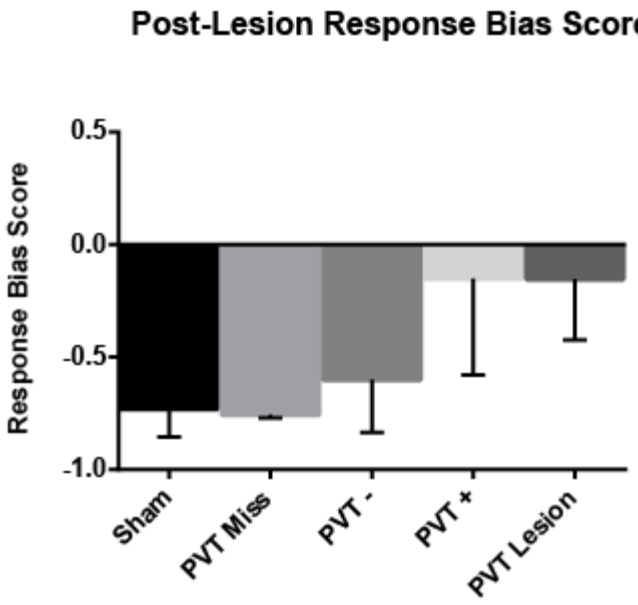
Supporting Figure S2.1. Effects of PVT lesion on the acquisition of "off target" behaviors in bHR and bLR animals. Mean + SEM for A) number of food cup contacts, B) probability of food cup contact, and C) latency to food cup contact for bHR animals (Lesion, n=9; Sham, n=12). For bLR animals (Lesion, n=12; Sham, n=10), mean + SEM for D) number of lever contacts, E) probability of lever contact, and F) latency to lever contact.



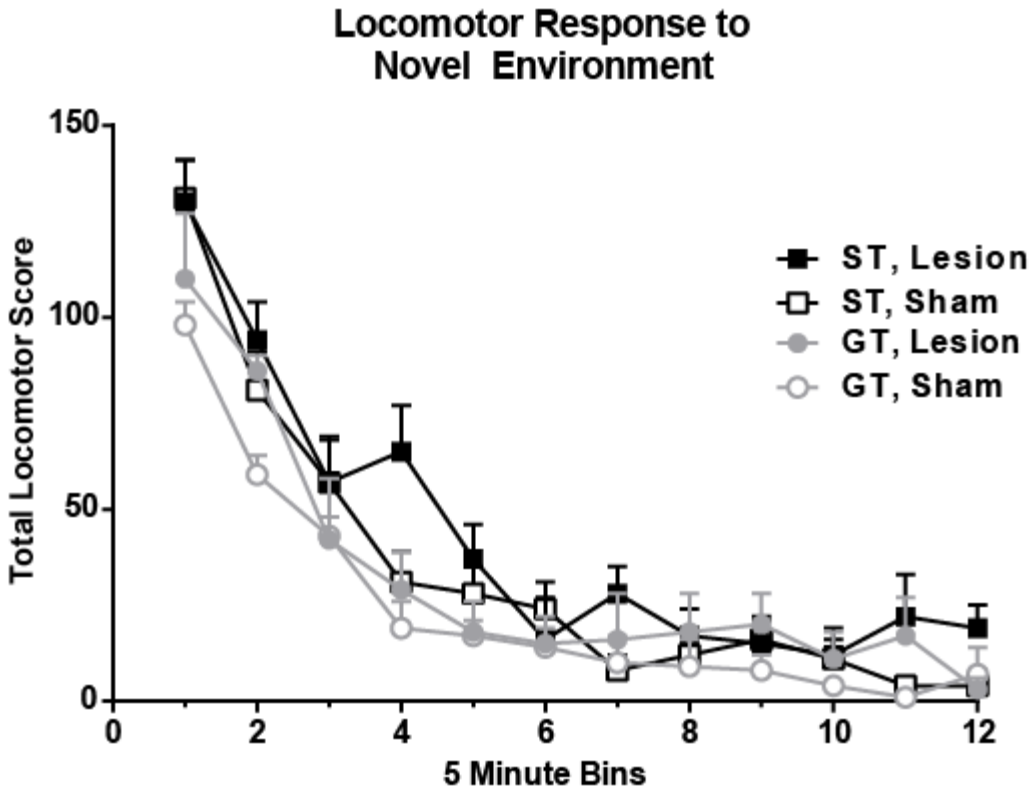
Supporting Figure S2.2. Activity during the inter-trial interval for bLR rats. Mean + SEM for the number of food cup contacts during the inter-trial interval. bLR Sham (n=10) and bLR Lesion (n=12) groups significantly differed on the number of food cup entries during the inter-trial interval only on session 8 (P = 0.01).



Supporting Figure S2.3. The acquisition of sign- and goal-tracking conditioned responses across 7 PCA training sessions. Mean + SEM for A) number of food cup contacts, B) probability of food cup contact, C) latency to food cup contact, D) number of lever contacts, E) probability of lever contact, and F) latency to lever contact. (ST Lesion n = 11, ST Sham n =13, GT Lesion n =9, GT Sham n =12)



Supporting Figure S2.4. The extent of PVT lesion underlies change in response bias score. Mean + SEM of post-lesion response bias score (average of sessions 19-21) for GTs from Experiment 2 that were included in the study (Sham, n=12; PVT Lesion, n=9) or did not meet inclusion criteria (PVT Miss, n=3; PVT -, n=4; PVT +, n=4).



Supporting Figure S2.5. PVT lesions do not affect locomotor response to a novel environment. The line graph represents the mean + SEM for total locomotor score (lateral + rearing activity) across 5 minute time bins (GT Lesion, n=4; GT Sham, n=8; ST Lesion, n=5; ST Sham, n=8).

Chapter 3

A food predictive cue engages subcortical afferents and efferents of the paraventricular nucleus of the thalamus if it is attributed with incentive salience

Note: At the time of submission, the following text, as well as the figures, were under review for publication in the journal Neuroscience.

Introduction

The paraventricular nucleus of the thalamus (PVT) has gained increasing attention for its involvement in a variety of motivated behaviors (Kirouac, 2015; Vertes *et al.*, 2015), including reward-seeking behaviors (Martin-Fardon & Boutrel, 2012; James & Dayas, 2013; Urstadt & Stanley, 2015). Much of the research surrounding the PVT and reward-seeking behaviors has focused on the role of the PVT in mediating responses to food- or drug-paired cues. For example, repeated Pavlovian pairings of a discrete cue-light with a water reward leads to greater c-Fos induction in the PVT, relative to controls who received unpaired presentations of the cue and reward (Igelstrom *et al.*, 2010). Enhanced levels of c-Fos are also found in the PVT in response to presentation of cocaine-paired cues (Matzeu *et al.*, 2015a), as well as following cue-induced reinstatement of ethanol- and cocaine-seeking behavior (Wedzony *et al.*, 2003; Dayas *et al.*, 2008; James *et al.*, 2011). In addition, transient inactivation of the PVT attenuates cue-induced reinstatement of cocaine seeking behavior (Matzeu *et al.*, 2015b) and the expression of cocaine conditioned place preference (Browning *et al.*, 2014). While these findings demonstrate that the PVT is involved in mediating cue-motivated behaviors, its specific role in these processes is less well known.

Identifying the neural mechanisms underlying cue-motivated behaviors has been complicated by the fact that Pavlovian-conditioned cues can act as both predictive and incentive stimuli (Robinson & Berridge, 1993). Initially, it was thought that if a cue was predictive of reward delivery (i.e. a predictive stimulus) it was also imbued with incentive properties, capable of eliciting complex motivational states (de Wit & Stewart, 1981; Childress *et al.*, 1993). Upon

further study, however, it was discovered that individuals differ in the extent to which they attribute incentive motivational value or incentive salience to reward-predictive stimuli (Flagel *et al.*, 2009; Robinson & Flagel, 2009). To study this phenomenon, we use a Pavlovian conditioned approach (PCA) procedure that allows us to capture individual variation in the propensity to attribute incentive salience to reward cues, and to thereby explore the underlying neural mechanisms. In this model, where presentation of a discrete lever-cue (conditioned stimulus, CS) is followed by presentation of a food reward (unconditioned stimulus, US), some rats develop a sign-tracking conditioned response (CR). These rats, referred to as "sign-trackers (STs)" (Hearst & Jenkins, 1974), approach and engage the lever-CS upon presentation, and will work for presentation of the lever-CS, even in the absence of a food reward (Robinson & Flagel, 2009). Other rats develop a goal-tracking CR, and these rats, referred to as "goal-trackers (GTs)" (Boakes, 1977), rapidly approach the location of food delivery upon lever-CS presentation, and are less motivated than STs to work for lever presentation in the absence of food reward. The remaining rats develop a mixed CR, vacillating between engagement with the lever-CS and the location of food delivery. Thus, for all individuals, the lever-CS serves as a predictive stimulus, since it elicits a CR, but only for STs does the lever-CS also become an incentive stimulus (Robinson & Flagel, 2009).

Using the sign-tracker/goal-tracker animal model, it has been shown that cortico-thalamic-striatal circuitry is engaged only when a reward cue is attributed with incentive value—that is, to a greater extent in sign-trackers than goal-trackers (Flagel *et al.*, 2011a; Yager *et al.*, 2015). The PVT seems to represent a central node of this differential activity, as there are robust phenotypic differences in food- and drug-cue induced c-Fos in this region, and distinct patterns of correlated neural activity involving the PVT. In sign-trackers, food-cue induced c-fos mRNA is correlated between the PVT and the shell of the nucleus accumbens (NAc); whereas in goal-trackers, cue-induced c-fos mRNA is correlated between areas of the prefrontal cortex (PFC), particularly the prelimbic cortext (PrL) and the PVT (Flagel *et al.*, 2011b; Haight & Flagel, 2014). Additional evidence supporting a role for the PVT in mediating the propensity to attribute incentive salience to reward cues comes from a lesion study in which we found that PVT lesions attenuate a goal-tracking CR, while concomitantly increasing a sign-tracking CR (Haight *et al.*, 2015). These findings demonstrate a causal link between the PVT and the attribution of incentive

salience to a reward cue, suggesting that the PVT may act as a "brake" on incentive salience attribution.

To better understand the functional role of the PVT in mediating the propensity to attribute incentive salience to reward cues, it is crucial to examine the afferent and efferent circuitry of this nucleus. The PVT is situated on the dorsal midline of the thalamus in the rat, directly underneath the 3rd ventricle, and has numerous connections with cortical, limbic and motor areas. Specifically, the PVT receives dense cortical input from the entire anterior-posterior gradient of the PrL, as well as the infralimbic (IL) and cingulate cortices (Vertes, 2004; Li & Kirouac, 2012). Subcortical afferents are widely distributed, and arise from the hypothalamus, ventral subiculum (vSub), and the central and medial amygdala, among other areas (Chen & Su, 1990; Van der Werf *et al.*, 2002; Kirouac *et al.*, 2005; 2006; Vogt *et al.*, 2008; Hsu & Price, 2009; Li & Kirouac, 2012; Li *et al.*, 2014). In addition to its diverse inputs, the PVT sends efferent fibers to a variety of cortical and subcortical structures, including the PrL and IL, NAc core and shell, parts of the bed nucleus of the stria terminalis, and the central and basolateral amygdala, among other areas (Jones *et al.*, 1989; Berendse & Groenewegen, 1990; Su & Bentivoglio, 1990; Moga *et al.*, 1995; Van der Werf *et al.*, 2002; Pinto *et al.*, 2003; Parsons *et al.*, 2006; Parsons *et al.*, 2007; Li & Kirouac, 2008; Vertes & Hoover, 2008). Importantly, many of the sources of afferents, as well as the efferent targets, of the PVT have been implicated in cue-motivated behaviors, including the PrL and IL (Willcocks & McNally, 2013; Moorman & Aston-Jones, 2015), hypothalamus (Petrovich *et al.*, 2012; Cole *et al.*, 2015), amygdala (Parkinson *et al.*, 2000; Mahler & Berridge, 2009), ventral subiculum (Sun & Rebec, 2003; Kufahl *et al.*, 2009), and NAc (Cardinal *et al.*, 2002; Bossert *et al.*, 2007).

The neuroanatomical location of the PVT allows it to integrate cortical and subcortical inputs and send this information to the NAc to control motivated behavior (Kelley *et al.*, 2005b). We postulate that the PVT acts as a central node to modulate the attribution of incentive salience to reward cues, with STs being more susceptible to subcortical motivational processes and GTs being biased towards greater cortical control of behavior (Haight & Flagel, 2014). Specifically, given that GTs perform better than STs on behavioral tests dependent on cortical processes, including sustained attention (Paolone *et al.*, 2013) and impulsive action (Flagel *et al.*, 2010; Lovic *et al.*, 2011), we hypothesize that these rats will show greater activation of PrL afferents to the PVT, representing greater top-down control of behavior. In contrast we hypothesize that STs

will show greater activation of subcortical inputs to the PVT, including those from the hypothalamus, amygdala, and ventral subiculum. In addition, we expect greater activation of PVT efferents to the NAc in STs, as the sign-tracking, but not the goal-tracking, response has been shown to be dependent on dopamine transmission in the nucleus accumbens (Flagel *et al.*, 2011b; Saunders & Robinson, 2012), which can be influenced by projections from the PVT (Parsons *et al.*, 2006). These hypotheses were examined by assessing specific PVT afferent and efferent circuits that are engaged in response to presentation of a predictive (i.e. for STs and GTs) or incentive (i.e. for STs only) stimulus associated with a food reward.

Materials and methods

All experiments were conducted in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals: Eighth Edition, revised in 2011. In addition, all procedures were approved by the University of Michigan Institutional Animal Care and Use Committee.

PVT afferent labeling

Subjects

Forty male Sprague-Dawley rats were obtained from Envigo (formerly Harlan, Haslett, MI) at approximately 8 weeks of age (~230-300g body weight). Upon arrival, rats were pair housed in standard acrylic cages (46 x 24 x 22 cm) in a climate-controlled room and allowed to acclimate to the new environment for 10 days prior to any experimental manipulations. Throughout the experiment, rats were maintained on a 12-hour light:dark cycle (lights on at 07:00 hours), and food and water were available *ad libitum*. All behavioral training was conducted in the light cycle between 12:00 and 17:00 hours.

Surgical procedures

Following the 10 day acclimation period, all rats underwent stereotaxic surgery in order to infuse the retrograde tracer fluorogold (FG; Fluorochrome, Denver, CO, USA) into the PVT. All surgery was performed under aseptic conditions. First, a surgical plane of anesthesia was induced with inhalation of 5% isoflurane. Once rats were fitted into the stereotaxic frame, anesthesia was maintained throughout the procedure with inhalation of 1-2% isoflurane. The

scalp was shaved and sterilized with alternating swabs of 70% alcohol and Betadine solution (Betadine, Stamford, CT, USA). A small incision was made in the scalp to expose bregma and lambda coordinates, and the skull was leveled within +/- 0.1 mm. Small burr holes were then drilled above the PVT, and a 0.5 µl Hamilton Neuros syringe (Hamilton Company, Reno, NV, USA) mounted 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, which was the smallest volume that could be reliably injected. Some have argued that FG can be taken up by damaged axons of passage, leading to 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, minimizing damage to the injection site and limiting 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 (stereotaxic arm angled at 10^{o} towards the midline). Each injection lasted approximately 2 minutes, and the syringe was left in place for 5 minutes following the injection to minimize diffusion of FG solution up the injection track. The syringe was then slowly retracted, and the scalp was closed with wound clips. Immediately prior to surgery, and 24 hours after, rats 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 from surgery for 8-9 days prior to any handling or behavioral testing.

PVT efferent labeling

Subjects

Sixty male Sprague-Dawley rats were obtained from Envigo (formerly Harlan, Haslett, MI) at approximately 8 weeks of age (~225-275g body weight). Upon arrival, rats were pair housed in conditions identical to those above, and were allowed to acclimate to the new environment for one week prior to any experimental manipulations. Throughout the experiment, rats were maintained on a 12-hour light:dark cycle (lights on at 06:00 hours), and food and water were available *ad libitum*. All behavioral training was conducted in the light cycle between 12:00 and 17:00 hours.

Surgical procedures

Surgical procedures were identical to those described for the PVT afferent experiment, with the exception of the location of FG injection. Bilateral 50 nl injections were made into the border of the NAc core/shell (coordinates from bregma: AP 1.7, ML +/- 1.0, DV -7.2; stereotaxic arm perpendicular to the skull surface).

Behavioral testing

Pavlovian conditioned approach training

Following recovery from surgery, animals underwent PCA procedures similar to those previously described (Flagel *et al.*, 2011a; Haight *et al.*, 2015; Fraser *et al.*, 2016). Standard behavioral test chambers were used (MED Associates, St. Albans, VT, USA). All chambers were housed in sound-attenuating boxes that were equipped with ventilation fans that provided a constant flow of air, as well as background noise. All behavioral data was collected using MED PC software (Med Associates. St. Albans, VT, USA).

Each chamber was equipped with a food cup connected to a pellet dispenser located in the middle of one wall. Operation of the pellet dispenser resulted in the delivery of one 45-milligram banana-flavored grain pellet (Bio-Serve, Flemington, NJ, USA). Each food cup was equipped with an infra-red photo beam, and breaks of the beam were 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. All levers were set so that 10 grams of force would cause a deflection of the lever and register as a lever contact. A white house light was situated on the upper middle portion of the wall directly across from the food cup and lever, and was illuminated for the duration of each of the Pavlovian conditioning sessions.

For two days prior to 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 delivered in the home cage, to familiarize the rats with the experimenters and the novel food. Following these two days, all rats underwent one pretraining session in the test chambers. Prior to the start of the session, each food cup was primed with 3 banana-flavored pellets, to direct the rats' attention to the location of reward delivery. At the beginning of the session, the house light remained off for 5 minutes, to allow the rats to adjust to the training chamber. Following 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 0-60s). The lever remained retracted for the entirety of the session, which lasted an average of 12.5 minutes. After pretraining, rats went through 5 sessions of Pavlovian conditioned approach training, one session per day. Each session consisted of 25 trials 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 seconds). In addition to the rats receiving PCA training, a small subset of rats from each experimental group (Afferent Experiment n=6, Efferent Experiment n=8) were used as an unpaired control group. These rats received the same number of CS and US presentations, but in an unpaired fashion. Each session (PCA and Unpaired Control) lasted approximately 40 minutes. The following data was recorded per trial during each session, in order to quantify Pavlovian conditioned approach behaviors: (1) the number of food cup entries during the 8 second 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.

Following session 5 of Pavlovian training, rats (in the "paired" group) were classified as STs, GTs, or intermediate responders (INs) based on their average PCA Index scores (Meyer *et al.*, 2012) from sessions 4 and 5. The PCA Index is a compound score that is used to measure whether an individual's behavior is directed towards the lever-CS or food cup (location of US delivery) using 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 PCA Index score, which ranges from -1.0 to 1.0, with -1.0 representing an individual whose behavior is directed solely at the food cup, and 1.0 representing an individual whose behavior is directed solely at the lever-CS.

Context habituation and re-exposure to the CS

Following Pavlovian conditioned approach 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

(UNs) were placed into the reconfigured test chambers on three consecutive days. Following an initial 5-minute acclimation period, the house light was illuminated and the animals remained in the chambers for another 30 minutes, with the lever retracted. On the fourth day (i.e. test day), rats were placed into the chambers, and, following the 5 minute acclimation period, the house light was illuminated, and the illuminated lever-CS was inserted into the cage for 2 seconds, once a minute, over a period of ten minutes, for a total of 10 lever-CS presentations. Importantly, these presentations were not paired with pellet delivery. 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 minutes. 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 0.1M sodium phosphate buffer; Fisher Scientific, Hampton, NH, USA).

Tissue processing and quantification

Tissue preparation

Following perfusion, brains were extracted and post-fixed overnight in 4% formaldehyde at 4^{o}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^{o}C. Following cryoprotection, brains were sectioned at 40 µm on a frozen cryostat (Leica Biosystems Inc, Buffalo Grove, IL, USA). Starting with the anterior PrL 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. Towards the hindbrain, where the vSub is located, sections were collected in 48-well plates, one section per well. All sections were stored in 0.1M NaPB at 4^{o}C.

Immunohistochemistry

One series from each brain, as well as the appropriate vSub section, was processed for detection of FG and c-Fos via free-floating immunohistochemistry. All immunohistochemical processing took place at room temperature with gentle agitation. Free floating sections were

washed 3-5 times (5 min each wash) in 0.1M phosphate-buffered saline (PBS) in between incubations. Sections were first incubated in 1% hydrogen peroxide (H_2O_2; diluted in 0.1M PBS) for 10 minutes. Sections were then incubated in a blocking solution containing 2.5% normal donkey serum (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) and 0.4% Triton X-100 (Acros Organics, Geel, Belgium), diluted in 0.1M PBS, for 1 hour. Following incubation with the blocking solution sections were incubated overnight in primary antibody solution containing 1:500 goat anti-c-Fos antibody (lot H2214, Santa Cruz Biotechnology, Inc., Dallas, TX, USA), 1% normal donkey serum, and 0.4% Triton X-100. The next day, sections were incubated in secondary antibody solution containing 1:500 donkey anti-goat antibody (lots 118762 and 119956, Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA), 1% normal donkey serum, and 0.4% Triton X-100 in 0.1M PBS, for 1 hour. Sections were then 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, Burlingame, CA, USA). This stain was then visualized by incubating the tissue in a solution containing 0.02% 3,3'-diaminobenzidine tetrahydrochloride, 0.08% nickel sulfate hexahydrate, and 0.012% H_2O_2, diluted in 0.1M NaPB, for 10 minutes. This caused a dark black precipitate to form at the location of c-Fos detection. Following development for c-Fos staining, the tissue was processed for FG staining. Tissue was again incubated in 1% H_2O_2 for 10 minutes, and then incubated overnight in rabbit anti-FG primary antibody (1:50,000; 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) with 1% normal donkey serum and 0.4% Triton X-100 in 0.1M PBS. On the third day, sections were incubated in secondary antibody solution containing 1:500 donkey anti-rabbit antibody (lots 119063 and 124459, Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA), 1% normal donkey serum, and 0.4% Triton X-100 in 0.1M PBS, for 1 hour. Sections were then 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, Burlingame, CA, USA). This stain was then visualized by incubating the tissue in a solution containing 0.02% 3,3'-diaminobenzidine tetrahydrochloride and 0.012% H_2O_2, diluted in 0.1M NaPB, for 10 minutes. This caused a brown precipitate to form at the location of FG detection. The sections were then mounted onto SuperFrost Plus microscope slides (Fisher Scientific, Hampton, NH,

USA), dehydrated in graduated ethanol solutions followed by xylenes, and coverslipped with Permount medium (Fisher Scientific, Hampton, NH, USA).

FG/c-Fos quantification

All immunohistochemical analysis was conducted by an experimenter blind to experimental conditions using the software program Stereo Investigator (MBF Bioscience, Williston, VT, USA) which was connected to a Zeiss Axiophot microscope (Zeiss, Oberkochen, Germany) and an Optronics Microfire digital camera (Optronics International, Tulsa, OK, USA). For each section, a contour was traced around the area of interest at 2.5x magnification using recognizable landmarks from the Rat Brain Atlas (Paxinos & Watson, 2007). Three measurements were then collected at either 10x or 20x magnification: the total number of FG positive cells (distinguishable by a brown cytosolic stain), the total number of c-Fos positive cells (distinguishable by a dark black nuclear stain), and the total number of FG/c-Fos double-labeled cells (distinguishable by a black nuclear stain surrounded by a brown cell body; see Figure 3.2C for representative image). Target areas were identified for analysis using the patterns of retrograde labeling observed here, as well as published anterograde and retrograde tracing reports by others (Chen & Su, 1990; Van der Werf *et al.*, 2002; Kirouac *et al.*, 2005; Parsons *et al.*, 2006; Li & Kirouac, 2008; Hsu & Price, 2009; Li & Kirouac, 2012; Li *et al.*, 2014). For the Afferent Experiment, areas of quantification included layer 6 of the anterior PrL (approximate bregma level AP +4.2), layer 6 of the posterior PrL and IL (approximate bregma level AP +3.0), the central amygdala and medial amygdaloid complex (approximate bregma level AP -2.6), the hypothalamus (A13 cell group, dorsomedial nucleus, ventromedial nucleus and lateral hypothalamus, which included the perifornical area; approximate bregma level AP -2.8) and ventral subiculum (approximate bregma level AP -5.6). For the Afferent Experiment, one section was chosen at the appropriate bregma level for each area of interest, and both hemispheres were quantified individually and then summed for one total count for each measure per section. For the Efferent Experiment, three PVT sections were identified and analyzed separately for quantification for each subject; one from the anterior PVT (approximate bregma AP -1.6), one from the mid PVT (approximate bregma AP -2.8), and one from the posterior PVT (approximate bregma AP -3.6)

Statistical analyses

To analyze differences on each measure of PCA behavior, linear mixed-effects models were used, with Session as the repeated variable and Group as the between subjects variable. To analyze differences in overall levels of activity in a given region, a one-way ANOVA was used with the total number of c-Fos positive cells as the dependent variable, and Group (UN, GT, or ST) as the independent variable. To assess differences in the activity of specific populations of PVT efferents/afferents, a binary logistic regression model analysis was used, with the dependent variable consisting of (the total number of FG and c-Fos double labeled cells) ÷ (the total number of FG positive cells) while Group was used as the independent variable. Modeling the dependent variable in this fashion controls for the subtle variability within the total population of FG-labeled cells across individuals, allowing for a more robust assessment of activity within each population of PVT efferents/afferents. When significant main effects were observed, Bonferroni post-hoc comparisons were performed. For all analyses, significance was set at $p \leq 0.05$.

Results

Afferent experiment

Pavlovian conditioned approach behavior

Similar to previous reports (Flagel *et al.*, 2009; Meyer *et al.*, 2012; Haight *et al.*, 2015), considerable variation was seen in the CRs acquired by individual rats following 5 sessions of PCA training (Figure 3.1). Some rats directed their behavior towards the lever-CS, and were classified as STs (n = 15; PCA Index range +0.3 to +0.93; Figure 3.1A-C), while others directed their behavior towards the location of US delivery upon lever-CS presentation, and were classified as GTs (n = 12; PCA Index range -0.48 to -0.9; Figure 3.1D-F). In addition, some rats showed an intermediate response, and were excluded from the study (n = 7; PCA index range 0.29 to -0.29; data not shown). Last, control rats (UNs; n = 6) receiving CS-US presentations in an unpaired fashion did not acquire a sign- or goal-tracking CR (Figure 3.1) Note that additional animals were excluded due to missed FG injections (*see below*), and their data is not included in Figure 3.1.

Linear mixed-effects models showed significant overall effects of Session and Group, as well as a significant Session x Group interaction, on all measures of sign-tracking behavior (all $p < 0.001$; Figure 3.1A-C). For all three measures (contacts, probability, and latency), Bonferroni

post-hoc analyses showed a significant within-group effect of Session for STs ($p < 0.001$), but not for GTs or UNs ($p > 0.05$). STs began to demonstrate lever directed behavior as early as session 2, while GTs and UNs did not develop lever-directed behavior across sessions. For goal-tracking behavior, there was a significant effect of Session, as well as a significant Session x Group interaction, for all three measures (contacts, probability, latency; $p \leq 0.002$; Figure 3.1D-F). In addition, there was a significant effect of Group for food cup contacts ($p = 0.018$; Figure 3.1D) and Bonferroni post-hoc analyses revealed a significant within-group effect of Session for GTs and STs ($p < 0.001$), but not for rats in the UN group ($p > 0.05$). That is, GTs increased their number of food cup contacts during the 8-second CS period across training sessions, while STs decreased responding directed at the food cup, and the behavior of rats in the UN group did not significantly change across sessions. For probability of food cup contact, as well as latency to food cup contact, Bonferroni post-hoc comparisons showed a significant within-group effect of Session for all three groups (all $p \leq 0.032$; Figure 3.1E-F). GTs increased their food-cup-directed behavior across training sessions, while STs and rats in the UN group decreased their food-cup-directed behavior following session 3 and 4, respectively. Taken together, these results indicate that STs developed lever-directed behavior, while GTs developed food-cup-directed behavior, and rats in the UN group developed neither a sign-tracking nor a goal-tracking conditioned response.

