Involvement of Corticotropin-Releasing Factor Signaling in Food Deprivation Stress-induced Heroin Seeking Following Punishment-Imposed Abstinence in Male Rats

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A Thesis
in
The Department
of Psychology

Presented in Partial Fulfillment of the Requirements for the Degree of Master of Arts (Psychology) at Concordia University

Montreal, Quebec, Canada

July 2025

CONCORDIA UNIVERSITY
SCHOOL OF GRADUATE STUDIES

This is to certify that the thesis prepared
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Entitled: Involvement of Corticotropin-Releasing Factor Signaling in Food Deprivation Stress-induced Heroin Seeking Following Punishment-Imposed Abstinence in Male Rats

and submitted in partial fulfillment of the requirements for the degree of
Master of Arts (Psychology)
complies with the regulations of the University and meets the accepted standards with respect to originality and quality.
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Abstract

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Danna Morales

Relapse remains a major challenge in treating opioid use disorder, often triggered by stress-related factors that reinstate drug-seeking after abstinence. Corticotropin-releasing factor (CRF), a neuropeptide central to the stress response, has been implicated in stress-induced relapse, particularly via the mesolimbic dopamine system. However, the neural sources of relapse-driving CRF remain unclear. This thesis investigates CRF signaling in stress-induced heroin seeking and examines the paraventricular nucleus of the hypothalamus (PVN), a CRF-expressing region, as a potential driver.

In the first experiment, male rats were trained to self-administer heroin using a seek-take chain schedule modeling human drug use. After stable intake, rats underwent punishment-imposed abstinence, where footshocks were probabilistically paired with drug-seeking responses, leading to suppression. During relapse testing, each rat was evaluated under two conditions: sated and food deprived. Heroin-seeking behavior was significantly higher under food deprivation than when sated. This increase was blocked by intracerebroventricular administration of a CRF receptor antagonist, supporting CRF's role in stress-induced relapse and validating the seek-take punishment model.

In the second experiment, the PVN was targeted to explore its contribution to relapse. Rats underwent the same protocol and received either a ligand to chemogenetically inhibit PVN neurons or vehicle before relapse testing. PVN inhibition modestly reduced heroin seeking under

food deprivation but did not reach strong statistical certainty. These results suggest the PVN may contribute to CRF-driven relapse, though further studies are needed.

Together, these findings highlight CRF's role in stress-induced relapse and suggest involvement of multiple CRF-releasing regions.

Acknowledgments

My sincerest appreciation to the members of the Center for Studies in Behavioral Neurobiology, to my thesis committee members Dr. Richard Courtemanche and Dr. Andrew Chapman, and to everyone else who contributed to the development of this thesis and its author, both personally and professionally. Thank you to my thesis supervisor, Dr. Uri Shalev, for his guidance and feedback throughout this process. His input helped me navigate the challenges of this project and complete this thesis.

A special thank you to Erin Page. Erin, your collaboration, clarity, and humor made the long days so much easier. Thank you for being a thoughtful colleague and a good friend, always offering your help, whether in data analysis or life advice. I am proud of the work we have done together.

To the incredible undergraduate students, I have had the pleasure of working with, Samantha Dorrance, Cassandra Soules, Alessia Macri, and Anika Michalko: thank you for your enthusiasm, dedication, and attention to detail. Your help was instrumental in keeping the work moving forward, and it was a joy to watch you grow into budding scientists.

I am deeply grateful to my parents, Javier and Dora Morales, whose unconditional support, encouragement, and sacrifices have been the foundation of everything I have achieved. Your belief in me gave me strength during the most challenging moments, and I dedicate this work to you. This is as much yours as it is mine. Hats off to you.

I would also like to thank my dear friends Bhoomi, Sepideh, Elizabeth, Kiera, and Ana. Your unwavering support, encouragement, and kindness have been invaluable throughout this journey. Thank you for always being there, cheering me on, and reminding me to find balance and joy outside of my work.

Finally, to everyone who has been part of this experience in any way, your contributions have shaped this thesis and my growth as a researcher and person. I am deeply grateful for your support.

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Introduction

The Opioid Epidemic

Since the 1990s, the world has been caught in the grip of an opioid epidemic that shows no signs of slowing down. What began with an increase in prescription painkillers has escalated into a global crisis involving heroin and more recently potent synthetic opioids that are easy to manufacture and widely available (Cook, 2022). The reasons behind this ongoing crisis are complex and interconnected, including chronic pain, mental health issues, substance use disorders, and the widespread impact of the COVID-19 pandemic (Cook, 2022).

Women have been especially affected by the opioid crisis, both in terms of biological vulnerability and the social consequences of opioid use, making it essential for healthcare providers and researchers to consider sex specific factors in understanding and treating opioid use disorder (Cook, 2022). Despite decades of scientific research and public health efforts, the rates of opioid use disorder, overdose deaths, and related health impacts continue to rise (Cook, 2022).

The numbers speak for themselves. In 2024, Canada reported 5,626 apparent opioid toxicity deaths, an average of 21 lives lost every day (Public Health Agency of Canada, 2024). In the United States, more than 100,000 people have died from opioid overdoses each year since 2021, almost three times the number in 2015 (Spencer et al., 2024). That is nearly 300 deaths every single day, the equivalent of a Boeing 737 crashing daily. These alarming trends demand a deeper look into the underlying factors that have fueled the growth and persistence of the opioid epidemic.

Why are we having an opioid epidemic?

The opioid epidemic didn't fall from the sky; it was built, prescribed, and marketed into existence. What began as a medical solution for pain spiraled into a public health disaster fueled by overprescription, misinformation, and a misplaced faith in the safety of opioids. For decades, opioids were handed out like candy after surgeries and for routine pain, often with little consideration for their addictive potential (Makary et al., 2017). The notion that opioids were barely addictive when treating pain gave rise to a generation of aggressive prescribing practices (Becker & Fiellin, 2017; Leung et al., 2017).

Compounding this was the flawed belief that pain should be treated as the "fifth vital sign." Suddenly, patient satisfaction scores were influencing opioid doses more than clinical judgment (Scher et al., 2018). Between 1999 and 2010, opioid prescriptions in the United States quadrupled, conveniently mirroring the surge in overdose deaths (Centers for Disease Control and Prevention [CDC], 2016). The result? Far too many patients left hospitals with more pills than they needed, 70 to 80 percent of which went unused and often ended up misused (Hill et al., 2017).

But it is not just the system. There is a deeply human side to this crisis. Opioid use disorder (OUD) is a complex interplay of biology, environment, and trauma. Genetics, neurotransmitter imbalances, adverse childhood experiences, and mental health conditions all contribute to vulnerability (Cook, 2022). Once dependence sets in, whether through a legitimate prescription or diverted medication, it can progress into a chronic, relapsing condition with life-threatening consequences (World Health Organization, 2022).

In response, public health efforts have focused on curbing overprescribing, improving clinical education, and expanding access to naloxone and medication assisted treatments. Yet

even today, only a small fraction of those living with OUD receive the care they need (World Health Organization, 2022).

In many ways, the opioid crisis began in the pharmacy. But as prescriptions became harder to obtain and tolerance increased, people did not stop. They switched. And that is where heroin enters the story.

