The role of the paraventricular nucleus of the thalamus in the augmentation of heroin seeking induced by chronic food restriction

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ABSTRACT

The role of the paraventricular nucleus of the thalamus in the augmentation of heroin seeking induced by chronic food restriction

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North America is in the midst of an opioid crisis. Many individuals who suffer from addiction to opioids attempt to stop using the drug. However, many of these individuals often find it difficult to maintain abstinence and ultimately end up relapsing, starting the vicious cycle of addiction all over again. In both human and animal models of drug addiction, chronic food restriction increases rates of relapse. Our laboratory has previously demonstrated a robust increase in drug-seeking following a period of abstinence in chronically food-restricted rats compared to their sated counterparts. To date, the neural mechanisms that mediate the effect of chronic food restriction on drug-seeking remain elusive. However, the paraventricular nucleus of the thalamus (PVT) appears to be a promising candidate to investigate. The PVT is uniquely situated to contribute to homeostatic and drug-seeking systems. Thus, this thesis aimed to examine the role of the PVT in the augmentation of heroin seeking induced by chronic food restriction using a chemogenetic approach. In the first series of experiments, we sought to determine if the PVT plays a role in heroin seeking in chronically food-restricted rats following a period of abstinence. Here, we showed that chemogenetically activating the PVT abolishes heroin seeking in chronically food-restricted rats indicating that the PVT does play a role in this phenomenon. Next, we investigated the role of the input from the prelimbic cortex to the PVT. Here, we did not observe any changes in heroin seeking behaviour in chronically food-restricted or sated rats when we activated or inhibited this pathway despite verifying that chemogenetic

manipulations were sufficient to alter neuronal activity. In the last series of experiments, we assessed the role of the output from the PVT to the nucleus accumbens (NAc). We demonstrated that chemogenetically activating PVT projections to the NAc shell, but not core, abolished the augmentation of heroin seeking in chronically food-restricted rats. Taken together, the findings presented in this thesis indicate that the PVT and its projection to the NAc shell play a critical role in the regulation of heroin seeking in chronically food-restricted rats following a period of abstinence.

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CONTRIBUTION OF AUTHORS

Chapter 1: The general introduction was written by Alexandra Chisholm and revised by Dr. Uri Shalev.

Chapter 2: The general methodology was written by Alexandra Chisholm and revised by Dr. Uri Shalev.

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Chapter 6: The general discussion was written by Alexandra Chisholm and revised by Dr. Uri Shalev.

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LIST OF ABBREVIATIONS

AAV ..... adeno-associated virus
ACh ..... acetylcholine
AgRP ..... agouti-related protein
ARC ..... activity-regulated cytoskeleton-associated protein
ACSF ..... artificial cerebrospinal fluid
AMPA ..... α-amino-3 hydroxy-5-methylisoazole-4-proprionic acid
AMPAR ..... α-amino-3 hydroxy-5-methylisoazole-4-proprionic acid receptor
ANOVA ..... analysis of variance
BDNF ..... brain derived neurotrophic factor
BLA ..... basolateral amygdala
B/M ..... baclofen/muscimol
BNST ..... bed nucleus of the stria terminalis
CART ..... cocaine- and amphetamine-regulated transcript
CeA ..... central nucleus of the amygdala
CNO ..... clozapine-N-oxide
CPP ..... conditioned place preference
CRE ..... Cre recombinase
DAB 3,3-diaminobenzidine
D1/D1R ..... dopamine-1 receptor
D2/D2R ..... dopamine-2 receptor
DA ..... dopamine
DREADDs ..... designer receptors exclusively activated by designer drugs
DMSO ..... dimethyl sulfoxide
EPM ..... elevated plus maze
FDR ..... food restricted
FI ..... fixed interval
FLEX ..... flip excision
GABA ..... gamma-aminobutyric acid
GAL ..... galanin
hSyn ..... human synapsin

IC.....insular cortex
IL.....infralimbic cortex
i.p. ..... intraperitoneal
IR..... immunoreactivity
IU. .....International unit
i.v. .....intravenous
KHz..... kilohertz
LHA ..... lateral hypothalamus
mGluR.....metabotropic glutamate receptor
mPFC .....medial prefrontal cortex
mCherry ..... mCherry red fluorescent protein
mRNA ..... messenger ribonucleic acid
MSNs ..... medium spiny neurons
MTN.....midline thalamic nuclei
NAc .....nucleus accumbens
NAcC .....nucleus accumbens core
NAcS.....nucleus accumbens shell
NBQX .....2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione
NMDA ..... N-methyl-D-aspartate
NMDAR..... N-methyl-D-aspartate receptor
nmol .....nanomole
OX1R .....orexin-1 receptor
OX2R .....orexin-2 receptor
pA.....picoampere
PBS ..... phosphate-buffered saline
PrL..... prelimbic cortex
PVT ..... paraventricular nucleus of the thalamus
aPVT ..... anterior paraventricular nucleus of the thalamus
mPVT..... mid paraventricular nucleus of the thalamus
pPVT ..... posterior paraventricular nucleus of the thalamus
s.c. .....subcutaneous

TBS ..... Tris-buffered saline

THC delta-9-tetrahydrocannabinol

TCSOX229 ..... (2*S*)-1-(3,4-Dihydro-6,7-dimethoxy-2(1*H*)-isoquinolinyl)-3,3-dimethyl-2-[(4-pyridinylmethyl)amino]-1-butanone hydrochloride

TTX ..... tetrodotoxin

VTA ..... ventral tegmental area

ZI ..... zona incerta

CHAPTER 1: GENERAL INTRODUCTION

"Opioids reach every part of society: Blue collar, white collar, everybody. It's nonstop. It's every day. And it doesn't seem like it's getting any better."

- Walter Bender (Deputy Sherriff in Montgomery County, Ohio)

Anyone reading or watching the news today is aware of how easy, cheap and widely available drugs are. In the United States of America, the number of drug overdose deaths has dramatically risen over the past 15 years. In 2017, 70 237 Americans died from drug overdose (Scholl, Seth, Kariisa, Wilson, & Baldwin, 2018). Of these 70 237 deaths, opioids were responsible for claiming 47 600 lives representing over 65% of all deaths. Comparably, since 2016 more than 13 900 Canadians have died due to opioid overdose (Special Advisory Committee on the Epidemic of Opioid Overdoses, 2020). To put this into perspective, every day, approximately 130 people in the United States and 11 people in Canada die from opioid overdose (Hedegaard, Warner, & Miniño, 2018; Special Advisory Committee on the Epidemic of Opioid Overdoses, 2020). Before you read any further, take a look at "The Opioid Diaries," published in Time Magazine (Nachtwey, 2018). If you haven't witnessed the devastation that opioids cause in our society, this is a good place to start. However, I will warn you; it is graphic.

As I write this dissertation, the opioid crisis continues to rage. But how did we get here? Opioids are potent analgesics typically prescribed for pain management. In 2015, in the United States, over 240 million opioid prescriptions were dispensed for a population of 320 million people (Makary, Overton, & Wang, 2017). That means that 75% of the US population had a legal prescription for opioids! Canada is not much better. In 2016, over 20 million prescriptions for opioids were dispensed for a population of 37 million people,

meaning 55% of the Canadian population had a legal prescription for opioids (Belzak & Halverson, 2018). No wonder we have a problem!

While opioids are potent analgesics, the inability to stop using them once the pain has ceased can result in opioid misuse. Opioid misuse refers to drug use other than how it was prescribed and includes snorting/injecting the drug or taking higher doses of the drug. Opioids are often misused due to their analgesic and rewarding effects (Volkow, Benveniste, & McLellan, 2018). Additionally, physically dependent individuals may misuse opioids to avoid withdrawal symptoms. It is therefore not surprising that misuse of prescription opioids has noticeably escalated. While the opioid crisis was initially driven by over-prescription of opioids that facilitated misuse, the current crisis reflects a shifting scenario.

Today, prescription opioid use is being replaced by heroin and other synthetic opioids. But why did the pattern shift? Individuals dependent on prescription opioids transitioned to heroin due to its widespread availability and inexpensiveness (Volkow et al., 2018). This transition from prescription opioid misuse to heroin is particularly evident in young individuals (Mars et al., 2015). Moreover, this shift from prescription opioid misuse towards heroin contributes to the continually rising number of opioid-related fatalities in our society.

Living with Addiction

Not all individuals who misuse opioids will become addicted. However, a small number of users will go on to develop an addiction. Frank, a deceased heroin addict, reveals that struggling with heroin addiction is like having a "*one-way ticket on a Hellbound train*" (Maté, 2008). But what is drug addiction? For an individual dependent

on heroin, addiction is a cyclic pattern that involves periods of drug use and abstinence that the individual feels compelled to persist despite negative life consequences. Simply put, addiction is a cycle (Figure 1.1).

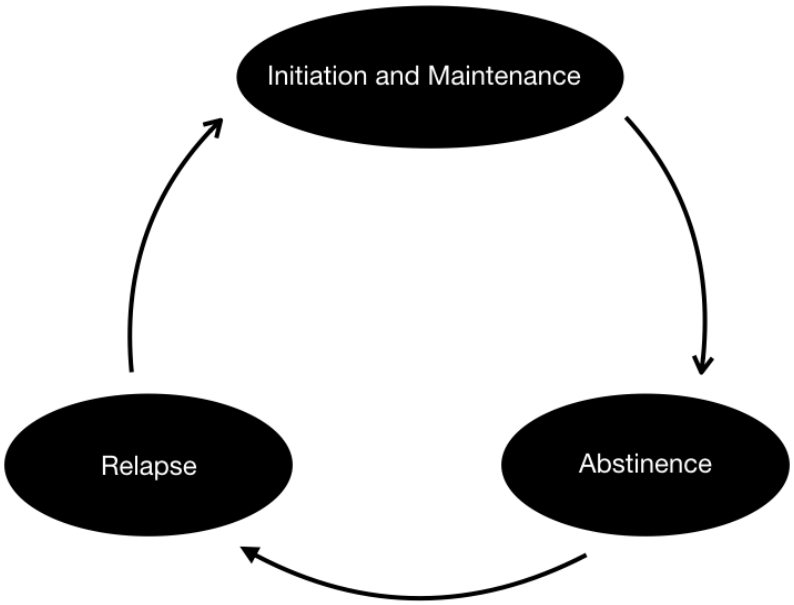

Figure 1. 1. Drug addiction is a vicious cycle involving three main stages: (1) the initiation and/or maintenance of drug use, (2) a period away from the drug, known as abstinence, and (3) a return to drug use, known as relapse.

One major issue in the treatment of drug addiction is relapse, a return to drug use following a period of non-use. "Once you're into heroin, it's almost like a relationship with a person you love. And letting go of that, the thought of never seeing someone I love again - I couldn't imagine giving it up forever" (Nachtwey, 2018). Unfortunately, 40-50% of individuals use heroin long-term (Hser, 2007; Hser, Hoffman, Grella, & Anglin, 2001). An alarmingly high relapse rate occurs in abstinent heroin users with estimates ranging from 70-91% (Hunt, Barnett, & Branch, 1971; Smyth, Barry, Keenan, & Ducray, 2010).

Perhaps not surprisingly, even after 15 years of abstinence, approximately 25% of heroin users relapse (Hser et al., 2001).

Triggers to Relapse

In abstinent drug users, relapse is triggered by three key factors. First, re-exposure to the abused drug can increase drug craving and desire, triggering relapse (De Wit, 1996). For example, if an abstinent drug user is exposed to morphine to treat pain, this may trigger relapse. Second, re-exposure to cues associated with drug availability and use increases subjective drug craving and relapse (Childress et al., 1993). For instance, if an abstinent heroin user is around paraphernalia previously used to take heroin, this may trigger relapse. Lastly, stressors increase drug craving and relapse (Sinha, 2001). Acute and chronic stressors increase relapse (Brown, Vik, Patterson, Grant, & Schuckit, 1995; Preston & Epstein, 2011; Sinha, 2001). For example, in an abstinent drug user, any stressful life event (e.g., death of a loved one) can trigger relapse.

Detoxing from opioids is an unpleasant and challenging process that can be tolerated due to its relatively short time course (i.e., 24-48 hours). But managing physical withdrawal symptoms is not the real challenge. The real challenge is maintaining abstinence. Many people relapse during opioid abstinence because they have such intense cravings for the drug. Many abstinent drug users report increased craving for cigarettes, alcohol, and methamphetamine over the abstinence period (Li, Caprioli, & Marchant, 2015), a phenomenon known as incubation of drug craving (Grimm, Hope, Wise, & Shaham, 2001). Consequently, in an abstinent drug user, exposure to any of the three triggers may increase drug craving and relapse risk.

Caloric Restriction and Drug Craving

Caloric restriction during abstinence can modulate the three relapse triggers mentioned above. In abstinent drug users, caloric restriction increases the user's tendency to seek out and use drugs. For example, dieting increases drug craving and relapse rates in abstinent cigarette users (Cheskin, Hess, Henningfield, & Gorelick, 2005; Hall, Tunstall, Vila, & Duffy, 1992; Ockene et al., 2000). Furthermore, dieting severity is positively associated with cigarette, alcohol, and marijuana use (Krahn, Kurth, Demitrack, & Drewnowski, 1992).

Poor diet is a common problem in many drug users. Heroin users exhibit nutritional deficiencies and dysfunctional eating patterns (el-Nakah, Frank, Louria, Quinones, & Baker, 1979; Nazrul Islam, Jahangir Hossain, Ahmed, & Ahsan, 2002; Best et al., 1998; Neale, Nettleton, Pickering, & Fischer, 2012; Noble & McCombie, 1997). While abstinent heroin users initially report pleasure eating and gain weight, over time, these same users become anxious over gaining weight and controlling their appetite (Neale et al., 2012). However, dietary restriction during a period of non-use can increase drug craving and rates of relapse. As such, abstinent drug users who have concerns over weight gain and controlling appetite may try to compensate by engaging in restrictive behaviours (i.e., eating less). Abstinent drug users engaging in restrictive behaviours may face unintended consequences - increased drug craving and relapse - that may hinder their ability to abstain from drug use and remain in treatment. But what causes the increased drug craving and relapse?

We Do Not Know

At the present time, we do not know why restricted food intake increases drug craving and relapse in abstinent drug users. Studies using human participants are limited

in identifying causal mechanisms when examining the effect of caloric restriction on drug craving and relapse. Many studies are correlational - it is impossible to determine the causative nature of events. Drug craving may be subject to inaccurate reporting (Rosenberg, 2009; Sayette et al., 2000). Additionally, drug craving does not always lead to relapse (Droungas, Ehrman, Childress, & O'Brien, 1995). Ethical limitations minimize the type of research conducted using human participants. Due to these limitations, it is difficult to identify causal neural mechanisms involved in relapse using human participants. Fortunately, animal models are a useful alternative: they provide greater experimental control and allow the study of underlying neural mechanisms involved in relapse (Shaham, Shalev, Lu, de Wit, & Stewart, 2003).

Animal Models of Relapse

In animal models of relapse, animals learn to self-administer a drug by performing an operant response (i.e., lever pressing or nose poke) to obtain a dose of that drug. Animals then undergo a period of abstinence – the drug is not available, and finally, are tested for relapse by examining their drug-seeking behaviour. Abstinence can be achieved using three methods: (1) extinction, (2) forced abstinence, or (3) voluntary abstinence (Reiner, Fredriksson, Lofaro, Bossert, & Shaham, 2019).

In extinction-based abstinence models, animals (for example, rats) learn to self-administer a drug by performing an operant response in the presence of discrete cues (e.g., tone and/or light paired with the delivery of the drug infusion) and then operant responding for the drug is extinguished. During extinction training, performing the operant response no longer results in drug delivery. In forced abstinence models, rats learn to self-administer a drug by performing an operant response in the presence of

discrete cues and are then removed from the drug-taking context and placed into another context (i.e., home cage) for a specified period of time – the rats no longer have access to the drug or exposure to the drug-associated cues. In the voluntary abstinence model, rats learn to self-administer a drug by performing an operant response in the presence of discrete cues. Then the same operant responding is associated with an aversive consequence (i.e., electrical footshock), or an alternative choice for a nondrug reward is provided (i.e., social interaction, palatable food) – resulting in voluntary abstinence from performing the operant response that results in drug administration.

In extinction-based abstinence models, reinstatement of drug-seeking is achieved by presenting discrete, discriminative, or contextual cues (i.e., re-exposure to the drug-taking environment) by drug re-exposure or by exposure to stressors (e.g., food deprivation). Unfortunately, this model's major limitation is its ecological validity; active extinction is not a cause of abstinence in drug users. The forced and voluntary abstinence models of relapse attempt to solve this issue by attempting to mimic the human condition – drug users are forced to abstain from drug use (e.g., incarceration), or they choose to abstain. In these two models, relapse testing is performed by presenting discrete cues previously associated with drug-taking and by examining the number of drug-associated operant responses the animal makes under extinction conditions. The number of operant responses on the drug-associated lever serves as a measure of drug-seeking and drug craving. Using these two models, relapse testing can occur at any point during the abstinence period.

Acute Food Deprivation in Animal Models of Drug Use and Relapse

Dietary manipulations affect drug-taking and drug-seeking in rodent models of relapse. Acute food deprivation (e.g., 24- 48 h) and chronic food restriction (e.g., prolonged period of time with limited access to food) increase oral and intravenous drug intake for numerous illicit substances (Carroll, France, & Meisch, 1979; Carroll & Meisch, 1981; Carroll & Meisch, 1984). Similarly, not only does acute food deprivation increase drug intake, but it also increases drug-seeking. For example, acute food deprivation reinstates extinguished heroin and cocaine seeking (Shalev, Highfield, Yap, & Shaham, 2000; Shalev, Robarts, Shaham, & Morales, 2003b). Interestingly, administration leptin, which is a hormone released from peripheral adipocytes and is involved in energy balance, reduces heroin seeking in food-deprived rats (Shalev, Yap, & Shaham, 2001). However, administration of leptin does not affect drug prime or stress-induced reinstatement, suggesting that drug-seeking induced by dietary manipulation is mediated by a distinct neural circuit (Shalev et al., 2001). However, in humans, the existing literature indicates that only prolonged food restriction, but not acute food deprivation is associated with increased drug intake and risk for relapse (Cheskin et al., 2005; Zacny & de Wit, 1992; Hall et al., 1992). These findings suggest that chronic food restriction is a more ecologically relevant stressor for studying relapse in rodent models.

Chronic Food Restriction in Animal Models of Drug Use and Relapse

Experimental evidence using animal models indicates that chronic food restriction alters drug-related behaviours. Chronic food restriction enhances the acquisition of both oral and intravenous drug self-administration (Carroll et al., 1979). Chronic food restriction increases the reinforcing properties of electrical brain stimulation reward (Fulton, Woodside, & Shizgal, 2000), potentiates threshold-lowering effects of lateral

hypothalamic brain stimulation reward of amphetamine, phencyclidine, MK-801 and cocaine (Cabeza De Vaca & Carr, 1998; Carr, 2007) and increases the locomotor-stimulating effects of opioids and psychostimulants (Deroche, Piazza, Casolini, Le Moal, & Simon, 1993; Marinelli, Le Moal, & Piazza, 1996; Campbell & Fibiger, 1971).

Evidence indicates that chronic food restriction increases the rewarding properties of drugs. Chronic food restriction increases the persistence of cocaine, and morphine-induced conditioned place preference established under ad libitum conditions (Zheng, Cabeza de Vaca, & Carr, 2012; Jung et al., 2016). Moreover, ten days of chronic food restriction but not seven days reinstates extinguished heroin seeking (Shalev, 2012). These findings indicate that chronic food restriction can alter the rewarding and conditioned reinforcing effects of drugs of abuse.

Augmentation of Heroin Seeking by Chronic Food Restriction

Our laboratory investigates relapse using an abstinence procedure. In this procedure, rats are trained to self-administer heroin for ten days (i.e., self-administration phase). Then, rats are moved to the animal colony for a 15-day forced abstinence period (i.e., withdrawal phase). During this phase, rats have restricted access to food (FDR group) or unrestricted access to food (sated group). On the last day of the withdrawal phase, rats are returned to the self-administration chamber for a drug-seeking test under extinction conditions (i.e., drug-seeking test phase). Using this procedure, our laboratory has demonstrated a robust increase in heroin seeking in chronically food-restricted rats. FDR rats seek heroin up to 250% more than sated controls (D'Cunha, Sedki, Macri, Casola, & Shalev, 2013)! To date, the neural mechanisms involved in food restriction-induced augmentation of heroin seeking remain unknown. However, one interesting

neural candidate that may be involved in mediating this effect is the paraventricular nucleus of the thalamus (PVT) due to its role in drug-related behaviours and homeostatic control.

Paraventricular Nucleus of the Thalamus

Over 50 years ago, the PVT first emerged as a prospective mediator of motivated behaviour when it was observed that rats would bar press for intracranial stimulation of electrodes placed in and near the PVT (Cooper & Taylor, 1967). About 15 years later, these findings were corroborated when it was reported that intracranial self-stimulation scores were higher when electrode placement approached the midline, which included the PVT (Clavier & Gerfen, 1982).

Due to the complex nature of drug addiction, much research has attempted to elucidate the neurobiological substrates that contribute to addiction-related behaviours. However, most of this research has been focused on the mesolimbic dopamine system, while other potential contributors have been overlooked (Ikemoto, 2010). One particular structure that was previously not considered to be part of the addiction circuitry but has received a sudden surge of attention in the last few years is the PVT (see Zhou & Zhu, 2019 for review). The PVT has garnered much attention due to its unique location, extensive and diverse neural network and for its role in mediating motivated behaviours (Martin-Fardon & Boutrel, 2012; James & Dayas, 2013).

PVT Circuitry

The PVT is a midline nucleus located directly below the third ventricle and is optimally positioned to act as an interface for neural integration of subcortical, cortical, limbic and motor circuits and relay this information to other areas of the brain –

specifically the striatum (Kelley, Baldo, & Pratt, 2005). The PVT receives an extensive set of subcortical afferents that arise from areas including, but not restricted to, the hypothalamus, locus coeruleus, hippocampus, amygdala, dorsal raphe and ventral tegmental area (Van der Werf, Witter, & Groenewegen, 2002; Vogt, Hof, Friedman, Sikes, & Vogt, 2008; Hsu & Price, 2009; Kirouac, 2015; Li & Kirouac, 2012). The PVT also receives innervation from the medial prefrontal cortex (mPFC). Overall, the densest source of innervation from the mPFC arises from the prelimbic cortex (PrL), which innervates anterior and posterior aspects of the PVT. In addition, the PVT receives innervation from the infralimbic (IL) and cingulate cortices (Vertes & Hoover, 2008; Li & Kirouac, 2012).

The PVT receives an expansive set of subcortical and cortical afferents, and PVT efferents, mainly glutamatergic, extensively project to subcortical and cortical structures including the PrL, IL, nucleus accumbens (NAc) core and shell, bed nucleus of the stria terminalis (BNST), central amygdala (CeA), basolateral amygdala (BLA), hippocampus and hypothalamus (Li & Kirouac, 2008; Jones, Kilpatrick, & Phillipson, 1989; Pinto, Jankowski, & Sesack, 2003; Vertes & Hoover, 2008).

aPVT and pPVT Circuitry

Evidence from studies examining the PVT and its circuitry suggests that the anterior PVT (aPVT) and posterior PVT (pPVT) may play functionally distinct roles in regulating behaviour due to their selective connectivity with other regions. The aPVT and pPVT are innervated by the PrL and IL, with aPVT innervation arising from the IL and pPVT innervation arising from the PrL (Li & Kirouac, 2012; Millan, Ong, & McNally, 2017). PrL-aPVT projectors arise from the posterior region of the PrL, while PrL-pPVT

projectors arise from the anterior region of the PrL (Li & Kirouac, 2012). In addition, the aPVT is preferentially innervated by the arcuate nucleus of the hypothalamus, suprachiasmatic nucleus, ventral hippocampus and ventral subiculum (Li & Kirouac, 2012; Kirouac, 2015; Lee, Lee, & Lee, 2015; Millan et al., 2017).

The aPVT and pPVT also exhibit distinct topographical patterns in their projections to other brain regions. While the aPVT and pPVT both innervate the NAc shell to a similar degree, where these projectors end up within the NAc shell is different. aPVT projectors innervate the dorsomedial NAc shell while the pPVT projectors innervate the ventromedial NAc shell (Li & Kirouac, 2008; Dong, Li, & Kirouac, 2017). In addition, compared to the aPVT, the pPVT more densely innervates the NAc core (Li & Kirouac, 2008). Neurons in the aPVT project more heavily to the NAc shell than the core, while in the pPVT, there does not appear to be a difference between the proportion of neurons innervating the NAc core or shell (Dong et al., 2017). Aside from the NAc, the aPVT projects most heavily to the IL, PrL, and insular cortex, while the pPVT strongly innervates the CeA, BLA and BNST (Li & Kirouac, 2008). Together, these findings indicate that the aPVT and pPVT exhibit divergent input and output patterns; however, the exact functions related to these differences are still not understood.

PVT and Food Intake

About 15 years ago, the PVT was proposed to function as part of a hypothalamic-thalamic-striatal circuit that integrated energy balance, arousal and food intake (Kelley et al., 2005). The first study to demonstrate a role for the PVT in food intake showed that PVT lesions increase food intake and body weight in rodents (Bhatnagar & Dallman, 1999). Consistent with these findings, pharmacological inactivation or chemogenetic

inhibition of the PVT increases food consumption (Stratford & Wirtshafter, 2013; Zhang & van den Pol, 2017), while optogenetic activation of PVT neurons alone reduces food consumption (Zhang & van den Pol, 2017). Stimulation of lateral hypothalamus (LHA) GABAergic neurons projecting to the PVT, Zona Incerta GABAergic neurons projecting to the PVT, or activation of ARC^{AGRP} neurons projecting to the PVT increases food consumption (Zhang & van den Pol, 2017; Betley, Cao, Ritola, & Sternson, 2013). These findings indicate that the PVT and its circuitry play a critical role in the regulation of feeding behaviour.

Empirical evidence suggests that LHA orexin/ hypocretin projections innervating the PVT play a role in the regulation of food intake. Neurons expressing the orexin-1 receptor (OX1R) are activated by cues previously associated with food rewards, and activation of OX1R in the pPVT increases sucrose intake (Choi, Davis, Fitzgerald, & Benoit, 2010; Barson, Ho, & Leibowitz, 2015). Genetic knockdown of the OX1R in the PVT attenuates overconsumption of a food reward in sated rats (Choi et al., 2012) while activation of PVT OX1R-expressing neurons increases dopamine in the NAc. Overall, these findings suggest that the PVT may control food intake via hypothalamic-thalamic-striatal circuitry that is mediated by altering dopamine signalling in the NAc.

Food predictive stimuli activate the PVT. Food-restricted rats display greater PVT neuronal activation before food delivery (Poulin & Timofeeva, 2008). Moreover, cues and contexts that signal the availability of food rewards increase PVT neuronal activation, and some of these cells are OX1R expressing (Igelstrom, Herbison, & Hyland, 2010; Schiltz, Bremer, Landry, & Kelley, 2007; Choi et al., 2010). Together, these

findings indicate that the PVT plays an important role in the regulation of cue-induced feeding.

PVT and Reward Predictive Cues

Reward predictive cues engage PVT neurons to a greater degree in rodents who attribute incentive and predictive value to the reward predictive cues (e.g., sign tracking) in comparison to animals who only attribute predictive value to the reward predictive cue (e.g., goal tracking) or cue-naïve control animals (Flagel et al., 2011; Haight, Fuller, Fraser, & Flagel, 2017). In line with these findings, the PVT appears to function very differently in animals that attribute incentive salience to cues. Lesioning the PVT increases sign tracking but decreases goal-tracking behaviour (Haight, Fraser, Akil, & Flagel, 2015). The increase in sign-tracking behaviour that occurs in response to lesioning the PVT suggests that the PVT acts as a gatekeeper for the expression of cue driven behaviours (Haight et al., 2015). In support of this idea, transient inactivation of the PVT selectively enhances cue-induced reinstatement of cocaine-seeking in goal trackers but does not affect sign trackers (Kuhn, Klumpner, Covelo, Campus, & Flagel, 2018). These findings suggest that the PVT plays a critical role in controlling cue motivated behaviours.

It has been suggested that the PVT plays a critical role in the integration of cortical and subcortical hypothalamic signals to regulate cue motivated behaviour (Campus et al., 2019; Otis et al., 2019). Recently, it was reported that chemogenetic activation of the PrL-PVT pathway decreases the incentive value of a reward-paired cue (Campus et al., 2019). In contrast, chemogenetic inhibition of the PrL-PVT pathway enhances the incentive value of a reward-paired cue (Campus et al., 2019). The authors

also reported that inhibition of the PrL-PVT pathway increases dopamine levels in the nucleus accumbens shell while activation does not. These findings suggest that the PrL-PVT-NAc shell pathway may play a critical role in regulating the incentive value of reward-paired cues.

Most recently, using a Pavlovian conditioning paradigm, Otis et al. (2019) reported that PVT-NAc activity is determined by inputs from glutamatergic PrL and GABAergic LHA projectors. Specifically, PrL-PVT neurons show reduced activity to reward-paired cues following training while, LHA-PVT neurons show increased activity during behavioural output. Furthermore, optogenetically stimulating PrL-PVT projectors interfered with PVT-NAc cue encoding and behaviour. Together, these findings suggest that PVT-NAc neurons encode a multiplexed signal that integrates cortical and subcortical input to guide behavioural output (Otis et al., 2019).

PVT and Drug Addiction

The PVT is uniquely positioned to integrate subcortical and cortical information regarding environmental, emotional and homeostatic state to guide motivated behaviour. In recent years, the PVT has become a significant focus for researching the neural circuitry of drug addiction (James & Dayas, 2013). The surge of attention garnered by the PVT is likely due to its vast inputs and outputs that have been documented to play a role in drug intake and relapse (Zhou & Zhu, 2019). To date, the PVT and some of its circuitry has been reported to play a role in many stages of addiction, including drug-taking, withdrawal, and relapse (Neumann et al., 2016; Zhu, Wienecke, Nachtrab, & Chen, 2016; Giannotti, Barry, Siemsen, Peters, & McGinty, 2018).

PVT and Drug Taking

The PVT is recruited by acute administration of psychostimulants, alcohol, morphine, nicotine, and THC as evidenced by increased c-fos mRNA or protein expression in the PVT (Deutch, Ongür, & Duman, 1995; Deutch, Bubser, & Young, 1998; Stephenson, Hunt, Topple, & McGregor, 1999; Barson et al., 2015; Ryabinin & Wang, 1998; Garcia, Brown, & Harlan, 1995; Gutstein, Thome, Fine, Watson, & Akil, 1998; Ren & Sagar, 1992; Allen, McGregor, Hunt, Singh, & Mallet, 2003). Moreover, cues and contexts previously associated with psychostimulants or alcohol increase c-fos expression in the PVT (Brown, Robertson, & Fibiger, 1992; Johnson, Revis, Burdick, & Rhodes, 2010; Franklin & Druhan, 2000; Rhodes, Ryabinin, & Crabbe, 2005; Dayas, McGranahan, Martin-Fardon, & Weiss, 2008; Perry & McNally, 2013; Wedzony et al., 2003). Taken together, these findings suggest that the PVT is recruited by many different classes of drugs and by cues and contexts that signal the availability of drugs.

A limited number of studies have examined the role of the PVT in drug self-administration. In a comprehensive study by Barson et al. (2015) voluntary home cage alcohol intake recruits the PVT as evidenced by an increase in Fos protein expression in the anterior PVT. However, these authors also reported that home cage alcohol intake increases OX2R expression in the aPVT. In addition, following an oral gavage with alcohol, there is an increase in the number of double labelled OX2R-cFos immunoreactive neurons in the aPVT, suggesting that the changes observed are due to exposure to alcohol. In addition, Barson et al. (2015) reported that microinfusing an OX2R antagonist into the aPVT reduces home cage alcohol intake up for up to 2 hours, but this effect is only significant at 30 minutes when compared to controls (Barson et al., 2015). However, microinfusing orexin-A or orexin-B in the aPVT but not pPVT

increases home cage alcohol intake while microinfusing orexin-A in the pPVT but not aPVT increases sucrose intake.