Injection screening and retrograde labeling

Following immunohistochemistry, all brains were screened for FG injection and retrograde labeling accuracy. In general, anterior and posterior injections were centered on the midline and targeted directly at the PVT, completely filling the structure (Figure 3.2A-B). Despite the small volume of the injection (50 nl), all subjects had some degree of FG staining in the surrounding nuclei: laterally in portions of the mediodorsal and paratenial nucleus, ventrally in portions of the centromedial nucleus and interanteromedial nuclei (anterior PVT sections) or intermediodorsal and centromedial nuclei (posterior PVT sections), and dorsally in portions of the habenula (Figure 3.2A-B). This is due to the small size of the PVT, making it extremely difficult to isolate. Two subjects ($n = 1$ UN, 1 GT) with accurate PVT injections had FG staining that appeared slightly larger around the anterior injection site, spreading ventrally through the interanteromedial nucleus into the borders of the rhomboid/reuniens nucleus on some sections,

but the retrograde labeling from these subjects did not appear to differ from the other subjects in their respective groups, so they were included in the study. In addition, 7 subjects were excluded from the study for seemingly innacurate FG injections. These subjects either had had FG injections that missed the PVT (*n* = 1 GT, 1 ST); had injections that filled the PVT but were heavily biased towards the left hemisphere (*n* = 1 UN, 1 GT, 2 ST); or had dense dorsomedial hippocampal staining above the PVT (*n* = 1 ST). The final number of subjects included in the study was: 5 UNs, 10 GTs, and 11 STs.

While many of the surrounding thalamic nuclei also receive similar afferent connections as the PVT, our injections entirely filled the PVT, while only partially filing the surrounding nuclei, so the majority of retrogradely labeled cells quantified in the current study are likely PVT afferents. In addition, we have previously shown that lesions of the PVT affected the expression of PCA behavior, while damage to the surrounding area in the absence of PVT damage did not appear to affect PCA performance (Haight *et al.*, 2015), supporting that it is indeed PVT afferents, and not afferents to nearby nuclei, that were activated by stimulus presentation in the current study. Furthermore, the pattern of retrograde labeling observed following FG injection into the PVT was consistent with previously published findings (Chen & Su, 1990; Van der Werf *et al.*, 2002; Kirouac *et al.*, 2005; 2006; Vogt *et al.*, 2008; Hsu & Price, 2009; Li & Kirouac, 2012; Li *et al.*, 2014).

Quantification results

In order to evaluate differences in c-Fos protein expression between STs, GTs, and UNs in response to cue presentation, two different assessments were made. The first was an assessment of the overall amount of cue-induced c-Fos in a given region, quantified as the total number of c-Fos positive nuclei. The second was an assessment of c-Fos specifically in PVT afferents from a given region, which was quantified as (the total number of FG and c-Fos double labeled cells) ÷ (the total number of FG positive cells). Of note, some brain areas could not be quantified for certain subjects, due to damage that occurred during brain extraction and processing (see figure legend for the *n* for each region).

Prelimbic and infralimbic cortex

Following PVT FG injection, a dense number of retrogradely labeled cells was seen in layer 6 of the PrL. This labeling was observed throughout the anterior-posterior axis of the PrL. Therefore, two different regions of the PrL were quantified: one anterior at approximately AP +4.2, and one posterior at approximately AP +3.0. The pattern of group differences was similar in both the anterior and posterior PrL, so the counts from these sections were averaged for each subject to assess PrL c-Fos expression (Figure 3.3A). A one-way ANOVA showed no difference between groups in the amount of cue-induced c-Fos in layer 6 of the PrL (effect of Group; $F_{(2,21)} = 0.05$, $p = 0.95$; Figure 3.3B). In order to assess differences in c-Fos counts specifically in PVT afferent cells (i.e. double-labeled cells), a binary logistic regression model analysis was used. Results show an overall effect of Group (Wald $\chi^2_{(2,n=24)} = 8.340$, $p = 0.02$; Figure 3.3C). Bonferroni post-hoc comparisons show that both GTs ($p = 0.02$) and STs ($p < 0.01$) have a small, yet significantly greater engagement of PVT afferents from the PrL compared to UNs (Figure 3.3C). These results indicate that presentation of a reward-paired stimulus can evoke activity in PVT afferents from the PrL, regardless of its incentive motivational value.

Beneath the posterior PrL, dense retrograde labeling was seen in layer 6 of the IL, so this area was also quantified (Figure 3.3D). For overall c-Fos count, a one-way ANOVA showed no effect of Group within this region ($F_{(2,21)} = 0.10$, $p = 0.91$; Figure 3.3E). For c-Fos counts specifically in PVT afferents from the IL, binary logistic regression model analysis also showed no effect of Group (Wald $\chi^2_{(2,n=24)} = 1.87$, $p = 0.39$; Figure 3.3F), demonstrating that cue presentation did not evoke differential activity in the IL between STs, GTs and UNs.

Amygdala

Next, the amount of cue-induced c-Fos was quantified in the CeA (Figure 3.4A). A one-way ANOVA showed a trend towards an effect of Group in overall c-Fos count ($F_{(2,18)} = 0.11$, $p = 0.07$; Figure 3.4B), with a tendency for STs to have more c-Fos expression than the other groups. Only a few brains had any observable FG/c-Fos double-labeled cells, and a binary logistic regression model analysis revealed no effect of Group (Wald $\chi^2_{(2,n=21)} = 2.64$, $p = 0.28$; Figure 3.4C). These data demonstrate that while an incentive stimulus tends to evoke c-Fos expression in cells in the CeA, this expression is not in cells projecting to the PVT.

Retrograde labeling was seen throughout the MeA (Figure 3.4D), and the pattern of c-Fos expression here was quite different from that in the CeA. A one-way ANOVA showed no effect of Group in cue-induced c-Fos induction in the MeA ($F_{(2,16)} = 1.88$, $p = 0.19$; Figure 3.4E), indicating that cue presentation did not lead to differences in overall activation of this structure. However, when looking specifically at PVT afferents from the MeA, binary logistic regression model analysis shows an overall effect of Group (Wald $\chi^2_{(2,n=19)} = 8.99$, $p = 0.01$; Figure 3.4F). Bonferroni post-hoc comparisons revealed that STs have greater cue-induced c-Fos in MeA afferents to the PVT compared to UNs ($p < 0.01$; Figure 3.4F), indicating that a reward-paired cue must be attributed with incentive value for it to evoke activity in PVT afferent cells from the MeA.

Hypothalamus

A large number of retrogradely labeled cells was observed throughout the hypothalamus, second in number only to the density of retrogradely labeled cells in the PrL. These cells were found in several of the hypothalamic nuclei including the A13 cell group, the dorsomedial nucleus (DMD), the ventromedial nucleus (VMH), and the lateral hypothalamus (LH), which included the perifornical region (PF). In the A13 cell group (Figure 3.5A), there were no differences in overall c-Fos counts (effect of Group; $F_{(2,18)} = 2.39$, $p = 0.12$; Figure 3.5B). In addition, there was no effect of Group c-Fos in specific PVT afferents from the A13 cell group following cue presentation (Wald $\chi^2_{(2,n=21)} = 2.73$, $p = 0.26$; Figure 3.5C), indicating that presentation of a predictive or incentive stimulus does not lead to increased activity in this nucleus.

Similar to the A13 cell group, cue presentation did not lead to differences in overall c-Fos expression in the VMH (Figure 3.5D), as there was not a significant effect of Group ($F_{(2,12)} = 0.02$, $p = 0.99$; Figure 3.5E). In addition, very few FG/c-Fos cells were seen in this area ($n < 3$ per subject), and there was no effect of Group for double-labeled cells (Wald $\chi^2_{(2,n=15)} = 2.04$, $p = 0.36$; Figure 3.5F). These results demonstrate that cue presentation has little effect on activity of PVT afferents from the VMH.

In the DMD (Figure 3.5G), cue presentation again did not lead to differences in overall c-Fos expression (effect of Group; $F_{(2,17)} = 0.65$, $p = 0.54$; Figure 3.5H). However, there was a trend for an effect of Group for c-Fos expression in PVT afferents from the DMD (Wald $\chi^2_{(2,n=20)}$

= 5.75, p = 0.06; Figure 3.5I). Bonferroni post-hoc comparisons show that there was a tendency for STs to have greater c-Fos expression in this circuit compared to UNs (p = 0.08).

Cue presentation did lead to robust c-Fos expression in the LH, which included the PF (Figure 3.5J), specifically in STs. A one-way ANOVA showed an overall effect of Group ($F_{(2,15)}$ = 7.86, p < 0.01; Figure 3.5K). Bonferroni post-hoc comparisons revealed that STs have significantly greater c-Fos counts in the LH compared to GTs (p < 0.01), and a trend towards greater c-Fos counts compared to UNs (p = 0.07). A similar effect was seen in PVT afferents from the LH. Binary logistic regression analysis revealed an overall effect of Group (Wald $\chi^2_{(2,n=18)}$ = 21.64, p < 0.01; Figure 3.5L), with Bonferroni post-hoc comparisons indicating that cue presentation led to greater c-Fos expression in this circuit in STs compared to both UNs (p = 0.01) and GTs (p < 0.01).

Since the LH and DMD afferents to the PVT showed similar patterns of c-Fos expression in PVT afferents, with STs tending to have more expression in this circuit, the total counts from these areas were summed in order to get a general picture of activity in dorsomedial/lateral hypothalamic afferents to the PVT. There was an overall effect of Group (Wald $\chi^2_{(2,n=18)}$ = 29.01, p < 0.01) for double-labled cells in the combined DMD/LH afferents to the PVT (*data not shown*). Bonferroni post-hoc comparisons indicate that STs have greater c-Fos expression in these hypothalamic afferents compared to both GTs (p < 0.01) and UNs (p < 0.01). These results confirm that presentation of an incentive, but not a reward predictive stimulus alone, leads to increased activity in PVT afferents from the dorsomedial/lateral hypothalamus (see Figure 3.10).

Ventral subiculum

In the VSub there was no effect of Group for overall levels of cue-induced c-Fos expression ($F_{(2,16)}$ = 1.09, p = 0.36; Figure 3.6B), and no significant differences in c-Fos expression in PVT afferents from the VSub cell group following cue presentation (Wald $\chi^2_{(2,n=19)}$ = 3.83, p = 0.15; Figure 3.6C). These results suggest that cells in the VSub that project to the PVT do not encode either the predictive or incentive motivational value of the reward cue.

Efferent experiment

Pavlovian conditioned approach behavior

Similar to the Afferent Experiment, there were differences in the CRs that were acquired following 5 days of PCA training (Figure 3.7). Rats that directed their behavior towards the lever-CS were classified as STs (n = 23; PCA Index range +0.54 to +0.93; Figure 3.7A-C). Due to physical experimental limitations, 17 were selected to continue on in the study with PCA scores ranging from +0.6 to +0.93. Rats that exhibited a CR that was directed towards the location of US delivery (food cup) upon lever-CS presentation were classified as GTs (n = 11; PCA Index range -0.57 to -0.92; Figure 3.7D-F). Rats classified as intermediates vacillated between the lever and the food cup, and they were excluded from the study (n = 18; PCA Index range +0.45 to -0.49; data not shown). In addition, 8 unpaired control rats (UN) received an equivalent number of CS-US presentations, but in an unpaired fashion, and they did not acquire either a sign- or goal-tracking phenotype (n = 8; Figure 3.7). Note that additional subjects were excluded due to missed FG injections (see below), and their data is not included in Figure 3.7.

Linear mixed effects models showed significant overall effects of Session and Group, as well as a significant Session x Group interaction, on all measures of sign-tracking behavior (all $p \leq 0.001$; Figure 3.7A-C). For all three measures, Bonferroni post-hoc analyses showed a significant within-group effect of Session for STs ($p < 0.001$), but not for GTs or UNs ($p > 0.05$). STs began to demonstrate lever-directed behavior as early as session 2, while GTs and rats in the UN group did not develop this behavior across sessions. For goal-tracking behavior, linear mixed effects models showed significant overall effects of Session and Group, as well as a significant Session x Group interaction, on all three measures (all $p \leq 0.001$; Figure 3.7D-F). Bonferroni post-hoc analyses revealed a significant within-group effect of Session for food cup contacts for GTs and STs ($p < 0.001$), but not for UNs ($p > 0.05$;Figure 3.7D). Thus, GTs increased their food cup contacts during the 8-second CS period across training sessions, whereas STs decreased their food cup contacts, and rats in the UN group did not alter their behavior directed towards the food cup over the course of training. For probability of food cup contact, as well as latency to food cup contact, Bonferroni post-hoc comparisons showed a significant within-group effect of Session for all three groups (all $p \leq 0.032$; Figure 3.7E-F). Again, GTs increased their food-cup-directed behavior across training sessions, indicating the development of a goal-tracking CR. In contrast, STs began to decrease their food-cup-directed behavior after session 2, and rats in the

UN group began to decrease their food-cup-directed behavior following session 3. Taken together, these results indicate that STs developed lever-directed behavior, while GTs developed food-cup-directed behavior, and rats in the UN group developed neither a sign-tracking nor a goal-tracking conditioned response.

Injection screening and retrograde labeling

Following immunohistochemistry, all brains were screened for accuracy of the FG injection and pattern of retrograde labeling. For the majority of animals, FG staining at the injection site was contained within the NAc (Figure 3.8A). A small number of rats had unilateral injections, or injections that missed or extended beyond the borders of the NAc and they were excluded from the study (n= 2 UNs, 3 GTs, and 2 STs). In some rats, there was FG staining that followed up the injection track through the lateral septum/dorsal striatum. While the PVT does send projections to this area, these projections are primarily limited to the anterior PVT (Moga *et al.*, 1995; Li & Kirouac, 2008), and appear slightly less dense than those to the NAc (Li & Kirouac, 2008).

The pattern of retrograde labeling following the injection was consistent with previous published findings (Li & Kirouac, 2008). Dense FG labeling was seen throughout the rostro-caudal axis of the PVT, and thus one section from the anterior, middle, and posterior PVT was quantified for each brain. Importantly, FG labeling was observed in the PVT in all of the subjects that had a successful surgery, except for one, which was excluded from the study due to minimal FG staining in the PVT (n = 1 GT). The remaining brains (n = 6 UNs, 7 GTs, 15 STs) were further processed and quantified as described above.

Quantification results

Similar to the Afferent Experiment, two different assessments were made. First, the total amount of c-Fos positive nuclei in a given PVT section was measured. Second, the expression of c-Fos in cells that projected specifically to the NAc was assessed, which was quantified as (the total number of FG and c-Fos double labeled cells) ÷ (the total number of FG positive cells) within each PVT section.

Anterior PVT

Within the anterior PVT (Figure 3.9A), cue presentation did not lead to differences in overall c-Fos, as there was no effect of Group ($F_{(2,25)} = 0.18$, $p = 0.83$; Figure 3.9B). There was also no effect of Group for the expression of c-Fos in anterior PVT neurons that project directly to the NAc (Wald $\chi^2_{(2,n=28)} = 2.92$, $p = 0.23$; Figure 3.9C). These results suggest that cells in the anterior PVT that project to the NAc do not encode either the predictive or incentive motivational qualities of reward cues.

Mid PVT

There was also not an an effect of Group on overall cue-induced c-Fos activation in the mid-PVT ($F_{(2,25)} = 0.16$, $p = 0.86$; Figure 3.9E), nor was there an effect on c-Fos expression specifically in the efferents from this region to the NAc (Wald $\chi^2_{(2,n=28)} = 0.54$, $p = 0.76$; Figure 3.9F). Thus, similar to the anterior PVT, cue presentation did not lead to differential levels of activity in the mid-PVT, suggesting that this part of the PVT also does not encode the predictive or incentive properties of reward cues.

Posterior PVT

Similar to the anterior and mid PVT sections, there was no effect of Group on cue-induced c-Fos expression in the posterior PVT ($F_{(2,25)} = 1.60$, $p = 0.22$; Figure 3.9H). However, c-Fos expression specifically in the NAc afferents in this region was significantly different between groups (effect of Group; Wald $\chi^2_{(2,n=28)} = 6.16$, $p < 0.05$; Figure 3.9I). Bonferroni post-hoc comparisons revealed that STs have significantly more c-Fos counts in cells projecting to the NAc relative to UN controls ($p < .05$). These data suggest that a reward-paired cue must be attributed with incentive salience for it to elicit activity in posterior PVT efferents to the NAc. Of note, the posterior PVT was the only region quantified where a significant correlation between total FG cells quantified and the % activity in the circuit (calculated by the total number of FG and c-Fos double labeled cells ÷ the total number of FG positive cells) was observed ($r(26) = -.40$, $p = 0.04$). This indicates that subjects with higher FG counts also had lower % activity measurements. Importantly, this significant correlation was only present when subjects were collapsed into a single group for analysis, and not divided by phenotype.

Discussion

The current study measured c-Fos expression in specific PVT afferent and efferent neuron populations in response to presentation of a predictive and incentive stimulus (in STs), or a predictive-only stimulus (in GTs). This was accomplished using a combination of retrograde tracing and immunohistochemical analyses in a rat model that captures individual variation in Pavlovian conditioned approach behavior. Results indicate that presentation of a reward-predictive stimulus increases activation of PrL cells that project directly to the PVT, evidenced by increased activity in this circuit in both STs and GTs. However, when a reward-predictive stimulus becomes imbued with incentive motivational value, its presentation is able to evoke greater activity in subcortical structures that project to the PVT, mainly the MeA and the dorsomedial/lateral hypothalamus, shown by increased activity in these circuits in STs only. In addition, presentation of an incentive stimulus also leads to greater activity in PVT cells that project to the NAc in STs relative to unpaired controls (Figure 3.10). While changes in activity in these circuits were significant, they were subtle in nature, with the average percentage of c-Fos/FG double-labeled cells ranging from approximately 1-9%. These results bring to mind recent work surrounding the role of neuronal ensembles in motivated behavior (Cruz *et al.*, 2013; Cruz *et al.*, 2015), and suggest that only a small number of neurons in a given circuit are necessary for the expression of motivated behaviors.

These findings extend the theories put forth by Ann Kelley and colleagues (Kelley *et al.*, 2005a; Kelley *et al.*, 2005b), implicating the involvement of a hypothalamic-thalamic-striatal axis underlying the ability of reward-paired cues to motivate behavior. Specifically, these results highlight the PVT as a potential modulator of incentive salience attribution, via integration of signals from subcortical structures, including the hypothalamus, and sending a coordinated output to the NAc, an area critical for motivated behavior. We previously hypothesized that GTs are more biased towards top-down control of behavior through cortical input to the PVT (Haight & Flagel, 2014; Haight *et al.*, 2015), and this hypothesis was only partially supported by the data in the current study, as both GTs and STs showed enhanced c-Fos expression in layer 6 PrL cells that project to the PVT, relative to unpaired controls. Previous work from our lab and others has shown that Pavlovian conditioned food cues do not elicit greater c-Fos expression in the PrL in either GTs or STs relative to unpaired controls (Flagel *et al.*, 2011a; Yager *et al.*, 2015), an effect that the current study has replicated. Here we build on these findings, by looking specifically at

layer 6 PrL neurons that project directly to the PVT. Contrary to our hypothesis, we found that presentation of a Pavlovian conditioned food cue elicits increased activity in this specific circuit for both GTs and STs, despite a lack of overall changes in total c-Fos expression. This was somewhat surprising, since we have previously shown that cue-induced c-fos mRNA levels in the PrL are correlated with those in the PVT in GTs, but not STs (Haight & Flagel, 2014). This, and the fact that goal-trackers appear to have better top-down cognitive control (Lovic *et al.*, 2011; Paolone *et al.*, 2013) led us to hypothesize that we would find an increase in activity in the PrL to PVT circuit in GTs only. Furthermore, the prelimbic cortex has previously been implicated in other goal-directed behaviors (Balleine & Dickinson, 1998), including cue-motivated behaviors (Sangha *et al.*, 2014; West *et al.*, 2014; Moorman & Aston-Jones, 2015). It is important to note, however, that work in this area has often focused on the PrL connections with different parts of the striatum (Baker & Ragozzino, 2014; Stefanik *et al.*, 2015), so little is known about the role of the PrL to PVT pathway in appetitive cue-motivated behaviors, despite the relatively dense connection (Li & Kirouac, 2012). One possible explanation for the current findings is that the PrL is sending information to the PVT regarding the predictive value of the reward-paired cue for both STs and GTs. Then, only for STs, is this information combined with signals from subcortical structures signaling the incentive value of the cue, overriding communication from the PrL to the PVT. Another possible explanation could depend on the post-synaptic targets of the PrL afferents to the PVT. It has been previously shown that the anterior and posterior regions of the PVT have overlapping, but differential inputs from the anterior and posterior regions of the PrL (Li & Kirouac, 2012). Since we injected FG into both the anterior and posterior portions of the PVT, it is possible that we were not able to detect subtle differences in PrL activity across these connections. Future studies will investigate this further, to determine if the increased PrL activity in STs is targeting a different region of the PVT than that of GTs, and therefore potentially communicating different information about the cue.

The second part of model assumes that STs would show greater activity in subcortical afferents to the PVT, and this hypothesis was supported by the data. The PVT receives a number of subcortical inputs from the hypothalamus, including dopamine, orexin, and cocaine-and-amphetamine-regulating-transcript, and neuropeptide-Y (Kirouac *et al.*, 2005; 2006; Li *et al.*, 2014; Lee *et al.*, 2015; Urstadt & Stanley, 2015). These peptides project from a heterogeneous group of hypothalamic nuclei, including, but not limited to, the A13 cell group, the DMD, the

VHM and LH. In the current study, PVT afferents from the DMD and LH showed similar patterns of activity in response to lever-CS presentation, so these regions were combined and assessed as the dorsomedial/lateral hypothalamus. It was found that lever-CS presentation leads to greater activation in dorsomedial/lateral hypothalamic afferents to the PVT in STs, compared to GTs and unpaired controls. While these data indicate that these projections may be specifically involved in processing the incentive value of reward-paired cues, the molecular identity of the cells specifically sending this signal is not known. One hypothalamic input of particular interest is orexin, because of its known role in motivated behavior (Mahler *et al.*, 2012; Sakurai, 2014). Orexinergic-positive cells that project to the PVT are primarily found distributed throughout lateral hypothalamus, including the perifornical area (Kirouac *et al.*, 2005; Lee *et al.*, 2015), but there appears to be slight orexinergic innervation from the dorsomedial hypothalamus as well (Kirouac *et al.*, 2005). These cells have been found to terminate in close proximity to the PVT cells that project to the NAc (Parsons *et al.*, 2006), and administration of orexin in the PVT can elicit dopamine efflux in the NAc (Choi *et al.*, 2012), indicating that this circuit can directly influence motivated behavior. In addition, presentation of food-paired cues and contexts activates orexin-positive neurons in the hypothalamus (Choi *et al.*, 2010; Petrovich *et al.*, 2012). These studies, combined with the results of the current experiment, highlight the possible involvement of this orexin circuit in incentive salience attribution. We hypothesize that presentation of an incentive stimulus activates orexinergic cells in the lateral/perifornical hypothalamus, and possibly the dorsomedial hypothalamus, that project to the PVT, where they can influence the activity of PVT cells that project to the NAc and affect sign-tracking behavior. Further studies utilizing functional methods, such as pharmacology or optogenetics, are needed to fully examine this hypothesis.

Lever-CS presentation also evoked greater c-Fos expression in MeA cells that project to the PVT in STs, compared to UNs. Interestingly, the MeA is a brain area that is far from wholly understood. Early work demonstrated that animals are willing to self-stimulate an electrode that has been planted in the MeA (Kane *et al.*, 1991). More recently, the MeA has been implicated in fear processing (Cousens *et al.*, 2012; Tsuda *et al.*, 2015). Apart from these findings, little else is known about the role of the MeA in motivated behaviors. Here we identify a novel role for the MeA, with MeA afferents to the PVT potentially underlying incentive salience attribution to reward paired cues. Presumably, these afferents are directed towards the anterior pole of the

PVT, since retrograde labeling has only been seen in this area following tracer injection into the anterior PVT (Chen & Su, 1990). It is possible, though, that these cells are also targeting the parataenial nucleus of the thalamus. In the previous study (Chen & Su, 1990), as well as our own, some of the tracer injection did leak into the parataenial nucleus, which is a small thalamic nucleus adjacent to the anterior PVT. On the other hand, an anterograde tracing study showed a dense efferent connection from the MeA to the PVT and medial portions of the mediodorsal nucleus, with only sparse innervation of the parataenial nucleus (Canteras *et al.*, 1995). Nonetheless, the majority of retrograde tracer was injected into the PVT, thus increasing the likelihood that the retrogradely labeled MeA cells are indeed targeting the PVT.

In the last part of the model, it was hypothesized that STs would show greater activity in NAc afferents from the PVT, where they could influence dopamine transmission. Importantly, sign-tracking behavior is dependent on dopamine transmission in the PVT, while goal-tracking behavior is not (Flagel *et al.*, 2011b; Saunders & Robinson, 2012). The results of the current study lend support to this hypothesis, and indicate that the posterior PVT may play a more prominent role in incentive salience attribution relative to other areas of the PVT. While the anterior and posterior aspects of the PVT send similarly dense efferents to the NAc (Li & Kirouac, 2008), the posterior PVT was the only area where an increase in activity in PVT efferents to the NAc was observed. In agreement, it was in the posterior PVT that an increase in c-Fos mRNA expression upon cue presentation in STs was also observed (Flagel *et al.*, 2011a). However, these findings do not preclude a role for the other areas of the PVT, since there could be different levels of activation in efferent PVT populations that were not measured in the current study.

While the results presented here lend further support to the theory that the PVT is important for influencing cue motivated behavior (Kelley *et al.*, 2005b; Martin-Fardon & Boutrel, 2012; James & Dayas, 2013; Matzeu *et al.*, 2014; Urstadt & Stanley, 2015), and in particular the propensity to attribute incentive salience to a reward cue (Haight & Flagel, 2014; Haight *et al.*, 2015), they are not in complete agreement with the literature. Previous work has demonstrated that presentation of an incentive stimulus can elicit robust c-Fos expression in the PVT (Flagel *et al.*, 2011a; Yager *et al.*, 2015). To our surprise, in the current study, there were no differences between phenotypes in cue-induced c-Fos in the PVT. These discrepant findings may be due to slight but significant differences in methodology between the current and previous

work. In one study, rats received 7 sessions of PCA training (Flagel *et al.*, 2011a), instead of the 5 sessions used in the current study. Thus, it is possible that the two studies were capturing brain states at different stages of acquiring the conditioned response. Also, Flagel et al. (2011a) quantified c-Fos mRNA expression using *in situ* hybridization, rather than c-Fos protein using immunohistochemistry, which was measured here. Although mRNA and protein levels are most often positively correlated, they measure two different substrates and do not always show the same trends (Guo *et al.*, 2008). In addition, *in situ* hybridization quantifies mRNA concentration, and not necessarily the number of cells expressing mRNA; whereas, in the current study, using immunohistochemistry, the number of cells expressing c-Fos protein was quantified. Thus, while our data suggest that the number of cells engaged in the PVT did not significantly differ between groups, the concentration of c-Fos signal within specific cells might have changed to a different degree in STs vs. GTs or UN rats in response to incentive stimulus presentation. Although c-Fos protein was measured in a second study by Yager et al (2015), there were other important methodological differences that need to be considered. In the Yager et al (2015) study the context that was utilized for context habituation and the lever-cue test day prior to sacrifice included the food cup (Yager *et al.*, 2015); whereas in the current study, the context for habituation and the lever-cue test day did not contain the food cup. Given that the PVT is known to play a role in both context- and cue-motivated behaviors (Wedzony *et al.*, 2003; Schiltz *et al.*, 2007; Hamlin *et al.*, 2009; Igelstrom *et al.*, 2010; James *et al.*, 2011; Browning *et al.*, 2014), having the food cup present upon the cue re-exposure might have led to differences in c-Fos induction in the PVT, especially since GTs and STs are differentially responsive to contextual cues (Morrow *et al.*, 2011; Saunders *et al.*, 2014).

