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Assessing the Involvement of Projections from the Prelimbic Prefrontal Cortex to the Paraventricular Nucleus of the Thalamus in Cocaine Withdrawal-Induced Anxiety

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Assessing the involvement of projections from the prelimbic prefrontal cortex to the paraventricular nucleus of the thalamus in cocaine withdrawal-induced anxiety.

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List of Abbreviations

AAV: adeno-associated virus

ANOVA: Analysis of Variance

A/P: Anterior/Posterior

aPVT: Anterior Paraventricular nucleus of the Thalamus

DREADD: Designer Receptor Exclusively Activated by Designer Drugs

D/V: Dorsal/Ventral

DA: Dopamine

FR1: Fixed-Ratio 1

GABA: Gamma Aminobutyric Acid

GPCR: G Protein-Coupled Receptor

IP: Intraperitoneal

M/L: Medial/Lateral

NAc: Nucleus Accumbens

NIH: National Institute of Health

PL: Pre-limbic medial prefrontal cortex

PFC: Prefrontal Cortex

pPVT: Posterior Paraventricular nucleus of the Thalamus

PVT: Paraventricular nucleus of the Thalamus

SUD: Substance Use Disorder

SC: Subcutaneous

VTA: Ventral Tegmental Area

VP: Ventral Pallidum

Abstract

CLINTON S. COELHO. Assessing the involvement of projections from the prelimbic prefrontal cortex to the paraventricular nucleus of the thalamus in cocaine withdrawal-induced anxiety. (Under the direction of JACQUELINE F. MCGINTY)

Withdrawal from cocaine induces an immediate and powerful negative affective state which is characterized by feelings of agitation and anxiety in humans and anxiety-like behaviors in rodents. It has been hypothesized that the anxiogenic effects associated with cocaine withdrawal play a major role in continued cocaine use and drive relapse to cocaine seeking. The prelimbic (PL) medial prefrontal cortex is not only involved in the processing and regulation of negative emotions such as anxiety but also mediates relapse to cocaine-seeking. Subcortical hubs of the reward system that receive projections from the PL neurons are known to be dysregulated during different stages of cocaine addiction, including withdrawal. One of the emerging and severely understudied subcortical targets of the PL cortex implicated in cocaine reward and relapse to cocaine seeking is the paraventricular nucleus of the thalamus (PVT). PL inputs to the PVT have been implicated in fear-induced anxiogenic behaviors as well as relapse to cocaine seeking. However, little is known about the contribution of PL→PVT in cocaine withdrawal-induced anxiety. Therefore, in this study, we explored the implications of chemogenetically inhibiting PL→PVT projections using a combinatorial AAV vector approach in the attenuation of cocaine-induced anxiogenic behaviors during early withdrawal from cocaine using an elevated zero maze (EZM) and an open field test (OFT). We hypothesized that cocaine withdrawal-induced anxiety-like behavior would be greater than that in saline control rats on the EZM and OFT tasks. Further, inhibition of the PL→PVT projection would attenuate cocaine withdrawal-induced anxiogenic behaviors and therefore decrease the probability of subsequent relapse to cocaine seeking.

Additionally, we predicted that PL→PVT inhibition would not affect anxiety-like behavior in saline control rats. Results from our study demonstrate that PL→pPVT inhibition decreased the latency to enter open arms but increased the number of explorations of the open arms in rats with cocaine experience compared to yoked-saline controls. In the same animals, inhibition of PL→pPVT increased the time spent in the center of the open field in rats with cocaine experience. Together, our results demonstrate that the PL→pPVT projection influences aspects of the anxiety phenotype associated with exploratory behaviors. Inhibition of the PL→PVT pathway during early withdrawal from cocaine attenuated withdrawal-induced anxiogenic behaviors and this inhibition did not affect baseline anxiety-like behaviors in control rats. Therefore, this study provides evidence for the involvement of the PL→PVT pathway in mediating cocaine withdrawal-induced anxiety.

Chapter 1: Specific Aims

A high rate of relapse after a period of abstinence is one of the primary factors driving cocaine use disorder (CUD). Withdrawal from cocaine induces an immediate and powerful negative affective state which is characterized by feelings of agitation and anxiety in humans (Gawin and Kleber, 1986) and anxiety-like behaviors in rodents (Barbee and Gourley, 2022). It has been posited that this anxiety leads to continued cocaine use and drives relapse to cocaine seeking. Indeed, trait anxiety (in humans) and increased anxiety-like behaviors (in rodents) is an influencing factor for future cocaine use and a strong predictor for reinstatement to cocaine seeking, respectively (Woicik et al., 2009; Erb, 2010; Liu et al., 2020; Barbee and Gourley, 2022). We therefore proposed that targeting the negative affective states, specifically anxiety, during this early withdrawal period would be an innovative approach in the development of novel therapeutics for the prevention of CUD.

The medial prefrontal cortex (mPFC), specifically, the prelimbic (PL) but not the infralimbic mPFC, is involved in the processing and regulation of negative emotions such as anxiety (El Hage et al., 2012; Suzuki et al., 2016; Jacobs and Moghaddam, 2021a). Furthermore, the PL cortex has been demonstrated to mediate relapse to cocaine-seeking (Koob and Volkow, 2016). Several subcortical hubs of the reward system that receive projections from the PL neurons are known to be dysregulated in cocaine addiction. For example, the PL to nucleus accumbens (NA) core pathway has been implicated most often in all major forms of drug seeking after abstinence or extinction (McFarland and Kalivas, 2001; McLaughlin and See, 2003; Berglind et al., 2009; Ma et al., 2014; Stefanik et al., 2016). One of the emerging and severely understudied subcortical targets of the PL implicated in drug and non-drug associated reward seeking is the paraventricular nucleus of the thalamus (PVT) (McGinty and Otis, 2020). For example, the PVT contributes to reinstatement of cocaine seeking (James et al., 2010; Matzeu et al., 2015) and inhibition of PL→PVT during early

withdrawal decreased subsequent cocaine seeking (Giannotti et al., 2018). Additionally, cocaine and exposure to cocaine-conditioned cues/contexts has been shown to activate PVT, specifically, posterior (p) PVT (Millan et al., 2017). Furthermore, PVT neurons are recruited by stress, and activation of hypothalamic input to the PVT induces fear and anxiety-like behaviors indicating a role for PVT in the regulation of negative emotional states (Kirouac, 2015, 2021). Therefore, it is possible that withdrawal-induced anxiogenic effects of cocaine and subsequent increase in relapse probability involves recruitment of PVT neurons. While it is known that major cortical inputs to the PVT come from PL cortex (Li and Kirouac, 2012) and that a projection from PL to PVT is critical in retrieval of remote fear memories (Do-Monte et al., 2015; Do Monte et al., 2016), little is known about the contribution of PL→PVT in cocaine withdrawal-induced anxiety. Therefore, in this study, we investigated the role of the PL→pPVT pathway in male and female rats during cocaine withdrawal-induced anxiety-like behaviors using an elevated zero maze (EZM) and an open field test (OFT). We explored the implications of chemogenetically inhibiting PL→PVT projections using a combinatorial AAV viral vector approach to inhibit cocaine-induced anxiogenic behaviors during early withdrawal.

We hypothesized that anxiety-like behaviors would be greater in animals with cocaine experience than in yoked-saline controls during acute withdrawal. Further, chemogenetic inhibition of PL→PVT immediately after the last cocaine self-administration session would decrease anxiety-like behaviors at that time. We compared the EZM and OFT behavior of male and female rats in which the PL→PVT pathway had been chemogenetically inhibited or not 2 hr after the end of cocaine SA or yoked saline. We also assessed the influence of inhibiting this pathway on cue-induced relapse to cocaine seeking after one week of abstinence.

Chapter 2: Introduction

Drug addiction is a behaviorally cyclical, chronically relapsing, complex, and multistage disorder. Behaviorally, it is characterized by a compulsion to seek and consume the drug, loss of control over drug consumption and the development of a negative emotional and physiological state when drug consumption is interrupted as well as access to the drug is prevented (Koob and Volkow, 2016). Addiction has been conceptualized in the form of a three-stage recurring cycle (Kalivas and Volkow, 2005; Koob and Schulkin, 2019) – binge/intoxication, withdrawal/negative affect, and preoccupation/anticipation (craving). This cycle repeats itself and worsens over time. In the binge/intoxication stage, drug consumption is initiated. The consumption of drugs leads to feelings of pleasure and gratification, increased sexual arousal, and enhanced hedonic states. Discontinuation of drug consumption leads to the emergence of withdrawal/negative affect stage and is characterized by negative motivational states such as chronic irritability, emotional pain, malaise, dysphoria, alexithymia, states of stress and anxiety, and loss of motivation for natural rewards. This is followed by the preoccupation/anticipation stage which develops after periods of abstinence. In this stage drug-seeking behavior is reinstated and has been hypothesized to be a key element of relapse in humans (Koob and Volkow, 2016). Neurobiologically, each stage of the addiction process originates from drug-induced dysregulation of brain structures that normally function to process reward, emotions, and executive functions in the brain.

Neurobiological mechanisms of drug addiction

Drugs of abuse activate brain reward systems and induce changes in these systems which initiate the development of addiction. Intoxicating doses of drugs activate dopaminergic neurons of the ventral tegmental area (VTA) and release dopamine (DA) and opioid peptides into the ventral striatum (Volkow et al., 2007; Mitchell et al., 2012). This leads to a fast and steep increase

in DA release and activation of low-affinity dopamine D1 receptors (Drd1) in the ventral striatum (Volkow et al., 2003) and is associated with subjective sensations and rewarding effects felt after drug consumption inducing appetitive drug-related behaviors (Caine et al., 2007). This initial activation of the VTA to ventral striatum reward circuit in turn influences several neural inputs and outputs that interact with it leading to drug-induced neuroadaptations within these circuits. The circuits of the reward system form essential components regulating reward and motivation, stress (via hypothalamic-pituitary-adrenal axis; HPA axis), mood regulation – including stress reactivity (via amygdala, hypothalamus, and habenula) and interoception (via insula and anterior cingulate cortex), memory (via hippocampus), and executive functions – including inhibitory control and decision making (via prefrontal cortex). Drug-induced neuroadaptations alter the functioning of these circuits and lead to dysregulation of reward and motivation, mood regulation and executive function via multiple neurotransmitter systems, which include not only DA and opioid peptides but also γ-aminobutyric acid (GABA), glutamate, serotonin, acetylcholine, norepinephrine, endocannabinoids, enkephalins, dynorphins, neuropeptide Y, and corticotrophin-releasing factor (CRF) (Koob and Volkow, 2016). Another key feature of drugs of abuse is their ability to reinforce behaviors previously associated with neutral, non-rewarding contextual stimuli and conditioning them to become rewarding during drug consumption (Koob and Volkow, 2016; Werner et al., 2019). Drug-induced activation and release of DA in ventral striatum also leads to development of incentive salience. During the emergence of incentive salience, environmental and interoceptive cues become associated with the subjective sensations and rewarding effects of drugs and in turn lead to phasic release of DA when an individual is exposed to these drug-associated cues (Berridge, 2012). This phasic release of DA induced by drug-associated cues recruits basal ganglia circuits. Thereafter, activation of ventral striatum leads to the recruitment of striatal-

pallidal thalamocortical loops that activate the dorsal striatum resulting in habit formation (Belin et al., 2009). Together, these processes lead to habit-like, compulsive drug seeking in humans (Belin and Everitt, 2008) and cue-induced drug seeking and drug self-administration (SA) in preclinical animal models of addiction (Koob and Volkow, 2016). Therefore, persistent drug-induced dysregulation of different reward system circuits coupled with conditioned reinforcement and incentive salience of drug-related behaviors and drug-associated cues lead to the development of motivationally rewarding, compulsive drug-related seeking and consummatory behaviors. During acute or protracted abstinence from drug consumption, the development of negative affect (characterized by chronic irritability, malaise, dysphoria, states of aversion, stress and anxiety, and loss of motivation for natural rewards) indicates progression through the addiction cycle (Koob and Volkow, 2016). Neurochemical changes associated with this stage include decrease in DAergic and serotonergic transmission in the nucleus accumbens (NAc) (Weiss et al., 1992) causing decreases in reward system function for drug and non-drug rewards (Volkow et al., 1997; Martinez et al., 2007; Volkow et al., 2007; Volkow et al., 2014). Emotional dysregulation observed during this stage are induced by elevated HPA axis function and increased CRF in the amygdala by chronic drug exposure (Piazza and Le Moal, 1996; Koob et al., 2014). As the period of abstinence increases, brain stress systems continue to be recruited in the extended amygdala leading to negative emotional states (Delfs et al., 2000; Koob et al., 2014). Aversive states developed during acute and protracted withdrawal are partially mediated in the lateral habenula (Hikosaka, 2010) via its projection to the rostromedial tegmental nucleus (Jhou, 2013) by decreasing DA neuron firing in the ventral tegmental area (VTA) (Kimura et al., 2007; Matsumoto and Hikosaka, 2007). Decreased reward function and increased stress function in the motivational circuits of the ventral striatum, extended amygdala, and habenula trigger negative reinforcement

and contribute to compulsive drug-seeking behaviors (Werner et al., 2019). Thus, development of enduring aversive negative affective states due to overactivation of stress and anti-reward systems have the potential to drive relapse to drug-seeking in individuals abstaining from drug consumption. The preoccupation/anticipation stage is the stage of addiction in which, after abstinence, the reinstatement of drug-seeking behavior occurs. This stage has been the primary focus for identifying neurobiological mechanisms of relapse and developing pharmacotherapies. Preclinical rodent studies have derived three major modalities of reinstatement – drug-induced, cue-induced, and stress-induced. Drug- and cue-induced reinstatement involves the glutamatergic projection of prelimbic prefrontal cortex (PL) to ventral striatum circuit (McFarland and Kalivas, 2001) and glutamatergic projection from the PL, basolateral amygdala, and ventral subiculum to the NAc (Vorel et al., 2001; Everitt and Wolf, 2002), respectively. However, stress-induced reinstatement is dependent on the activation of CRF and norepinephrine circuits in the extended amygdala (Pich et al., 1995; Shaham et al., 1998; Delfs et al., 2000; Shaham et al., 2000; Shalev et al., 2002; Shaham et al., 2003) and the VTA. Similarly, human studies have demonstrated activation of the prefrontal cortex (PFC) (Lee et al., 2005; Risinger et al., 2005; Volkow et al., 2005; Jasinska et al., 2014; Kober et al., 2016) and increased DA release in striatum, amygdala, and PFC by drug-associated cues (Volkow et al., 2006; Koob and Volkow, 2010; Fotros et al., 2013; Milella et al., 2016). The other behaviors characteristic of this stage include excessive salience to drug-associated rewards, decreased responsiveness to non-drug rewards, and decreased ability to inhibit maladaptive behaviors (Volkow et al., 2003; Goldstein and Volkow, 2011). These are derived from deficits in executive functions which are largely controlled by the frontal cortex. Indeed, individuals with a history of drug addiction show decreased frontal cortex activity during decision making, self-regulation, inhibitory control, and working memory (Volkow et al., 2011).