The switch to heroin and synthetic opioids

A key turning point in the opioid crisis occurred when many individuals, initially dependent on prescription opioids, began transitioning to heroin and later to synthetic opioids such as fentanyl. As prescription opioids became less accessible due to tighter regulations and reformulations aimed at reducing abuse (e.g., abuse deterrent formulations of OxyContin), many users sought alternatives that were cheaper and more readily available (Cicero et al., 2014; Mars et al., 2014). Around 2016, about 80 percent of people who used heroin had first misused prescription opioids, highlighting a clear trajectory from prescribed substances to illicit drugs (Carlson et al., 2016; Cicero et al., 2014). Heroin emerged as a prevalent substitute, often costing less on the street and providing a comparable or stronger euphoric effect. However, the shift did not stop there. In recent years, synthetic opioids, particularly illicitly manufactured fentanyl, have flooded drug markets, significantly amplifying the lethality of opioid use (O'Donnell et al., 2017). These synthetic opioids are not only more potent than heroin but are frequently mixed into other substances without the user's knowledge, increasing the risk of overdose (Gladden et al., 2016). This progression from prescription drugs to heroin and then to synthetic opioids marks a dangerous evolution of the crisis, one that calls attention to the need for policies that address both supply side control and the underlying demand driving opioid misuse.

Defining Substance Use Disorder

Here's the silver lining: not everyone who misuses opioids ends up addicted. Vulnerability to addiction arises from a complex interaction of brain systems, social and cultural environment, genetic predisposition, and individual traits such as impulsivity. These factors differ by drug type and can influence the likelihood of addiction (Ouzir & Errami, 2016). Substance use disorder (SUD) is a chronic condition marked by compulsive drug seeking and use despite negative consequences (American Psychiatric Association, 2023). It develops gradually, beginning with early rewarding drug experiences that reinforce continued use. Over time, this behavior becomes increasingly difficult to control (McAuliffe & Gordon, 1980).

Drugs tap into brain systems that evolved to encourage survival behaviors, such as seeking food or avoiding danger (Volkow & Morales, 2015). They activate dopamine circuits involved in reward and motivation, overriding natural controls and encouraging repeated use (Volkow & Morales, 2015). With repeated exposure, brain networks responsible for reward processing, decision making, mood regulation, and self-awareness are disrupted, making drug use harder to resist (Koob & Volkow, 2016).

The Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition (DSM 5), defines SUD using eleven criteria grouped into impaired control, social impairment, risky use, and physiological effects like tolerance and withdrawal. The disorder is classified as mild, moderate, or severe depending on how many criteria are met (American Psychiatric Association, 2013).

Historically, addiction was viewed as a moral failing, but neuroscience has reframed it as a treatable brain disorder involving long lasting changes to neural function (Volkow et al., 2023). The speed at which SUD develops depends on a drug's pharmacological effects (Lopez-Quintero et al., 2011), its availability and legal status, and how socially accepted its use is (McNeely et al.,

2016). Among opioids, heroin shows one of the highest transition rates, with about 23 percent of users developing a use disorder (Lopez-Quintero et al., 2011).

SUD is rarely linear. Most individuals alternate between periods of drug use and abstinence, often experiencing relapse even after treatment. (American Psychiatric Association, 2023).

Understanding Relapse

Relapse remains one of the most difficult and frustrating parts of the recovery journey. Even after periods of abstinence, many individuals with substance use disorder find themselves returning to drug use. This return can bring serious consequences that can be physical, psychological, and social. Physically, relapse can lead to organ damage, cognitive decline, chronic illnesses such as cancer or hepatitis C, and even death (Marlatt & Donovan, 2005). Psychologically, individuals often experience guilt, shame, and hopelessness. These emotions are part of what is known as the abstinence violation effect, where one lapse triggers a negative emotional spiral that increases the risk of a full relapse (Larimer et al., 1999). Socially, relapse may cause job loss, strain relationships, and create legal challenges (Miller, 1996).

Despite these severe outcomes, relapse is very common. Heroin relapse rates are especially high, ranging from 70 to 91 percent, and even after 15 years of abstinence, around 25 percent of individuals relapse (Hunt et al., 1971; Hser et al., 2001; Smyth et al., 2010). Most relapses occur within the first year of stopping drug use (Kadam et al., 2017).

Three key triggers are consistently linked to relapse. The first is drug preexposure, where even a small amount of the previously used drug can reignite cravings and drive renewed use (De Wit, 1996). For example, a former heroin user given morphine in a medical setting might experience intense urges to return to heroin. The second trigger is exposure to cues associated

with past drug use, such as people, places, or paraphernalia, which can quickly bring back strong cravings (Childress et al., 1993; Carter & Tiffany, 1999). The third, and perhaps most powerful, is stress. Both acute and chronic stressors such as the loss of a loved one or financial problems can lead to increased craving and relapse (Sinha, 2001; Brown et al., 1995; Preston & Epstein, 2011). Stress has consistently been cited by individuals in recovery as a major reason for returning to substance use (Sinha, 2001). Recent research even found that 13.3 percent of Americans reported starting or increasing substance use during the COVID19 pandemic to cope with stress and emotional discomfort (Czeisler et al., 2020).

Cheskin et al. (2005) show that stress not only affects individuals psychologically but also triggers physiological responses that increase cravings. A range of environmental stressors, including calorie restriction, have been found to heighten drug seeking behavior. Understanding these triggers and how they interact is key to making sense of why relapse is so common, even after extended periods of abstinence.

Translational Approaches to Studying SUD

Relapse is one of the hardest parts of addiction to study, especially in humans. Since researchers can't ethically test relapse triggers in people, they rely heavily on animal studies to explore the brain mechanisms behind it. Most of these models follow a basic structure: animals first learn to take the drug, then go through a period without it, and finally are exposed to a trigger to see if they start seeking the drug again (De Wit & Stewart, 1983; Shaham et al., 2003) The most common model is the reinstatement model. In this setup, animals stop taking the drug because it is no longer available, not because they choose to stop. After the extinction of drug seeking, relapse is tested with a drug trigger, a cue, or stress (Shaham et al., 2003; Stewart & de Wit, 1987). This is different from what happens in people, where abstinence usually comes from

a decision to stop, even though the drug is still available and rewarding (Epstein et al., 2006; Marlatt, 2002). Furthermore, operant extinction of drug seeking does not occur in human drug users. Because of this difference, the model doesn't reflect real-life experiences very well (Reiner et al., 2019).

Another model is forced abstinence, where animals are just taken away from the drug environment (Reiner et al., 2019). This can represent situations like jail or inpatient rehab, but only a small number of people with substance use disorders go through those kinds of programs (Apsley et al., 2024). Most people stop using drugs by choice. Importantly, brain activity in forced and voluntary abstinence is not the same (Reiner et al., 2019), which makes the model less useful for understanding human relapse.

To improve this, researchers developed voluntary abstinence models. In these, animals stop using the drug either because they choose another reward like food or social interaction (Venniro et al 2016), or because drug-seeking leads to a negative outcome like a mild electric footshock (Deroche-Gamonet et al., 2004; Pelloux et al., 2007). This setup is closer to what people experience. In real life, people often quit because of health problems, family issues, or legal trouble (Burman, 1997; Cunningham et al., 2000; Klingemann, 1991). Punishing drug-seeking in animal models helps reflect the consequences people try to avoid. Even though a shock isn't the same as real-life outcomes, it works as a mental deterrent, which makes the model more realistic (Cooper et al., 2007; Fredriksson et al., 2020).