In conclusion, the current data lend further support to the theory that a hypothalamic-thalamic-striatal axis underlies cue-motivated behavior, by showing that presentation of an incentive stimulus elicits activity specifically in the dorsomedial/lateral hypothalamic-PVT-NAc circuit. In addition, inputs from the MeA likely contribute to the neural circuitry underlying these behaviors. Last, it seems that the PrL to PVT circuit is activated by the predictive, and not the incentive, qualities of a conditioned stimulus. Since the current study was anatomical in nature, follow up studies utilizing functional technologies such as local pharmacology, chemogenetics and optogenetics will be imperative in fully understanding how these circuits contribute to cue-motivated behavior.

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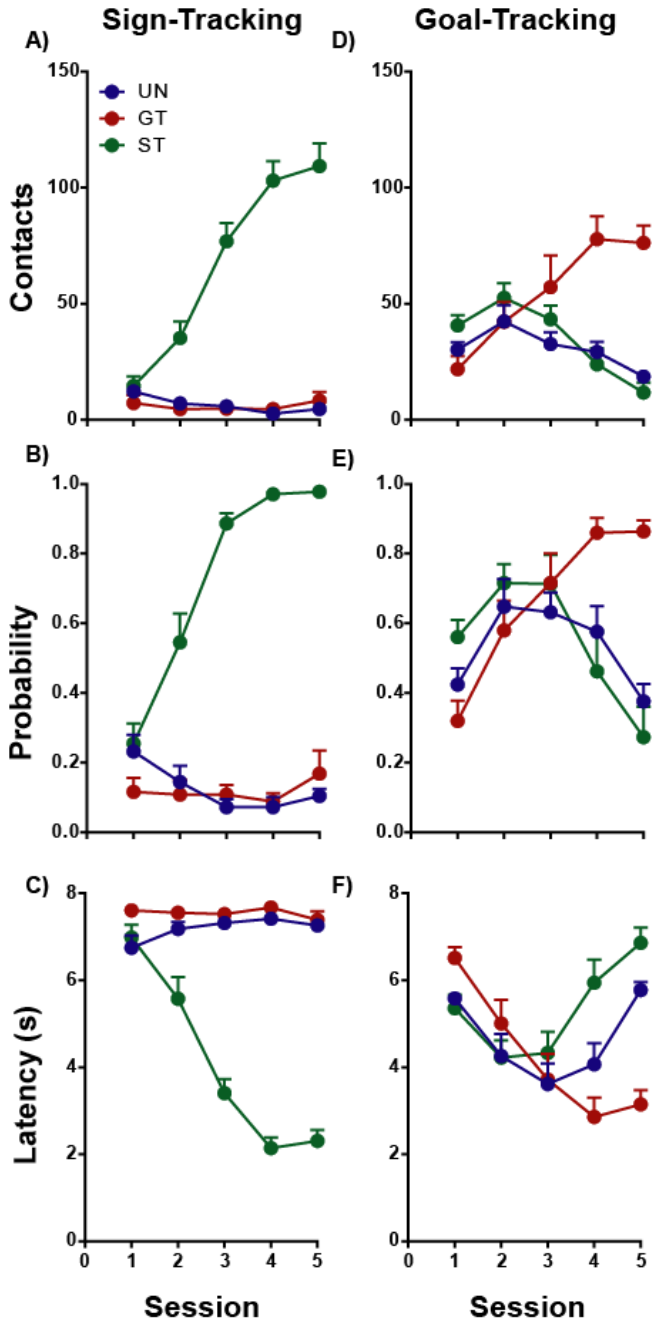

Figure 3.1. Acquisition of the sign- and goal-tracking conditioned response following 5 Pavlovian conditioning sessions (afferent experiment). Mean + SEM for A) lever contacts, B) probability of lever contact, C) latency to lever contact, D) food cup contacts, E) probability of food cup contact, and F) latency to food cup contact. Rats that displayed lever-directed behavior were classified as sign-trackers (STs; n = 11), while those that directed their behavior towards the food cup were classified as goal-trackers (GTs; n = 10). Rats who received unpaired CS-US presentations did not develop a conditioned response (UNs; n = 5).

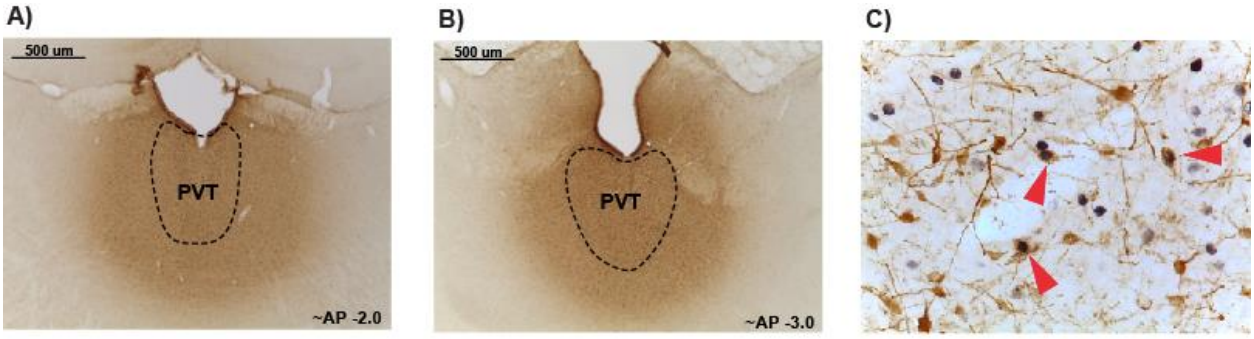

Figure 3.2. Representative images of fluorogold (FG) injections into the PVT and immunohistochemical labeling. FG injection shown in A) anterior and B) posterior portions of the PVT, shown at 5x magnification. Dashed line represents the approximate boundaries of the PVT. C) FG/c-Fos double-labeling following immunohistochemical staining, shown at 40x. Red arrowheads indicate FG/c-Fos double-labeled cells.

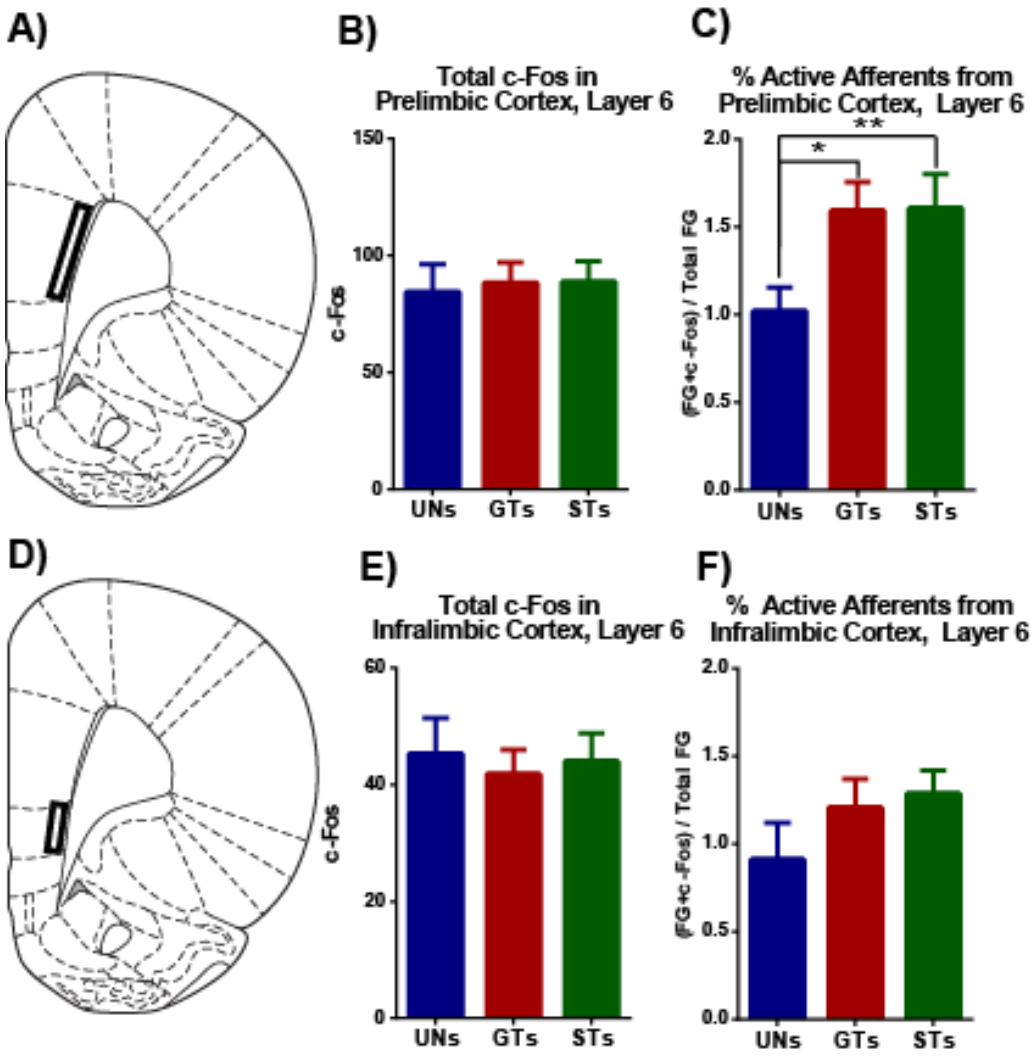

Figure 3.3. c-Fos expression in the medial prefrontal cortex following cue presentation. A) Schematic representing the approximate area of quantification for PrL at AP +3. (AP +4.2 not shown). Mean + SEM for B) overall c-Fos levels in layer 6 of the PrL and C) percent activity specifically in PVT afferents from the prelimbic cortex following stimulus presentation (UNs = 5, GTs = 8, STs = 11). There was an overall effect of phenotype and post-hoc revealed that both GTs (*p = 0.02) and STs (**p < 0.01) have greater engagement of PVT afferents from the PrL compared to UNs. D) Schematic representing the approximate area of quantification for IL. Mean + SEM for E) overall c-Fos levels in layer 6 of the IL and F) percent activity specifically in PVT afferents from the infralimbic cortex following stimulus presentation (UNs = 5, GTs = 8, STs = 11). Percent activity was calculated as (FG + c-Fos double-labeled cells) / (total FG labeled cells). Atlas images adapted from Paxinos and Watson (2007).

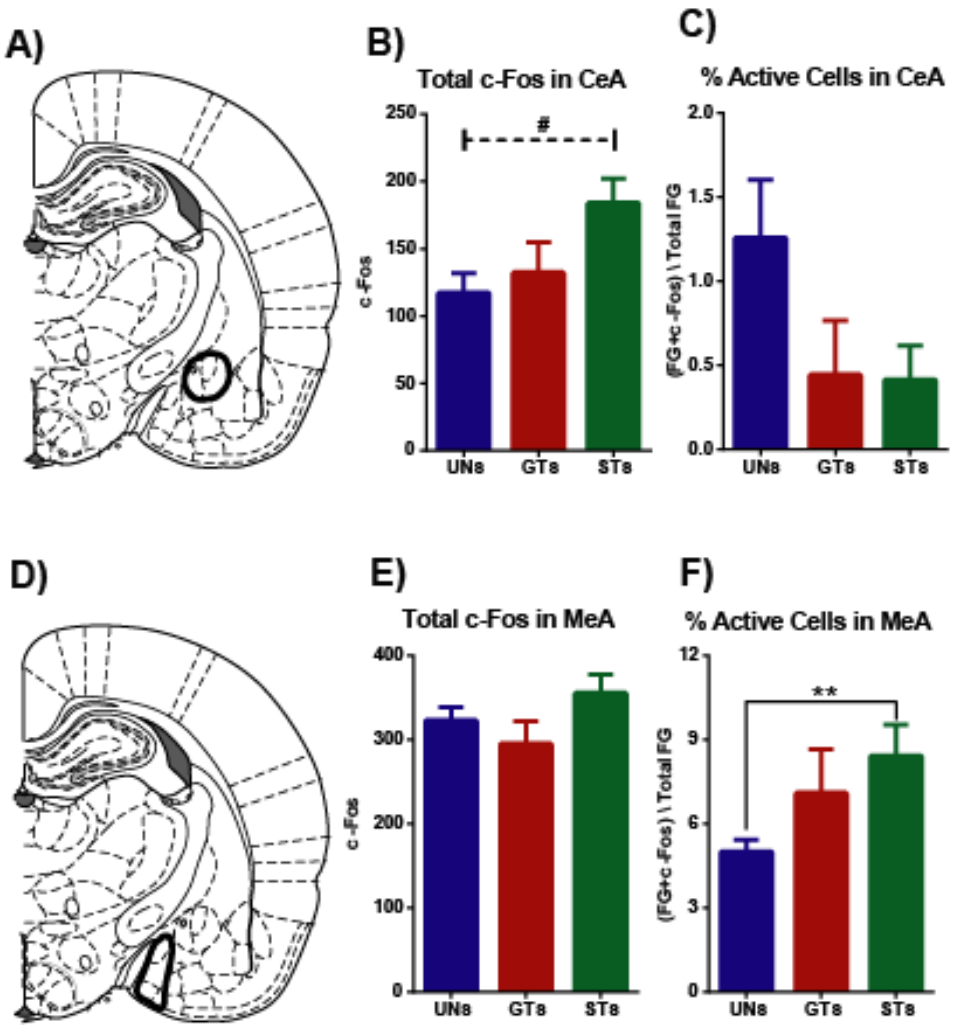

Figure 3.4. c-Fos expression in the central and medial amygdala following cue presentation. A) Schematic representing the approximate area of CeA quantification. Mean + SEM for B) overall c-Fos levels in the CeA and C) percent activity specifically in PVT afferents from the CeA following stimulus presentation (UNs = 5, GTs = 7, STs = 9). There was a trend towards a significant effect of phenotype ($F(2,18) = 3.171$, #$p = 0.07$) for overall c-Fos expression in the CeA. D) Schematic representing the approximate area of MeA quantification. Mean + SEM for E) overall c-Fos levels in the MeA and F) percent activity specifically in PVT afferents from the MeA following stimulus presentation (UNs = 5, GTs = 6, STs = 8). There was an overall effect of phenotype and post-hoc comparisons reveal that STs show greater activity in this circuit compared to UNs (**$p < 0.01$). Percent activity was calculated as (FG + c-Fos double-labeled cells) / (total FG labeled cells). Atlas images adapted from Paxinos and Watson (2007).

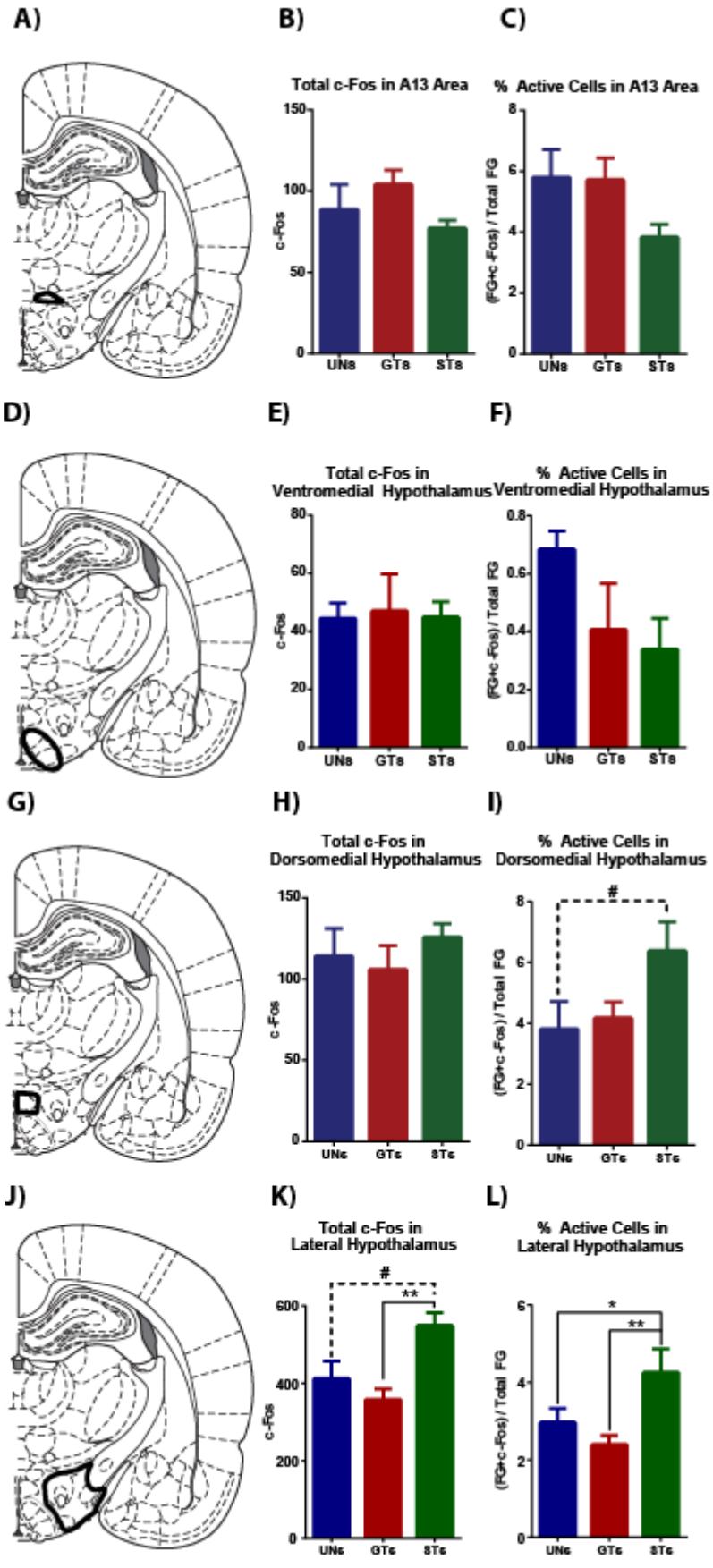
A)
B)
C)
Total c-Fos in A13 Area
% Active Cells in A13 Area
c-Fos
(FG+c-Fos) / Total FG
UNs
GTs
STs
D)
E)
F)
Total c-Fos in Ventromedial Hypothalamus
% Active Cells in Ventromedial Hypothalamus
G)
H)
I)
Total c-Fos in Dorsomedial Hypothalamus
% Active Cells in Dorsomedial Hypothalamus
J)
K)
L)
Total c-Fos in Lateral Hypothalamus
% Active Cells in Lateral Hypothalamus

Figure 3.5. c-Fos expression in the hypothalamus following cue presentation. A) Schematic representing the approximate area of the A13 cell group quantified. Mean + SEM for B) overall c-Fos levels in the A13 cell group and C) percent activity specifically in PVT afferents from the A13 cell group following stimulus presentation (UN = 5, GTs = 8, STs = 8). D) Schematic representing the approximate area of VMH quantified. Mean + SEM for E) overall c-Fos levels in the VMH and F) percent activity specifically in PVT afferents from the VMH following stimulus presentation (UNs = 3, GTs = 7, STs = 5). G) Schematic representing the approximate area of the DMD quantified. Mean + SEM for H) overall c-Fos levels in the DMD and I) percent activity specifically in PVT afferents from the DMD following stimulus presentation (UN = 5, GTs = 7, STs = 8). There was a trend towards an overall effect of phenotype, and post-hoc comparisons show that STs tend to have greater activity in this circuit compared to UNs (#$p = 0.08$). J) Schematic representing the approximate area of LH quantified. Mean + SEM for K) overall c-Fos levels in the LH and L) percent activity specifically in PVT afferents from the LH following stimulus presentation (UNs = 5, GTs = 7, STs = 6). There was an effect of phenotype on overall c-Fos levels in the LH, and post-hoc comparisons show that STs have higher levels of c-Fos compared to GTs (**$p < 0.01$), and trend towards an increase compared to UNs (#$p = 0.07$). For percent activity in PVT afferents from the LH, there was an effect of phenotype, and post-hoc comparisons show that STs have greater activity in this circuit compared to GTs (**$p < 0.01$) and UNs (*$p = 0.01$). Percent activity was calculated as (FG + c-Fos double-labeled cells) / (total FG labeled cells). Atlas images adapted from Paxinos and Watson (2007).

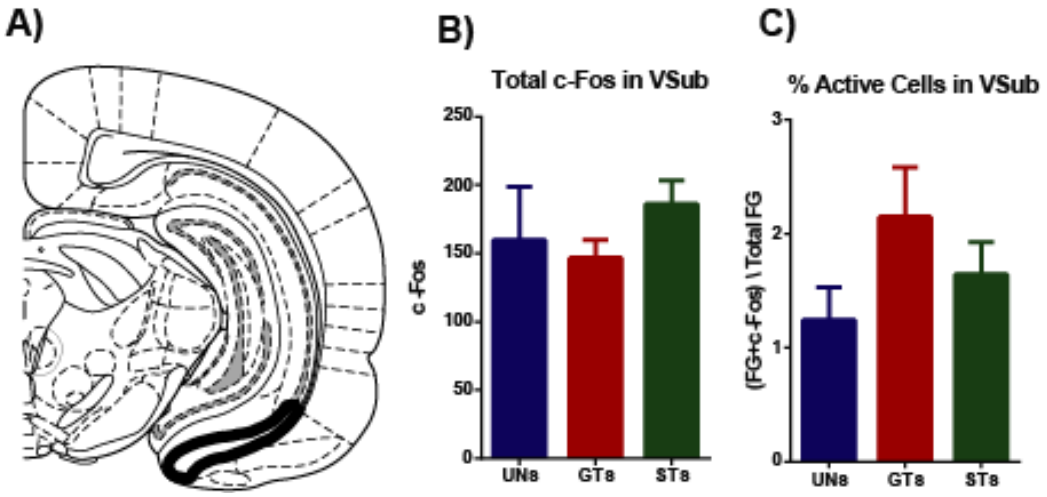

Figure 3.6. c-Fos expression in the ventral subiculum following cue presentation. A) Schematic representing the approximate area of VSub quantification. Mean + SEM for B) overall c-Fos levels in the VSub and C) percent activity specifically in PVT afferents from the VSub (depicted as [FG + c-Fos double-labeled cells] / [total FG labeled cells]) following stimulus presentation (UNs = 4, GTs = 9, STs = 6). Atlas image adapted from Paxinos and Watson (2007).

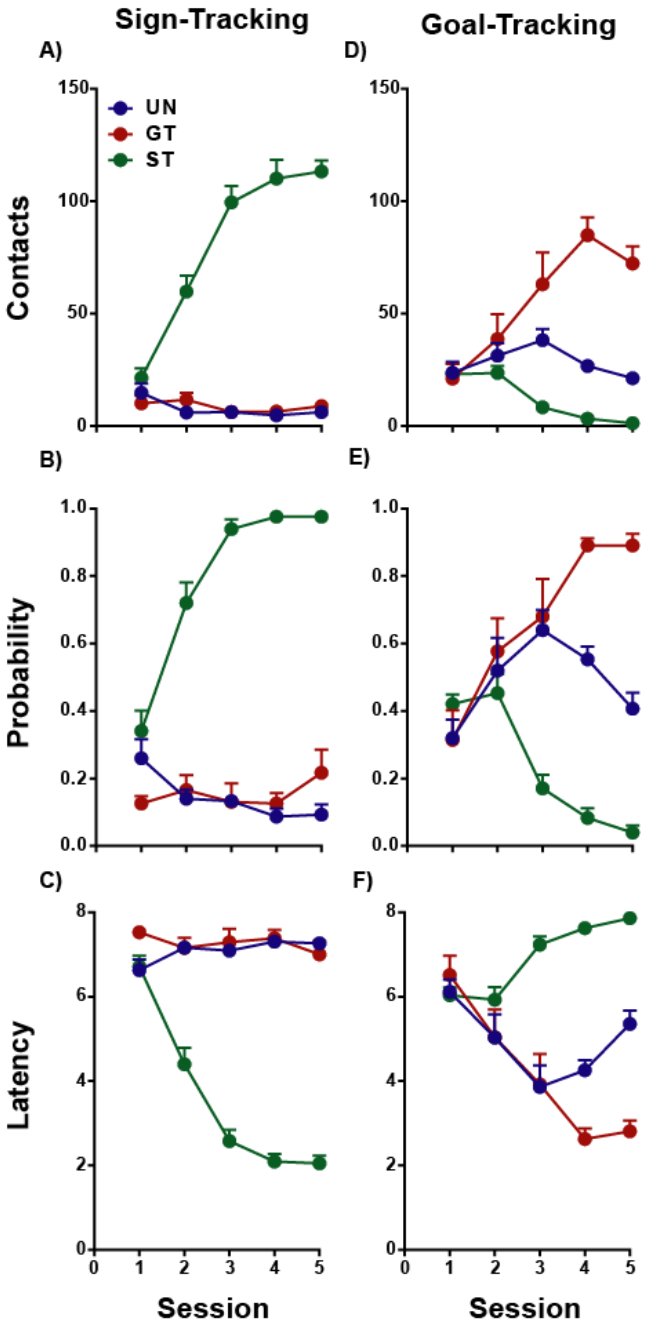

Figure 3.7. Acquisition of the sign- and goal-tracking conditioned response following 5 Pavlovian conditioning sessions (efferent experiment). Mean + SEM for A) lever contacts, B) probability of lever contact, C) latency to lever contact, D) food cup contacts, E) probability of food cup contact, and F) latency to food cup contact. Rats that displayed lever-directed behavior were classified as sign-trackers (STs; n = 15), while those that directed their behavior towards the food cup were classified as goal-trackers (GTs; n = 7). Rats who received unpaired CS-US presentations did not develop a conditioned response (UNs; n = 6).

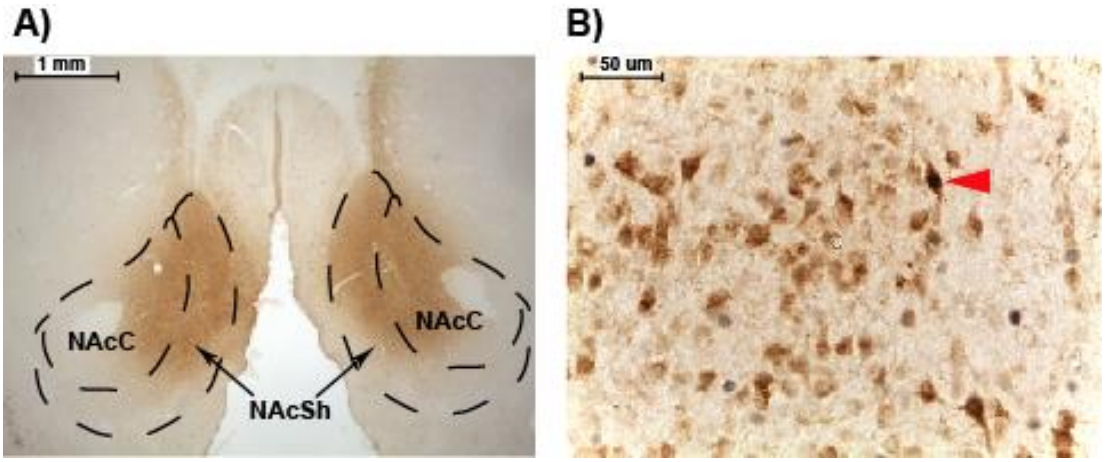

Figure 3.8. Representative images of fluorogold (FG) injections into the NAc and immunohistochemical labeling. A) FG injection shown in the NAc, shown at 2.5x magnification. Dashed lines represent the approximate boundaries of the NAc core (NacC) and shell (NacSh). B) FG/c-Fos double-labeling following immunohistochemical staining, shown at 40x magnification. Red arrowhead indicates a PVT cell double-labeled for FG/c-Fos.