Therefore, drug-induced deficits in executive function via dysregulation of neuronal networks within the PFC, coupled with heightened incentive salience for reinstatement of drug-related behaviors against a background of low reward system function and high stress system function induce a powerful drive for pathological drug seeking.

Cocaine Use Disorder (CUD) abstinence and relapse

From a clinical point of view, drug addiction is now termed substance use disorder (SUD) and defined in a range from mild to moderate to severe, with the severity of the SUD depending upon the number of established criteria applying to an individual's condition (American Psychiatric Association and Association, 2013). SUDs are further classified based on the class of drug that an individual is addicted to. For example, development of addiction to alcohol is termed as alcohol use disorder (AUD), to opioids is termed as opioid use disorder (OUD), and to cocaine is termed as cocaine use disorder (CUD). In the United States alone, of the 61.2 million users of illicit drugs, 4.8 million people aged 12 older used cocaine in 2020 (Center for Behavioral Health Statistics, 2021). Of the 4.8 million people using cocaine, 1.4 million people had CUD. Therefore, CUD affects millions of people worldwide and is a significant public health issue that negatively affects social relationships and leads to substantial economic burden on the society. Despite these staggering numbers, there are no approved pharmacotherapies and behavioral interventions remain ineffective in a significant population of patients (Kampman, 2019). One of the primary factors driving CUD is the high rate of relapse after a period of abstinence. Interestingly, number of days of continuous abstinence is one of the best outcome predictors preventing relapse to cocaine use (Carroll et al., 2014). Therefore, promoting abstinence by mitigating cocaine-withdrawal-induced symptoms has the potential to decrease relapse probability and promote recovery. Preclinical studies demonstrate that dysregulation of reward and stress function and emotional dysregulation

remain malleable during acute withdrawal and can be interrupted to increase reward thresholds and therefore attenuate compulsive drug seeking. For example, CRF receptor antagonists administered during acute withdrawal block both anxiety-like and stress-like effects of cocaine withdrawal and increased reward threshold by reduced drug taking during compulsive cocaine seeking (Koob et al., 2014). Compulsive drug-seeking and negative affective states get solidified during withdrawal and protracted abstinence (Koob and Volkow, 2016). Additionally, during acute withdrawal, goal-directed behaviors are intact (George and Koob, 2010). Therefore, targeting acute withdrawal phase for attenuating withdrawal-induced symptoms provides greater promise for ensuring recovery.

Cocaine abstinence-induced anxiety

Early work by Gawin and Kleber classifies cocaine abstinence into three phases - the first phase is "crash" which lasts from 9 hours to 4 days post cocaine, followed by "withdrawal", lasting from 1-10 weeks, and lastly "extinction". The authors noted anxiety in the first two phases and proposed that alleviation of anxiety was necessary to prevent relapse (Gawin and Kleber, 1986). While the intensity and duration of cocaine withdrawal-induced anxiety remains debatable, a relationship between cocaine withdrawal-induced anxiety and craving continues to persist (Barbee and Gourley, 2022). Therefore, it has been posited that this anxiety leads to continued cocaine use and drives relapse to cocaine seeking. Indeed, DSM-5 diagnostic criteria for abstaining cocaine users includes anxiety sensitivity tests as an influencing factor for future cocaine use (Woicik et al., 2009; Liu et al., 2020). Several preclinical studies have demonstrated an association between trait anxiety and cocaine consumption, duration of cocaine withdrawal-induced anxiety and association between cocaine withdrawal-induced anxiety and reinstatement of cocaine-seeking behaviors (Erb, 2010; Barbee and Gourley, 2022). In these studies, the duration of cocaine

abstinence-induced anxiety lasted from 24 hours to 8 days after last cocaine administration (de Oliveira Citó et al., 2012; El Hage et al., 2012). This could be enhanced for up to 28 days when coupled with a stressful stimulus (Aboutalebi et al., 2018). Furthermore, cocaine withdrawal-induced anxiety was also affected by the duration of cocaine exposure. For example, male rats trained on long access cocaine SA schedule demonstrated anxiety-like behaviors for up to 42 days post-cocaine when compared to saline controls or rats trained on short access cocaine SA schedule (Aujla et al., 2008). Buffalari and colleagues demonstrated that cocaine intake positively correlated with cocaine withdrawal-induced anxiety. In the same study, the authors also demonstrated that increased anxiety-like behavior correlated with greater cocaine-primed reinstatement (Buffalari et al., 2012).

Involvement of PL cortex in cocaine – related behaviors

The preoccupation/anticipation stage of CUD is driven by the PFC. Executive functions are regulated by PFC. Deficits in executive functions including decision-making, self-regulation, inhibitory control and working memory underlie uncontrolled cocaine-seeking, decreased responsiveness to non-drug rewards, and decreased ability to inhibit maladaptive behaviors (Volkow et al., 2003; Goldstein and Volkow, 2011). The PL cortex has long been demonstrated to mediate relapse to cocaine-seeking (Koob and Volkow, 2016). PL neurons project to several sub-cortical hubs of reward circuitry that are known to be dysregulated in cocaine addiction. The PL to nucleus accumbens (NA) core pathway has been implicated most often in all major forms of drug seeking after abstinence and/or extinction (McFarland and Kalivas, 2001; McLaughlin and See, 2003; Berglind et al., 2009; Ma et al., 2014; Stefanik et al., 2016). However, neurobiological mechanisms underlying anxiety during cocaine abstinence are poorly understood.

Impaired cognitive and attentional processes occurring during anxiety and anxiety disorders

have been associated with the PFC (Basten et al., 2011, 2012). Both clinical and preclinical studies on processing of anxiety and anxiety-like behaviors are associated with circuits between the regions of the PFC and a set of limbic structures which includes the amygdala, insula as well as interconnected structures such as the hypothalamus (Jacobs and Moghaddam, 2021b). Interestingly, neuroimaging studies in abstinent cocaine-dependent patients and studies in rodents have consistently pointed to persistent structural and functional alterations in the PFC (especially in the orbitofrontal, cingulate and insular cortices) (Franklin et al., 2002; Bolla et al., 2004; Robinson and Kolb, 2004; Tanabe et al., 2009; Ersche et al., 2011; Goldstein and Volkow, 2011) and some of its connected subcortical areas (amygdala, thalamus and hippocampus) (Makris et al., 2004; Goussakov et al., 2006; Tomasi et al., 2007). The high overlap between the brain regions affected by chronic cocaine exposure and those that are parts of the proposed anxiety circuits suggests that some of them may contribute to the impaired expression and/or regulation of anxiety during cocaine abstinence. Indeed, a notable study by El Hage and colleagues demonstrated altered reactivity in anterior cingulate and PL cortices, paraventricular nucleus of the thalamus (PVT), and areas of the hypothalamus in cocaine-withdrawn rats demonstrating exacerbated anxiety (El Hage et al., 2012).

Involvement of PVT in cocaine - related behaviors

One of the emerging and severely understudied subcortical targets of the PL implicated in negative affect associated with withdrawal from cocaine use is the PVT. The PVT is a critical interface between the PFC and the subcortical circuitry which has been shown to regulate both appetitive and aversive behavior (Kirouac, 2015; McGinty and Otis, 2020; Kirouac, 2021). A recent study from our lab has demonstrated inhibition of PL projections to posterior PVT during early withdrawal decreased subsequent cocaine seeking (Giannotti et al., 2018). Additionally, the

PVT contributes to reinstatement of cocaine seeking (James et al., 2010; Matzeu et al., 2015). Exacerbated anxiety observed in cocaine-withdrawn rats placed in an anxiogenic environment was accompanied by dysfunctions within a restricted set of neuroanatomical regions including the dorsal mPFC and anatomically related subcortical regions such as the PVT and specific regions of the hypothalamus. The role of subcortical afferents to PVT in many of these behaviors has been explored (James et al., 2010; Martin-Fardon and Boutrel, 2012; Matzeu et al., 2017) but little is known about the contribution of PL projections to PVT in these behaviors.

Anatomical organization and connectivity of the PVT

The PVT is part of the dorsal midline thalamic nuclei. Anatomically, PVT lies adjacent to the dorsal aspect of the third ventricle. In rodents, the PVT primarily consists of excitatory neurons and extends over the entire rostrocaudal length of the midline thalamus. The shape of the PVT varies along the anterior-posterior extent of the thalamus due to the presence of other groups of midline nuclei (intermediodorsal and centromedial nuclei) and is the dorsal most member of the midline group of thalamic nuclei. Further, the anterior aspect of the PVT (aPVT) is bordered by the paratenial nucleus laterally, while the posterior aspect of PVT (pPVT) is bordered by the mediodorsal nucleus (Kirouac, 2015).

Retrograde tracing studies have identified significant input from the nucleus of solitary tract, locus coeruleus, laterodorsal tegmental nucleus, pedunculopontine tegmental nucleus, parabrachial nucleus, periaqueductal gray, raphe nuclei and the deep mesencephalic nucleus of brainstem to the PVT (Kirouac, 2015). Interestingly, except the afferents from the nucleus of solitary tract, all the projections mentioned above are not unique to the PVT in that they also target other midline and intralaminar nuclei (Kirouac, 2015). The only thalamic nucleus providing inputs to the PVT is the ventromedial part of the reticular nucleus (Li and Kirouac, 2012). The PVT

receives afferents from multiple areas of the hypothalamus. Most robust hypothalamic innervations to the PVT come from the dorsomedial nucleus and suprachiasmatic nuclei (Li and Kirouac, 2012). Other sources of hypothalamic inputs to the PVT include ventromedial, paraventricular, arcuate nuclei, and preoptic, anterior, zona incerta and lateral hypothalamic areas (Kirouac, 2015). The PFC is another major source of input to the PVT. These prefrontal cortical projections to the PVT originate in layer 6 of PL, infralimbic (IL), and insular cortical areas (Li and Kirouac, 2012). The inputs from insular cortical areas are modest in comparison to those from the PL and IL. Furthermore, inputs from the PL and IL are topographically organized in the pPVT and aPVT, respectively (Millan et al., 2017). The subiculum of the hippocampus also projects to the PVT along with minor projections from the septal area and bed nucleus of stria terminalis and these preferentially innervate the aPVT (Kirouac, 2015; Millan et al., 2017).

The PVT sends out dense projections to subcortical areas extending from the NAc to the bed nucleus of stria terminalis (BNST), and ventral regions of caudate putamen (CPu) to central nucleus of the amygdala (CeA) (Kirouac, 2015). Within the NAc, PVT sends dense projections to both core and shell subregions of the NAc (Otake and Nakamura, 1998; Li and Kirouac, 2012) and these projections originate from the rostrocaudal aspect of PVT (i.e. both aPVT and pPVT) (Li and Kirouac, 2008). However, it has been noted that aPVT preferentially projects to NAc shell while pPVT preferentially targets NAc core (Millan et al., 2017). The PVT projects heavily to dorsolateral BNST and lateral and capsular subnuclei of the CeA (Li and Kirouac, 2008; Vertes and Hoover, 2008). Although these projections originate from the rostrocaudal aspect of PVT, there is a predominance of these projections originating from the pPVT (Li and Kirouac, 2008). Other areas of the amygdala that receive much weaker innervations from the PVT include basomedial, basolateral, medial, and lateral nuclei of the amygdala (Moga et al., 1995; Vertes and

Hoover, 2008). The PVT also projects to the PFC and other subcortical structures but these projections are less dense (Li and Kirouac, 2008). The PVT projects to all layers of the IL and ventral region of the PL cortex (Li and Kirouac, 2008; Vertes and Hoover, 2008). Additionally, PVT innervates the insular cortex and ventral subiculum of the hippocampus (specifically aPVT projects to subiculum) (Kirouac, 2015). Weak to moderate projections from the PVT to suprachiasmatic, arcuate, dorsomedial, and ventromedial nuclei of hypothalamus as well as lateral hypothalamus and lateral septal area have been identified (Moga et al., 1995; Li and Kirouac, 2008; Vertes and Hoover, 2008).

