# Characterizing neuronal orexinergic projections from the lateral hypothalamus to the paraventricular nucleus of the thalamus involved in encoding incentive salience

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# Abstract

Environmental cues serve as predictors of biologically relevant rewards by associative learning. However, these cues can become also imbued with incentive value, which can gain excessive and maladaptive control of behavior. Such attribution of incentive value can parallel many psychopathologies including substance use disorders and externalizing disorders. We use a Pavlovian conditioned approach (PavCA) training paradigm in which the presentation of a lever cue (conditioned stimulus, CS) directly precedes delivery of a food reward (unconditioned stimulus, US). Some rats (sign-trackers) will develop a conditioned response towards the lever-CS when it is presented and will attribute it with incentive and predictive value (i.e., sign-trackers, STs). On the other hand, another subset of animals will instead approach the food magazine when the lever-CS is presented, seeing the lever-CS only as a predictor of reward (i.e., goal-trackers). We know that STs show more engagement of "bottom-up" circuitry than GTs, with the pathway from the lateral hypothalamus (LH) to the paraventricular nucleus of the thalamus (PVT) being a major subcortical pathway underlying this behavior. We also know that STs show more activity in the LH in response to cue presentation, lesions of the LH attenuate sign-tracking behavior, and that antagonism of receptors of orexin (OX), a neuropeptide largely expressed in the LH, appears to attenuate sign-tracking. Here, we characterize the neuronal projections from the LH to the PVT using a novel combination of techniques: hairpin chain reaction in-situ hybridization (HCR FISH) and immunofluorescence (IF). We confirm that STs show more engagement of the LH, and of the LH-PVT pathway, and we discover that they also show more activity in the OXergic LH-PVT projections relative to GTs. We also compare subregions of the LH and find that the posterior LH appears to send more OXergic projections to the PVT than the anterior LH. Future studies should confirm the unique activities of OXergic projections in STs relative to GTs, which could be achieved through pathway manipulations (ex. chemogenetics and optogenetics) to OXergic transmission. Further it is important to continue indexing rostrocaudal differences in the LH.

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# Scientific Acknowledgements

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| **Figure #** | **Contributions** |
|---|---|
| 1-2 | Behavioral experiments were conducted by Dr. Joshua Haight and colleagues. |
| 3 | Probes were designed by Allison Johnson & Amanda Iglesias. |
| 4 | All tissue processing was conducted by Amanda Iglesias and I. |
| 5A, C | Image was collected by Dr. Aram Parasegian. |
| 5B, D, E | Images were collected by Amanda Iglesias and I. |
| 6-8 | All data were collected, analyzed, graphed, and interpreted by Amanda Iglesias and I. |

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# 1. Introduction

For animals and humans alike, environmental cues serve as an indicator of valuable resources or an upcoming event. However, in some individuals, the cue itself becomes desirable. We can assess the value placed upon a reward-associated cue utilizing a classical Pavlovian conditioning paradigm in which the presentation of a lever (conditioned stimulus) precedes the presentation of a food pellet (unconditioned stimulus). Through this approach, we see two distinct conditioned responses emerge: sign-tracking and goal-tracking. Sign-trackers (STs) interact with the lever while it is presented, whereas goal-trackers (GTs) immediately approach the food cup. Importantly, upon lever retraction and pellet delivery, both groups will move towards the food cup and consume the reward. In other words, while GTs attribute the lever cue primarily with predictive value, STs assign both predictive and incentive value (i.e., incentive salience) to such reward-predictive cues.

We hypothesize that subcortical circuits are involved in mediating the incentive salience attributed to reward cues, and therefore that STs will show more engagement of such circuitry compared to GTs. One specific pathway that has been implicated in sign-tracking behavior (i.e., incentive salience attribution) is that from the lateral hypothalamus (LH) to the paraventricular nucleus of the thalamus (PVT). To better characterize the involvement of this pathway in encoding the incentive value of reward cues, we assessed neuronal markers in the projections from the LH to the PVT in STs and GTs. Since orexin (OX), a neuropeptide implicated in motivated behavior, originates primarily from the LH, one possibility is that the LH-PVT projections that encode the incentive motivational value of reward cues are OXergic. To determine whether OX plays a role in the attribution of incentive value to reward cues, we assessed what proportion of the OXergic projections from the LH to the PVT are engaged in response to a reward cue in STs relative to GTs. To do so, we used two tissue processing techniques to visualize and label OXergic projections that are active in response to cue presentation.

## 1.1 Drug addiction and related psychopathologies

By further characterizing the neuronal subtypes in the LH-PVT pathway in incentive salience attribution, we hope to better understand more about what neurobiology underlies maladaptive motivational behavior. Our interest in this experiment is in part due to the wide-

ranging impacts of drug addiction and other related motivational phenotypes on morbidity and mortality around the world (McGinnis & Foege, 1999; Scott et al., 2020). Data from 2020 suggest that the prevalence of substance use disorders has increased due to the COVID-19 pandemic, as approximately 30 million past users of alcohol and 11 million past users of drugs perceived that their use of substances had increased relative to before the pandemic (NSDUH, 2020). Furthermore, research shows that individuals with externalizing disorders tend to experience substance use, depression, and social difficulties (Fariba & Gokarakonda 2023). Furthermore, individuals with impulse control disorders appear to also be at risk of obesity and suicidality (Tsukayama et al., 2010; Scott et al., 2020). Overall, the incidence and morbidity of substance use disorders and related psychopathologies highlight the importance of determining their neurobiological underpinnings to develop potential treatments.

While many biological and psychosocial factors can contribute to an individual's likelihood of facing substance use or impulse control disorders, there are certainly some characteristics of these disorders that can be modeled through differential motivation and behavior towards environmental cues. Indeed, some cues that precede rewards can elicit psychopathology including not only substance abuse but also pathological gambling, eating disorders, and post-traumatic stress disorder (PTSD) in certain individuals (Small et al., 2001, Limbrick-Oldfield, et al., 2017, Shin et al., 2004).

### 1.2 Environmental cues

In any environment, visual, auditory, olfactory, and other types of cues exist to signal an upcoming event. Not only are cues ever present in our environment, but animals and humans alike need to be able to recognize and interpret the meaning associated with these environmental cues to better prepare for survival. For instance, for prey in the wild, a rustling in the bushes may indicate that a predator is nearby and able to attack at any second, so it is essential that the preyed animal understand and interpret the sound as a sign of danger and aptly flee the situation. Nonetheless, besides taking on the role as a signal of prediction, a cue itself can gain its own value and can potentially motivate behavior directed toward it. Assigning excessive value to a cue itself can be counterproductive to an animal's survival in the wild, as the cue's motivational value (i.e., the incentive value) can detract from the importance of its predictive value. If an

animal were to instead be motivated to interact with a cue predictive of its predator's arrival, this excessive incentive value, which we call incentive salience, can distract it from its own survival.

### 1.3 Incentive salience

The Incentive Sensitization Theory of drug addiction proposed by Berridge and Robinson first introduced the concept of incentive salience (1993). The theory postulates that continuous bouts of drug use lead to a series of neuroanatomical changes (also known as sensitization) and subsequent drug effects. Incentive salience attribution is a process by which cues that predict reward are themselves assigned with incentive value, such that these cues become "wanted" and desirable in and of themselves. Repeated drug use can cause sensitization in the neural circuitry underlying "wanting behavior", ultimately resulting in an increased motivation towards the cue due to the excessive value assigned to it.

Incentive stimuli possess three unique properties (Berridge, 2001; Cardinal et al., 2002) Robinson et al., 2014h). First, discrete cues attributed with incentive salience bias attention and elicit approach towards them. Second, animals will work to gain access to incentive stimuli (i.e., they can act as conditioned reinforcers). Finally, these stimuli evoke a motivational state in subjects, either initiating or maintaining reward-seeking behavior. The way in which animals attribute reward cues with incentive salience seem to parallel the way in which individuals with substance abuse disorders respond to drug cues, as we see those interactions with incentive stimuli "increase the vigor with which they are sought", a phenomenon that can be seen in how drug cues trigger relapse (Berridge et al., 2009). As a result, elucidating mechanisms underlying behavioral endophenotypes could allow researchers to develop a better understanding of the neural mechanisms of addiction to drugs of abuse and other related impulse control disorders.

### 1.4 Individual variation in cue-motivated behavior

Individuals vary in their behavior and the propensity to attribute incentive value to reward cues (Robinson & Flagel 2009). Cue-motivated behavior can be modeled in a number of ways, one of which is through a Pavlovian conditioned approach (PavCA) paradigm. In a typical Pavlovian conditioning experiment, the training period involves repeatedly pairing an unconditioned stimulus (US), such as a food pellet, with a neutral stimulus, such as a lever. Over training, this once neutral stimulus is transformed into a conditioned stimulus (CS). After sufficient pairing between the CS and the US, the CS will now elicit behavior in the subject that

was once induced solely by the US, also known as a conditioned response (CR). Within the realm of Pavlovian classical conditioning, the CS is seen to only be assigned predictive value, as the subject observes the CS as a signal of upcoming US presentation. However, it appears that a cue (i.e., CS) can also be attributed with another type of value, incentive value, in addition to being a predictor of reward (i.e., US).

The PavCA procedure is performed with 1) presentation of a lever (CS) for 8 seconds, 2) followed by delivery of a food pellet in a food cup that is in a different location from the lever regardless of the rat's response or lack thereof towards the cue (Flagel et al., 2009). Importantly, reward delivery occurs independent of the animals' response. Using a PavCA paradigm, we are able to observe different behavioral endophenotypes in response to the presentation of a lever-CS that precedes the delivery of a reward-US. Some individuals, upon presentation of the lever-CS will orient and interact with it, even though interaction with the CS is not necessary to gain access to the reward-US (Robinson & Flagel, 2009). These individuals, termed "sign-trackers" (STs), attribute not only predictive value, but also incentive value to the CS. Another group of individuals do not avidly engage with the lever-CS. These individuals are called "goal-trackers" (GTs), and they only assign predictive value to the CS. Another subset of individuals does not show an inclination to either sign-tracking or goal-tracking behavior and are referred to as "intermediates" (IN). Previous data have shown that each of the three phenotypes represent approximately one third of the subject population (2009). These phenotypes were described in dogs that had underwent Pavlovian conditioning and learned to associate the sound of a tone (CS) and food delivery (US) (Pavlov 1937). After repeated CS-US pairings, some dogs directed themselves to the food pan when the tone (CS) was presented, which we would now call goal-tracking. However, interestingly, some of the dogs instead directed themselves toward the tone-cue itself during its presentation, which we would call sign-tracking.

GTs, during lever presentation will not interact with it, seeing it only as a predictor of upcoming food delivery, and going to the location of reward delivery upon its presentation. STs, on the other hand, will approach the lever-CS, gnaw on it, bite it ("consummatory behavior") in a manner like that displayed upon food consumption. Both STs and GTs will approach and consume the food in the food cup, however, the differences in their behaviors toward the CS illustrate differences in the types of value they attribute to it.