In contrast to these findings, lesioning the PVT does not alter instrumental responding for alcohol (Hamlin, Clemens, Choi, & McNally, 2009). Disrupting synaptic transmission in PVT-NAc shell projecting neurons reduces the acquisition of cocaine self-administration, but not incubation of cocaine craving during abstinence (Neumann et al., 2016). However, lesioning the PVT prevents psychomotor sensitization induced by repeated cocaine administration but enhances acute exposure-induced hyper locomotor activity (Young & Deutch, 1998). Overall, these findings suggest that the PVT may contribute to alcohol and cocaine intake.

PVT and Opiate Dependence

The only study to date investigating the role of the PVT in withdrawal, suggests that the PVT-NAc pathway is a critical circuit that mediates opiate dependence (Zhu et al., 2016). Photoactivation of the PVT-NAc shell pathway induces place aversion that is dependent on glutamatergic transmission in the NAc. Next, using an acute morphine dependence model, the authors reported that spontaneous or precipitated opiate withdrawal results in increased c-Fos expression in PVT-NAc shell projecting neurons. Moreover, the authors reported that the PVT-NAc shell pathway is required for the expression of aversive withdrawal symptoms induced by naloxone (i.e., jumps, rearing, tremors) as photoinhibition of this pathway blocks withdrawal symptoms and conditioned place aversion. Chemogenetic inhibition of this pathway also reduced conditioned place aversion. The authors also demonstrate that chronic morphine exposure strengthened PVT inputs onto medium spiny neurons (MSN) in the NAc expressing the dopamine D2

receptor via the insertion of synaptic AMPARs. Furthermore, optogenetic depotentiation of the PVT-NAc D2 MSN synapses attenuated aversive morphine withdrawal symptoms. In line with these findings, microinfusing OX1R or OX2R antagonists into the PVT impaired the expression of morphine-induced conditioned place avoidance (Li et al., 2011). These findings suggest that the PVT and its projection to the NAc may play an important role in the negative symptoms associated with drug withdrawal.

PVT and Relapse

There is extensive literature demonstrating a role for the PVT in the regulation of alcohol and cocaine-seeking following periods of abstinence. The PVT is recruited during cue and context-induced reinstatement of alcohol-seeking (Dayas et al., 2008; Hamlin et al., 2009; Marchant, Furlong, & McNally, 2010; Perry & McNally, 2013; James, Charnley, Flynn, Smith, & Dayas, 2011a). Hamlin et al. (2009) reported that PVT- NAc shell projecting neurons were recruited during context-induced reinstatement and that lesions of the PVT prevented context-induced reinstatement of alcohol-seeking. Microinfusing a kappa-opioid receptor agonist (U50488) into the PVT, mimicking the activation of an extinction-related pathway from the medial hypothalamus, prevents context-induced reinstatement of alcohol-seeking (Marchant et al., 2010). Together, these results suggest a vital role for the PVT in the renewal of alcohol seeking.

Several studies have reported a role for the PVT in the reinstatement of cocaine-seeking. The PVT is recruited during cue-induced reinstatement of cocaine-seeking (James et al., 2011a; James et al., 2011b; Matzeu, Cauvi, Kerr, Weiss, & Martin-Fardon, 2017). Consistent with these findings, inactivation of the PVT via baclofen/muscimol inhibits the expression of cocaine conditioned place preference and cue-induced

reinstatement of cocaine-seeking (Browning, Jansen, & Sorg, 2014; Matzeu, Weiss, & Martin-Fardon, 2015). However, blocking OX1R in the PVT does not affect cue-induced reinstatement of cocaine-seeking (James et al., 2011b). Microinfusing tetrodotoxin (TTX), a sodium channel blocker, or the calcium channel blocker neuropeptide, cocaine and amphetamine-regulated transcript (CART) into the PVT attenuates cocaine primed reinstatement of cocaine-seeking (James et al., 2010). Microinfusing orexin-A into the pPVT is sufficient to prime cocaine-seeking; however, co-administration of orexin-A and TCSOX229 (OXR2 antagonist) prevents this effect, suggesting that orexin signalling in the pPVT plays a role in cocaine-seeking (Matzeu, Kerr, Weiss, & Martin-Fardon, 2016).

Rationale for Current Studies

In the available literature, three key neural circuits have been repeatedly reported to play a role in both food intake and drug-seeking: (1) PrL-PVT (2) LHA-PVT and (3) PVT-NAc. These findings indicate that the PVT functions to integrate both cortical (PrL-PVT) and subcortical (LHA-PVT) information to guide future behaviour via output projections to downstream targets, specifically in this case, the nucleus accumbens. However, despite a large amount of research indicating a role for the PVT in drug addiction and food intake, most research has focused on psychostimulants and alcohol. In contrast, opiates have been largely ignored, making it difficult to speculate on the role of the PVT and its circuitry in opiate seeking. At the time I started this research project, all studies examining the role of the PVT in drug-seeking utilized an extinction-based model of relapse. However, extinction is not the main reason why an individual abstains from drug use; instead, they are forced to refrain from drug use (i.e., incarcerated), or they

voluntarily abstain. Again, this makes it difficult to speculate on the role of the PVT in drug-seeking using an abstinence-based procedure.

At the present time, the neural mechanisms that mediate the augmentation of heroin seeking induced by chronic food restriction remain elusive. Due to its extensive neural network and brain location, the PVT is a unique candidate to regulate food intake and drug-seeking simultaneously. Thus, the objective of this dissertation was to investigate the role of the PVT and its circuitry in the augmentation of heroin seeking following a period of withdrawal in sated and food restricted rats. Designer Receptors Exclusively Activated by Designer Drugs (DREADD) technology was used to excite or inhibit the PVT and its projections following the withdrawal period during the drug-seeking test in sated and food-restricted rats.

In Chapter 3, the goal of the experiments was to ascertain the role of the PVT in the augmentation of heroin seeking following chronic food restriction. Based on the existing literature, we hypothesized that inactivating the PVT would reduce heroin seeking in both sated and food-restricted rats, but that this effect would be stronger in the food-restricted rats. To test our hypothesis, we inactivated the PVT using DREADD technology and pharmacological inactivation before the heroin-seeking test. In a separate experiment, we activated the PVT using DREADDs before the heroin-seeking test and verified DREADD functionality using in vitro slice electrophysiology.

In Chapter 4, the goal of the experiments was to examine the role of glutamatergic projections from the medial prelimbic cortex to the PVT in the augmentation of heroin seeking induced by chronic food restriction. We hypothesized that if we activated PrL-PVT projections, we would block the augmentation of heroin seeking in chronically

food-restricted rats. To test this hypothesis, we chemogenetically excited or inhibited PrL-PVT projections immediately before the heroin seeking test. We sought to validate DREADD functionality by examining Fos immunoreactivity following excitation or inhibition of PrL-PVT projections to ensure that our manipulation worked properly.

In Chapter 5, the goal of the experiments was to examine the role glutamatergic projections from the PVT to the NAc, core and shell, in the augmentation of heroin seeking induced by chronic food restriction. We hypothesized that activation of PVT-NAc projections would reduce the augmentation of heroin seeking observed in our chronically food-restricted rats. As in Chapter 4, to test this hypothesis, we chemogenetically activated or inhibition PVT- NAc shell or core projections immediately before the heroin seeking test. As in Chapter 4, we sought to validate DREADD functionality by examining Fos immunoreactivity following activation of PVT-NAc core and shell projections.

These studies will help to provide a better understanding of the role of the PVT and its circuitry involved in opiate seeking and drug addiction in general. Further, these studies will directly contribute to our understanding of the neurobiological mechanisms underlying the augmentation of heroin seeking induced by chronic food restriction.

CHAPTER 2: GENERAL METHODOLOGY

Investigating the function of specific neural circuits involved in addiction-related behaviours in freely moving animals requires a method that is minimally invasive so as not to interfere with complex behaviour while simultaneously allowing for selective control of the circuit being examined. In our case, the use of DREADDs (Designer Receptors Exclusively Activated by Designer Drugs) best suits our needs. DREADD technology utilizes an existing class of receptors known as G-protein coupled receptors that have been modified so that they are only activated by a biologically inert ligand known as clozapine-N-oxide (CNO) (Armbruster, Li, Pausch, Herlitze, & Roth, 2007). Activation of DREADDs by delivering CNO allows for reversible control of cellular activity. Upon administration, CNO binds to the DREADD receptor and alters cellular activity by engaging specific intracellular signalling cascades (Ferguson & Neumaier, 2015). Additionally, DREADDs are tagged with a fluorescent protein that allows researchers to examine the exact spread of the manipulation.

Methodological Considerations

One major problem with studying the PVT is that a moderate to high degree of its projecting axons collateralize to innervate multiple brain areas including but not limited to the NAc core, NAc shell, CeA, BLA, BNST, and mPFC (Dong et al., 2017; Freedman & Cassell, 1994; Bubser & Deutch, 1998; Su & Bentivoglio, 1990). This means that a traditional dual-virus "Retrograde-DREADD" approach to target a specific neural projection is not appropriate as systemic administration of CNO will also activate or inactivate collaterals (Urban & Roth, 2015). In this technique, a retrograde recombinase-encoding viral vector is infused into the terminal region and a flip excision (FLEX)-switch virus that encodes a DREADD is infused into the cell body region. Then, the

recombinase-encoding virus is retrogradely transported back to the cell body and flipped into position by the FLEX DREADD to allow for DREADD expression in a pathway-specific manner. Unfortunately, any of the projectors infected with the DREADD may also have collaterals projections that innervate other brain regions. Thus, activating the DREADD with CNO may result in activation of the specific projection but may also activate collaterals to other brain regions, limiting the conclusions that can be drawn about pathway specificity. To get around this problem, it is possible to infuse a viral vector carrying an excitatory or inhibitory DREADD into the brain region containing the cell bodies (i.e., PVT) and then implant guide cannulae into the target region (i.e., NAc shell). CNO can then be microinfused directly into the target region to selectively excite or inhibit the specific projector without altering neural activity in collateral projections. In Chapter 3, we infused a DREADD into the PVT and then excited or inhibited the PVT using systemic CNO injections. In Chapters 4 and 5, to ensure pathway-specific chemogenetic manipulation, we excited or inhibited specific neural pathways by microinfusing CNO into the terminal region.

In the following paragraphs, I will describe the general procedure used. Specific details that delineate each experiment will be presented in the corresponding chapter.

Subjects

Male Long Evans rats (Raleigh, North Carolina, U.S.A.), weighing 250-275 g at the beginning of all experiments were used. Before surgery, animals were pair-housed for 1 week under a reverse 12 hour light/dark cycle (9:30 am light OFF) with *ad libitum* access to chow (Agribran Purina Canada Inc., Woodstock, Ontario) and water unless otherwise specified. Following surgery, rats were individually housed in plastic shoebox

cages for 2 days before being moved to the operant conditioning chambers for drug self-administration. Following drug self-administration training, rats were returned to the animal care facility and individually housed for the drug withdrawal phase. All experiments were approved by the Animal Research Ethics Committee of Concordia University, and were carried out in accordance with the recommendations of the Canadian Council on Animal Care.

Surgical Procedures

Intravenous Catheterization and Intracranial Surgery

Across all experiments described in this dissertation, rats underwent surgery to implant an intravenous catheter to allow for heroin self-administration. Before surgery, rats were subcutaneously (s.c.) administered 2 ml of 0.9% saline, penicillin (450 000 IU/rat; s.c.), and atropine (0.1 mg/kg/rat) to aid in hydration and prevent infection. Under 2-3% isoflurane anesthesia, rats were implanted with an intravenous (i.v.) Silastic catheter (Dow Corning, Midland, MI, USA) 3 cm into the right jugular vein that was held in place with silk sutures, as previously described (Sedki et al., 2013). The tip of the intravenous catheter was attached to a modified 22-gauge cannula (5-up, Plastics One, Roanoke, VA) and anchored to the skull of the rat using five screws and dental cement (Parkell Inc., Edgewood, NY). Throughout self-administration training, catheters were flushed once a day with gentamicin and heparin (7.5 IU + 40.0 ug per day) in sterile saline to maintain catheter patency.

Immediately following intravenous catheterization, rats were infused with a viral vector and/or were implanted with guide cannulae aimed at a specific brain region. Specific details for viral vector infusion and cannulae implantation for each experiment

are described in each chapter. Rats were administered 0.9% saline (2 ml, s.c.) and Ketoprofen (2.0 mg/kg; Merial Canada Inc., Baie-d'Urfe, QC) immediately following surgery, and postoperatively for 48 hours.

Apparatus

Operant conditioning chambers (Coulbourn Instruments, Allentown, PA, USA; 29.0 cm X 29.0 cm x 25.5 cm) enclosed in sound-attenuating boxes were used. Each chamber was equipped with a red house light, a food hopper, and a water bottle. The 'active' lever was positioned 9 cm above the floor and was located on the wall opposite the house light. An 'inactive' lever was positioned 9 cm above the floor, on the same wall as the 'active' lever. Responses on the active lever activated an infusion pump (Razel Scientific Instruments, Stamford, CT), which was positioned inside the sound-attenuating cabinet. A white cue light and tone generator (Coulborn Instruments, Sonalert, 2.9KHz) was located directly above the active lever. Presses on the inactive lever were recorded but had no programmed consequence. The infusion pump was connected to the catheter through a liquid swivel (Lomir Biomedical Inc., Notre-Dame-de-l'Île-Perrot, QC, Canada) and Tygon tubing (Saint-Gobain, Courbevoie, France) shielded by a metal spring.

Drugs

Heroin HCl (Contribution from the National Institute for Drug Abuse, Research Triangle Park, NC, USA) was dissolved in 0.9% sterile saline (5.0 mg/ml). This solution was further diluted with 0.9% saline based on the bodyweight of each individual rat in order to yield 0.1 mg/kg/infusion. In Chapter 3, Clozapine-*N*-Oxide (CNO; Contribution from the National Institute for Drug Abuse) was dissolved in 5% dimethyl sulfoxide

(DMSO) and 95% sterile saline solution to a concentration of 6.0 mg/ml to allow for systemic administration. Muscimol (0.03 nmol; Sigma-Aldrich; M153) + baclofen (0.3 nmol; Sigma-Aldrich, B5399) were dissolved in saline and injected using a 28-gauge injector (Plastics One) that extended 2 mm below the guide cannula, at a rate of 0.3 μl/minute, over 1 minute. The injector was left in place for 2 minutes following the infusion to allow for optimal drug diffusion. In Chapters 4 and 5, Clozapine-*N*-Oxide (CNO; Contribution from the National Institute for Drug Abuse, Research Triangle Park, NC, USA) was dissolved in 1% DMSO and 99% sterile saline to allow intracranial microinfusion. CNO was injected using a 10 μl Hamilton syringe placed in a microinfusion pump (Harvard Apparatus, Holliston, MA, USA). The syringe was attached to polyethylene-20 tubing to a 28-gauge injector (Plastics One) that extended 2 mm below the guide cannulae. CNO was infused at a rate of 0.30 μl/minute, over 1 minute to yield a final concentration of 1mM. The injector was left in place for 2 minutes following the infusion to allow for optimal drug diffusion.

General Procedure

All experiments presented within this dissertation that assessed drug-seeking followed the same general procedure: (1) heroin self-administration, (2) withdrawal, (3) heroin seeking test and (4) locomotor activity test.

Heroin Self-Administration

Rats were trained to self-administer heroin for 10 days, in three 3-hour sessions per day separated by 3-hour intervals, under a fixed-interval-20 second (FI-20) schedule of reinforcement. The first daily session began shortly after the onset of the dark period and was indicated by the illumination of the house light, entry of the active lever, and

activation of the cue light and the tone for 30 seconds or until the active lever was pressed. Pressing the active lever resulted in a 0.13 ml infusion of heroin over 5 seconds and the initiation of a 20 second timeout period during which the house light was turned off, and the cue light and tone were activated. Responses made on the active lever during the timeout were recorded but did not result in an additional infusion. Presses on the inactive lever were recorded but had no programmed consequence.

Drug Withdrawal

Following heroin self-administration training, rats were transferred back to the animal care facility and housed in individual cages. Following a 24-hour drug washout period, rats were assigned into one of two groups, food-restricted (FDR) or sated, matched by the average number of infusions taken, active lever responses made, and body weight over the last five days of training. Consequently, no statistical analyses were conducted on self-administration acquisition data. Throughout the withdrawal period, the amount of food given to FDR rats was titrated to maintain the rats at 90% of their body weight on the drug washout day.

Heroin-Seeking Test

On day 14 of withdrawal, rats were returned to the operant conditioning chambers for a heroin-seeking test session. During testing, all conditions were identical to self-administration training except that rats were tested under extinction conditions, and an empty food hopper was placed in the chamber of FDR rats. Specific details for each experiment are described in each chapter.

Locomotor Activity Test

On the 16th day of withdrawal, rats were placed in a locomotor activity chamber to assess general locomotor activity. Locomotor activity data were collected using Tru Scan 2.0 activity monitoring system (Coulbourn Instruments). Total distance moved (cm) was collected during a period of 1 hour. Specific details for each experiment are described in each chapter.

Histology and Immunohistochemistry

Following all experiments except for those that assessed DREADD functionality, rats were transcardially perfused with PBS followed by 4% paraformaldehyde. Brains were extracted, and post-fixed for 24 hours, cryoprotected with 30% sucrose at 4°C for 48 hours, stored in -80°C, and coronal sections (40 μm) were sliced on a cryostat. In Chapter 3, slices were stained for mCherry to detect DREADD expression (primary antibody: 1:1000, mouse host, Abcam Inc., Cambridge, MA; secondary antibody: 1:200, Anti-Mouse Alexa 594, Jackson Immuno Research Labs, West Grove, PA). However, in Chapters 4 and 5, DREADD expression was strong and therefore staining for mCherry was not required. DREADD expression was determined under a fluorescent microscope using the TX2 filter with reference to the brain atlas of Paxinos and Watson (2004). When verification of DREADD expression was not required, but cannula placement was, coronal sections (40 μm) were sliced on a cryostat. Injector placement was verified using a light microscope with reference to the brain atlas of Paxinos and Watson (2004).

Fos Immunohistochemistry

In Chapters 4 and 5, rats were intracranially infused with CNO (1mM) or vehicle into the PVT (Exp. 1 & Exp. 2), NAc shell (Exp. 1) or NAc core (Exp. 2), and 90 minutes later were overdosed with sodium pentobarbital and transcardially perfused with PBS

(pH 7.4) followed by 4% paraformaldehyde. Brains were extracted and post-fixed in 4% paraformaldehyde for 24 hours, followed by cryoprotection in a 30% sucrose solution at 4°C for 48 hours. Brain tissue was collected in 40 μm coronal slices using a Leica cryostat and PrL, or PVT sections were mounted onto super frost plus microscope slides (Fisher Scientific, St. Laurent, QC, Canada) depending on the experiment. Depending on the experiment, 40 μm sections for the PVT, NAc shell, or NAc core were collected and stored at -20°C in cryoprotectant.

First, free-floating sections containing the PVT, NAc shell or NAc core were washed 12 x 5 minutes in TBS. Sections were then blocked in 3% normal goat serum and 0.20% Triton-X in TBS for 2 hours at 4°C. Next, sections were incubated for 48 hours at 4°C with the primary rabbit anti-Fos antibody (Cell Signalling #2250S; 1:2000), in 3% Normal Goat Serum and 0.15% Triton-X in TBS. Following primary incubation, sections were washed 5 x 5 minutes in TBS and then quenched in a 0.3% TBS hydrogen peroxide 30 minutes at 4°C. Following quenching, sections were washed 5 x 5 minutes and were then incubated in the secondary antibody solution containing biotinylated goat anti-rabbit IgG antibody (Vector Laboratories; 1:200), 3% normal goat serum and 0.2% Triton-X at 4°C for 48 hours. Following secondary incubation, sections were washed 3 x 5 minutes in TBS, and were then incubated for 1 hour in a Vectastain Elite ABC solution (Vector Laboratories) at 4°C. Sections were washed 3 x 5 minutes in TBS. Next, Fos reactive cells were visualized by reacting with DAB and Nickle Cl (Vector Laboratories) for 1.5 minutes. The reaction was briefly paused by placing the sections in TBS. The reaction was stopped by placing the sections in tap water for 5 minutes. Sections were washed 3 x 5 minutes in TBS and mounted onto SuperFrost Plus microscope slides and, cover-

slipped with Permount medium (Fisher Scientific). Injection placement and viral expression were determined under a confocal microscope (Leica, DMRA2) using the TX2 filter with reference to the brain atlas of Paxinos and Watson (2004) as previously described (Chisholm et al., 2020).

Fos Immunoreactivity Quantification

Fos immunoreactivity (IR) was conducted by an experimenter blind to conditions. Images were taken using the software program ToupView (Hangzhou ToupTek Photonics Co., Ltd.) that was connected to a ToupTek LCMOS digital camera and a Leica microscope (DM4000). ImageJ software (National Institute of Health) was used for Fos labeled cell counting. In Chapter 4, one image per section was taken at 20x objective. Three images from the PVT (bregma AP: -2.76 to AP: -3.48; Exp.1 & Exp. 2) with the highest number of Fos reactive cells were averaged for each subject. In Chapter 5, in each brain section, for each hemisphere, two images at 20x objective were captured. For each subject, counts from three bilateral sections from the NAc shell (bregma: AP 1.68 to AP 1.92; Exp. 1A) or core (bregma AP: 2.28 and AP: 2.76; Exp. 2) with the highest number of Fos reactive cells were averaged.

CHAPTER 3: THE ROLE OF THE PARAVENTRICULAR NUCLEUS OF THE THALAMUS IN THE AUGMENTATION OF HEROIN SEEKING INDUCED BY CHRONIC FOOD RESTRICTION

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Abstract

Drug addiction is a chronic disorder that is characterized by compulsive drug seeking and involves cycling between periods of compulsive drug use, abstinence, and relapse. In both human addicts and animal models of addiction, chronic food restriction has been shown to increase rates of relapse. Previously, our laboratory has demonstrated a robust increase in drug seeking following a period of withdrawal in chronically food-restricted rats compared to sated rats. To date, the neural mechanisms that mediate the effect of chronic food restriction on drug seeking have not been elucidated. However, the paraventricular nucleus of the thalamus (PVT) appears to be a promising target to investigate. The objective of the current study was to examine the role of the PVT in the augmentation of heroin seeking induced by chronic food restriction. Male Long-Evans rats were trained to self-administer heroin for 10 days. Rats were then removed from the training chambers and experienced a 14-day withdrawal period with either unrestricted (sated) or mildly restricted (FDR) access to food. On day 14, rats underwent a 1-hour heroin-seeking test under extinction conditions, during which neural activity in the PVT was either inhibited or increased using pharmacological or chemogenetic approaches. Unexpectedly, inhibition of the PVT did not alter heroin seeking in food-restricted or sated rats, while enhancing neural activity in the PVT attenuated heroin seeking in food-restricted rats. These results indicate that PVT activity can modulate heroin seeking induced by chronic food restriction.

Introduction

Drug addiction is a chronic disorder that is characterized by compulsive drug seeking and involves recurrent periods of drug abstinence and relapse (O'Brien, 1997; O'Brien & Gardner, 2005). In abstinent drug users, relapse to drug use and drug craving can be triggered by three key factors: (1) re-exposure to drugs of abuse, (2) re-exposure to drug associated cues, and (3) stressors (Childress et al., 1993; Sinha, 2001; De Wit, 1996). In humans, a common stressor is restricted food intake, which results in increased use of coffee and tobacco products (Franklin, Schiele, Brozek, & Keys, 1948). Moreover, restricted food intake or caloric restriction results in increases in subjective drug craving and higher rates of relapse. For example, dieting during smoking cessation has been shown to increase rates of relapse (Hall et al., 1992; Pirie et al., 1992) and caloric restriction increases cigarette use in adult smokers (Cheskin et al., 2005).

In laboratory animals, the effects of restricted food intake on drug-related behaviours are well established. Both the initiation and maintenance of drug intake increase following periods of restricted food intake (Carroll & Meisch, 1984; Lu, Shepard, Scott Hall, & Shaham, 2003). In addition, both acute food deprivation (24-48 hours) and chronic food restriction result in reinstatement of extinguished drug seeking in rats with a history of cocaine or heroin self-administration (Shalev, 2012; Shalev et al., 2000; Shalev, Marinelli, Baumann, Piazza, & Shaham, 2003a).

An important critique of the reinstatement procedure as a model for drug relapse is the involvement of extinction training, which is not a cause for a reduction in drug taking in humans (Katz & Higgins, 2003). An alternative model, the 'abstinence procedure', has been suggested to have better validity as a relapse model (Fuchs,

Lasseter, Ramirez, & Xie, 2008). Briefly, a period of "forced withdrawal" follows the drug self-administration phase, typically in a different context, and a drug seeking test under extinction conditions is performed at the end of this abstinence period. Importantly, partial dissociation in neuroanatomical substrates has been demonstrated for cue-induced cocaine seeking following an abstinence period versus reinstatement of extinguished cocaine seeking (Fuchs, Branham, & See, 2006). Using the abstinence procedure, our laboratory has demonstrated a robust increase in heroin seeking following a 14-day period of withdrawal in chronically food-restricted rats (D'Cunha et al., 2013). To date, the neural mechanisms that underlie the augmentation of heroin seeking induced by chronic food restriction remain unknown.

The paraventricular nucleus of the thalamus (PVT) is uniquely placed to contribute to drug seeking and food intake systems, as it receives input and innervates mesolimbic and cortical regions, as well as areas associated with homeostatic control (Kirouac, 2015). PVT neurons are activated by acute and repeated exposure to drugs of abuse (e.g., opioids, psychostimulants, ethanol) (Barson et al., 2015; Dayas et al., 2008; Garcia et al., 1995) and by cues and contexts that have been previously paired with psychostimulant drugs (Brown et al., 1992; Rhodes et al., 2005). In addition, cue-induced reinstatement of alcohol and cocaine seeking is associated with neuronal activation in the PVT (Dayas et al., 2008; James et al., 2011a). Consistent with a potential role of the PVT in drug seeking, lesions of the PVT, activation of *k*-opioid receptors in the PVT, or transient inactivation of the PVT attenuated context-induced reinstatement of alcohol seeking (Hamlin et al., 2009; Marchant et al., 2010), expression of cocaine conditioned place preference (Browning et al., 2014), discriminative cue-induced reinstatement of

extinguished cocaine seeking (Matzeu et al., 2015), and drug-induced reinstatement of cocaine seeking (James et al., 2010). More recently, it was reported that chemogenetic inhibition of the midline thalamic nuclei (MTN, including the PVT) attenuated cue- and priming-induced reinstatement of extinguished cocaine, but not sucrose seeking (Wunsch et al., 2017). Taken together, these studies indicate that the PVT is activated by exposure to drugs of abuse and drug-associated cues, and that this activation is important for drug seeking.

Several lines of evidence also support a role for the PVT in the control of food intake. The PVT is activated by cues and contexts that have been previously associated with palatable foods (Igelstrom et al., 2010). Interestingly, cue-induced activation of the PVT seems to occur only in rats that have attributed incentive salience to a food cue (Flagel et al., 2011). Both pharmacological inactivation and lesions to the PVT result in increased food intake in rats (Bhatnagar & Dallman, 1999; Stratford & Wirtshafter, 2013). Taken together, this evidence indicates that the PVT plays a role in food intake, particularly in situations involving highly palatable foods.

The objective of this study was to investigate the role of the PVT in the augmentation of heroin seeking following a period of withdrawal in food-restricted rats. We hypothesized that since PVT activation seems to play an important role in drug seeking, PVT inactivation would attenuate heroin seeking in abstinent rats. Additionally, considering the involvement of the PVT in the control of food intake and seeking, it was hypothesized that this effect would be stronger in food-restricted rats. We tested these hypotheses using chemogenetic (Urban & Roth, 2015) and pharmacological approaches to inhibit or excite the PVT.

Materials and Methods

Subjects

Male Long Evans rats (Charles River, St. Constant, Quebec, Canada; n = 115) were used in three different experiments. Before surgery, animals were pair-housed in standard clear cages under a reverse 12-hour light/dark cycle (9:30 am light OFF), with ad libitum access to chow (Agribran Purina Canada Inc., Woodstock, Ontario) and water. Following recovery from surgery, rats were housed individually in operant conditioning chambers for drug self-administration training. Subsequently, rats were returned to the animal care facility and individually housed for the drug withdrawal phase. All experiments were approved by the Animal Research Ethics Committee of Concordia University, and were carried out in accordance with the recommendations of the Canadian Council on Animal Care.

Intravenous and Intracranial Surgery

Rats were implanted with an intrajugular Silastic catheter (Dow Corning, Midland, MI, USA) under 2% isoflurane anaesthesia, as previously described (Sedki, Gardner Gregory, Luminare, D'Cunha, & Shalev, 2015). During the same surgery, rats were injected with 0.6 µl of viral vector (Experiment 1: AAV8-hSyn-hM4D(Gi)-mCherry, University of North Carolina, Chapel Hill, NC; Figure 3.1A; Experiment 3: AAV8-hSyn-hM3D(Gq)-mCherry, Canadian Neurophotonic Platform/viral vector team, Québec, QC; Figure 3.1C) into the PVT (-3.0 AP, 0.0 ML, -5.4 DV relative to Bregma) at a rate of 0.1 µl /minute. The injector was left in place for an additional 10 minutes. For intra-PVT injections of muscimol + baclofen solution (Experiment 2; Figure 3.1B), rats were implanted with a guide cannula aimed at the PVT (AP: -3.0, 0.0 ML, -3.2 DV). Rats

were administered 0.9% saline (2 ml, s.c.) and Ketoprofen (2.0 mg/kg; Merial Canada Inc., Baie-d'Urfe, QC) immediately following surgery, and once daily over the next 2 days (see Appendix). A general viral vector approach and timeline for each experiment are presented in Figure 3.1. General procedures are described in Chapter 2.

Apparatus

Operant conditioning chambers with two retractable levers, a house light, white cue lights above the levers, and a tone generator (Coulbourn Instruments, Allentown, PA, USA; 29.0 cm X 29.0 cm x 25.5 cm) enclosed in sound-attenuating boxes were used (see Appendix).

Drugs

Heroin HCl (provided by the National Institute for Drug Abuse, Research Triangle Park, NC, USA) was dissolved in 0.9% sterile saline and administered at a dose of 0.1 mg/kg/infusion. Clozapine-*N*-Oxide (CNO; Contribution from the National Institute for Drug Abuse) was dissolved in 5% dimethyl sulfoxide (DMSO) and 95% sterile saline solution to a concentration of 6.0 mg/ml. Muscimol (0.03 nmol; Sigma-Aldrich; M153) + baclofen (0.3 nmol; Sigma-Aldrich, B5399) were dissolved in saline and injected using a 28-gauge injector (Plastics One) that extended 2 mm below the guide cannula, at rate of 0.3 µl/minute, over 1 minute, and the injector was left in place for 2 minutes following the infusion.

Procedure

Heroin Self-Administration

Rats were trained to self-administer heroin for 10 days, in three 3-hour sessions per day separated by 3-hour intervals, under a fixed-interval-20 second (FI-20) schedule

of reinforcement. The first daily session began shortly after onset of the dark period and was indicated by the illumination of the house light, entry of the active lever, and activation of the cue light and the tone for 30 seconds or until the active lever was pressed. Pressing the active lever resulted in a 0.13 ml infusion of heroin over 5 seconds and the initiation of a 20-second timeout period during which the house light was turned off, and the cue light and tone were activated. Responses made on the active lever during the timeout were recorded but did not result in an additional infusion. Presses on the inactive lever were recorded but had no programmed consequence.