Functional role of PVT in addiction related behaviors

Originally, the PVT along with other groups of midline and intralaminar thalamic nuclei were hypothesized to function as a thalamocortical arousal system and regulate arousal, attention, and awareness (Bentivoglio et al., 1991; Groenewegen and Berendse, 1994; Van der Werf et al., 2002). While these functions remain true for the PVT afferents from brainstem regions and certain cortical and subcortical regions discussed above, the PVT, through its projections to the PFC, NAc and amygdala places it in a unique position to affect cortico-striatal and cortico-limbic mechanisms involved in reward and motivation (McGinty and Otis, 2020). Indeed, single unit recording experiments have demonstrated increased inhibitory tone in aPVT neurons to sucrose reward (Do-Monte et al., 2017). Similarly, single-cell calcium imaging experiments have demonstrated increased inhibitory tone in pPVT to NAc projections in response to sucrose-predictive cues (Otis et al., 2019). Additionally, excitatory glutamatergic afferents from PL to PVT were also inhibited in response to reward-predicting cues (Otis et al., 2017; Otis et al., 2019). The PVT projects to brain regions that are implicated in the control of drug-taking and drug-seeking behavior, such as the NAc, amygdala, BNST, and PFC (Moga et al., 1995; Kirouac, 2015). Although not initially

included in the neurocircuitry of addiction, recent evidence implicates the PVT in the modulation of drug-directed behavior. The PVT is activated by a single exposure to addictive drugs (ethanol, cocaine, amphetamine, morphine, cannabinoids, nicotine), exposure to cues/context previously paired with addictive drugs, and by reinstatement of drug seeking behaviors (Zhou and Zhu, 2019). Earlier findings demonstrated ethanol seeking selectively activated the PVT (Dayas et al., 2008; Hamlin et al., 2009). Additionally, potent, and selective activation of the PVT has been demonstrated during cocaine seeking that does not occur during natural reward seeking (Martin-Fardon and Boutrel, 2012). Functional inactivation of PVT interferes with addiction-related behaviors, including drug seeking, indicating recruitment of the PVT in motivational aspects of drug addiction. For example, PVT lesions prevented the psychomotor sensitization produced by repeated administrations of cocaine (Young and Deutch, 1998) and attenuated context-induced reinstatement of alcohol seeking (Hamlin et al., 2009). While expression of cocaine-conditioned place preference was attenuated by injecting GABA agonists into the PVT (Browning et al., 2014), injecting the sodium channel blocker, tetrodotoxin, into the PVT attenuated drug-primed reinstatement of cocaine seeking (James et al., 2010). Further evidence for the involvement of PVT in drug-dependent behaviors come from pathway-specific transient chemogenetic inactivation of PFC→pPVT neurons. When these projections were inhibited immediately after the end of cocaine self-administration, context-induced cocaine-seeking after 7 days of abstinence, and cue-induced reinstatement of cocaine-seeking after extinction was inhibited (Giannotti et al., 2018). A recent report in which transient inhibition of PVT neurons at the time of reinstatement testing enhanced cue-induced cocaine-seeking in goal-tracking rats (Kuhn et al., 2018). Additionally, specific inhibition of PL→PVT in sign-tracking rats decreased cue-induced reinstatement but not cocaine-induced reinstatement of cocaine-seeking (Kuhn et al., 2022) further corroborates functional

regulation of drug-seeking behaviors by the PVT. However, involvement of PL→PVT in anxiety-like behaviors during early withdrawal from cocaine has not been explored. Therefore, in the present study we assessed the involvement of the PL→PVT projection in cocaine-induced anxiety-like behavior during early withdrawal.

Assessing anxiety-driven behaviors

Anxiety-like behaviors have been evaluated frequently using two major paradigms – elevated plus maze (EPM) and open field test (OFT) (La-Vu et al., 2020). A modification of the EPM is the elevated zero maze (EZM) that is a continuous circle with two open areas and two closed areas. Increases in anxiety-like behaviors are indicated by decreased time spent in open areas, increased latency to enter open areas, and fewer entries into open areas (Shepherd et al., 1994). In the OFT, thigmotaxis, time spent in the center, entries into the center, increased latency to enter the center are measures of anxiety-like behavior (Seibenhener and Wooten, 2015; La-Vu et al., 2020). Therefore, in this study, we investigated the role of the PL→pPVT pathway in male and female rats during cocaine withdrawal-induced anxiety-like behaviors using these two tasks. We hypothesized that chemogenetic inhibition of PL→pPVT immediately after the last cocaine self-administration session will decrease anxiety-like behaviors 2 hr later. We compared the anxiety-like behavior of male and female Sprague Dawley rats in whom the PL→pPVT pathway was chemogenetically inhibited using EZM and OFT. We also assessed the influence of inhibiting this pathway/anxiogenic effects of cocaine withdrawal on cue-induced relapse to cocaine seeking.

Chapter 3: Materials and Methods

Animals: A total of 66 (34 male and 32 female) wildtype Sprague Dawley rats (WT SD) were assigned for this study. Of these, 13 animals did not survive surgery, and 4 animals did not complete self-administration due to catheter patency failing or self-administration not acquired. Finally, 23 males and 26 females (N = 49) weighing approximately 300g at study onset were used for cocaine or yoked-saline SA training. All animals were purchased from Charles River/Envigo and were single housed upon arrival in a humidity/temperature-controlled colony room within the MUSC Division of Laboratory Animal Resources (MUSC DLAR) facility. All animals were maintained on a 12:12 h reversed light-dark cycle (lights off at 0700 h). Animals were provided with *ad libitum* food and water upon arrival, while recovering from surgical procedures, and during home cage abstinence. Animals were food-restricted to 20 g rat chow 24 h prior to, and throughout cocaine SA training. Surgical and experimental procedures were conducted in accordance with the rules and regulations set forth by the Institutional Care and Use Committee (IACUC) of the Medical University of South Carolina and were in accordance with the National Institutes of Health guidelines.

Chemicals/Drugs and viral vectors: Cocaine hydrochloride (cocaine HCl) was obtained through the National Institute on Drug Abuse (NIDA) Drug Supply Program (NIH NIDA, Baltimore MS, USA). Chemicals/drugs used during surgeries included ketamine (VetOne, Boise ID, USA), xylazine (Thermo Fisher Scientific, Ward Hill MA, USA), ketorolac (Sigma-Aldrich, St. Louis MO, USA), cefazolin (Sandoz, Princeton NJ, USA), isoflurane (Piramal Pharma, Telangana, India), taurolidine citrate lock solution (TCS; Access Technologies, Skokie IL, USA), and sterile saline (Baxter, Deerfield IL, USA). Clozapine *N*-oxide dihydrochloride (CNO; Hello Bio.com) was used as a ligand for activating the Gi-coupled hM4D DREADD. Viruses used included AAV1-hSyn-DIO-hM4Di-mCherry (pAAV-hSyn-DIO-hM4D(Gi)-mCherry, RRID:Addgene_44362), AAV2-hSyn-DIO-mCherry (pAAV-hSyn-DIO-mCherry, RRID:Addgene_50459) and AAVrg-Ef1a-Cre-mCherry

(pAAV-Ef1a-mCherry-IRES-Cre, RRID: Addgene_55632).

Intrajugular catheterization and intracranial viral Infusions: All surgeries were performed after at least 3 days of homecage acclimation and prior to the start of cocaine or yoked-saline SA training. For chemogenetic inhibition of PL→pPVT pathway, a combinatorial AAV vector approach was used. This approach combined intracranial administration of a retrograde cre-expressing AAV viral vector in the pPVT and double-floxed AAV viral vector in the PL for targeted expression of Gi-coupled hM4Di DREADD (N = 23) or control virus (N = 26) in PL neurons projecting to the pPVT. Intrajugular catheterization and intracranial viral infusion procedures were performed as previously described (Giannotti et al., 2018; Siemsen et al., 2019).

Briefly, rats were intraperitoneally (i.p.) injected with a ketamine (66 mg/kg)/xylazine (1.33 mg/kg) cocktail for initial induction of anesthesia and maintained on 0.5-1% isoflurane throughout the surgical procedures. A silastic catheter was implanted into the right jugular vein attached to a catheter access button (SAI infusion technologies, Lake Villa IL, USA). To maintain catheter patency during recovery and throughout SA training, i.v. catheters were flushed with TCS daily. Immediately following intrajugular catheter implantation, anesthetized animals were head-fixed in a stereotaxic instrument (Model 940; David Kopf Instruments, Tujunga CA, USA). Burr holes were drilled at appropriate locations on the skull. 0.75 µL/hemisphere of AAV1-hSyn-DIO-hM4Di-mCherry or AAV2-hSyn-DIO-mCherry was bilaterally injected into PL (coordinates from bregma: +2.8 mm AP, ±0.6 mm ML, -3.8 mm DV) over 5 minutes using a Nanoject II microinjector (Drummond Scientific, Broomall PA, USA). Injectors were left in place for an additional 5 minutes to allow for viral diffusion. 0.75 µL of AAVrg-Ef1a-Cre-mCherry was unilaterally injected into the pPVT (coordinates from bregma: −3.2 mm AP, 2.42 mm ML, −5.70 mm DV at an angle of 25°) using a Nanoject II microinjector and left in place for an additional 5 minutes for the virus to diffuse.

Immediately after surgeries, animals were removed from the sterotaxic apparatus and placed in a heated, clean cage until sternally recumbent and demonstrating voluntary movements. Post surgeries, animals were returned to their home cages and allowed to recover for 7-10 days. During recovery animals were handled, checked for health issues, appropriate recovery, and flushed with 0.05 mL of TCS to maintain catheter patency daily.

Behavior:

Self-Administration. After recovery, all rats underwent cocaine/yoked-saline SA training for 14 days. The SA procedure was followed as previously described (Giannotti et al., 2018; Siemsen et al., 2019) and took place 6 days/week between 1100 and 1300 hours. Briefly, cocaine SA (N = 27) or yoked-saline infusions (N = 22) were conducted in standard operant chambers equipped with two retractable levers, a cue light, and a tone generator (Med Associates, Fairfax VT, USA). Each SA session began with the illumination of the house light and extension of the two levers. Rats were trained for 14d (2 h/day) to lever press for cocaine on a fixed ratio 1 reinforcement schedule. Pressing the active lever resulted in delivery of the light and tone followed by an intravenous (i.v.) infusion of 200µg/50µL (days 1-14) cocaine HCl dissolved in sterile saline. This was followed by a 20s time-out period during which pressing of either lever did not result in any programmed consequences. Both active and inactive lever presses, and number of infusions received were recorded. Yoked-saline rats were run on a prerecorded program to receive 10 i.v. infusions of sterile saline at random intervals over 2 h. Active and inactive lever presses were also recorded in these rats. Criteria for acquisition of cocaine SA was ≥ 10 infusions/session for 14 days. Animals that received less than 10 infusions on any day were trained for additional days. During the time course of cocaine SA training, if trend in lever pressing was altered inexplicably, animals were tested for catheter patency with a single 0.05 – 0.10 mL i.v. infusion of brevital (sodium methohexital;

10mg/ml) to validate catheter patency. Any animal failing to respond to brevital administration underwent recatheterization followed by a period of recovery. If recatherterization was performed, cocaine SA training in animals was continued or restarted depending on their progress and performance across the SA training procedure.

Elevated zero maze*:* Two hours following the last cocaine/yoked saline SA session, all animals were tested for anxiety-like behaviors using the EZM test for 6 minutes. This time duration for EZM test was used following original EZM test protocol established by Shepherd and colleagues (Shepherd et al., 1994). The EZM apparatus consists of an annular elevated platform with two enclosed areas and two open areas. Animals were placed at the junction of open and enclosed areas facing the open area and allowed to freely explore the maze. Behaviors were recorded and anxiety-like behaviors were quantified both digitally and manually. Between animals, the platform was cleaned using 10% ethanol/water solution and dried thoroughly.

The design of the maze was based on that originally proposed by Shepherd and colleagues (Shepherd et al., 1994). Briefly, the maze consisted of a black acrylic annular platform with a diameter of 105 cm diameter and width of 10 cm. This platform was elevated to 65 cm from the ground. Two opposite quadrants of the platform were enclosed with black acrylic walls (27 cm in height) on either edge. The remaining two quadrants were left open. The maze was enclosed using 100% light blocking curtains and was only illuminated with dim lighting (intensity of 40 lux).

A camera connected to a computer was suspended from the ceiling and recorded the behavior of the animal for subsequent analyses (Shepherd et al., 1994). Ethovision software (Noldus, Leesburg VA, USA) was used to quantify anxiety-like behaviors from video recordings of animals undergoing the EZM test. Behaviors measured digitally included latency to enter open area, number of explorations of the open area (number of times animal crosses the open-closed divide), and the

total time spent in the open areas. Number of entries into the open areas (number of times animal moves from closed areas into open areas) was scored manually from recorded videos. Criteria used for this measure was when all four paws of the animal had crossed over the open-close divide into the open area.

Open field test. Immediately following the EZM test, anxiety-like behaviors in animals were tested using OFT for 30 minutes where thigmotaxis revealed high anxiety and more time spend in the center revealed less anxiety. The OFT is one of the most commonly used behavioral testing methods for assessing anxiety-like behaviors. The OFT apparatus consists of square or cylindrical arena with clear walls and an open ceiling and uses time spent in the center of the arena as an assessment of anxiety-like behavior (La-Vu et al., 2020).

In this study, we used square-shaped, plexiglass-covered locomotor boxes with open tops for open field testing. The locomotor boxes (Omnitech Electronics Inc., Columbus OH, USA) were approx. 12x12 inches, enclosed by clear plexiglass walls with infrared sensors that recorded the location of the animal within the arena of the box. This location data of the animal was digitally analyzed using VersaMax software (Omnitech Elctronics Inc., Columbus OH, USA). Prior to the start of OFT, the software was programmed to identify central 8x8 inches of the arena as zone 1 (center) and an internal path (4 inch wide) along each side as zone 2 (periphery) to delineate the center of the box from the periphery. Animals were placed in the center of the box and allowed to freely explore for 30 minutes. The software recorded the location of the animal and analyzed the time spent by the animal in each zone. Individual locomotor boxes were used for all animals. Between animal cohorts, locomotor boxes were cleaned with 70% ethanol/water solution and thoroughly dried and ventilated.

Cocaine abstinence and post-abstinence testing. After anxiety testing was completed, animals were

returned to their home cages and underwent forced abstinence for approximately 6 days. On the 7th day, all animals underwent a 2 h cue-induced relapse test under extinction conditions as previously described (Giannotti et al., 2018). Briefly, rats were placed in the same operant chamber where they had previously undergone cocaine/yoked-saline SA training for 2 h. During the test, both light and tone cues accompanied active lever presses, but no drug was delivered. Active and inactive lever presses were recorded. Active lever pressing during cue test was compared to the average of active lever presses on last three days of SA training. Rats from the cocaine SA group that pressed the active lever more than or equal to the number of average active lever presses on the last three days of cocaine SA were considered to have relapsed to cocaine seeking.