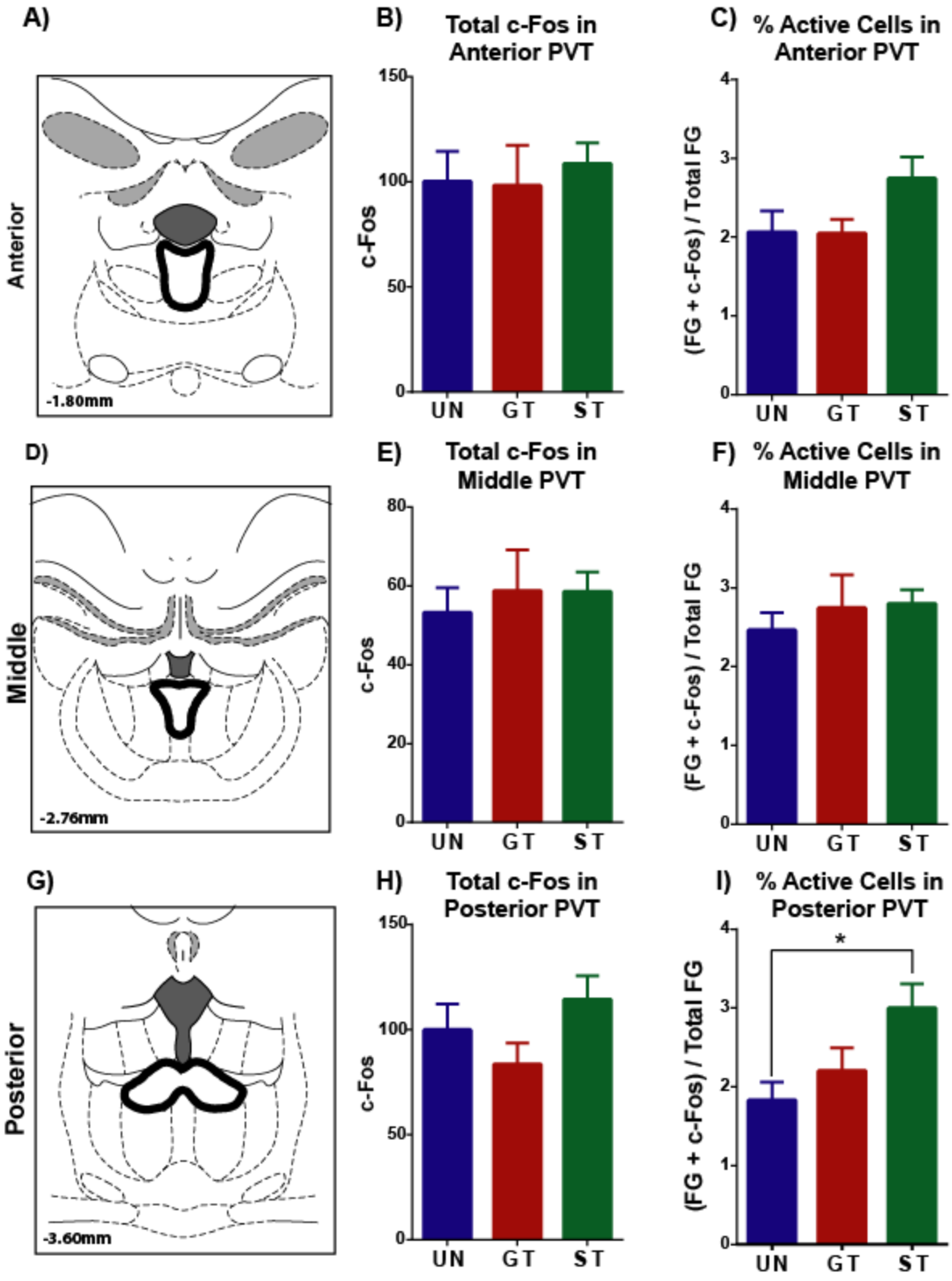
A)
Anterior
-1.80mm
B)
Total c-Fos in Anterior PVT
c-Fos
150
100
50
0
UN
GT
ST
C)
% Active Cells in Anterior PVT
(FG + c-Fos) / Total FG
4
3
2
1
0
UN
GT
ST
D)
Middle
-2.76mm
E)
Total c-Fos in Middle PVT
c-Fos
80
60
40
20
0
UN
GT
ST
F)
% Active Cells in Middle PVT
(FG + c-Fos) / Total FG
4
3
2
1
0
UN
GT
ST
G)
Posterior
-3.60mm
H)
Total c-Fos in Posterior PVT
c-Fos
150
100
50
0
UN
GT
ST
I)
% Active Cells in Posterior PVT
*
(FG + c-Fos) / Total FG
4
3
2
1
0
UN
GT
ST

Figure 3.9. c-Fos expression in the PVT following cue presentation. A) Schematic representing the approximate area of anterior PVT quantified. Mean + SEM for B) overall c-Fos levels in the anterior PVT and C) percent activity specifically in anterior PVT efferents to the NAc following stimulus presentation. D) Schematic representing the approximate area of middle PVT quantified. Mean + SEM for E) overall c-Fos levels in the mid PVT and F) percent activity specifically in mid PVT efferents to the NAc following stimulus presentation. G) Schematic representing the approximate area of the posterior PVT quantified. Mean + SEM for H) overall c-Fos levels in the posterior PVT and I) percent activity specifically in posterior PVT efferents to the NAc following stimulus presentation. There was an overall effect of phenotype, and post-hoc comparisons showed that STs have a greater percent activity in posterior PVT cells projecting to the NAc relative to UN controls (*$p < .05$). P activity was calculated as (FG + c-Fos double-labeled cells) / (total FG labeled cells). Atlas images adapted from Paxinos and Watson (2007).

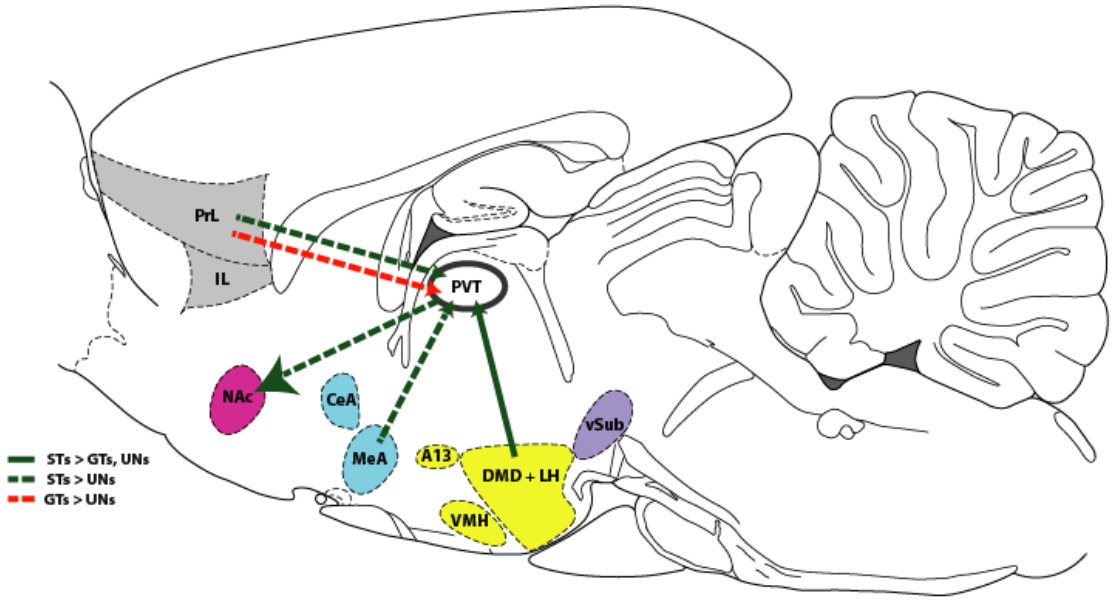

Figure 3.10. Schematic demonstrating the efferent and afferent circuits of the PVT engaged by cue presentation in STs vs. GTs. Sagittal schematic representing the efferent and afferent connections of the PVT that had significantly different levels of cue-induced c-Fos between STs and GTs or unpaired controls. Solid green arrows represent connections where STs had greater % activity compared to GTs and UNs. Dashed green and red arrows represent connections where STs or GTs had greater % activity than UNs, respectively. Abbreviations: A13, A13 cell group; CeA, central nucleus of the amygdala; DMD, dorsomedial nucleus of the hypothalamus; IL, infralimbic cortex; LH, lateral hypothalamus; MeA, medial amygdaloid complex; NAc, nucleus accumbens; PrL, prelimbic cortex; PVT, paraventricular nucleus of the thalamus; VMH, ventromedial hypothalamus; vSub, ventral subiculum. Atlas images adapted from Paxinos and Watson (2007).

Chapter 4

Investigating the role of orexin receptor 2 in the paraventricular nucleus of the thalamus in cue-motivated behavior

Introduction

Recently, the paraventricular nucleus of the thalamus (PVT) has been identified as a key brain region underlying cue-motivated behaviors (Hsu *et al.*, 2014; Kirouac, 2015). However, the specific role of the PVT and its efferent and afferent circuitry in these behaviors has been difficult to uncover, since reward-paired cues can simultaneously act as predictive and incentive stimuli (Robinson & Berridge, 1993). While some cues merely act as predictors of reward delivery, reward-paired cues that have been attributed with incentive salience can evoke complex motivational states (Childress *et al.*, 1993; Robinson & Berridge, 1993). It is thought that cues acquire incentive qualities, in part, through Pavlovian learning mechanisms (Robinson & Berridge, 2000). Importantly, individuals differ in the extent to which they attribute incentive salience to reward paired cues. Taking advantage of this individual variability, an animal model has been developed that allows us to parse the incentive from the predictive qualities of reward reward-paired cues (Robinson & Flagel, 2009). In this model, animals that attribute both predictive and incentive value to the cue, called sign-trackers (STs), rapidly approach and vigorously interact with the cue (Flagel *et al.*, 2009). In addition, these animals will perform a novel instrumental action for presentation of the Pavlovian reward cue, even in the absence of the reward that the cue was initially paired with (Robinson & Flagel, 2009). Other animals, termed goal-trackers (GTs), treat the cue merely as a predictive stimulus, and will rapidly approach the location of impending reward delivery upon cue presentation (Flagel *et al.*, 2009).

Using this model, we have begun to elucidate the neural circuitry underlying sign- and goal-tracking behavior, and thus the systems underlying the attribution of incentive salience to reward-paired cues. The PVT has been identified as a central node that mediates these behaviors (Haight & Flagel, 2014; Haight *et al.*, 2015). The PVT is a small midline thalamic nucleus that is

connected with cortical, limbic and motor circuitries. Specifically, the PVT receives innervation from the prelimbic and infralimbic cortices, amygdala, hypothalamus, and ventral subiculum, among other brain regions (Chen & Su, 1990; Canteras *et al.*, 1995; Van der Werf *et al.*, 2002; Vertes, 2004; Kirouac *et al.*, 2005; 2006; Vogt *et al.*, 2008; Hsu & Price, 2009; Li & Kirouac, 2012; Li *et al.*, 2014; Lee *et al.*, 2015). The PVT also projects to a number of brain areas critical for motivated behavior, including the nucleus accumbens (Berendse & Groenewegen, 1990; Su & Bentivoglio, 1990; Pinto *et al.*, 2003; Parsons *et al.*, 2006; Parsons *et al.*, 2007; Li & Kirouac, 2008; Vertes & Hoover, 2008). Using the sign-tracker/goal-tracker animal model, previous studies have shown that presentation of an incentive, but not a predictive stimulus is capable of eliciting robust c-fos expression in the PVT (Flagel *et al.*, 2011a; Yager *et al.*, 2015). In addition, sign- and goal-trackers differ in their 'functional connectivity', assessed by correlating levels of c-fos mRNA across brain regions. Specifically, sign-trackers showing strong correlations of c-fos mRNA between the PVT and nucleus accumbens (NAc) shell, indicating that these animals may be relying on sub-cortical communication (Flagel *et al.*, 2011a; Haight & Flagel, 2014). In support, stimulation of PVT neurons that project to the NAc can elicit dopamine efflux independent of VTA activation (Jones *et al.*, 1989; Parsons *et al.*, 2007), and dopamine transmission in the NAc is critical for sign-, but not goal-tracking behavior (Flagel *et al.*, 2011b; Saunders & Robinson, 2012). In contrast, GTs showed correlations of c-fos mRNA between the medial prefrontal cortex and the PVT, showing that these animals might be relying more on cortical control of their behavior (Flagel *et al.*, 2011a; Haight & Flagel, 2014), which is also supported by previous studies (Lovic *et al.*, 2011; Paolone *et al.*, 2013). While these results suggest that the PVT may be serving as a locus for mediating incentive salience attribution, it wasn't until recently that a causal link between the PVT and sign- and goal-tracking behavior was established, with PVT lesions leading to an overall decrease in goal-tracking behavior and concomitant increase in sign-tracking behavior (Haight *et al.*, 2015).

In addition to establishing a role for the PVT in sign- and goal-tracking behavior, we have been exploring the role of specific PVT efferent and afferent circuits in these behaviors as well. Using the retrograde tracer fluorogold (FG), combined with double-labeled immunohistochemistry for c-fos, we have demonstrated that presentation of an incentive stimulus evokes greater activity in specific PVT afferents from the dorsomedial/lateral hypothalamus, as well as PVT efferents to the NAc (Chapter 3). These data support the theory

put forth by Kelley and colleagues, and built upon by others, that a hypothalamic-thalamic-striatal axis underlies motivated behavior (Kelley *et al.*, 2005; Martin-Fardon & Boutrel, 2012; James & Dayas, 2013; Urstadt & Stanley, 2015). We have further hypothesized that this circuitry may be specifically involved in mediating incentive salience attribution to reward-paired cues (Haight & Flagel, 2014). While our data suggest that the dorsomedial/lateral hypothalamus-PVT circuit may play an important role in incentive-salience attribution to reward-paired cues, the molecular identity of the cells or transmitter systems contributing to this process remains unknown. Here we aim to investigate the role of orexin in this circuit, as it has been hypothesized that transmission of orexin from the lateral hypothalamus to the PVT is critical for motivated behaviors (Kelley *et al.*, 2005).

The orexin family consists of two neuropeptides, orexin-A and orexin-B, which are derived from the same precursor peptide, called prepro-orexin (de Lecea *et al.*, 1998; Sakurai *et al.*, 1998). Prepro-orexin positive neurons originate exclusively in the lateral, perifornical, and dorsomedial hypothalamic regions (de Lecea *et al.*, 1998; Sakurai *et al.*, 1998). Orexin peptides bind to two distinct G-protein coupled receptors, orexin receptor 1 (OX-1R) and orexin receptor 2 (OX-2R). OX-1R has a higher affinity for orexin-A, while OX-2R binds both orexin-A and orexin-B with similar affinities (Sakurai *et al.*, 1998). Functionally, orexin has been implicated in cue-motivated behaviors (Mahler *et al.*, 2012; Sakurai, 2014). Previous work has demonstrated that exposure to contextual stimuli previously paired with a food reward activated orexinergic neurons in the perifornical region of the hypothalamus (Choi *et al.*, 2010). Presentation of a tone cue previously paired with a food reward in a Pavlovian fashion also led to an increase is c-fos expression in orexinergic neurons in the hypothalamus (Petrovich *et al.*, 2012), and this activation is only apparent following extended conditioning, and not a single training session (Cole *et al.*, 2015). In addition, activation of orexin neurons in the lateral hypothalamus is positively correlated with the degree of expression of a conditioned place preference for a context associated with food reward (Harris *et al.*, 2005). Moreover, systemic blockade of OX-1Rs attenuated the expression of a conditioned response following Pavlovian pairings of a tone cue with a food reward (Keefer *et al.*, 2016); and cue-induced reinstatement of sucrose- and saccharin-seeking behavior is also attenuated following OX-1R blockade (Cason & Aston-Jones, 2013b; a). These studies clearly indicate a role for orexin signaling in food-cue-motivated behaviors.

Some progress has been made in identifying the specific neurobiological circuits that underlie orexin's role in motivated behavior. Much of this functional anatomy work has focused on the role of orexin transmission in the ventral tegmental area [for review see (Aston-Jones *et al.*, 2010; Thompson & Borgland, 2011; Mahler *et al.*, 2012)]. Recently, this focus is beginning to shift towards the role of orexin in the PVT in mediating motivated behaviors (Martin-Fardon & Boutrel, 2012; James & Dayas, 2013; Matzeu *et al.*, 2014). The PVT receives dense orexinergic innervation from the hypothalamus (Kirouac *et al.*, 2005; Lee *et al.*, 2015). Similar levels of OX-1R and OX-2R mRNA are found equally distributed throughout the PVT (Trivedi *et al.*, 1998; Marcus *et al.*, 2001). Studies utilizing slice electrophysiology have found that administration of both orexin peptides, orexin-A and orexin-B, depolarize post-synaptic PVT cells (Kolaj *et al.*, 2007), and it is thought that this effect is mainly mediated by OX-2Rs (Ishibashi *et al.*, 2005; Huang *et al.*, 2006). OX-2Rs in the PVT have been shown to mediate a number of different behaviors, including alcohol consumption (Barson *et al.*, 2015), morphine withdrawal-induced conditioned place aversion (Li *et al.*, 2011), and anxiety-related behaviors (Li *et al.*, 2010). In addition, blockade of OX-1Rs in the PVT does not block cue-induced reinstatement of cocaine seeking (James *et al.*, 2011), but the role of OX-2Rs in this behavior was not tested, potentially implicating OX-2Rs as the critical target for orexin in the PVT for cue-mediated behaviors. Last, administration of orexin into the PVT elicits efflux of dopamine in the nucleus accumbens (Choi *et al.*, 2012), and it has been previously demonstrated that dopamine transmission in this structure is important for sign- but not goal-tracking behavior (Saunders & Robinson, 2012).

Based on the evidence described above, we hypothesize that orexinergic activity at OX-2Rs in the PVT is critical for sign-, but not goal-tracking behavior. To test this hypothesis, the specific OX-2R antagonist TCS OX2 29 was administered directly into the PVT of rats, and its effects on Pavlovian conditioned approach behavior, as well the conditioned reinforcing properties of a food-paired stimulus, were examined. In addition, the effects of OX-2R antagonism in the PVT on feeding behavior were examined, since a role for orexin transmission at OX-1Rs in the PVT in feeding has been established (Choi *et al.*, 2012), but it is not known if this behavior is also modulated by OX-2Rs in this brain area.

Materials and methods

All of the following procedures were approved by The University of Michigan Institutional Animal Care and Use Committee, and all experiments were conducted in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals: Eighth Edition, revised in 2011. Two experiments utilizing two separate cohorts of subjects are described in detail below, following a general description of procedures common to both experiments.

Pharmacological compound

In order to assess the role of OX-2Rs in the PVT on the behaviors of interest, the OX-2R antagonist TCS OX2 29 (Lot # 2A/175266 for Experiment 1, Lot # 2A/179223 for Experiments 2 and 3; Tocris Bioscience, Avonmouth, Bristol, UK) was used. TCS OX2 29 was dissolved in 0.9% sterile saline at a concentration of either 10, 15 or 30µg per 300nl. TCS OX2 29 or vehicle (saline) was delivered directly into the PVT via a chronic indwelling double guide cannula (1mm center to center gap, cut 6mm below pedestal, 26 gauge, part # C235G-1.0-SP, Plastics One, Roanoke, VA).

Housing

Male Sprague-Dawley rats were pair-housed in standard acrylic cages (46 x 24 x 22 cm) in a climate-controlled room, and food and water were available ad libitum. The housing room was maintained on a 12-hour light:dark cycle (lights on at 07:00 hours), and all behavioral training was conducted during the light cycle. Following arrival, rats were allowed to acclimate to the new environment a minimum of 8 days prior to any manipulations

Cannulation and infusion

A double injector extending 1mm beyond the guide cannula was used to allow for two simultaneous infusions at two injection sites, one targeted towards the anterior PVT and another targeted towards the posterior PVT, to be performed simultaneously. Infusions into the PVT lasted 2 minutes, at a flow rate of 150nl per minute, for a total infusion volume of 300nl per injection site. Infusions were following by a 2-minute incubation period to minimize diffusion up the guide cannula upon injector removal.

Surgery

Following the initial acclimation period, all rats underwent surgical implantation of a chronic indwelling double cannula targeted at the PVT. All surgery was performed under aseptic conditions. A surgical plane of anesthesia was induced with inhalation of 5% isoflurane, and anesthesia was maintained throughout the procedure with inhalation of 1-2% isoflurane. Rats were fitted into the stereotaxic frame and the scalp was shaved and sterilized with alternating swabs of 70% alcohol and Betadine solution (Betadine, Stamford, CT, USA). A small incision was then made in the scalp, and the skull was leveled within +/- 0.1 mm using bregma and lambda coordinates. Two small holes were created above the PVT using a hand-held micro-drill and a stainless steel double cannula with the arms spaced 1mm apart (PlasticsOne, Roanoke, VA) was inserted at the following coordinates relative to bregma: AP -2.0, ML -1.0, DV -4.7 and AP -3.0, ML -1.0, DV -4.7 (stereotaxic arm angled at 10^0 towards the midline). Four screws were then implanted in the skull, and the cannula was fixed in place using dental cement. Once the cement was dry, the incision was closed around the cement with stainless steel wound clips. In addition, the cannula was plugged with a dummy injector that was flush with the end of the cannula, and covered with a dust cap. Immediately prior to surgery, and 24 hours after, rats 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) for pain management. Following surgery, all rats were single-housed for the remainder of the study, and were allowed to recover a minimum of 8 days prior to any behavioral testing.

Pavlovian conditioned approach training

Similar to the behavioral testing described in detail in the two previous chapters, all subjects underwent Pavlovian conditioned approach training following recovery from surgery. MED Associates behavioral test chambers housed in sound-attenuating cabinets were used, and all behavioral data were collected using MED PC software (MED Associates, St. Albans, VT). Each cabinet was outfitted with a ventilation fan that provided a constant flow of air, as well as background noise. Each chamber was equipped with a food cup connected to a pellet dispenser located in the middle of the right wall. Each time the pellet dispenser was triggered, one 45-mg banana-flavored grain pellet (Bio-Serve, Flemington, NJ) was delivered into the food cup. Head entries were recorded by breaks in an infra-red photo beam inside the food cup. A retractable,

illuminated lever was located either to the left or right of the food cup. All levers were set so that approximately 10 grams of force would cause a deflection of the lever and register as a lever contact. A white house light was situated on the upper middle portion of the left wall, opposite the food cup and lever, and was illuminated for the duration of each of each behavioral session.

For two days prior to 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 delivered in the home cage, to familiarize the rats to the experimenters and the novel food. Following these two days, all rats underwent one pretraining session, followed by 5 Pavlovian autoshaping sessions. Prior to each pretraining and autoshaping session (except the last post-test session in Experiments 1 and 2), rats were transported to a separate room and handled by the experimenters, increasing in time from approximately 30 seconds (pretraining) to 4 minutes (autoshaping session 5), during which time all rats had their dust caps screwed on and off. This was done in order to acclimate the rats to the to the infusion procedure. Following handling and/or infusions rats were transported to the testing room where they sat for 10 minutes in their home cages, under red light, prior to being placed in the testing chambers. This was to allow the rats to acclimate following the infusion procedure.

For pretraining, all rats were placed into the training chambers. Prior to the start of the session, each food cup was primed with 3 banana-flavored pellets, to direct the rats' attention to the location of reward delivery. At the beginning of the session, the house light remained off for 5 minutes, to allow the rats to acclimate to the chamber. Following 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 0-60s). The lever remained retracted for the entirety of the session, which lasted an average of 12.5 minutes. Rats typically consumed all of the pellets delivered into the food cup during the pretraining session. After pretraining, rats went through 5-7 sessions of Pavlovian autoshaping, one session per day. Each autoshaping session consisted of 25 trials in which the illuminated lever (CS) was inserted into the test chamber for 8 seconds. Upon lever retraction, one banana-flavored pellet (US) was delivered into the food cup. Lever (CS)-pellet (US) trials occurred on a variable interval 90-second schedule (range 30-150 seconds), with each session lasting approximately 40 minutes. The following data were recorded during each session, in order to quantify Pavlovian conditioned approach behaviors: (1) the number of food cup entries during the 8 second 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.

Experiment 1

Subjects

Forty-eight male Sprague Dawley rats (~7-8 weeks of age) were obtained from Charles River Laboratories (Wilmington, MA). Following arrival, rats were allowed to acclimate 8-11 days prior to any manipulation.

PCA classification and vehicle infusions

All subjects went through 1 session of pretraining, followed by 7 sessions of Pavlovian conditioning (for experimental timeline, see Figure 4.1A). Prior to sessions 6 and 7, each rat had the dust cap and dummy injector removed, and a double injector protruding 1mm beyond the guide cannula was inserted (final infusion coordinates ~AP -2.0, ML -1.0, DV -5.7 and AP -3.0, ML -1.0, DV -5.7). The injector was connected via P50 tubing to two 1-ul Hamilton syringes housed in a Harvard Apparatus double syringe pump. Each rat was then infused with vehicle (0.9% sterile saline) over 2 minutes. The injector was subsequently left in place for 2 minutes to minimize diffusion up the guide cannula upon removal. An experimenter gently held each rat for the 4-minute duration of the infusion. After the injector was removed, the dummy injector and dust cap was replaced, and the rat was placed back into its home cage and transported to the testing room, where it sat for 10 minutes prior to being placed in the testing chambers. Following session 7 of Pavlovian training, rats were classified as STs, GTs, or intermediate responders (INs) based on their average PCA Index scores (Meyer *et al.*, 2012) from sessions 6 and 7. The PCA Index is a composite score that is used to measure the degree to which an individual's behavior is directed towards the lever-CS or food cup (location of US delivery) using 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 were averaged together and rounded to the nearest tenth of a decimal place to create the PCA Index score, which ranges from -1.0 to 1.0, with -1.0 representing an individual whose behavior is directed

solely at the food cup, and 1.0 representing an individual whose behavior is directed solely at the lever-CS.

The effects of PVT OX-2R antagonism on Pavlovian conditioned approach behavior in goal-trackers

Following 7 days of Pavlovian conditioning, rats were split into drug and vehicle groups, which were counterbalanced based on PCA Index score. They then went through two more Pavlovian autoshaping sessions (sessions 8 & 9) in order to assess the effects of OX-2R antagonism in the PVT on Pavlovian conditioned approach behavior. Prior to the training sessions, each rat was infused with vehicle, 10ug, or 15ug of TCS OX2 29 per injection site, in a manner identical to the infusion procedures described above. On the last day (session 10), all rats went through an additional Pavlovian conditioning session with no drug or vehicle infusions to assess any lasting effects of drug infusion on PCA behavior.

Experiment 2

Subjects

Sixty male Sprague Dawley rats weighing approximately 200-250g (~7-8 weeks of age) were obtained from Charles River Laboratories (Wilmington, MA). Following arrival, rats were allowed to acclimate 15-19 days prior to any manipulation.

PCA classification

All subjects went through 1 session of pretraining, followed by 5 sessions Pavlovian conditioning, one session per day (for experimental timeline, see Figure 4.1B). Prior to each pretraining and conditioning session, rats were transported to a separate room and handled by the experiments, increasing in time from approximately 30 seconds (pretraining) to 4 minutes (autoshaping session 5). In addition, during the handling prior to sessions 4 and 5, all rats had their dust caps screwed on and off, in order to acclimate them to the infusion procedure. Following handling, rats were transported to the testing room, where they sat for 10 minutes in their home cages, under red light, prior to being placed in the testing chambers. Following session 5 of Pavlovian training, rats were classified as STs, GTs, or INs based on their average PCA Index scores (Meyer *et al.*, 2012) from sessions 4 and 5.