STs are of particular interest because they attribute incentive value in a way that seems to parallel individuals with substance use disorders, externalizing disorders, and other psychopathologies. The relationship between the propensity to sign-track and addiction-related behaviors has been assessed using drug self-administration, cue-induced reinstatement, and conditioned place preference. It has been reported that STs attribute drug-paired cues with incentive salience (Flagel et al., 2010; Yager et al., 2015; Yager & Robinson, 2013), display long lasting impacts of cocaine-induced psychomotor sensitization after repeated cocaine treatment (Flagel et al., 2008), show a higher propensity for relapse or reinstatement of drug-seeking behavior (Saunders & Robinson, 2010, 2011; Saunders et al., 2013), display an exaggerated fear response to discrete aversive cues (Morrow et al., 2011; Morrow et al., 2015), attribute more incentive salience to discrete localizable and interoceptive cues (Robinson et al., 2014), show enhanced impulsive action (Lovic et al., 2011), are more novelty-seeking (Beckmann et al., 2011), make riskier decisions (Olshavksy et al., 2014), and have relatively poor attentional control (Paolone et al., 2013). On the other hand, individuals identified as GTs have been shown to display other distinct behavioral characteristics including more susceptibility to context-induced drug seeking behavior (Saunders et al., 2014) as well as enhanced fear response to aversive contexts, relative to GTs (Morrow et al., 2011).

The ST/GT model has several implications in human correlates of psychopathology. Many individuals experiencing pathological drug taking behavior find themselves attracted to people, places, and paraphernalia associated with their experiences with drugs of abuse, and such behavior can stimulate relapse to occur (Flagel et al., 2009). Drug cues, including glassware that the individual consumed alcohol in, the place at which the individual consumed drugs/alcohol, and pipes used to consume cocaine, can reinstate and/or maintain drug taking in individuals with substance abuse disorders. Similarly, drug-taking and seeking behavior among STs is also influenced by discrete cues (2009). Furthermore, STs exhibit more PTSD-like abnormal fear responses that incubate or increase over time, potentially explaining the high comorbidity between PTSD and substance abuse (Morrow et al., 2015; Cottler, Compton, Mager, Spitznagel, & Janca, 1992; Kulka et al., 1990). On the other hand, GTs tend to show symptoms of disorders of "over-control" including obsessive compulsive personality disorder and restrictive eating disorders such as anorexia nervosa (Tomie & Morrow 2018). Importantly, sign-tracking and goal-tracking behavior is not limited to animals, as we have recently found that human subjects

also exhibit similar endophenotypes phenotypes towards a food predictive cue (Colaizzi et al., 2022). Overall, the ST/GT model allows us to examine individual differences in the propensity to attribute predictive versus incentive value to a reward cues, and studying these cue-learning strategies can reveal neurobiological characteristics of substance abuse disorder, impulse control disorders, and other related psychopathologies.

### 1.5 The neurobiology underlying individual differences in cue-motivated behavior

In addition to the distinct behavioral characteristics of STs and GTs, there appears to be some neurobiological differences. Indeed, it appears that only STs, and not GTs, show engagement in motivation circuitry previously suggested to be activated by reward cues, illustrating that this reward circuitry is only active upon incentive salience attribution to reward cues (Flagel et al., 2011). One region of interest is the paraventricular nucleus of the thalamus (PVT), which receives many different subcortical and cortical inputs within the brain. Our interest in the PVT stems from several experiments highlighting its role as a critical mediator of sign- and goal-tracking behavior. One of the first studies purporting a differential role of the PVT in STs and GTs found that after PavCA training, re-exposure to the lever-CS induced c-Fos mRNA levels in midline thalamic structures, including the PVT, in STs but not GTs (Flagel et al., 2011). This study illustrates that thalamic nuclei like the PVT were activated during cue presentation only when incentive salience was attributed, highlighting an important role of the PVT in cue-motivated behavior. Furthermore, the PVT appears to inhibit cue-directed behavior in GTs as inactivation of this nucleus resulted in a significant increase in sign-tracking behavior in GTs (Kuhn et al., 2018).

### 1.6 “Top-down” cortical control vs. “bottom-up” subcortical drive

Overall, functional connectivity studies have illustrated that the PVT is an important mediator of both “top-down” cortical processing and “bottom-up” subcortical processing. Importantly, this feature of the PVT is relevant to the discussion of individual differences in the propensity to attribute incentive salience to reward cues, as STs and GTs show differential activation of unique PVT pathways (Campus et al., 2019; Flagel, Cameron et al., 2011; Haight et al., 2014). In general, STs rely more on “bottom-up” PVT pathways, while GTs rely more on “top-down” PVT pathways.

### 1.6.1 "Top-down" cortical processing

The pathway between the prelimbic cortex (PrL) and the PVT plays a crucial role in "top-down" processing and is hypothesized to modulate the predictive facets cue-motivated behavior. Stimulation of this pathway decreases incentive salience attribution to reward cues in STs while inhibition increases the incentive value of reward cues in GTs (Campus et al., 2019). One study showed that PVT lesions in GTs resulted in more sign-tracking behavior (Haight et al., 2015). In other words, GTs attribute more incentive value to reward cues when the PrL-PVT pathway is not active. On the other hand, STs attribute less incentive salience to reward cues when the PrL-PVT pathway is stimulated. Overall, this research suggests that the PrL-PVT pathway encodes "top-down" cortical control that relays the predictive value of cues and enhanced engagement of the pathway can put a brake on incentive salience attribution to reward cues (Campus et al., 2019).

### 1.6.2 The lateral hypothalamus (LH) and "bottom-up" processing

We postulate that the "top-down" cortical processing mediated by the PVT opposes "bottom-up" subcortical circuitry. Implicated heavily in this "bottom-up" processing is the lateral hypothalamus (LH) which projects to the PVT and shows greater neuronal engagement in STs relative to GTs (Haight et al., 2014). The LH is a region in the posterior hypothalamus that is anatomically positioned to send and receive a variety of cortical and subcortical projections, and it expresses a variety of neuropeptides and neurotransmitters including GABA, glutamate, galanin, and orexins (Fakhoury et al., 2020). It can also be neuroanatomically divided into three sections based on its efferents: anterior (aLH), tuberal (tLH), and posterior (pLH) (Saper et al., 1979). The LH is largely known to be a key area responsible for guiding behavior to maintain homeostasis. It was first implicated in feeding behavior, as rats and cats that underwent bilateral LH lesions displayed complete cessation of food-seeking behavior and eating, while electrical stimulation induced feeding (Anand & Brobeck, 1951; Delgado & Anand, 1952). In addition to feeding, the LH is involved in a variety of other homeostatic and motivated behaviors including other appetitive behaviors and arousal.

In addition to its connectivity to the PVT, the LH communicates with a number of other reward-related brain regions, including the nucleus accumbens (NAc) and ventral pallidum (Bonnavion et al., 2016, Castro et al., 2015). Previous research has shown that animals will work to receive electrical stimulation of the LH, which appears to be largely influenced by the

mesolimbic dopamine systems, leading some to name it as a “pleasure center” (Fakhoury et al., 2016; Ide et al., 2017; Koob et al.,1978; Olds, J, 1956). Interestingly, however, LH stimulation that is too intense can become aversive (Bower & Miller, 1958). A recent study has implicated specific LH cell populations in guiding behavioral choices according to nutritional and social needs (Petzold et al., 2023). Furthermore, some LH pathways are involved in mediating mesolimbic dopamine release and cocaine-seeking behavior in response to nociception (Lee et al., 2022). The LH is also required for context-induced reinstatement of seeking of alcoholic beer and a sucrose reward in rats, and the pathway from the LH to the nucleus accumbens (NAc) shell is specifically active during this reinstatement (Marchant et al., 2009).

Data from our laboratory thus far suggests that the LH-PVT pathway plays a primary role in the individual differences we see in cue-motivated behaviors. We have found that lesions of the LH attenuate the propensity to sign-track, without no effect on goal-tracking behavior (Haight et al., 2020). STs also express greater cue-induced c-Fos, an immediate early gene, specifically in LH-PVT projection neurons compared to GTs, suggesting that the LH-PVT pathway is active during sign-tracking behavior as opposed to goal-tracking (Haight et al., 2017). Taken together, it appears that STs rely more heavily on the LH-PVT pathway as compared to GTs, although it is not yet fully understood what neurotransmitters/neuropeptides might mediate this.

### 1.6.3 Orexin and psychopathology

Orexin (OX), also known as hypocretin, is a neuropeptide expressed largely in the posterior hypothalamus and appears to be involved in many neuroendocrine processes. OX originates largely from the LH and is composed of two main subtypes: orexin A and orexin B (Sakurai et al., 1998, de Lecea et al.,1998). Both subtypes bind two G-protein coupled receptors: Orexin-1 receptor (OX1r) and Orexin-2 receptor (OX2r), which are widely found throughout the central nervous system including in the neocortex, amygdala, basal forebrain, ventral tegmental area, thalamus (Moore et al., 2000; Sakurai, 2014; Aston-Jones et al., 2010). OX release appears to occur with other neurotransmission including serotonergic and dopaminergic transmission (Fadel et al., 2005, Horvath et al., 1999). OX was originally found to be involved in regulating feeding behavior (Sakurai et al., 1998), but research has since shown its involvement in sleeping, wakefulness, and arousal (Sakurai, 2007). These “multi-tasking” neurons are also responsible for

maintaining energy homeostasis and for generating appropriate stress-responsive behavior, as well as regulation of reward systems (Stuber & Wise, 2016).

Orexinergic neurons are wide-ranging and have been implicated in a number of behaviors of relevance to psychopathology. For example, OX projections to the PVT have been implicated in negative emotional states likely via interactions with the limbic arousal system (Li et al., 2010; Kirouac et al., 2005). One recent study shows that administration of an acute OX antagonist effects anticipatory anxiety in humans (Gorka et al., 2022). In addition, reduced orexin-A concentrations are associated with decision-making in patients with anorexia nervosa (Steward et al., 2019). Using optogenetics in mice, it was shown that stimulation of OX neurons in the LH increases impulsivity in a Go/No-go task (Tyree et al., 2023). It has also been shown that OX neurons are activated in response to drugs of abuse, including nicotine and amphetamine. In addition, OX1r antagonism prior to cocaine self-administration has led to reduced drug seeking behavior compared to controls (Hutscheson et al., 2011).

The role of OX in drug-taking and drug-seeking has not yet been fully determined either. Furthermore, it appears that the OXergic innervation of the posterior PVT undergoes neuroadaptive alterations during periods of abstinence, indicating that the OX system is involved in the long-term effects of drugs of abuse and potentially relapse behavior (Matzeu, & Martin-Fardon, 2021).

Of particular relevance to this research, OXergic neurons from the LH have been implicated in reward-related behaviors. OXergic projections from the LH to the ventral tegmental area dopamine neurons play an important role in reward-seeking behaviors (Thomas et al., 2022). As sign-tracking behavior relies on dopaminergic transmission (Flagel et al., 2011), the fact that OX neurons play a role in dopamine release provides a potential mechanism through which incentive salience could be encoded. OX cells also are responsive to stimuli associated with rewards including drugs of abuse as well as discrete and contextual food cues (Mahler et al., 2012). One of our studies found that antagonism of OX-1r or OX-2r selectively in the PVT attenuates sign-tracking behavior and the conditioned reinforcing properties of reward cues (Haight et al., 2020). Moreover, since LH lesions prevent the development of sign-tracking, as previously mentioned, and the LH sends dense OXergic projections to the PVT, we hypothesize that OXergic signaling within the PVT may play an important role in assigning incentive value to reward cues. The current research aims to shed light on this hypothesis, investigating

differences in neuronal activity in orexinergic LH-PVT neurons in response to a cue with incentive value (i.e. in STs), relative to one with only predictive value (i.e. in GTs).