Perfusion, histology, mapping, and imaging: Immediately after the cue-induced relapse test, rats were anesthetized with 5 mg/kg, i.p. of 30% urethane (Thermo Fisher Scientific, NJ, USA) and perfused with 100 ml PBS followed by buffered paraformaldehyde (PFA: 4% PFA in 0.1M PBS; Sigma-Aldrich, St. Louis MO, USA) and the brains were extracted. Brains were postfixed in PFA for 24 h and then the fixative was replaced with 1X PBS. Coronal brain slices of the PL and PVT were cut in ice-cold 1X PBS at 100 μm thickness using a vibratome (Leica 1200S, Leica Biosystems, Nussloch, Germany). The slices were stored and preserved in 1X PBS containing sodium azide. PL- and PVT-containing sections were mounted and imaged using a Leica Thunder microscope and Leica imaging software (Leica Microsystems, Wetzlar, Germany) for mCherry expression to verify intracranial viral injection sites in both PL and PVT, and the expression of Gi-coupled hM4D DREADD or control virus in the PL. Sections with maximal mCherry expression were mapped using Adobe Illustrator software (Adobe, USA). Intracranial injection placements were anatomically verified using a standard rat atlas (Paxinos and Watson, 2018).

Experimental Design: Figure 1 shows a schematic of the experimental design. Following recovery from intracranial and i.v. catheter surgeries, the rats were allowed to recover for 4-7 days. To assess the influence of PL→pPVT inhibition on cocaine withdrawal-induced anxiety, the rats underwent cocaine SA for 14 days while their yoked-saline counterparts received saline infusions. Immediately after the last cocaine or yoked saline session, all animals were injected i.p. with 5 mg/kg of CNO. Two hours after CNO administration, animals were put in to the EZM for 5 minutes. Latency to enter open arms, number of explorations, and time spent in open arms of the EZM were digitally computed using Ethovision software (Noldus). Immediately after the EZM test, animals were put into the OFT apparatus and allowed to explore for 30 minutes. Time spent in the center versus time spent in the peripheral zone were digitally acquired using VersaMax software. Cocaine withdrawal-induced anxiety was assessed 2 h after the last cocaine or yoked-saline SA session based on a previous study from our group which showed that rescuing a cocaine SA-induced decrease in plasticity-related phosphoproteins in PL cortex at this time point decreased subsequent cocaine seeking (Whitfield et al., 2011). Following OFT, all animals were returned to their homecages and underwent 6 d forced homecage abstinence. Data from the EZM test and OFT were used to assess anxiogenic behaviors in both PL→pPVT-inhibited animals and controls.

On the seventh day of abstinence, all animals underwent a 2 h cue-induced relapse test to assess the relationship between inhibition of cocaine withdrawal-induced anxiety during acute withdrawal on relapse to cocaine seeking. Immediately following the cue test, rats were exsanguinated and perfused with 4% PFA in PBS under anesthesia. Post perfusion, rats were decapitated, and brains were extracted and collected for verification of DREADD or control expression in PL and accuracy of intracranial injections in both PL and pPVT.

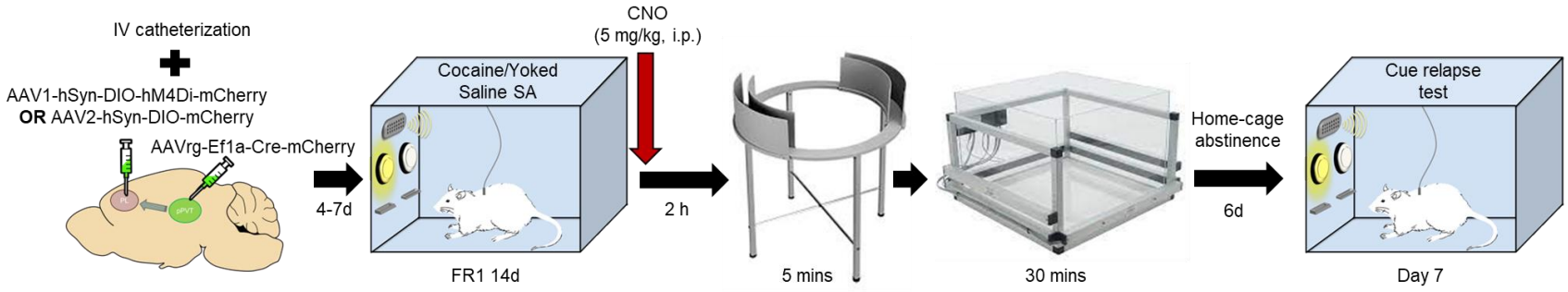

Figure 1. Schematic of experimental design.

Data processing and statistical analyses: Data from SA, EZM, and OFT were compiled onto Excel spreadsheets. These data were imported and graphed using GraphPad Prism software (Dotmatics, Boston MA, USA). Prior to statistical analyses, data from 10 animals with off-target viral infusions were excluded from EZM, OFT and cue-induced relapse test datasets. Outliers were assessed by employing ROUT outlier test in GraphPad Prism software, and individually excluded from datasets of anxiety measurements prior to statistical analyses. Final sample sizes of datasets for each group used for statistical analyses have been included in figure legends. Statistical analyses were conducted using SPSS software (IBM, Armonk NY, USA) and included mixed and factorial ANOVAs followed by Bonferroni-corrected pairwise comparisons when appropriate. Wherever assumptions of sphericity were violated, Greenhouse-Geisser corrected degrees of freedom have been reported. Post-hoc power analyses conducted using G*power software demonstrated that our study was not sufficiently powered to assess sex differences. Therefore, data from both males and females have been pooled and analyzed together.

Chapter 4: Results

Validation of intracranial AVV viral vector placements

Most injections for PL were verified to be within +2.5 to +4.2 mm from bregma (Figure 2A) and those for pPVT lay within -3.00 to -3.72 mm from bregma (Figure 2B). mCherry expression in PL cortex for most animals was at AP coordinates +3.7 mm from bregma (Figure 2C) while that for pPVT was between AP coordinates -3.2 to -3.4 mm from bregma (Figure 2D). A total of 11 rats had infusions that missed the target or lacked fluorophore expression and were excluded from analysis for anxiety-like behavioral measurements and cue-induced relapse test.

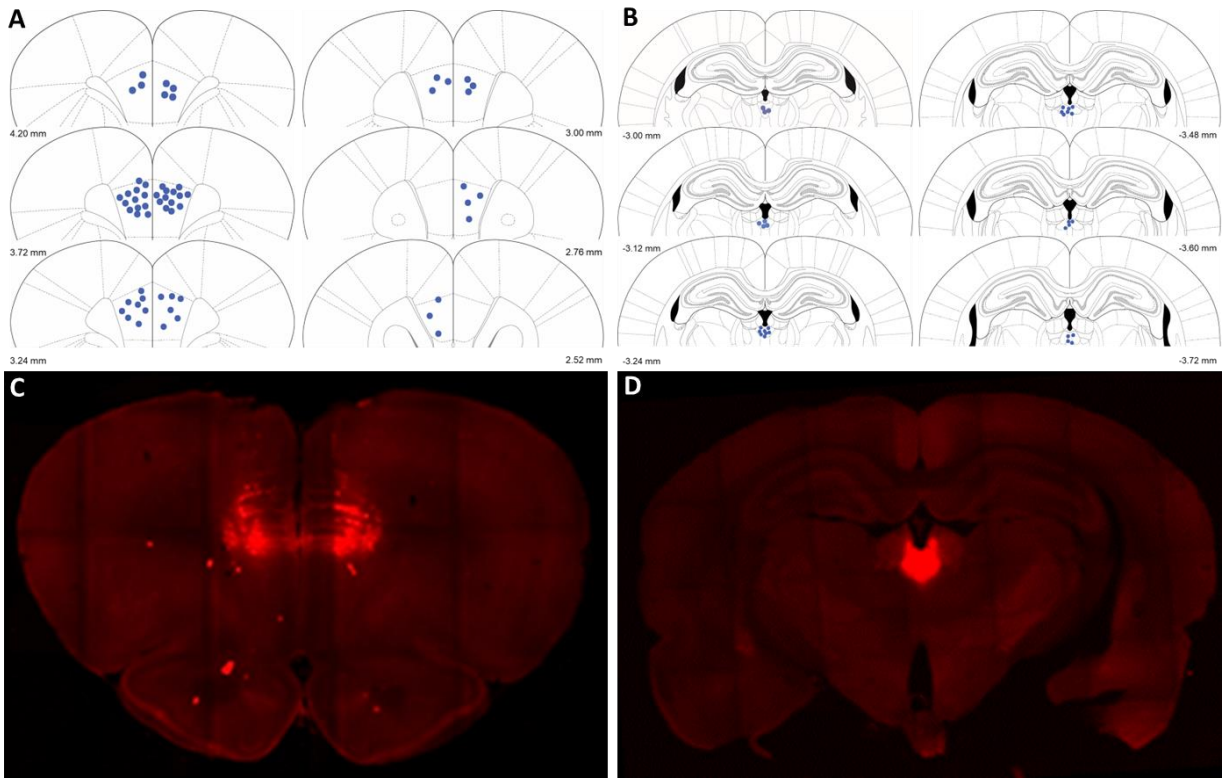

Figure 2. Mapping of viral expression in PL cortex and pPVT.

A. Schematic mapping of PL cortex coronal sections (Paxinos and Watson, 2018) with viral injection sites (blue dots). All viral injection sites were confirmed to be within the PL cortex (anteroposterior coordinates from bregma: +2.5 to +4.2 mm). **B**. Schematic mapping of pPVT coronal sections (Paxinos and Watson, 2018) with viral injection sites (blue dots). All viral injection sites were confirmed to be within the pPVT (anteroposterior coordinates from bregma: -3.0 to -3.8 mm). **C**. Sample image of AAV1-hSyn-DIO-hM4Di-mCherry or AAV2-hSyn-DIO-mCherry viral expression in PL cortex. **D**. Sample image of AAVrg-Ef1a-Cre-mCherry viral expression in pPVT.

Acquisition and maintenance of cocaine self-administration

To determine if animals acquired cocaine SA, we compared active versus inactive lever pressing in both DREAAD-expressing and non-expressing rats that underwent cocaine and yoked saline SA training across the 14 days of training procedure. A four-way (Within-subjects factors – Number of lever presses: Active/Inactive; Number of SA days – 1-14, and between-subjects factors – Drug: Saline/Cocaine; Group: DREADD/Non-DREADD) mixed ANOVA was used to assess statistically significant differences.

Analyses revealed that lever pressing was significantly different between cocaine versus saline treated rats (significant interaction between number of lever presses and cocaine vs saline treatments; $F_{(1,\ 45)} = 17.185$, $p < 0.001$; Figure 3A). Specifically, active lever presses were significantly greater than inactive lever presses in both DREADD-expressing and non-expressing rats that underwent cocaine SA training compared to yoked-saline controls. However, active, or inactive lever pressing did not significantly differ between DREADD-expressing and non-expressing cocaine and yoked-saline groups (no significant interaction between lever pressing and DREADD/Non-DREADD groups; $F_{(1,\ 45)} = 1.369$, $p = 0.248$). There was also a significant main effect of number of SA days ($F_{(2.09,\ 93.91)} = 3.328$, $p = 0.038$) indicating that within groups, saline and cocaine treated rats differed in their lever pressing across the 14 SA training days. Specifically, number of active lever presses remained significantly greater in rats undergoing cocaine SA training compared to yoked-saline controls across the days of SA training ($p < 0.001$). Whereas the number of inactive lever presses did not differ between cocaine- vs yoked-saline SA rats across the 14 days of SA training ($p > 0.05$; Figure 3A). Additionally, there was no significant difference in lever pressing behavior between DREADD-expressing or non-expressing rats across the 14 days

of SA training (no significant interaction effect of DREADD expression between cocaine vs saline rats in lever pressing across SA days; $F_{(2.00,\ 90.18)} = 0.512$, $p = 0.609$; Figure 3A). These data demonstrate that both DREADD-expressing and non-expressing rats acquired and maintained cocaine SA. As expected DREADD expression did not alter cocaine SA behavior, nor did it affect acquisition or maintenance of cocaine SA.

A separate three-way (Within-subjects factor – Number of infusions across 14 days of SA training, and between-subjects factors – Drug: Saline/Cocaine; Group: DREADD/Non-DREADD) mixed ANOVA revealed that there were no significant differences in the number of cocaine or saline infusions received between DREADD-expressing and non-expressing rats across the 14 days of SA training (no significant interaction between number of cocaine or saline infusions received between DREADD-expressing and non-expressing rats; $F_{(4.10,\ 184.32)} = 0.613$, $p = 0.685$; Figure 3B).

Together, these data demonstrate that DREADD expression did not alter cocaine or yoked saline SA behavior in animals. This was expected since during SA training, DREADDs were not activated.

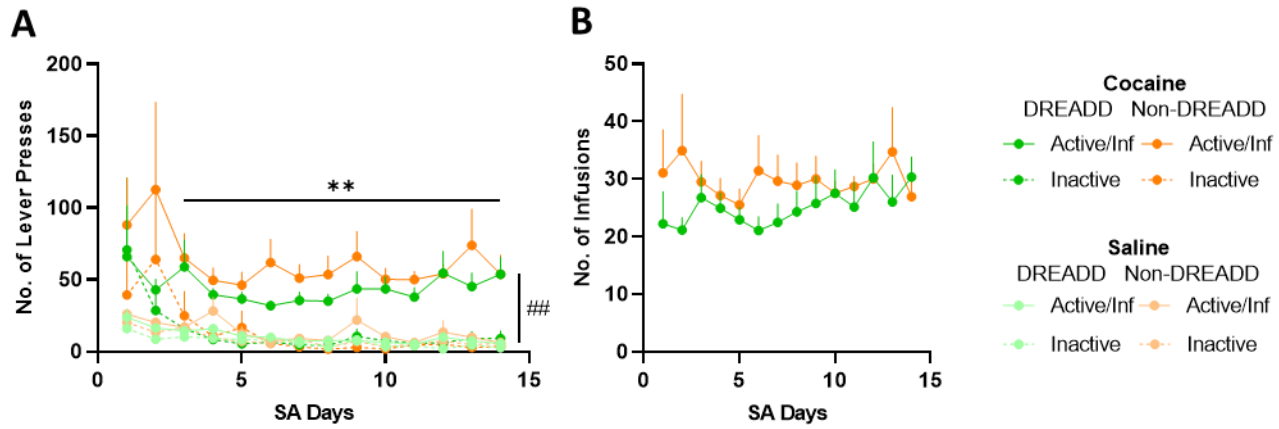

Figure 3. Cocaine and yoked saline SA.