The effects of PVT OX-2R antagonism on Pavlovian conditioned approach behavior in sign-trackers

Following 5 days of Pavlovian autoshaping, all rats were split into drug and vehicle groups counterbalanced based on PCA Index score. They then went through another Pavlovian autoshaping session (session 6; i.e. test session) in order to assess the effects of OX2R antagonism in the PVT on Pavlovian conditioned approach behavior. Prior to the test session, each rat was infused with vehicle or 15ug TCS OX2 29 per injection site as described in detail above. After the injector was removed, the dummy injector and dust cap was replaced, and the rat was placed back into its home cage and transported to the testing room, were it sat for 10 minutes prior to being placed in the testing chambers. The next day, all rats went through an additional Pavlovian conditioning session (session 7) with no drug or vehicle infusions to assess any lasting effects of drug infusion on PCA behavior.

Conditioned reinforcement

Following the completion of PCA training, all subjects were tested in a conditioned reinforcement (CRf) paradigm, in order to assess the effects of OX-2R antagonism in the PVT on the conditioned reinforcing properties of the lever-CS. Importantly, all treatment groups remained consistent from the conditioning experiment to the CRf experiment, so that subjects that received infusion of TCS OX2 29 during conditioning also received infusion of TCS OX2 29 during the CRf test, and likewise for the vehicle group. Similar to the Pavlovian conditioned approach experiment, prior to the CRf test session, each rat was infused with vehicle or 15ug of TCS OX2 29. After the injector was removed, the dummy injector and dust cap was replaced, and each rat was placed back into its home cage and transported to the testing room, were it sat for 10 minutes prior to being placed in the testing chambers.

For the CRf test, the chambers were rearranged so that the food cup and pellet dispenser were removed, and the lever-CS was moved to the center of the right side wall. Two nose poke ports were then installed to the right and left of the lever. The nose port installed opposite the previous position of the lever-CS was designated the “active” nose port, and pokes into this port resulted in the brief 2-second presentation of the illuminated lever-CS on a fixed-ratio 1 schedule. Pokes into the other nose port, designated the “inactive”, did not result in lever-CS

presentation. Once the rats were placed into the test chambers, the house light remained off for 1 minute. After the 1-minute acclimation period, the house light was illuminated, and the CRf test session began. The session lasted 40 minutes, and the Med PC software program recorded the following measures for analysis: 1) the number of pokes into the active nose port, 2) the number of pokes into the inactive nose port, and 3) the number of lever contacts.

Experiment 3

Subjects

Subjects from Experiment 2 were used in Experiment 3, including 3 rats who were not sign-trackers (2 GT and 1 IN). Vehicle and treatment groups remained consistent from Experiment 2 to Experiment 3, except that subjects receiving TCS OX2 29 received 30ug infusions for Experiment 3, instead of 15ug infusions (Experiment 2).

Food restriction

The effects of OX-2R antagonismin the PVT on food consumption under food restriction conditions were tested. Water was available ad limibtum throughout the experiment, but all food was removed from the homecage at 5 PM (2 hours before lights-off) the day prior to the baseline test session. The next day, following the baseline test session, all subjects were given 4 pellets of chow for the night with water available ad libitum, in order to continue the light food restriction.

Feeding test

The baseline test session occurred between the hours of 11:30 and 17:30. All rats were placed into a novel environment containing ~25g of standard pellet chow one hour. Importantly, the exact amount of food delivered to each subject was weighed and recorded. After the hour, animals were returned to their home cages, and the remaining food was weighed, in order to assess total consumption. The following day, between the hours of 13:00 to 19:30 all rats went through the test session, in order to assess the effects of PVT OX-2R antagonism on food consumption. The procedures for the Test Session were similar to those during the Baseline Session, except immediately prior to being placed into the test chambers, subjects were infused with vehicle or 30ug of TCS OX2 29 per injection site. After the injector was removed, the dummy injector and dust cap were replaced, and each rat was immediately placed into the same

test chamber as the day before and given ~25g of standard pellet chow. Again, the exact amount of food delivered to each subject was weighed and recorded. After one hour, animals were returned to their home cages, and the remaining food was weighed, in order to assess total consumption. Following completion of the experiment, all subjects were returned to ad libitum feeding.

Tissue processing

Following the completion of the experiments, brains were collected to assess the accuracy of cannula placement. Rats were deeply anesthetized with an intraperitoneal injection of ketamine/xylazine. Prior to perfusion, an injector was inserted into the guide cannula and 300nl of 1% pontine sky blue dye dissolved in 0.9% sterile saline was infused over two minutes at each injection site, in order to aid in detection of the injection site. The injector was then left in place for 2 minutes to minimize diffusion up the guide cannula upon removal. Following dye infusion, rats were 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 0.1M sodium phosphate buffer; Fisher Scientific, Hampton, NH). Brains were then extracted and post-fixed overnight in 4% formaldehyde at 4°C. Brains were cryoprotected over three nights in graduated sucrose solutions (10%, 20%, and 30%, dissolved in 0.1M sodium phosphate buffer, pH=7.3-7.4) at 4°C. Following cryoprotection, brains were sectioned at 40 µm on a frozen cryostat (Leica Biosystems Inc, Buffalo Grove, IL). Brain sections were serially collected in 6-well plates. Each well contained a full series of sections through the thalamus, with each section approximately 200 µm caudal from the previous section. All sections were stored in 0.1M NaPB at 4°C. One or two wells of tissue were then mounted onto SuperFrost Plus microscope slides (Fisher Scientific) and visually analyzed for cannula placement using a Leica microscope.

Statistical analyses

To assess differences in the acquisition of PCA behavior, linear mixed effects model analyses were used with Session as the repeated variable, Treatment as the between-subjects variable, and each behavioral PCA measure as the dependent variable. In order to analyze the effects of TCS OX2 29 administration on PCA behavior, a two-way repeated measures ANOVA

was used, with Session and Treatment as the independent variables, and each behavioral PCA measure as the dependent variable. In Experiment 2, an additional analysis was conducted using the test session (session 6) normalized to baseline behavior [(test ÷ baseline)*100] for all sign-tracking measures. Using this measure, differences were assessed between Treatment groups (Vehicle or 15ug TCS OX2 29) using unpaired t-tests. For the conditioned reinforcement test, differences in nosepoke behavior were analyzed using a two-way ANOVA with Noseport (active vs. inactive) and Treatment as independent variables; and differences in lever contacts was analyzed using an unpaired t-test. To better capture differences in the incentive qualities of the lever-CS during the CRf session, an index of Incentive Value of the lever-CS was calculated as: [(Active Nosepokes + Lever Contacts) – Inactive Nosepokes], and differences on this measure were analyzed using an unpaired t-test. Last, food pellet consumption in Experiment 3 was normalized to baseline [(test ÷ baseline)*100], and differences between Treatment groups (Vehicle or 30ug TCS OX2 29) were analyzed using an unpaired t-test.

Results

Experiment 1: The effects of PVT OX-2R antagonism on PCA behavior in goal-trackers

Pavlovian conditioned approach behavior

Similar to previous reports (Robinson & Flagel, 2009; Meyer *et al.*, 2012), variation was seen in the CRs acquired following 7 sessions of PCA training. However, the majority of rats directed their behavior towards the food cup, and were classified as GTs (n = 25), with average PCA index scores from sessions 6 and 7 ranging between -0.3 and -1.0. Some rats displayed lever directed behavior, and were classified as STs (n = 9), with PCA Index scores ranging from 0.3 to 1.0. A small number vacillated between the lever and food magazine and were classified as INs (n = 6), with PCA index scores ranging from -0.2 to 0.2. In addition, 8 rats did not make it through the experiment due to health or technical issues. Due to the small numbers of STs and INs, we were not able to collect enough data to adequately assess the effects of TCS OX2 29 administration in these groups, so only the data from GTs is described below. Note that additional GTs were eliminated due to missed cannula placements (as indicated below) and these subjects were also not included in the following analyses.

Across training sessions, GTs developed a bias towards food-cup directed behavior over lever-CS directed behavior (Figure 4.2). Linear mixed effects models showed a significant effect

of Session for lever-CS contacts ($F_{(6,7)} = 7.69$, $p = 0.008$; Figure4.2A), as well as a significant effect of Session ($F_{(6,7)} = 33.41$, $p < 0.001$) and a significant Session x Treatment interaction ($F_{(12,7)} = 5.70$, $p = 0.014$) for probability of lever-CS contact (Figure 4.2B). Bonferroni post hoc comparisons revealed a significant effect of Session for both GT Vehicle and GT 15ug TCS OX2 29 groups (both $p < 0.001$), but not for the GT 10ug TCS OX2 29 group ($p = 0.353$), on the probability of lever-CS contact measure across the 7 training days prior to drug administration. There were no significant effects of Session or Treatment, and no significant Session x Treatment interaction for latency to lever contact (Figure 4.2C). Taken together, these results indicate that there was a small yet significant increase in lever-directed behavior for GTs across training sessions. In contrast, GTs showed a robust increase in food-cup directed behavior across sessions. Linear mixed effects models showed a significant effect of Session for food cup contacts ($F_{(6,17)} = 3.83$, $p = 0.013$; Figure 4.2D), probability of food cup contact ($F_{(6,42)} = 10.26$, $p < 0.001$; Figure 4.2E), and latency to food cup contact ($F_{(6,12)} = 14.84$, $p < 0.001$; Figure 4.2F). Importantly, there were no significant differences between treatment groups on the acquisition of goal-tracking behavior (i.e. no effect of Treatment and no Treatment x Session interaction). These results demonstrate that GTs developed robust food-cup directed behavior across training sessions.

Cannula placement verification

Similar to other studies (Dong *et al.*, 2015), cannula placements were verified for all subjects to ensure accuracy of infusions. Subjects with injector tracts abutting the dorsal border of the PVT, within the PVT, or immediately adjacent to the lateral or ventral borders of the PVT were considered accurate injections, and remained in the study (for an example of acceptable cannula placement, see Figure 4.3). Subjects with injections that did not touch the top of the PVT, or were completely contained within the mediodorsal nucleus or the habenula, were excluded from the study. Following cannula screening, 15 GTs were excluded due to missed placements, and the remaining subjects were as follows: GT vehicle, n=4; GT 10ug TCS OX2 29, n=2, and GT 15ug TCS OX2 29, n=4.

Antagonism of OX-2Rs in the PVT does not affect PCA behavior in goal-trackers

In order to assess the effects of OX-2R antagonism on PCA behavior in GTs, autoshaping data from sessions 6 & 7 and 8 & 9 were averaged to create two data points, one 'baseline' and one 'test', respectively. These data were then analyzed, along with the data from 'post-test' session 10, using a two-way repeated measures ANOVA. Results show that there were no significant overall effects of Session or Treatment, and no significant Treatment x Session interactions, for any measures of sign- (Figure 4.4A-C) or goal-tracking behavior (Figure 4.4D-F). In addition, there were no differences across sessions within Treatment groups. Although the n's are low, these results indicate that TCS OX2 29 administration did not affect PCA behavior in GTs.

Experiment 2: The effects of PVT OX-2R antagonism on PCA behavior and conditioned reinforcement in sign-trackers

Pavlovian conditioned approach behavior

As described above for Experiment 1, variation was seen in types of conditioned responses acquired following 5 sessions of PCA training, prior to drug administration. In Experiment 2, however, the majority of rats directed their behavior towards the lever-CS, and were classified as STs (n = 32) with average PCA index scores from sessions 4 and 5 ranging between 0.5 and 1.0. Some rats displayed food cup-directed behavior, and were classified as GTs (n = 9) with PCA index scores from -0.5 to -1.0. Some subjects vacillated between the lever and food magazine and were classified as INs (n = 12), with PCA index scores between -0.2 and 0.4. In addition, 7 rats did not make it through the experiment due to health or technical issues. Due to the small numbers of GTs and INs, we were not able to collect enough data to adequately assess the effects of TCS OX2 29 administration in these groups, so only the data from STs is described below. Additional STs were eliminated from the following analyses due to missed cannula placements as described below.

Subjects classified as STs showed a bias towards lever-CS directed behavior over food cup directed behavior across 5 sessions of Pavlovian conditioning (Figure 4.5). Linear mixed effects models showed a significant overall effect of Session for lever-CS contacts ($F_{(4,45)} = 7.02$, $p < 0.001$; Figure 4.5A), probability of lever-CS contact ($F_{(4,21)} = 14.76$, $p < 0.001$; Figure 4.5B), and latency to lever-CS contact ($F_{(4,46)} = 13.67$, $p < 0.001$; Figure 4.5C). Concomitant with this

increase in lever-CS directed behavior, there was a slight decrease in food cup directed behavior, shown by a significant overall effect of Session on food cup contacts ($F_{(4,20)} = 4.48$, $p = 0.009$; Figure 4.5D), probability of food cup contact ($F_{(4,30)} = 3.68$, $p = 0.015$; Figure 4.5E), and latency to food cup contact ($F_{(4,27)} = 3.23$, $p = 0.027$; Figure 4.5F). Importantly, there were no differences between treatment groups for either sign- or goal-tracking behavior during the 5-day Pavlovian training phase prior to drug treatment (i.e. no effect of Treatment and no Session x Treatment interactions).

Cannula placement verification for Experiments 2 and 3.

Using the same criteria as Experiment 1, cannula placements were verified for all subjects to ensure accuracy of infusions. Following cannula screening, 18 STs were excluded due to missed placements, and the remaining subjects were as follows: ST vehicle, n=6 and ST 15ug TCS OX2 29, n=8.

Antagonism of OX-2Rs in the PVT attenuates the escalation of sign-tracking behavior.

Similar to the analyses performed in Experiment 1, autoshaping data from sessions 4 & 5 were averaged to into a single baseline data point. This data point was then included in an analysis with data from session 6 (test session) and session 7 (post-test session) to compare the different test phases using a two-way repeated measures ANOVA (Figure 4.6). Results showed that there was a trend towards a significant Session x Treatment interaction for lever-CS contacts ($F_{(1.27, 15.26)} = 3.744$, $p = 0.064$; Figure 4.6A), as well as a significant Session x Treatment interaction on probability of lever-CS contact ($F_{(2,24)} = 3.301$, $p = 0.054$; Figure 4.6B). Bonferroni post-hoc comparisons revealed a significant difference between the baseline and test session for lever-CS contacts ($p = 0.017$) and probability of lever contact ($p = 0.039$) for the ST Vehicle group, but not the treatment group. On the latency to lever-CS contact measure (Figure 4.6C), as well as all goal-tracking measures (Figure 4.6D-F), there was no significant overall effect of Session or Treatment, and no significant Session x Treatment interactions. These findings suggest that treatment with the OX-2R antagonist prevented any further increase in sign-tracking behavior.

To better visualize the changes in the behavioral data all sign-tracking measures were normalized [(test session/baseline) x 100] (Figure 4.7). Unpaired t-tests revealed a significant

difference between TCS OX2 29 and vehicle groups on all 3 measures: lever-CS contacts ($t_{(12)}$ = -3.661, p = 0.003, Figure 4.7A), probability of lever-CS contact ($t_{(12)}$ = -2.338, p = 0.038, Figure 4.7B), and latency to lever-CS contact ($t_{(12)}$ = 2.397, p = 0.034, Figure 4.7C). In agreement with the analysis above, sign-tracking behavior increased on the test session compared to baseline for the ST Vehicle group, while sign-tracking behavior stayed constant or slightly decreased in STs that received 15ug TCS OX2 29.

Antagonism of OX-2Rs in the PVT appears to alter the conditioned reinforcing properties of the lever-CS.

Following PCA training subjects were tested in CRf paradigm, in order to assess the effects of OX-2R antagonism in the PVT on the conditioned reinforcing properties of the lever-CS. A 2-way ANOVA showed that there was a significant effect of Noseport (Active vs. Inactive; $F_{(1,24)}$ = 14.54, p = 0.001), but no effect of Treatment (Drug vs. Vehicle; $F_{(1,24)}$ = 0.349, p = 0.56) or Treatment x Noseport interaction ($F_{(1,24)}$ = 1.488, p = 0.23; Figure 4.8A). However, an unpaired t-test showed there was a trend towards reduced lever-CS contacts during the CRf session following drug administration ($t_{(12)}$ = -2.029, p = 0.065; Figure 4.8B). That is, even though the lever-CS is presented only very briefly (2 sec) upon pokes into the active port, vehicle-treated rats tended to engage with it to a greater extent than TCS OX2 29 treated rats during the CRf session. To better capture differences in the incentive qualities of the lever-CS, we calculated an index of Incentive Value as: [(Active Nosepokes + Lever Contacts) – Inactive Nosepokes]. Similar to lever contacts, an unpaired t-test demonstrated a trend ($t_{(12)}$ = -1.984, p = 0.071; Figure 4.8C) towards a reduction in the incentive motivational value of the lever-CS in STs that received 15ug TCS OX2 29 prior to the test session, relative to those that received vehicle. Overall, these results indicate that antagonism of OX-2Rs in the PVT tends to reduce the conditioned reinforcing properties or the incentive motivational value of the lever-CS.

Experiment 3: The effects of PVT OX-2R antagonism on food consumption

Following the conclusion of conditioned reinforcement test, the effects of PVT OX-2R antagonism on food consumption were tested under food-deprived conditions. A repeated measures 2-way ANOVA showed no effect of Treatment (Vehicle, n = 5 vs. 30ug TCS OX2 29, n = 6) and no Treatment x Session interaction on pellet consumption from baseline to the test

session (Figure 4.9A). There were also no significant differences when the data were normalized to baseline and compared using an unpaired t-test (Figure 4.9B). Thus, feeding behavior does not appear to be dependent on OX-2Rs in the PVT; rather, orexin transmission in the PVT may mediate feeding behavior exclusively via OX-1Rs (Choi *et al.*, 2012).

Discussion

The experiments described above tested the hypothesis that orexinergic activity at OX-2Rs in the PVT is critical for incentive salience attribution to reward-paired cues. Specifically, we examined the effects of administration of the OX-2R antagonist, TCS OX2 29 in the PVT on sign- and goal-tracking behavior. In addition, we sought to characterize the role of PVT OX-2Rs in feeding behavior. Due to the smaller size of the experimental groups, as well as differences in methodology, the studies are better assessed as three separate 'pilot' studies and specifics will be discussed individually below, followed by a more general discussion.

Experiment 1

The first study sought to assess the role of orexin transmission in goal-tracking behavior. It was hypothesized that orexin transmission at OX-2Rs in the PVT is not critical for goal-tracking behavior, as GTs do not assign incentive motivational value to the reward cue (Robinson & Flagel, 2009). As the data shows, administration of TCS OX2 29 into the PVT did not alter the expression of goal-tracking behavior, regardless of dose. Although these findings do not support a role for orexin transmission at OX-2Rs in the PVT in the behavior of goal-trackers, there are some concerns with experimental design that need to be addressed. In this experiment, each subject received 4 infusions (2 vehicle, followed by 2 vehicle or TCS OX2 29). When sections were visualized for cannula placement verification, it was evident that some damage had been done to the PVT, most likely due to the repeated number if infusions. It is possible that this damage attenuated any pharmacological effect that might have otherwise been observed. When performing follow-up studies, it will be important to limit the number of infusions (as was done in Experiment 2) to keep PVT damage to a minimum.

Experiment 2

The more interesting results obtained come from Experiment 2, where the effects of PVT administration of TCS OX2 29 on sign-tracking behavior, as well as the conditioned reinforcing properties of the lever-CS, were assessed. It was hypothesized that orexin transmission at OX-2Rs in the PVT is critical for incentive salience attribution, and thus, blocking these receptors should reduce sign-tracking behavior. The results from experiment 2 demonstrated that the ST Vehicle group continued to increase their sign-tracking behavior across sessions, while the ST TCS OX2 29 group did not show a change in behavior. Thus, TCS OX2 29 administration appears to have prevented the further enhancement of sign-tracking behavior, possibly by blunting the incentive motivational value of the lever-CS.

In addition to the effects observed in the PCA experiment, there appeared to be a decrease in the incentive motivational value of the lever-CS during the CRf procedure following TCS OX2 29 administration. CRf testing is another way to assess the motivational value of the lever-CS (Cardinal *et al.*, 2002), as the rats are required to perform an instrumental response for presentation of the CS in the absence of the food reward. Although there were no significant differences between treatment groups in instrumental responding during the CRf test, the fact that the drug-treated STs tended to approach and contact the lever less once it was presented suggests that the incentive motivational value of the lever was attenuated for these rats.

Another possible explanation for the decrease in behavior observed during CRf testing has to do with the fact that role orexin signaling is especially important for maintaining motivation when increased effort is required (Thompson & Borgland, 2011; Mahler *et al.*, 2014). For example, administration of orexin-A into the rostral lateral hypothalamic area increases responding for a sweet pellet reward on a progressive ratio (PR) schedule, as well as a fixed ratio (FR) 20 schedule, where substantial effort is required (Thorpe *et al.*, 2005). Also, a number of studies have demonstrated that blockade of OX-1Rs decreases motivation to work for food or drug rewards when high, but not low levels of effort are required to obtain the reward (Hollander *et al.*, 2008; Borgland *et al.*, 2009; España *et al.*, 2010; Bentzley & Aston-Jones, 2015; Brodnik *et al.*, 2015). While all of these studies implicate orexin transmission at OX-1Rs in motivational situations requiring high amounts of effort, this does not preclude a role for OX-2Rs, since these studies did not use OX-2R specific compounds. Thus, the trend in reduced incentive value of the lever-CS observed during the CRf test session in the current study may reflect the role of orexin

transmission at OX-2Rs in the PVT under conditions that require more effort than standard PCA training. Due to the nature of the trend, as well as the moderately low number of subjects in the study, follow-up replications are needed to confirm this hypothesis.

Experiment 3

In Experiment 3, the role of orexin transmission at OX-2Rs in the PVT on feeding behavior was assessed. Previous work has demonstrated that administration of orexin-A into the posterior PVT leads to increased consumption of 2% sucrose solution (Barson *et al.*, 2015), while knockdown of OX-1Rs in the PVT reduces hedonic feeding of high-fat chow in rats (Choi *et al.*, 2012). In the current study, we demonstrated that OX-2R antagonsim in the PVT did not reduce consumption of normal chow in food-restricted rats. These data further implicate a specific role for OX-1Rs in the PVT in mediating food consumption. One important difference, though, is that the current experiment did not utilize a highly palatable food, such as sucrose or high-fat chow. Follow-up studies will be needed to assess whether PVT OX-2R antagonism affects consumption of highly palatable foods.

General discussion

The studies here assessed the role of orexin transmission at OX-2Rs in the PVT on food-cue-motivated behavior. Interestingly, however, the PVT is engaged by both food- and drug-associated incentive cues (Yager *et al.*, 2015), and the role of orexin in cue-motivated behaviors is not limited to food, but extends to drugs of abuse as well. Presentation of a discrete odor stimulus previously linked to ethanol availability increased activity in dorsomedial and perifornical/lateral hypothalamic orexin neurons (Dayas *et al.*, 2008). Activation of orexin neurons specifically in the lateral hypothalamus is also positively correlated with reward-seeking behavior following CPP training for cocaine and morphine (Harris *et al.*, 2005). Antagonist studies have shown that systemic administration of the OX-2R antagonist 2-SORA 18 blocked cue-induced reinstatement of nicotine-seeking behavior (Uslaner *et al.*, 2014). In addition, systemic administration of the OX-1R antagonist SB-334867 blocks discrete cue- and context-induced reinstatement of cocaine-seeking (Smith *et al.*, 2009; Smith *et al.*, 2010), discrete cue-induced reinstatement of morphine seeking (Smith & Aston-Jones, 2012), morphine CPP (Harris *et al.*, 2005; Sharf *et al.*, 2010), and olfactory cue-induced ethanol seeking (Lawrence *et al.*,

2006). These studies clearly show that orexin transmission is involved in drug-cue-motivated behavior, and it is likely that orexin transmission in the PVT is at least partially responsible. Follow-up studies similar to the ones contained here, but utilizing drug-paired cues instead, will be able to confirm this hypothesis.

One alternative explanation for the results described above that has yet to be explored has to do with the interactions of the PVT, orexin, the hypothalamic-pituitary-adrenal (HPA) axis, and stress (Hsu *et al.*, 2014). An early study demonstrated that the posterior PVT shows increased c-fos expression to a novel stressor *following* a chronic stress paradigm (Bhatnagar & Dallman, 1998). Follow-up studies confirmed that the posterior PVT is important for habituation to chronic stress (Bhatnagar *et al.*, 2000; Bhatnagar *et al.*, 2002). Specifically, it was shown that the posterior PVT mediates the elevated HPA-axis activity observed in response to a novel stressor in animals that have already been chronically stressed (Bhatnagar & Dallman, 1998). It was later demonstrated that administration of an OX-1R antagonist in the posterior PVT prior to each chronic stress session, but not prior to the subsequent novel stress session, attenuated the elevated HPA response to a novel stressor following chronic stress (Heydendael *et al.*, 2011). While this study concluded that orexin transmission in the PVT only during the chronic stress paradigm, and not in response to the novel stressor, was critical for mediating the HPA-axis response, the role of OX-2Rs was not examined, leaving the door open for OX-2R involvement in the PVT during periods of acute stress. Importantly, Pavlovian autoshaping procedures have been shown to increase plasma corticosterone levels (Tomie *et al.*, 2004). This is especially true for rats that have a higher frequency of lever contacts during autoshaping (Tomie *et al.*, 2000), or rats that will develop a sign-tracking phenotype (Flagel *et al.*, 2009), implicating an interaction between the stress system and sign-tracking behavior. Thus, it is possible that on the Test session during Experiment 2, the infusion procedure served as a novel stressor, which led to increased sign-tracking behavior in the vehicle control group, and this effect was blocked by OX-2R antagonism in the PVT. This explanation could be extended to the CRf session as well, with TCS OX2 29 administration blunting an increase in HPA-axis function in response to the novel task, and thus reducing responding for the incentive stimulus. Follow-up studies investigating the role of OX-2Rs in the PVT in mediating HPA-axis activity will be needed to further explore this theory.

While it was hypothesized that orexin transmission specifically at OX-2Rs is important for mediating the incentive motivational value of cues, the role of OX-1Rs was not tested. Importantly, the PVT contains both OX-1 and OX-2 receptors, and it has been hypothesized that OX-1 and OX-2 receptors may have divergent roles, due in part to their different expression patterns throughout the brain (Mieda *et al.*, 2013; Sakurai, 2014). It is unclear how this relates to the PVT, though, since distinct patterns of OX-1R and OX-2R mRNA expression in the PVT have not been identified. While the results here support the hypothesis above, it is not possible to completely rule out a functional role for OX-1Rs in the PVT in mediating incentive salience attribution. Follow up studies utilizing OX-1R specific, or dual orexin receptor antagonists, will aid in determining whether the role of orexin transmission in the PVT in incentive-salience attribution to reward-paired cues is exclusively mediated by OX-2Rs.

In conclusion, this work suggests that orexin transmission at OX-2Rs in the PVT is important for mediating behavior motivated by presentation of an incentive stimulus. In addition, a number of questions have been raised regarding the interactions surrounding PVT orexin transmission in motivated behaviors that require increased effort, as well as the interactions of orexin and stress systems in the PVT, and how these phenomena relate to sign-tracking behavior. Further work is needed to fully understand the complex role of orexin transmission in the PVT, specifically by dissecting the neural circuitry involved and any divergent roles for OX-1Rs and OX-2Rs in these behaviors.