### Thesis Goals

In an attempt to elucidate the role of OX projections from the LH to the PVT in incentive salience attribution, we are using a novel combination of techniques. First, we will use hybridized chain reaction fluorescent *in situ* hybridization (HCR FISH) to label cells containing prepro-orexin, a precursor of both orexin-a and b, the two identified forms of OX in the brain (Sakurai et al., 1998; De Lecea et al., 1998). Second, we will use an immunofluorescence (IF) assay to label cells expressing fluorogold (FG) and c-Fos, an immediate early gene and marker of neuronal activation. After examining fluorescent images, we will then compare the proportion of LH cells that express FG, cFos, and OX in STs to that of GTs. We hypothesize that STs will show a higher proportion of these triple-labeled neurons relative to GTs, which would suggest that OXergic projections from the LH to the PVT are active during incentive salience attribution.

## 2. Materials and Methods

The following methods, up until tissue processing, are adapted from Haight et al., 2017.

### 2.1 Subjects

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

### 2.2 Surgery

After the 10-day acclimation period, the retrograde tracer fluorogold (FG; Fluorochrome, Denver, CO, USA) was infused into the PVT via stereotaxic surgery. Aseptic conditions were maintained throughout the surgery. First, a surgical plane of anesthesia was induced with inhalation of 5% isoflurane. The scalp was then shaved and sterilized with swabs of a 70%

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

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

### 2.3 Behavioral Testing

**2.3.1 PavCA Training**: After surgical recovery, all rats underwent Pavlovian conditioned approach (PavCA) procedures like those previously described (Flagel et al., 2011; Haight et al., 2015; Fraser et al., 2016). Standard behavioral test chambers (MED Associates, St. Albans, VT, USA) were enclosed in sound-attenuating boxes that were equipped with ventilation fans that provided constant air flow and background noise. All behavioral data were collected using MED PC software (Med Associates, St. Albans, VT, USA).

A food cup was connected to a pellet dispenser located in the middle of one of the walls in each chamber. Upon activation of the pellet dispenser, one 45-milligram banana-flavored grain pellet (Bio-Serve, Flemington, NJ, USA) was delivered. Each food cup was equipped with an infra-red photo beam and breaks of this beam were registered and recorded as head entries.

Flanking the food cup to the left or right was a retractable, illuminated lever, positioned at equal height with the food cup. Chambers alternated in whether the lever was to the left or right of the food cup. All levers were calibrated such that approximately 10-15 grams of force would cause a deflection of the lever and would be registered as a lever contact. A white house light was placed on the upper middle portion of the wall directly across from the food cup and lever. This light was illuminated for the duration of each Pavlovian conditioning session.

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

After session 5 of PavCA, rats in the "paired" group were classified as STs, GTs, or intermediate responders (INs) based on their average PavCA Index scores from sessions 4 and 5. The PavCA Index is a composite score that is used to assess the propensity of a rat to approach

the lever-CS vs. the food cup (location of US delivery) upon CS presentation. This Index relies on three different metrics: response bias [(total lever contacts – total food cup contacts)/(sum of total contacts)], probability difference score [$prob_{(lever)} - prob_{(food\ cup)}$], and latency difference score [– (lever contact latency – food cup entry latency/8]. These three measures are then averaged together to create the PavCA Index score, which ranges from -1.0 to 1.0, with -1.0 representation an individual whose behavior is solely directed toward the lever-CS.

**2.3.2 CS re-exposure**: After PavCA training, the test chambers were reconfigured such that the food cup and pellet dispenser were removed, the lever was placed in the center of the wall it was previously located on, and new metal grate flooring was inserted. To minimize the influence of contextual cues, rats classified as STs, GTs, and the unpaired control groups (UNs) were placed into the reconfigured test chambers on three consecutive days. During these sessions, following an initial 5-min acclimation period, the house light was illuminated, and the animals remained in the chambers for another 30 min, with the lever retracted. On the fourth day (i.e., cue-test session), rats were placed into the chambers, and following the 5-min acclimation period, the house light was illuminated, and the illuminated lever-CS was inserted into the cage for 2 sec, 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, but lever contacts were recorded during the session. Following the 10th lever presentation, rats were placed back into their home cages and transferred to the housing room, where they were left undisturbed for 60 min. Following this 60-min period, the rats were deeply anesthetized with an intraperitoneal injection of a cocktail containing ketamine (90 mg/kg) and xylazine (10 mg/kg) and transcardially perfused with approximately 100 mL of room temperature 0.9% saline, followed by approximately 200 mL of room-temperature 4% formaldehyde (pH = 7.3–7.4, diluted in 0.1 M sodium phosphate buffer; Fisher Scientific, Hampton, NH, USA).

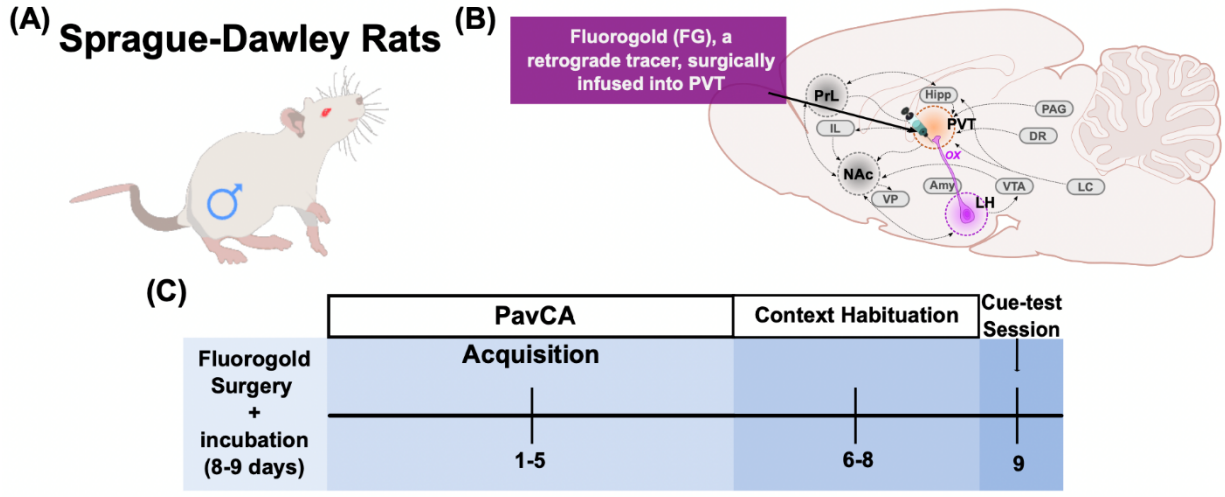


**Figure 1: Fluorogold (FG) surgery and experimental timeline**. A) Male Sprague-Dawley rats underwent surgical infusion (n=40) prior to behavioral testing. B) FG infusion into the PVT via stereotaxic surgery to trace PVT efferents. C) Experimental timeline prior to tissue processing.

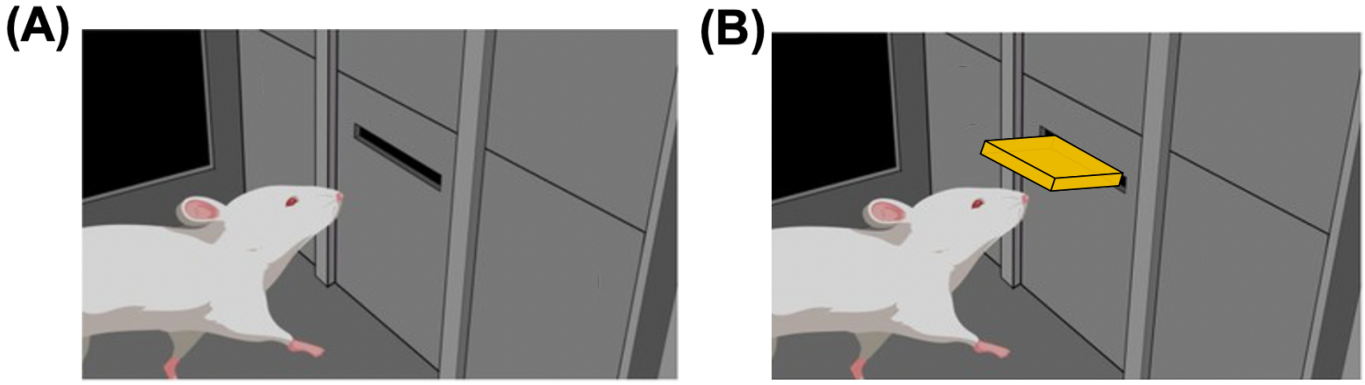


**Figure 2: Context habituation and cue-test session.** A) Context Habituation: rats placed into novel context for three days to ensure that cFos captured after cue-test session is due to cue presentation alone. B) Cue-test Session: lever-CS presentation to induce cue-elicited cFos expression in active neurons.

### 2.4 Tissue processing

Following perfusion, brains were extracted and post-fixed overnight in 4% formaldehyde at 4° C. Brains were then cryoprotected over three nights in graduated sucrose solutions (10%, 20%, and 30%, dissolved in 0.1M sodium phosphate buffer, NaPB; pH =7.3-7.4) at 4° C. Next, brains were sectioned at 40$\mu$m on a frozen cryostat (Leica Biosystems Inc, Buffalo Grove, IL,

USA). Starting with the anterior prelimbic cortex and continuing through the thalamus, brain sections were serially collected in 6-well plates. Each well contained a full brain series, with each section approximately 200 $\mu$m caudal from the previous section. Toward the hindbrain, where the ventral subiculum is located, sections were collected in 48-well plates, one section per well. All sections were stored in 0.1 M NaPB at 4° C. For long-term storage, tissue was stored in cryoprotectant at -20 °C.

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

Brain tissue of 10 STs and 9 GTs were chosen from the aforementioned behavioral study based on amount of tissue available to process. The unpaired group was not quantified here due to time constraints. The tissue underwent the free-floating hairpin chain reaction fluorescent in-situ hybridization (HCR FISH) protocol in order to detect orexin mRNA. Using FASTA sequence, a text-based format that represents nucleotide or peptide sequences, approximately 20 pairs of short oligonucleotide probes (~40 nucleotides) were designed to bind to prepro-orexin mRNA via complementary binding. The following prepro-orexin probe sequence was used:

```
  1 ggctcggcgg cctcagactc cttgggtatt tggaccactg caccgaagat accatctctc
 61 cggattgcct ctccctgagc tccagacacc atgaaccttc cttctacaaa ggttccctgg
121 gccgccgtga cgctgctgct gctgctactg ctgccgccgg cgctgctgtc gcttggggtg
181 gacgcgcagc ctctgcccga ctgctgtcgc cagaagacgt gttcctgccg tctctacgaa
241 ctgttgcacg gagctggcaa ccacgccgcg ggcatcctca ctctgggaaa gcggcgacct
301 ggacccccag gcctccaagg acggctgcag cgcctccttc aggccaacgg taaccacgca
361 gctggcatcc tgaccatggg ccgccgcgca ggcgcagagc tagagccata tccctgccct
421 ggtcgccgct gtccgactgc aaccgccacc gctttagcgc cccggggcgg atccagagtc
481 tgaacccgtc ttctatccct gtcctagtcc taactttccc ctctcctcgc cggtccctag
541 gcaataaaga cgtttctctg ctaaaaaaaa aaaaaaaaaa aaaaa
```