Drug Withdrawal

Following heroin self-administration training, rats were transferred back to the animal care facility and housed in individual cages. Following a 24-hour drug washout period, rats were assigned into one of two groups, food restricted (FDR) or sated, matched by average number of infusions taken, active lever responses made, and body weight over the last 5 days of training. Consequently, no statistical analyses were conducted on self-administration acquisition data. During the withdrawal period, the amount of food given to FDR rats was titrated to maintain the rats at 90% of their body weight on the drug washout day.

Heroin-Seeking Test

Experiment 1: PVT inactivation using inhibitory DREADDs

On day 14 of food restriction, rats were returned to the operant conditioning chambers for one 1-hour heroin-seeking test session. Twenty minutes prior to the beginning of the test, rats were injected with either CNO (6.0 mg/kg, i.p.) or vehicle (1 ml/kg, 5% DMSO in sterile saline, i.p.). During testing, all conditions were identical to

self-administration training, except that rats were tested under extinction conditions (i.e.,, responding on the active lever did not result in an infusion of heroin) with all drug-associated cues present.

Experiment 2: PVT inactivation using baclofen + muscimol

Conditions were similar to the ones described for Experiment 1, except that rats were intracranially injected with either 0.3 µl baclofen + muscimol (0.3 + 0.03 nmol) or 0.3 µl vehicle (sterile saline), 5-10 minutes prior to the beginning of the heroin-seeking test session.

Experiment 3: PVT activation using excitatory DREADDs

Conditions were similar to the ones described for Experiment 1.

Elevated plus maze: PVT inactivation using baclofen + muscimol

PVT inactivation results in increased levels of emotional behaviours (Barson & Leibowitz, 2015). To validate the efficacy the PVT inactivation in our hands, anxiety-related behaviour was assessed using an elevated plus maze (EPM). Rats were intracranially injected with either 0.3 µl baclofen + muscimol (0.3 + 0.03 nmol) or 0.3 µl vehicle (sterile saline) 5-10 minutes prior to testing. Rats were then placed in the center of the EPM for a 10-minute video recorded test. Time spent in the open arm throughout the test and time spent in the open arm following the last entry into the open arm were scored "offline" from the video recordings, as previously described (Mahmud, Gallant, Sedki, D'Cunha, & Shalev, 2017); see Appendix).

Locomotor Activity Test

On the 16th day of food restriction, rats that participated in Experiment 3 were injected with either CNO (6.0 mg/kg, i.p.) or vehicle, 30 minutes prior to being placed into a locomotor activity monitoring chamber (Coulbourn Instruments). Total distance traveled (m) was recorded during a period of 1 hour using the TruScan software (Coulbourn Instruments).

Electrophysiological validation of excitatory DREADD activation in the PVT

Whole cell in vitro electrophysiological recordings in 11 to 16 weeks-old-rats were used to assess the effects of CNO on the excitability of PVT neurons, 3 to 6 weeks following AAV8-hSyn-hM3D(Gq)-mCherry infusion (see Appendix).

Histology

Experiment 1 and 3: PVT inactivation and PVT activation using CNO

Following the locomotor activity test, rats were transcardially perfused with PBS followed by 4% paraformaldehyde. Brains were extracted and post-fixed for 24 hours, cryoprotected with 30% sucrose at 4°C for 48 hours, stored in -80°C, and coronal sections (40 µm) were sliced on a cryostat. Slices were stained for mCherry to detect DREADD expression (primary antibody: 1:1000, mouse host, Abcam Inc., Cambridge, MA; secondary antibody: 1:200, Anti-Mouse Alexa 594, Jackson Immuno Research Labs, West Grove, PA). DREADD expression was determined under a fluorescent microscope using the TX2 filter with reference to the brain atlas of (Paxinos & Watson, 2004).

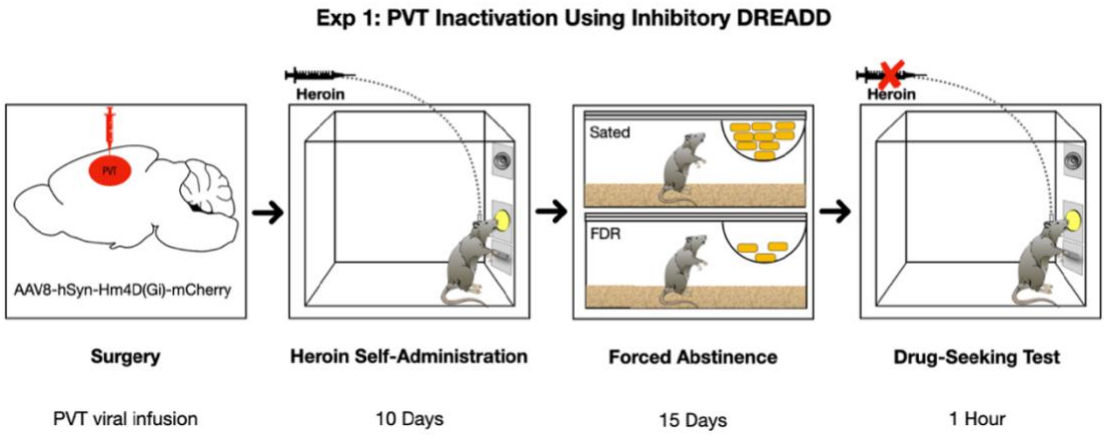
Experiment 2: PVT inactivation using baclofen + muscimol

Following the heroin-seeking tests, brains were extracted, and coronal sections (40 μm) were sliced on a cryostat. Injector placement was verified using a light microscope with reference to the brain atlas of Paxinos and Watson (2004).

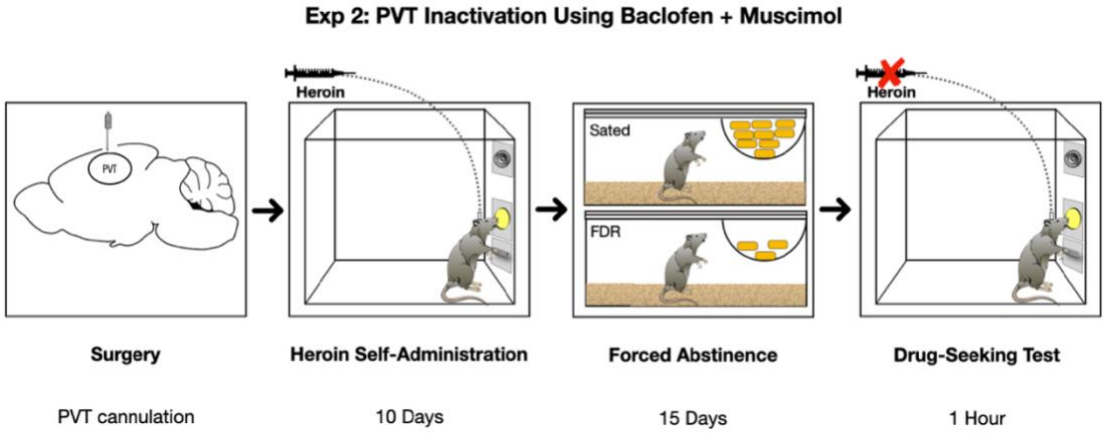
Statistical Analyses

The critical threshold for statistically significant results was set at $p < 0.05$. Body weights were compared using t-tests, corrected for unequal variance when necessary. The number of lever presses made during the heroin-seeking test was initially analyzed using a three-way ANOVA with *drug treatment* (Vehicle, CNO; B/M) and *feeding condition* (FDR, Sated) as between subject factors, and *lever type* (active, inactive), as a within subject factor. However, in all experiments, ANOVAs revealed a statistically significant main effect of lever type, and thus further analyses were conducted using separate two-way ANOVAs. The number of responses on the active and inactive levers during the heroin-seeking test session were analyzed separately using two-way ANOVAs with *CNO treatment* (vehicle, CNO) and *feeding condition* (FDR, sated) as between subject factors for Experiment 1 and 3, and *B/M treatment* (Vehicle, B/M) and *feeding condition* (FDR, sated) as between subject factors for Experiment 2. Significant interactions were followed by multiple comparisons with Holm-Sidak correction. Active lever responses over the test session were also analyzed using a repeated measures ANOVA with *drug treatment* (vehicle, CNO; B/M) and *feeding condition* (FDR, sated) as between subjects factors and *time* (6 x 10 minute bins) as the within subjects. Planned *t* tests were used to assess changes in electrophysiological measurements. The *n*'s in these analyses were the total number of neurons from which recording was made.

(A)


Exp 1: PVT Inactivation Using Inhibitory DREADD
PVT
AAV8-hSyn-Hm4D(Gi)-mCherry
Heroin
Sated
FDR
Heroin
Surgery
Heroin Self-Administration
Forced Abstinence
Drug-Seeking Test
PVT viral infusion
10 Days
15 Days
1 Hour

(B)


Exp 2: PVT Inactivation Using Baclofen + Muscimol
PVT
Heroin
Sated
FDR
Heroin
Surgery
Heroin Self-Administration
Forced Abstinence
Drug-Seeking Test
PVT cannulation
10 Days
15 Days
1 Hour

(C)

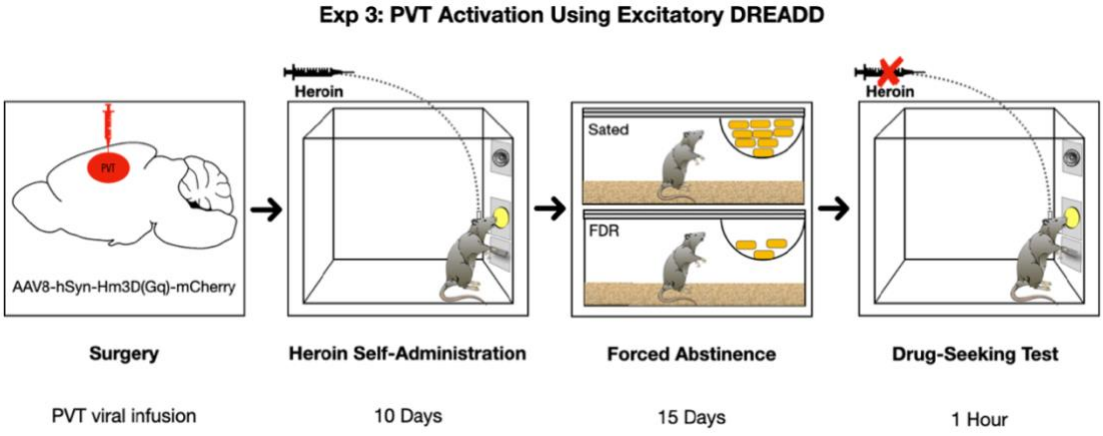

Exp 3: PVT Activation Using Excitatory DREADD
PVT
AAV8-hSyn-Hm3D(Gq)-mCherry
Heroin
Sated
FDR
Heroin
Surgery
Heroin Self-Administration
Forced Abstinence
Drug-Seeking Test
PVT viral infusion
10 Days
15 Days
1 Hour

Figure 3. 1. Diagram of viral vector approach and general experimental timelines. (A) Diagram of viral vector approach and the experimental timeline for Experiment 1. (B) Diagram of viral vector approach and the experimental timeline for Experiment 2. (C) Diagram of viral vector approach and the experimental timeline for Experiment 3.

Results

Mean ± SEM number of active lever responses, inactive lever responses, and infusions made on the last day of heroin self-administration training, for each treatment group in each experiment, are presented in Tables 3.1, 3.2, and 3.3. There were no statistically significant differences in these measures between the different experimental groups.

Table 3. 1. Mean ± SEM number of active lever responses, inactive lever response and heroin infusions made on the last day of training (9 hours), and body weight on test day in Experiment 1. * $p < 0.0001$, sated compared to food-restricted (FDR) rats.

	Infusions	Active lever	Inactive lever	Body weight
Sated- vehicle	35.15 ± 6.15	97.38 ± 25.45	6.75 ± 1.52	435.7 ± 11.1 *
Sated- CNO	44.00 ± 5.54	116.30 ± 26.25	11.00 ± 2.41	427.3 ± 11.9
FDR- vehicle	36.86 ± 3.75	110.43 ± 20.70	10.14 ± 2.87	327.7 ± 9.4
FDR- CNO	39.22 ± 5.98	97.78 ± 25.04	15.44 ± 8.27	316.8 ± 5.5

Table 3. 2. Mean ± SEM number of active lever responses, inactive lever response and heroin infusions made on the last day of training (9 hours), and body weight on test day in Experiment 2. * $p < 0.0001$, sated compared to food-restricted (FDR) rats.

	Infusions	Active lever	Inactive lever	Body weight
Sated- vehicle	34.13 ± 4.46	92.13 ± 23.49	2.63 ± 0.78	416.4 ± 10.4 *
Sated- B+M	45.90 ± 8.89	126.60 ± 32.26	18.40 ± 7.74	439.8 ± 9.5
FDR- vehicle	41.30 ± 5.42	115.30 ± 21.36	11.10 ± 2.59	331.3 ± 3.6
FDR- B+M	31.08 ± 4.03	81.23 ± 14.85	12.23 ± 5.48	332.9 ± 5.2

Table 3. 3. Mean ± SEM number of active lever responses, inactive lever response and heroin infusions made on the last day of training (9 hours), and body weight on test day in Experiment 3. * $p < 0.0001$, sated compared to food-restricted (FDR) rats

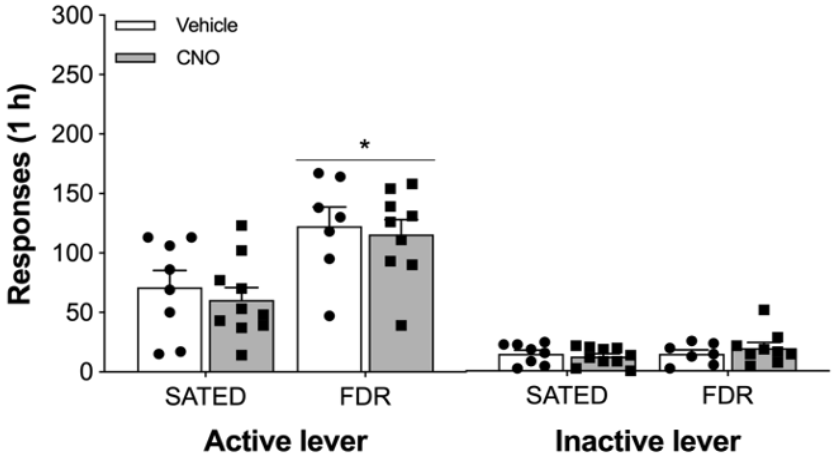
	Infusions	Active lever	Inactive lever	Body weight
Sated- vehicle	38.10 ± 5.69	100.10 ± 18.90	25.60 ± 11.33	442.6 ± 5.5 *
Sated- CNO	59.60 ± 9.87	227.30 ± 59.56	25.40 ± 11.41	455.6 ± 11.3
FDR- vehicle	46.33 ± 6.85	173.33 ± 68.10	24.50 ± 13.32	346.4 ± 4.3
FDR- CNO	35.43 ± 4.33	83.14 ± 13.72	9.57 ± 5.53	330.1 ± 5.6

Experiment 1: PVT inactivation using inhibitory DREADD

Four rats were removed due to catheter leakage, failure to train, health issues or incorrect viral vector placement. Therefore, the final analysis included 34 rats in the 4 experimental conditions: FDR-Vehicle (n = 7), FDR-CNO (n = 9), Sated-Vehicle (n = 8), and Sated-CNO (n = 10). On test day, FDR rats (n = 16; 321.6 ± 5.1 g) weighed statistically significantly less than sated rats (n = 18; 431.1 ± 8.1 g, $t_{(28.341)}$ = -11.461, p < 0.0001, d = -4.306). The three-way ANOVA revealed significant main effects of *lever type* ($F_{(1, 30)}$ = 159.215, p < 0.0001, η^2 = 0.772) and *feeding condition* ($F_{(1, 30)}$ = 15.045, p = 0.001, η^2 = 0.058)**,** and a significant *lever type* x *feeding condition* ($F_{(1, 30)}$ = 16.386, p < 0.0001, η^2 = 0.079) interaction. Further analyses were conducted using separate two-way ANOVAs for active and inactive lever responses.

FDR rats pressed more on the active lever during the heroin-seeking test, compared to the sated rats (*feeding condition*: $F_{(1, 30)}$ = 16.625, p < 0.0001, η^2 = 0.353; Figure 3.2A). However, no statistically significant effect for *CNO treatment* ($F_{(1, 30)}$ = 0.451, p = 0.507, η^2 = 0.01)*,* or *feeding condition* x *CNO treatment* interaction *(*$F_{(1, 30)}$ = 0.018, p = 0.895, η^2 = 0.000)*,* was found. Similarly, analysis of active lever responses over the test session (10 minute bins) revealed a statistically significant effect of *time* ($F_{(5,150)}$ = 27.19, p < 0.0001), but no significant interaction with any of the other factors (Figure 3.2B). No statistically significant effects were found for inactive lever responding (Figure 3.2A).

(A)



(B)

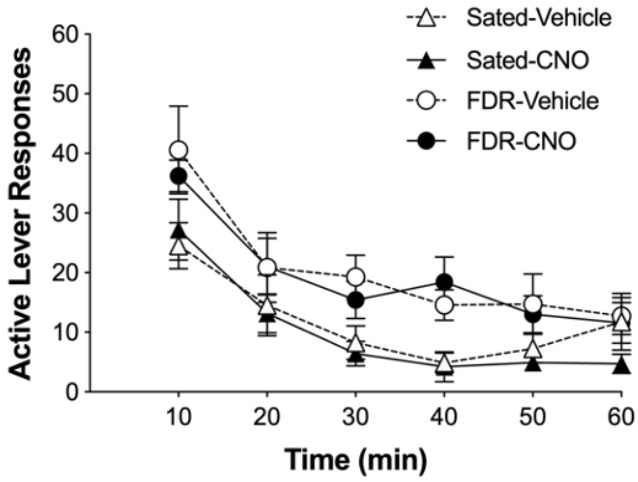


Figure 3. 2. Chemogenetic inhibition of the PVT had no effect on food restriction-induced augmentation of heroin seeking. (A) Mean ± SEM number of active (left) and inactive (right) lever responses made during the 1-hour heroin-seeking test on day 14 of food restriction in the sated and food restricted (FDR) groups. (B) Mean ± SEM number of active lever responses made over the 1-hour test, presented in 10-minute time intervals. Rats were administered with CNO (6.0 mg/kg, i.p.) or vehicle 20 minutes before the test. * $p < 0.0001$ compared to sated groups.

Experiment 2: PVT inactivation using baclofen + muscimol

Nine rats were removed due to catheter leakage, failure to train, health issues or incorrect injector placement. One rat (FDR-B/M group) was considered an outlier due to an extreme number of active lever presses performed during the test (> 2.5 SD above group average). Therefore, the final analysis included 41 rats in the four experimental conditions: FDR-Vehicle (n = 10), FDR-B/M (n = 13), Sated-Vehicle (n = 8), and Sated-B/M (n = 10). On test day, food-restricted (n = 23; 332.3 ± 3.3 g) rats weighed statistically significantly less than sated rats (n = 18; 429.4 ± 7.4 g); $t_{(23.676)}$ = -12.046, p < 0.0001, d = -4.951). The three-way ANOVA revealed a significant main effect of *lever type* ($F_{(1, 37)}$ = 83.343, p < 0.0001, η^2 = 0.656) and *feeding condition* ($F_{(1, 37)}$ = 8.48, p = 0.006, η^2 = 0.058), and a significant *lever type* x *feeding condition* ($F_{(1, 37)}$ = 5.383, p = 0.026, η^2 = 0.042) interaction. Further analyses were conducted using separate two-way ANOVAs for active and inactive lever responses.

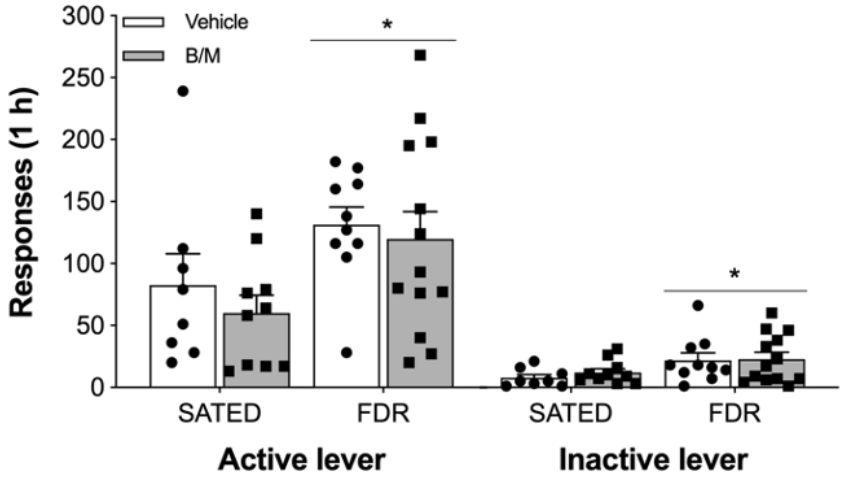
Rats in the food restricted group pressed significantly more on the active lever during the heroin-seeking test, compared to the sated group (*feeding condition*: $F_{(1, 37)}$ = 7.352, p = 0.01, η^2 = 0.163; Figure 3.3A). However, no statistically significant effect of *B/M treatment* ($F_{(1, 37)}$ = 0.714, p = 0.403, η^2 = 0.016) or *feeding condition* x *B/M treatment* ($F_{(1, 37)}$ = 0.007, p = 0.783, η^2 = 0.002) interaction was found. Analysis of active lever responses over the test session (10 minute bins) revealed a statistically significant effect of *time* ($F_{(5,185)}$ = 12.73, p < 0.0001), but no significant interaction with any of the other factors (Figure 3.3B).

A small, but statistically significant, increase in responses on the inactive lever during the heroin-seeking test was observed in the FDR rats, compared to the sated group

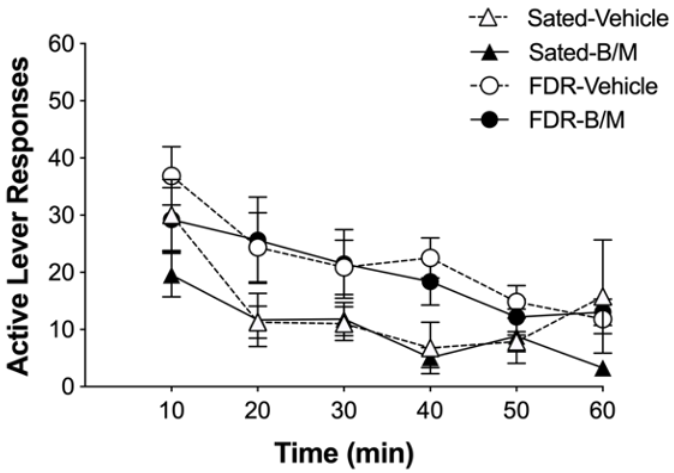
(*feeding condition*: $F_{(1, 37)} = 6.284$, $p = 0.02$, $\eta^2 = 0.144$; Figure 3.3A). No statistically significant effect of *B/M treatment* ($F_{(1, 37)} = 0.293$, $p = 0.591$, $\eta^2 = 0.007$) or *B/M treatment* x *feeding condition* interaction ($F_{(1, 37)} = 0.112$, $p = 0.740$, $\eta^2 = 0.003$) was found.

To validate the efficacy of the PVT inactivation manipulation, performance on an EPM was assessed. Intra-PVT injection of baclofen + muscimol (n = 6 per group) resulted in a considerable reduction (38%) in the time spent in the open arm of the elevated plus maze compared to vehicle administration, but the difference was not statistically significant ($t_{(10)} = 1.718$), $p = 0.116$). However, further analysis revealed that PVT inactivation resulted in a shorter time spent in the open arm upon the last entry into the open arm ($t_{(10)} = 2.53$, $p = 0.029$), suggesting slower habituation to the maze (Figure 3.3C).

(A)


Vehicle
B/M
Responses (1 h)
300
250
200
150
100
50
0
*
*
SATED
FDR
SATED
FDR
Active lever
Inactive lever

(B)


Sated-Vehicle
Sated-B/M
FDR-Vehicle
FDR-B/M
Active Lever Responses
60
50
40
30
20
10
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10
20
30
40
50
60
Time (min)

(C)

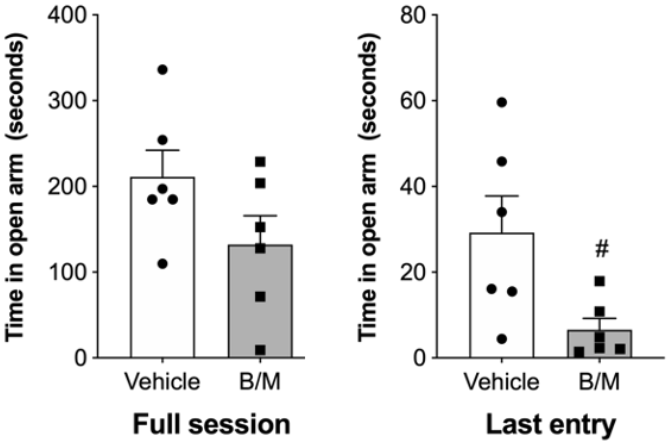

Time in open arm (seconds)
400
300
200
100
0
Vehicle
B/M
Full session
Time in open arm (seconds)
80
60
40
20
0
#
Vehicle
B/M
Last entry

Figure 3. 3. Pharmacological inhibition of the PVT had no effect on food restriction-induced augmentation of heroin seeking. (A) Mean ± SEM number of active (left) and inactive (right) lever responses made during the 1-hour heroin-seeking test on day 14 of food restriction in the sated and food-restricted (FDR) groups. (B) Mean ± SEM number of active lever responses made over the 1-hour test, presented in 10-minute time intervals. Rats were administered with baclofen + muscimol (B/M; 0.3 + 0.03 nmol) or vehicle 5 minutes before the test session. (C) Mean ± SEM time (seconds) spent in the open arm of the elevated plus maze following administration of baclofen + muscimol or vehicle throughout the 10 minute test (left), and following the last entry into the open arm (right) * $p \leq 0.02$ compared to sated groups; # $p = 0.03$.

Experiment 3: PVT activation using excitatory DREADD

Sixteen rats were removed due to catheter leakage, failure to train, health issues or incorrect viral placement. Therefore, the final analysis included 39 rats in the 4 experimental conditions: FDR-Vehicle ($n = 12$), FDR-CNO ($n = 7$), Sated-Vehicle ($n = 10$), and Sated-CNO ($n = 10$). On test day, food restricted (n = 19; 340.4 ± 3.8 g) rats weighed statistically significantly less than sated rats (n = 20; 449.1 ± 6.3 g; $t_{(37)} = -14.785$, $p < 0.0001$, $d = -5.297$). The three-way ANOVA revealed a significant main effect of *lever type* ($F_{(1, 35)} = 67.530$, $p < 0.0001$, $\eta^2 = 0.545$). Additionally, a statistically significant *feeding condition* x *drug condition* x *lever type* interaction was observed ($F_{(1, 37)} = 7.542$, $p = 0.009$, $\eta^2 = 0.061$). Further analyses were conducted using separate two-way ANOVAs for active and inactive lever responses.

Food restriction augmented heroin seeking in vehicle-treated rats, as indicated by the increase in active lever presses. However, CNO dramatically attenuated heroin

seeking in the FDR-CNO group, while having a minor effect in the Sated-CNO group (*feeding condition* x *CNO treatment*: $F_{(1, 35)} = 6.695$, $p = 0.014$, $\eta^2 = 0.115$; Figure 3.4A). There was no statistically significant main effect of *feeding condition* ($F_{(1,35)} = 1.02$, $p = 0.319$, $\eta = 0.018$) on active lever responding, but there was a significant main effect of *CNO treatment* ($F_{(1,35)} = 15.52$, $p < 0.0001$, $\eta^2 = 0.266$). Post hoc analyses revealed that rats in the FDR-Vehicle group pressed statistically significantly more on the active lever compared to the Sated-Vehicle ($p = 0.045$) and the Sated-CNO groups ($p = 0.003$). Importantly, active lever presses were statistically significantly lower in the FDR-CNO group compared to the FDR-Vehicle group ($p < 0.0001$), but there was no significant difference in active lever responding between the Sated-CNO and Sated-Vehicle groups ($p = 0.758$). Repeated measures ANOVA revealed a statistically significant effect of *time* ($F_{(5,175)} = 20.63$, $p < 0.0001$), and no significant interaction with any of the other factors, suggesting that all groups decreased their active lever presses at similar rate over the test session. However, visual inspection of active lever responses made over the test session (10 minute bins; Figure 3.4B) suggests that the response rate in the FDR-CNO was consistently low, compared to the FDR-Vehicle group, for the duration of the session.

Rats in the CNO-treated groups pressed significantly less on the inactive lever during the heroin seeking test, compared to the Vehicle-treated groups (*CNO treatment*: $F_{(1, 35)} = 10.16$, $p = 0.003$, $\eta^2 = 0.224$; Figure 3A). No statistically significant effect of *feeding condition* ($F_{(1, 35)} = 0.04$, $p = 0.847$, $\eta^2 = 0.001$) or *CNO treatment* x *feeding condition* interaction ($F_{(1, 35)} = 0.167$, $p = 0.685$, $\eta^2 = 0.004$) was found.

Data from the locomotor activity test was lost for one animal in the Sated-Vehicle group. Mean ± SEM distance traveled (m) were: Sated-Vehicle 8.37 ± 0.46; Sated-CNO 7.10 ± 1.10; FDR-Vehicle 8.31 ±0.76; FDR-CNO 5.55 ± 0.95. CNO treatment resulted in a statistically significant reducted distance traveled compared to vehicle injections (*CNO treatment*: $F_{(1,34)} = 5.461$, $p = 0.025$, $\eta^2 = 0.133$). However, no statistically significant effect of *feeding condition* or *feeding condition* x *CNO treatment* interaction was found for locomotor activity during the 1-hour locomotor activity test.

Histology and electrophysiological effects of DREADD activation

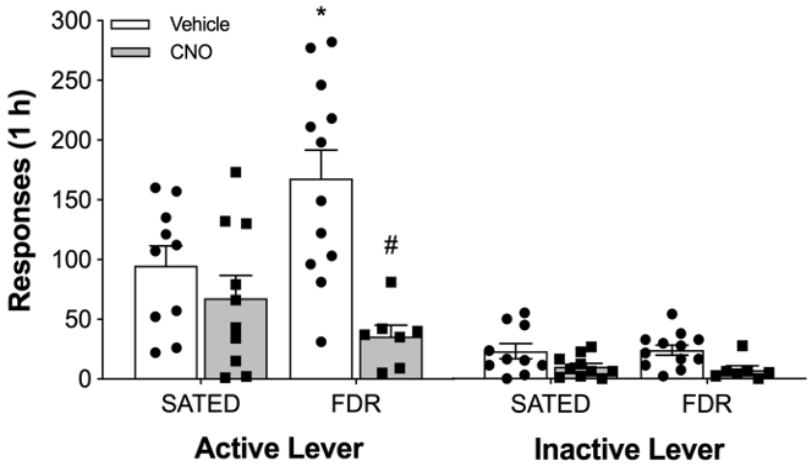
The extent of expression of hM4D(Gi) and hM3D(Gq) in the PVT is presented in Figure 3.5A and 3.5C, respectively. Anatomical positions of the injector tips used for intra-PVT B/M microinjections are shown in Figure 3.5B.