As mentioned above, relapse triggers in both animals and humans include re-exposure to the drug, drug-related cues, and stress (Sinha, 2001). Of these, stress is especially relevant because it is nearly impossible to avoid. In animal research, physical stress like footshock or restraint is often used to trigger relapse (Mantsch et al., 2016; Mercier et al., 2003), but these

don’t really match the types of emotional stress that lead to relapse in people (Eisenberger, 2012).

Emotional stress in animals has been modeled with things like isolation or early-life stress, but these methods can be hard to control. One alternative is to use caloric stress, which is easier to apply and more relevant. Many people who use opioids, especially heroin, report not eating enough or having unstable access to food (Best et al., 1998; el-Nakah et al., 1979; Neale et al., 2012; Noble & McCombie, 1997). There is also a link between hunger and drug craving (Cheskin et al., 2005; Hall et al., 1992), which makes food deprivation an important variable to study.

Both long-term food restriction and short-term food deprivation have been shown to bring back drug-seeking behavior. Long-term restriction increases how rewarding the drug feels and promotes relapse after both extinction and abstinence (D’Cunha et al., 2013; Shalev, 2012). But short-term food deprivation, like going without food for 24 hours, may be more relevant to real-world drug users who might often skip meals for long stretches (Shalev et al., 2000). This kind of deprivation raises stress hormones and activates brain areas involved in stress and motivation (Shalev et al., 2003; Moscarello et al., 2009).

Interestingly, food deprivation seems to trigger relapse through different brain pathways than other types of stress. For example, giving leptin (a hormone related to hunger) reduces heroin seeking only after food deprivation, not when relapse is triggered by drugs or physical stress (Shalev et al., 2001).

While classic reinstatement models have taught us a lot about relapse across different drugs like heroin, cocaine, and alcohol (Erb et al., 1996; Le et al., 1998; Conrad et al., 2010), they fall short in capturing the complexity of human relapse. To address this, our group

developed a new model that puts together three key ideas: separating drug-seeking from drug-taking with a two-step task, using punishment to create voluntary abstinence, and using short-term food deprivation as the stressor (Borges et al., 2023). This model aims to better reflect what people go through and will serve as the basis for this thesis.

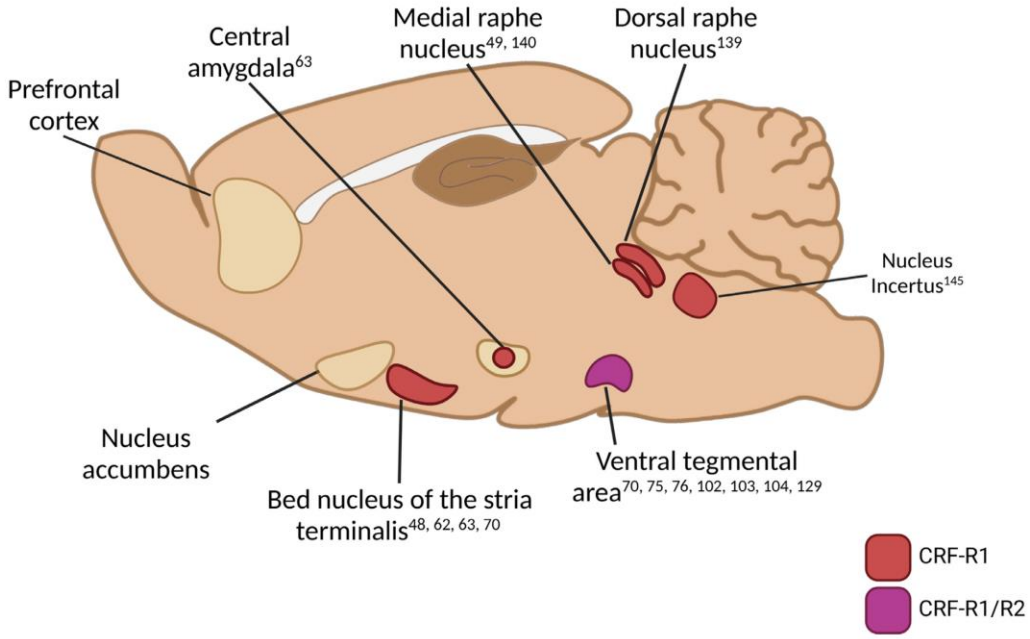
Corticotropin-Releasing Factor

In addiction research using animal models, corticotropin releasing factor (CRF), a neuropeptide integral to the brain's stress response, has emerged as a key modulator of stress-induced relapse (Shaham et al., 1997). CRF is best known for initiating the hormonal stress cascade through its actions on the hypothalamic pituitary adrenal (HPA) axis, but its role extends well beyond that. CRF's role in the stress response and addiction includes activating extrahypothalamic brain areas involved in emotional regulation, motivation, and reward. Intriguingly, CRF administration in the ventricles has been shown to reinstate drug seeking behavior in animals, even in the absence of actual drug availability (Shaham et al., 1997).

Prior studies have established CRF as a key mediator of stress-induced relapse, showing that it drives reinstatement triggered by various stressors including footshock, acute food deprivation, and anxiogenic drugs in animals with a history of heroin (Shalev et al., 2006), cocaine (Erb et al., 1996), and alcohol (Le et al., 1998) use. Blocking CRF signaling via intracerebroventricular (ICV) infusion of CRF receptor antagonists reliably suppresses this relapse behavior (Shaham et al., 1997; Le et al., 2000), highlighting CRF's central role in relapse mechanisms. CRF-producing neurons are found across several brain regions, including the central amygdala (CeA), the bed nucleus of the stria terminalis (BNST), nucleus accumbens (NAc), and paraventricular nucleus (PVN) of the hypothalamus, forming a distributed network

that links emotional regulation, motivation, and stress reactivity to addiction-related behaviors (Pomrenze et al., 2021).

A broad body of literature has identified multiple brain regions where CRF exerts its effects. Notably, while the CeA plays a role, it is the CRF projections from the CeA to the BNST that have been implicated in stress-induced drug seeking, likely through the generation of negative affective states (Koob & Schulkin, 2019). Together, these CRF-sensitive regions form a network through which stress can drive drug-seeking behavior. The diagram below illustrates the key nodes of this drug relapse network in rodents.



Why does revisiting CRF Still Matter?

Despite the robust literature implicating CRF in relapse, gaps in current literature remain, particularly concerning its role in more translationally valid models of abstinence. As addressed previously, the widely used extinction reinstatement model, though foundational, lacks face and predictive validity (Mantsch et al., 2016).

The role of CRF in food-deprivation-induced relapse after punishment is one such gap. While CRF's involvement in food-deprivation-induced reinstatement of heroin seeking has been previously shown (Shalev et al., 2006), no study has yet examined this within a model of punishment-imposed abstinence, which better mimics human patterns of drug cessation. The study conducted will addresses this gap by investigating whether CRF antagonism reduces relapse in rats exposed to food deprivation following punishment-based abstinence; a more ecologically valid scenario (Borges et al., 2023).