**Figure 3:** Prepro-orexin mRNA sequence used to design the oligonucleotide probes for HCR FISH procedure.

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

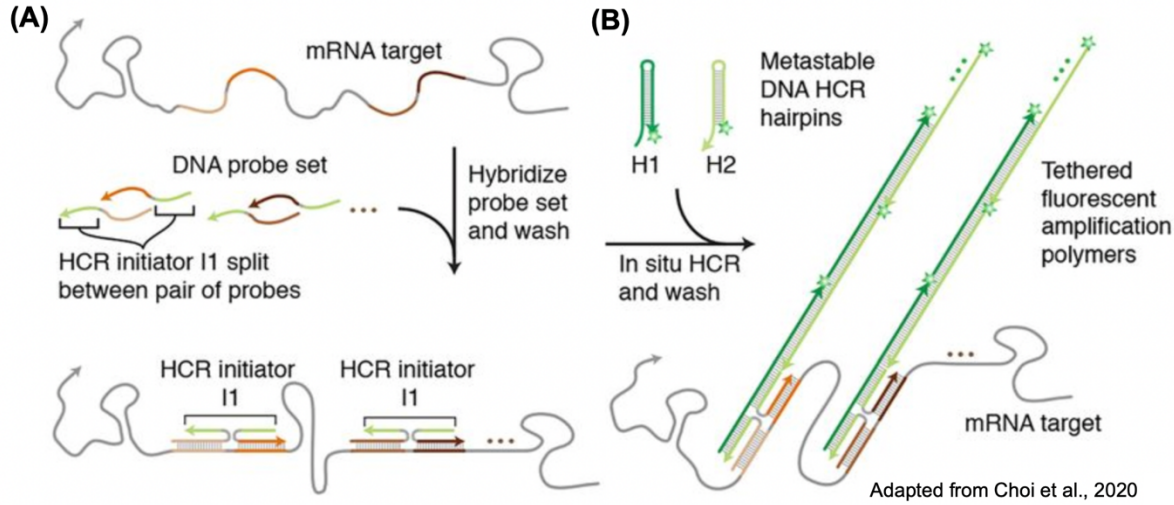


**Figure 4: HCR FISH mechanism**: HCR DNA hairpins hybridize to probes that bind to target mRNA present in the tissue. A) HCR probes bind to the target mRNA B) HCR DNA hairpins containing fluorophores hybridize to the probes bound to the target mRNA, creating a chain of fluorescent DNA hairpins that amplify the fluorescent signal emitted by the target mRNA. (Adapted from Choi et al., 2020).

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

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

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

### 2.4.2 Immunofluorescence (IF)

Immediately following HCR FISH amplification, the same tissue was processed for detection of FG and c-Fos via free-floating immunofluorescence (IF). All IF procedures took place at room temperature on a shaker providing gentle agitation. Free-floating sections were rinsed 3-4 times for 5 minutes each in 0.1 M PBS in between incubations. Tissue was blocked in 2.5% normal donkey serum (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA), 0.4% Triton-X (Acros Organics, Geel, Belgium), and diluted in 0.1 M PBS for 1 h. Sections were then incubated overnight in a double-antibody cocktail. The solution contained 1:500 mouse anti-c-Fos antibody (GR3454884-1, abcam) and 1:1000 rabbit anti-FG primary antibody (this antibody was a generous gift from Dr. Stanley Watson's Laboratory at the University of Michigan and is commercially available from Fluorochrome, Denver, CO, USA) diluted in 1% normal donkey serum and 0.4% Triton X in 0.1 M PBS. Both primaries were diluted in sterile water. The next day, sections were again rinsed three times in 0.1 M PBS. After this, sections were incubated in a secondary antibody solution containing 1:500 donkey anti-mouse antibody conjugated with Alexa Fluor 594 (lot 153991 Invitrogen, Thermo Fisher Scientific), biotinylated donkey anti-rabbit antibody (lots 162263 and 159880 Invitrogen, Thermo Fisher Scientific). Sections were then rinsed with 0.1 M PBS. Finally, all sections were incubated in 1:1000 Streptavidin conjugated with Alexa Fluor 488 (lot 1802442 Invitrogen, Thermo Fisher Scientific), each diluted in 1% normal donkey serum, and 0.4% Triton X-100 in

0.1 M PBS, for 2 h in the dark. Sections were then rinsed in 0.1 M PBS. Prior to mounting, sections were incubated with 4',6-diamidino-2-phenylindole (DAPI), a fluorescent DNA stain for cellular nuclei, diluted 1:2000 in PBS for 20 minutes. The sections were then mounted onto SuperFrost Plus microscope slides (Fisher Scientific, Hampton, NH, USA) and coverslipped with ProLong Gold Antifade Mountant (lot 2406594, Thermo Fisher Scientific), which is a hard mount that preserves fluorescent signal.

**2.5 Imaging and Image Analysis**

After the tissue processing protocols were completed, the tissue was then imaged using the FV3000 confocal microscope and the FV31S-SW Viewer software (OLYMPUS Microscopes, Center Valley, PA, USA). The following range of settings was used in the program when imaging: 1.0% Gain, 600-700 HV, 2-8% Offset. Anterior LH was defined between dorsal/ventral coordinates -1.72 to -2.16 (relative to Bregma, Paxinos & Watson, 2007) and cell counting areas were chosen directly lateral to the fornix. Posterior LH was defined between dorsal/ventral coordinates -3.24 to -3.48 (relative to Bregma, Paxinos & Watson, 2007) and cell counting areas were chosen around the perifornical part of the LH. Sample images are included in Figure 5.

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

individual neuronal cell bodies and then converted to mask (MaxEntropy algorithm) suitable for automated cell courting which was performed using 'Analyze Particles' tool ("size=25-500 circularity=0.60-1.00 show=Masks display exclude summarize). Finally, image calculator tool 'AND operator' was used to quantify the overlapping population for the FG + cFos, FG + OX, cFos + OX, and FG + cFos + OX comparisons. The resulting intersection image containing common pixel data was then used with 'analyze particles' tool (size=15-500 circularity=0.00-1.00 show=Masks display exclude summarize) to count the overlapping cells. Using a maximum thresholding value, an area of each ROI was calculated and then used to estimate the neuronal number per unit area (number density). Percent of colocalized cells was then calculated and used for histograms. Statistical analysis was performed on both raw and percent data.

Cell counting was done by raters naïve to the behavioral phenotypes of the subjects using ImageJ. All cell counts and image analyses were conducted on images representing 824 × 824 μm square section of the LH. First, the total amounts of c-Fos+ nuclei, FG+ nuclei, and OX+ nuclei in a given LH section were quantified according to their anteroposterior region in the LH. Next, a series of colocalization counts were completed to determine the number of FG+/OX+, FG+/cFos+, and OX+/cFos+ cells in the anterior and posterior LH. Afterwards, the number of triple-labeled cells were quantified as the number of cells expressing FG, cFos, and OX.

Then, a series of percentages were calculated. First, the percentage of active lateral hypothalamic cells that projected specifically to the PVT was assessed as: (the total number of FG and c-Fos double labeled cells) ÷ (the total number of FG+ cells) × 100% within each LH section. The percentage of neurons that project from the LH to the PVT and are OXergic was measured as (the total number of FG and OX double labeled cells) ÷ (the total number of FG+ cells) × 100% within each LH section. Finally, the percentage of neurons that project from the LH to PVT and are cellularly active and OXergic was calculated as: (the number of triple-labeled cells expressing c-Fos, FG, and OX) ÷ (the total number of FG+ cells) × 100% within each LH section.

Once all the cell counts were conducted, the anterior and posterior quantities of single labeled cells (FG, OX, and cFos cells), double labeled cells (either OX+/FG+, FG+/cFos+, and OX+/cFos+), and triple labeled cells (OX+/FG+/cFos+) were compared between STs and GTs.

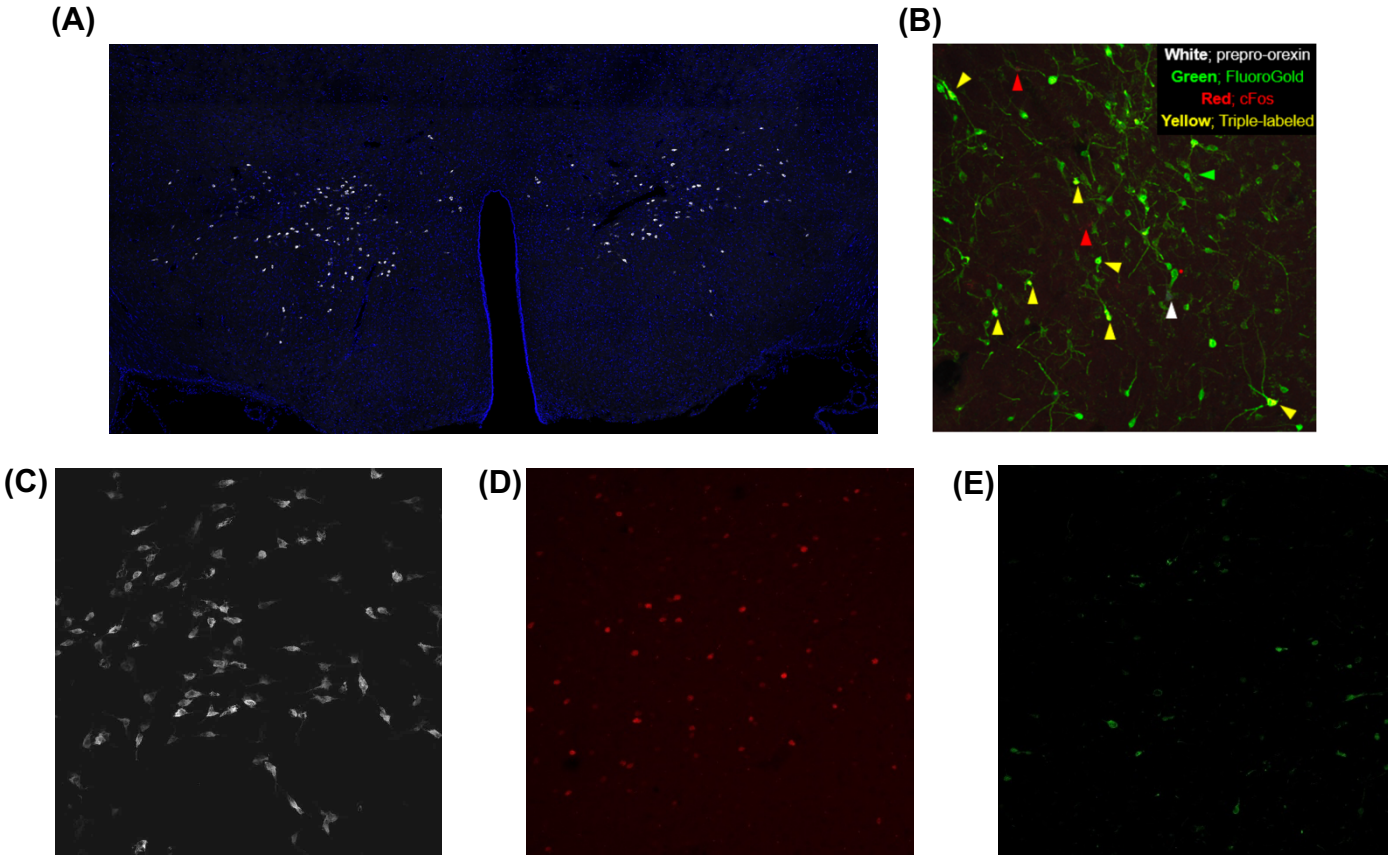


**Figure 5: Sample images validating expression of orexin (OX), cFos, and Fluorogold (FG),**. A) Hybridization of OX probes to the target prepro-orexin mRNA sequence in the lateral hypothalamus (white), imaged with a DAPI counterstain (blue) for cellular nuclei B) Validation of immunofluorescence labelling. White arrows indicate prepro-orexin mRNA, green arrows indicate fluorogold (FG) expression, red arrows indicate cFos expression, and yellow arrows indicate cells expressing prepro-orexin, FG, and cFos. C) 20x magnification of OX hairpins. D) 20x magnification of cFos expression. E) 20x magnification of FG expression.