To confirm that CNO administration activated PVT neurons in hM3D(Gq) expressing rats, we used in vitro electrophysiological recordings from neurons in the PVT, before and during CNO exposure. CNO increased the number of action potentials evoked by 500 ms-duration positive current steps in PVT neurons held near an initial membrane potential of -60 mV. The mean number of spikes evoked was statistically significantly increased during 25 pA steps ($n = 7$, $t_6 = 2.65$, $p = 0.038$) and 50 pA steps, ($t_6 = 2.65$, $p = 0.038$), but the increase did not reach statistical significance during 75 pA steps ($t_6 = 1.69$, $p = 0.152$; Figure 3.5D$_{1\text{-}2}$). The increased spiking was not due to a tonic depolarizing influence of CNO, because cells were held near -60 mV using constant current injection both before and after application of CNO. Further, the amount of negative current required to keep cells at -60 mV was also not increased during application of CNO (-44 ±20 pA in ACSF and -42 ±18 pA in CNO). However, the

increased spiking induced by positive current steps during CNO application was associated with an increase in both peak input resistance ($t_6 = 4.51$, $p = 0.004$; Figure 3.5D$_3$) and steady-state input resistance (54.7 ± 5.9 vs. 73.5 ± 7.1 MΩ; $t_6 = 4.59$, $p = 0.004$) which likely underlies the enhanced spiking.

The increased electroresponsiveness of PVT neurons during CNO application was also expressed in response to prolonged 5-second depolarizing current steps of 25 or 50 pA (Figure 3.5D$_4$). The intensity of firing observed during recordings in normal ACSF varied among cells, but there was a reliable increase in mean number of spikes evoked during application of CNO (t_6=2.97, p=.025; Figure 3.5D$_5$). The increase in input resistance induced by CNO in PVT neurons is therefore associated with increased evoked spiking during both brief and longer lasting depolarizing current injection.

(A)



(B)

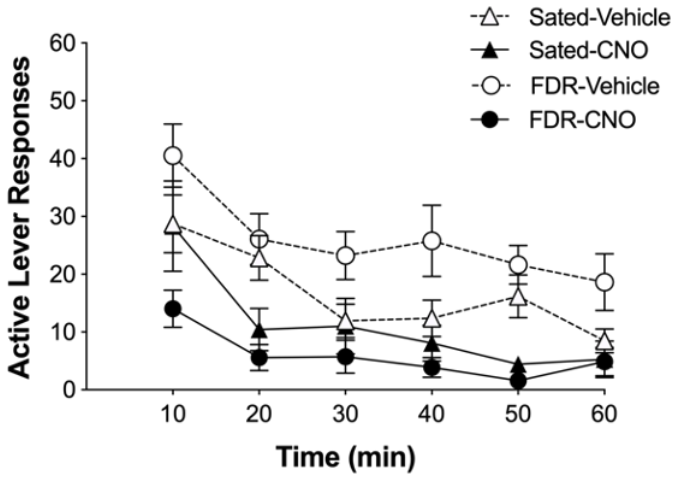


Figure 3. 4. Chemogenetic activation of the PVT blocked food restriction-induced augmentation of heroin seeking. (A) Mean ± SEM number of active (left) and inactive (right) lever responses made during the 1-hour heroin-seeking test on day 14 of food restriction in the sated and food-restricted (FDR) groups. (B) Mean ± SEM number of active lever responses made over the 1-hour test, presented in 10-minute time intervals. Rats were administered with CNO (6.0 mg/kg, i.p.) or vehicle 20 minutes before the test. * $p = 0.045$ compared to sated-vehicle group, $p = 0.003$ compared to sated-CNO group, # $p < 0.0001$ compared to the FDR-CNO group

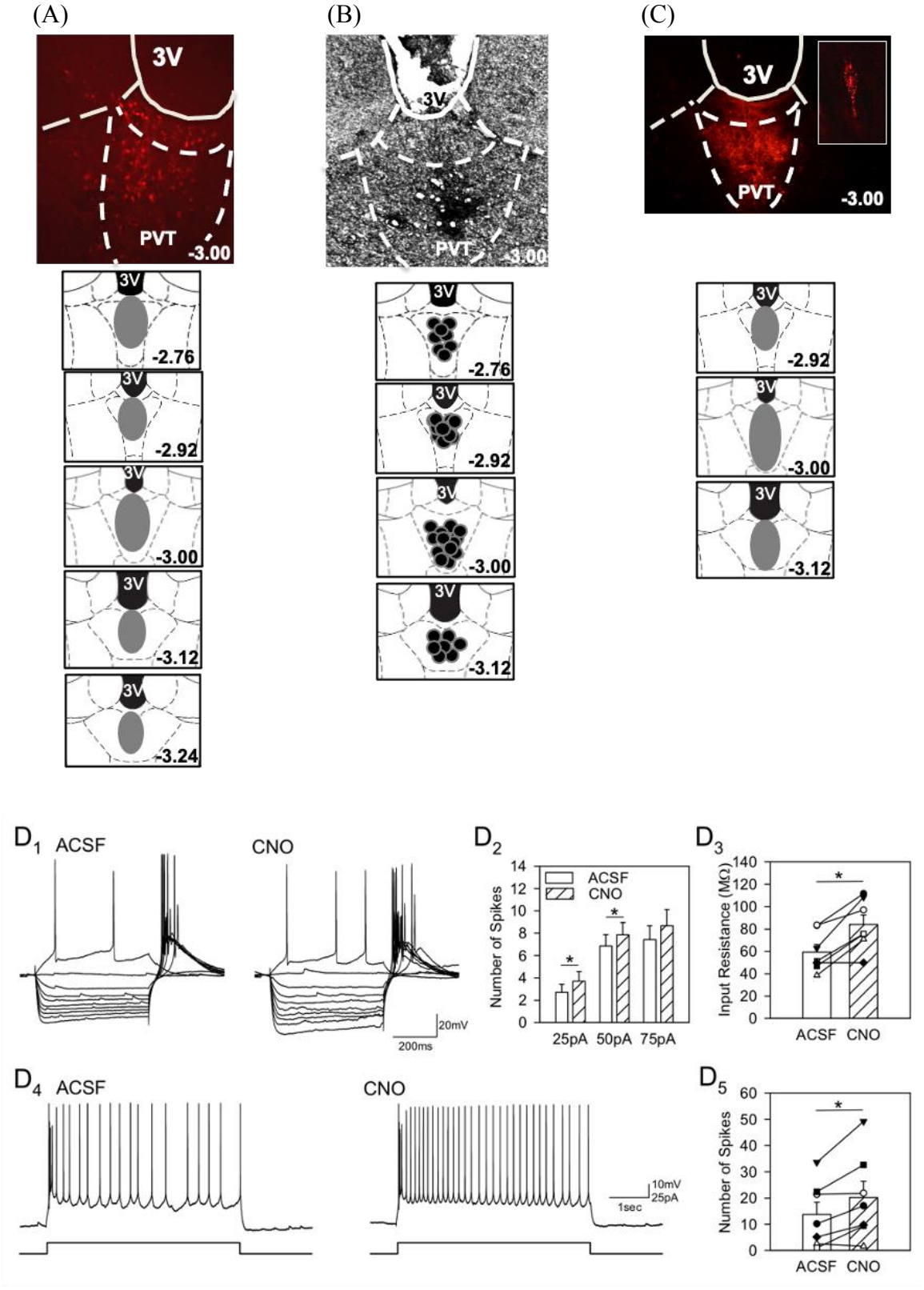

(A)
(B)
(C)
3V
PVT
-3.00
-2.76
-2.92
-3.12
-3.24
D1 ACSF
CNO
20mV
200ms
D2
ACSF
CNO
Number of Spikes
25pA 50pA 75pA
D3
Input Resistance (MΩ)
ACSF CNO
D4 ACSF
CNO
10mV
25pA
1sec
D5
Number of Spikes
ACSF CNO

Figure 3. 5. Representative mCherry tagged immunofluorescence in the PVT (A) Top: Representative section of mCherry tagged hM4D(Gi) immunofluorescence in the PVT for Experiment 1 (third ventricle (3V)). Bottom: Spread of hM4D(Gi)-mCherry expression in the PVT. (B) Top: Representative section of injector tip placement in the PVT for Experiment 2. Bottom: Anatomical positions of the injector tips used for intra-PVT B/M microinjections. (C) Top: Representative section of mCherry tagged hM3D(Gq) immunofluorescence in the PVT for Experiment 3 (insert: expression of mCherry tagged hM3D(Gq) in a single neuron in the PVT under high magnification). Bottom: Spread of hM3D(Gq)-mCherry expression in the PVT. (D) CNO increases action potential firing evoked by depolarizing current steps in PVT neurons from rats infused with AAV8-hSyn-hM3D(Gq)-mCherry (D_1) Superimposed membrane potential responses to hyperpolarizing and depolarizing current steps are shown for a representative neuron recorded in normal ACSF and after application of 1 μM CNO. (D_2) The number of spikes evoked by the 25 and 50-pA current pulses was significantly increased under the CNO condition (n=7; * $p < 0.05$). (D_3) Application of CNO was also associated with an increase in cellular input resistance that reflects the increased hyperpolarization during negative current steps in D_1. Connected symbols in D_3 show data from individual cells. The number of spikes evoked by 5-second duration positive current steps was also increased during application of CNO: (D_4) Representative traces from one cell. (D_5) The group means and data from individual cells (* $p < 0.05$).

Discussion

In this series of experiments, we examined the role of the PVT in the augmentation of heroin seeking induced by chronic food restriction following a period of withdrawal. To our knowledge, ours is the first study to examine the role of the PVT in heroin seeking. As demonstrated in our previous reports (e.g., D'Cunha et al., 2013; Sedki et al., 2015), rats that were food restricted over a withdrawal period displayed a significant increase in heroin seeking compared to sated controls. Contrary to our initial hypothesis, we found that PVT inactivation did not affect heroin seeking, while PVT chemogenetic activation abolished the augmentation of heroin seeking in chronically food restricted rats.

Previous evidence indicates that PVT inactivation interferes with context-induced reinstatement of alcohol seeking, cue- and drug-induced reinstatement of alcohol and cocaine seeking, and with cocaine CPP (Hamlin et al., 2009; Browning et al., 2014; Matzeu et al., 2015; James et al., 2010; Wunsch et al., 2017). Moreover, PVT neurons are recruited when learning about cue-reward associations (Flagel et al., 2011), and when seeking alcohol (Dayas, Liu, Simms, & Weiss, 2007), or following re-exposure to drug associated context (Brown et al., 1992; Rhodes et al., 2005). Consequently, we hypothesized that inactivation of the PVT would attenuate heroin seeking. Moreover, since the PVT plays a role in the control of feeding (Igelstrom et al., 2010; Bhatnagar & Dallman, 1999; Stratford & Wirtshafter, 2013; Millan et al., 2017), we expected the attenuating effect to be more robust in the food-restricted rats.

The lack of effect for PVT inactivation on heroin seeking is therefore quite intriguing. However, it is consistent with previous reports on the effects of anatomically

specific inactivation of either the anterior (aPVT) or posterior PVT (pPVT) on cue-induced drug seeking. Distinct PVT afferent and efferent innervations have been identified for the aPVT and pPVT, which suggests a functional distinction (Kirouac, 2015). For example, compared to the aPVT, the pPVT receives more robust input from the prefrontal cortex and projects more extensively to the central amygdala, the bed nucleus of the stria terminalis, and NAc core (Kirouac, 2015). Examination of the histological data in the current study indicates that DREADD were primarily expressed in the pPVT. Many of the studies that report an effect of PVT inactivation on drug seeking or CPP identified the aPVT as a critical area (e.g., Browning et al., 2014; James et al., 2010), but note, however, that Matzeu et al. (2015) found that pPVT inactivation blocked discriminative stimulus-induced reinstatement of cocaine seeking. Conversely, chemogenetic inactivation of aPVT-NAc projections enhanced cue-induced reinstatement of cocaine seeking, while inactivation of the pPVT-NAc projections had no effect (Wunsch et al., 2017). Similarly, combined inactivation of aPVT and pPVT augmented cue-induced reinstatement of extinguished cocaine seeking, but specifically in "goal tracking" rats, with no significant effects on reinstatement in "sign tracking" rats (Kuhn et al., 2018). Unfortunately, the effects of PVT activation on drug seeking were not assessed in any of the studies cited above.

An interesting complement to the reported effects of PVT inactivation on reward seeking is the recent demonstration that photoactivation of the aPVT abolished (while photoinhibition increased) sucrose seeking, particularly when reward was omitted (Do-Monte, Minier-Toribio, Quiñones-Laracuente, Medina-Colón, & Quirk, 2017). Furthermore, Do-Monte et al. (2017) also showed that selective photoactivation of the

PVT to NAc or PVT to central amygdala pathways, two of the major PVT targets (Kirouac, 2015), inhibits sucrose seeking. However, the relevance of Do-Monte et al.'s (2017) findings to our study should be assessed with caution considering their focus was on the aPVT, and the use of a natural reward.

The discrepancy from previous findings might also be explained by the fact that addiction to psychostimulant and opiate drugs are distinct phenomena that have different underlying neurobiological as well as behavioural and cognitive characteristics (Badiani, Belin, Epstein, Calu, & Shaham, 2011). In addition, most studies examining the role of PVT in drug seeking have utilized a reinstatement procedure. As mentioned above, the neural mechanisms underlying reinstatement of extinguished drug seeking are different from those that underlie drug seeking following a period of withdrawal (Fuchs et al., 2008; Fuchs et al., 2006).

Recently, Flagel et al. suggested that the PVT is important for the process of attributing incentive salience to reward-associated cues (Kuhn et al., 2018; Haight et al., 2015; Haight et al., 2017). Thus, at least in a subset of individuals, the PVT may act to "hide" the learned incentive value of a conditioned cue. Inhibition of PVT activity, then, may "unmask" this suppressed incentive value (Kuhn et al., 2018). Our findings are in agreement with this theory. It is possible, although not directly assessed by Kuhn et al. (2018), that activation of the PVT would robustly act to suppress a conditioned incentive value that was previously acquired (i.e., during the heroin self-administration training), resulting in the attenuation of cue-induced heroin seeking. Food restriction has been shown to increase incentive salience of reward-associated cues (Anderson, Bush, &

Spear, 2013), possibly resulting in higher propensity to observe an effect for PVT activation in the FDR group.

The neural mechanisms that underlie this effect are not clear, but one possible explanation could be selective plasticity in PVT inputs onto NAc MSNs expressing the dopamine D2 receptor (D2-MSNs). Such plasticity has been demonstrated in morphine-dependent mice, by an increased AMPA/NMDA ratio in D2-MSNs, but not the D1-MSNs, directly innervated by the PVT (Zhu et al., 2016). Activation of D2-MSNs has been associated with punishment and aversion (Kravitz, Tye, & Kreitzer, 2012), and this effect may have opposed heroin seeking during the test. In fact, Zhu et al. (2016) suggest that the input from the PVT to the NAc is aversive and might be instrumental for the negative symptoms of drug withdrawal. It is, however, important to note that the behavioural and functional effects observed by Zhu et al. (2016), were associated with acute morphine withdrawal. It is not yet clear if similar adaptations are present following prolonged withdrawal from heroin, as in our study. Moreover, the reason for the specificity of the attenuating effect to the food-restricted rats in the current study is not clear. One possibility is that food restriction may result in a further increase in the AMPA/NMDA ratio on MSNs due to synaptic incorporation of calcium-permeable AMPA receptors in the food-restricted rats (Ouyang et al., 2017). However, Ouyang et al. (2017) also present data suggesting that this plasticity occurs exclusively in D1-MSNs.

Methodological Considerations

The lack of effect for inhibition of the PVT using DREADD could be the result of insufficient virus infection, low DREADD expression, or low efficiency of DREADD-induced inhibition. However, histology revealed strong levels of DREADDs expression,

reflected by robust expression of mCherry. Moreover, pharmacological inhibition of the PVT, using behaviourally effective doses of GABA receptor agonists (e.g., Browning et al., 2014), also had no effect on heroin seeking (Experiment 2). Finally, transient inactivation of the PVT using the same dose of GABA receptors agonists used in Experiment 2 resulted in a considerable anxiogenic effect, as previously reported (Barson & Leibowitz, 2015), supporting the efficacy of this manipulation.

DREADD-independent behavioural effects for CNO have been recently reported (MacLaren et al., 2016). However, the attenuation of heroin seeking presented in Experiment 3 seems to be specific to the activation of the hM3D(Gq) receptor, since a similar dose of CNO had no behavioural results in rats expressing hM4D(Gi) (Experiment 1). An additional consideration is that the attenuated drug seeking found in Experiment 3 might be the result of non-specific locomotor effect due to the hM3D(Gq) activation. Indeed, we observed a small statistically significant attenuation of inactive lever responses in both feeding groups during the test, and hM3D(Gq) activation also decreased locomotion in an open field. Similarly, Do-Monte et al. (2017) reported that activation of PVT projections to the central amygdala resulted in impaired locomotion in an open field. However, a non-specific locomotor effect cannot fully explain the robust, and selective, attenuation in active lever responses in the food restricted rats. More targeted activation of particular PVT projections (e.g., to NAc) could be used in future studies to minimize locomotor effects.

Conclusions

In summary, this is the first study to examine the role of the PVT in heroin seeking using an ‘abstinence procedure’. Our results indicate that activation of the PVT

can strongly inhibit the augmentation of heroin seeking induced by chronic food restriction. This report adds to the accumulating evidence suggesting an important role for the PVT in drug addiction. However, understanding the precise function of the PVT in drug seeking remains a major challenge.

CHAPTER 4: INVESTIGATNG THE ROLE OF CORTICO-THALAMIC PROJECTIONS IN THE AUGMENTATION OF HEROIN SEEKING INDUCED BY CHRONIC FOOD RESTRICTION

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Data presented in this chapter were included in a paper publish in J Neurosci.

Abstract

Drug addiction is a chronic disorder characterized by compulsive drug seeking and involves cycling between periods of compulsive drug use, abstinence, and relapse. In humans and animals, chronic food restriction increases rates of relapse. Previously, our laboratory has demonstrated a robust increase in drug-seeking in chronically food-restricted rats compared to sated rats, following a period of withdrawal. To date, the neural mechanisms that mediate the effect of chronic food restriction-induced drug-seeking remain elusive. However, recent evidence from our laboratory indicates that the paraventricular nucleus of the thalamus (PVT) plays a key role in this effect. Specifically, activating the PVT reduces heroin seeking in food-restricted animals. A significant excitatory input to the PVT arises from the prelimbic cortex (PrL), and previous data suggest a role for this pathway in drug-seeking. The objective of the current study was to assess the effect of chemogenetically activating and inhibiting PrL-PVT neurons in the food restriction-induced heroin seeking effect. Male Long Evans rats were injected with a viral vector carrying an excitatory Designer Receptor Exclusively Activated by Designer Drug (DREADD) into the PrL and implanted with a guide cannula aimed at the PVT. Next, rats were trained to self-administer heroin over 10 days (0.1 mg/kg/infusion; i.v.). Following training, rats were removed from the operant conditioning chambers and placed into drug withdrawal for 15 days. Over the withdrawal period, rats were exposed to a mild food restriction (90% of baseline body weight) or were given unrestricted access to food. On the 15th day of the withdrawal period, a drug-seeking test was conducted in which rats were intracranially injected with CNO (1.0 mM) or vehicle into the PVT to activate or inhibit the PrL-PVT pathway. As expected, food-restricted rats

demonstrated an augmented heroin seeking during the heroin-seeking test in comparison to sated controls. However, chemogenetic activation or inhibition of the PrL-PVT pathway did not alter heroin seeking in food-restricted or sated rats. These results suggest that the cortical input from the PrL to the PVT does not play a role in heroin seeking following a period of abstinence.

Introduction

In abstinent drug users, relapse is often triggered by exposure to cues (i.e., drug paraphernalia) that have been associated with drug-taking, re-exposure to the drug that was previously abused, and by stress (Childress et al., 1993; Sinha, 2001; De Wit, 1996). These triggers induce intense drug craving that can elicit drug-seeking (Shaham et al., 2003). Similarly, caloric restriction increases drug use, subjective drug craving and rates of relapse. For example, caloric restriction increases the use of coffee and tobacco products (Franklin et al., 1948) and increases cigarette use in adult smokers (Cheskin et al., 2005). Also, relapse rates increase when individuals diet while they attempt to stop smoking (Hall et al., 1992; Pirie et al., 1992).

In laboratory animals, caloric restriction increases the initiation and maintenance of drug intake (Carroll & Meisch, 1984; Lu et al., 2003). Acute food deprivation (24-48 h) and chronic caloric restriction reinstates extinguished cocaine and heroin seeking (Shalev, 2012; Shalev et al., 2000; Shalev et al., 2003a). Our laboratory has demonstrated a robust increase in heroin seeking following 14 days of withdrawal in chronically food-restricted rats (D'Cunha et al., 2013). Most recently, we reported that the paraventricular nucleus of the thalamus (PVT) plays a key role in this effect. Specifically, chemogenetically activating the PVT abolishes heroin seeking in chronically food-restricted abstinent rats (Chisholm et al., 2020). However, the neural mechanism by which the PVT attenuates heroin seeking in chronically food-restricted rats remains a mystery.

The PVT is densely innervated by medial prefrontal cortical (mPFC) glutamatergic neurons arising from layer VI of the prelimbic (PrL), infralimbic (IL), and

insular cortices (IC) (Li & Kirouac, 2012). Across the anterior-posterior axis, the anterior PrL provides the greatest source of innervation to the pPVT. In contrast, the posterior PrL more heavily innervates the aPVT (Li & Kirouac, 2012). Here we chose to focus on PrL innervation of the PVT instead of the IL or IC for three key reasons. First, the PrL provides the strongest source of excitatory innervation to the pPVT, the region of the PVT that we previously activated resulting in an attenuation of heroin-seeking in our chronically food-restricted rats, making the PrL-PVT projection a prime candidate to mediate this effect. Second, a role for the PrL-PVT pathway in the regulation of motivated behaviour has been proposed (Otis et al., 2017; Otis et al., 2019; Giannotti et al., 2018; Campus et al., 2019; Kuhn et al., 2020). Additionally, no reports indicating a role for the IL-PVT pathway in drug-seeking have been presented.

The PrL is a critical brain region implicated as a neural substrate underlying drug addiction and in the regulation of drug-seeking behaviour. The PrL is activated during cue-induced reinstatement of cocaine and heroin seeking in a polydrug use model (Rubio et al., 2019). Food deprivation enhances c-fos expression in the PrL in stress-induced reinstatement of heroin-seeking (Shalev et al., 2003b). The PrL is also activated during non-drug reward-seeking (Burgos-Robles, Bravo-Rivera, & Quirk, 2013; Moorman & Aston-Jones, 2015). For example, the PrL is activated during the reinstatement of discriminative stimulus-induced sucrose seeking (Moorman & Aston-Jones, 2015). These findings suggest that inactivating the PrL can attenuate both drug and non-drug reward-seeking. In line with this idea, PrL inactivation attenuates both cue- and heroin prime-induced reinstatement of heroin seeking (LaLumiere & Kalivas, 2008; Rogers, Ghee, & See, 2008). Furthermore, PrL inactivation attenuates food-seeking as well as stress-

induced reinstatement of food-seeking (Sangha, Robinson, Greba, Davies, & Howland, 2014; Calu et al., 2013). Together, these data support a role for the PrL in drug as well as non-drug reward seeking.

To date, only a handful of studies have investigated the role of PrL-PVT projections in motivated behaviour, including fear memory (Do-Monte, Quiñones-Laracuente, & Quirk, 2015), reward-seeking (Otis et al., 2017; Otis et al., 2019; Campus et al., 2019) and drug-seeking (Giannotti et al., 2018). Inhibiting PrL-pPVT neurons immediately following the last cocaine self-administration session attenuates cocaine-seeking following abstinence and cue-induced reinstatement of extinguished cocaine-seeking (Giannotti et al., 2018). In contrast, evidence examining the role of PrL-PVT neurons in cue-motivated behaviour indicates that inhibiting PrL-PVT projections increases the incentive value of reward-paired cues in animals who attribute a predictive value to the cue (i.e., goal trackers), while activating PrL-PVT neurons reduces the incentive value of reward-paired cues in animals who attribute both predictive and incentive value to the cue (i.e., sign trackers), suggesting that the PrL-PVT pathway exerts top-down control of incentive salience attribution (Campus et al., 2019). In addition, PrL-PVT neurons show distinct cue encoding during appetitive learning; little response occurs to reward-paired cues before learning, but the majority of PrL-PVT neurons acquire new inhibitory responses following learning (Otis et al., 2017). The development of these inhibitory responses appears to be critical for the encoding of reward-predictive cues, and the expression of cue-induced reward-seeking as photostimulation of PrL-PVT neurons dampens both of these effects (Otis et al., 2017;

Otis et al., 2019). Together, these findings suggest the PrL-PVT neurons contribute to reward and drug-seeking.

The objective of this study was to investigate the role of PrL-PVT projections in the augmentation of heroin seeking following a period of withdrawal in sated and food-restricted animals. To answer this question, we infused a viral vector carrying excitatory or inhibitory DREADD (Designer Receptors Exclusively Activated by Designer Drugs) into the PrL. Next, we cannulated the PVT to manipulate PrL-PVT projections selectively. Based on the results of our previous study, we hypothesized that chemogenetically activating PrL-PVT projections would attenuate heroin seeking in our chronically food-restricted abstinent rats.

Materials and Methods

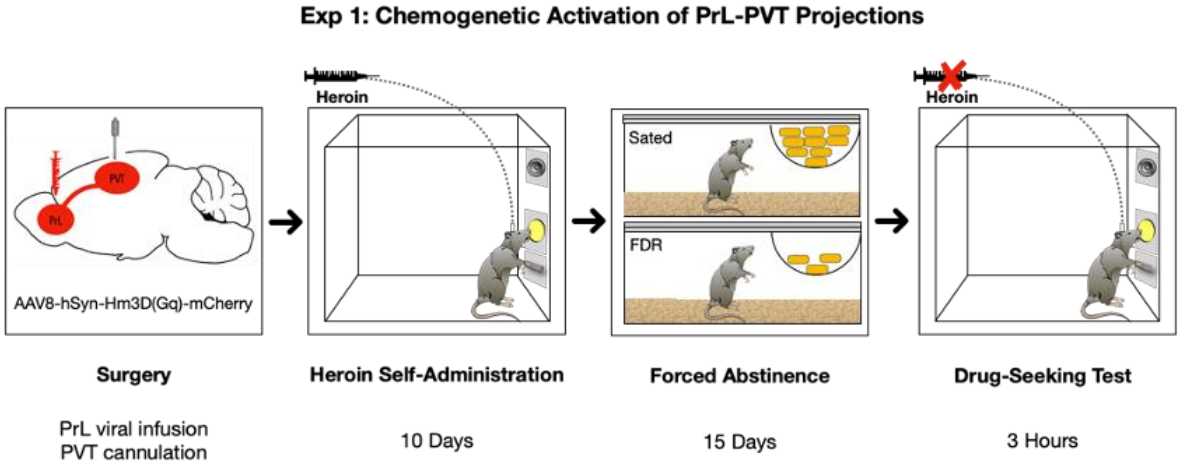
Subjects

Eighty-three male Long Evans rats (Raleigh, North Carolina, U.S.A.), weighing 250-275 g at the beginning of the experiments, were used in two different experiments.

Intravenous and Intracranial Surgery

Immediately following intravenous catheterization, across both experiments, rats were infused with a viral vector in the PrL and were implanted with a guide cannula aimed at the PVT. General procedures are described in Chapter 2. A general viral vector approach and timeline for each experiment are presented in Figure 4.1.

(A)



(B)

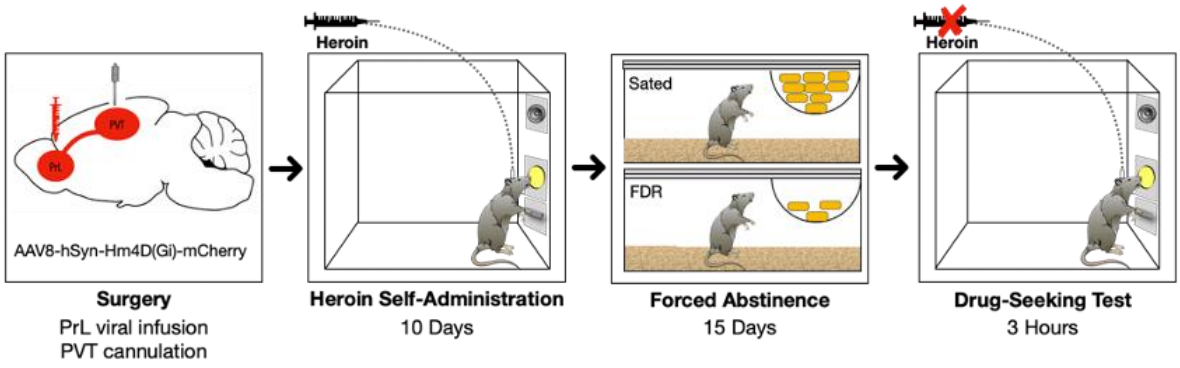


Figure 4. 1. Diagram of viral vector approach and general experimental timelines. (A) Diagram of viral vector approach and the experimental timeline for Experiment 1. (B) Diagram of viral vector approach and the experimental timeline for Experiment 2.

Experiment 1: Chemogenetic activation of PrL-PVT projections

Rats were bilaterally infused with 0.75 µl of viral vector (AAV8-hSyn-hM3D(Gq)-mCherry, Canadian Neurophotonic Platform/viral vector team, Québec, QC) into the PrL (3.0 AP, 1.0 ML, -3.5 DV relative to Bregma) at a rate of 0.1 µl /minute. Next, rats were implanted with a guide cannula aimed at the PVT (-3.0 AP, 0.0 ML, -3.2 DV, relative to Bregma) to allow for intra-PVT micro infusions (Figure 4.1A).

Experiment 2: Chemogenetic inhibition of PrL-PVT projections

Rats were bilaterally injected with 0.75 µl of viral vector (AAV8-hSyn-hM4D(Gi)-mCherry, Canadian Neurophotonic Platform/viral vector team, Québec, QC) into the PrL (3.0 AP, 1.0 ML, -3.5 DV relative to Bregma) at a rate of 0.1 µl /minute. Rats were given four weeks to recover to ensure optimal viral infection. Following these four weeks, rats were implanted with an intravenous catheter as described above. Immediately following intravenous catheterization, rats were implanted with a guide cannula aimed at the PVT (-3.0 AP, 0.0 ML, -3.2 DV, relative to Bregma) to allow for intracranial micro infusions (Figure 4.1B).

Heroin-Seeking Test

On the 14th day of food restriction, rats were returned to the operant conditioning chambers for one 3-hour heroin-seeking test session. Rats were intracranially injected with either 0.30 µl of CNO (1 mM) or 0.30 µl vehicle, 10 minutes prior to the beginning of the heroin-seeking testing. During testing, all conditions were identical to self-administration training except that rats were tested under extinction conditions. At the time of the test, all rats had a minimum of 5 weeks of viral incubation.

Locomotor Activity Test

To assess possible non-specific motor effects of the chemogenetic manipulations, on the 16th day of food restriction, rats were intracranially injected with either 0.30 µl of CNO (1 mM) or 0.30 µl vehicle, 10 minutes prior to the beginning of a locomotor activity test. Locomotor activity data were collected using Tru Scan 2.0 activity monitoring system (Coulbourn Instruments). Total distance covered (cm) was recorded for 1 hour.

Validation of DREADD Functionality

Rats that underwent behavioural testing were used to assess DREADD functionality in PrL-PVT projection inhibition (Exp. 2). Due to technical issues with brain collection, a new group of 12 rats was used to validate DREADD functionality in PrL-PVT projection activation (Exp. 1.).