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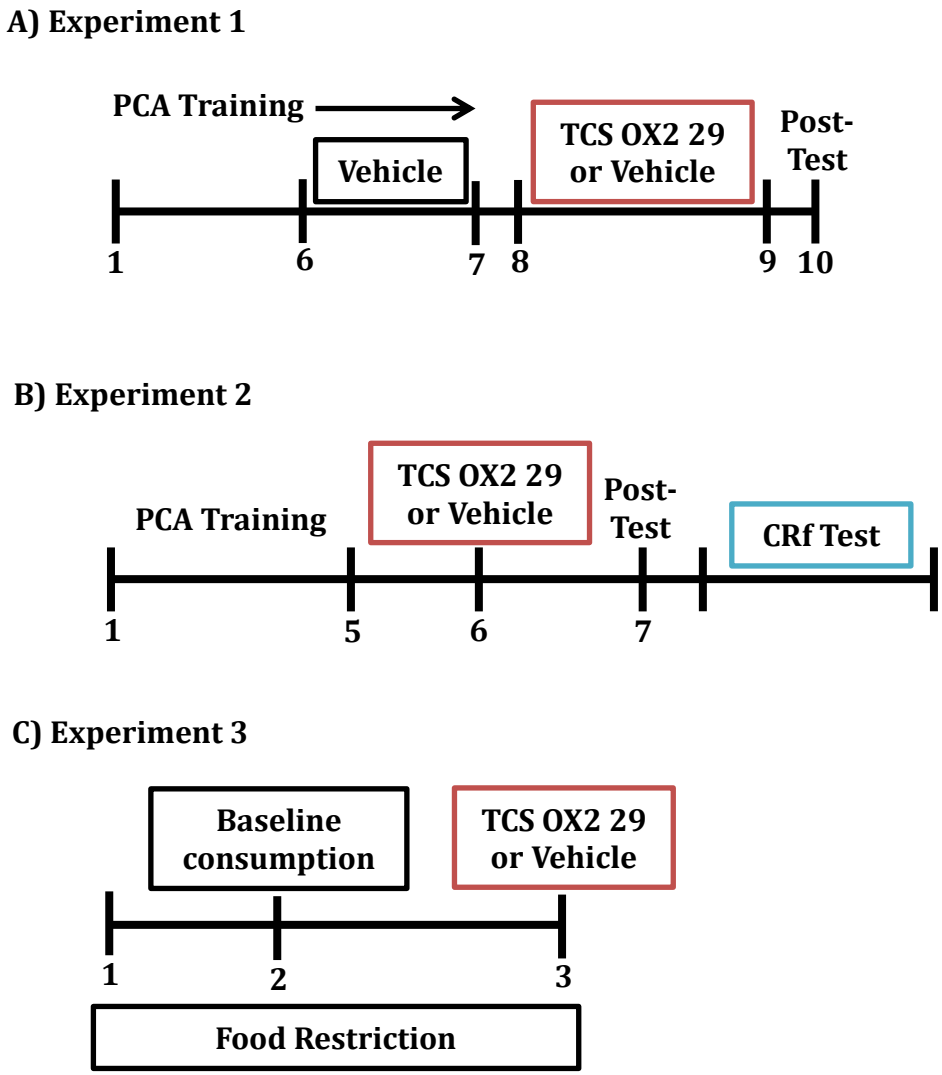

Figure 4.1. Timeline for Experiments 1, 2 and 3. Schematic timeline for A) Experiment 1(goal-tracking study), B) Experiment 2 (sign-tracking study), and Experiment 3 (feeding study). Numbers indicate session.

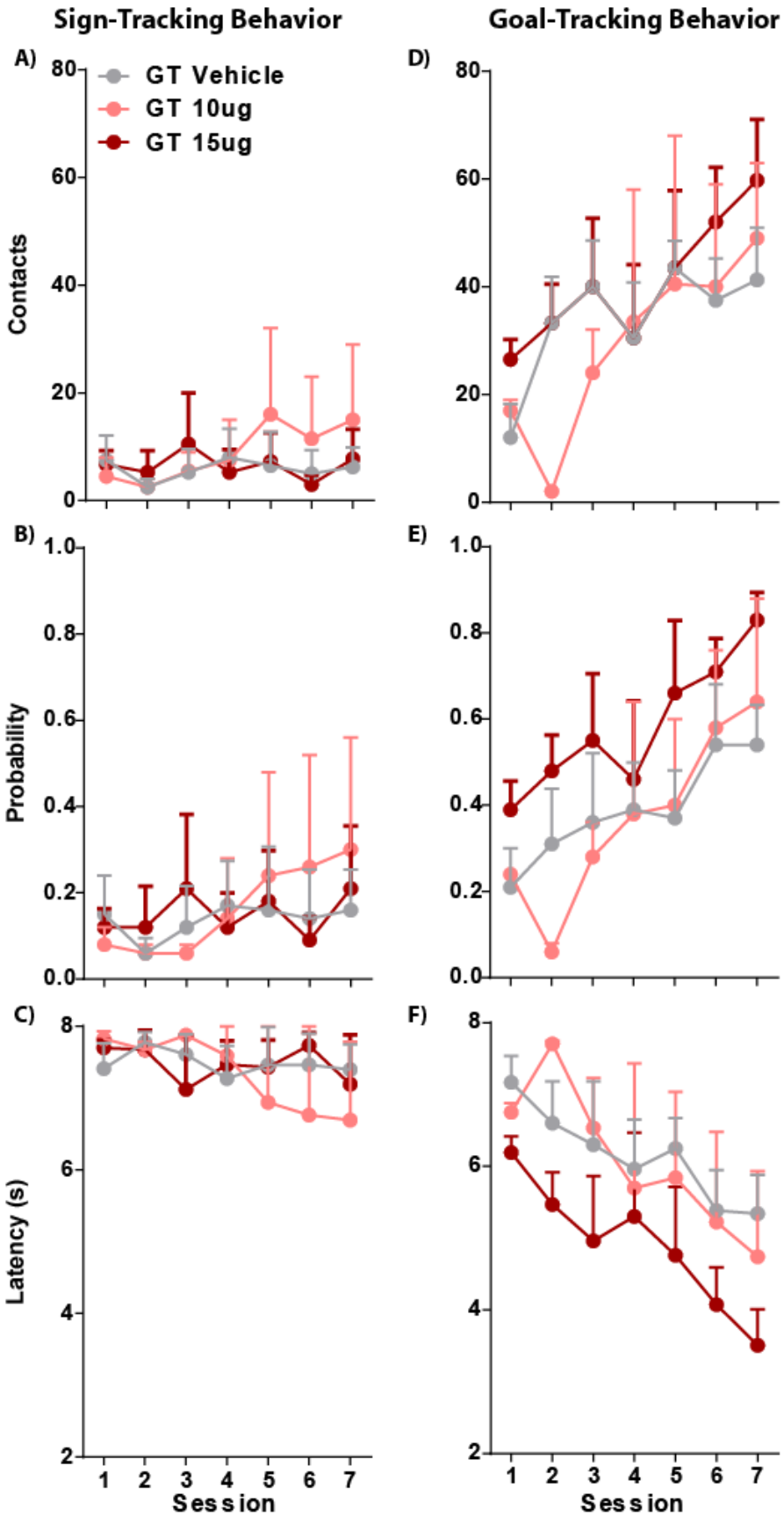
Sign-Tracking Behavior
Goal-Tracking Behavior
A)
D)
GT Vehicle
GT 10ug
GT 15ug
Contacts
B)
E)
Probability
C)
F)
Latency (s)
Session
Session

Figure 4.2. Acquisition of goal-tracking behavior across 7 sessions of Pavlovian conditioned approach training. Mean + SEM for A) lever contacts, B) probability of lever contact, C) latency to lever contact, D) food cup contacts, E) probability of food cup contact, and F) latency to food cup contacts during the CS period. Subjects acquired food cup directed behavior (D-F) in response to CS presentation during the 7 Pavlovian conditioning sessions prior to drug treatment. There were no significant differences between the treatment groups in goal-tracking behavior during this training phase.

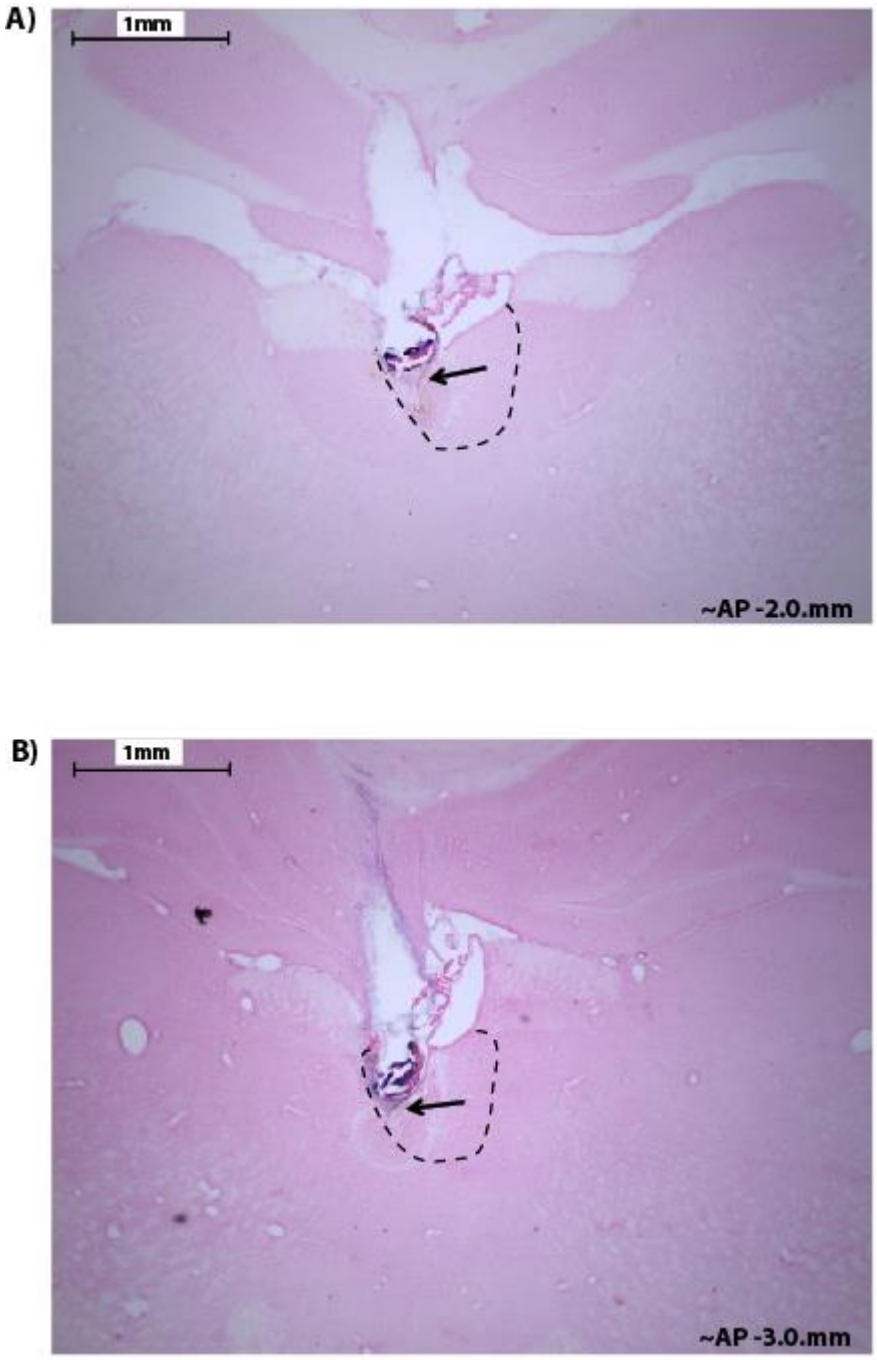

Figure 4.3 Cannula placement verification. Images showing examples of acceptable A) anterior cannula placement (approximate bregma level AP -2.0) and B) posterior cannula placement (approximate bregma level AP -3.0) within the PVT (2.5x magnification). Black arrow indicates the injector tract, evidenced by some damage/gliosis in the surrounding tissue. Dotted line represents the approximate borders of the PVT.

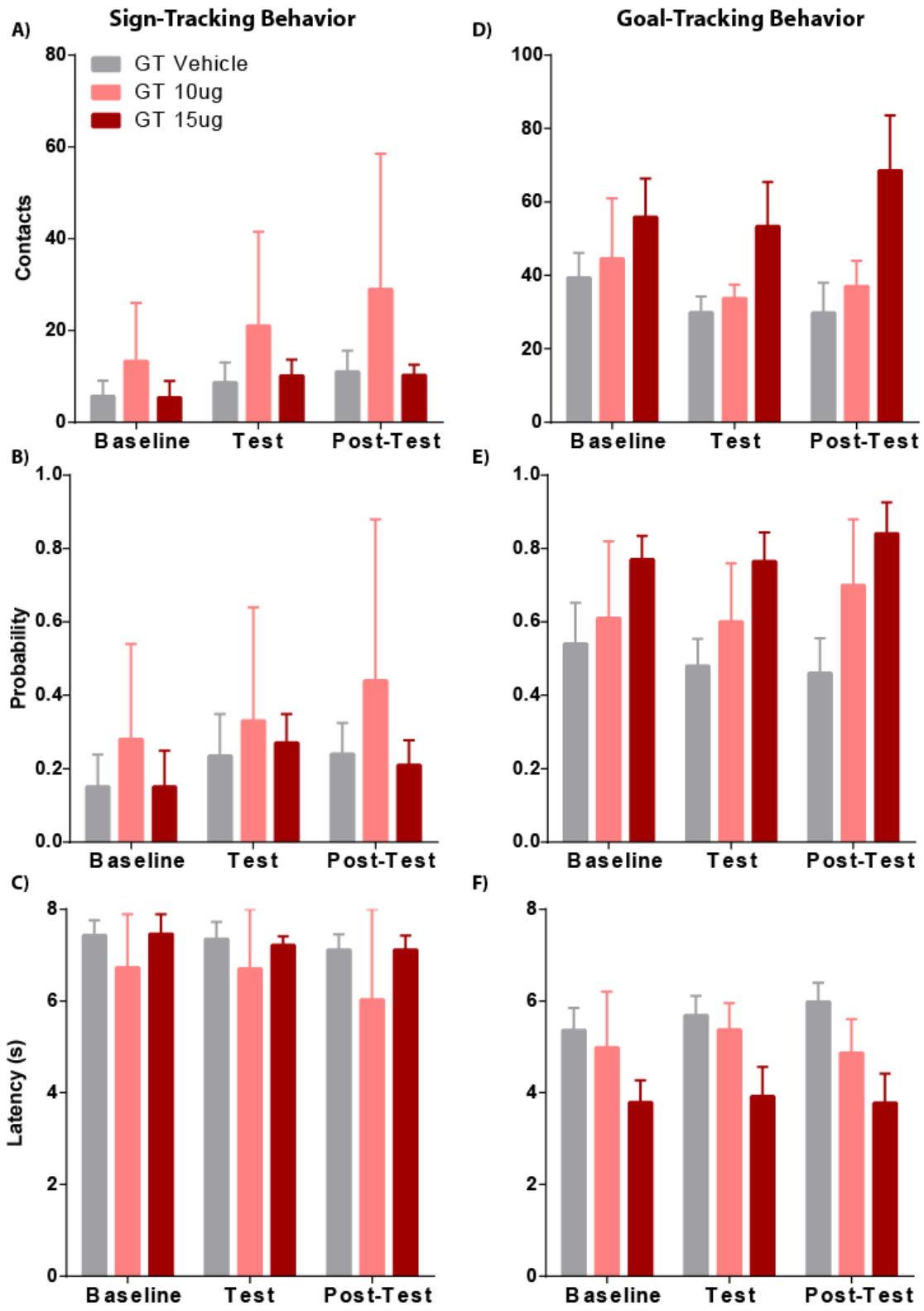
A)
Sign-Tracking Behavior
GT Vehicle
GT 10ug
GT 15ug
Contacts
Baseline
Test
Post-Test
D)
Goal-Tracking Behavior
B)
Probability
E)
C)
Latency (s)
F)

Figure 4.4. Antagonism of OX-2 receptors in the PVT does not alter Pavlovian conditioned approach behavior in goal-trackers. Baseline (average of session 6-7), test (average of sessions 8-9) and post-test (session 10) performance of Pavlovian conditioned approach behavior in GTs. Bar graphs represent mean + SEM for A) lever contacts, B) probability of lever contact, C) latency to lever contact, D) food cup contacts, E) probability of food cup contact, and F) latency to food cup contacts during the CS period. Either dose of TCS OX2 29 did not alter Pavlovian conditioned approach behavior. GT Vehicle, n = 4; GT 10ug TCS OX2 29, n = 2; GT 15ug TCS OX2 29, n = 4.

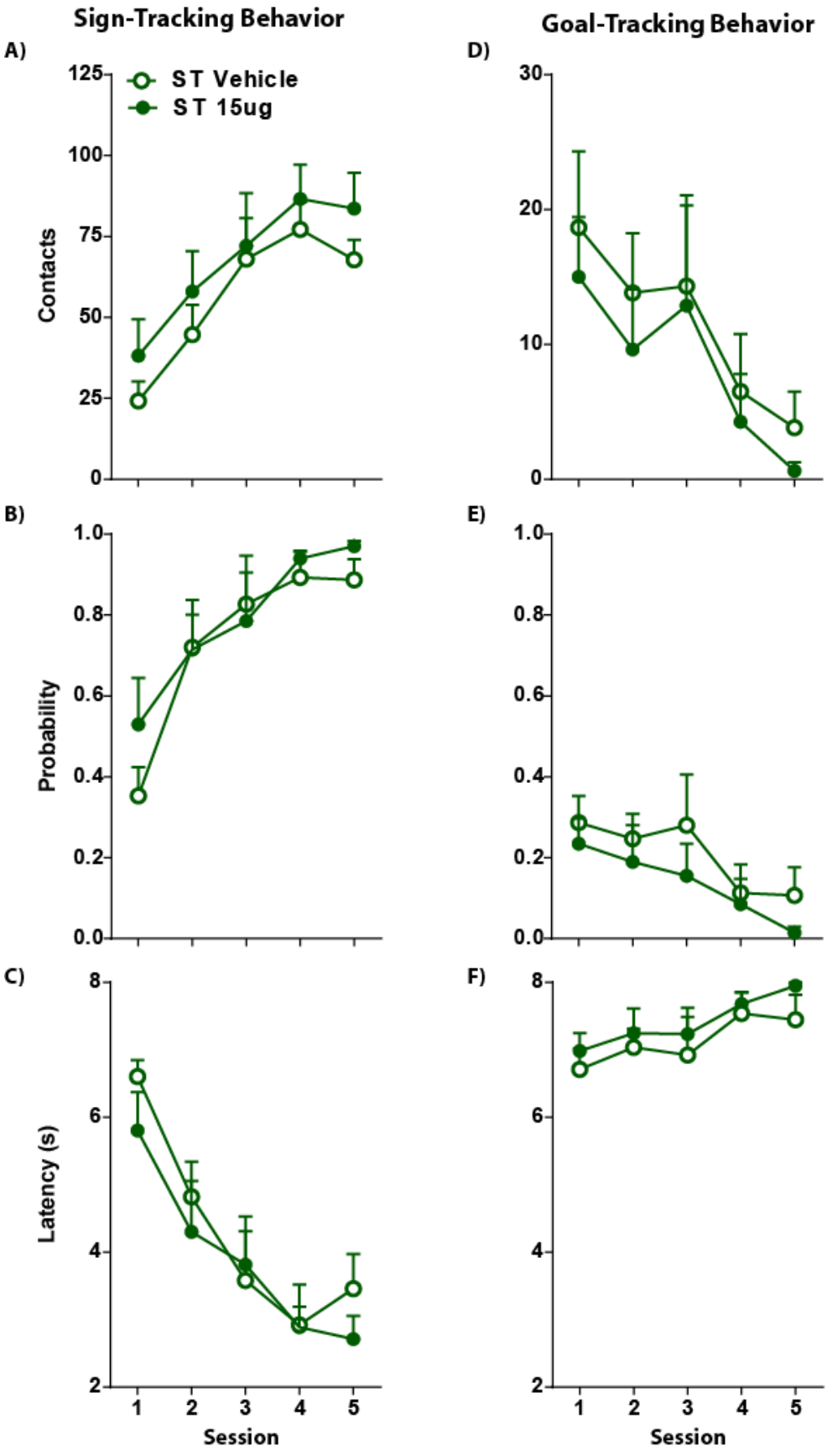
Sign-Tracking Behavior
Goal-Tracking Behavior
A)
D)
ST Vehicle
ST 15ug
Contacts
125
100
75
50
25
0
30
20
10
0
B)
E)
Probability
1.0
0.8
0.6
0.4
0.2
0.0
C)
F)
Latency (s)
8
6
4
2
1
2
3
4
5
Session

Figure 4.5. Acquisition of sign-tracking behavior across 5 sessions of Pavlovian conditioned approach training. Mean + SEM for A) lever contacts, B) probability of lever contact, C) latency to lever contact, D) food cup contacts, E) probability of food cup contact, and F) latency to food cup contacts during the CS period. Subjects acquired lever directed behavior (A-C) during the 5 Pavlovian conditioning sessions prior to drug treatment. There were no significant differences between the treatment groups in sign- or goal-tracking behavior during this training phase.

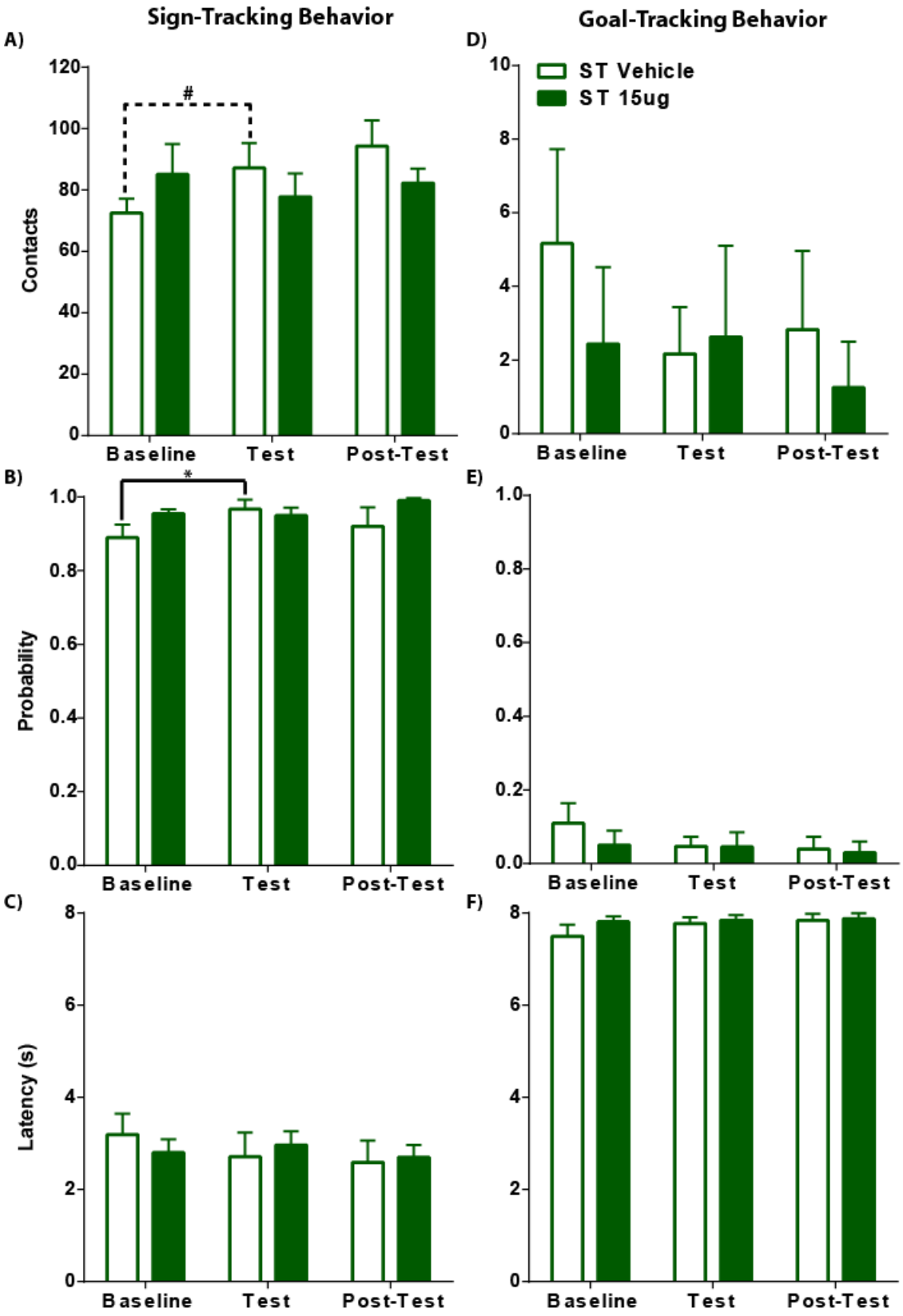
Sign-Tracking Behavior
Goal-Tracking Behavior
A)
D)
ST Vehicle
ST 15ug
Contacts
#
Baseline
Test
Post-Test
B)
E)
Probability
*
C)
F)
Latency (s)

Figure 4.6. Antagonism of OX-2 receptors in the PVT attenuates Pavlovian conditioned approach behavior in sign-trackers. Baseline (average of session 4-5), test (sessions 6) and post-test (session 7) performance of Pavlovian conditioned approach behavior in STs. Bar graphs represent mean + SEM for A) lever contacts, B) probability of lever contact, C) latency to lever contact, D) food cup contacts, E) probability of food cup contact, and F) latency to food cup contacts during the CS period. Post-hoc analyses revealed that subjects in the ST Vehicle group had a greater number of lever contacts (**p = 0.017) and probability of lever contact (*p = 0.039) on the test session compared to baseline. ST Vehicle, n = 6; ST 15ug TCS OX2 29, n = 8.

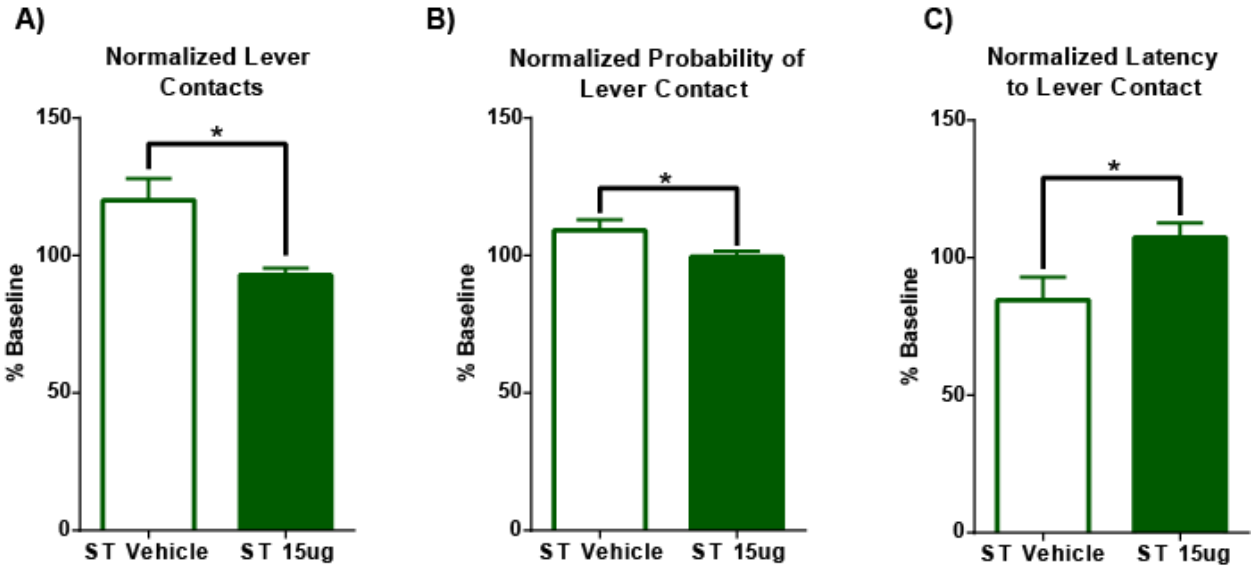

Figure 4.7. Antagonism of OX-2 receptors in the PVT attenuates the escalation of sign-tracking behavior. Mean + SEM for percent baseline [(test session / baseline)*100] for A) lever contacts, B) probability of lever contact, and C) latency to lever contact. Unpaired t-tests revealed significant differences between ST Vehicle (n = 6) and ST 15ug TCS OX2 29 (n = 8) on A) lever contacts (**p = 0.003), B) probability of lever contact (*p = 0.038), and C) latency to lever contact (*p = 0.034).