### 2.6 Statistical Analysis

The Statistical Package for the Social Sciences (SPSS) program version 28.0 (IBM, Armok, NY, USA) was used to analyze behavioral outcome measures as well as the cell counts and percentages. When neuroanatomical region and phenotype were directly compared a two-way ANOVA was performed, as described below. When total cell counts (anterior and posterior combined) and phenotype were directly compared, an independent samples t-test was performed, as described below. For all analyses, statistical significance was set at $p < 0.05$, and Bonferroni *post hoc* comparisons were made when significant main effects or interactions were detected. A

two-way ANOVA was conducted when region (“anterior LH” vs “posterior LH”) was directly compared to phenotype (“ST” vs “GT”), with region (“anterior LH” vs “posterior LH”) as the within/between subject independent variable and phenotype (“ST” vs “GT”) as the between subject independent variable. Differences in total number of cells (anterior and posterior counts combined, dependent variable) between phenotype (“ST” vs “GT”) were analyzed using an unpaired t-test (ST total number of cells vs. GT total number of cells).

## 3. Results

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

First, the number of cells labeled for FG, cFos, and OX were assessed and compared between phenotypes and anterior vs posterior subregions. The number of FG cells did not significantly differ between phenotypes, and this was true across anterior – posterior subregions (Figure 6A) and when the total number of FG+ cells were considered (Figure 6B). The same was true for the number of OX+ cells (Figure 6E-F). Neither FG+ cells nor OX+ cells differed between anterior vs. posterior subregions. Thus, the density of projections from the LH to the PVT and the number of orexinergic neurons appear to be similar between neuroanatomical subregions and between STs and GTs. Relative to GTs, however, STs expressed more cFos+ cells in the LH (Figure 6C, Effect of Phenotype, $F_{(1,33)} = 12.500$, $p=0.001$; Figure 6D, $t_{(17)} = 3.202$, $p=0.005$) and there were a greater number of cFos+ cells in the posterior region of the LH relative to the anterior region (Figure 6C, Effect of Region; $F_{(1,33)} = 4.460$, $p=0.042$).

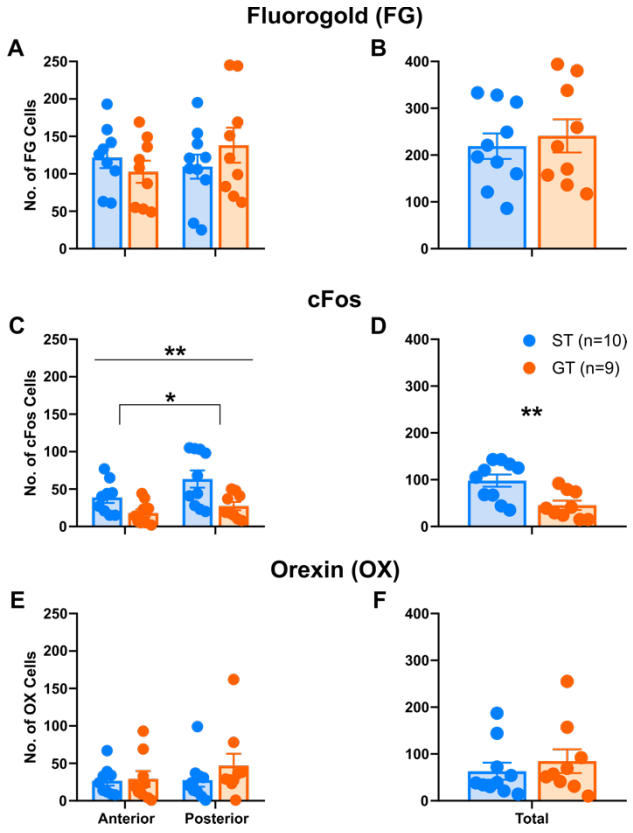


**Figure 6: Number of FG+, cFos+, and OX+ cells in the LH.** Left panels show counts across the anterior and posterior subregions, while panels on the right display the total counts. A-B) No significant differences observed between subregions or phenotypes in the number of FG+ cells in the LH. C) Effect of phenotype (STs > GTs; $F_{(1,33)}$ =12.500, p=0.001) and effect of subregion (posterior > anterior; $F_{(1,33)}$ =4.460, p=0.042) observed for the number of cFos+ cells. D) Significant difference in the number of cFos+ cells between phenotype (STs>GTs; $t_{(17)}$ =3.202, p=0.005). E-F) No significant differences observed between subregions or phenotypes in the number of OX+ cells in the LH.

Upon analysis of colocalization, we found that, relative to GTs, STs have a greater number of cFos+ cells in LH-PVT projecting neurons and this is true across anterior-posterior subregions (Figure 7A, Effect of Phenotype, $F_{(1,33)}$ =12.514, p=0.001) and when comparing the total number of co-labeled FG+ and cFos+ cells (Figure 7B, $t_{(17)}$ =2.558, p=0.020). While the distribution of colocalized FG+ and cFos+ cells did not differ across subregions, colocalization of FG+ and OX+ occurred to a greater extent in the posterior subregion relative to the anterior subregion (Figure 7C, Effect of Region, $F_{(1,33)}$=12.514, p=0.001), suggesting that orexinergic projections from the LH to the PVT are more abundant in the posterior subregion of the LH. Orexinergic cells are also more active in STs relative to GTs, as revealed by colocalization of

OX and c-Fos across anterior-posterior subregions (Figure 7E, Effect of Phenotype, $F_{(1,33)}$ =22.812, p=0.001) and when considering total cell count (Figure 7F; $t_{(17)}$=3.511, p=0.003). When analyzing the triple labelled cells, we found that STs show more colocalization of FG+, cFos+, OX+ cells relative to GTs, and this is true across the anterior-posterior gradient (Figure 7G, Effect of Phenotype, $F_{(1,33)}$ =14.236, p<0.001) and when considering the total number of triple-labeled cells (Figure 7H, $t_{(17)}$ =2.820, p=0.012).

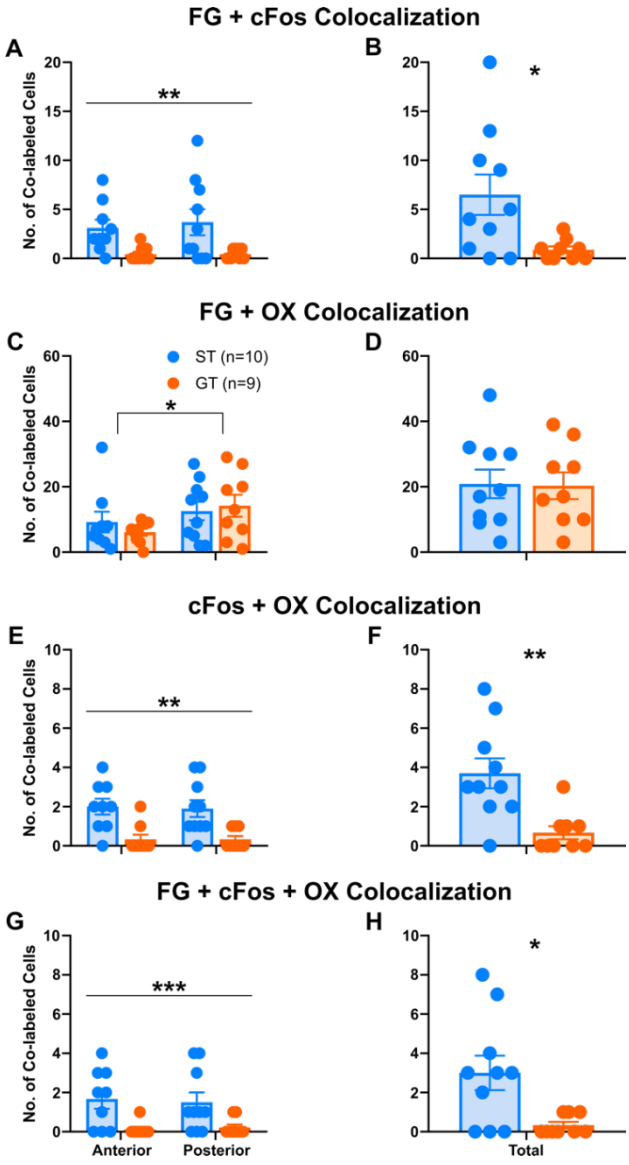


**Figure 7: Number of colocalized cells in the LH.** Left panels show counts for colocalization across the anterior and posterior subregions of the LH, while panels on the right display the total counts. A) Effect of phenotype observed in number of cells with FG+ and cFos+ colocalization (STs > GTs; $F_{(1,33)}$ =12.514, p=0.001). B) Significant difference observed in the total number of FG+ and cFos+ cells in the LH (ST > GT; $t_{(17)}$ =2.558, p=0.020). C) Effect of subregion observed in the number of cells with colocalization of FG and OX in the LH (posterior > anterior; $F_{(1,33)}$=12.514, p=0.001). D) Significant difference observed in the total number of FG+ and OX+ cells in the LH (ST > GT; $t_{(17)}$ =3.511, p=0.003). E) Effect of phenotype observed in number of cells with colocalization of cFos and OX+ (STs > GTs; $F_{(1,33)}$ =22.812, p=0.001). F) Significant difference observed in the total number of cFos+ and OX+ cells in the LH (ST > GT; $t_{(17)}$=3.511, p=0.003). G) Effect of phenotype observed in number of cells with colocalization of FG, cFos, and OX (STs > GTs; $F_{(1,33)}$ = 14.236, p<0.001). H) Significant difference observed in the number of triple-labeled cells in the LH (ST > GT; $t_{(17)}$ =2.820, p=0.012).

Only when we assessed the percentage of cells with double- or triple-labeling did we observe a significant interaction between subregion and phenotype. The percentage of FG+ cells that also expressed cFos differed significantly between phenotypes and subregion (Figure 8A, Phenotype x Region, $F_{(1,33)}$=4.841, p=0.035). Pairwise comparisons revealed that STs showed a greater percentage of FG cells with cFos expression in the posterior LH relative to the anterior (Figure 8A, $F_{(1,33)}$=11.621, p=0.002), and only in the posterior subregion did the two phenotypes significantly differ (Figure 8A, $F_{(1,33)}$=18.752, p<0.001). While approximately 50% of LH-PVT projecting neurons were active in STs, only ~25% were active in GTs (Figure 8B, $t_{(17)}$=3.811, p=0.001). The percentage of FG+ cells that were both OXergic and active in response to cue presentation also differed between phenotypes, with STs displaying a higher percentage than GTs across subregions (Figure 8E, Effect of Phenotype, $F_{(1,33)}$ =14.250, p<0.001) and when the total percentage within the LH was considered (Figure 8F ($t_{(17)}$=2.871, p=0.011). Together, these data suggest that the incentive motivational value of a reward cue is encoded, at least in part, by orexinergic neurons projecting from the LH to the PVT, and perhaps to a greater extent in projecting neurons originating in the posterior portion of the LH.