Fos Immunohistochemistry and Quantification

Specific details for Fos immunohistochemistry and immunoreactivity quantification procedures are described in Chapter 2.

Statistical Analyses

The critical threshold for statistically significant results was set at $p < .05$. Body weights were compared using t-tests, corrected for unequal variance when necessary. The total number of active and inactive lever responses made during the heroin-seeking test was analyzed using two separate two-way ANOVAs with *drug treatment* (vehicle; CNO) and *feeding condition* (FDR, sated) as between-subjects factors. The number of active lever responses made over the course of the heroin-seeking test was analyzed using a three-way ANOVA with *drug treatment* (vehicle; CNO) and *feeding condition* (FDR, sated) as between subjects factors and *time* (3 x 1-hour segments) as the within subjects factor. Significant interactions were analyzed using three separate two-way ANOVAs with *drug treatment* (vehicle; CNO) and *feeding condition* (FDR, sated) as factors at hour 1, hour 2 and hour 3 of the heroin seeking test. The number of Fos immunoreactive cells in CNO and vehicle-treated subjects were compared using a t-test, corrected for unequal variance when necessary.

Results

Mean ± SEM number of active lever responses, inactive lever responses, and infusions made on the last day of heroin self-administration training, for each treatment group are presented in Tables 4.1 and 4.2. There were no statistically significant differences in these measures between the different experimental groups during the last five days of self-administration training for any experiment.

Experiment 1: Activation of PrL-PVT projections did not change food restriction-induced augmentation of heroin seeking

Twenty-two rats were removed due to catheter leakage, failure to train, health issues, incorrect viral vector placement/expression, or incorrect injector placement. One rat (Sated-CNO group) was considered an outlier due to an extreme number of active lever presses performed during the test (> 2.5 SD above group average). Therefore, the final analysis included 33 rats in the four experimental conditions: FDR-vehicle (n = 8), FDR-CNO (n = 8), sated-vehicle (n = 7) and sated-CNO (n = 10). On test day, FDR rats (n = 16; 346.81 ± 5.42g) weighed statistically significantly less than sated rats (n = 17; 426.82 ± 8.43g, $t_{(31)} = 7.874$, $p < 0.0001$, $d = 2.83$).

Table 4. 1. Mean ± SEM number of active lever responses, inactive lever response and heroin infusions made on the last day of training (9-hours), and body weight on test day in Experiment 1. * $p < 0.0001$, sated compared to food-restricted (FDR) rats.

	Infusions	Active lever	Inactive lever		Body weight
Sated- vehicle	28.57 ± 2.52	89.29 ± 25.17	8.14 ± 2.84	*	435.29 ± 11.53
Sated- CNO	35.00 ± 3.07	94.90 ± 10.54	9.00 ± 2.99		420.90 ± 11.98
FDR-vehicle	32.63 ± 5.75	73.25 ± 20.41	6.13 ± 2.14		352.63 ± 9.21
FDR- CNO	39.88 ± 5.32	111.88 ± 28.65	25.38 ± 21.85		341.00 ± 5.58

Table 4. 2. Mean ± SEM number of active lever responses, inactive lever response and heroin infusions made on the last day of training (9-hours), and body weight on test day in Experiment 2. * $p < 0.0001$, sated compared to food-restricted (FDR) rats.

	Infusions	Active lever	Inactive lever		Body weight
Sated- vehicle	38.88 ± 5.88	111.00 ± 30.70	14.13 ± 4.37	*	521.13 ± 13.89
Sated- CNO	46.24 ± 5.24	126.89 ± 24.86	17.00 ± 4.32		518.56 ± 13.94
FDR-vehicle	47.48 ± 8.66	161.62 ± 48.18	9.82 ± 2.83		399.40 ± 10.06
FDR- CNO	54.64 ± 5.35	138.73 ± 22.06	19.18 ± 7.66		397.27 ± 8.33

Heroin Seeking Test

As expected, food restricted rats pressed more on the active lever during the heroin seeking test, when compared to their sated counterparts (*feeding condition*: $F_{(1, 29)} = 12.32$, $p = 0.002$, $\eta^2 = 0.29$; Figure 4.2A). However, no statistically significant effects for *CNO treatment* ($F_{(1,29)} = 1.11$, $p = 0.301$, $\eta^2 = 0.03$), or *feeding condition* x *CNO treatment* interaction ($F_{(1,29)} = 0.72$, $p = 0.402$, $\eta^2 = 0.02$), were found. No statistically significant effects were found for inactive lever responding.

Analysis of active lever responding over the test session (1-hour bins) revealed a statistically significant effects of *time* ($F_{(1.362, 39.49)} = 71.43$, $p < 0.001$, $\eta^2 = 0.39$), *feeding condition* ($F_{(1, 29)} = 12.32$, $p = 0.002$, $\eta^2 = 0.33$), *time x feeding condition* interaction ($F_{(2, 58)} = 5.00$, $p = 0.010$, $\eta^2 = 0.03$), and *time x feeding condition x CNO treatment* interaction ($F_{(2, 58)} = 4.64$, $p = 0.014$, $\eta^2 = 0.03$). However, no statistically significant effects for *CNO treatment* ($F_{(1,29)} = 1.11$, $p = 0.301$, $\eta^2 = 0.03$), *time* x *CNO treatment* interaction ($F_{(2,58)} = 2.64$, $p = 0.078$, $\eta^2 = 0.01$), or *feeding condition* x *CNO treatment* interaction ($F_{(1,29)} = 0.72$, $p = 0.402$, $\eta^2 = 0.02$), were found. Post hoc two-way ANOVAs revealed a statistically significant main effect of *feeding condition* in each time point, indicating that food restricted rats displayed higher levels of responding at hour 1 ($F_{(1,29)} = 11.16$, $p = 0.002$, $\eta^2 = 0.25$), hour 2 ($F_{(1,29)} = 10.40$, $p = 0.003$, $\eta^2 = 0.26$), and hour 3 ($F_{(1,29)} = 12.13$, $p = 0.002$, $\eta^2 = 0.29$) compared to sated rats (Figure 4.2B). No other statistically significant main effects or interactions were observed.

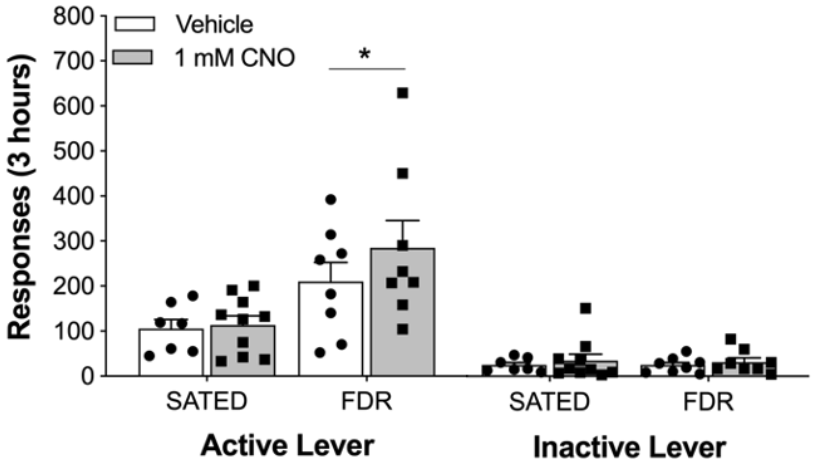
Locomotor Activity Test

Data from the locomotor activity test was lost for one animal in the sated-CNO group. Mean ± SEM distance traveled (cm) was sated-vehicle (7266.53 ± 363.18), sated-CNO (6406.17 ± 810.16), FDR-vehicle (6570.35 ± 1191.36) and FDR-CNO (6571.64 ± 980.48). No statistically significant effects were observed for distance traveled.

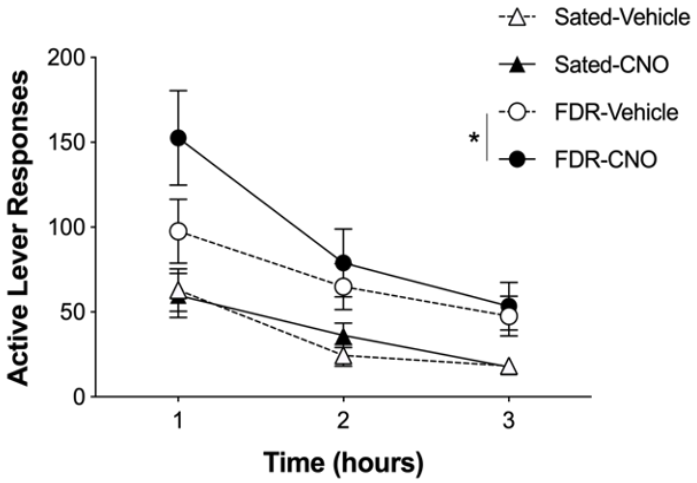
Validation of DREADD Functionality

CNO treatment (n = 6) statistically significantly increased the number of Fos-IR cells in the PVT compared to vehicle controls (n = 6; $t_{(10)} = 1.85$, $p = 0.047$, $d = 1.17$; Figure 4.2C), indicating that DREADD-mediated excitation of the PrL-PVT pathway successfully increased neuronal activity in the PVT. Representative DREADD expression in the PrL and PVT is presented in Figures 4.4A and 4.4B, respectively.

(A)


Vehicle
1 mM CNO
*
Responses (3 hours)
800
700
600
500
400
300
200
100
0
SATED
FDR
SATED
FDR
Active Lever
Inactive Lever

(B)


Sated-Vehicle
Sated-CNO
FDR-Vehicle
FDR-CNO
*
Active Lever Responses
200
150
100
50
0
1
2
3
Time (hours)

(C)

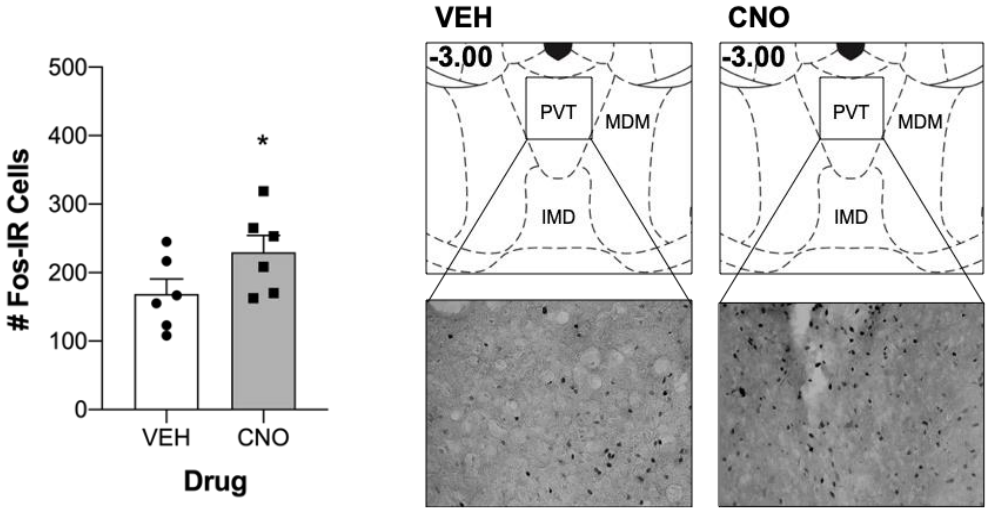

Fos-IR Cells
500
400
300
200
100
0
*
VEH
CNO
Drug
VEH
CNO
-3.00
-3.00
PVT
MDM
IMD
PVT
MDM
IMD

Figure 4. 2. Chemogenetic activation of PrL-PVT projections did not alter food restriction-induced augmentation of heroin seeking. Rats were intracranially administered CNO (1 mM) or vehicle into the PVT 5-10 minutes before the test. (A) Mean ± SEM number of active (left) and inactive (right) lever responses made during the 3-hour heroin-seeking test on day 14 of food restriction in the sated and food-restricted (FDR) groups. * $p < 0.01$, compared with sated groups. (B) Mean ± SEM number of active lever responses made over the 3-hour test, presented in 1-hour time intervals. * $p < 0.01$, compared with sated groups. (C) Chemogenetic activation of PrL-PVT projections increased the number of Fos immunoreactive (Fos-IR) cells in the PVT of CNO-treated subjects compared to vehicle. Rats were intracranially administered CNO (1 mM) or vehicle into the PVT 90 minutes before the perfusion. Data are Mean ± SEM number of Fos-IR cells in the PVT of vehicle- and CNO-treated rats who had their PrL-PVT projections activated (left). * $p < 0.05$, vehicle compared to CNO treatment. Example Fos-IR in the PVT in vehicle- and CNO-treated subjects are also presented (right). All images were taken at 20X magnification.

Experiment 2: Inhibition of PrL-PVT projections did not change food restriction-induced augmentation of heroin seeking

Ten rats were removed due to catheter leakage, failure to train, health issues, incorrect viral vector placement/expression, or incorrect injector placement. Two rats (sated-veh and FDR-veh groups) were considered outliers due to an extreme number of active lever presses performed during the test (> 2.5 SD above group average). Therefore, the final analysis included 38 rats in the four experimental conditions: FDR-vehicle (n = 10), FDR-CNO (n = 11), sated-vehicle (n = 8) and sated-CNO (n = 9). On test day, FDR

rats (n = 21; 398.29 ± 6.32g) weighed statistically significantly less than sated rats (n = 17; 519.76 ± 9.55g, $t_{(36)} = 10.96$, $p < 0.0001$, $d = 3.65$).

Heroin-Seeking Test

Food restriction augmented heroin seeking as indicated by the high active lever responses when compared to sated controls (*feeding condition*: $F_{(1, 34)} = 20.82$, $p < 0.0001$, $\eta^2 = 0.36$; Figure 4.3A). However, no statistically significant effects for *CNO treatment* ($F_{(1, 34)} = 2.20$, $p = 0.147$, $\eta^2 = 0.04$), or *feeding condition* x *CNO treatment* interaction ($F_{(1, 34)} = 0.03$, $p = 0.853$, $\eta^2 = 0.00$) were found indicating that inhibition of PrL-PVT projections did not alter heroin seeking regardless of the feeding condition. There were no significant effects for inactive lever responding. Analysis of active lever responding over the test session (1-hour bins; Figure 4.3B) revealed statistically significant effects of *time* ($F_{(1.336, 45.43)} = 64.08$, $p < 0.0001$, $\eta^2 = 0.41$), *feeding condition* ($F_{(1, 34)} = 20.80$, $p < 0.0001$, $\eta^2 = 0.30$), and *time x feeding condition* interaction ($F_{(2, 68)} = 8.09$, $p = 0.001$, $\eta^2 = 0.05$). However, no statistically significant effects for *CNO treatment* ($F_{(1,34)} = 2.14$, $p = 0.153$, $\eta^2 = 0.03$), *time* x *CNO treatment* interaction ($F_{(2,68)} = 0.14$, $p = 0.871$, $\eta^2 = 0.00$), *feeding condition* x *CNO treatment* interaction ($F_{(1,34)} = 0.03$, $p = 0.866$, $\eta^2 = 0.00$), or *time x feeding condition x CNO treatment* interaction ($F_{(2, 68)} = 0.14$, $p = 0.869$, $\eta^2 = 0.00$) were found. Post hoc two-way ANOVAs revealed a statistically significant main effect of *feeding condition* in each time point indicating that food restricted rats displayed higher levels of responding at hour 1 ($F_{(1,34)} = 16.23$, $p < 0.001$, $\eta^2 = 0.32$), hour 2 ($F_{(1,34)} = 23.39$, $p < 0.001$, $\eta^2 = 0.38$), and hour 3 ($F_{(1,34)} =$

11.65, $p = 0.002$, $\eta^2 = 0.24$) compared to sated rats. No other statistically significant main effects or interactions were observed.

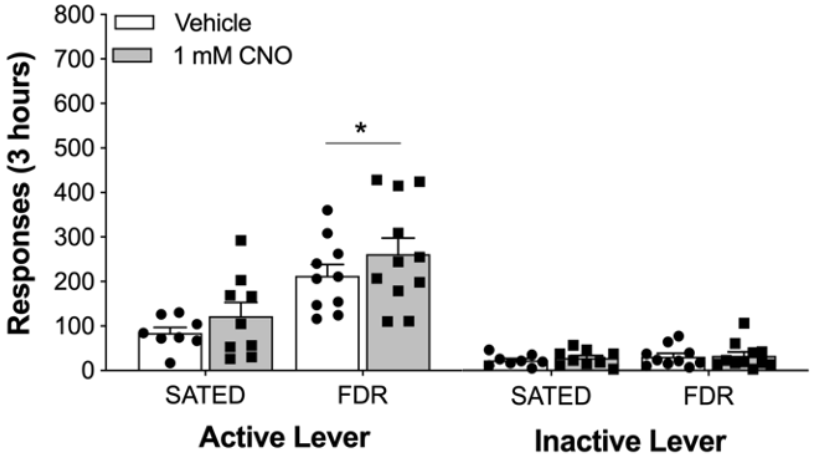
Locomotor Activity Test

Data from the locomotor activity test were lost for one animal in the sated-vehicle group. Mean ± SEM distance traveled (cm) was sated-vehicle (6855.91 ± 563.59), sated-CNO (6282.86 ± 327.21), FDR-vehicle (5756.77 ± 789.03) and FDR-CNO (7302.05 ± 723.92). No statistically significant effects were observed for distance traveled.

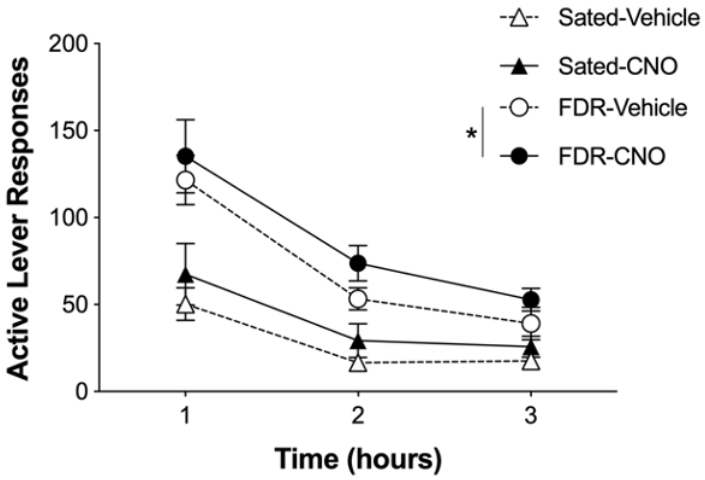
Validation of DREADD Functionality

CNO treatment (n = 6) statistically significantly reduced the number of Fos-IR cells in the posterior PVT compared to vehicle controls (n = 6; t(10) = 2.06, p = 0.033, d = 1.31) indicating that DREADD-mediated inhibition of the PrL-PVT pathway successfully decreased neuronal activity in the PVT (Figure 4.3C).

(A)


Vehicle
1 mM CNO
Responses (3 hours)
800
700
600
500
400
300
200
100
0
*
SATED
FDR
SATED
FDR
Active Lever
Inactive Lever

(B)


Sated-Vehicle
Sated-CNO
FDR-Vehicle
FDR-CNO
*
Active Lever Responses
200
150
100
50
0
1
2
3
Time (hours)

(C)

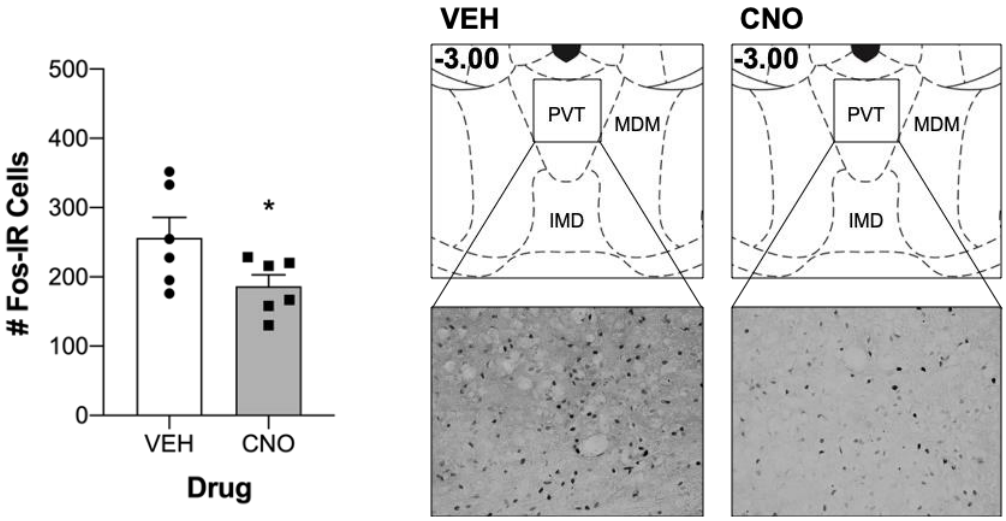
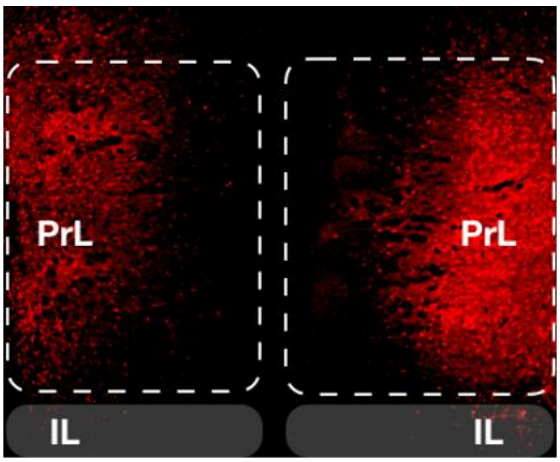

Fos-IR Cells
500
400
300
200
100
0
*
VEH
CNO
Drug
VEH
CNO
-3.00
-3.00
PVT
MDM
IMD
PVT
MDM
IMD

Figure 4. 3. Chemogenetic inhibition of PrL-PVT projections did not alter food restriction-induced augmentation of heroin seeking. Rats were intracranially administered CNO (1 mM) or vehicle into the PVT 5-10 minutes before the test. (A) Mean ± SEM number of active (left) and inactive (right) lever responses made during the 3-hour heroin-seeking test on day 14 of food restriction in the sated and food-restricted (FDR) groups. * $p < 0.0001$, compared with sated groups. (B) Mean ± SEM number of active lever responses made over the 3-hour test, presented in 1-hour time intervals. * $p < 0.01$, compared with sated groups. (C) Chemogenetic inhibition of PrL-PVT projections reduced the number of Fos-IR cells in the PVT of CNO-treated subjects compared to vehicle. Rats were intracranially administered CNO (1 mM) or vehicle into the PVT 90 minutes before the perfusion. Data are Mean ± SEM number of Fos-IR cells in the PVT of vehicle- and CNO-treated rats who had their PrL-PVT projections inhibited (left). * $p < 0.05$, vehicle compared to CNO treatment. Example Fos-IR in the PVT of vehicle- and CNO-treated subjects are also presented (right). All images were taken at 20X magnification.

(A)



(B)

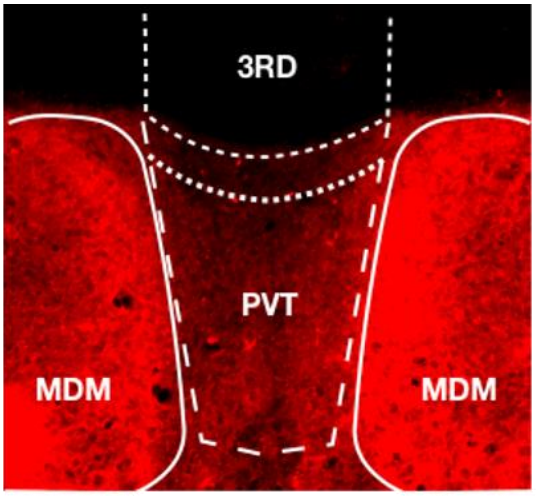


Figure 4. 4. Representative sections of mCherry tagged immunofluorescence in PrL cell bodies and PVT terminals. (A) Representative section of mCherry tagged immunofluorescence in PrL cell bodies (Bregma: +3.00) taken at 2.5X magnification from Experiment 2. (B) Representative section of mCherry tagged immunofluorescence in PrL-PVT projections in the terminal region taken at 5X magnification from Experiment 2.

Discussion

In this series of experiments, we examined the role of PrL-PVT neurons in the augmentation of heroin seeking in chronically food-restricted rats following a period of abstinence by chemogenetically exciting or inhibiting PrL-PVT projections immediately before a heroin seeking test. To our knowledge, this is the first study to examine the role of PrL-PVT projections in heroin seeking. As we have previously reported, following a period of abstinence, chronically food-restricted rats displayed a robust increase in heroin seeking in comparison to their sated counterparts (D'Cunha et al., 2013; Sedki et al., 2013; Sedki et al., 2015; D'Cunha et al., 2017; Chisholm et al., 2020). Based on our previous findings indicating that chemogenetically activating the PVT blocks heroin seeking in chronically food-restricted rats, we hypothesized that chemogenetic activation of PrL-PVT neurons would replicate this effect. Contrary to our hypothesis, activating or inhibiting PrL-PVT neurons did not alter heroin seeking in chronically food-restricted or sated rats. Importantly, we verified that chemogenetically activating or inhibiting PrL-PVT neurons significantly increases and decreases neural activity in PrL-PVT projectors.

The role of the PrL-PVT pathway in reward-seeking is ambiguous. Exposure to reward-associated cues appears to result in an inhibitory response in PrL-PVT neurons, and activation of this pathway suppressed acquisition and expression of conditioned reward-seeking (Otis et al., 2017). In contrast, inhibition of the PrL-PVT pathway attenuated cue-induced cocaine-seeking (Giannotti et al., 2018). Consequently, we explored the effects of PrL-PVT projection inhibition and excitation. PrL-PVT projections arising from layer VI reportedly participate in fear learning, drug-seeking, in the attribution of incentive salience to reward-associated cues and cue reward learning

(Otis et al., 2017; Giannotti et al., 2018; Campus et al., 2019; Otis et al., 2019). Thus, the lack of effect for PrL-PVT projection manipulations on heroin seeking in our chronically food-restricted rats is surprising.

Food restriction can increase the incentive value of reward-paired cues (Anderson et al., 2013). Unpublished data from our laboratory indicate that food restriction increases the incentive value of heroin-associated cues in second-order conditioning and choice procedures (Sedki et al. 2016, SfN abstract). Non-selective inhibition of the PVT resulted in a robust increase in cue-induced reinstatement of cocaine-seeking in rats that attribute predictive value to reward cues (goal trackers), suggesting that the PVT inhibits cue-triggered drug-seeking (Kuhn et al., 2018). The same group reported that chemogenetic inhibition of PrL-PVT projections enhanced the incentive value of reward-paired cues in goal trackers, while activation of PrL-PVT projections decreased the incentive value in rats that attribute motivational value to reward cues (sign trackers) (Campus et al., 2019). Accordingly, we hypothesized that activation of PrL-PVT projections would reduce the incentive value of the heroin-associated cue in our chronically food-restricted rats leading to dampened heroin seeking. However, we did not pre-screen our rats to identify individual differences in the attribution of incentive salience to cues. Thus, one possible reason for the lack of an effect for PrL-PVT manipulations is that the different effects in sign-trackers and goal-trackers blurred the overall effect.

Although the input from the PrL input to the PVT plays a major role in response to reward-associated cues (Otis et al., 2017), including relapse to cocaine-seeking (Giannotti et al., 2018), other excitatory and inhibitory inputs integrate with cortical input to control behaviour (Otis et al., 2019). For example, a dense input from the

hypothalamus to the PVT carries inhibitory GABAergic, as well as peptidergic signals that are implicated in arousal, feeding, energy homeostasis, and stress responses (Millan et al., 2017). Future studies will investigate the potential role of other PVT afferents.

Another explanation for our findings is a delayed incorporation of the PrL-PVT pathway in memory retrieval. (Giannotti et al., 2018) have demonstrated a time-dependent effect, where chemogenetic inhibition of PrL-PVT projections immediately following the last cocaine self-administration session blocked cue-induced reinstatement of cocaine-seeking seven days later. The authors reported that PrL and pPVT neurons were activated early in withdrawal from cocaine (i.e., 2-hours following the last cocaine self-administration session). These findings suggest that PrL-PVT neurons exhibit time-dependent reorganization that may be necessary for cue-induced drug-seeking that interacts with the impact of exposure to food restriction. Therefore, it is possible that we did not observe changes in heroin seeking during the test due to the time at which the PrL-PVT projection was manipulated (i.e., immediately before the heroin-seeking test).

Our results suggest that the PrL-PVT pathway may not be the critical input to the PVT that mediates the attenuation of heroin seeking in chronically food-restricted abstinent rats. Although speculative, we believe that the subcortical input from the lateral hypothalamus may play a role in the augmentation of heroin seeking in chronically food-restricted rats. Recent evidence indicates that the PVT synthesizes cortical and subcortical information related to the internal state and external environment that is encoded and communicated in PVT-NAc neurons to guide reward-seeking (Otis et al., 2019). PrL neurons projecting to the PVT appear to communicate cue-association information while LHA neurons projecting the PVT appear to communicate information

related to behavioural output. If this is true, we might expect that in our chronically food-restricted rats, inhibitory input from the LHA to the PVT is amalgamated and is communicated in PVT-NAc neurons that drive heroin-seeking at test. Future studies will assess the role of PVT-NAc neurons in the augmentation of heroin-seeking induced by chronic food-restriction.

Methodological Considerations

Our findings indicate that the PrL-PVT pathway does not appear to play a role in the augmentation of heroin seeking induced by chronic food restriction. The lack of effect for activating or inhibiting the PrL-PVT pathway using DREADDs could be explained by insufficient viral infection, inadequate DREADD expression or a lack of DREADD-induced efficacy. However, these explanations seem unlikely. Only animals that had strong levels of DREADD expression verified by robust mCherry expression were included in this experiment, suggesting that the lack of effect is not due to insufficient viral infection or DREADD expression. Importantly, we verified that chemogenetic activation and inhibition alter neural activity in PrL-PVT neurons by assessing Fos immunoreactivity, which indicates that the lack of effect is not the result of a lack of DREADD efficacy.

Conclusion

In conclusion, this is the first study to examine the role of PrL-PVT neurons in heroin seeking using an abstinence procedure. Our results indicate that chemogenetically activating or inhibiting PrL-PVT neurons is not sufficient to alter heroin seeking in sated or chronically food-restricted rats following a period of abstinence. These findings add to the knowledge of PVT circuitry involved in drug addiction. However, due to the lack of

effect, examining other inputs to the PVT (i.e., LHA) and outputs from the PVT (i.e., NAc) may help to elucidate the circuitry involved in this effect.

CHAPTER 5: INVESTIGATING THE ROLE OF THALAMO-ACCUMBENS PROJECTIONS IN THE AUGMENTATION OF HEROIN SEEKING INDUCED BY CHRONIC FOOD RESTRICTION

Alexandra Chisholm, Damaris Rizzo, Émilie Fortin, Nour Quietshat, Vanessa Moman, Assunta Romano, Jean-Phillipe Manoliadis, Adamo Ghezzo, Joséphine Gasparri, Tanya Cappolichio, Pasquale Esposito, Amalia Mignelli, Elisabeth Sylvain, Liyah Teller, Paris Parsa, Catarina Borges Jordan Charles, Fatima Inigo, Paria Shafiei and Uri Shalev

Data presented in this chapter were included in a paper publish in J Neurosci.