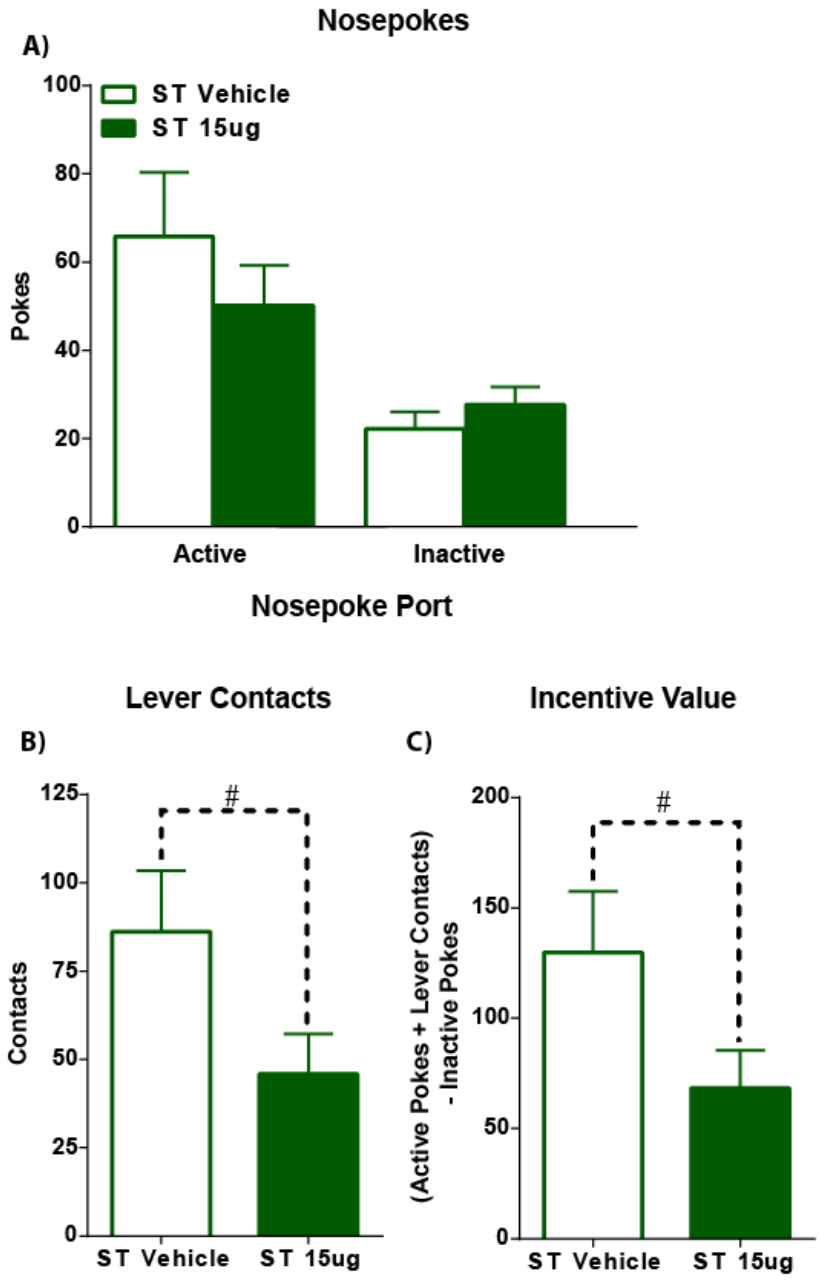

Figure 4.8. Antagonism of OX-2 receptors in the PVT may alter the conditioned reinforcing properties of the lever-CS. Mean + SEM for A) active and inactive nosepokes and B) lever contacts during the CRf test session, as well as C) the incentive value index, calculated as [(active nosepokes + lever contacts) – inactive nosepokes]. Unpaired t-tests revealed a trend towards significant differences between ST Vehicle (n = 6) and ST 15ug TCS OX2 29 (n = 8) on B) lever contacts (#p = 0.065) and C) incentive value (#p = 0.071), calculated as [(active nosepokes + lever contacts)*100].

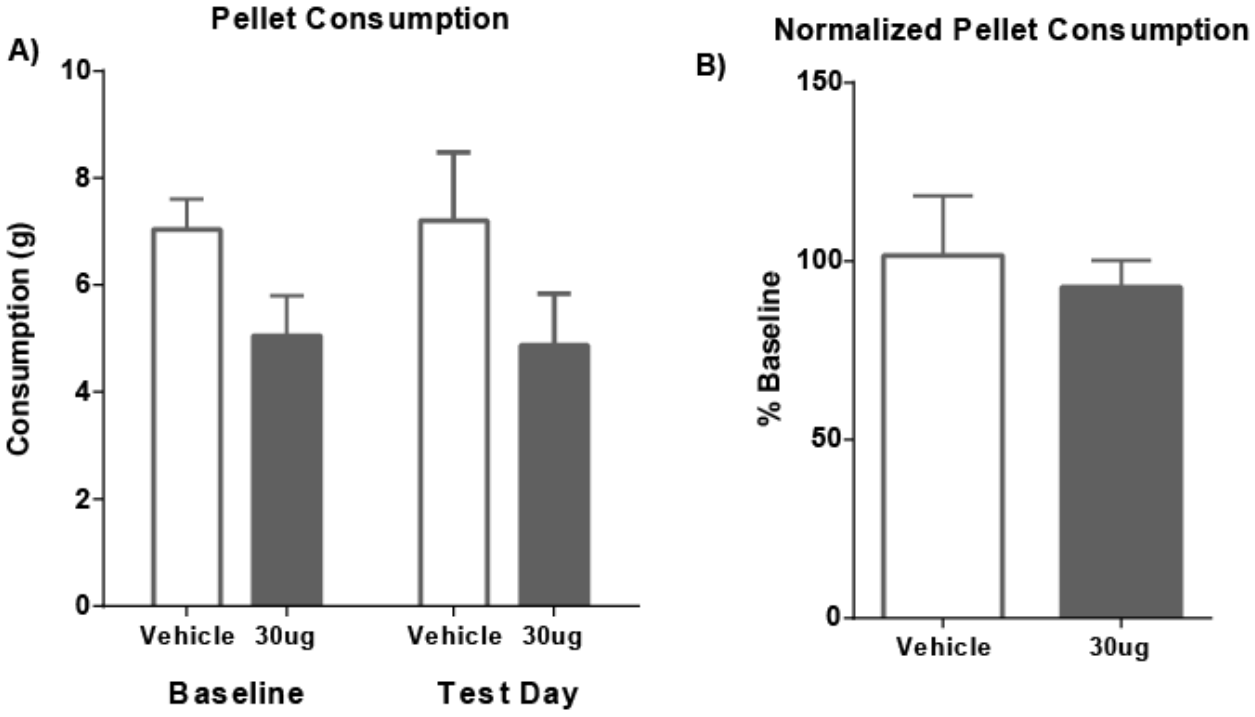

Figure 4.9. Antagonism of OX-2 receptors in the PVT does not alter feeding behavior. Mean + SEM for A) pellet consumption during the baseline and test sessions, as well as B) percent baseline [(test session / baseline)*100] pellet consumption. Vehicle, n = 5; 30ug TCS OX2 29, n = 6.

Chapter 5

General Discussion

This thesis set out to examine the role for the paraventricular nucleus of the thalamus in cue-motivated behavior. Specifically, the hypothesis that the PVT is a critical part of the neural circuitry mediating incentive salience attribution to reward-paired cues was tested. This was accomplished through a series of studies using a variety of methods, including lesions, anatomical tracing combined with c-Fos immunohistochemistry, and local pharmacology. The results from these studies have confirmed that the PVT, along with select efferent and afferent circuits, are involved in mediating sign- and goal-tracking behavior, and thus the propensity to attribute incentive salience to reward-paired cues. The results of each specific experiment are discussed in detail in the preceding Chapters, 2-4. Following is a more general discussion of the role of the PVT in mediating the propensity to attribute incentive salience to reward cues, as well as a proposed model of the PVT-circuitry that is involved.

The role of the PVT in the propensity to attribute incentive salience to reward cues: Updating the model

The primary goal of the work contained in this thesis was to establish a role for the PVT in incentive salience attribution, with the hypothesis that the PVT is critical for the acquisition and expression of sign- and goal-tracking behavior. Early work by Ann Kelley (2005) and colleagues hypothesized that the PVT was part of a hypothalamic-thalamic-striatal axis that underlies motivated behavior, including cue-motivated behavior (Kelley *et al.*, 2005). This notion was widely supported, and built upon, by studies demonstrating that the PVT is engaged during a number of motivated behaviors, including cue-motivated behavior (for review see (Martin-Fardon & Boutrel, 2012; James & Dayas, 2013; Urstadt & Stanley, 2015). This idea was then taken one step further, with work from our lab and others (Flagel *et al.*, 2011a; Yager *et al.*, 2015), demonstrating that presentation of a reward-predictive stimulus alone is not enough to

elicit c-Fos expression in the PVT, but that the cue must be attributed with *incentive salience* for it to evoke neuronal activity in this area. In addition, follow-up analyses looking at 'functional connectivity', defined here as correlated levels of c-fos mRNA across brain regions within phenotypes, revealed a strong correlation between the PVT and NAc shell in STs, indicating that sign-tracking behavior may be a product of sub-cortical processing involving the PVT and NAc (Flagel *et al.*, 2011a; Haight & Flagel, 2014). This idea is supported by the fact that subcortical dopamine transmission in the NAc is required for sign-, but not goal-tracking behavior (Flagel *et al.*, 2011b; Saunders & Robinson, 2012). For GTs, the 'functional connectivity' analyses revealed correlations between the prelimbic cortex and PVT (Haight & Flagel, 2014). This suggested to us that the goal-tracking response may be a product of top-down cognitive control. This theory had been suggested previously, since GTs are less impulsive than STs (Lovic *et al.*, 2011). In addition, GTs perform better on sustained attention tasks, and this is related to greater cholinergic release in the medial PFC (including the PrL) in GTs compared to STs (Paolone *et al.*, 2013).

The culmination of this research led us to propose a model in which the PVT is a central mediator of the propensity to attribute incentive salience to reward-paired cues, and thus sign- and goal-tracking CRs (Introduction; (Haight & Flagel, 2014). In this model, the PVT is biased towards responding to incentive stimuli via sub-cortical inputs from areas such as the hypothalamus that are known to regulate responses to reward-paired cues. The inputs from these areas synapse onto PVT projection cells to drive activity in other subcortical structures, mainly the NAc. Thus, this subcortical circuit ultimately influences dopamine transmission, a critical component of the sign-tracking response. Then, selectively for GTs, it was proposed that activity in this sub-cortical circuit is attenuated through top-down cortical inputs from the PrL to the PVT. A potential mechanism for this cortical control includes PrL afferents terminating on inhibitory post-synaptic metabotropic glutamate receptors, or presynaptic modulation of GABAergic terminals in the PVT. Using this model, it was hypothesized that lesions of the PVT would either selectively disrupt the sign-tracking response, or disrupt both sign- and goal-tracking conditioned response. Much to our surprise, however, in outbred GTs, PVT lesions led to a decrease in goal-tracking behavior and a concomitant *increase* in sign-tracking behavior, while STs remained largely unaffected by the lesion (Chapter 2;(Haight *et al.*, 2015). In support, recent work by a fellow graduate student in our lab, Brittany Kuhn, has shown that inactivation

of the PVT increases cue-induced reinstatement of cocaine-seeking behavior specifically in GTs, rendering them indistinguishable from STs during the test session. In conjunction with the lesion study results, these data suggest that overall disruption of the PVT pushes GTs to behave more like STs in response to cue presentation, and this seems to be the case for both food- and drug-paired cues. Furthermore, in agreement with the 'functional connectivity' data described above, these data support the notion that STs and GTs may be relying on separate neural circuitries that converge on the PVT, although in ways that are different from those originally hypothesized (Haight & Flagel, 2014).

The data described above have led to revisions in the working model of the PVT in sign- and goal-tracking behavior (Figure 5.1), and it is now proposed that the PVT is biased towards acting as a 'brake' on incentive salience attribution through top-down cognitive control, mediated by the PrL to PVT circuit (Haight *et al.*, 2015), and when overall PVT function is disrupted, this 'brake' is released. In support of this hypothesis, both STs and GTs exhibit greater levels of cue-evoked c-Fos protein in PVT afferents from the PrL (Chapter 3), indicating that presentation of cues that are predictive of reward delivery, but not necessarily attributed with incentive salience, engage this circuit. This also suggests that both STs and GTs have similar levels of cognitive engagement in response to cue presentation. The exact mechanism of this top down control is not known, but there are several possibilities. One possibility is that prelimbic projection neurons, which are presumed to be glutamatergic in nature (Bannister, 2005), cause inhibition of PVT neurons that project to the NAc through inhibitory post-synaptic metabotropic glutamate receptors or presynaptic modulation of GABAergic terminals. Another possibility is that prelimbic neurons excite PVT cells that project to other brain areas, such as to the medial PFC, where they innervate GABAergic interneurons that in turn cause overall inhibition of PFC cells that project to the NAc, in a form of negative feedback. While these ideas are purely speculative, they illustrate the varied ways in which the PrL to PVT circuit may be acting as a brake in incentive salience attribution, and illustrate the need for further research aimed at understanding this mechanism.

While both GTs and STs show engagement of the PrL to PVT pathway in response to cue presentation, it is further hypothesized that specifically in STs, sub-cortical inputs to the PVT override this top-down cortical control. This subcortical drive would then, presumably, engage PVT cells projecting to the NAc, allowing a reward-paired stimulus to be attributed with

incentive salience. This aspect of the model is also supported by the data described in Chapter 3, in which presentation of an incentive stimulus leads to increased engagement of dorsomedial/lateral hypothalamic, as well as medial amygdala, inputs to the PVT, and PVT efferents projecting to the NAc. Additional support for this model is found in Chapter 4, where blocking orexin transmission at OX-2 receptors in the PVT has a tendency to attenuate sign-tracking behavior, as well as the conditioned reinforcing properties of an incentive stimulus, without affecting goal-tracking behavior. This view is also congruent with the lesion data, based on the assumption that the contributions of the subcortical inputs to the PVT, as well as PVT efferents to the NAc, are necessary for incentive salience attribution only when the PVT is intact and functioning as a 'brake'. Once the PVT is lesioned or inactivated, the overall brake is released, and a behavioral shift towards sign-tracking is possible, presumably mediated by other pathways that support the attribution of incentive salience to reward-paired cues, such as dopaminergic transmission from the ventral tegmental area to the NAc. Importantly, this is only the start of our understanding of the specific role of the PVT in incentive salience attribution. Following is a detailed discussion of specific afferent and efferent pathways of the PVT, and how they contribute (hypothetically at times) to incentive salience attribution in light of this revised working model. Future work utilizing sophisticated functional anatomy techniques, including Designer Receptors Exclusively Activated by Designer Drugs (DREADDs) and optogenetics, as well as specific genetic targeting of neuronal populations such as dopamine, using transgenic (i.e. TH:cre) rat lines, will be integral in further exploring the proposed model.

The role of specific PVT afferents in sign- and goal-tracking behavior

Medial prefrontal cortical afferents to the PVT

The PVT receives its densest afferents from the medial prefrontal cortex, specifically the PrL and IL (Li & Kirouac, 2012). For some time it was thought that the PrL and IL function in opposing roles in motivated behavior, but this dichotomy has been recently called into question (Moorman *et al.*, 2015), as it seems that the PrL and IL can function in a similar capacity (Moorman & Aston-Jones, 2015). In this thesis, the activation of both the PrL and IL in response to reward-predictive and incentive stimuli was examined (Chapter 3). Similar to previous work (Flagel *et al.*, 2011a; Yager *et al.*, 2015), presentation of a reward-predictive or incentive stimulus did not lead to differences in overall c-Fos expression in the PrL and IL compared to

unpaired controls, suggesting that these brain regions are not involved in sign- and goal-tracking behavior. Here, this analysis was taken one step further, by looking at c-Fos expression specifically in PVT afferents from the PrL and IL. With this analysis there were no differences in the engagement of specific IL afferents to the PVT following the presentation of reward-predictive or incentive stimulus, suggesting that the IL to PVT pathway is not part of the reward-seeking circuitry. However, a reward-predictive stimulus was sufficient to engage PrL afferents to the PVT, since both sign- and goal-trackers had increased c-Fos expression in this pathway relative to unpaired controls. These data suggest that PrL afferents to the PVT are part of the circuitry that promotes reward-seeking behavior, potentially by sending information about the predictive qualities of the cue to the PVT.

These data are congruent with the hypothesis laid out in the revised model, i.e. that the nature of the PrL to PVT pathway in reward-seeking behavior seems to be one of top-down control, generally acting as a brake on incentive salience attribution, and instead promoting a more goal-directed learning strategy. This top-down control signal is in direct competition with bottom-up signals coming from sub-cortical areas such as the hypothalamus and medial amygdala, which appear to be stronger in subjects that attribute incentive salience to reward-paired cues (Chapter 3). Recent data from our laboratory further supports this hypothesis (Covelo *et al.*, 2015). By using a novel flex-DREADD technique, in which DREADD receptors are expressed specifically in the PrL to PVT pathway, the functional role of this pathway in sign- and goal-tracking behavior could be investigated. In GTs, 'turning off' the PrL to PVT pathway, via stimulation of inhibitory DREADD receptors, led to an increase in lever-directed behavior during PCA training. In contrast, in STs, 'turning on' this top-down control, by activating excitatory DREADD receptors in the PrL to PVT pathway, led to an increase in food-cup-directed behavior. These data, together with the proposed model, suggest that the PrL to PVT pathway is working in a 'push-pull' relationship with sub-cortical afferents to the PVT, which show greater engagement in STs compared to GTs (Chapter 3). That is, in STs, there exists a sub-cortical drive that normally overrides the top-down cortical control of the PVT, and promotes sign-tracking behavior. However, activation of the PrL-PVT pathway in these individuals allows the top-down control aspect to better compete with the subcortical drive, which ultimately leads to an increase in goal-tracking behavior. In contrast, in GTs, inhibition of the PrL to PVT pathway allows the weaker sub-cortical drive in these animals to override the

top-down control from the now attenuated PrL pathway, leading to an increase in sign-tracking behavior. Further research using DREADD receptors expressed simultaneously in both the PrL to PVT pathway, as well as select sub-cortical afferents to the PVT, will allow us to further test this hypothesis and refine the working model of the PVT in sign- and goal-tracking behavior.

Of note, the PrL to PVT pathway has been shown to be critical for other motivated behaviors, specifically processing of fear cues and retrieval of fear memories. A recent study by Do-Monte and colleagues (2015) demonstrated that inhibition of the PrL to PVT pathway attenuates freezing behavior in response to a fear-conditioned tone cue. Interestingly, this attenuation was observed at 7 days, and not 6 hours, after fear conditioning (Do-Monte *et al.*, 2015), suggesting that the PrL to PVT pathway is necessary for the retrieval of a long-term, but not short-term fear memory. Whether the PrL to PVT pathway functions in a similar manner during appetitive conditioning remains to be determined. It is possible that the role of the PrL to PVT pathway described in the experiments above is also limited to retrieval of long-term appetitive memories, since all PrL testing (c-Fos quantification, DREADD manipulation) was performed following multiple days of PCA training. Future experiments could explore this possibility by manipulating the PrL to PVT pathway following a single PCA training session, to see if this pathway is also involved in the acquisition of appetitive conditioning, or if this pathway is only involved in the later stages (expression) of PCA training. It is important to note, though, that these types of studies are difficult to interpret, since it is difficult to know what CR an individual will develop following a single PCA training session.

In sum, the results contained in this thesis, combined with those in literature, suggest that the PrL to PVT pathway plays a role in reward-seeking behavior. Furthermore, this pathway is engaged by a food-predictive, and not necessarily an incentive, stimulus. Based on these data, it is hypothesized that the PrL to PVT pathway is involved in attenuating sign-tracking behavior by acting as a 'brake' on incentive-salience attribution through top-down cortical control, and that sub-cortical afferents to the PVT can override this control, leading to the attribution of incentive salience to reward cues and thereby the development and expression of a sign-tracking phenotype.

Subcortical afferents to the PVT

Hypothalamus The PVT receives a dense set of afferents from a heterogeneous group of thalamic nuclei, including but not limited to the A13 cell group, the dorsomedial nucleus, the ventromedial nucleus, and the lateral hypothalamic area. The molecular identities of these inputs have been heavily explored (Lee *et al.*, 2015; Urstadt & Stanley, 2015), and they include orexin, dopamine, cocaine-and-amphetamine-regulating-transcript (CART), dynorphin, NPY, and corticotrophin releasing factor (CRF) (Otake & Nakamura, 1995; Kirouac *et al.*, 2005; 2006; Parsons *et al.*, 2006; Marchant *et al.*, 2010; Li *et al.*, 2014; Lee *et al.*, 2015). Early evidence also demonstrated the presence of melatonin concentrating hormone (MCH) receptors in the PVT (Saito *et al.*, 2001), but it has since been shown that there are minimal MCH terminals in the PVT (Lee *et al.*, 2015), suggesting that this peptide has a negligible role in PVT function (Urstadt & Stanley, 2015).

The role of orexin transmission in the PVT has already been discussed in depth (Chapter 4), and will only be discussed briefly here. Our work demonstrated that presentation of an incentive, but not a predictive stimulus engages hypothalamic afferents from the dorsomedial/lateral hypothalamus to the PVT (Chapter 3). Importantly, the PVT receives orexinergic input from these areas of the hypothalamus (Kirouac *et al.*, 2005; Lee *et al.*, 2015), and orexin is known to play a role in cue-motivated behavior (Thompson & Borgland, 2011; Mahler *et al.*, 2012; Mahler *et al.*, 2014). Orexinergic-positive fibers are also found in close proximity to PVT cells that project to the NAc (Parsons *et al.*, 2006; Lee *et al.*, 2015). This led us to hypothesize that orexinergic transmission from the hypothalamus to the PVT was critical for incentive salience attribution to reward-paired cues, and thus sign-tracking behavior. Presumably, this circuit functions by overriding 'top-down' cortical control input from the PrL to the PVT, allowing for the animals to attribute incentive salience to a reward-paired cue through 'bottom-up' processing. We directly tested this hypothesis (Chapter 4), and found that blocking OX-2 receptors in the PVT attenuated the incentive-motivational value of a reward-paired cue. Importantly, this study did not rule out an additional role for orexin receptor 1 (OX-1) in these behaviors. While one previous study demonstrated that OX-1 receptor antagonism in the PVT did not prevent cue-induced reinstatement of cocaine seeking, this study limited its infusion to one location in the center of the PVT (James *et al.*, 2011). The PVT is a long structure, and both orexin receptors are expressed throughout the anterior-posterior axis (Trivedi *et al.*, 1998;

Marcus *et al.*, 2001). In addition, blocking OX-1Rs in the posterior PVT has been shown to affect stress-related behavior (Heydendael *et al.*, 2011), which may interact with sign-tracking behavior (Tomie *et al.*, 2000; Tomie *et al.*, 2004; Flagel *et al.*, 2009). Thus, it is possible that activity at OX-1Rs in other PVT locations might also play a role in incentive-salience attribution, and this possibility warrants further investigation.

Discovering the exact location of the orexinergic cell bodies projecting to the PVT that are influencing sign-tracking behavior might also aid in our understanding of their role in incentive salience attribution. Previous work has begun to demonstrate that there is a possible dichotomy in orexin function based on neuroanatomical location (Harris & Aston-Jones, 2006), with orexinergic neurons in the dorsomedial and perifornical regions of the hypothalamus involved in arousal and feeding, and orexinergic neurons in the lateral hypothalamus involved more broadly in reward-based behaviors. It has been argued, though, that more evidence is needed before this distinction is clearly drawn (Sakurai, 2014). If there is indeed a distinction in orexinergic function based on anatomical location, it is possible that cells in either population could be influence sign-tracking behavior, either through increasing arousal states in response to an incentive stimulus, or by driving the incentive aspects of the stimulus itself. Further anatomical work could be used to determine the exact location of orexinergic neurons that project to the PVT that are active in response to presentation of an incentive stimulus. Unfortunately, it will be hard to functionally assess their role, since specific orexinergic manipulations are difficult due to the heterogenous cellular nature of the hypothalamus. One possibility would be to use a transgenic model with cre recombinase expressed selectively in orexin neurons, which would allow for selective expression of DREADD or optogenetic receptors in specific orexinergic cell populations following local virus injections into the dorsomedial/perifornical hypothalamus, or the lateral hypothalamic area. While an orexin-cre rat line is not widely available, an orexin-cre mouse line has been established (Matsuki *et al.*, 2009). Importantly, a recent report has been able to differentiate sign- and goal-tracking in mice (Campus *et al.*, 2016). Combining these approaches would allow for a dissection of specific orexinergic inputs to the PVT, and their role in sign-tracking behavior.

Another subcortical input of interest to the PVT is dopamine. It was originally thought that this input was coming from the VTA (Takada *et al.*, 1990), but is has since been demonstrated that a large proportion of this input arises in the hypothalamus, including the A13

cell group (Sánchez-González *et al.*, 2005; Li *et al.*, 2014). Little is known about dopamine transmission in the PVT, other than the fact that the PVT preferentially expresses the D3 dopamine receptor (Mansour & Watson, 1995). Previous work has demonstrated that the D3 receptor is important for cue-motivated behavior (Khaled *et al.*, 2010), and imaging studies in humans has shown that dopamine transmission in the thalamus may be related to activation of the medial orbital PFC, which is significantly correlated with drug craving (Volkow *et al.*, 2005). This led us to hypothesize that dopamine transmission in the PVT is another one of the sub-cortical afferents needed to override the top-down cognitive control coming from the PrL to the PVT (Introduction). Interestingly, in Chapter 3, we did not observe differences in overall c-fos expression in the A13 cell group, nor did we observe differences in c-fos expression specifically in A13 cells that project to the PVT. While these data indicate that the A13 cell group might not be involved in mediating sign- or goal-tracking behavior, it does not rule out a potential role for dopamine in the PVT in these behaviors. Aside from the A13 cell group, tyrosine hydroxylase-positive PVT afferents can be found heterogeneously distributed throughout other parts of the hypothalamus, as well as the periaqueductal gray (Sánchez-González *et al.*, 2005; Li *et al.*, 2014). In order to more completely assess the role of PVT dopamine in PCA behavior, future studies using local pharmacological experiments could be conducted, taking advantage of the fact that the PVT predominately expresses D3 receptors (Mansour & Watson, 1995; Haight & Flagel, 2014). In addition, rats expressing cre recombinase under the control of tyrosine hydroxylase (TH:cre rats), a marker of dopaminergic neurons, are becoming more widely available. These rats could be used to specifically manipulate hypothalamic dopamine transmission to the PVT, by injecting a cre-dependent optogenetic virus into the hypothalamus, and then targeting a laser at the terminals of these neurons in the PVT. This would allow for specific activation or inhibition of TH-positive cells that project to the PVT during PCA testing, leading to an increased understanding of the role of dopamine in the PVT in mediating incentive salience attribution.

The role of CART in the PVT has also gained some attention in the literature, although its specific role in cue-motivated behavior has yet to be established. CART innervation of the PVT arises primarily in the hypothalamic arcuate nucleus, along with sparser innervation from the lateral hypothalamus, zona incerta, and paraventricular hypothalamus (Kirouac *et al.*, 2006; Lee *et al.*, 2015). Slice electrophysiology studies have shown that administration of CART

peptides reduces the excitability of anterior PVT neurons, but this was not tested on posterior PVT cells (Yeoh *et al.*, 2014). Consistent with the idea that CART decreases excitability of PVT neurons, it was demonstrated that administration of CART directly into the PVT attenuates cocaine-primed reinstatement of drug-seeking behavior (James *et al.*, 2010). Interestingly, CART positive fibers, along with orexin positive fibers, are often found in close proximity to PVT cells that project to the NAc (Parsons *et al.*, 2006; Lee *et al.*, 2015). It is thought-provoking that PVT cells projecting to the NAc would receive excitatory transmission from orexin neurons, while simultaneously receiving inhibitory input from CART neurons. This suggests that orexin and CART are potentially acting in opposing roles in the PVT, at least on cells that project to the NAc. If this is true, it is possible that animals that develop a goal-tracking phenotype have greater activation of CART positive cells that project to the PVT, while sign-trackers have greater engagement of orexin cells, as proposed above.