Summary graph of lever pressing behavior (**A**) and cocaine or saline infusions received (**B**) in all animals during cocaine and yoked-saline SA training. **A**. Differences in active (solid lines) and inactive (dotted lines) lever presses between DREADD-expressing cocaine (green; $N = 13$) and yoked-saline (light green; $N = 10$) rats, and non-DREADD expressing cocaine (orange; $N = 14$) and yoked-saline (light orange; $N = 12$) rats across 14 days of SA training have been plotted. Active lever presses for both DREADD-expressing and non-expressing cocaine SA rats were significantly greater than inactive lever presses ($^{**}p < 0.01$) across the 14 days of SA training. Active lever presses for both DREADD-expressing and non-expressing cocaine SA rats were significantly greater than active and inactive lever presses in yoked-saline DREADD-expressing and non-expressing rats ($^{\#\#}p < 0.01$). **B**. Cocaine infusions between DREADD-expressing (green) and non-expressing (orange) rats did not significantly differ across the 14 days of SA training.

Chemogenetic inhibition of PL→pPVT altered anxiety-like behaviors in animals with cocaine experience but not in yoked saline controls.

We hypothesized that anxiety-like behaviors would be greater in animals with cocaine experience than in yoked-saline controls. Further, chemogenetic inhibition of PL→pPVT during acute withdrawal from cocaine would attenuate cocaine withdrawal-induced anxiety-like behaviors. We used EZM and OFT behavioral paradigms to test these hypotheses.

To assess differences in anxiety-like behaviors in the EZM, a multivariate two-way (Between-subjects factors – Drug: Saline/Cocaine; Group: DREADD/Non-DREADD) ANOVA was conducted. The number of entries into the open arms (no significant interaction effect of DREADD expression between cocaine vs saline rats in number of open arm entries; $F_{(1, 28)} = 0.312$, $p = 0.518$; Figure 4A) and time spent in open arms (no significant interaction effect of DREADD expression between cocaine vs saline rats in time spent in open arms; $F_{(1, 28)} = 1.339$, $p = 0.257$; Figure 4B) did not significantly differ between DREADD-expressing or non-expressing cocaine or yoked-saline SA rats. Overall, latency to enter open arms did not significantly differ between DREADD-expressing and non-expressing cocaine or yoked-saline SA rats (no significant interaction effect of DREADD expression between cocaine vs saline rats in time spent in open arms; $F_{(1, 28)} = 1.739$, $p = 0.198$). However, latency to enter open arms was significantly lower in DREADD-expressing cocaine SA rats compared to DREADD-expressing yoked-saline SA rats ($p = 0.046$; Figure 4C). The number of explorations of the open arms was significantly different between DREADD-expressing and non-expressing cocaine or yoked-saline SA rats (significant interaction effect of DREADD expression between cocaine vs saline rats in time spent in open arms; $F_{(1, 28)} = 5.827$, $p = 0.023$; Figure 4D). Specifically, and paradoxically, chemogenetic inhibition of PL→pPVT significantly decreased the number of explorations during acute

withdrawal from cocaine in cocaine SA rats compared to yoked-saline controls (p = 0.002). Additionally, anxiety-like behaviors assessed during the EZM test were not altered by chemogenetic inhibition of PL→pPVT in DREADD-expressing or non-expressing yoked-saline SA rats (Figure 4A-D). No significant differences were observed in latency to enter open arm, time spent in open arm, number of entries into the open arm, number of open arm explorations between non-DREADD expressing cocaine or yoked-saline SA rats, suggesting that anxiety-like behaviors in rats with a cocaine history did not differ from saline control rats in the EZM.

To assess the influence of chemogenetic inhibition of PL→pPVT during acute cocaine withdrawal using the OFT, a separate two-way (Between-subjects factors – Drug: Saline/Cocaine; Group: DREADD/Non-DREADD) ANOVA was used. Analyses revealed that DREADD expression had a significant effect on time spent in the center of the open field (significant main effect of group: DREADD/Non-DREADD on time spent in center; $F_{(1, 35)}$ = 7.892, p = 0.008; Figure 5). Specifically, chemogenetic inhibition of PL→pPVT significantly increased time spent in the center of the open field in DREADD-expressing cocaine SA animals compared to non-expressing cocaine SA animals (p = 0.022). Time spent in the center of the open field did not significantly differ between DREADD-expressing or non-expressing yoked-saline controls (p = 0.114; Figure 5). We found no significant differences in time spent in the center of the open field (p = 0.940) between non-DREADD expressing cocaine or yoked-saline SA rats, suggesting that anxiety-like behaviors in rats with a cocaine history did not differ from saline control rats in the OFT.

Therefore, our data demonstrate that PL→pPVT inhibition decreased the latency to enter open arms but increased the number of explorations of the open arms in rats with cocaine experience compared to rats that did not receive cocaine. Additionally, this inhibition also

increased the time spent in the center of the open field in rats with cocaine experience. Together, these data suggest that PL→pPVT may be involved in aspects of the anxiety phenotype associated with exploratory behaviors and that inhibition of this pathway attenuates anxiety-induced decrease in exploratory behaviors. A major caveat of this finding was that acute withdrawal from cocaine did not cause more anxiety-like behavior than in saline-treated rats.

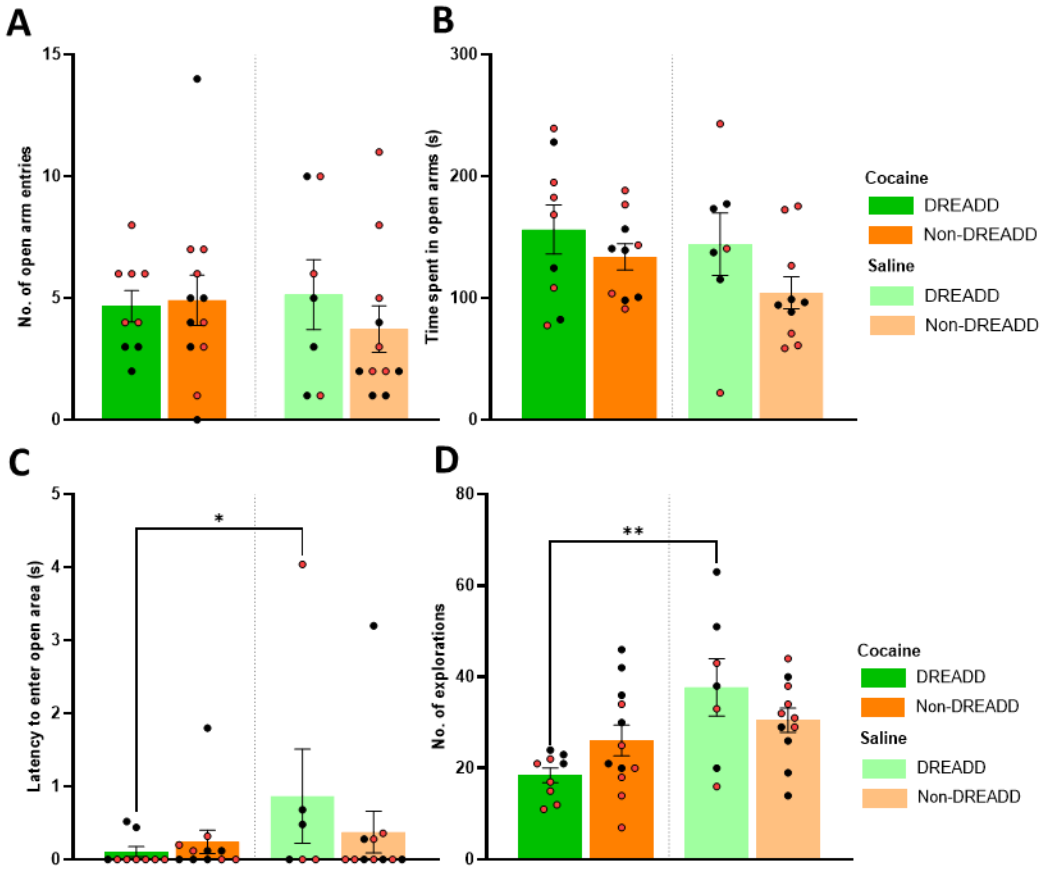

Figure 4. Effect of chemogenetic inhibtion of PL→pPVT during EZM.

Summary graphs of anxiety-like behaviors during EZM test. **A**. Number of open arm entries did not differ between DREADD-expressing (green; *N* = 9) and non-expressing (orange; *N* = 12) cocaine SA rats or DREADD-expressing (light green; *N* = 7) and non-expressing (light orange; *N* = 11) yoked-saline rats. **B**. Time spent in open arms did not differ between DREADD-expressing (green; *N* = 9) and non-expressing (orange; *N* = 10) cocaine SA rats or DREADD-expressing (light green; *N* = 7) and non-expressing (light orange; *N* = 10) yoked-saline rats. **C**. Latency to enter open arms was significantly decreased in DREADD-expressing cocaine SA rats (green; *N* = 9) compared to DREADD-expressing yoked-saline controls (light green; *N* = 6; $^{*}p < 0.05$). Latency to enter open arms did not significantly differ between non-DREADD expressing cocaine SA rats (orange; *N* = 11) and DREADD-expressing (light green; *N* = 6) and non-

expressing (light orange; $N = 11$) yoked-saline SA rats. **D**. Number of explorations of the open arms was significantly decreased in DREADD-expressing cocaine SA rats (green; $N = 9$) compared to DREADD-expressing yoked-saline controls (light green; $N = 7$; $^{*}p < 0.05$). Number of explorations of the open arms did not significantly differ between non-DREADD expressing cocaine SA rats (orange; $N = 12$) and DREADD-expressing (light green; $N = 7$) and non-expressing (light orange; $N = 11$) yoked-saline SA rats. Red circles represent data points for female rats and black circles represent data points for male rats.

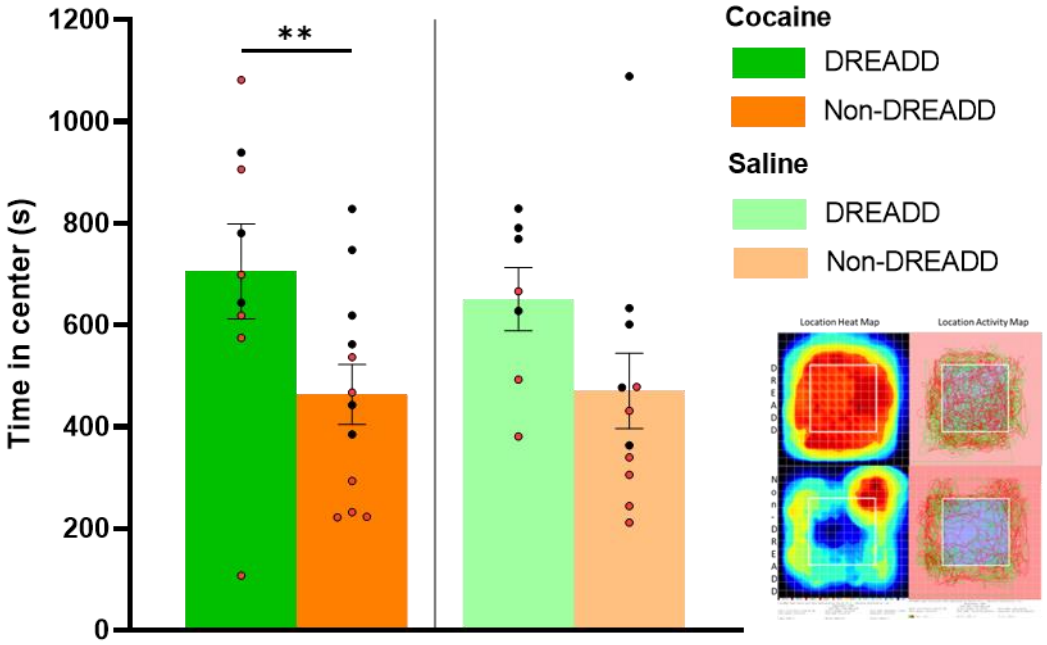

Figure 5. Chemogenetic inhibtion of PL→pPVT decreased anxiety-like behavior during OFT. Inhibition of PL→pPVT in DREADD-expressing (green; $N = 9$) cocaine SA rats significantly increased time spent in the center of the open field arena compared to non-DREADD expressing (orange; $N = 12$) cocaine SA rats ($^{**}p < 0.01$). Time spent in the center of the open field arena did not significantly differ between DREADD-expressing (light green; $N = 7$) and non-expressing (light orange; $N = 11$) yoked-saline SA rats and non-DREADD expressing cocaine SA rats. Insert demonstrates an example of location heat map (left) and location activity map (right) of DREADD-expressing (top) and non-expressing (bottom) rats. Red circles represent data points for female rats and black circles represent data points for male rats.

Chemogenetic inhibition of PL→pPVT during acute withdrawal from cocaine attenuated cue-induced relapse to cocaine seeking after one week of abstinence.

To confirm that inhibition of PL→pPVT immediately after the last cocaine SA session attenuated relapse to cocaine seeking after 7 days of forced home-cage abstinence as previously described (Giannotti et al., 2018), we compared average active lever presses during the last 3 cocaine SA sessions to active lever presses during a single cue-induced relapse test. During the cue-induced relapse test, drug infusions were not a consequence associated with discriminatory lever pressing. Therefore, we did not compare the differences in inactive lever presses between groups. A three-way (Within-subjects factors – Active lever presses: Average active lever presses during last 3 SA sessions/Active lever presses during cued relapse test; Between-subjects factors – Drug: Saline/Cocaine; Group: DREADD/Non-DREADD) mixed ANOVA was used to assess these differences.