The VTA and the Search for CRF Input

The ventral tegmental area (VTA) has emerged as a critical neural hub in relapse, due to its role in reward, motivation, and dopaminergic signaling. The VTA as a relapse hub is supported by evidence showing that stress-induced relapse is associated with increased glutamate, dopamine, and CRF release in this region (Wang et al., 2005). Moreover, local infusion of CRF antagonists into the VTA has been shown to suppress stress-induced reinstatement of drug seeking (Wang et al., 2005).

However, uncertainty about the CRF source persists. Although CRF release in the VTA has been observed following stress exposure, it remains unclear which upstream brain regions are driving this release. Understanding where CRF is coming from and how it modulates the VTA remains a key unanswered question.

The Paraventricular Nucleus to Ventral Tegmental Area Circuit

Recent research by Xu and colleagues (2024) offers important new insight into how CRF circuits influence behavior. Their study showed that neurons in the paraventricular nucleus (PVN) of the hypothalamus that release corticotropin releasing factor (CRF) send signals to the ventral tegmental area (VTA), and that activating this pathway can lead to reward seeking

behaviors. Interestingly, these effects happened without any changes in corticosterone levels or stress related behaviors, suggesting the reward response was not driven by activation of the stress hormone system. Instead, when these PVN CRF neurons were stimulated, animals showed a preference for the area where stimulation occurred and were willing to work to activate the pathway, showing it was reinforcing. These findings suggest that CRF neurons in the PVN may play a direct role in promoting reward and motivation, independent of their usual role in stress.

This opens a new direction for studying relapse. Although the Xu et al. (2024) study was done in drug-free animals, it raises the possibility that these same neurons may also be involved in relapse behavior after drug use. If activating PVN CRF neurons can drive reward seeking, it makes sense to ask whether these neurons also play a role in stress induced drug seeking. Consequently, in the current study we began the investigation of the role of the PVN to VTA pathway using transient inhibition of the PVN.

Why start with the PVN?

Recent work has shown that CRF's effects depend on the brain region. For example, when CRF neurons are activated in the BNST, animals tend to avoid that experience and lose motivation. But when similar neurons are activated in the NAc or CeA, animals are more motivated and work harder to get rewards (Pomrenze et al., 2021). All these regions interact with the PVN, which plays a key role in both the hormonal and behavioral responses to stress (Pomrenze et al., 2021).

As a follow-up to our initial pharmacological CRF antagonism experiment, in the second experiment, we non-selectively inhibited activity in the PVN to see whether shutting down this major stress responsive area would affect relapse behavior. Since the PVN contains many CRF neurons and helps kickstart the stress response, reducing its activity may also disrupt the broader

CRF systems involved in relapse (Koob & Le Moal, 2005; Pomrenze et al., 2021). Additionally, since we know that CRF has different effects depending on where in the brain it is activated, it becomes even more important to understand how upstream areas like the PVN influence downstream structures.

Building on evidence that CRF mediates stress-induced relapse, we hypothesized that disrupting stress-related signaling would reduce heroin seeking following punishment-imposed abstinence. Specifically, we predicted that blocking CRF signaling during relapse testing would attenuate heroin seeking triggered by acute food deprivation (Experiment 1), and that chemogenetic inactivation of the PVN would similarly reduce stress-induced heroin seeking (Experiment 2). Together, these approaches aim to clarify the neurobiological mechanisms underlying stress-induced relapse and identify potential targets for therapeutic intervention.

1. General Methods

Subjects

Male (250-275g; N=35) Long Evans rats (Charles River, St. Constant, Quebec, Canada) were initially double housed in the animal care facility (ACF) at Concordia University, Montreal, Canada. Rats were maintained under a reverse light cycle (lights off: 9:30 am, lights on: 9:30 pm). Two days following surgery, rats were transferred to self-administration chambers and singly housed for the remainder of the study. Outside of designated food-deprivation days, they had ad libitum access to chow (Teklad 2018C, Inotiv) and water. Routine care involved daily monitoring of body weight, health assessments (e.g., posture, grooming behavior), and maintenance of intravenous catheters. All experimental procedures were conducted by trained personnel and approved by the Concordia University Animal Research Ethics Committee

(protocol #30000301), in accordance with the Canadian Council on Animal Care (CCAC) guidelines.

Intravenous surgery

Rats underwent surgery to implant an intravenous (i.v.) catheter for heroin self-administration. Prior to surgery, animals were weighed to ensure they met the minimum required weight (≥300 g). They were administered penicillin (180 mg/kg, s.c.), carprofen (5.0 mg/kg, s.c.), and 0.9% saline (3 mL, s.c.), all under 2% isoflurane anesthesia. A 2.0 cm segment of polyurethane catheter tubing (SAI, Lake Villa, IL, USA; assembled in-house) was inserted into the right jugular vein through a small incision and secured with silk sutures (Sedki et al., 2013). The distal end of the catheter was tunneled subcutaneously to the skull and connected to a modified 22-gauge cannula (5-up, made in-house). The cannula was affixed to the skull using five jeweler's screws and dental cement (Parkell Inc., Edgewood, NY). Postoperatively, rats were administered carprofen (5.0 mg/kg, s.c.) for two days following surgery. Throughout self-administration training, catheters were flushed daily with a solution of gentamicin and heparin (7.5 IU + 800 µg; 0.2–0.3 mL) in sterile saline to maintain catheter patency.

Drugs

Heroin hydrochloride (HCl), provided by the National Institute on Drug Abuse (NIDA; Research Triangle Park, NC, USA), was dissolved in 0.9% sterile saline. The solution was prepared to deliver a dose of 0.05 mg/kg per infusion, with the concentration adjusted daily based on each rat's body weight.

For Experiment 1, α-helical corticotropin-releasing factor (α-helical CRF), a CRF receptor antagonist, was obtained from Cayman Chemical Company (Ann Arbor, MI, USA) and dissolved in distilled water at a concentration of 5 µg/µL. The dose of α-helical CRF was based

on previous studies demonstrating its efficacy in modulating stress-induced drug-seeking behavior (Shalev et al., 2006). For experiment 2, Deschloroclozapine dihydrochloride (DCZ) was diluted in saline to a final concentration of 0.1 mg/mL.

Apparatus

Behavioral experiments were conducted in standard operant conditioning chambers (Coulbourn Instruments, Allentown, PA, USA; 29.0 cm × 29.0 cm × 25.5 cm), each housed within a sound-attenuating enclosure. Each chamber was equipped with two retractable levers positioned on the same wall, 9 cm above the grid floor, a house light, white cue lights located above each lever, and a tone generator (2.9 kHz; Sonalert, Coulbourn Instruments). One lever was designated the *seek* lever, which, when pressed, granted access to the *take* lever. Pressing the *take* lever was paired with a heroin infusion. Drug delivery was initiated by an infusion pump connected to the rat's catheter via a liquid swivel (Lomir Biomedical, Notre-Dame-de-l'Île-Perrot, QC, Canada) and Tygon tubing (Saint-Gobain, Courbevoie, France) encased in a protective metal spring.

Self-administration

Self-administration with taking lever under fixed ratio 1 (FR1):

During the first phase of heroin self-administration training (3 days), rats were trained using a fixed ratio 1 (FR1) schedule with only the take lever available. Each daily session lasted 6 hours and began at the onset of the dark phase of the reversed light cycle (approximately 9:30 AM). At the start of each trial, the take lever was inserted and the houselight was turned on to signal drug availability.