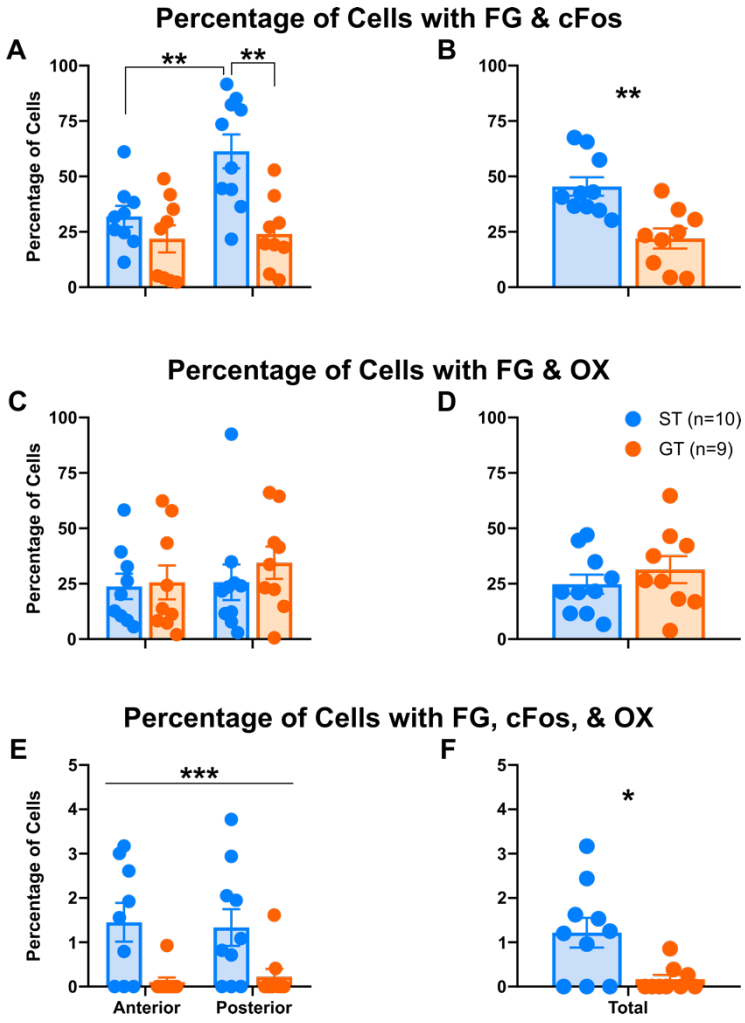


**Figure 8: Percentage of FG+ cells expressing cFos, OX, and cFos + OX.** Left panels show the percentage of cells across anterior and posterior subregions of the LH, while panels on the right display the total percentage within the LH. A) A Phenotype x Region interaction was observed in the percentage of FG+ cells expressing cFos showing that STs have different expression relative to themselves (based on region) and from GTs (STs posterior LH > STs anterior LH, $F_{(1,33)}$=11.621, p=0.002; and STs posterior LH > GTs posterior LH; $F_{(1,33)}$=18.752, p<0.001). ($F_{(1,33)}$=4.841, p=0.035). B) Significant difference observed in the total percentage of FG+ cells expressing cFos in the LH (STs >GTs; $t_{(17)}$=3.811, p=0.001. C-D) No significant differences observed between subregion or phenotype in the percentage of FG+ that express OX+ in the LH. E) Effect of phenotype observed in the percentage of FG+ cells expressing cFos and OX (STs > GTs; $F_{(1,33)}$=14.250, p<0.001). F) Significant difference observed in the total percentage of FG+ cells expressing cFos and OX (STs >GTs; $t_{(17)}$=2.871, p=0.011).

## 4. Discussion

Our objectives for this experiment were to confirm previous literature regarding the activity of the LH-PVT pathway and additionally determine whether OXergic projections play a role in incentive salience attribution encoded through this pathway. We were successfully able to support previous research demonstrating that activity of the LH-PVT pathway differs between STs and GTs. Furthermore, we found differences between anterior and posterior projections of the LH-PVT in their activity of OX neurons in response to a reward cue. Importantly, we show that there are no significant differences in the LH in the FG or OX expression between STs and GTs, suggesting that the OX innervation and connectivity of the LH to the PVT are not different between the phenotypes.

First, we confirmed that the amount of cue-induced activity (number of cFos+ cells) in the LH alone and percent of active cells projecting from the LH to the PVT (amount of FG cells expressing cFos) is increased in STs relative to GTs. These findings were previously shown in a study conducted by Haight et al, wherein STs show greater colocalization of cFos and FG in the LH (2017). It is important to note that the histological methods we used are different from the previous work that inspired this study. In this case, we use immunofluorescence to identify the cFos+ and FG+ neuronal populations in the LH, but Haight and colleagues utilized 3,3'-Diaminobenzidine (DAB) immunohistochemistry to do so. Nonetheless, these different histological methods appear to yield similar results. Thus, these data bolster the finding that, relative to GTs, STs show greater cue-induced activity in the LH-PVT pathway.

Next, we introduced OX into our study of STs and GTs. Our hypothesis that OXergic LH neurons could be involved in encoding the incentive value of reward cues is based, in part, on experiments indicating that animals will work to receive stimulation of the LH (Koob et al.,1978; Olds, J, 1956) and that OXergic projections to the PVT is implicated in motivated behavior (Li et al., 2009; Barson et al., 2015). Some research supporting a role for OXergic neurons in cue-motivated behavior showed that exogenous stimulation of LH OXergic neurons led to the reinstatement of drug-seeking behavior (Harris et al., 2007), and that antagonism decreased morphine preference (Harris et al., 2005). Early studies also showed the OX administration increased intake of a high-fat diet in sated rats and that OX antagonism reduced cue-induced reinstatement of food rewards (Cason et al., 2010). Recent research from our lab has also determined that the LH is necessary for sign-tracking behavior to develop and that OX

antagonism attenuate sign-tracking (Haight et al., 2020). Now, given our results, we further this line of research by suggesting that OXergic innervation from the LH-PVT does play a role in cue-elicited sign-tracking responses. We saw that the overall amount of colocalization between FG and OX in response to cue presentation did not differ between STs and GTs, although we did find that the posterior LH may send more OXergic projections to the PVT than the anterior LH. We also assessed colocalized cells that are OXergic, cellularly active, and project from the LH to the PVT and determined that STs show a greater number of these triple labelled cells than GTs. These data revealed that OX in the LH-PVT pathway may play a role in encoding the incentive value of a reward cue.

We also uncovered regional differences in the number of engaged neurons, number of OXergic LH-PVT projections, and the percentage of active LH-PVT projection neurons. Previous literature has shown that the anterior and posterior regions of the LH receive and send projections from different brain areas (Berk & Finkelstein, 1982; Barone et al., 1981). One study found that while anterior LH neurons terminate in regions including the lateral septum, only the posterior LH projects considerably to the NAc, caudate, and putamen, which all play a role in the reward system (Villalobos & Ferssiwi, 1987). Moreover, the LH sends denser OXergic projections to the posterior PVT as opposed to the anterior PVT (Goto & Swanson, 2004; Kirouac et al., 2005). Interestingly, we found that only a portion of the cells that project to the PVT were cellularly active, suggesting that most of the active LH cells during cue presentation terminate onto other brain areas. Our results indicate that the posterior LH neurons show more activation upon cue presentation relative to anterior LH neurons, which could suggest that projections from the posterior LH to the PVT are possibly more involved in encoding incentive value of reward cues than those from the anterior LH. Importantly, our study also revealed that STs show a greater percentage of cellularly active projections to the PVT in the LH than GTs, and that the posterior region of the LH displays more projections relative to the anterior LH.

There are a few limitations to our study of this pathway. First, the tissue that we are using was gathered several years ago, so the levels of expression may be different than at the time of perfusion. We also are using cFos as a marker of neuronal activity, however, it has been suggested that Fos protein may actually be elevated for extended periods of time (Schwartz et al., 1994). Additionally, our image analysis was conducted on a subsection of the LH, so we were not able to quantify the whole lateral hypothalamic area in our study. Because we only saw a

small amount of colocalization between OX, cFos, and FG, we suspect that other neuronal subtypes in the LH-PVT pathway that we did not study are playing a role in incentive salience attribution.

At this moment, we know that the LH expresses many other neurochemicals, some of which are known to be expressed differentially along the anteroposterior gradient (Bilbao et al., 2022; Bonnavion et al., 2016; Fakhoury et al., 2020). However, there is still limited information regarding many of the different neuronal subtypes in the LH in relation to their neuroanatomical location. Unfortunately, many functional studies have considered the LH as one single area without accounting for the heterogeneity that could lie within (Diaz et al., 2023). For example, one study found that in a conditioned place preference paradigm, rats show increased Fos expression in OX neurons when they are in the test session without drug or food rewards (Aston-Jones et al., 2010). However, it is not known whether this same experiment would yield differences between the amount of OX+/Fos+ neurons in the anterior versus posterior LH. Therefore, future studies should continue to investigate the unique neurochemical profiles and circuitry of the anterior and posterior LH individually.

Future areas of study could also determine the role of OX in reward processing by studying other lateral hypothalamic projections to reward-related brain areas. For example, the ventral tegmental area (VTA) receives dense OXergic projections from the LH and expresses OX1r (Berthoud & Münzberg, 2011), and we know OX1 receptor signaling is necessary for cue-driven cocaine demand (Pantazis et al., 2022). It has been found that administration of a selective OX receptor antagonist in the VTA reduced the rewarding properties of morphine, suggesting that OX projections to the VTA are important in encoding the rewarding aspects of drug cues (Narita et al., 2006). The VTA also receives PVT projections and is a main production site of dopamine, which, as previously mentioned, is necessary for sign-tracking behavior (Flagel et al., 2011). We also know that recent literature has implicated a role of VTA dopaminergic neurons in encoding both predictive and incentive value of reward cues, highlighting its relevance to the study of cue motivated behavior (Ferguson et al., 2020). Because it has been reported that either OX1r or OX2r receptor antagonism in the PVT attenuates sign-tracking (Haight et al., 2020), it would be interesting to determine whether OX receptor antagonism in the VTA would also yield similar behavioral outcomes.

Another area of research expanding from this study could be to investigate sex differences in OXergic activation in the LH in response to reward cues. It has been previously reported has that female rats show higher orexin-a and prepro-orexin mRNA levels compared to males and that this difference seems to be due to glucocorticoid receptor action (Taheri et al., 1999; Grafe et al., 2017). We know that glucocorticoids are critical for sign-tracking behavior as well, indicating another way in which OX neurons could be involved in cue-motivated behavior (Flagel et al., 2008; Rice et al., 2018; Rice et al., 2019). Further research is needed to determine whether the OX system is sex-dependent, whether it can be manipulated via glucocorticoid action, and whether males and females differentially send OXergic projections to the LH when presented with reward predictive cues.

Taken together, these differences in OXergic activation in the LH-PVT pathway between these different behavioral endophenotypes suggest that OX is mediating some of the "bottom-up" processing in sign-tracking behavior (Kuhn et al., 2018; Sarter & Phillips, 2018). Additionally, we see that the anatomical area of activation in the LH is important to consider in this regard. This information is useful for future researchers when studying reward circuitry in the LH, as the anteroposterior location that is studied could yield different or conflicting findings. Finally, as we successfully were able to characterize neurons via both HCR-FISH and IF, we hope to use this novel combination of techniques to identify other neuronal subtypes that play a role in encoding incentive value of reward cues.