Our analysis revealed that active lever presses during the last 3 SA sessions and cue-induced relapse test were significantly different between cocaine vs yoked-saline rats (significant interaction between active lever presses and drug groups; $F_{(1, 32)} = 8.667$, $p = 0.006$). Specifically, both DREADD-expressing ($p = 0.002$) and non-expressing ($p < 0.001$) cocaine SA rats had a significantly greater average number of active lever presses during the last 3 SA sessions than yoked-saline controls (Figure 6). Similarly, the average number of active lever presses was significantly greater in DREADD-expressing ($p = 0.016$) and non-expressing ($p < 0.001$) cocaine SA rats during the cue-induced relapse test than yoked-saline controls. While non-DREADD-expressing cocaine SA rats had a significantly greater number of active lever presses during the cue-induced relapse test compared to those during the last 3 cocaine SA sessions ($p < 0.001$), DREADD-expressing rats did not (Figure 6). Additionally, non-DREADD-expressing cocaine SA

rats had a significantly greater number of active lever presses during the cue test compared to DREADD-expressing cocaine SA rats ($p = 0.038$; Figure 6).

Together, these data demonstrate that cocaine SA and cue-induced relapse to cocaine seeking increased active lever pressing. Additionally, non-DREADD expressing cocaine SA rats reliably relapse to cue-induced cocaine seeking after 7 days of forced home cage abstinence. Furthermore, PL→pPVT inhibition immediately after the last cocaine SA session decreased cue-induced relapse to cocaine seeking after 7 days of forced home-cage abstinence, confirming the previous findings of (Giannotti et al., 2018).

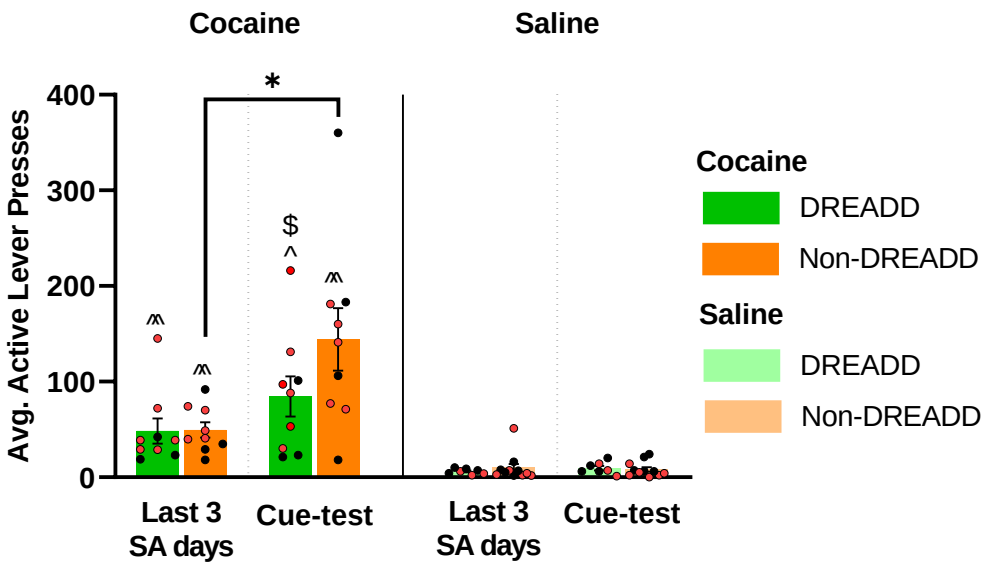

Figure 6. Inhibition of PL→pPVT prevented cue-induced relapse to cocaine seeking. Summary graph of differences in active lever presses during last 3 SA sessions and cue-induced relapse test between DREADD-expressing and non-expressing cocaine and yoked-saline SA rats. Dark colored bars represent the average number of active lever presses of DREADD expressing (green; $N = 9$) and non-expressing (orange; $N = 9$) cocaine SA rats, and light-colored bars represent DREADD-expressing (light green; $N = 7$) and non-expressing (light orange $N = 11$) yoked-saline SA rats. The average number of active lever presses in all cocaine SA rats was significantly greater than those of yoked-saline SA rats ($^{\wedge, \wedge\wedge}p < 0.05, 0.01$). The average number of active lever presses during the cue-induced relapse test was significantly greater than the average number of active presses during the last 3 cocaine SA sessions in non-DREADD expressing cocaine SA rats ($^{*}p < 0.05$). The average number of active lever presses during the cue-induced relapse test was significantly lower in DREADD-expressing cocaine SA rats than in non-DREADD expressing cocaine SA rats ($^{\$}p < 0.05$). Red circles represent data points for female rats and black circles represent data points for male rats.

Chapter 5: Discussions

While there is evidence implicating both PL and PVT separately in behaviors associated with cocaine addiction and anxiety (Kirouac, 2015; Koob and Volkow, 2016; Millan et al., 2017; Jacobs and Moghaddam, 2021a; Kirouac, 2021), no study to our knowledge has assessed the involvement of PL→pPVT projections in cocaine withdrawal-induced anxiety. Here, we have demonstrated that the PL→pPVT projection is recruited during acute withdrawal from cocaine and influences approach and exploratory behaviors in rats. Specifically, inhibition of this projection is necessary for the attenuation of decreased approach and exploratory behaviors in rats. This effect was not seen in yoked-saline controls suggesting specific recruitment of this projection following cocaine experience. Additionally, we corroborated the involvement of this projection in promoting cue-induced relapse to cocaine seeking post 7 days of forced home-cage abstinence as shown previously (Giannotti et al., 2018).

Assays of anxiety-like behaviors in rodents typically leverage the balance between exploratory behaviors of a novel environment ("approach") and innate desire to avoid unsafe, threatening environments (avoidance). Increase in reluctance to explore novel environments or avoidance of unsafe, threatening environments has been associated with an anxiety-like phenotype. OFT is one of the commonly used assays in testing anxiety-like behaviors in rodents (La-Vu et al., 2020). In the OFT, natural exploratory instincts versus aversion of exploring novel open environments is leveraged. Increased anxiety in rodents would deter them from exploring the center of the open field. During acute withdrawal from cocaine, inhibition of PL→pPVT increased the time spent by rats exploring the center, suggesting the involvement of this projection in cocaine withdrawal-induced anxiogenic effects. Unlike the OFT, which uses approach-avoidance conflict associated with the exploration of a novel environment, the EZM test leverages distinct conflicting behaviors. Specifically, the EZM test leverages natural exploration of a novel environment and the

innate aversion to heights and open spaces to assess anxiety-like behaviors. Because of this innate aversion, rodents prefer to spend more time in enclosed arms (Shepherd et al., 1994; La-Vu et al., 2020) and the proportion of exploratory behaviors of the open arm provides a measure of anxiety. Additionally, the EZM test provides more modalities to measure approach-avoidance conflicts. In the EZM test, PL→pPVT inhibition attenuated approach behaviors by reducing latency to enter the open arm but did not influence exploratory behaviors in animals experiencing acute cocaine withdrawal. However, exploratory behavior in the OFT was enhanced by inhibition of the PL→pPVT projection, in animals experiencing acute cocaine withdrawal. This finding suggests that: 1. PL→pPVT is recruited during more emotionally motivated risk assessment behaviors in the EZM but influences exploratory behaviors in OFT, and 2. PL→pPVT influences these behaviors in a cocaine-dependent manner, such that withdrawal from cocaine or previous cocaine experience is essential in its recruitment. Exactly how the PL→pPVT projection is involved in risk assessment (approach-avoidance) behaviors versus exploratory behaviors during cocaine withdrawal needs further investigation. Analyses of head dips and stretched attend postures during the EZM test should be included in future studies to validate the specific involvement of this pathway in risk assessment behaviors during the EZM test. Indeed, frequency of stretch attend postures, head dipping, and latency to enter the open arm are considered to be more sensitive indices of changes in emotional aspects of anxiety-like behaviors (Shepherd et al., 1994).

Based on findings from our study as well as previous research on cue-induced cocaine seeking, it seems likely that withdrawal from cocaine activates PL→pPVT since its inhibition during acute withdrawal from cocaine attenuated cue-induced cocaine seeking (Giannotti et al., 2018). Notably, inhibition of PL→pPVT immediately after the last cocaine experience was significant in inducing prolonged reduction in cocaine seeking. It is well known that rats with a

history of cocaine experience have motivational deficits. Inhibition of PL→pPVT immediately after the last cocaine experience may reduce these motivational deficits during cocaine withdrawal and therefore decrease cue-induced relapse to cocaine seeking. In contrast, during the EZM test and OFT, inhibition of PL→pPVT during early withdrawal from cocaine attenuated innate motivation to avoid "unsafe" open areas. Therefore, it is possible that PL→pPVT differentially modulates motivational aspects associated with risk taking behaviors, including cocaine seeking during early withdrawal and later abstinence.

Limitations and alternative future directions

Both clinical and preclinical investigations have proposed that negative mood states, characterized by irritability, anxiety, and reduced ability to experience pleasure, during withdrawal from cocaine contributes to increased probability for relapse to cocaine-seeking. A major caveat of this study was the lack of heightened anxiety induced by acute cocaine withdrawal. We have a few explanations for this result. Firstly, there is evidence suggesting inconsistencies in the intensity and duration of cocaine-withdrawal induced anxiety (Barbee and Gourley, 2022). To our knowledge, anxiety-like behaviors have never been measured 2 h after the last cocaine experience. The shortest duration after cocaine exposure that has demonstrated cocaine withdrawal-induced anxiety-like behaviors assessed with a light-dark box test was reported in mice a minimum of 6 h after repeated, non-contingent binge-like cocaine administration (Gasparyan et al., 2021). Perhaps for the development of cocaine withdrawal-induced anxiety, the 2 h time point or the 2 hr SA sessions were too short. Differences in anxiety-like behaviors become stronger after a longer duration of cocaine SA and withdrawal (Erb, 2010). Secondly, cocaine contingencies used to induce cocaine withdrawal-induced anxiety and behavioral assays used to assess it are inconsistent. For example, EPM/EZM provided most consistent assessments of cocaine withdrawal-induced

anxiety following non-contingent cocaine administration (Sarnyai et al., 1995; DeVries and Pert, 1998; Erb et al., 2006; de Oliveira Citó et al., 2012; El Hage et al., 2012). Studies that used a cocaine SA paradigm were inconsistent in their ability to detect cocaine withdrawal-induced anxiety using OFT or EPM/EZM (Mantsch et al., 2008; Buffalari et al., 2012; Bolton et al., 2018). Lastly, other tasks, such as a defensive burying task, is an alternative that may demonstrate cocaine withdrawal-induced anxiety with different modalities since it does not involve anxiogenic effects associated with novel environments (Barbee and Gourley, 2022). Notably, the defensive burying task was the only anxiety assay which was consistent in detection of cocaine withdrawal-induced anxiety in rats across different cocaine contingencies, albeit at later time points during withdrawal (Basso et al., 1999; Harris et al., 2001; Aujla et al., 2008; Buffalari et al., 2012; El Hage et al., 2012). Therefore, it is possible that anxiety-like behaviors measured by the defensive burying task may have higher sensitivity to detect cocaine withdrawal-induced anxiety phenotype in rodents and therefore be able to detect cocaine-withdrawal induced anxiety at earlier time-points. Future studies should include a defensive burying test as one of the tests assessing anxiety-like behaviors or conduct cocaine withdrawal-induced anxiety assessments at later time points during withdrawal.

Additionally, anxiety-like behaviors differ between male and female rats (Scholl et al., 2019) and it may be possible that sex differences exist in the regulation of cocaine withdrawal-induced anxiety mediated by the PL→pPVT projection. Table 1 has been included to show differences in anxiety measurements between males and females. Post-hoc power analyses for the final sample size of 39 animals conducted using G-power software demonstrated that our study was not sufficiently powered to assess sex differences (power = 0.388) in anxiety measurements. To assess sex differences, for an effect size of 0.08 with power of 0.8, we would need a total sample size of 81. A higher sample size should be able to rectify this limitation of our study. Surgical

complications and inconsistencies in viral vector expression in both PL and pPVT contributed to reduced sample sizes. Furthermore, the viruses used in our combinatorial approach to label and express DREADDs in PL→pPVT projections used the same mCherry fluorophore. Hence, validation of projection specific DREADD expression could not be conducted. Therefore, it is possible that some inconsistencies in our findings may arise from the non-specific expression of DREADD. Nevertheless, our study was able to provide preliminary evidence implicating the involvement of PL→pPVT in cocaine withdrawal-induced anxiety.