A single press on the take lever (FR1) resulted in the delivery of a heroin infusion (0.1 mg/kg in 0.13 mL over 5 s), and the retraction of the lever. A cue light above the lever and an auditory tone were activated during the infusion, while the houselight was turned off. Afterward, a 30-s

inter-trial interval (ITI) ensued, during which no cues were presented, the houselight remained off, and the lever stayed retracted. At the start of the next trial, the take lever was reinserted and the houselight turned back on.

Animals were required to reach a performance criterion of at least 10 take lever presses in a single session on or before the third day to proceed to the next phase of training (seek-take chain schedule).

Self-Administration with a Seek-Take Chain Under Fixed Ratio 1 (FR1)

In this phase, a seek-take chain schedule was implemented over two days of training, each daily session lasting six hours.

At the start of each daily session, the operant training system was programmed to insert the seek lever and turn on the houselight, signaling the beginning of the trial. The take lever remained retracted during this time. Once the rat pressed the seek lever (FR1), the seek lever was retracted and the take lever was immediately inserted. The rat was required to press the take lever (FR1). Upon the rat's response, a cue light and tone were activated for a 5 s, the houselight was turned off and a heroin infusion (0.05 mg/kg in 0.13 mL over 5 s) was delivered. The take lever remained extended for a continuous 5 minutes, allowing additional heroin infusions. At the end of the 5 min period, the take lever retracted, and a 30-s inter-trial interval (ITI) ensued, during which no cues were presented, the houselight remained off, and both levers were retracted. The next trial began with the re-insertion of the seek lever and the houselight turned on.

If the rat failed to complete the seek-take chain within 10 minutes (i.e., by not pressing either the seek or take lever), the 30-s ITI was automatically initiated to signal the end of the trial and the loss of the opportunity to self-administer heroin. After the ITI, the seek lever was extended again to start a new trial.

Self-administration with a seek-take chain under Variable Intervals (VI5, VI30 and VI60): In this phase of the experiment, rats were trained under seek-take chain reinforcement schedules using Variable Interval (VI) schedules, following the progression from Fixed Ratio 1 (FR1). Each trial began with the insertion of the seek lever and illumination of the houselight, signaling the start of the session. The take lever remained retracted. The first press on the seek lever initiated a variable interval timer, with the software randomly selecting a time from a list to determine the duration of the interval before the first seek lever press was followed by the extension of the take lever. For the VI5 schedule, the interval was randomly chosen from 0.1 s, 5 s, and 10 s, resulting in an average interval of 5 s; for VI30, the system selected from 15 s, 30 s, and 45 s (average 30 s); and for VI60, the options were 45 s, 60 s, and 75 s (average 60 s). After the appropriate interval elapsed, the next response on the seek lever resulted in retraction of the lever, and the take lever was extended. Once the rat pressed the take lever, the cue light and tone were activated for 5 s, the houselight turned off, and a heroin infusion (0.05 mg/kg in 0.13 mL over 5 s) was delivered. The take lever remained extended for 5 minutes. Following this, a 30-s inter-trial interval (ITI) occurred, during which no cues were presented, and the lever was retracted. The ITI duration gradually increased each day, starting from 30 s and up to 7 minutes. The next trial began with the insertion of the seek lever and illumination of the houselight. If the rat failed to complete the seek-take chain within 10 minutes (i.e., not pressing the seek or take lever), the trial ended, and the ITI was initiated, signaling the loss of the opportunity to self-administer heroin. Training under the VI5 schedule lasted for 3 days, followed by 1 day under the VI30 schedule and 9 days under the VI60 schedule. Training continued for approximately 22 days.

Punishment-Imposed Abstinence

After reaching the self-administration criterion which was defined as a stable number of heroin infusions across four consecutive days under a VI60 schedule, rats entered a punishment-imposed abstinence phase designed to suppress drug-seeking behavior through probabilistic punishment. Each 6-hour daily session began with the insertion of the seek lever and activation of the houselight, with the seek link still operating under the VI60 schedule. Upon completion of the seek link, the operant system either administered a mild 0.5 s footshock (in 30% of trials) or extended the take lever for drug delivery (in 70% of trials). In footshock trials, the seek lever retracted, footshock was delivered, and a 7-minute inter-trial interval (ITI) followed, during which the houselight and all cues remained off. In drug-available trials, pressing the take lever resulted in the delivery of heroin (0.05 mg/kg in 0.13 mL), and activation of the cue light and tone for 5 s. The take lever remained extended for 5 continuous minutes, regardless of when the rat pressed it. After 5 min, the take lever retracted, and a 7 min ITI ensued. If a rat failed to complete the seek-take chain within 10 minutes, a 7-minute ITI was triggered to signify a missed opportunity.

For experiment 1, the footshock intensity began at 0.2 mA and increased by 0.1 mA every two days, up to 0.6 mA, with regular supervision to ensure animal welfare. If rats failed to reduce seeking after 6 days at 0.6 mA, the intensity increased by 0.1 mA each day up to a maximum of 1.0 mA. For experiment 2, the footshock intensity began at 0.4 mA and increased by 0.1 mA every day up to 1.0 mA. Punishment phase ended only after rats reached two consecutive days with no infusions.

Heroin relapse test

One day following the final punishment-imposed abstinence session, rats were divided into two groups based on feeding conditions: Sated and Food Deprived (FD). Groups were

counterbalanced and matched for body weight, average seek lever presses, and infusions over the self-administration training days and the first three days of punishment. The relapse tests occurred 48 hours apart in a counterbalanced order.

For the Food Deprived condition, food hoppers were removed at approximately 9:30 AM the day prior to testing, while sated animals had continuous access to food. Water remained freely available for all animals throughout. During the deprivation period, rats were monitored to ensure they did not exhibit any signs of health deterioration. Animals were housed under habituation-like conditions (no house light, no levers or heroin syringes) and remained attached to their metal spring.

On test day, animals received their assigned treatment 15 minutes prior to the relapse session, according to the experiment that was carried out. A 3-hour heroin-seeking test was then conducted with the seek lever under a VI60 schedule, and the take lever available for 5 min, with a 7-minute inter-trial interval. During the session, no heroin or footshock was administered. Cue light and tone were presented contingent on take lever presses. After the session, food was returned to previously food-deprived rats.

On the following day, feeding conditions were reversed: animals that were previously sated underwent 24-hour food deprivation, and previously food-deprived animals had ad libitum access to food. The same testing protocol was repeated under these reversed conditions.

Locomotor test

The day following the heroin-seeking test, animals underwent a locomotor activity assessment to evaluate potential treatment-related effects on general motor behavior. Animals received their assigned treatment (e.g., ligand, antagonist, or vehicle) prior to testing, according to the specific experimental protocol. After a 15-minute time interval, they were placed in a locomotor activity

monitoring chamber (Coulbourn Instruments). Locomotor activity was recorded over a 1-hour period using TruScan software (Coulbourn Instruments), with total distance traveled (in meters) used as the primary measure of activity.

Perfusion and Brain Tissue Processing

Following completion of behavioral testing (e.g., locomotor activity assessment), animals were transcardially perfused with phosphate-buffered saline (PBS), followed by 4% paraformaldehyde to fix the brain tissue. Brains were extracted and post-fixed in 4% paraformaldehyde for 2-4 hours, then cryoprotected in a 30% sucrose solution at 4°C for 48 hours. After cryoprotection, brains were wrapped in labeled aluminum foil and stored at −80°C until sectioning.