## 5. References

Aston-Jones, G., Smith, R. J., Sartor, G. C., Moorman, D. E., Massi, L., Tahsili-Fahadan, P., & Richardson, K. A. (2010). Lateral hypothalamic orexin/hypocretin neurons: A role in reward-seeking and addiction. *Brain Research*, *1314*, 74–90. https://doi.org/10.1016/j.brainres.2009.09.106

Anand, B. K., & Brobeck, J. R. (1951). Hypothalamic control of food intake in rats and cats. The Yale journal of biology and medicine, 24(2), 123–140.

Barone, F. C., Wayner, M. J., Scharoun, S. L., Guevara-Aguilar, R., & Aguilar-Baturoni, H. U. (1981). Afferent connections to the lateral hypothalamus: a horseradish peroxidase study in the rat. *Brain research bulletin*, *7*(1), 75-88.

Berk, M. L., & Finkelstein, J. A. (1982). Efferent connections of the lateral hypothalamic area of the rat: an autoradiographic investigation. *Brain research bulletin*, *8*(5), 511-526.

Berridge, K. C. (2009). 'Liking' and 'wanting' food rewards: brain substrates and roles in eating disorders. *Physiology & Behavior*, *97*(5), 537-550. https://doi.org/10.1016/j.physbeh.2009.02.044

Berridge KC. Reward learning: reinforcement, incentives and expectations. In: Medin D, editor. *Psychology of learning and motivation*. Academic Press; 2001. pp. 223–278.

Berridge, K. C., Robinson, T. E., & Aldridge, J. W. (2009). Dissecting components of reward: 'liking', 'wanting', and learning. *Current Opinion in Pharmacology*, *9*(1), 65–73. https://doi.org/10.1016/j.coph.2008.12.014

Berthoud, H. R., & Münzberg, H. (2011). The lateral hypothalamus as integrator of metabolic and environmental needs: from electrical self-stimulation to opto-genetics. *Physiology & behavior*, *104*(1), 29-39.

Bilbao, M. G., Garrigos, D., Martinez-Morga, M., Toval, A., Kutsenko, Y., Bautista, R., Barreda, A., Ribeiro Do-Couto, B., Puelles, L., & Ferran, J. L. (2022). Prosomeric Hypothalamic Distribution of Tyrosine Hydroxylase Positive Cells in Adolescent Rats. *Frontiers in neuroanatomy*, *16*, 868345. https://doi.org/10.3389/fnana.2022.868345

Bonnavion, P., Mickelsen, L. E., Fujita, A., De Lecea, L., & Jackson, A. C. (2016). Hubs and spokes of the lateral hypothalamus: cell types, circuits and behaviour. The Journal of physiology, 594(22), 6443-6462.

Bossert JM, Mejias-aponte CA, Saunders T, Altidor L, Emery MA, Fredriksson I, Batista A, Claypool S, Caldwell K, Foltz M, Kumar V, Seasholtz A, Hughes E, Filipiak W, Harvey B, Richie CT, Vautier F, Michaelides M, Gomez JL, Kieffer BL, Watson SJ Jr., Akil H, Shaham Y. Effect of Selective Lesions of Nucleus Accumbens $\mu$-Opioid Receptor-Expressing Cells on Heroin Self-Administration in Male and Female Rats: A Study with Novel Oprm1-Cre Knock-in Rats. The Journal of Neuroscience. 2023 Mar; 43 (10) 1692-1713

Bower, G. H., & Miller, N. E. (1958). Rewarding and punishing effects from stimulating the same place in the rat's brain. Journal of comparative and physiological psychology, 51(6), 669–674. https://doi.org/10.1037/h0038925

Campus P, Covelo IR, Kim Y, Parsegian A, Kuhn BN, Lopez SA, Neumaier JF, Ferguson SM, Woods LCS, Sarter M (2019) The paraventricular thalamus is a critical mediator of top-down control of cue-motivated behavior in rats. Elife 8.

Cardinal RN, Parkinson JA, Hall J, Everitt BJ. Emotion and motivation: the role of the amygdala, ventral striatum, and prefrontal cortex. *Neurosci Biobehav Rev*. 2002;26(3):321–352.

Castro, D. C., Cole, S. L., & Berridge, K. C. (2015). Lateral hypothalamus, nucleus accumbens, and ventral pallidum roles in eating and hunger: interactions between homeostatic and reward circuitry. Frontiers in systems neuroscience, 9, 90. https://doi.org/10.3389/fnsys.2015.00090

Choi, H. M., Schwarzkopf, M., Fornace, M. E., Acharya, A., Artavanis, G., Stegmaier, J., ... & Pierce, N. A. (2018). Third-generation in situ hybridization chain reaction: multiplexed, quantitative, sensitive, versatile, robust. *Development*, *145*(12). https://doi.org/10.1242/dev.165753

Colaizzi, J. M., Flagel, S. B., Gearhardt, A. N., Borowitz, M. A., Kuplicki, R., Zotev, V., ... & Paulus, M. P. (2022). The propensity to sign-track is associated with externalizing behaviour and distinct patterns of reward-related brain activation in youth. https://doi.org/10.1101/2022.01.29.477945

Dado, R. J., Burstein, R., Cliffer, K. D., & Giesler Jr, G. J. (1990). Evidence that Fluoro-Gold can be transported avidly through fibers of passage. Brain research, 533(2), 329-333.

de Lecea, L., Kilduff, T. S., Peyron, C., Gao, X., Foye, P. E., Danielson, P. E., Fukuhara, C., Battenberg, E. L., Gautvik, V. T., Bartlett, F. S., 2nd, Frankel, W. N., van den Pol, A. N., Bloom, F. E., Gautvik, K. M., & Sutcliffe, J. G. (1998). The hypocretins: hypothalamus-specific peptides with neuroexcitatory activity. *Proceedings of the National Academy of Sciences of the United States of America*, *95*(1), 322–327. https://doi.org/10.1073/pnas.95.1.322

Delgado, J. M., & Anand, B. K. (1952). Increase of food intake induced by electrical stimulation of the lateral hypothalamus. American Journal of Physiology-Legacy Content, 172(1), 162-168.

Fakhoury, M., Rompre, P. P., and Boye, S. M. (2016). Role of the dorsal diencephalic conduction system in the brain reward circuitry. Behav. Brain Res. 296, 431–441. doi: 10.1016/j.bbr.2015.10.038

Fariba KA, Gokarakonda SB. Impulse Control Disorders. [Updated 2022 Aug 22]. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2023 Jan-. Available from: https://www.ncbi.nlm.nih.gov/books/NBK562279/

Flagel, S. B., Akil, H., & Robinson, T. E. (2009). Individual differences in the attribution of incentive salience to reward-related cues: Implications for addiction. *Neuropharmacology*, *56*, 139-148. https://doi.org/10.1016/j.neuropharm.2008.06.027

Flagel, S. B., Cameron, C. M., Pickup, K. N., Watson, S. J., Akil, H., & Robinson, T. E. (2011). A food predictive cue must be attributed with incentive salience for it to induce c-fos mRNA expression in cortico-striatal-thalamic brain regions. *Neuroscience*, *196*, 80–96. https://doi.org/10.1016/j.neuroscience.2011.09.004

Flagel, S., Clark, J., Robinson, T. et al. A selective role for dopamine in stimulus–reward learning. Nature 469, 53–57 (2011). https://doi.org/10.1038/nature09588

Flagel, S. B., Watson, S. J., Akil, H., & Robinson, T. E. (2008). Individual differences in the attribution of incentive salience to a reward-related cue: influence on cocaine sensitization. *Behavioural Brain Research*, *186*(1), 48-56. https://doi.org/10.1016/j.bbr.2007.07.022

Fraser, K. M., Haight, J. L., Gardner, E. L., & Flagel, S. B. (2016). Examining the role of dopamine D2 and D3 receptors in Pavlovian conditioned approach behaviors. *Behavioural brain research*, *305*, 87-99.

Grafe, L. A., Cornfeld, A., Luz, S., Valentino, R., & Bhatnagar, S. (2017). Orexins mediate sex differences in the stress response and in cognitive flexibility. Biological psychiatry, 81(8), 683-692.

Gorka, S.M., Khorrami, K.J., Manzler, C.A. et al. Acute orexin antagonism selectively modulates anticipatory anxiety in humans: implications for addiction and anxiety. Transl Psychiatry 12, 308 (2022). https://doi.org/10.1038/s41398-022-02090-x
Steward, T., Mestre-Bach, G., Granero, R., Sánchez, I., Riesco, N., Vintró-Alcaraz, C., ... & Fernández-Aranda, F. (2019). Reduced plasma orexin-A concentrations are associated with cognitive deficits in anorexia nervosa. Scientific Reports, 9(1), 1-7.

Haight, J. L., Campus, P., Maria-Rios, C. E., Johnson, A. M., Klumpner, M. S., Kuhn, B. N., ... & Flagel, S. B. (2020). The lateral hypothalamus and orexinergic transmission in the paraventricular thalamus promote the attribution of incentive salience to reward-associated cues. *Psychopharmacology*, *237*(12), 3741-3758. https://doi.org/10.1007/s00213-020-05651-4

Haight, J. L., Fuller, Z. L., Fraser, K. M., & Flagel, S. B. (2017). A food-predictive cue attributed with incentive salience engages subcortical afferents and efferents of the paraventricular nucleus of the thalamus. *Neuroscience*, *340*, 135–152. https://doi.org/10.1016/j.neuroscience.2016.10.043

Haight, J. L., & Flagel, S. B. (2014). A potential role for the paraventricular nucleus of the thalamus in mediating individual variation in Pavlovian conditioned responses. *Frontiers in Behavioral Neuroscience*, *8*, 79. https://doi.org/10.3389/fnbeh.2014.00079

Ide, S., Takahashi, T., Takamatsu, Y., Uhl, G. R., Niki, H., Sora, I., et al. (2017). Distinct roles of opioid and dopamine systems in lateral hypothalamic intracranial self-stimulation. Int. J. Neuropsychopharmacol. 20, 403–409. doi: 10.1093/ijnp/pyw113

Iglesias, A. G., & Flagel, S. B. (2021). The Paraventricular Thalamus as a Critical Node of Motivated Behavior *via* the Hypothalamic-Thalamic-Striatal Circuit. *Frontiers in integrative neuroscience*, *15*, 706713. https://doi.org/10.3389/fnint.2021.706713

Johnson, A. (2020). *Elucidating the Role of the Orexinergic Projections from the Lateral Hypothalamus to the Paraventricular Nucleus of the Thalamus in Incentive Motivational Processes* [Unpublished bachelor's thesis]. University of Michigan

Kirouac, G. J., Parsons, M. P., & Li, S. (2005). Orexin (hypocretin) innervation of the paraventricular nucleus of the thalamus. *Brain Research*, *1059*(2), 179-188. https://doi.org/10.1016/j.brainres.2005.08.035

Koob, G. F., Fray, P. J., and Iversen, S. D. (1978). Self-stimulation at the lateral hypothalamus and locus coeruleus after specific unilateral lesions of the dopamine system. Brain Res. 146, 123–140. doi: 10.1016/0006-8993(78)90222-6

Kuhn, B. N., Campus, P., & Flagel, S. B. (2018). Chapter 3: The neurobiological mechanisms underlying sign-tracking behavior. *Sign-tracking and drug addiction (Arthur T and Morrow J, ed). Ann Arbor: Michigan Publishing, University of Michigan Library*.