Abstract

Drug addiction is a chronic disorder that is characterized by compulsive drug seeking and involves switching between periods of compulsive drug use, abstinence, and relapse. In both human and animal models of addiction, chronic food restriction increases rates of relapse. Previously, our laboratory has demonstrated a robust increase in drug-seeking following a period of withdrawal in chronically food-restricted rats compared to sated rats. To date, the neural mechanisms that mediate the effect of chronic food restriction on drug-seeking remain a mystery. Evidence from our laboratory indicates that the paraventricular nucleus of the thalamus (PVT) is involved in this effect, specifically activating the PVT abolishes heroin seeking in chronically food-restricted rats. The PVT extensively innervates the nucleus accumbens (NAc). Thus, the objective of the current study was to study the effect of chemogenetic activation of the PVT- NAc shell and core projections on heroin seeking under food restriction conditions. Male Long Evans rats were injected with a viral vector carrying an excitatory Designer Receptor Exclusively Activated by Designer Drug (DREADD) into the PVT and were implanted with a guide cannulae aimed at the NAc shell or core. Next, rats were trained to self-administer heroin over 10 days (0.1 mg/kg/infusion; i.v.). Following training, rats were removed from the operant conditioning chambers and placed into drug withdrawal for 15 days. Over the withdrawal period, rats were exposed to a mild food restriction (90% of baseline body weight) or were given unrestricted access to food. On the 15th day of the withdrawal period, a drug-seeking test was conducted in which rats were intracranially injected with CNO (1.0 mM) into the NAc shell or core, to activate the PVT-NAc pathway or vehicle. All rats' reliably learned to self-administer heroin. As expected, food-restricted rats

demonstrated an augmented heroin seeking during the heroin-seeking test. Activation of the PVT-NAc shell but not core pathway attenuated heroin seeking in chronically food-restricted rats. These results indicate that PVT-NAc shell plays a role in mediating heroin seeking induced by chronic food restriction.

Introduction

Drug addiction is a chronic relapsing disorder characterized by compulsive drug seeking and involves cycling between periods of drug use, abstinence and relapse. In abstinent drugs users, relapse is trigger by three main factors: (1) re-exposure to the drug previously abused, (2) re-exposure to drug-associated cues, and (3) stressors (Childress et al., 1993; De Wit, 1996; Sinha, 2001). In humans, one common stressor is caloric restriction, which increases the use of coffee/ tobacco products, subjective drug craving and rates of relapse (Franklin et al., 1948). For example, in adults who are attempting to quit smoking, dieting during abstinence increases rates of relapse and cigarette use (Cheskin et al., 2005; Hall et al., 1992).

In animal models of relapse, restricted food intake affects drug-related behaviours. Restricted food intake increases the initiation of drug use and maintenance of drug intake. Acute food deprivation (24 - 48 hours) and chronic caloric restriction reinstate extinguished drug-seeking in rats with a history of cocaine or heroin self-administration (Lu et al., 2003; Shalev, 2012). Previously, our laboratory has demonstrated a robust increase in heroin seeking in chronically food-restricted rats following a 14-day withdrawal period (D'Cunha et al., 2013; Sedki et al., 2013; D'Cunha et al., 2017; Chisholm et al., 2020).

The paraventricular nucleus of the thalamus (PVT) has become increasingly recognized as a critical locus in the neural circuitry underlying drug addiction. The PVT is a midline thalamic nucleus that is uniquely placed to integrate subcortical and cortical information regarding motivational, emotional and homeostatic state to guide behaviour. Recently, we reported that the PVT is critically involved in the augmentation of heroin

seeking induced by chronic food restriction (Chisholm et al., 2020). Specifically, chemogenetically activating the PVT attenuates heroin seeking in chronically food-restricted rats. However, understanding the precise function and circuitry of the PVT involved in this effect remains a major challenge.

The PVT may guide reward-seeking behaviour via excitatory outputs to downstream targets. One downstream target of the PVT is the nucleus accumbens (NAc), which functions as a "limbic-motor interface" integrating available cues to guide behaviour via downstream targets (Mogenson, Jones, & Yim, 1980). The NAc is composed of two different subregions: the NAc shell and the NAc core, both of which are innervated by the PVT. Perhaps not surprisingly, the NAc is a critical area implicated in the neural circuitry underlying drug-prime, cue-induced, context-induced, and stress-induced opioid seeking. However, the roles of the specific subregions of the NAc appear to differ depending on the trigger used to incite opioid-seeking and is likely explained by differences in anatomical connectivity to downstream targets (Reiner et al., 2019). Recently, our laboratory published results indicating that chronic food restriction enhances dopaminergic signalling within the NAc core and NAc shell (D'Cunha et al., 2017). Interestingly, infusion of a D1 antagonist, SCH 23390, into the NAc core selectively attenuated heroin-seeking in chronically food-restricted rats while infusion of a D1-antagonist into the NAc shell attenuated heroin-seeking in both sated and chronically food-restricted rats. These findings support a role for the NAc in the regulation of heroin-seeking induced by chronic food-restriction.

The PVT-NAc pathway is a crucial circuit that mediates opiate dependence. Activation of the PVT-NAc pathway is required for the expression of opioid withdrawal

symptoms, and its inhibition attenuates these symptoms (Zhu et al., 2016). Moreover, activating this pathway induces behavioural aversion (Zhu et al., 2016; Do-Monte et al., 2017). Other evidence indicates that PVT-NAc neurons develop inhibitory responses to reward-predictive cues following repeated presentations and that these responses correlate with behavioural output (Otis et al., 2019). Consistent with these findings, reduced excitatory input from the PVT to the NAc shell promotes feeding (Reed et al., 2018) while activation of anterior PVT-NAc projections attenuates cued sucrose seeking but only under conditions where the reward is omitted (Do-Monte et al., 2017). These findings suggest that PVT-NAc neurons develop an inhibitory response to cues associated with rewards that may promote reward-seeking and consumption while activating this pathway may reduce reward-seeking and consumption.

Therefore, the objective of this study was to investigate the role of PVT-NAc projections in the augmentation of heroin seeking in chronically food-restricted rats following a period of withdrawal. Based on previous results, we hypothesized that activation of PVT-NAc projections would reduce heroin seeking. To test these hypotheses, we used a chemogenetic approach to excite projections from the PVT to the NAc core and NAc shell.

Materials and Methods

Subjects

Ninety-six male Long Evans rats (Raleigh, North Carolina, U.S.A.), weighing 250-275 g at the beginning of the experiments were used in three different experiments.

Intravenous and Intracranial Surgery

Immediately following intravenous catheterization, across all experiments, rats were infused with a viral vector in the PVT and were implanted with a guide cannulae aimed at the NAc shell or core. General procedures are described in Chapter 2. The general viral vector approach and timeline for each experiment are presented in Figure 5.1.

Experiment 1A: Chemogenetic activation of PVT-NAc shell projections

Rats were infused with 0.75 µl of viral vector (AAV8-hSyn-hM3D(Gq)-mCherry, Canadian Neurophotonic Platform/viral vector team, Québec, QC) into the PVT (-3.0 AP, 0.0 ML, -5.4 DV relative to Bregma) at a rate of 0.1 µl /minute. Next, rats were implanted with guide cannulae aimed at the NAc shell (+1.70 AP, ± 3.70 ML, -4.60 DV, 20-degree angle, relative to Bregma) (Figure 5.1A).

Experiment 1B: Specificity of CNO effect in rats expressing DREADDs in PVT- NAc shell projections

Rats were infused with 0.75 µl of viral vector (AAV8-hSyn-mCherry, Canadian Neurophotonic Platform/viral vector team, Québec, QC) into the PVT (-3.0 AP, 0.0 ML, -5.4 DV relative to Bregma) at a rate of 0.1 µl /minute. Next, rats were implanted with guide cannulae aimed at the NAc shell (+1.70 AP, ± 3.70 ML, -4.60 DV, 20-degree angle, relative to Bregma) (Figure 5.1B).

Experiment 2: Chemogenetic activation of PVT-NAc core projections

Rats were infused with 0.75 µl of viral vector (AAV8-hSyn-hM3D(Gq)-mCherry, Canadian Neurophotonic Platform/viral vector team, Québec, QC) into the PVT (-3.0 AP, 0.0 ML, -5.4 DV relative to Bregma) at a rate of 0.1 µl /minute. Next, rats were implanted

with guide cannulae aimed at the NAc core (AP: +2.52, ML: ± 2.50 DV: -4.50, 6-degree angle, relative to Bregma) to allow for intra-NAc micro infusions (Figure 5.1C).

Heroin-Seeking Test

On the 14th day of withdrawal, rats were returned to the operant conditioning chambers for one 3-hour heroin-seeking test session. Rats were intracranially injected with either 0.30 μl of CNO (1mM) or 0.30 μl vehicle (1% DMSO in 99% sterile saline) into the NAc shell (Exp. 1A) or NAc core (Exp. 2), 10 minutes prior to the beginning of the heroin-seeking test. Rats expressing only the mCherry fluorescent protein were intracranially injected with 0.30 μl of CNO (1mM) into the NAc shell (Exp. 1B), 10 minutes prior to the beginning of the heroin-seeking test. During testing, all conditions were identical to self-administration training except that rats were tested under extinction conditions. At the time of test, all rats had a minimum of 5 weeks of viral incubation.

Locomotor Activity Test

To assess possible non-specific motor effects of chemogenetic manipulations, on the 16th day of withdrawal, a locomotor activity test was conducted. Procedures are identical to those described above. Locomotor activity data were collected using Tru Scan 2.0 activity monitoring system (Coulbourn Instruments). Total distance covered (cm) was recorded for 1 hour.

Validation of DREADD Functionality

Due to technical issues with brain collection, a separate group of 12 rats were used to determine the functionality of PVT-NAc shell chemogenetic activation (Exp. 1A). The same animals from Experiment 2 were used to assess the functionality of PVT-NAc core chemogenetic activation.

Fos Immunohistochemistry and Immunoreactivity Quantification

Specific details for Fos immunohistochemistry and immunoreactivity quantification procedures are described in Chapter 2.

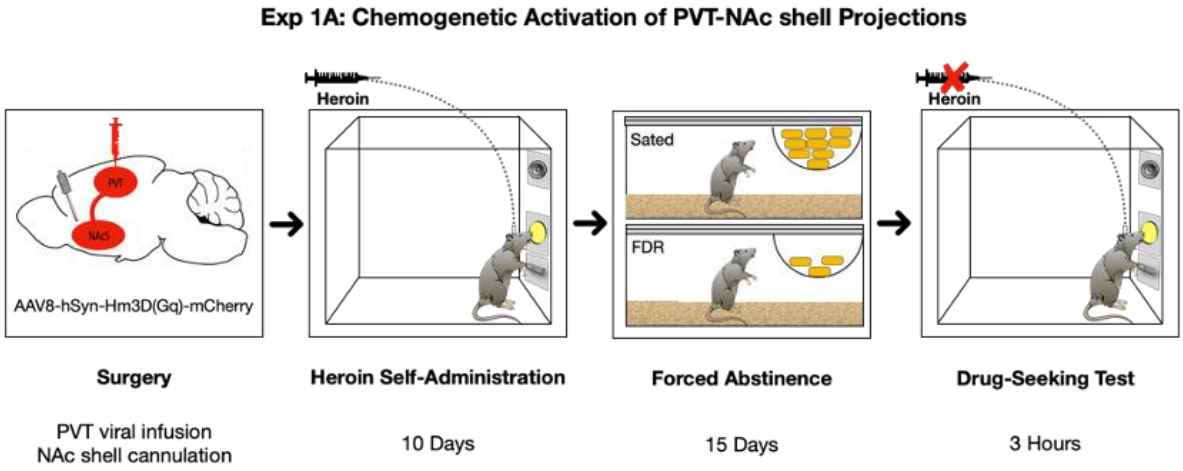
Statistical Analyses

The critical threshold for statistically significant results was set at $p < .05$. Body weights were compared using t-tests, corrected for unequal variance when necessary. In Experiments 1A and 2, the total number of active and inactive lever responses made during the heroin-seeking test was analyzed using two separate two-way ANOVAs with *drug treatment* (vehicle; CNO) and *feeding condition* (FDR, sated) as between-subjects factors and planned comparisons. The number of active lever responses made over the course of the heroin-seeking test was analyzed using a three-way ANOVAs with *drug treatment* (vehicle; CNO) and *feeding condition* (FDR, sated) as between subjects factors and *time* (3 x 1-hour segments) as the within subjects factor. Significant interactions were analyzed using three separate two-way ANOVAs with *drug treatment* (vehicle; CNO) and *feeding condition* (FDR, sated) as factors at hour 1, hour 2 and hour 3 of the heroin seeking test. The number of Fos immunoreactive cells in CNO and vehicle-treated subjects were compared using t-tests, corrected for unequal variance when necessary.

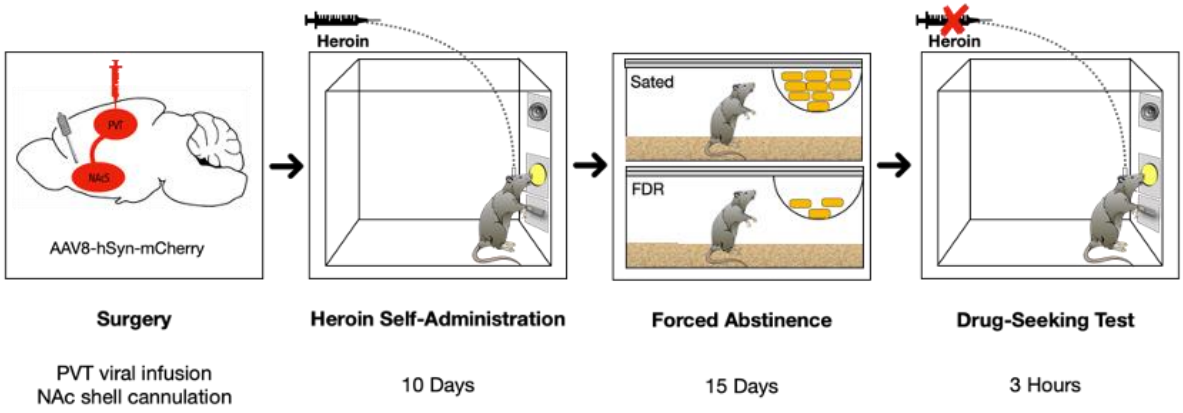
In Experiment 1B, the number of active and inactive lever responses made during the heroin-seeking test was analyzed using two separate t-tests with *feeding condition* (FDR, sated) as the between-subjects' factor. The number of active lever responses made throughout the heroin-seeking test was analyzed using a two-way ANOVA with *feeding condition* (FDR, sated) as the between-subjects factor and *time* (1 hour, 2 hour and 3

hour) as the within-subjects factor. Post hoc analyses were applied where necessary, using the Bonferroni correction.

(A)


Exp 1A: Chemogenetic Activation of PVT-NAc shell Projections
PVT
NAcS
AAV8-hSyn-Hm3D(Gq)-mCherry
Heroin
Sated
FDR
Heroin
Surgery
Heroin Self-Administration
Forced Abstinence
Drug-Seeking Test
PVT viral infusion
NAc shell cannulation
10 Days
15 Days
3 Hours

(B)


Exp 1B: CNO Effect in Rats Expressing DREADDs in PVT-NAc shell Projections
PVT
NAcS
AAV8-hSyn-mCherry
Heroin
Sated
FDR
Heroin
Surgery
Heroin Self-Administration
Forced Abstinence
Drug-Seeking Test
PVT viral infusion
NAc shell cannulation
10 Days
15 Days
3 Hours

(C)

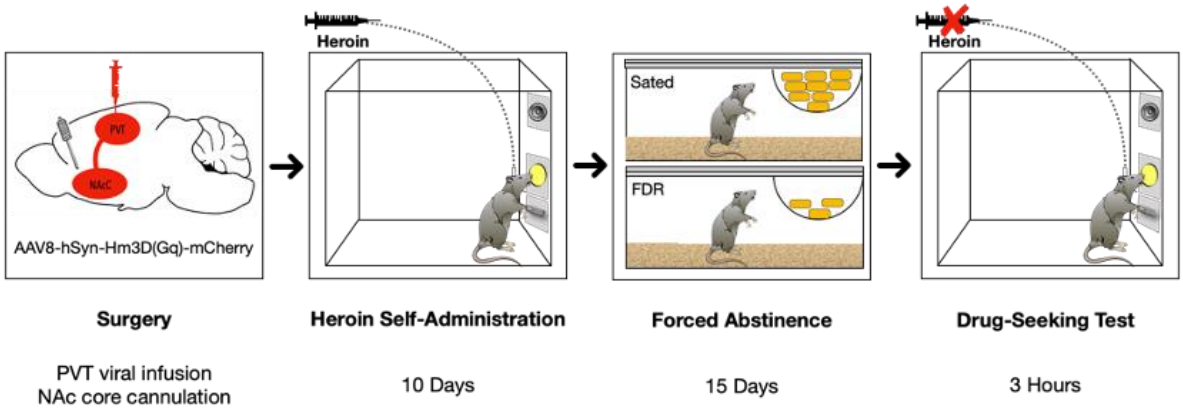

Exp 2: Chemogenetic Activation of PVT-NAc core Projections
PVT
NAcC
AAV8-hSyn-Hm3D(Gq)-mCherry
Heroin
Sated
FDR
Heroin
Surgery
Heroin Self-Administration
Forced Abstinence
Drug-Seeking Test
PVT viral infusion
NAc core cannulation
10 Days
15 Days
3 Hours

Figure 5. 1. Diagram of viral vector approach and general experimental timelines. (A) Diagram of viral vector approach and the experimental timeline for Experiment 1A. (B) Diagram of viral vector approach and the experimental timeline for Experiment 1B. (C) Diagram of viral vector approach and the experimental timeline for Experiment 2.

Results

Mean ± SEM number of active lever responses, inactive lever responses, and infusions made on the last day of heroin self-administration training for each treatment group are presented in Tables 5.1, 5.2 and 5.3. There were no statistically significant differences in these measures between the different experimental groups during the last five days of self-administration training for any experiment.

Table 5. 1. Mean ± SEM number of active lever responses, inactive lever response and heroin infusions made on the last day of training (9-hours), and body weight on test day in Experiment 1A. * $p < 0.0001$, sated compared to food-restricted (FDR) rats.

	Infusions	Active lever	Inactive lever	Body weight
Sated- vehicle	44.78 ± 4.76	133.44 ± 27.15	27.67 ± 8.77	446.56 ± 13.34
Sated- CNO	49.63 ± 9.08	144.50 ± 47.64	16.00 ± 5.45 *	446.13 ± 17.03
FDR-vehicle	53.50 ± 6.49	162.75 ± 36.49	13.75 ± 2.50	344.25 ± 8.54
FDR- CNO	48.00 ± 11.41	150.43 ± 56.80	11.43 ± 4.47	340.71 ± 9.60

Table 5. 2. Mean ± SEM number of active lever responses, inactive lever response and heroin infusions made on the last day of training (9-hours), and body weight on test day in Experiment 1B. * $p < 0.0001$, sated compared to food-restricted (FDR) rats.

	Infusions	Active lever	Inactive lever	Body weight
Sated- CNO	56.43 ± 13.24	181.43 ± 63.57	24.71 ± 10.64	442.86 ± 10.27 *
FDR- CNO	53.14 ± 10.38	146.43 ± 49.65	10.57 ± 2.40	314.43 ± 11.57

Table 5. 3. Mean ± SEM number of active lever responses, inactive lever response and heroin infusions made on the last day of training (9-hours), and body weight on test day in Experiment 2. * $p < 0.0001$, sated compared to food-restricted (FDR) rats.

	Infusions	Active lever	Inactive lever	Body weight
Sated- vehicle	45.63 ± 8.63	133.63 ± 39.60	19.63 ± 4.70	445.88 ± 25.52 *
Sated- CNO	39.20 ± 9.15	126.90 ± 43.74	14.30 ± 5.44	432.90 ± 22.74
FDR-vehicle	42.30 ± 11.60	117.70 ± 47.04	24.60 ± 7.49	330.70 ± 15.77
FDR- CNO	46.55 ± 5.67	107.91 ± 21.21	9.73 ± 3.90	385.55 ± 19.51

Experiment 1A: Chemogenetic activation of PVT-NAc shell projections blocked food restriction-induced augmentation of heroin seeking

Eighteen rats were removed due to catheter leakage, failure to train, health issues, incorrect viral vector placement, or incorrect injector placement. One rat (FDR-CNO) was considered an outlier due to an extreme number of active lever presses performed during the test (> 2.5 SD above group average). Therefore, the final analysis included 32 rats in the four experimental conditions: FDR-vehicle (n = 8), FDR-CNO (n = 7), sated-vehicle (n = 9) and sated-CNO (n = 8). On test day, FDR rats (n = 15; 342.60 ± 6.17 g) weighed statistically significantly less than sated rats (n = 17; 446.35 ± 10.37 g, $t_{(30)}$ = 8.31, $p < 0.0001$, $d = 3.04$).

Heroin-Seeking Test

Chemogenetic excitation of the PVT-NAc shell pathway in the FDR, but not the sated, group resulted in a dramatic attenuation of active lever responses compared to vehicle treated rats (Figure 5.2A). The two-way ANOVA revealed a statistically significant main effect of *CNO treatment* ($F_{(1, 28)} = 5.71$, $p = 0.024$, $\eta^2 = 0.14$). No statistically significant effects of *feeding condition* ($F_{(1, 28)} = 3.48$, $p = 0.073$, $\eta^2 = 0.09$) or *CNO treatment x feeding condition* ($F_{(1, 28)} = 2.92$, $p = 0.098$, $\eta^2 = 0.07$) interaction were found. Planned comparisons revealed a statistically significant difference between FDR-vehicle and sated- vehicle rats ($p = 0.014$; $d = 1.06$), and this effect was reversed by CNO treatment (FDR-vehicle vs. FDR-CNO: $p = 0.009$, $d = 0.99$). No statistically significant main effects or interaction were found for inactive lever responding during the heroin-seeking test.

Analysis of active lever responding over the test session (1-hour bins) revealed a statistically significant main effect of *time*, ($F_{(1.165, 32.61)} = 36.37$, $p < 0.0001$, $\eta^2 = 0.34$) and *CNO treatment* ($F_{(1, 28)} = 5.71$, $p = 0.024$, $\eta^2 = 0.16$; Figure 5.2B). However, no statistically significant effects for *feeding condition* ($F_{(1, 28)} = 3.48$, $p = 0.073$, $\eta^2 = 0.10$), *time* x *feeding condition* ($F_{(2, 56)} = 2.65$, $p = 0.08$, $\eta^2 = 0.02$), *time* x *CNO treatment* interaction ($F_{(2,56)} = 2.41$, $p = 0.099$, $\eta^2 = 0.02$), *feeding condition* x *CNO treatment* interaction ($F_{(1,28} = 2.92$, $p = 0.098$, $\eta^2 = 0.08$), or *time x feeding condition x CNO treatment* interaction ($F_{(2, 56)} = 2.17$, $p = 0.124$, $\eta^2 = 0.02$) were found. Visual inspection of active lever responses made over the test session indicates that the response rate of the FDR-CNO group was consistently low from the outset of the heroin seeking test session when compared with the FDR-vehicle group.

Locomotor Activity Test

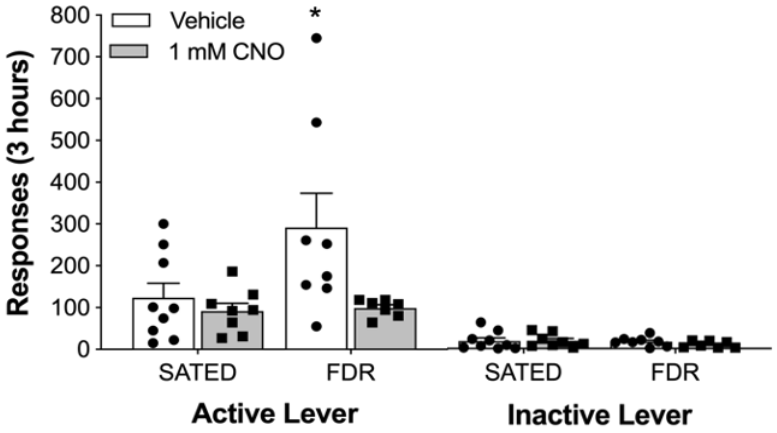
Data from the locomotor activity test were lost for one animal in the sated-CNO group. Mean ± SEM distance traveled (cm) were: sated-vehicle 6038.10 ± 769.51; sated-CNO 5387.84 ± 603.44; FDR-vehicle 8829.25 ± 538.85; FDR-CNO 9954.77 ± 1090.33. Food restriction statistically significantly increased total distance traveled compared to sated rats (*feeding condition*: $F_{(1,27)} = 22.57$, $p < .0001$, $\eta^2 = 0.44$). No statistically significant effect of *CNO treatment* or *feeding condition* x *CNO treatment* interaction was observed during the 1-hour locomotor activity test.

Validation of DREADD Functionality

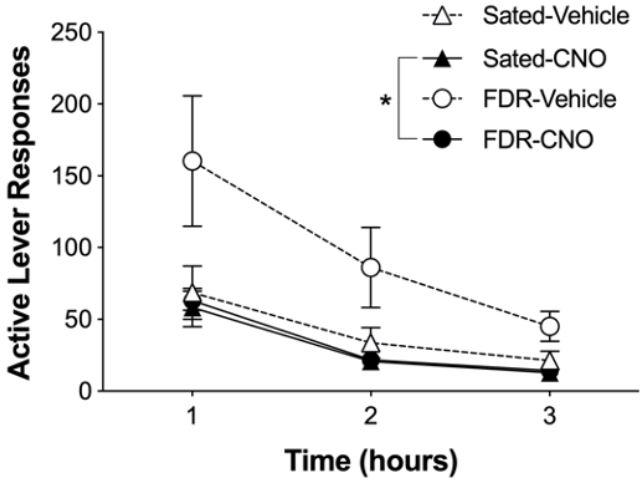
CNO treatment (n = 5) statistically significantly increased the number of Fos-IR cells in the NAc shell compared to vehicle controls (n = 6; $t_{(9)} = 2.09$, $p = 0.033$, $d = 1.39$; Figure 5.2C), indicating that DREADD-mediated excitation of the PVT-NAc shell

pathway successfully increased neuronal activity in the NAc shell. The extent of DREADD expression in the PVT is presented in Figure 5.5A. The extent of DREADD expression in the terminal region of the PVT-NAc shell projection neurons is presented in Figure 5.5B.

(A)


Vehicle
1 mM CNO
Responses (3 hours)
800
700
600
500
400
300
200
100
0
*
SATED
FDR
SATED
FDR
Active Lever
Inactive Lever

(B)


Sated-Vehicle
Sated-CNO
FDR-Vehicle
FDR-CNO
*
Active Lever Responses
250
200
150
100
50
0
1
2
3
Time (hours)

(C)

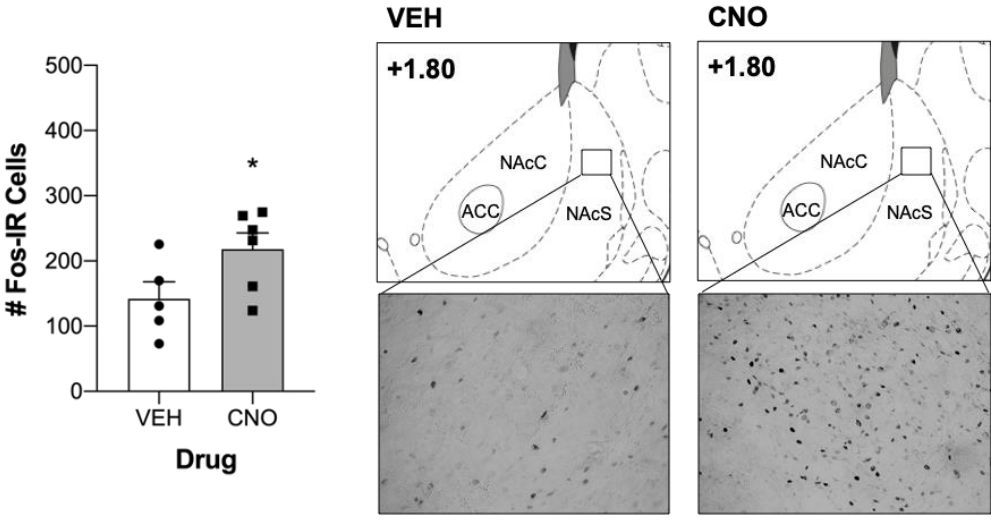

Fos-IR Cells
500
400
300
200
100
0
*
VEH
CNO
Drug
VEH
CNO
+1.80
+1.80
NAcC
ACC
NAcS
NAcC
ACC
NAcS

Figure 5. 2. Chemogenetic activation of PVT-NAc shell projections blocked food restriction-induced augmentation of heroin seeking. Rats were intracranially administered CNO (1 mM) or vehicle into the NAc shell 5-10 minutes before the test. (A) Mean ± SEM number of active (left) and inactive (right) lever responses made during the 3-hour heroin-seeking test on day 14 of food restriction in the sated and food-restricted (FDR) groups. * $p < 0.05$, compared with sated vehicle and FDR CNO groups. (B) Mean ± SEM number of active lever responses made over the 3-hour test, presented in 1-hour time intervals. * $p < 0.05$, compared with vehicle-treated groups (C) Chemogenetic activation of PVT-NAc shell projections increased the number of Fos-IR cells in the NAc shell of CNO-treated subjects compared to vehicle. Rats were intracranially administered CNO (1 mM) or vehicle into the NAc shell 90 minutes before the perfusion. Data are the Mean ± SEM number of Fos-IR cells in the NAc shell of vehicle- and CNO-treated rats who had their PVT-NAc shell projections activated (left). * $p < 0.05$, vehicle compared to CNO treatment. Example Fos-IR in the NAc shell of vehicle- and CNO-treated subjects are also presented (right). All images were taken at 20X magnification.

Experiment 1B: CNO infusion did not alter food-restriction induced heroin-seeking in rats expressing only mCherry in PVT-NAc shell projections

Six rats were removed due to catheter leakage, failure to train, excessive drug intake, health issues, incorrect viral vector placement, or incorrect injector placement. Therefore, the final analysis included 14 rats in the two experimental conditions: FDR-CNO (n = 7), and sated-CNO (n = 7). On test day, FDR rats (n = 7; 314.43 ± 11.57 g) weighed statistically significantly less than sated rats (n = 7; 442.86 ± 10.27 g, $t_{(12)} = 8.31$, $p < 0.0001$, $d = 4.80$).

Heroin Seeking Test

On test day, FDR rats made statistically significantly more responses on the active lever than sated rats ($t_{(12)} = 3.01$, $p = 0.011$, $d = 1.73$; Figure 5.3A). No statistically significant differences in inactive lever responding during the test were observed. Notably, heroin seeking in the sated and FDR groups was similar to the levels observed in Experiment 1A. Analysis of active lever responding over the test session (1-hour bins) revealed statistically significant main effects of *time*, ($F_{(1.582,18.99)} = 43.66$, $p < .0001$, $\eta^2 = 0.54$) and *feeding condition*, ($F_{(1,12)} = 9.03$, $p = 0.011$, $\eta^2 = 0.24$), and a *time by feeding condition* interaction ($F_{(2, 24)} = 6.07$, $p = 0.007$, $\eta^2 = 0.08$). Post hoc analyses revealed that FDR rats pressed statistically significantly more on the active lever at hour 1 ($p = 0.03$, $d = 2.46$) when compared to sated rats (Figure 5.3B). These data indicate that the infusion of CNO in the absence of an active DREADD did not alter heroin seeking in chronically food-restricted rats.

Locomotor Activity Test

Data from the locomotor activity test was lost for two rats in the FDR group and one animal in the sated group. Mean ± SEM distance traveled (cm) were: Sated-CNO 6504.60 ± 496.51; FDR-CNO 4587.52 ± 975.37. No statistically significant effects were observed for locomotor activity.