Coronal brain sections (40 μm thick) were prepared using a cryostat. These sections were used for downstream histological analyses, such as verification of anatomical targeting or molecular markers, according to the specific aims of each experiment. The Paxinos and Watson Rat Brain Atlas (2005) was used to guide anatomical identification.

2. Experiment 1: Role for Corticotropin-Releasing Factor in Acute Food Deprivation-Induced Relapse to Heroin Seeking after Punishment-Imposed Abstinence in Rats

2.1 Surgery

As described in the General Methods, all animals underwent intravenous catheter implantation into the right jugular vein for heroin self-administration.

Immediately after the IV catheter implantation surgery, animals in Experiment 1 were also implanted with a unilateral intracranial guide cannula targeting the lateral ventricles. Under 2% isoflurane anesthesia, a 22-gauge guide cannula (RWD Life Sciences, Shenzhen, China) was stereotaxically implanted at the following coordinates relative to bregma: −0.9 mm posterior, +1.4 mm lateral, and −2.0 mm ventral. The cannula was secured in place using an acrylic dental resin head cap anchored to the skull with jeweler screws. This configuration allowed for central

administration of α-helical CRF or vehicle directly into the cerebroventricular system for diffusion throughout the brain.

Post-operative care was administered as described in the General Methods.

2.2 Cannula Placement Verification Test

Following the post-surgical recovery period, an angiotensin test was conducted to confirm accurate placement of the intracranial cannula within the cerebral ventricles. Angiotensin II, when administered intracerebroventricularly (ICV), elicits a characteristic increase in water intake, serving as a behavioral indicator of correct ventricular targeting.

To begin, the guide cannula was accessed and checked for patency. A solution of angiotensin II (MilliporeSigma) was prepared in sterile saline at a concentration of 50 ng/µL. An injector (RWD, 28G) was attached to a Hamilton syringe connected to an infusion pump (Harvard Apparatus 11 Plus) via PE20 tubing. The injector was inserted into the guide cannula, and 2.00 µL of the angiotensin II solution was delivered ICV at a controlled rate of 1.0 µL per minute.

A robust drinking response typically occurred within 30–60 s of infusion, confirming successful cannula placement. Rats that did not exhibit this response were excluded from further testing.

2.3 Relapse Test

In this experiment, relapse to heroin seeking was examined under two feeding conditions: food deprived and sated, following punishment-imposed abstinence. To evaluate the role of corticotropin releasing factor (CRF) signaling, rats received intracerebroventricular (ICV) infusions of either α helical CRF (10.0 µg; MilliporeSigma, Canada) or 0.9% sterile saline prior to each test session.

Infusions were administered 10 minutes before the 3-h relapse test using a 28G injector (RWD) inserted into the preimplanted guide cannula, connected to a Hamilton syringe and an infusion

pump (Harvard Apparatus 11 Plus) via PE20 tubing. A total volume of 2.00 µL was delivered at a rate of 1.0 µL per minute. To ensure proper diffusion, the injector remained in place for one additional minute following infusion. Each rat received the same treatment (α helical CRF or saline) for both relapse tests.

The feeding conditions were counterbalanced across test days. Rats experienced one test while food deprived for 24 hours and another while sated, allowing within subject comparisons of drug effects across metabolic states.

3. Experiment 2: The Role of the Paraventricular Nucleus of the Hypothalamus in Stress-Induced Relapse to Heroin Seeking

3.1 Surgery

As described in the General Methods, all animals received intravenous catheterization prior to behavioral testing. In this experiment, rats also underwent bilateral intracranial surgery targeting the paraventricular nucleus (PVN) of the hypothalamus for viral vector delivery, immediately following the IV catheter implantation.

A total volume of 0.3 µL per hemisphere of AAV2/8-hSyn-hM4D(Gi)-mCherry viral vector (Canadian Neurophotonic Platform/Viral Vector Core, Québec, QC) was delivered bilaterally to the PVN using a 33-gauge injector connected to a Hamilton syringe via PE20 tubing. The injection was administered at a rate of 0.1 µL per minute (coordinates relative to bregma: AP -1.5 mm, ML ±0.35 mm, DV -7.8 mm). Following infusion, the injector was left in place for an additional 10 minutes to allow for diffusion of the viral solution before withdrawal.

Postoperative care was administered as described in the General Methods.

3.2 Relapse Test

In this experiment, relapse to heroin seeking was examined under two feeding conditions: food deprived and sated, following punishment-imposed abstinence. To investigate the contribution of

specific neural circuit inhibition, rats assigned to the experimental condition received intraperitoneal (IP) injections of a chemogenetic ligand (DCZ; 0.1 mg/kg) 15 minutes prior to the 3-hour relapse test. Control animals received an equal volume of vehicle solution.

Feeding conditions were counterbalanced across test days. Each rat completed one test under food-deprived conditions (24-hour deprivation) and one test while sated, allowing within-subject comparisons of chemogenetic inhibition across metabolic states. Each animal received the same treatment (DCZ or vehicle) for both test sessions.

4. Statistical Analyses

All statistical analyses were performed using GraphPad Prism 10, with significance set at $p < 0.05$. Repeated measures ANOVA was used to analyze self-administration and punishment performance across days, with seek lever responses, take lever responses, and number of infusions as dependent variables. For relapse tests, a two-way mixed ANOVA was conducted to assess heroin-seeking behavior, with feeding condition (food deprived, sated) as the within-subject factor and treatment condition (e.g., α-helical CRF vs. saline or DCZ vs. vehicle) as the between-subject factor. The main variables of interest in relapse tests were the number of responses on the seek and levers, and the number of "infusions". Specific group comparisons were done using Bonferroni's correction for multiple comparisons. Total distance traveled during the locomotor activity test was analyzed using independent samples t-tests, with corrections for unequal variance when necessary. Body weight comparisons were also assessed using t-tests. Effect sizes are reported as Cohen's d or partial eta squared (η^2), where appropriate.

Results

Experiment 1. Role for Corticotropin-Releasing Factor in Acute Food Deprivation-Induced Relapse to Heroin Seeking after Punishment-Imposed Abstinence in Rats

Placement Pre-Screening: All 20 rats showed increased water intake after angiotensin II administration, confirming correct ventricular cannula placement.

Data Integrity: During the experiment, two rats were euthanized due to health complications. Additionally, two rats were excluded from the study because they obtained very low number of infusions by the end of the training during the self-administration phase.

Self-Administration training: All remaining rats (N = 16) demonstrated reliable heroin self-administration. As the variable interval increased over training days, rats showed a progressive increase in heroin-seeking (seek lever presses), heroin-taking (take lever presses), and the number of heroin infusions obtained (Fig. 1). A one-way repeated measures ANOVA revealed a significant effect of training day on seek responses ($F(3.72, 55.82) = 6.66$, $p = <.001$, $\eta^2 = 0.31$), take responses ($F(3.99, 59.90) = 2.85$, $p = <.001$, $\eta^2 = 0.16$), and infusions ($F(4.18, 62.64) = 2.98$, $p = 0.02$, $\eta^2 = 0.17$), indicating that heroin-directed behaviors increased significantly across the training period.