There are a number of other hypothalamic inputs to the PVT of potential interest, but minimal research has been conducted on the functional role of these transmitters/peptides in the PVT in motivated behavior. One is neuropeptide-Y (NP-Y). NP-Y cells that project to the PVT are found exclusively in the arcuate nucleus of the hypothalamus (Lee *et al.*, 2015). Similar to CART and orexin, NP-Y positive fibers are also found in close proximity to PVT cells that project to the NAc. While NP-Y is considered an orexinergic peptide, having similar effects as orexin itself, CART is considered anorexinergic (Lee *et al.*, 2015). Thus, it is possible that orexin and NP-Y cells combined are working in opposition to CART in the PVT. This is hypothetical, though, and further work is needed to assess the roles of these peptides in the PVT in cue-motivated behavior.

Another transmitter of interest is dynorphin, which originates in part from mediodorsal hypothalamic cells that project to the PVT (Marchant *et al.*, 2010). Dynorphin binds to PVT cells expressing kappa opioid receptors, and decreases neuronal excitability (Chen *et al.*, 2015). One functional study observed that administration of a kappa opioid receptor agonist into the PVT attenuated context-induced reinstatement of alcohol seeking behavior (Marchant *et al.*, 2010), indicating a potentially similar inhibitory role as CART peptides in the PVT (James *et al.*, 2010). It is unknown whether this pathway serves a similar role for discrete cue-induced reinstatement of reward-seeking behavior. This warrants further investigation, especially in relation to sign- and goal-tracking behaviors.

Neurons containing corticotrophin releasing factor (CRF) also innervate the PVT. These cells bodies originate in various brain regions, including the lateral and paraventricular hypothalamic nuclei (Otake & Nakamura, 1995). Due to its known role in the stress response (McEwen *et al.*, 2015), the potential interactions between CRF, sign-tracking, HPA-axis acivity and the PVT (as described in Chapter 4), the almost unknown functional role of these inputs warrants further investigation.

In sum, the hypothalamus is a diverse source of inputs to the PVT, and the complicated ways in which these inputs interact in the PVT is far from wholly understood. While our data suggests a role for orexinergic transmission in the PVT in mediating incentive salience attribution, the functional role of the other transmitters described above have yet to be confirmed. Interestingly, some of these peptides appear to function in opposing roles, and PVT cells that project to the NAc seems to be a sight of convergence for many of these distinct transmitter systems, indicating a complex role for this circuitry in motivated behavior.

Amygdala Previous studies have identified afferents to the PVT arising from the CeA and MeA (Chen & Su, 1990; Canteras *et al.*, 1995; Van der Werf *et al.*, 2002; Li & Kirouac, 2012). Here, we confirmed these studies using PVT injections of the retrograde tracer Fluorogold (Chapter 3). Previous work also suggested that the CeA is involved in sign-, but not goal-tracking behavior, since presentation of incentive stimuli are able to evoke greater c-fos expression in this area (Yager *et al.*, 2015). This led us to hypothesize that CeA inputs to the PVT are part of the sub-cortical input that is driving incentive salience attribution to reward-paired cues. This hypothesis was assessed in Chapter 3. Similar to the previous work, we observed a trend towards an overall increase in c-fos expression in STs in the CeA, indicating that this nucleus is likely engaged by presentation of incentive stimuli. Contrary to our hypothesis, however, we observed minimal activation of cells in the CeA communicating with the PVT in response to either reward-predictive *or* incentive stimuli. These results indicate that, while the CeA is possibly mediating incentive salience attribution, it is doing so through neural circuitry independent of its projections to the PVT. To our surprise, though, we did observe increased activity in MeA afferents to the PVT following cue presentation in STs, compared to GTs and unpaired controls, identifying a novel pathway involved in incentive salience attribution (discussed in detail in Chapter 3). It is likely that these amygdalar afferents are combining with other sub-cortical

afferents from the hypothalamus in the PVT, to override the top-down PrL input and mediate sign-tracking behavior. Future studies utilizing chemogenetic techniques will greatly advance our understanding of this circuit and its role in mediating incentive salience attribution. Specifically, utilizing different combinations of DREADD receptor expression in both MeA and hypothalamic inputs simultaneously will enable us to directly test whether either one of these inputs alone is enough to drive sign-tracking behavior, or if they are both required.

Ventral subiculum Ventral subiculum inputs from the hippocampus to the PVT were also examined in Chapter 3. It was originally hypothesized that this circuit would be engaged by presentation of an incentive stimulus, due to the known role of the ventral subiculum in cue-motivated behavior (Sun & Rebec, 2003; Kufahl *et al.*, 2009). Contrary to our hypothesis, however, presentation of the reward-predictive or incentive cue did not lead to increased engagement of this circuit. This brings about an interesting possibility that was briefly discussed in Chapter 2: that the ventral subiculum to PVT pathway might be more involved in mediating context-motivated behaviors. Both the PVT and the ventral subiculum have been shown to be critical for other context-motivated behaviors, including context-induced reinstatement of drug-seeking behavior (Hamlin *et al.*, 2009; Bossert & Stern, 2014). In addition, recent work has demonstrated that animals who exhibit a goal-tracking phenotype might be more susceptible to context-induced reinstatement of reward-seeking behavior, compared to STs (Robinson *et al.*, 2014; Saunders *et al.*, 2014). This brings about the possibility that the ventral subiculum to PVT pathway might be more involved in context-motivated behavior, compared to discrete cues. It follows, then, that this circuit would be more active in GTs, compared to STs, during context-induced reinstatement testing. This line of research warrants attention and will be especially important for furthering our understanding of the PVT in discrete cue- vs. context-mediated behaviors.

Other inputs The PVT receives a number of other inputs that may be important circuits for mediating incentive salience attribution to reward-paired cues. They include inputs from the periaqueductal gray, dorsal raphe nucleus, and bed nucleus of the stria terminalis (Van der Werf *et al.*, 2002; Hsu & Price, 2009; Li & Kirouac, 2012; Li *et al.*, 2014), areas known to be involved in emotional behaviors. A couple studies have suggested that PVT inputs from the

periaqueductal gray and/or dorsal raphe may be important for response to acute stress (Bhatnagar *et al.*, 2000; Otake *et al.*, 2002), but little is known about the role PVT inputs from the bed nucleus of the stria terminalis. While a detailed discussion of the role of these various inputs would be purely speculative at this point, as research surrounding the neural circuitry of the PVT increases in popularity, these pathways may prove to be interesting candidates for investigation in sign- and goal-tracking behaviors.

Summary: PVT afferents The PVT receives a diverse array of inputs from cortical and sub-cortical structures, many of which have been implicated in motived behavior, including incentive salience attribution to reward-paired cues. While this thesis has increased our knowledge about specific sub-circuits of the PVT, this realm of research is still in its infancy. Future research further dissecting both the anatomical inputs to the PVT, as well as the effects of specific transmitters and peptides on PVT neurons, and how these circuits relate to cue-motivated behavior promises to be a fruitful and interesting for years to come.

The role of specific PVT efferents in sign- and goal-tracking behavior.

Nucleus accumbens The NAc, divided into the core and shell sub regions, has a long and rich history in motivated behavior (for review see (Floresco, 2015; Salamone *et al.*, 2016). Much of the research surrounding the role of the NAc in motivated behavior has focused on the role of dopamine, and has demonstrated time and again that dopamine transmission in this brain area is critical for many different types of motivated behavior, including the conditioned properties of rewards (Baik, 2013). The PVT sends dense projections to the NAc shell, and to a lesser extent NAc core (Moga *et al.*, 1995; Li & Kirouac, 2008; Vertes & Hoover, 2008), implicating the involvement of the PVT in motivated behaviors through its projections to the NAc. Early work investigating the physiological properties of the PVT to NAc pathway revealed that chemical excitation of PVT cell bodies increased the concentration of dopamine metabolites in the NAc, indicating that the PVT influences dopamine release (Jones *et al.*, 1989). This was later confirmed by Parsons and colleagues (2007), who observed that electrical excitation of PVT cells leads to dopamine release in the NAc independent of ventral tegmental area activity. These results confirmed that the PVT can directly cause release of dopamine, and it was hypothesized that this was through presynaptic modulation of VTA terminals in the NAc (Parsons *et al.*,

2007). In addition, this effect is at least partially mediated by orexin transmission, since administration of orexin-A into the PVT leads to increased levels of dopamine in the NAc (Choi *et al.*, 2012). This data, combined with the fact that sign-tracking, but not goal-tracking behavior is dependent on dopamine transmission in the NAc, led us to hypothesize that the PVT to NAc pathway is critical for incentive salience attribution to reward-paired cues, and thus sign-tracking behavior. In support of this model, we observed greater engagement of posterior PVT efferents to the NAc in response to cue presentation in STs, but not GTs, indicating that this circuit is activated selectively by the presentation of an incentive stimulus. Presumably, PVT efferents to the NAc are causing increased dopamine release in the NAc in response to incentive stimuli, leading to sign-tracking behavior.

Recent studies investigating the role of the PVT to NAc pathway have added several layers of complexity to the role of this circuit in motivated behavior, and thus revisiting the hypothesis above is necessary. While it was originally hypothesized that the PVT to NAc circuit exerts its influence through presynaptic dopamine modulation, it has also been shown that PVT efferents to the NAc mediate a number of different behaviors through direct interactions with post-synaptic NAc medium-spiny neurons (MSNs) (Labouebe *et al.*, 2016; Neumann *et al.*, 2016; Zhu *et al.*, 2016), but not cholinergic interneurons in the NAc (Ligorio *et al.*, 2009). Some of these studies support the hypothesis that PVT inputs to the NAc drive reward seeking, and possibly incentive-salience attribution. For example, it was demonstrated that the PVT to NAc pathway mediates cocaine-seeking behavior, and it is believed that this is facilitated, in part, by synaptic connections with MSNs in the NAc shell (Neumann *et al.*, 2016). In addition, activation of PVT to NAc projection neurons that contain the glucose transporter Glut2 increased the motivation to obtain a sucrose reward on a progressive ratio (PR) schedule of responding, where increased responding is required over time to obtain each subsequent reward, and this too is believed to be mediated by PVT input to NAc MSNs (Labouebe *et al.*, 2016). In contrast to these appetitive behaviors, however, recent work has demonstrated that stimulation of the PVT to NAc pathway using optogenetics leads to the development of conditioned place avoidance in mice, and that this effect was mediated by PVT input onto MSNs in the NAc, and was independent of dopamine transmission (Zhu *et al.*, 2016). These findings demonstrate that the PVT pathway can carry aversive, as well as appetitive, information through direct inputs to NAc MSNs.

The culmination of this work supports the notion that PVT efferents to the NAc can affect NAc activity in distinct ways, by modulating dopamine terminals (Jones *et al.*, 1989; Parsons *et al.*, 2007; Choi *et al.*, 2012) or by activating post-synaptic medium spiny neurons (Labouebe *et al.*, 2016; Neumann *et al.*, 2016; Zhu *et al.*, 2016). Thus, it is possible that specific neuronal populations of PVT efferents to the NAc are mediating different aspects (appetitive vs. aversive) of motivated behavior. In our studies, only a small population of PVT efferents to the NAc was active in response to presentation of an incentive stimulus (Chapter 3). Hypothetically, these neurons are also expressing OX-2 receptors (Chapter 4), and synapse onto DA terminals in the NAc, where they modulate dopamine levels, thus mediating sign-tracking behavior and incentive salience attribution. On the other hand, it may be the case that a separate set of PVT neurons, possibly containing other receptor subtypes (OX-1, CART, etc.) and projecting to specific MSN populations may mediate the aversive response observed in Zhu et al. (2016).

The anatomical location of the PVT cell populations that are innervating the NAc, as well as the region of NAc innervation, may also play a role in mediating these different behaviors. While both anterior and posterior aspects of the PVT project heavily to the NAc shell, the posterior PVT has been shown to have a denser projection to the NAc core than the anterior PVT (Li & Kirouac, 2008). Importantly, it was the posterior PVT efferents to the NAc that were engaged by incentive stimulus presentation (Chapter 3). In addition, it was in the NAc core where it was observed that dopamine transmission was critical for sign-tracking behavior (Saunders & Robinson, 2012), indicating that it might be posterior PVT cells projecting to the NAc core that were engaged in response to incentive stimulus presentation. In Zhu et al. (2016), optical stimulation was targeted above the medial shell of the NAc, and not the core, indicating that the PVT to NAc shell pathway is responsible for aversive behavior. Thus, it is possible that the region of NAc innervated by the PVT may also influence the behavioral role of the PVT to NAc connection. More research into the specific sub-circuits in the PVT to NAc pathway will indeed be helpful in substantiating these claims.

Other sub-cortical projections In addition to the NAc, the PVT sends projections to other sub-cortical brain regions that have been implicated in motivated behavior. One interesting target is the amygdala. While the PVT sends some projections to the basolateral amygdala, the CeA is more heavily innervated (Li & Kirouac, 2008; Vertes & Hoover, 2008). Recent functional

studies have demonstrated that the PVT to CeA pathway is critical for the expression of freezing behavior in response to a fear-conditioned cue (Do-Monte *et al.*, 2015; Penzo *et al.*, 2015). As described previously, the CeA is also engaged by presentation of an appetitive incentive stimulus (Yager *et al.*, 2015). Thus, it would be interesting to examine the role of the PVT to CeA pathway in behavioral responding to appetitive-conditioned cues, and to determine whether or not this pathway is selective only for fear-conditioned cues. The PVT also sends some projections to the ventral pallidum (VP) (Li & Kirouac, 2008; Vertes & Hoover, 2008), a brain region that is critical for cue-elicited lever responding to obtain a sucrose reward (Richard *et al.*, 2016). More specifically, VP neurons appear to encode the incentive value of a cue (Ahrens *et al.*, 2016) and inhibition of the VP prevents the acquisition of sign- but not goal-tracking behavior (Chang *et al.*, 2015). While the PVT to VP pathway has been shown to be activated during context-induced reinstatement of alcohol-seeking behavior (Perry & McNally, 2013), it is unknown if this pathway contributes to discrete cue-motivated behavior, and specifically sign-tracking, as well. Again, future studies using optogenetic and chemogenetic techniques will allow for select excitation or inhibition of this circuit during the acquisition or expression of Pavlovian conditioned responses, identifying a possible role of the PVT to VP pathway in sign- and goal-tracking behavior.

Cortical projections In addition to the sub-cortical targets described above, the PVT sends efferents to the medial prefrontal cortex (mPFC), including the prelimbic and infralimbic cortices (Li & Kirouac, 2008; Vertes & Hoover, 2008). Interestingly, a small proportion of these cortically projecting neurons have bifurcating axons that extend to the NAc (Bubser & Deutch, 1998; Otake & Nakamura, 1998). Little is known about the functional roles of cortically projecting PVT neurons. One study demonstrated that PVT neurons that project to the mPFC are engaged following foot shock (Bubser & Deutch, 1999). Another study demonstrated that PVT neurons in this circuit are excited by orexin administration, and it was shown that this was primarily though the actions of orexin at OX-2 receptors (Huang *et al.*, 2006). In addition, the work presented in Chapter 4 suggests that orexin activity at OX-2 receptors in the PVT may be important for sign-tracking behavior. Thus, it is possible that the effects of PVT orexin transmission on sign-tracking behavior are being mediated in part through the PVT to mPFC pathway, and specifically through PVT cells that collaterize to the mPFC and NAc. Presumably,

this pathway would exert its influence on other mPFC circuits known to be involved in motivated behavior, such as the mPFC to NAc pathway.

Summary: PVT efferents Although the roles of a wide variety of PVT afferents have been investigated, the study of PVT efferent circuits has been limited, and mainly focused on the NAc and CeA. Here we identified a potential role for the PVT to NAc pathway in incentive salience attribution has been identified (Chapter 3). Little else is known about the role of the other PVT efferent pathways in cue-motivated appetitive behavior. While roles for the PVT to CEA and PVT to VP pathways in incentive salience attribution seem likely, a potential role for the PVT to mPFC pathway in this phenomenon is less clear.

Special note on anterior vs. posterior aspects of the paraventricular nucleus of the thalamus

The PVT is a long structure, extending over 2mm along the anterior-posterior axis in the rat brain. In general, PVT neurons are a relatively homogenous group of glutamatergic projection neurons with similar morphological structure, although some distinct sub-populations have been identified (for review see (Kolaj *et al.*, 2014; Kirouac, 2015). While there is overlap between the inputs and outputs of the PVT along this axis (Li & Kirouac, 2008; Vertes & Hoover, 2008; Li & Kirouac, 2012), variations in the anatomical connections between the anterior and posterior aspects of this nucleus have prompted a division of the nucleus into the anterior PVT (aPVT), mid PVT, and posterior PVT (pPVT). While the majority of research studying the PVT has either ignored this differentiation, or only investigated one aspect of the PVT, a number of studies have emerged promoting the idea that the distinct regions of the PVT may have differential roles in behavior. The first of these studies demonstrated that the pPVT, but not the aPVT, is activated in response to a novel stressor following a chronic stress paradigm (Bhatnagar & Dallman, 1998). This discovery spurred a line of research that has thoroughly identified a role for the pPVT is responding to stress, and anxiety-related behaviors (Bhatnagar *et al.*, 2000; Bhatnagar *et al.*, 2002; Bhatnagar, 2003; Heydendael *et al.*, 2011). Another study revealed that lesions of the aPVT, but not pPVT, altered the entrainment of circadian rhythms to light (Salazar-Juárez *et al.*, 2002), and this is believed to be through reciprocal connections with the suprachiasmatic nucleus (Alamilla & Aguilar-Roblero, 2010; Alamilla *et al.*, 2015). Follow up

studies have since revealed that the PVT may be involved in regulating different aspects of circadian rhythms and homeostasis, but it is unclear if this role if specific to the aPVT (for review see (Colavito *et al.*, 2015). Another study identified that the majority of aPVT, but not pPVT neurons are responsive to dynorphin (Chen *et al.*, 2015), which is in part coming from afferents from the mediodorsal hypothalamus (Marchant *et al.*, 2010), implicating an anatomical division for dynorphin's role in mediating behavior through the PVT.

More recently, Barson and colleagues have been exploring the role of the aPVT and pPVT in a variety of emotional and appetitive behaviors. Specifically, they have demonstrated that inactivation of the aPVT and pPVT decrease the locomotor response to a novel environment, but the observed decrease was larger following pPVT inactivation (Barson & Leibowitz, 2015). In addition, pPVT, but not aPVT inactivation led to an increase in anxiety-like behavior, evidenced by a decrease in time spent in the open arms of an elevated plus maze (Barson & Leibowitz, 2015). A second study revealed that blocking OX-2 receptors in the aPVT attenuated alcohol consumption, while administration of orexin-A into the pPVT increased sucrose consumption (Barson *et al.*, 2015). Combined, these studies have begun to demonstrate that distinct regions of the PVT may differentially mediate behavior.

While it was decided *a priori* to simultaneously manipulate multiple sites along the anterior-posterior axis of the PVT in the majority of studies contained in this thesis (Chapter 2; Chapter 3, Afferent Experiment; Chapter 4), the nature of the Efferent Experiment contained in Chapter 2 did differentiate between distinct aspects of the PVT, and thus allows us to add to this body of literature. Specifically, we observed that presentation of an incentive stimulus was able to evoke greater c-fos expression in pPVT neurons that project to the NAc, compared to unpaired control animals. This difference was not observed in the aPVT, or sections in the middle of the PVT, indicating that the pPVT efferents to the NAc are selectively activated by incentive stimuli, and may be part of the neural circuitry underlying incentive salience attribution. As mentioned in the discussion in Chapter 3, though, we cannot yet rule out a role for the aPVT in incentive salience attribution. Additional research will allow us to verify if other efferent projections, including those from the aPVT, are involved.

Concluding remarks and future directions

Through this dissertation work, the PVT has been identified as a critical modulator of incentive salience attribution to reward-paired cues, and a hypothetical model for the PVT in incentive-salience attribution has been developed and refined. In this model, the PVT serves as a 'brake' on incentive salience attribution, resulting from top-down cortical control coming from the PrL. Then, in select individuals, sub-cortical input from the hypothalamus and medial amygdala is able to override this cortical control, which results in an increase in activity in PVT efferents to the NAc, allowing for the attribution of incentive-salience to reward-paired cues. While this model is currently supported by the data contained in this thesis, additional work will be critical to further evaluate the role of the PVT and related circuitry in cue-motivated behavior, and, specifically, the propensity to attribute incentive salience to reward cues.

One interesting aspect to come out of the work contained in this dissertation revolves around the subtlety of behavioral changes following PVT manipulations. Unlike previous work demonstrating that blocking dopamine transmission causes a large attenuation of sign-tracking behavior (Flagel *et al.*, 2011b; Saunders & Robinson, 2012), the manipulations here caused changes in behavior that were relatively small in comparison. This is not surprising, given that thalamic nuclei have been traditionally thought to serve as 'relay' stations, accumulating signals from many different brain areas and coordinating them into behavior. Thus, while manipulating one afferent pathway to the PVT results in small changes in behavior (Chapter 4; (Covelo *et al.*, 2015), manipulating multiple pathways simultaneously may lead to larger behavioral changes. For instance, while reducing top-down control by inhibiting the PrL to PVT pathway in GTs leads to a subtle increase in lever-directed behavior (Covelo *et al.*, 2015), concomitantly exciting the subcortical mediodorsal/lateral hypothalamus to PVT pathway during this manipulation might be able to cause a full shift towards sign-tracking behavior in GTs, indicating greater incentive-salience attribution to the reward-paired cue. Conversely, performing the opposite manipulations in STs may lead to a robust attenuation of incentive-salience attribution, evidenced by a strong shift towards goal-tracking behavior. These types of dual circuit manipulations are now possible with the advent of diverse chemogenetic tools, including different types of DREADD receptors that are activated by different ligands. For example, in the experiment described above, the newly developed inhibitory κ opioid-receptor based DREADD (KORD), which is activated by salvinorin B, could be expressed in the PrL to PVT pathway.

Simultaneously, the excitatory hM3Dq DREADD receptor, which is activated by clozapine-n-oxide (CNO), could be expressed in the dorsomedial/lateral hypothalamic to PVT pathway. This would allow for precise control of these distinct neural circuits in the same animal. By administering each of the ligands alone, you could assess the contribution of that particular pathway to incentive-salience attribution. You could then administer both simultaneously, and assess the combined effect. Future experiments utilizing these techniques will be critical in fully understanding how the greater PVT circuitry contributes to incentive-salience attribution.

A second possible explanation for the subtlety of the observed effects in the experiments contained in this thesis, and area of interest for future research, is the role of the greater PVT circuitry in the acquisition vs. the expression of PCA behavior. Aside from the Acquisition Lesion Experiment in Chapter 2, all of the manipulations performed here were done following the acquisition of PCA behavior. It is possible that, once acquired, PCA behavior is much harder to change than it would be during the learning of that behavior. It is also possible that, once acquired, PCA behavior is relying on different neural circuitry than it was during learning. In support, it was recently demonstrated that lesions of the ventral hippocampus, which included the ventral subiculum, attenuated the acquisition of a sign-tracking phenotype, but had no effect on the expression of sign-tracking behavior once it had been acquired (Fitzpatrick *et al.*, 2016). Engagement of PVT afferents from the ventral subiculum were examined in Chapter 3, and while no differences were observed between phenotypes in this area, this manipulation was performed after the acquisition of PCA behavior. Thus, had we performed the manipulation earlier, during acquisition of PCA behavior, we might have seen greater activity in ventral subiculum cell that project to the PVT in STs, compared to GTs and UNs. These differences in in acquisition vs. expression of PCA behavior, and thus the attribution of incentive salience to reward-paired cues, will be important to keep in mind when examining the roles of distinct neural circuits moving forward. Future studies utilizing newly developed chemogenetic techniques, as well as those exploring differences in aPVT and pPVT function, and the complex relationship between stress and the PVT, as described earlier, will be critical in fully understanding how the PVT contributes to the attribution of incentive salience to reward-paired cues.

As mentioned in the introduction, understanding the neural circuitry involved in stimulus-reward learning increases our knowledge regarding the fundamental psychological processes

underlying distinct forms of reward learning, including the attribution of incentive salience to reward-paired cues. Importantly, it is possible for these processes to awry, resulting in various psychological ailments, including addiction. Here, we have worked towards furthering our understanding of the neural circuitry underlying this phenomenon, and demonstrated novel pathways that are involved mediating incentive salience attribution. These findings have brought us one small step closer to building a full understanding of how differences in neurological function can underlie behavioral susceptibility to different forms of psychopathology.

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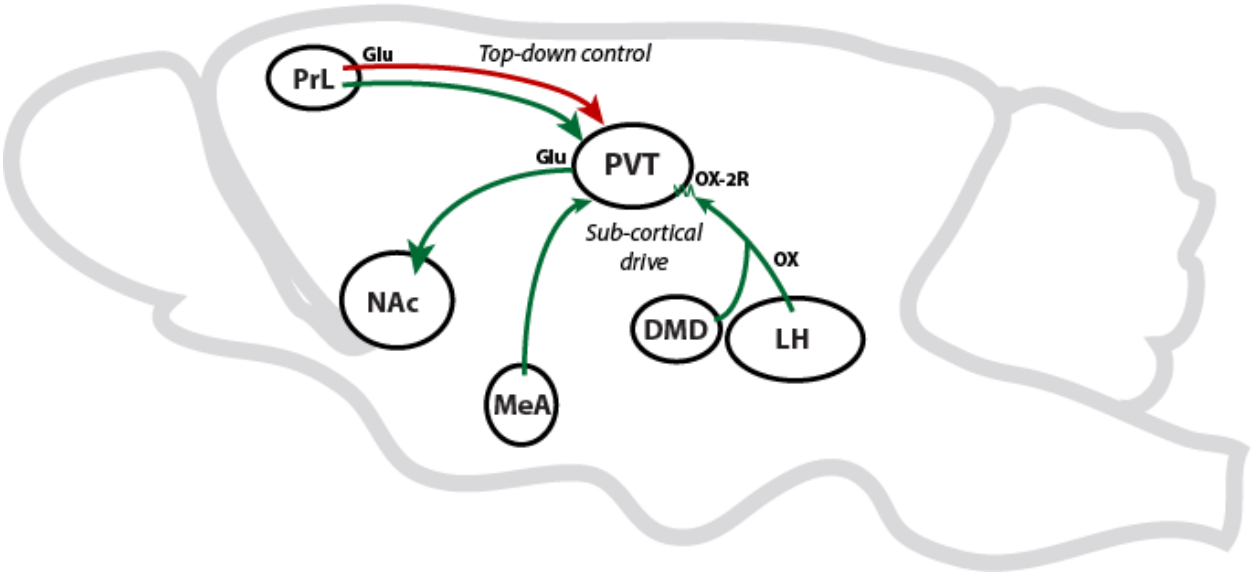

Figure 5.1. A proposed model for the role of the PVT, and select afferents and efferents, in mediating incentive salience attribution to reward-paired cues. Schematic representing the proposed role for the PVT in mediating incentive salience attribution. Top down cortical control coming from the PrL to the PVT acts as a 'brake' on incentive salience attribution for both sign-trackers (green arrows) and goal-trackers (red arrows). For sign-trackers, this top-down control in overridden by sub-cortical inputs from the MeA and hypothalamus. This input consists, in part, of orexinergic inputs from the DMD/LH to the PVT, where they activate post synaptic PVT neurons via OX-2Rs (wavy green line on PVT). This override allows PVT efferents to influence activity directly in the NAc by modulating medium spiny neurons, or by modulating dopamine terminals in the NAc, thus influencing the propensity to attribute incentive salience to reward-paired cues. Abbreviations: dorsomedial hypothalamic nucleus, DMD; glutamate, Glu; lateral hypothalamic area, LH; medial amygdala, MeA; nucleus accumbens, NAc; orexin, OX; orexin receptor 2, OX-2R; prelimbic cortex, PrL; paraventricular nucleus of the thalamus, PVT.