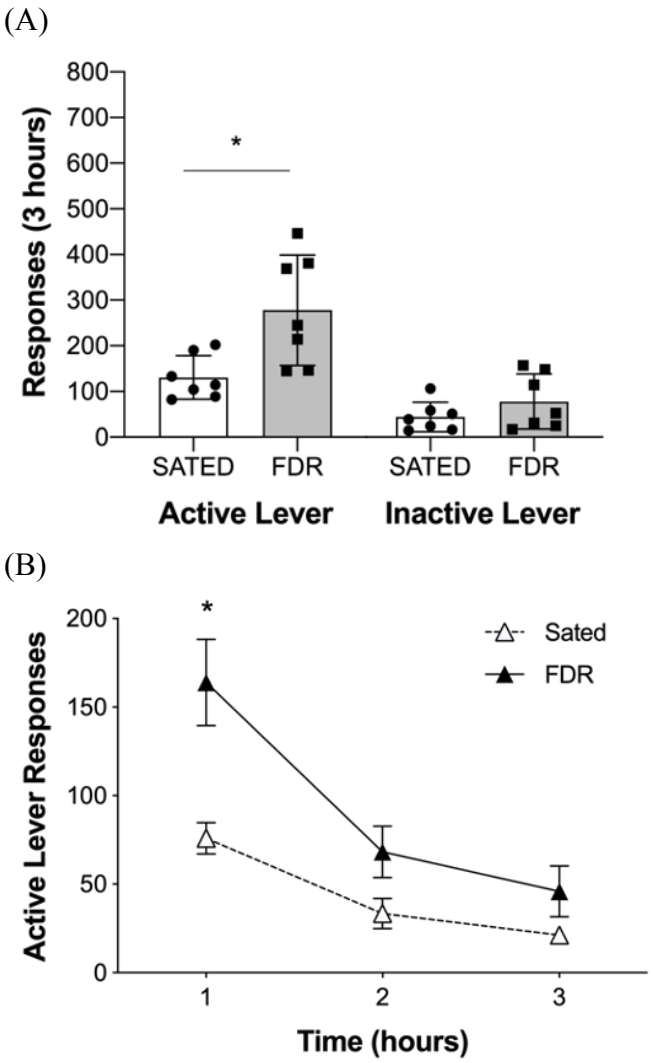


Figure 5. 3. CNO infusion in PVT-NAc shell projections expressing only mCherry did not alter food restriction-induced augmentation of heroin seeking. Rats were intracranially administered CNO (1 mM) into the NAc shell 5-10 minutes before the test. (A) Mean ± SEM number of active (left) and inactive (right) lever responses made during the 3-hour heroin-seeking test on day 14 of food restriction in the sated and food-restricted (FDR) group. * $p < 0.05$, compared with sated group. (B) Mean ± SEM number of active lever responses made over the 3-hour test, presented in 1-hour time intervals. * $p < 0.05$ compared with sated group.

Experiment 2: Chemogenetic activation of PVT-NAc core projections did not alter food-restriction induced heroin seeking

Eleven rats were removed due to catheter leakage, failure to train, health issues, incorrect viral vector placement, or incorrect injector placement. Thus, the final analysis included 39 rats in the four experimental conditions. FDR-vehicle (n = 10), FDR-CNO (n = 11), sated-vehicle (n = 8) and sated-CNO (n = 10). On test day, FDR rats (n = 21; 345.29 ± 12.76 g) weighed statistically significantly less than sated rats (n = 18; 438.67 ± 16.54 g, $t_{(37)} = 4.54$, $p < 0.001$, $d = 1.49$).

Heroin Seeking Test

Food restricted rats pressed significantly more on the active lever during the heroin-seeking test, compared to the sated group (*feeding condition*: $F_{(1,35)} = 23.03$, $p < 0.0001$, $\eta^2 = 0.39$; Figure 5.4A). No statistically significant effects of *CNO treatment* ($F_{(1, 35)} = 0.46$, $p = 0.502$, $\eta^2 = 0.01$) or *CNO treatment x feeding condition* interaction ($F_{(1, 35)} = 0.024$, $p = 0.877$, $\eta^2 = 0.00$) were found. Post hoc analyses revealed that FDR-vehicle and FDR-CNO treated subjects displayed elevated levels of responding on the active lever compared to Sated-vehicle and Sated-CNO subjects. These results indicate that chemogenetic excitation of the PVT-NAc core pathway did not attenuate heroin seeking in chronically food-restricted rats. A significant interaction (*feeding condition x CNO treatment*: $F_{(1,35)} = 6.18$, $p = 0.018$, $\eta^2 = 0.15$) was observed for inactive lever responding. However, post hoc tests revealed no statistically significant differences between the groups.

Analysis of active lever responding over the test session (1-hour bins) revealed statistically significant main effects of *time*, ($F_{(1.183,41.40)} = 64.68$, $p < 0.0001$, $\eta^2 = 0.40$)

and *feeding condition* ($F_{(1, 35)} = 24.11$, $p < 0.0001$, $\eta^2 = 0.28$), and a *time by feeding condition* interaction ($F_{(2, 70)} = 13.38$, $p < 0.0001$, $\eta^2 = 0.08$; Figure 5.4B). Post hoc analyses revealed that FDR rats pressed statistically significantly more on the active lever at hour 1 ($F_{(1,35)} = 22.27$, $p < 0.0001$, $\eta^2 = 0.39$), hour 2 ($F_{(1,35)} = 14.68$, $p = 0.001$, $\eta^2 = 0.28$) and hour 3 ($F_{(1,35)} = 20.43$, $p < 0.0001$, $\eta^2 = 0.36$) when compared to sated rats indicating that FDR rats were slower to extinguish their responding during the heroin seeking test.

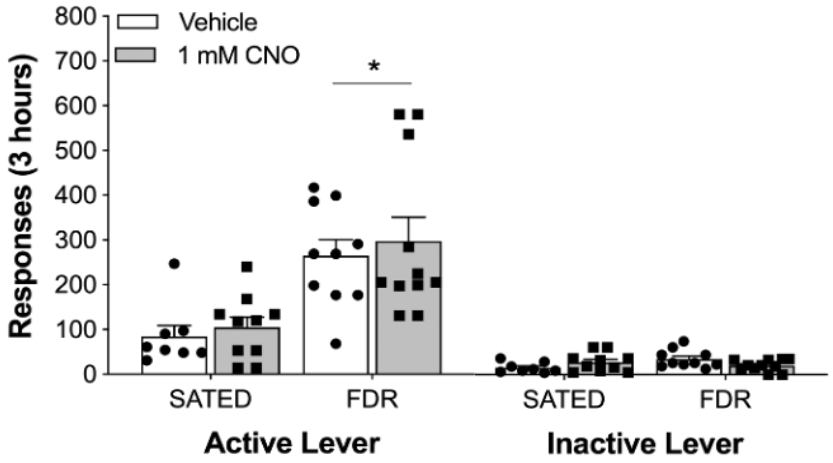
Locomotor Activity Test

Mean ± SEM distance traveled (cm) were: Sated-vehicle 6270.73 ± 583.83; Sated-CNO 4794.58 ± 359.83; FDR-vehicle 7204.42 ± 703.78; FDR-CNO 7163.25 ± 955.27. Food restriction statistically significantly increased distance traveled (*feeding condition*: $F_{(1,35)} = 5.22$, $p = 0.029$, $\eta^2 = 0.12$). No statistically significant effect of *CNO treatment* or *feeding condition* x *CNO treatment* interaction was observed during the 1-hour locomotor activity test.

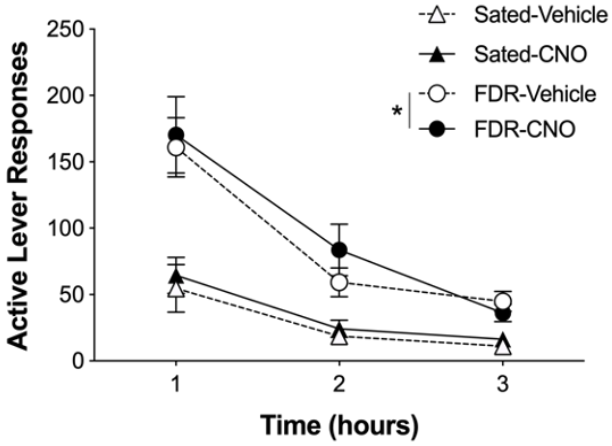
Validation of DREADD Functionality

CNO treatment (n = 6) statistically significantly increased the number of Fos-IR cells in the NAc core when compared to vehicle controls (n = 6; $t_{(10)} = 1.92$, $p = 0.042$, $d = 1.22$; Figure 5.4C). Representative DREADD expression in the terminal region of the PVT-NAc core projection neurons is presented in Figure 5.5C.

(A)


Vehicle
1 mM CNO
Responses (3 hours)
800
700
600
500
400
300
200
100
0
*
SATED
FDR
SATED
FDR
Active Lever
Inactive Lever

(B)


Sated-Vehicle
Sated-CNO
FDR-Vehicle
FDR-CNO
*
Active Lever Responses
250
200
150
100
50
0
1
2
3
Time (hours)

(C)

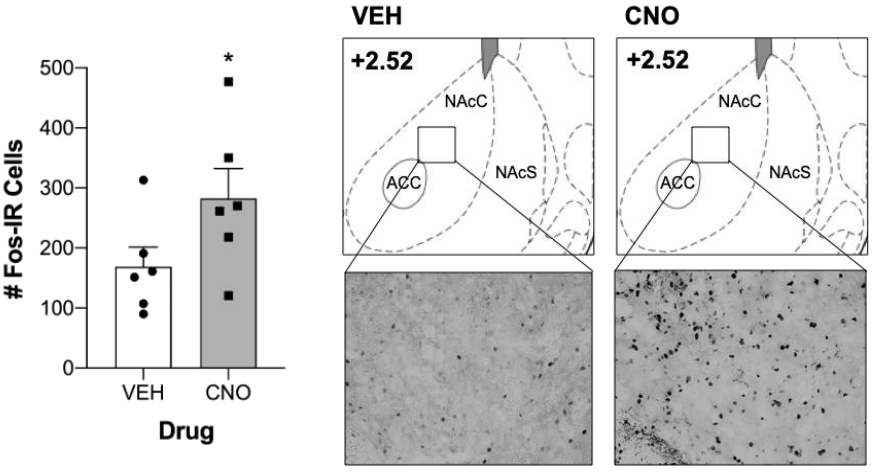
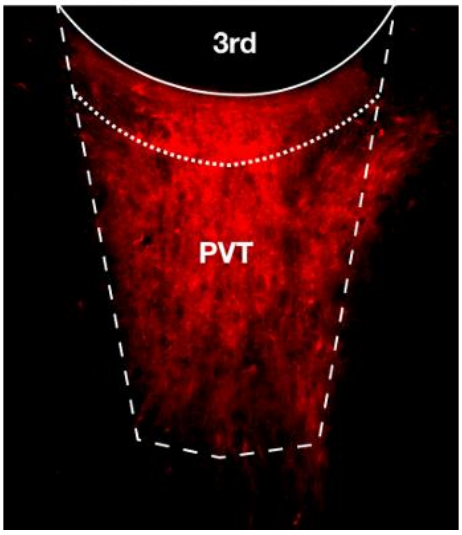
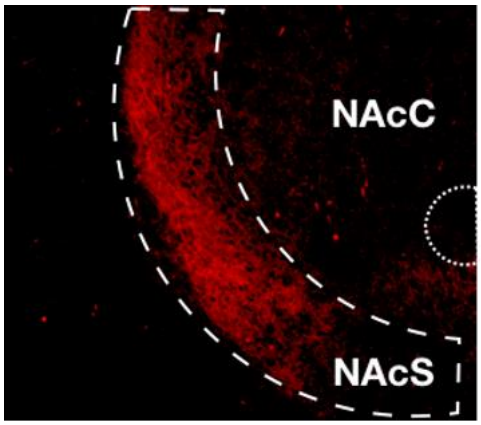

Fos-IR Cells
500
400
300
200
100
0
*
VEH
CNO
Drug
VEH
CNO
+2.52
+2.52
NAcC
NAcS
ACC
NAcC
NAcS
ACC

Figure 5. 4. Chemogenetic activation of PVT-NAc core projections did not alter food restriction-induced augmentation of heroin seeking. Rats were intracranially administered CNO (1 mM) or vehicle into the NAc core 5-10 minutes before the test. (A) Mean ± SEM number of active (left) and inactive (right) lever responses made during the 3-hour heroin-seeking test on day 14 of food restriction in the sated and food-restricted (FDR) groups. * $p < 0.0001$, compared with sated groups. (B) Mean ± SEM number of active lever responses made over the 3-hour test, presented in 1-hour time intervals. * $p < 0.001$, compared with sated groups. (C) Chemogenetic activation of PVT-NAc core projections increased the number of Fos-IR cells in the NAc core of CNO-treated subjects compared to vehicle. Rats were intracranially administered CNO (1 mM) or vehicle into the NAc core 90 minutes before the perfusion. Mean ± SEM number of Fos-IR cells in vehicle- and in CNO-treated rats who had their PVT-NAc core projections activated (left). * $p < 0.05$, vehicle compared to CNO treatment. Example Fos-IR in the NAc core of vehicle- and CNO-treated subjects are also presented (right). All images were taken at 20X magnification.

(A)



(B)



(C)

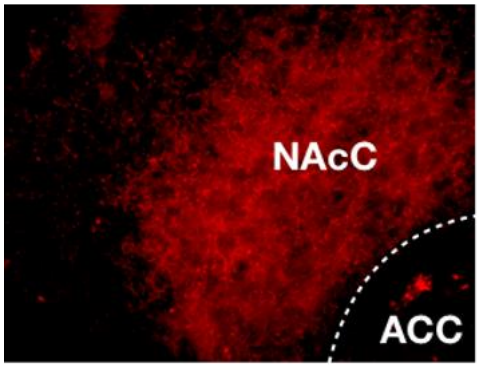


Figure 5. 5. Representative sections of mCherry tagged PVT cell bodies and NAc terminals. (A) Representative section of mCherry tagged immunofluorescence in PVT cell bodies taken at 10X magnification from Experiment 1A. (B) Representative section of mCherry tagged immunofluorescence in PVT- NAc shell projections in the terminal region taken at 10x magnification from Experiment 1A. (C) Representative section of mCherry tagged immunofluorescence in PVT- NAc core projections in the terminal region taken at 20X magnification from Experiment 2.

Discussion

In this series of experiments, we examined the role of PVT-NAc projections in the augmentation of heroin seeking induced by chronic food restriction following a period of withdrawal. Across both experiments, chronically food-restricted rats displayed augmented heroin seeking as indicated by the significantly higher number of active lever responses following a period of withdrawal compared to their sated counterparts. In line with our hypothesis, chemogenetically activating PVT-NAc shell neurons abolished the augmented heroin seeking normally observed in our chronically food-restricted rats. However, chemogenetically activating PVT-NAc core neurons had no effect on heroin seeking, suggesting that the PVT and its projection to the NAc shell plays a key role in mediating this effect.

The attenuation of food restriction-induced augmentation of heroin seeking observed following activation of the PVT-NAc shell pathway agrees with our previous report that chemogenetic activation of the PVT blocked heroin seeking in food-restricted rats (Chisholm et al., 2020). Our findings align with the idea presented by Kuhn et al. (2018) that the PVT output inhibits cue-induced drug (and probably non-drug rewards)

seeking, through attenuation of the incentive value of the cues. Accordingly, photoactivation of PVT-NAc shell neurons attenuated cued reward-seeking while photoinhibition enhanced it (Do-Monte et al., 2017). An inhibitory role for the PVT-NAc pathway in regulating drug-seeking is supported by the report that inhibition of this pathway enhanced cue-induced reinstatement of cocaine-seeking (Wunsch et al., 2017). Notably, in the latter two studies, the anterior PVT seems to be the critical area while we targeted a more posterior area of the PVT.

It is harder to reconcile our findings with the reports that inhibition of the PVT attenuates cue-induced drug-seeking. Transient pharmacological inhibition or lesion of the PVT attenuated discriminative cue-induced reinstatement of cocaine and alcohol-seeking (Matzeu et al., 2015) and context-induced reinstatement of alcohol-seeking (Hamlin et al., 2009; Marchant et al., 2010). Obvious differences from the current study include a different drug-seeking procedure, i.e., forced abstinence versus reinstatement of extinguished behaviour, and exposure to discrete versus discriminative or contextual cues. Additionally, rats in the current study were trained with heroin compared to cocaine or alcohol in Hamlin et al. (2009) and Marchant et al. (2010) reports, respectively. The neural circuits underlying different types of cue-induced drug-seeking procedures (e.g., Fuchs et al., 2006) and different drug categories (Badiani et al., 2011) are distinct to a large extent. Finally, inhibition of drug-seeking was demonstrated by non-selective inhibition or lesion of the whole PVT (Hamlin et al., 2009; Marchant et al., 2010; Matzeu et al., 2015). We were unable to identify any reports that indicate an attenuation of drug (or other rewards) seeking following targeted inhibition of the PVT-NAc pathway.

It is unclear how activation of NAc shell afferents from the PVT attenuates the augmentation of heroin seeking in food-restricted rats. It has been suggested that the PVT-NAc pathway carries negative valance, arguably through interaction with the DA D2 receptors-expressing medium spiny neurons (MSNs) in the NAc (Zhu et al., 2016). Interestingly, Zhu et al. (2016) demonstrated a morphine-dependence-induced potentiation of the transmission between the PVT and the D2 receptor-expressing neuron in the NAc. Thus, DREADD-induced activation of the PVT-NAc shell pathway could result in activation of the D2 receptor-expressing 'indirect pathway' neurons and a punishment effect (Kravitz et al., 2012). However, this mechanism cannot explain the specific effect on heroin seeking in food-restricted rats. Earlier reports suggested that excitatory inputs from the PVT interact with acetylcholinergic (ACh) interneurons in the NAc (Meredith & Wouterlood, 1990), which have a complex and potent impact on nucleus accumbens MSNs due to their extensive arborization (Contant, Umbriaco, Garcia, Watkins, & Descarries, 1996). Although a more recent investigation concluded that NAc afferents from the PVT do not synapse directly on ACh cells (Ligorio, Descarries, & Warren, 2009), there is strong evidence that PVT output can modulate (perhaps indirectly) ACh release in the NAc (Kelley et al., 2005). ACh transmission in the NAc has been implicated in food and drug-seeking, with increases in extracellular levels of acetylcholine associated with food satiety (Avena & Rada, 2012). Thus, activation of NAc shell ACh interneurons could counteract the food restriction-induced augmentation of heroin seeking.

Methodological Considerations

PVT neurons that project to the NAc are highly collateralized (Dong et al., 2017). Thus, it was essential to utilize a method whereby activation of PVT-NAc neurons did not activate collateral axons that project to other regions. To get around this problem, we utilized DREADD technology in a projection specific manner. Here, a viral vector carrying the excitatory DREADD is infused into a specific region (i.e., PVT), and the DREADD protein is carried to and expressed in the terminal region of the target region's projection neurons; in this case, the NAc shell and NAc core. We implanted guide cannulae aimed at the terminal target region (i.e., NAc shell or NAc core). Before the heroin-seeking test, CNO was intracranially administered into the terminal target region, ensuring that only DREADD-expressing terminals of PVT-NAc shell or PVT-NAc core projecting neurons were activated.

The lack of effect for PVT-NAc core projecting neurons could be explained by insufficient viral infection, inadequate DREADD expression or a lack of DREADD-induced efficacy. To address these concerns, we verified DREADD expression, cannulae placements and DREADD functionality. First, only animals that had strong levels of DREADD expression verified by robust m-Cherry expression were included in these experiments suggesting that the lack of effect for PVT-NAc core projections is not due to insufficient viral infection or DREADD expression. Second, only animals with correct placements were included in these experiments. Third, we verified that chemogenetic activation of PVT-NAc projecting neurons enhances neural activity in both PVT-NAc core and shell projecting neurons using c-fos immunoreactivity. Last, we confirmed that in subjects infused with a control DREADD, infusion of CNO alone does not alter heroin seeking behaviour in chronically food-restricted rats.

Conclusion

Our findings extend current knowledge about the role of the PVT and its circuitry involved in drug-seeking. This is the first study to examine the role of PVT-NAc projections in heroin seeking using an abstinence procedure. Our results indicate that activation of PVT-NAc shell but not PVT-NAc core strongly attenuates the augmentation of heroin seeking induced by chronic food restriction. Importantly, this report demonstrates that the PVT and its circuitry play a critical role in heroin seeking under a state of metabolic need. However, the exact molecular mechanisms underlying this effect remains to be determined.

CHAPTER 6: GENERAL DISCUSSION

The overall objective of this dissertation was to investigate the role of the PVT, cortical projections to the PVT, and thalamic projections to the nucleus accumbens in the augmentation of heroin seeking induced by chronic food restriction. We sought to investigate the role of the PVT and its inputs/outputs due to the extensive literature implicating a role for this brain region in drug-seeking and food intake. Initially, we hypothesized that inhibiting the PVT would block the augmentation of heroin seeking in chronically food-restricted rats. However, this did not turn out to be the case, rejecting our initial hypothesis. Our results indicate that activating the PVT robustly blocks the augmentation of heroin seeking in chronically food-restricted rats. Based on these findings, we assessed the role of excitatory input from the PrL to the PVT. We hypothesized that activation of the PrL-PVT pathway would attenuate heroin seeking in chronically food-restricted rats. Again, our results did not support our hypothesis, as neither activation nor inhibition of the PrL-PVT pathway had any effect on heroin seeking. Lastly, we sought to assess the role of excitatory output from the PVT to the NAc. Based on our previous results and the results of others, we hypothesized that activation of PVT-NAc projections would block the augmentation of heroin seeking in chronically food-restricted rats. Our results supported our final hypothesis, as activating PVT- NAc shell but not core projections blocked heroin seeking in chronically food-restricted rats. Our findings suggest that the PVT and its projections to the NAc shell play a vital role in the augmentation of heroin seeking induced by chronic food restriction. The role of the PVT and its projections in drug-seeking and food intake will be further discussed in this chapter.

The Role of the PVT in Drug Seeking and Food Intake: Reconciling the Differences

At the time that we started this project (2015), the conflicting observations that inhibiting the PVT could reduce drug-seeking, yet increase food intake made predictions about the effects of PVT manipulations difficult. Inhibiting the PVT might make our chronically food-restricted rats hungrier, which may increase heroin seeking, or it may block heroin seeking, according to the drug-seeking literature (Stratford & Wirtshafter, 2013; Hamlin et al., 2009). The findings from studies that target the two reward types do not completely overlap. For example, inhibiting the PVT reduces drug-seeking under certain circumstances (Hamlin et al., 2009; Browning et al., 2014; Matzeu et al., 2015). However, activating the PVT reduces food intake (Bhatnagar & Dallman, 1999; Stratford & Wirtshafter, 2013; Zhang & van den Pol, 2017). It should be noted that intake and seeking behaviours are fundamentally different, and this may explain some of the differences in findings on the role of the PVT in each reward type.

An additional factor that made interpreting these findings difficult was that no studies had examined the role of the PVT in opioid seeking. We based our studies on the findings that assessed the role of the PVT in drug-seeking utilizing psychostimulants or alcohol (Browning et al., 2014; Kuhn et al., 2018). Interestingly, we noticed that inhibition of the PVT appeared to considerably enhance heroin seeking in some chronically food-restricted and sated rats (Chapter 3, Figure 2A), however group means were not statistically significantly different. The results of the experiments presented in this dissertation align with findings on the role of the PVT in feeding-related behaviours, not with findings on the role of the PVT in drug-seeking (Zhang & van den Pol, 2017). However, the exact reason for this remains unclear.

One possible reason for these discrepancies may be due to the specificity of our effect. Across the experiments presented in this dissertation, activation of the PVT and its projection to the NAc shell selectively attenuated heroin-seeking in our chronically food-restricted rats. These findings suggest that the effects of chronic food restriction on heroin-seeking might be mediated by the neural circuitry implicated in the regulation of food intake or food-seeking in comparison to the neural circuitry implicated in drug-seeking. Moving forward, it will be paramount to understand how drug-seeking and feeding circuitry interact. This dissertation has started to pave the road for this to happen, and we hope that this work continues to move forward.

The PVT and Individual Differences

Evidence from the Flagel group indicates that the PVT is involved in mediating individual differences in the attribution of incentive salience to reward paired cues (Haight & Flagel, 2014). Transient inactivation of the PVT with baclofen/muscimol enhances cue-induced reinstatement of cocaine-seeking in goal-tracking, but not sign-tracking animals (Kuhn et al., 2018). The authors suggest that in goal-tracking animals, the PVT normally acts to inhibit cocaine seeking by reducing the incentive salience of cocaine cues. However, when the PVT is inhibited, it no longer acts to reduce the incentive value of cocaine cues, thereby resulting in increased cocaine seeking. These findings suggest that the PVT plays a role in encoding individual differences in the incentive value of drug paired cues. We did not assess individual differences in the attribution of incentive salience prior to conducting experiments. Moving forward, it will be essential to assess individual differences in the attribution of incentive value to drug paired cues before testing, as this may help to resolve conflicting data.

The PrL- PVT Pathway

Across its anterior-posterior axis, the PVT is densely innervated by the prefrontal cortex, specifically by the PrL and IL (Li & Kirouac, 2012). In Chapter 4, we wanted to investigate the role of one of the main excitatory inputs to the PVT in heroin seeking in chronically food-restricted rats. When we started designing these experiments, new literature had just come out implicating the PrL-PVT pathway in cue-motivated behaviour (Otis et al., 2017; Campus et al., 2019). Based on the findings from these studies as well as our results from Chapter 3, we hypothesized that activation of PrL-PVT projections would attenuate heroin seeking in chronically food-restricted rats. In Chapter 3, we report that neither chemogenetic activation nor inhibition of PrL-PVT neurons affected heroin seeking in chronically food-restricted rats. Importantly, we validated the utility of excitatory and inhibitory DREADDs using c-fos immunohistochemistry. These findings suggest that PrL-PVT projections do not play a role in mediating the augmentation of heroin seeking induced by chronic food restriction. In the next section, we will speculate on additional PVT inputs that may play a role in the augmentation of heroin-seeking induced by chronic food restriction.

The Wrong Input? The Potential Role for the LHA-PVT Pathway

The finding that chemogenetically activating or inhibiting PrL-PVT neurons did not alter heroin seeking behaviour in our chronically food-restricted rats was very surprising. It would be interesting to assess other inputs to the PVT. One potential input to the PVT that could be explored is the lateral hypothalamus (LHA). The lateral hypothalamus is a key part of the hypothalamic-thalamic-striatal axis proposed by Kelley et al. (2005). Although this axis was proposed to integrate and regulate arousal, energy

balance and feeding, the role of the LHA appears to be much more complex than previously thought (Bonnavion, Mickelsen, Fujita, De Lecea, & Jackson, 2016).

The LHA sends dense orexigenic projections to the PVT (Parsons, Li, & Kirouac, 2006). Orexin signalling has been implicated in the control of energy balance, arousal, stress, and addiction. Importantly, orexin signalling within the PVT has been implicated in the regulation of drug-seeking (Matzeu & Martin-Fardon, 2018). For example, orexin-A infusion into the PVT reinstates cocaine-seeking (Matzeu et al., 2016). Most of the research assessing the role of orexin in drug-seeking is confined to cocaine and alcohol, but new evidence has emerged implicating orexin signalling in the regulation of opioid seeking (Matzeu & Martin-Fardon, 2019). For example, OX1R blockade attenuates cue-induced reinstatement of oxycodone, remifentanil and fentanyl seeking (Matzeu & Martin-Fardon, 2019). However, in our case, we must also consider the impact of chronic food restriction on orexin signalling.

Importantly, orexins regulate behaviour under situations of high motivational relevance (i.e., physiological need) (Mahler, Smith, Moorman, Sartor, & Aston-Jones, 2012; Mahler, Moorman, Smith, James, & Aston-Jones, 2014). Orexin signalling is differentially altered depending on the type of stress encountered; acute stress is associated with an increase in orexin signalling while chronic stressors are associated with reduced orexigenic signalling (Yeoh, Campbell, James, Graham, & Dayas, 2014). At the present time, there is evidence indicating that hypothalamic orexin expression remains unchanged by chronic food-restriction (Cai et al., 1999; de Rijke, Hillebrand, Verhagen, Roeling, & Adan, 2005; Johansson et al., 2008).

In addition to providing orexigenic input, the LHA provides GABAergic innervation to the PVT (Otis et al., 2019). GABAergic LHA neurons projecting to the PVT show increased activity during behavioural output (Otis et al., 2019). These findings suggest that the PVT integrates both cortical and subcortical information to guide reward seeking via excitatory output signals in PVT-NAc neurons. These findings also align with Reed et al. (2018) recent report that reduced excitatory input to the rostral NAc shell from multiple sources, one being the PVT, is associated with increased food consumption.

Based on these findings, one might expect that chronic food restriction may reduce or leave unchanged orexigenic signalling into the PVT while GABAergic transmission during behavioural output is simultaneously increased, resulting in overall inhibition of the PVT and consequently, a reduction in PVT-NAc shell excitatory input. This idea aligns with the findings presented in this dissertation, whereby chemogenetically activating the PVT, or PVT-NAc shell pathway blocks the augmentation of heroin seeking in chronically food-restricted rats.

The same mechanism may explain why we did not observe any changes in heroin seeking in our chronically food-restricted rats when we manipulated the PrL-PVT pathway. If this hypothesis is correct (1) activating the PrL-PVT pathway alone may not be sufficient to override the enhanced GABAergic signal from LHA neurons, (2) inhibiting PrL-PVT neurons does not add any unique information beyond what is already communicated to the PVT by LHA inputs or, (3) inhibiting PrL-PVT neurons enhances the overall inhibitory signal that would be integrated by the PVT but this again, would likely not alter behavioural output.

The Wrong Input? A Potential Role for the ZI-PVT Pathway

An additional pathway that may explain the selectivity of our effect whereby activation of the PVT or PVT-NAc shell neurons attenuates heroin-seeking in our chronically food-restricted rats is the input to the PVT from the Zona Incerta (ZI). PVT glutamatergic neurons receive dense GABAergic input from the ZI (Zhang & van den Pol, 2017). Inhibitory input to the PVT is especially important considering that the PVT does not contain local interneurons to provide control over local circuits (Kirouac, 2015). Interestingly, neurons in the ZI are activated in response to food-deprivation, and food-deprivation lasting 24 hours enhances inhibitory input from ZI neurons onto PVT glutamate neurons (Zhang, Yang, Zhang, Wang, & Wang, 2011). Additionally, ZI neurons are activated by grehlin, an orexigenic hormone, and plasma ghrelin is elevated by chronic food-restriction suggesting that this may be a mechanism by which ZI-PVT neurons are selectively activated in our chronically food-restricted rats driving heroin seeking (Zhang & van den Pol, 2017; Toshinai et al., 2001).

At the present time, it is unclear how ZI or ZI-PVT neurons are altered by chronic food-restriction; however, if we extrapolate from existing data, it would be plausible to suggest that these neurons are likely activated in response to chronic food-restriction, perhaps by ghrelin, enhancing inhibitory input to the PVT. Optogenetically stimulating the ZI-PVT pathway stimulates food-intake and weight gain in mice who are food-restricted, suggesting that GABAergic input to the PVT can promote behavioural activation, perhaps via downstream projections to the NAc shell, an area known to be involved in drug as well as non-drug reward-seeking (Zhang & van den Pol, 2017; Otis et al., 2019). It is likely that this inhibitory input from the ZI to the PVT is communicated via PVT-NAc shell neurons in the form of reduced excitatory input to the NAc. In line

with this idea, reduced excitatory input to the NAc shell from the PVT promotes feeding and reward-seeking (Reed et al., 2018; Lafferty, Yang, Mendoza, & Britt, 2020). Therefore, we hypothesize that inhibiting the ZI-PVT pathway would attenuate heroin-seeking in our chronically food-restricted rats.