Punishment Phase: Over the 12-day punishment phase, rats were exposed to escalating footshock intensities while heroin remained available. As shock intensity increased, rats progressively decreased their seek lever presses, take lever presses, and number of heroin infusions (Fig. 2). However, one-way repeated measures ANOVAs conducted only on punishment days in which all 16 rats were still active in the procedure revealed no statistically significant effect of day on infusions ($F(2.02, 30.23) = 1.75$, $p = 0.19$, $\eta^2 = 0.10$), seek responses ($F(1.40, 20.97) = 2.91$, $p = 0.09$, $\eta^2 = 0.16$), or take responses ($F(2.59, 38.89) = 1.31$, $p = 0.29$, $\eta^2 = 0.08$). This lack of statistical significance is likely due to the limited subset of early punishment days included in the analysis, prior to full behavioral suppression. Ultimately, all rats (n = 16) reached voluntary abstinence by the end of the phase.

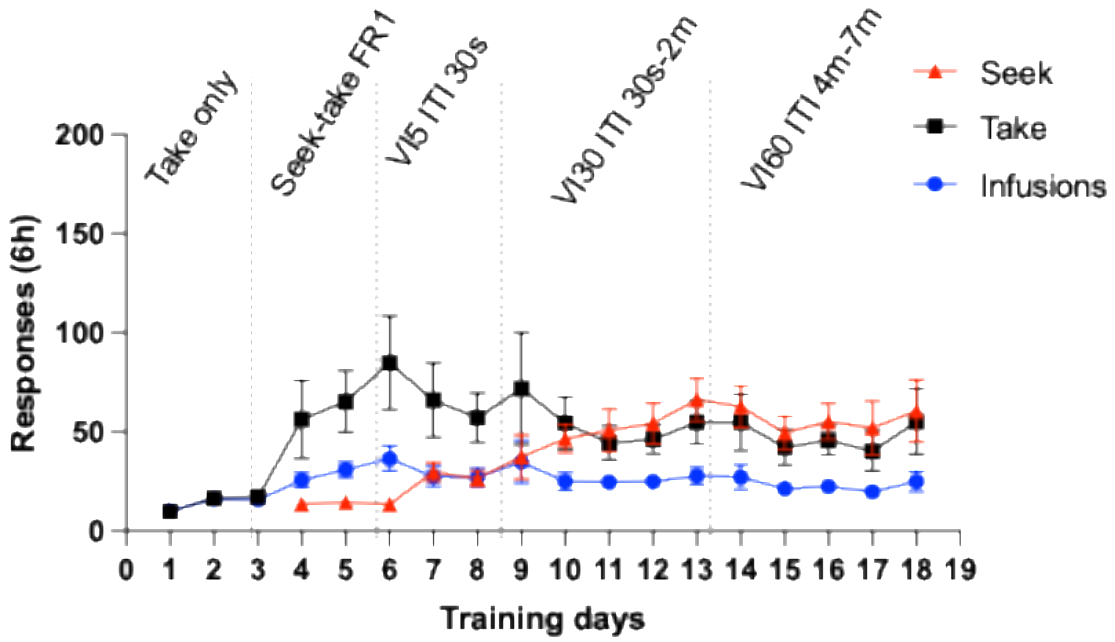


Figure 1.Self-administration training in male rats (N = 16). Mean (+ SEM) number of seek lever presses (red), take lever presses (black), and heroin infusions (blue) across training days. Data are presented across different phases of the reinforcement schedule: Take Only (no seek lever present), Fixed Ratio 1 (FR1), Variable Interval 5 s (VI5), Variable Interval 30 s (VI30), and Variable Interval 60 s (VI60). Vertical dashed lines indicate transitions between reinforcement schedule phases. ITI = inter-trial interval.

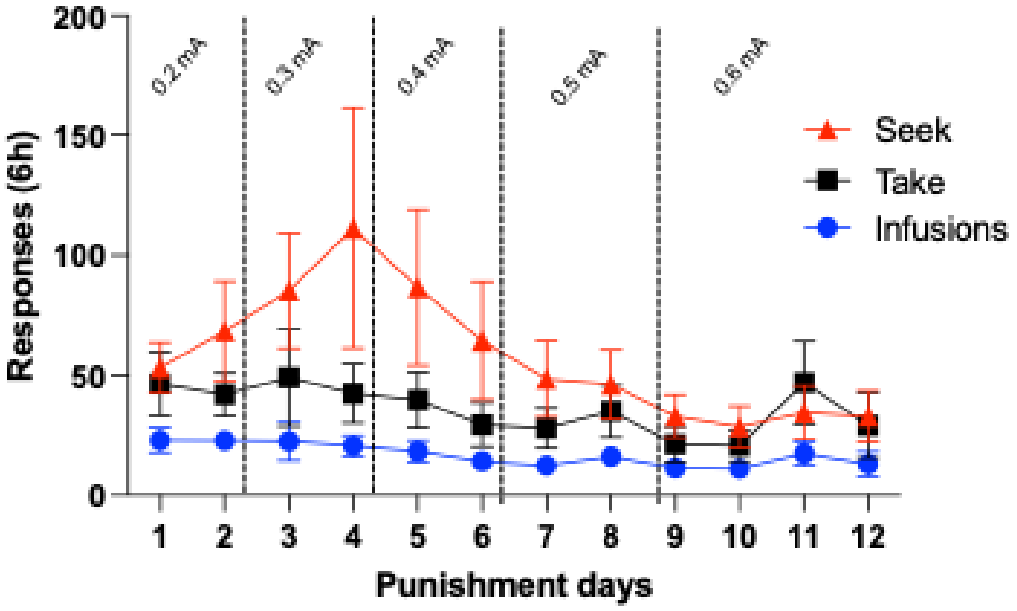


Figure 2. Punishment-imposed abstinence phase in male rats (N = 16). Mean (+ SEM) number of seek lever presses (red), take lever presses (black), and heroin infusions (blue) across 12 days of the punishment-imposed abstinence phase. All rats were included in this phase. Footshock intensity escalated by 0.1 mA every 2 days.

Heroin-seeking tests: Once abstinence was obtained, rats were assigned to either vehicle (n = 8) or CRF antagonist (n = 8) treatment groups. Food deprivation significantly increased seeking lever responses (M = 70.06, SD = 45.70) compared to sated conditions (M = 2, SD = 0.53), and administration of a CRF-receptor antagonist clearly attenuated this response, although the interaction effect did not reach statistical significance (Fig. 3). A two-way repeated measures ANOVA revealed a main effect of feeding condition ($F(1, 14) = 8.73$, $p = 0.011$, $\eta^2 = 0.21$. There was no significant main effect of treatment ($F(1, 14) = 1.85$, $p = 0.195$, $\eta^2 = 0.05$), and no treatment × feeding interaction ($F(1, 14) = 2.01$, $p = 0.178$, $\eta^2 = 0.05$). Close inspection of the data found a substantial attenuation of seeking responses in the CRF-receptor antagonist-treated rats. Bonferroni corrected comparisons revealed a statistically significant effect of food deprivation in the vehicle-treated rats ($t(14) = 3.09$, $p = 0.02$; Cohen's d = 1.09) but not in the CRF-receptor antagonist-treated rats ($t(14) = 1.09$, $p = 0.59$; Cohen's d = 0.39), indicating a large attenuation effect.