Kumar V, Krolewski DM, Hebda-Bauer EK, Parsegian A, Martin B, Foltz M, Akil H, Watson SJ. Optimization and evaluation of fluorescence in situ hybridization chain reaction in cleared fresh-frozen brain tissues. Brain Structure and Function. 2021 Jan; 226, 481–499

Li, Y., Li, S., Wei, C., Wang, H., Sui, N., & Kirouac, G. J. (2010). Changes in emotional behavior produced by orexin microinjections in the paraventricular nucleus of the thalamus. *Pharmacology, Biochemistry, and Behavior*, *95*(1), 121–128. https://doi.org/10.1016/j.pbb.2009.12.016

Limbrick-Oldfield, E., Mick, I., Cocks, R. *et al*. Neural substrates of cue reactivity and craving in gambling disorder. *Transl Psychiatry* 7, e992 (2017). https://doi.org/10.1038/tp.2016.256

Lindsay M. Ferguson, Allison M. Ahrens, Lauren G. Longyear, J. Wayne Aldridge Journal of Neuroscience 11 November 2020, 40 (46) 8951-8963; DOI: 10.1523/jneurosci.2947-19.2020

Lee, S. M., Jang, H. B., Fan, Y., Lee, B. H., Kim, S. C., Bills, K. B., Steffensen, S. C., & Kim, H. Y. (2022). Nociceptive Stimuli Activate the Hypothalamus-Habenula Circuit to Inhibit the Mesolimbic Reward System and Cocaine-Seeking Behaviors. The Journal of neuroscience: the official journal of the Society for Neuroscience, 42(49), 9180–9192. https://doi.org/10.1523/jneurosci.0577-22.2022

Mahler, S. V., Moorman, D. E., Smith, R. J., James, M. H., & Aston-Jones, G. (2014). Motivational activation: a unifying hypothesis of orexin/hypocretin function. *Nature neuroscience*, *17*(10), 1298–1303. https://doi.org/10.1038/nn.3810

Mahler, S. V., Smith, R. J., Moorman, D. E., Sartor, G. C., & Aston-Jones, G. (2012). Multiple roles for orexin/hypocretin in addiction. Progress in brain research, 198, 79–121. https://doi.org/10.1016/B978-0-444-59489-1.00007-0

Marchant, N. J., Hamlin, A. S., & McNally, G. P. (2009). Lateral hypothalamus is required for context-induced reinstatement of extinguished reward seeking. Journal of Neuroscience, 29(5), 1331-1342.

Matzeu, A., & Martin-Fardon, R. (2021). Cocaine-Seeking Behavior Induced by Orexin A Administration in the Posterior Paraventricular Nucleus of the Thalamus Is Not Long-Lasting: Neuroadaptation of the Orexin System During Cocaine Abstinence. *Frontiers in Behavioral Neuroscience*, *15*, 620868. https://doi.org/10.3389/fnbeh.2021.620868

McGinnis, J. M., & Foege, W. H. (1999). Mortality and morbidity attributable to use of addictive substances in the United States. *Proceedings of the Association of American Physicians*, *111*(2), 109–118. https://doi.org/10.1046/j.1525-1381.1999.09256.x

Meffre, J., Sicre, M., Diarra, M., Marchessaux, F., Paleressompoulle, D., & Ambroggi, F. (2019). Orexin in the Posterior Paraventricular Thalamus Mediates Hunger-Related Signals in the Nucleus Accumbens Core. *Current Biology : CB*, *29*(19), 3298–3306.e4. https://doi.org/10.1016/j.cub.2019.07.069

Narita, M., Nagumo, Y., Hashimoto, S., Narita, M., Khotib, J., Miyatake, M., ... & Suzuki, T. (2006). Direct involvement of orexinergic systems in the activation of the mesolimbic dopamine pathway and related behaviors induced by morphine. Journal of Neuroscience, 26(2), 398-405.

Olds, J. (1956). Pleasure centers in the brain. Scientific American, 195(4), 105-117.

Ollion J, Cochennec J, Loll F, Escudé C, & Boudier T. (2013). TANGO: a generic tool for high-throughput 3D image analysis for studying nuclear organization. Bioinformatics, 29(14), 1840–1841

Pantazis, C. B., James, M. H., O'Connor, S., Shin, N., & Aston-Jones, G. (2022). Orexin-1 receptor signaling in ventral tegmental area mediates cue-driven demand for cocaine. *Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology*, *47*(3), 741–751. https://doi.org/10.1038/s41386-021-01173-5

Petzold, A., van den Munkhof, H. E., Figge-Schlensok, R., & Korotkova, T. (2023). Complementary lateral hypothalamic populations resist hunger pressure to balance nutritional and social needs. Cell Metabolism, 35(3), 456-471.

Rice, B. A., Eaton, S. E., Prendergast, M. A., & Akins, C. K. (2018). A glucocorticoid receptor antagonist reduces sign-tracking behavior in male Japanese quail. *Experimental and clinical psychopharmacology*, *26*(4), 329–334. https://doi.org/10.1037/pha0000195

Rice, B. A., Saunders, M. A., Jagielo-Miller, J. E., Prendergast, M. A., & Akins, C. K. (2019). Repeated subcutaneous administration of PT150 has dose-dependent effects on sign tracking in male Japanese quail. *Experimental and clinical psychopharmacology*, *27*(6), 515–521. https://doi.org/10.1037/pha0000275

Robinson, T. E., & Berridge, K. C. (1993). The neural basis of drug craving: an incentive-sensitization theory of addiction. *Brain Research. Brain Research Reviews*, *18*(3), 247–291. https://doi.org/10.1016/0165-0173(93)90013-p

Robinson, T. E., Yager, L. M., Cogan, E. S., & Saunders, B. T. (2014). On the motivational properties of reward cues: individual differences. *Neuropharmacology*, *76*, 450-459. https://doi.org/10.1016/j.neuropharm.2013.05.040

Sakurai, T. The neural circuit of orexin (hypocretin): maintaining sleep and wakefulness. *Nat Rev Neurosci* 8, 171–181 (2007). https://doi.org/10.1038/nrn2092

Sakurai, T., Amemiya, A., Ishii, M., Matsuzaki, I., Chemelli, R. M., Tanaka, H., Williams, S. C., Richardson, J. A., Kozlowski, G. P., Wilson, S., Arch, J. R., Buckingham, R. E., Haynes, A. C., Carr, S. A., Annan, R. S., McNulty, D. E., Liu, W. S., Terrett, J. A., Elshourbagy, N. A., Bergsma, D. J., … Yanagisawa, M. (1998). Orexins and orexin receptors: a family of hypothalamic neuropeptides and G protein-coupled receptors that regulate feeding behavior. *Cell*, *92*(4), 573–585. https://doi.org/10.1016/s0092-8674(00)80949-6

Saper, C. B., Swanson, L. W., & Cowan, W. M. (1979). An autoradiographic study of the efferent connections of the lateral hypothalamic area in the rat. Journal of Comparative Neurology, 183(4), 689-706.

Sarter, M., & Phillips, K. B. (2018). The neuroscience of cognitive-motivational styles: Sign- and goal-trackers as animal models. *Behavioral neuroscience*, *132*(1), 1–12. https://doi.org/10.1037/bne0000226

Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, et al., 2012. Fiji: an open-source platform for biological-image analysis. Nature Methods 9, 676–682. https://doi.org/10.1038/nmeth.2019.

Schneider CA, Rasband WS, Eliceiri KW, 2012. NIH Image to ImageJ: 25 years of image analysis. Nature Methods 9, 671–675. https://doi.org/10.1038/nmeth.2089.

Schwartz, W. J., Takeuchi, J., Shannon, W., Davis, E. M., & Aronin, N. (1994). Temporal regulation of light-induced Fos and Fos-like protein expression in the ventrolateral subdivision of the rat suprachiasmatic nucleus. *Neuroscience*, *58*(3), 573–583. https://doi.org/10.1016/0306-4522(94)90082-5

Scott, K. M., de Vries, Y. A., Aguilar-Gaxiola, S., Al-Hamzawi, A., Alonso, J., Bromet, E. J., Bunting, B., Caldas-de-Almeida, J. M., Cía, A., Florescu, S., Gureje, O., Hu, C. Y., Karam, E. G., Karam, A., Kawakami, N., Kessler, R. C., Lee, S., McGrath, J., Oladeji, B., Posada-Villa, J., … World Mental Health Surveys collaborators (2020). Intermittent explosive disorder subtypes in the general population: association with comorbidity, impairment and suicidality. *Epidemiology and psychiatric sciences*, *29*, e138. https://doi.org/10.1017/S2045796020000517

Shin, L. M., Orr, S. P., Carson, M. A., Rauch, S. L., Macklin, M. L., Lasko, N. B., Peters, P. M., Metzger, L. J., Dougherty, D. D., Cannistraro, P. A., Alpert, N. M., Fischman, A. J., & Pitman, R. K. (2004). Regional cerebral blood flow in the amygdala and medial prefrontal cortex during traumatic imagery in male and female Vietnam veterans with PTSD. *Archives of General Psychiatry*, *61*(2), 168–176. https://doi.org/10.1001/archpsyc.61.2.168

Small, D. M., Zatorre, R. J., Dagher, A., Evans, A. C., & Jones-Gotman, M. (2001). Changes in brain activity related to eating chocolate: from pleasure to aversion. *Brain*, *124*(9), 1720-1733. https://doi.org/10.1093/brain/124.9.1720

Stuber, G. D., & Wise, R. A. (2016). Lateral hypothalamic circuits for feeding and reward. Nature neuroscience, 19(2), 198-205.

Taheri, S., Mahmoodi, M., Opacka-Juffry, J., Ghatei, M. A., & Bloom, S. R. (1999). Distribution and quantification of immunoreactive orexin A in rat tissues. FEBS letters, 457(1), 157-161.

Thomas, C. S., Mohammadkhani, A., Rana, M., Qiao, M., Baimel, C., & Borgland, S. L. (2022). Optogenetic stimulation of lateral hypothalamic orexin/dynorphin inputs in the ventral tegmental area potentiates mesolimbic dopamine neurotransmission and promotes reward-seeking behaviours. Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology, 47(3), 728–740. https://doi.org/10.1038/s41386-021-01196-y

Tomie, A., & Morrow, J. (2018). Sign-tracking and drug addiction. Maize Books.

Tsujino, N., & Sakurai, T. (2013). Role of orexin in modulating arousal, feeding, and motivation. *Frontiers in behavioral neuroscience*, *7*, 28.

Tsukayama, E., Toomey, S. L., Faith, M. S., & Duckworth, A. L. (2010). Self-control as a protective factor against overweight status in the transition from childhood to adolescence. *Archives of pediatrics & adolescent medicine*, *164*(7), 631–635. https://doi.org/10.1001/archpediatrics.2010.97

Tyree, S. M., Jennings, K. J., Gonzalez, O. C., Li, S. B., Nicholson, J. R., von Heimendahl, M., & de Lecea, L. (2023). Optogenetic and pharmacological interventions link hypocretin neurons to impulsivity in mice. Communications biology, 6(1), 74. https://doi.org/10.1038/s42003-023-04409-w

Watson, G. P. a. C. (2007). *The Rats Brain In Stereotaxic Coordinates 6th Edition*. Elsevier.

Wei Q, Kumar V, Moore S, Li F, Murphy GG, Watson SJ, Akil H. High emotional reactivity is associated with activation of a molecularly distinct hippocampal-amygdala circuit modulated by the glucocorticoid receptor. bioRxiv 2022.06.01.494356

Wiskerke, J., James, M. H., & Aston-Jones, G. (2020). The orexin-1 receptor antagonist SB-334867 reduces motivation, but not inhibitory control, in a rat stop signal task. Brain research, 1731, 146222. https://doi.org/10.1016/j.brainres.2019.04.017