Table 1. Sex differences in anxiety measurements between groups

Anxiety-like behavior	Drug	Sex	Virus	Mean	± SEM
Latency to enter open arm (s)	Cocaine	Male	AAV1-hM4Di-mCherry	0.32	0.60
			AAV2-mCherry	0.41	0.46
		Female	AAV1-hM4Di-mCherry	0.00	0.42
			AAV2-mCherry	0.13	0.46
	Saline	Male	AAV1-hM4Di-mCherry	1.23	0.52
			AAV2-mCherry	0.80	0.52
		Female	AAV1-hM4Di-mCherry	1.35	0.60
			AAV2-mCherry	0.11	0.42
No. of explorations of open arm	Cocaine	Male	AAV1-hM4Di-mCherry	22.67	5.55
			AAV2-mCherry	31.80	4.30
		Female	AAV1-hM4Di-mCherry	16.33	3.93
			AAV2-mCherry	22.20	4.30
	Saline	Male	AAV1-hM4Di-mCherry	43.00	4.81
			AAV2-mCherry	22.00	4.81
		Female	AAV1-hM4Di-mCherry	30.67	5.55
			AAV2-mCherry	34.67	3.93
No. of entries into open arm	Cocaine	Male	AAV1-hM4Di-mCherry	2.67	1.53
			AAV2-mCherry	3.40	1.18
		Female	AAV1-hM4Di-mCherry	5.67	1.08

			AAV2-mCherry	5.40	1.18
		Male	AAV1-hM4Di-mCherry	4.75	1.32
	Saline		AAV2-mCherry	1.50	1.32
		Female	AAV1-hM4Di-mCherry	5.67	1.53
			AAV2-mCherry	5.17	1.08
Time spent in the open arm (s)	Cocaine	Male	AAV1-hM4Di-mCherry	145.11	31.07
			AAV2-mCherry	127.12	24.07
		Female	AAV1-hM4Di-mCherry	161.95	21.97
			AAV2-mCherry	140.70	24.07
	Saline	Male	AAV1-hM4Di-mCherry	150.92	26.91
			AAV2-mCherry	94.63	26.91
		Female	AAV1-hM4Di-mCherry	135.33	31.07
			AAV2-mCherry	110.98	21.97
Time spent in center of OFT (s)	Cocaine	Male	AAV1-hM4Di-mCherry	787.93	116.59
			AAV2-mCherry	597.28	82.44
		Female	AAV1-hM4Di-mCherry	664.38	82.44
			AAV2-mCherry	329.09	82.44
	Saline	Male	AAV1-hM4Di-mCherry	754.20	100.97
			AAV2-mCherry	632.92	90.31
		Female	AAV1-hM4Di-mCherry	513.31	116.59
			AAV2-mCherry	335.13	82.44

In conclusion, we demonstrated that the PL→pPVT projection influences aspects of the anxiety phenotype associated with approach and exploratory behaviors only in animals with previous cocaine experience. Therefore, this study provides preliminary evidence for the involvement of the PL→PVT pathway in mediating cocaine withdrawal-induced anxiety. Additionally, we were also able to corroborate previous findings that demonstrated immediate inhibition of this pathway after cocaine experience in reducing relapse probability during abstinence.

References

Aboutalebi F, Alaei H, Oryan S, Radahmadi M (2018) Blockade of prelimbic glutamate receptor reduces the reinforcing effect of morphine. Can J Physiol Pharmacol 96:815-822.

American Psychiatric Association D, Association AP (2013) Diagnostic and statistical manual of mental disorders: DSM-5: American psychiatric association Washington, DC.

Aujla H, Martin-Fardon R, Weiss F (2008) Rats with extended access to cocaine exhibit increased stress reactivity and sensitivity to the anxiolytic-like effects of the mGluR 2/3 agonist LY379268 during abstinence. Neuropsychopharmacology 33:1818-1826.

Barbee BR, Gourley SL (2022) Brain systems in cocaine abstinence-induced anxiety-like behavior in rodents: A review. Addiction Neuroscience 2:100012-100012.

Basso AM, Spina M, Rivier J, Vale W, Koob GF (1999) Corticotropin-releasing factor antagonist attenuates the "anxiogenic-like" effect in the defensive burying paradigm but not in the elevated plus-maze following chronic cocaine in rats. Psychopharmacology 145:21-30.

Basten U, Stelzel C, Fiebach CJ (2011) Trait anxiety modulates the neural efficiency of inhibitory control. Journal of cognitive neuroscience 23:3132-3145.

Basten U, Stelzel C, Fiebach CJ (2012) Trait anxiety and the neural efficiency of manipulation in working memory. Cognitive, Affective, & Behavioral Neuroscience 12:571-588.

Belin D, Everitt BJ (2008) Cocaine seeking habits depend upon dopamine-dependent serial connectivity linking the ventral with the dorsal striatum. Neuron 57:432-441.

Belin D, Jonkman S, Dickinson A, Robbins TW, Everitt BJ (2009) Parallel and interactive learning processes within the basal ganglia: relevance for the understanding of addiction. Behavioural brain research 199:89-102.

Bentivoglio M, Balercia G, Kruger L (1991) Chapter 4 The specificity of the nonspecific thalamus:

The midline nuclei. In: Progress in Brain Research (Holstege G, ed), pp 53-80: Elsevier.

Berglind WJ, Whitfield TW, Jr., LaLumiere RT, Kalivas PW, McGinty JF (2009) A single intra-PFC infusion of BDNF prevents cocaine-induced alterations in extracellular glutamate within the nucleus accumbens. J Neurosci 29:3715-3719.

Berridge MJ (2012) Calcium signalling remodelling and disease. Biochemical Society Transactions 40:297-309.

Bolla K, Ernst M, Kiehl K, Mouratidis M, Eldreth D, Contoreggi C, Matochik J, Kurian V, Cadet J, Kimes A, Funderburk F, London E (2004) Prefrontal Cortical Dysfunction in Abstinent Cocaine Abusers. The Journal of Neuropsychiatry and Clinical Neurosciences 16:456-464.

Bolton JL, Ruiz CM, Rismanchi N, Sanchez GA, Castillo E, Huang J, Cross C, Baram TZ, Mahler SV (2018) Early-life adversity facilitates acquisition of cocaine self-administration and induces persistent anhedonia. Neurobiology of Stress 8:57-67.

Browning JR, Jansen HT, Sorg BA (2014) Inactivation of the paraventricular thalamus abolishes the expression of cocaine conditioned place preference in rats. Drug and Alcohol Dependence 134:387-390.

Buffalari DM, Baldwin CK, See RE (2012) Treatment of cocaine withdrawal anxiety with guanfacine: relationships to cocaine intake and reinstatement of cocaine seeking in rats. Psychopharmacology 223:179-190.

Caine SB, Thomsen M, Gabriel KI, Berkowitz JS, Gold LH, Koob GF, Tonegawa S, Zhang J, Xu M (2007) Lack of self-administration of cocaine in dopamine D1 receptor knock-out mice. Journal of Neuroscience 27:13140-13150.

Carroll KM, Kiluk BD, Nich C, DeVito EE, Decker S, LaPaglia D, Duffey D, Babuscio TA, Ball SA (2014) Toward empirical identification of a clinically meaningful indicator of treatment

outcome: features of candidate indicators and evaluation of sensitivity to treatment effects and relationship to one year follow up cocaine use outcomes. Drug and alcohol dependence 137:3-19.

Center for Behavioral Health Statistics S (2021) Key Substance Use and Mental Health Indicators in the United States: Results from the 2020 National Survey on Drug Use and Health. In.

Dayas CV, McGranahan TM, Martin-Fardon R, Weiss F (2008) Stimuli Linked to Ethanol Availability Activate Hypothalamic CART and Orexin Neurons in a Reinstatement Model of Relapse. Biological Psychiatry 63:152-157.

de Oliveira Citó MdC, da Silva FCC, Silva MIG, Moura BA, Macêdo DS, Woods DJ, de França Fonteles MM, de Vasconcelos SMM, de Sousa FCF (2012) Reversal of cocaine withdrawal-induced anxiety by ondansetron, buspirone and propranolol. Behavioural brain research 231:116-123.

Delfs J, Zhu Y, Druhan J, Aston-Jones G (2000) Noradrenaline in the ventral forebrain is critical for opiate withdrawal-induced aversion. Nature 403:430-434.

DeVries AC, Pert A (1998) Conditioned increases in anxiogenic-like behavior following exposure to contextual stimuli associated with cocaine are mediated by corticotropin-releasing factor. Psychopharmacology 137:333-340.

Do Monte FH, Quirk GJ, Li B, Penzo MA (2016) Retrieving fear memories, as time goes by…. Molecular psychiatry 21:1027-1036.

Do-Monte FH, Quinones-Laracuente K, Quirk GJ (2015) A temporal shift in the circuits mediating retrieval of fear memory. Nature 519:460-463.

Do-Monte FH, Minier-Toribio A, Quiñones-Laracuente K, Medina-Colón EM, Quirk GJ (2017) Thalamic Regulation of Sucrose Seeking during Unexpected Reward Omission. Neuron

94:388-400.e384.

El Hage C, Rappeneau V, Etievant A, Morel AL, Scarna H, Zimmer L, Bérod A (2012) Enhanced anxiety observed in cocaine withdrawn rats is associated with altered reactivity of the dorsomedial prefrontal cortex. PLoS One 7:e43535.

Erb S (2010) Evaluation of the relationship between anxiety during withdrawal and stress-induced reinstatement of cocaine seeking. Prog Neuropsychopharmacol Biol Psychiatry 34:798-807.

Erb S, Kayyali H, Romero K (2006) A study of the lasting effects of cocaine pre-exposure on anxiety-like behaviors under baseline conditions and in response to central injections of corticotropin-releasing factor. Pharmacology Biochemistry and Behavior 85:206-213.

Ersche KD, Barnes A, Jones PS, Morein-Zamir S, Robbins TW, Bullmore ET (2011) Abnormal structure of frontostriatal brain systems is associated with aspects of impulsivity and compulsivity in cocaine dependence. Brain 134:2013-2024.

Everitt BJ, Wolf ME (2002) Psychomotor stimulant addiction: a neural systems perspective. Journal of Neuroscience 22:3312-3320.

Fotros A, Casey KF, Larcher K, Verhaeghe JA, Cox SM, Gravel P, Reader AJ, Dagher A, Benkelfat C, Leyton M (2013) Cocaine cue-induced dopamine release in amygdala and hippocampus: a high-resolution PET [18F] fallypride study in cocaine dependent participants. Neuropsychopharmacology 38:1780-1788.

Franklin TR, Acton PD, Maldjian JA, Gray JD, Croft JR, Dackis CA, O'Brien CP, Childress AR (2002) Decreased gray matter concentration in the insular, orbitofrontal, cingulate, and temporal cortices of cocaine patients. Biological Psychiatry 51:134-142.

Gasparyan A, Navarrete F, Rodríguez-Arias M, Miñarro J, Manzanares J (2021) Cannabidiol

Modulates Behavioural and Gene Expression Alterations Induced by Spontaneous Cocaine Withdrawal. Neurotherapeutics 18:615-623.

Gawin FH, Kleber HD (1986) Abstinence symptomatology and psychiatric diagnosis in cocaine abusers. Clinical observations. Arch Gen Psychiatry 43:107-113.

George O, Koob GF (2010) Individual differences in prefrontal cortex function and the transition from drug use to drug dependence. Neurosci Biobehav Rev 35:232-247.

Giannotti G, Barry SM, Siemsen BM, Peters J, McGinty JF (2018) Divergent Prelimbic Cortical Pathways Interact with BDNF to Regulate Cocaine-seeking. J Neurosci 38:8956-8966.

Goldstein RZ, Volkow ND (2011) Dysfunction of the prefrontal cortex in addiction: neuroimaging findings and clinical implications. Nature reviews neuroscience 12:652-669.

Goussakov I, Chartoff EH, Tsvetkov E, Gerety LP, Meloni EG, Carlezon Jr WA, Bolshakov VY (2006) LTP in the lateral amygdala during cocaine withdrawal. European Journal of Neuroscience 23:239-250.

Groenewegen HJ, Berendse HW (1994) The specificity of the 'nonspecific' midline and intralaminar thalamic nuclei. Trends in Neurosciences 17:52-57.

Hamlin AS, Clemens KJ, Choi EA, McNally GP (2009) Paraventricular thalamus mediates context-induced reinstatement (renewal) of extinguished reward seeking. European Journal of Neuroscience 29:802-812.

Harris GC, Altomare K, Aston-Jones G (2001) Preference for a cocaine-associated environment is attenuated by augmented accumbal serotonin in cocaine withdrawn rats. Psychopharmacology 156:14-22.

Hikosaka O (2010) The habenula: from stress evasion to value-based decision-making. Nature reviews neuroscience 11:503-513.

Jacobs DS, Moghaddam B (2021a) Medial prefrontal cortex encoding of stress and anxiety. Int Rev Neurobiol 158:29-55.

Jacobs DS, Moghaddam B (2021b) Chapter Two - Medial prefrontal cortex encoding of stress and anxiety. In: International Review of Neurobiology (Brockett AT, Amarante LM, Laubach M, Roesch MR, eds), pp 29-55: Academic Press.

James MH, Charnley JL, Jones E, Levi EM, Yeoh JW, Flynn JR, Smith DW, Dayas CV (2010) Cocaine- and amphetamine-regulated transcript (CART) signaling within the paraventricular thalamus modulates cocaine-seeking behaviour. PLoS One 5:e12980.

Jasinska AJ, Stein EA, Kaiser J, Naumer MJ, Yalachkov Y (2014) Factors modulating neural reactivity to drug cues in addiction: a survey of human neuroimaging studies. Neuroscience & Biobehavioral Reviews 38:1-16.

Jhou TC (2013) Dopamine and anti-dopamine systems: polar opposite roles in behavior. The FASEB Journal 27:80.82-80.82.

Kalivas PW, Volkow ND (2005) The Neural Basis of Addiction: A Pathology of Motivation and Choice. American Journal of Psychiatry 162:1403-1413.

Kampman KM (2019) The treatment of cocaine use disorder. Science advances 5:eaax1532.

Kimura M, Satoh T, Matsumoto N (2007) What does the habenula tell dopamine neurons? Nature neuroscience 10:677-678.

Kirouac GJ (2015) Placing the paraventricular nucleus of the thalamus within the brain circuits that control behavior. Neurosci Biobehav Rev 56:315-329.

Kirouac GJ (2021) The Paraventricular Nucleus of the Thalamus as an Integrating and Relay Node in the Brain Anxiety Network. Front Behav Neurosci 15:627633.

Kober H, Lacadie CM, Wexler BE, Malison RT, Sinha R, Potenza MN (2016) Brain activity during

cocaine craving and gambling urges: an fMRI study. Neuropsychopharmacology 41:628-637.

Koob GF, Volkow ND (2010) Neurocircuitry of addiction. Neuropsychopharmacology 35:217-238.

Koob GF, Volkow ND (2016) Neurobiology of addiction: a neurocircuitry analysis. Lancet Psychiatry 3:760-773.

Koob GF, Schulkin J (2019) Addiction and stress: An allostatic view. Neurosci Biobehav Rev 106:245-262.

Koob GF, Buck CL, Cohen A, Edwards S, Park PE, Schlosburg JE, Schmeichel B, Vendruscolo LF, Wade CL, Whitfield Jr TW (2014) Addiction as a stress surfeit disorder. Neuropharmacology 76:370-382.