The PVT -NAc Pathway

The PVT densely innervates both the core and shell subregions of the NAc via glutamatergic projections (Li & Kirouac, 2008; Vertes & Hoover, 2008). In Chapter 5, we report that chemogenetically activating PVT-NAc shell neurons blocks heroin seeking in chronically food-restricted rats. Our findings are in agreement with recent publications indicating that activation of PVT-NAc shell neurons can interfere with reward-seeking behaviour (Do-Monte et al., 2017; Lafferty et al., 2020).

NAc shell in Extinction of Drug-Seeking

The NAc shell is implicated in the extinction of drug-seeking. For example, reversible inactivation of the NAc shell attenuates the expression of extinction of drug-seeking in animals previously trained to self-administer cocaine or alcohol (Peters, LaLumiere, & Kalivas, 2008; Millan, Furlong, & McNally, 2010). These findings suggest that the NAc shell exerts control over drug-seeking and that taking the NAc shell offline removes this control over drug-seeking, especially during extinction conditions. Importantly, the recruitment of the NAc shell aligns well with evidence implicating a role for glutamatergic neurotransmission in extinction of drug-seeking. For example, extinguished cocaine-seeking, but not sucrose seeking up-regulated mGluR1 and mGluR2/3 subunits of AMPARs in the NAc shell relative to subjects who underwent abstinence instead of extinction training and control subjects (Sutton, Schmidt, Choi,

Schad, & Whisler…, 2003). Importantly, this effect was observed specifically in the NAc shell but not in the NAc core. The same authors also reported that viral-mediated overexpression of GluR1 or GluR2 subunits in the NAc shell attenuated cocaine-seeking during extinction (Sutton et al., 2003). Again, these effects were not recapitulated in sucrose trained rats. Together, these findings indicate that the extinction of drug-seeking is capable of inducing neuroplasticity changes in the NAc shell. Importantly, as the NAc shell receives glutamatergic input from the PVT, it is possible that exciting these projections at test attenuated heroin-seeking in our chronically food-restricted rats.

The NAc shell also appears to gate non-drug reward-seeking. For example, inhibiting the NAc shell enhances reward-seeking during interstimulus intervals and to non-rewarded cues (Blaiss & Janak, 2009; Ambroggi, Ghazizadeh, Nicola, & Fields, 2011), and reduction in NAc shell activity is correlated with enhanced reward seeking (Basar et al., 2010; Floresco, 2015; Reed et al., 2018). On the flip side, increased NAc shell activity is observed when rodents are required to abstain from responding (Roitman & Loriaux, 2014; Ambroggi et al., 2011). As a whole, these findings suggest that NAc shell activity is important for the inhibition of reward-seeking, particularly under conditions when the reward is omitted, an idea consistent with the findings presented in this dissertation.

NAc shell in Context-Induced Reinstatement of Drug-Seeking

The NAc shell has also been critically implicated in context-induced reinstatement of drug-seeking (Gibson et al., 2018) and these data are particularly relevant for the experiments presented within this dissertation as our design includes contextual cue-induced heroin-seeking following a period of forced abstinence. Early

studies indicated that activating group II metabotropic glutamate receptors in the NAc shell dose-dependently attenuated context-induced reinstatement of heroin-seeking (Bossert, Gray, Lu, & Shaham, 2006). The same group also reported that blocking D1R in the NAc shell but not NAc core attenuated context-induced reinstatement of heroin-seeking (Bossert, Poles, Wihbey, Koya, & Shaham, 2007). Interestingly, systemic injection of D1R antagonist attenuated context-induced reinstatement of drug-seeking and attenuated c-fos expression in the NAc shell (Hamlin, Blatchford, & McNally, 2006). Context-induced reinstatement of sucrose and alcohol-seeking induces c-fos expression in the NAc shell, but not NAc core (Hamil et al., 2006; Hamlin, Newby, & McNally, 2007; Marchant et al., 2010). Temporary inactivation of the NAc shell or NAc core attenuated context-induced reinstatement of cocaine-seeking (Fuchs, Ramirez, & Bell, 2008). Additionally, D1R antagonist microinjections into the NAc shell and NAc core prior to testing attenuated context-induced reinstatement of ethanol seeking, however this effect appears to be more pronounced for the NAc core (Chaudhri, Sahuque, & Janak, 2009). Together these findings suggest a role for the NAc shell in context-induced reinstatement of drug-seeking across multiple classes of drugs.

NAc shell and Feeding

The NAc shell has been heavily implicated in the regulation of feeding behaviour. Inhibiting the NAc shell with GABA agonists stimulates feeding, a finding that aligns with the data presented above on the role of the NAc shell in drug-seeking (Stratford & Kelley, 1997; Stratford & Kelley, 1999; Söderpalm & Berridge, 2000; Ward, Somerville, & Clifton, 2000; Stratford, 2005). Glutamate receptors within the NAc shell appear to play a critical role in the regulation of feeding. For example, blocking glutamate

receptors in the NAc shell induces feeding while activation of glutamate receptors in the NAc shell suppresses feeding (Maldonado-Irizarry, Swanson, & Kelley, 1995; Stratford, Swanson, & Kelley, 1998; Reynolds & Berridge, 2001; Reynolds & Berridge, 2003). Furthermore, coordinated reductions in excitatory input into the NAc (including from the PVT) promotes feeding behaviour (Reed et al., 2018).

A Potential Downstream Target of the NAc shell

NAc shell neurons projecting to the LHA have been implicated in the regulation of feeding. NAc shell neurons projecting to the LHA are GABAergic (Mogenson, Swanson, & Wu, 1983; Thompson & Swanson, 2010). Activation of GABA receptors or inhibition of glutamate receptors in the NAc shell elicits feeding and induces neural activation in the LHA, as measured by c-fos expression (Baldo et al., 2004; Faure, Richard, & Berridge, 2010; Maldonado-Irizarry et al., 1995; Reynolds & Berridge, 2001; Reynolds & Berridge, 2003; Stratford & Kelley, 1997; Stratford et al., 1998; Stratford & Kelley, 1999; Stratford, 2005; Zheng et al., 2003; Zheng, Patterson, & Berthoud, 2007). This enhanced feeding is prevented by infusing a GABA agonist into the LHA (Maldonado-Irizarry et al., 1995; Urstadt, Kally, Zaidi, & Stanley, 2013).

Based on the above findings, a model was proposed whereby GABAergic projections from the NAc shell to the LHA can control feeding behaviours (Maldonado-Irizarry et al., 1995; Kelley et al., 2005; Kelley, Baldo, Pratt, & Will, 2005; Baldo & Kelley, 2007). In line with this model, activation of D1-MSN terminals targeting LHA GABA neurons stops ongoing food consumption in hungry animals while inhibition of NAc shell D1-MSN has the opposite effect (O'Connor et al., 2015). Together, these

findings suggest that the pathway from the NAc shell to LHA controls consumption and is capable of overriding metabolic needs.

The NAc shell-LHA pathway appears to play a critical role in drug-seeking, essentially promoting extinction of drug-seeking. NAc shell inactivation induces activation in orexin neurons in the LHA and reinstatement of alcohol-seeking, which is blocked by inactivation of the LHA (Millan et al., 2010). Based on these findings, the authors concluded that the NAc shell inhibits drug-seeking following extinction by inhibiting LHA neuropeptides. More recently, Gibson et al. (2018) reported that photostimulation of NAc shell-LH GABAergic neurons promotes extinction of alcohol-seeking. These results suggest that enhancing inhibitory input to the LHA can attenuate drug-seeking. Based on these findings, we can speculate that activation of D1- MSN NAc shell-LHA GABAergic neurons will result in extinction-like behaviour. In line with this idea, Keyes et al. (2020) recently reported that repeated morphine treatment induces a feedforward inhibition of NAc-LH neurons and that activating NAc-LHA projections prevents morphine-seeking in a conditioned place preference procedure. Thus, it is possible that activation of the PVT alone or activation of PVT-NAc shell neurons enhanced D1-MSN GABAergic input to the LHA and attenuated the food restriction-induced augmentation of heroin-seeking.

As a whole, these findings suggest that the NAc shell-LHA pathway may regulate drug seeking and feeding through a common mechanism, making it a prime candidate for future investigation. In our case, the NAc shell-LHA pathway may regulate the augmentation of drug-seeking induced by chronic food restriction. It is possible that in our chronically food-restricted rats, PVT neurons innervating the NAc shell provide

inhibitory signals, i.e., reduced excitatory input, that may cause NAc shell- LHA projecting neurons to become inactive, causing disinhibition of LHA neurons driving hunger/feeding and perhaps in the case of our chronically food-restricted rats driving heroin seeking. In support of this hypothesis, coordinated reductions in excitatory input from the PVT to the NAc promotes feeding and reward-seeking (Reed et al., 2018; Lafferty & Britt, 2020; Lafferty et al., 2020). Moreover, acute food deprivation induces synaptic depression at NAc shell D1-MSN neurons projecting to the LHA, and synaptic potentiation of this pathway reduces food consumption in these animals (Thoeni, Loureiro, O'Connor, & Lüscher, 2020). This idea may also explain why blocking excitatory transmission with AMPA receptors antagonists in the NAc shell and core did not alter heroin seeking behaviour in chronically food-restricted rats, in which excitatory input to the NAc shell was already reduced (D'Cuhna, Unpublished). Therefore, we hypothesize that activation of AMPA receptors in the NAc or activation of D1R-MSN in the NAc shell projecting to the LHA would block heroin seeking in chronically food-restricted rats.

Downstream of the LHA: The VTA

One major target downstream of the LHA is the VTA, a brain region known to be involved in motivation, reward, feeding, and drug-seeking (Leinninger et al., 2009; Tyree & de Lecea, 2017; Morales & Margolis, 2017; D'Cunha, Chisholm, Hryhorczuk, Fulton, & Shalev, 2020). Inhibition of the LHA-VTA pathway attenuates compulsive sucrose seeking but not food consumption in hungry mice, suggesting that this pathway is capable of interfering with reward-seeking (Nieh et al., 2015). Further to this point, the activation of LHA-VTA projections promotes compulsive sucrose seeking. A follow-up study

further disentangled how LHA-VTA projections work, showing that activating GABAergic LHA-VTA neurons disinhibits DA neurons in the VTA, increases DA release in the NAc, and promote behavioural activation (Nieh et al., 2016).

It is possible that activation of the PVT alone or activation of PVT-NAc shell D1-MSN projections targeting LHA GABAergic neurons enhanced inhibitory input onto LHA GABAergic neurons targeting the VTA, attenuating drug-seeking by increasing local inhibition of VTA DA neurons, thereby attenuating DA release in the NAc.

Putting It All Together

Together, the data presented throughout this dissertation indicate that the PVT is recruited in our chronically food-restricted rats and drives the augmentation of heroin-seeking. To our surprise, chemogenetically inhibiting or exciting the PVT did not alter heroin-seeking in our sated rats, nor did chemogenetically activating cortico-thalamic or thalamo-accumbens projectors. These experiments are the first to assess the role of the PVT in opioid-seeking following a period of abstinence and suggest that the PVT does not play a role in heroin-seeking following a period of abstinence under sated conditions.

Previous studies indicate that the PVT is recruited in the reinstatement of cocaine and alcohol-seeking (Hamlin et al., 2009; Marchant et al., 2010; James et al., 2011a; Perry & McNally, 2013; James et al., 2011b; Matzeu et al., 2017). However, to date, no studies have assessed the role of the PVT in the reinstatement of opioid seeking. Furthermore, the neural mechanisms underlying drug-seeking following a period of withdrawal are fundamentally different than the neural substrates mediating the reinstatement of extinguished drug-seeking (Fuchs et al., 2006; Fuchs et al., 2008). Taken together, these findings suggest that the PVT may be differentially recruited depending

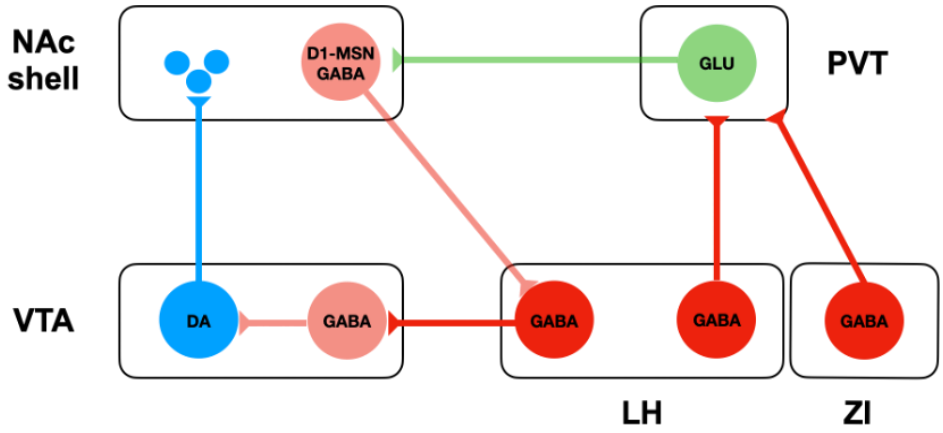
on the drug being studied and on the model of drug-seeking employed, and this is an area of study that warrants further investigation.

The idea that the PVT is selectively recruited in our chronically food-restricted rats but not in our sated rats aligns with previous findings indicating that inhibition of the PVT may only promote drug and non-drug reward-seeking under certain experimental conditions. Inhibiting the PVT enhances cue-induced reinstatement of cocaine-seeking but only in animals that attribute predictive value to cocaine-associated cues (i.e., goal trackers) but not in animals that attribute both predictive and incentive value to cocaine-associated cues (i.e., sign-trackers) (Kuhn et al., 2018). Additionally, inhibiting the PVT attenuates defensive but enhances reward-seeking behaviour (Choi & McNally, 2017). Furthermore, inhibiting the PVT enhances reward-seeking, but only when the expected reward is omitted (Do-Monte et al., 2017). Together, these findings suggest that under certain experimental conditions inhibiting the PVT promotes drug and non-drug reward-seeking, and we believe that chronic food restriction may be one of these conditions.

Here, we propose a circuit that can explain why activating the PVT or PVT-NAc shell neurons selectively attenuates heroin-seeking in our chronically food-restricted rats (Figure 6.1A). We believe that two feeding-related inputs described above, the LHA and ZI, are activated in response to chronic food restriction, providing enhanced inhibitory signals to the PVT. These inhibitory signals are then conveyed via PVT-NAc shell projectors in the form of reduced excitatory input to NAc shell D1-MSN. Consequently, the reduction in inhibitory input from D1-MSN NAc shell-LHA projecting neurons disinhibits LHA-VTA neurons, enhancing inhibitory input onto local VTA GABAergic neurons. This enhanced inhibitory input from LHA-VTA GABAergic neurons results in

disinhibition of local VTA GABAergic neurons driving the augmentation of heroin-seeking, perhaps by evoking dopamine release in the NAc shell via activation of VTA-NAc shell projectors. We believe that restoring excitatory input to the PVT or PVT-NAc shell neurons in our chronically food-restricted rats counteracts the inhibitory input from the LHA and ZI in response to chronic food-restriction attenuating the augmentation of heroin-seeking (Figure 6.1B).

(A)



(B)

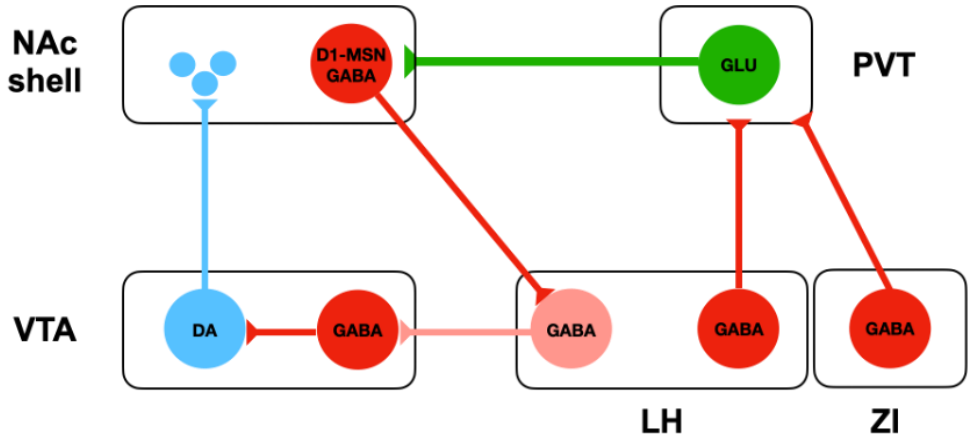


Figure 6. 1. (A) A proposed theoretical model to explain the augmentation of heroin-seeking induced by chronic food-restriction. (B) A proposed theoretical model to explain how activating PVT or PVT-NAc shell neurons attenuates the augmentation of heroin-seeking selectively in chronically food-restricted rats. Green indicates glutamatergic projections. Red indicates GABAergic projections. Blue indicates dopaminergic projections. Light colours indicate inhibited neurotransmission. Dark colours indicate increased neurotransmission.

Anterior and Posterior PVT Dichotomy

In rodents, the PVT is a lengthy structure, stretching over 2 mm across its anterior-posterior axis. The PVT is mainly composed of glutamatergic projections neurons. Across the anterior-posterior axis, there is considerable overlap in the inputs and outputs of the PVT. As a result, most early research focused on the PVT as a whole structure or on one division of the structure while largely ignoring the other aspect. However, recent evidence indicates that the anterior and posterior regions of the PVT are functionally distinct in their neuroanatomical innervations. These findings have encouraged investigation into the different regions of the PVT: the anterior PVT (aPVT), mid PVT (mPVT) and posterior PVT (pPVT). Newly emerging data support a differential role in the control of behaviour by the separate divisions of the PVT. In this dissertation, across all experiments, we focused our manipulations in a region that is between the mPVT and pPVT. To date, the role of the aPVT in heroin seeking behaviour has not been studied. Therefore, it would be interesting to see if similar manipulations in the aPVT block the augmentation of heroin seeking in chronically food-restricted rats or if the aPVT and pPVT are functionally distinct.

The notion that the aPVT and pPVT are functionally dichotomous has been challenged, and a newly proposed classification system has been put forth to clarify conflicting results in the field. Across its anterior-posterior axis, the PVT exhibits both homogeneity and heterogeneity when it comes to the anatomical and functional makeup of each region, with the mPVT serving as a bridge between the two areas. Within the PVT, two new functionally distinct neuronal subtypes have been identified: type I and type II neurons (Gao et al., 2020). Although type I and type II neurons populate the PVT across its entire axis, type II neurons compose the majority of the aPVT while type I neurons form the majority of the pPVT. These two functionally distinct neurons can be identified based on their genetic identity; type I neurons contain *Drd2* genetic markers, while type II contains *Gal* genetic markers. Type I and type II neurons are functionally distinct. In the PVT, type I neurons respond to the valence of salient appetitive and aversive stimuli in a bidirectional manner. Type I neuronal activity is enhanced by aversive stimuli and inhibited by appetitive stimuli. Salient stimuli inhibit type II neurons. Beyond the genetic and functional differences, type I and type II neurons display selectivity in the brain regions that they innervate. Type I neurons project to the NAc core, ventral aspect of the NAc shell and innervate the PrL. In contrast, type II neurons preferentially innervate the dorsomedial aspect of the NAc shell and the IL. These data suggest that characterizing the PVT based on the anatomy, functionality, and genetic identity of cell subtypes within and across each subregion of the PVT may be a better alternative to the original differentiation based on the region of the PVT.

Methodological Considerations

Animal Models of Relapse

The role of the PVT in drug seeking-behaviour has been extensively studied using the extinction-reinstatement model. Unfortunately, all studies assessing the role of the PVT utilizing this model have focused on psychostimulants or alcohol. To date, no studies have examined the role of the PVT in opiate seeking using this model. A critical problem with the use of the extinction-reinstatement model to study drug-seeking is that extinction is not a reason why an individual abstains from drug use, but rather the individual is forced to abstain (i.e., incarceration or hospitalization) or chooses to abstain from drug use (Epstein, Preston, Stewart, & Shaham, 2006). In the series of experiments presented in this dissertation, we have attempted to address this issue by utilizing a forced abstinence model that better mimics the human condition. At present, only one other study aside from those presented in this dissertation has assessed the role of the PVT employing an abstinence model, again using psychostimulants (Giannotti et al., 2018). Along with this challenge, psychostimulant and opiate addiction are cognitively, neurobiologically, and behavioural distinct phenomena (Badiani et al., 2011). It is tough to generalize findings from studies examining the role of the PVT and its circuitry in drug-seeking using psychostimulants.

To our knowledge, all studies assessing the role of the PVT in drug-seeking behaviour, including ours, have utilized male subjects. Based on the extensive evidence indicating a role for the PVT in addiction-related behaviour, it is critically important to determine if the PVT functions in a similar manner in female subjects. Accordingly, biological sex should be taken into account in all future studies assessing the role of the PVT in drug-seeking behaviour, and data should be pooled if no biological sex differences are observed (Diester, Banks, Neigh, & Negus, 2019).

Use of DREADDs to Examine Complex Behaviour

When employed correctly, DREADDs provides an innovative and minimally invasive tool to manipulate neural activity in a given brain region or pathway to study complex behaviour (i.e., drug-seeking). However, the use of DREADDs in experimental procedures requires appropriate control groups and validation of functionality to draw accurate inferences. First, it is critically important to include control groups (i.e., control virus with CNO treatment) to ensure that any changes in behaviour are not due to the infusion of CNO alone. In Chapter 5, we did this to verify that the infusion of CNO alone into the NAc shell in the presence of a control virus did not alter heroin seeking behaviour. When assessing a brain pathway in which axons collateralize to innervate multiple areas as is the case with the PVT, the use of CRE-dependent DREADDS is not an appropriate technique to employ as this technique cannot guarantee that any changes in behaviour are due to a specific pathway manipulation. Chemogenetic manipulation of collaterals to other brain regions may be involved in any effects observed. As described in Chapter 2, local infusion of a viral vector carrying a DREADD in the cell body region and cannulation of the target region (i.e., the terminal region of projecting cell body) coupled with local infusion of CNO gets around this issue. This method ensures that only neurons within a specific brain pathway will be inhibited or activated without affecting collaterals (Stachniak, Ghosh, & Sternson, 2014; Mahler et al., 2014). The use of DREADDs in such a precise manner requires validation in that activation or inhibition using DREADDs does alter neural activity. In the experiments presented in this dissertation, we verified changes in neural activity by ex vivo slice electrophysiology and by immunohistochemistry for the immediate early gene c-fos, and both resulted in

statistically significant changes in neural activity. Here, regardless of any behavioural outcomes, we have validated the use of DREADDs in PrL-PVT and PVT-NAc pathways for future study.

As more research has become available, it appears that there is a complex interplay and balance between cell types and pathways that are involved in guiding behaviour. Probing one pathway at a time is akin to suggesting that we live in a vacuum. When assessing complex circuitry, probing one pathway may be easier experimentally, but in doing so, we miss out on the complexity of circuitry interactions in behaviour, which ultimately may do a better job of answering the empirical question at hand. Moving forward, it will be important to assess multiple inputs to the same target (i.e., PVT) to determine how these potential pathways interact and what unique contribution they may provide in the augmentation of heroin seeking induced by chronic food restriction.

Concluding Remarks and Future Directions

The experiments presented in this dissertation sought to assess the role of the PVT, cortico-thalamic and thalamo-accumbens projections in the augmentation of heroin seeking induced by chronic food restriction. We established a role for the PVT in the augmentation of heroin seeking induced by chronic food restriction and highlight a role for PVT projections to the NAc shell in mediating this effect. At the time we started this project, no studies assessing the role of the PVT in opioid seeking existed. In addition, most investigation of the role of the PVT in drug-seeking had utilized a reinstatement model, which, as mentioned, may not be the most ecologically valid model to assess relapse behaviour in rodents. This work has filled a large gap in the empirical literature

and has enriched our knowledge about the role of the PVT and its circuitry in opioid seeking.

Future studies should further assess the role of different inputs to the PVT in the augmentation of heroin seeking induced by chronic food restriction. Future studies should investigate the role of the LHA-PVT and ZI-PVT pathways in the augmentation of heroin seeking induced by chronic food restriction. Additionally, it would be interesting to determine the contribution of D1-MSN NAc shell-LHA projectors in the augmentation of heroin-seeking induced by chronic food restriction. Prospective studies could also assess the role of the PVT and its projections utilizing the same forced abstinence procedure while evaluating different drugs of abuse. Future studies should seek to identify the role of the anterior vs. posterior PVT in the augmentation of heroin seeking induced by chronic food restriction.

An exciting alternative avenue to pursue in comparison to the work presented in this dissertation would be to assess the role of the PVT and its projections in heroin seeking using different relapse models. The role of the PVT and its projections should be assessed utilizing a reinstatement model. Given that different neurobiological circuits are recruited depending on the model of drug-seeking employed, it would be interesting to see if the PVT and the projections identified here function in a similar capacity in a reinstatement model assessing opioid seeking and might help to clarify conflicting data.

Motivationally conflicting situations arise when a subject must choose between two desirable/undesirable alternatives or when a subject must make a choice that has both positive and negative consequences. The PVT plays a critical role in the control of behaviour in motivationally conflicting situations (Choi, Jean-Richard-Dit-Bressel,

Clifford, & McNally, 2019; McNally, 2019). Future research should assess the role of the PVT utilizing a punishment-induced abstinence model. This model provides a better set up than traditional abstinence models (i.e., the model employed in this dissertation) to assess the role of the PVT in heroin seeking during a motivationally conflicting situation- a situation where the PVT should be highly relevant.

Conclusion

Overall, this work has helped us to understand the neural mechanisms by which chronic food restrictions augments heroin seeking. As a direct result of this work, we now know that the PVT and its projection to the NAc shell plays a critical role in the augmentation of heroin-seeking induced by chronic food-restriction. However, we have only begun to understand the complexity of this circuitry. Like a jigsaw puzzle, this dissertation has contributed a tiny puzzle piece towards our full understanding, but by adding more pieces over time, we will start to see the larger picture.

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APPENDIX: ONLINE SUPPLEMENTAL INFORMATION FOR CHAPTER 3

Materials and Methods

Intravenous and intracranial surgery

Rats were implanted with an intra-jugular Silastic catheter (Dow Corning, Midland, MI, USA) under 2% isoflurane anaesthesia, as previously described (Sedki et al., 2013). The other tip of the intravenous catheter was attached to a modified 22-gauge cannula (5-up, Plastics One, Roanoke, VA) and anchored to the skull using five screws and dental cement. Over the course of self-administration training, catheters were flushed once a day with gentamicin and heparin (7.5 IU + 40.0 μg per day) in sterile saline to maintain catheter patency. During the same surgery, rats were injected with 0.6 μl of viral vector (Experiment 1: AAV8-hSyn-hM4D(Gi)-mCherry, University of North Carolina, Chapel Hill, NC; Experiment 3: AAV8-hSyn-hM3D(Gq)-mCherry, Canadian Neurophotonic Platform/viral vector team, Québec, QC) into the PVT (-3.0 AP, 0.0 ML, -5.4 DV relative to Bregma) at a rate of 0.1 μl /minute. The injector was left in place for additional 10 minutes. For intra-PVT injections of muscimol + baclofen solution (Experiment 2), rats were implanted with a guide cannula aimed at the PVT (AP: -3.0, 0.0 ML, -3.2 DV). Rats were administered 0.9% saline (2 ml, s.c.) and Ketoprofen (2.0 mg/kg; Merial Canada Inc., Baie-d'Urfe, QC) immediately following surgery, and once daily over the next 2 days.

Apparatus

Operant conditioning chambers with two retractable levers (Coulbourn Instruments, Allentown, PA, USA; 29.0 cm X 29.0 cm x 25.5 cm) enclosed in sound-attenuating boxes were used. Each chamber was equipped with a red house light, a food

hopper, and a water bottle. Two retractable levers, an 'active' and an 'inactive' lever, were installed 9.0 cm above the floor on one wall. A white cue light and tone generator (Coulborn Instruments, Sonalert, 2.9 kHz) were located directly above the active lever. An infusion pump (Razel Scientific Instruments, Stamford, CT) was connected to the catheter through a liquid swivel (Lomir Biomedical Inc., Notre-Dame-de-l'Île-Perrot, QC, Canada) and Tygon tubing (Saint-Gobain, Courbevoie, France) shielded with metal spring.

The elevated plus-maze was located in a dimly lit room and was built out of wood and consisted of four arms (11.5×55.0 cm) positioned at right angles and elevated 50.0 cm above the floor. There were two opposed 'closed' arms, which had 40-cm high walls, and two 'open' arms, which had a slight raised edge (1.0 cm).

Elevated plus maze procedure

Rats were intracranially injected with either 0.3 μl baclofen + muscimol (0.3 + 0.03 nmol) or 0.3 μl vehicle (sterile saline) 5-10 minutes before a 10 min test on the elevated plus-maze. Test sessions were videotaped and behaviors were scored offline (randomly validated by a second coder). Two behaviors were analyzed: (1) time spent in the open arms, defined as a rat having its two front paws and half of the front body in the closed arm (2) time spent in the open arm, following the last open arm entry.

Electrophysiological Validation of Excitatory DREADD Activation in the PVT

Whole cell in vitro electrophysiological recordings in 11 to 16 weeks old rats were used to assess the effects of CNO on the excitability of PVT neurons, 3 to 6 weeks following AAV8-hSyn-hM3D(Gq)-mCherry infusion. Animals were anaesthetized with isoflurane, and perfused through the heart with high sucrose ACSF at 4°C containing, in

mM, 250.0 sucrose, 2.0 KCl, 1.25 NaH_2PO_4, 7.0 $MgCl_2$, 26.0 $NaHCO_3$, 0.5 $CaCl_2$ and 10 dextrose, saturated with 95% O_2/5% CO_2. Coronal slices 300 μm thick were obtained using a vibratome (Leica VT1200), and placed in normal ACSF containing 124.0 NaCl, 5.0 KCl, 1.25 NaH_2PO_4, 2.0 $MgSO_4$, 26.0 $NaHCO_3$, 2.0 $CaCl_2$, and 10.0 dextrose. Slices remained at 32˚C for 30 min and then at 22˚C for at least one hour prior to recordings. Slices were held submerged with a nylon net and perfused with ACSF at 2 ml/min, and visualized using an upright microscope with a 40X water-immersion objective and differential interference contrast optics (Leica, DML-FS). Borosilicate glass recording pipettes (4 to 6 MΩ) were filled with a solution containing 140.0 K-gluconate, 5.0 NaCl, 2 $MgCl_2$, 10.0 HEPES, 0.5 EGTA, 2.0 ATP-tris, 0.4 GTP-tris (pH adjusted to 7.25 using KOH). Tight seals onto soma were obtained (>1 GΩ), and there was a 10-min period in whole cell configuration prior to recordings. Mean series resistance was 27.8 ± 3.2 MΩ. Recordings were obtained using a Axopatch 200B amplifier, and were digitized (Digidata 1322A) and stored using pClamp 10.3 software (Molecular Devices).

PVT neurons typically fired at resting membrane potential (-42.1 ± 3.8 mV), and cells were therefore held near -60 mV using steady negative current injection (-44.6 ± 20.0 pA) to assess their electroresponsiveness before and after 5 to 10 min application of CNO (1.0 μM). Input resistance and firing in response to brief current steps was assessed by injection of 500 ms duration hyperpolarizing and depolarizing current steps between -200 and 75 pA in 25 pA intervals. Peak input resistance was measured by the largest voltage change in response to a -200 pA pulse, and steady state input resistance was assessed just prior to the end of the current step. The number of action potentials elicited by prolonged, 5 sec duration current steps was also determined. An amplitude of 25 pA

was used if repetitive firing could be evoked by this lower intensity (n = 5), and intensity was increased to 50 pA if needed to induce repetitive firing (n = 2). Five trials were recorded using an intertrial interval of 10 sec.