Kuhn BN, Klumpner MS, Covelo IR, Campus P, Flagel SB (2018) Transient inactivation of the paraventricular nucleus of the thalamus enhances cue-induced reinstatement in goal-trackers, but not sign-trackers. Psychopharmacology 235:999-1014.

Kuhn BN, Campus P, Klumpner MS, Chang SE, Iglesias AG, Flagel SB (2022) Inhibition of a cortico-thalamic circuit attenuates cue-induced reinstatement of drug-seeking behavior in "relapse prone" male rats. Psychopharmacology 239:1035-1051.

La-Vu M, Tobias BC, Schuette PJ, Adhikari A (2020) To Approach or Avoid: An Introductory Overview of the Study of Anxiety Using Rodent Assays. Front Behav Neurosci 14:145.

Lee J-H, Lim Y, Wiederhold BK, Graham SJ (2005) A functional magnetic resonance imaging (FMRI) study of cue-induced smoking craving in virtual environments. Applied psychophysiology and biofeedback 30:195-204.

Li S, Kirouac GJ (2008) Projections from the paraventricular nucleus of the thalamus to the

forebrain, with special emphasis on the extended amygdala. Journal of Comparative Neurology 506:263-287.

Li S, Kirouac GJ (2012) Sources of inputs to the anterior and posterior aspects of the paraventricular nucleus of the thalamus. Brain Structure and Function 217:257-273.

Liu Y, Ball JD, Elliott AL, Jacobs-Elliott M, Nicolette G (2020) Diagnostic sequence of cocaine use disorder in relation to other mental health conditions among college students. J Am Coll Health 68:575-578.

Ma YY, Lee BR, Wang X, Guo C, Liu L, Cui R, Lan Y, Balcita-Pedicino JJ, Wolf ME, Sesack SR, Shaham Y, Schluter OM, Huang YH, Dong Y (2014) Bidirectional modulation of incubation of cocaine craving by silent synapse-based remodeling of prefrontal cortex to accumbens projections. Neuron 83:1453-1467.

Makris N, Gasic GP, Seidman LJ, Goldstein JM, Gastfriend DR, Elman I, Albaugh MD, Hodge SM, Ziegler DA, Sheahan FS (2004) Decreased absolute amygdala volume in cocaine addicts. Neuron 44:729-740.

Mantsch JR, Baker DA, Francis DM, Katz ES, Hoks MA, Serge JP (2008) Stressor- and corticotropin releasing factor-induced reinstatement and active stress-related behavioral responses are augmented following long-access cocaine self-administration by rats. Psychopharmacology 195:591-603.

Martin-Fardon R, Boutrel B (2012) Orexin/hypocretin (Orx/Hcrt) transmission and drug-seeking behavior: Is the paraventricular nucleus of the thalamus (PVT) part of the drug seeking circuitry? Frontiers in Behavioral Neuroscience 0:1-28.

Martinez D, Narendran R, Foltin RW, Slifstein M, Hwang D-R, Broft A, Huang Y, Cooper TB, Fischman MW, Kleber HD (2007) Amphetamine-induced dopamine release: markedly

blunted in cocaine dependence and predictive of the choice to self-administer cocaine. American Journal of Psychiatry 164:622-629.

Matsumoto M, Hikosaka O (2007) Lateral habenula as a source of negative reward signals in dopamine neurons. Nature 447:1111-1115.

Matzeu A, Weiss F, Martin-Fardon R (2015) Transient inactivation of the posterior paraventricular nucleus of the thalamus blocks cocaine-seeking behavior. Neurosci Lett 608:34-39.

Matzeu A, Cauvi G, Kerr TM, Weiss F, Martin-Fardon R (2017) The paraventricular nucleus of the thalamus is differentially recruited by stimuli conditioned to the availability of cocaine versus palatable food. Addiction Biology 22:70-77.

McFarland K, Kalivas PW (2001) The circuitry mediating cocaine-induced reinstatement of drug-seeking behavior. J Neurosci 21:8655-8663.

McGinty JF, Otis JM (2020) Heterogeneity in the Paraventricular Thalamus: The Traffic Light of Motivated Behaviors. Front Behav Neurosci 14:590528.

McLaughlin J, See RE (2003) Selective inactivation of the dorsomedial prefrontal cortex and the basolateral amygdala attenuates conditioned-cued reinstatement of extinguished cocaine-seeking behavior in rats. Psychopharmacology (Berl) 168:57-65.

Milella MS, Fotros A, Gravel P, Casey KF, Larcher K, Verhaeghe JA, Cox SM, Reader AJ, Dagher A, Benkelfat C (2016) Cocaine cue–induced dopamine release in the human prefrontal cortex. Journal of Psychiatry and Neuroscience 41:322-330.

Millan EZ, Ong Z, McNally GP (2017) Paraventricular thalamus: Gateway to feeding, appetitive motivation, and drug addiction. Prog Brain Res 235:113-137.

Mitchell JM, O'Neil JP, Janabi M, Marks SM, Jagust WJ, Fields HL (2012) Alcohol consumption induces endogenous opioid release in the human orbitofrontal cortex and nucleus

accumbens. Science translational medicine 4:116ra116-116ra116.

Moga MM, Weis RP, Moore RY (1995) Efferent projections of the paraventricular thalamic nucleus in the rat. Journal of Comparative Neurology 359:221-238.

Otake K, Nakamura Y (1998) Single midline thalamic neurons projecting to both the ventral striatum and the prefrontal cortex in the rat. Neuroscience 86:635-649.

Otis JM, Namboodiri VMK, Matan AM, Voets ES, Mohorn EP, Kosyk O, McHenry JA, Robinson JE, Resendez SL, Rossi MA, Stuber GD (2017) Prefrontal cortex output circuits guide reward seeking through divergent cue encoding. Nature 543:103-107.

Otis JM, Zhu M, Namboodiri VMK, Cook CA, Kosyk O, Matan AM, Ying R, Hashikawa Y, Hashikawa K, Trujillo-Pisanty I, Guo J, Ung RL, Rodriguez-Romaguera J, Anton ES, Stuber GD (2019) Paraventricular Thalamus Projection Neurons Integrate Cortical and Hypothalamic Signals for Cue-Reward Processing. Neuron 103:423-431.e424.

Paxinos G, Watson C (2018) The Rat Brain in Stereotaxic Coordinates Compact, 7 Edition. San Diego: Elsevier Academic Press.

Piazza PV, Le Moal M (1996) Pathophysiological basis of vulnerability to drug abuse: role of an interaction between stress, glucocorticoids, and dopaminergic neurons. Annual review of pharmacology and toxicology 36:359-378.

Pich EM, Lorang M, Yeganeh M, De Fonseca FR, Raber J, Koob GF, Weiss F (1995) Increase of extracellular corticotropin-releasing factor-like immunoreactivity levels in the amygdala of awake rats during restraint stress and ethanol withdrawal as measured by microdialysis. Journal of Neuroscience 15:5439-5447.

Risinger RC, Salmeron BJ, Ross TJ, Amen SL, Sanfilipo M, Hoffmann RG, Bloom AS, Garavan H, Stein EA (2005) Neural correlates of high and craving during cocaine self-

administration using BOLD fMRI. Neuroimage 26:1097-1108.

Robinson TE, Kolb B (2004) Structural plasticity associated with exposure to drugs of abuse. Neuropharmacology 47:33-46.

Sarnyai Z, Bíró É, Gardi J, Vecsernyés M, Julesz J, Telegdy G (1995) Brain corticotropin-releasing factor mediates ‘anxiety-like’ behavior induced by cocaine withdrawal in rats. Brain Research 675:89-97.

Scholl JL, Afzal A, Fox LC, Watt MJ, Forster GL (2019) Sex differences in anxiety-like behaviors in rats. Physiology & Behavior 211:112670.

Seibenhener ML, Wooten MC (2015) Use of the Open Field Maze to measure locomotor and anxiety-like behavior in mice. J Vis Exp:e52434.

Shaham Y, Erb S, Leung S, Buczek Y, Stewart J (1998) CP-154,526, a selective, non-peptide antagonist of the corticotropin-releasing factor1 receptor attenuates stress-induced relapse to drug seeking in cocaine-and heroin-trained rats. Psychopharmacology 137:184-190.

Shaham Y, Highfield D, Delfs J, Leung S, Stewart J (2000) Clonidine blocks stress-induced reinstatement of heroin seeking in rats: An effect independent of locus coeruleus noradrenergic neurons. European Journal of Neuroscience 12:292-302.

Shaham Y, Shalev U, Lu L, De Wit H, Stewart J (2003) The reinstatement model of drug relapse: history, methodology and major findings. Psychopharmacology 168:3-20.

Shalev U, Grimm JW, Shaham Y (2002) Neurobiology of relapse to heroin and cocaine seeking: a review. Pharmacological reviews 54:1-42.

Shepherd JK, Grewal SS, Fletcher A, Bill DJ, Dourish CT (1994) Behavioural and pharmacological characterisation of the elevated "zero-maze" as an animal model of anxiety. Psychopharmacology (Berl) 116:56-64.

Siemsen BM, Giannotti G, McFaddin JA, Scofield MD, McGinty JF (2019) Biphasic effect of abstinence duration following cocaine self-administration on spine morphology and plasticity-related proteins in prelimbic cortical neurons projecting to the nucleus accumbens core. Brain Struct Funct 224:741-758.

Stefanik MT, Kupchik YM, Kalivas PW (2016) Optogenetic inhibition of cortical afferents in the nucleus accumbens simultaneously prevents cue-induced transient synaptic potentiation and cocaine-seeking behavior. Brain Struct Funct 221:1681-1689.

Suzuki S, Saitoh A, Ohashi M, Yamada M, Oka J, Yamada M (2016) The infralimbic and prelimbic medial prefrontal cortices have differential functions in the expression of anxiety-like behaviors in mice. Behav Brain Res 304:120-124.

Tanabe J, Tregellas JR, Dalwani M, Thompson L, Owens E, Crowley T, Banich M (2009) Medial orbitofrontal cortex gray matter is reduced in abstinent substance-dependent individuals. Biological psychiatry 65:160-164.

Tomasi D, Goldstein RZ, Telang F, Maloney T, Alia-Klein N, Caparelli EC, Volkow ND (2007) Thalamo-cortical dysfunction in cocaine abusers: implications in attention and perception. Psychiatry Research: Neuroimaging 155:189-201.

Van der Werf YD, Witter MP, Groenewegen HJ (2002) The intralaminar and midline nuclei of the thalamus. Anatomical and functional evidence for participation in processes of arousal and awareness. Brain Research Reviews 39:107-140.

Vertes RP, Hoover WB (2008) Projections of the paraventricular and paratenial nuclei of the dorsal midline thalamus in the rat. Journal of Comparative Neurology 508:212-237.

Volkow N, Wang G-J, Fowler J, Logan J, Gatley S, Hitzemann R, Chen A, Dewey S, Pappas N (1997) Decreased striatal dopaminergic responsiveness in detoxified cocaine-dependent

subjects. Nature 386:830-833.

Volkow N, Tomasi D, Wang G, Logan J, Alexoff D, Jayne M, Fowler J, Wong C, Yin P, Du C (2014) Stimulant-induced dopamine increases are markedly blunted in active cocaine abusers. Molecular psychiatry 19:1037-1043.

Volkow ND, Fowler JS, Wang G-J (2003) The addicted human brain: insights from imaging studies. The Journal of clinical investigation 111:1444-1451.

Volkow ND, Wang G-J, Fowler JS, Tomasi D, Telang F (2011) Addiction: beyond dopamine reward circuitry. Proceedings of the National Academy of Sciences 108:15037-15042.

Volkow ND, Wang G-J, Ma Y, Fowler JS, Wong C, Ding Y-S, Hitzemann R, Swanson JM, Kalivas P (2005) Activation of orbital and medial prefrontal cortex by methylphenidate in cocaine-addicted subjects but not in controls: relevance to addiction. Journal of Neuroscience 25:3932-3939.

Volkow ND, Wang G-J, Telang F, Fowler JS, Logan J, Childress A-R, Jayne M, Ma Y, Wong C (2006) Cocaine cues and dopamine in dorsal striatum: mechanism of craving in cocaine addiction. Journal of Neuroscience 26:6583-6588.

Volkow ND, Wang G-J, Telang F, Fowler JS, Logan J, Jayne M, Ma Y, Pradhan K, Wong C (2007) Profound decreases in dopamine release in striatum in detoxified alcoholics: possible orbitofrontal involvement. Journal of Neuroscience 27:12700-12706.

Vorel SR, Liu X, Hayes RJ, Spector JA, Gardner EL (2001) Relapse to cocaine-seeking after hippocampal theta burst stimulation. Science 292:1175-1178.

Weiss F, Markou A, Lorang MT, Koob GF (1992) Basal extracellular dopamine levels in the nucleus accumbens are decreased during cocaine withdrawal after unlimited-access self-administration. Brain research 593:314-318.

Werner CT, Gancarz AM, Dietz DM (2019) Chapter 10 - Mechanisms Regulating Compulsive Drug Behaviors. In: Neural Mechanisms of Addiction (Torregrossa M, ed), pp 137-155: Academic Press.

Whitfield TW, Jr., Shi X, Sun WL, McGinty JF (2011) The suppressive effect of an intra-prefrontal cortical infusion of BDNF on cocaine-seeking is Trk receptor and extracellular signal-regulated protein kinase mitogen-activated protein kinase dependent. J Neurosci 31:834-842.

Woicik PA, Stewart SH, Pihl RO, Conrod PJ (2009) The Substance Use Risk Profile Scale: a scale measuring traits linked to reinforcement-specific substance use profiles. Addict Behav 34:1042-1055.

Young CD, Deutch AY (1998) The Effects of Thalamic Paraventricular Nucleus Lesions on Cocaine-Induced Locomotor Activity and Sensitization. Pharmacology Biochemistry and Behavior 60:753-758.

Zhou K, Zhu Y (2019) The paraventricular thalamic nucleus: A key hub of neural circuits underlying drug addiction. Pharmacol Res 142:70-76.