A similar pattern was observed for heroin-taking behavior (Fig. 4), with food deprivation significantly increasing take lever responses (M = 251.81, SD = 188.71) compared to sated conditions (M = 13.06, SD = 6.45). A two-way repeated measures ANOVA revealed a main effect of feeding ($F(1, 14) = 10.16$, $p = 0.007$, $\eta^2 = 0.22$), but no main effect of treatment ($F(1, 14) = 2.74$, $p = 0.120$, $\eta^2 = 0.06$) and no significant interaction ($F(1, 14) = 3.40$, $p = 0.087$, $\eta^2 = 0.07$). Here again, close inspection of the data revealed that take responses were substantially attenuated in CRF-receptor antagonist-treated rats. Using Bonferroni-corrected comparisons, a statistically significant effect of food deprivation was observed in vehicle-treated rats, $t(14) = 3.56$, $p = 0.01$, Cohen's $d = 1.26$, but not in the CRF-receptor antagonist-treated rats, $t(14) = 0.95$, $p = 0.72$, Cohen's $d = 0.34$.

Food deprivation significantly increased the number of heroin "infusions" (M = 100.50, SD = 92.86) compared to sated conditions (M = 4.75, SD = 13.43). (Fig. 5). A main effect of feeding was observed ($F(1, 14) = 11.40$, $p = 0.005$, $\eta^2 = 0.22$), but no main effect of treatment ($F(1, 14) = 2.76$, $p = 0.119$, $\eta^2 = 0.06$), and the treatment × feeding interaction narrowly missed statistical significance ($F(1, 14) = 4.35$, $p = 0.056$, $\eta^2 = 0.08$). Close inspection of the data revealed that "infusion" responses were substantially attenuated in CRF-receptor antagonist-treated rats. Using Bonferroni-corrected comparisons, a statistically significant effect of food deprivation was observed in vehicle-treated rats, $t(14) = 3.86$, $p < .01$, Cohen's $d = 1.37$, but not in the CRF-receptor antagonist-treated rats, $t(14) = 0.91$, $p = 0.75$, Cohen's $d = 0.32$.

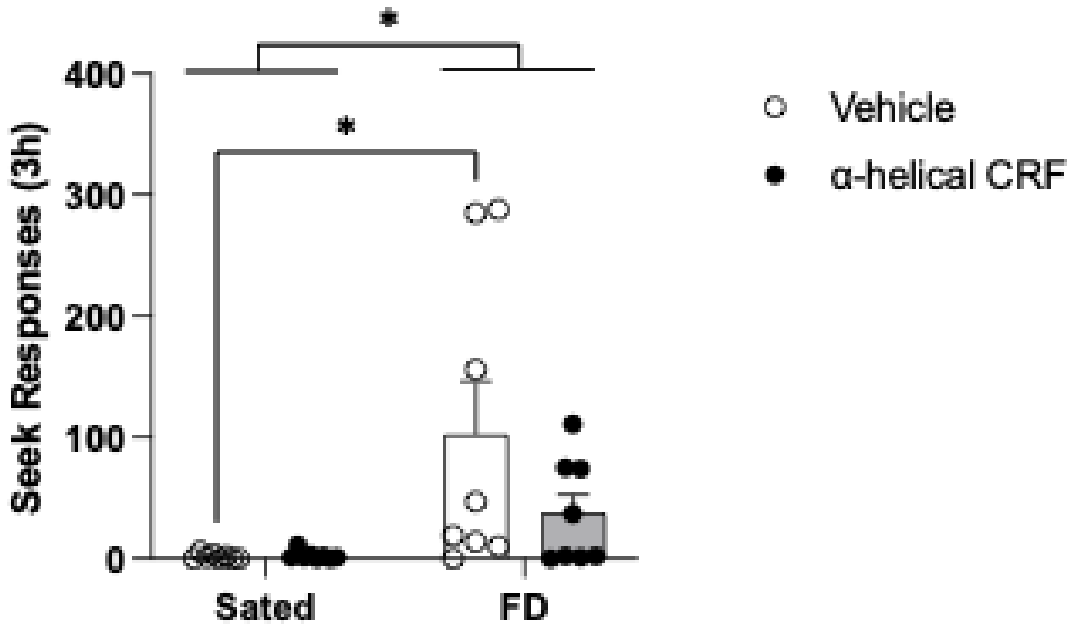


Figure 3. Seek lever responses during relapse test following punishment-imposed abstinence. Mean (+ SEM) number of seek lever presses during heroin-seeking tests under sated and food-deprived conditions for vehicle-treated (n = 8) and CRF antagonist-treated rats (n = 8). Asterisks indicate statistically significant differences ($p < 0.05$).

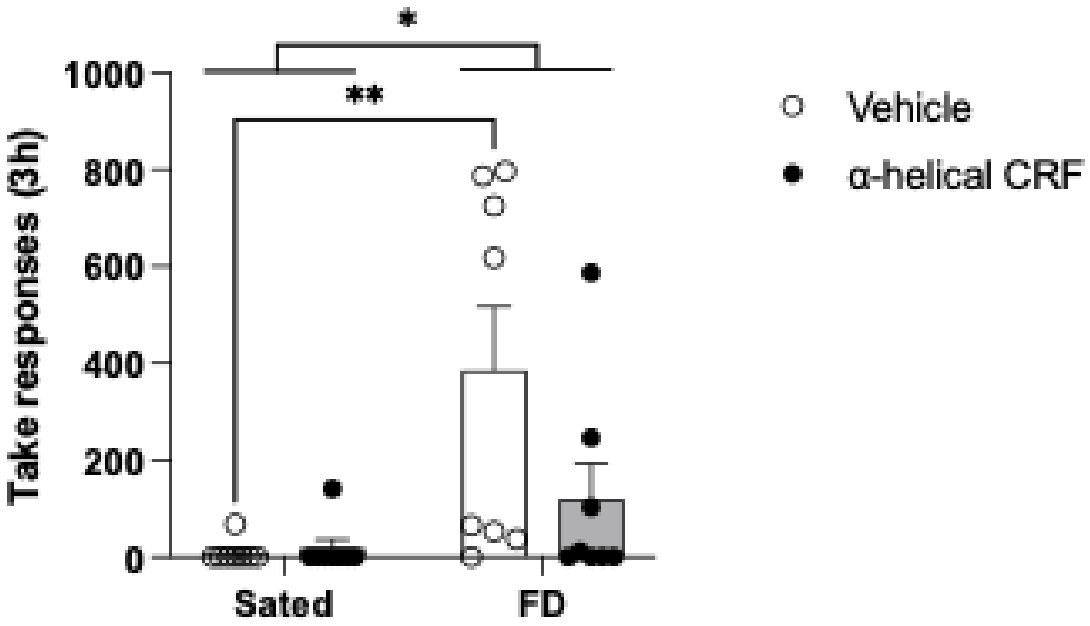


Figure 4. Take lever responses during relapse test following punishment-imposed abstinence. Mean (+ SEM) number of take lever presses during heroin-seeking tests under sated and food-deprived conditions for vehicle-treated (n = 8) and CRF antagonist-treated rats (n = 8). Asterisks indicate statistically significant differences ($p < 0.05$).

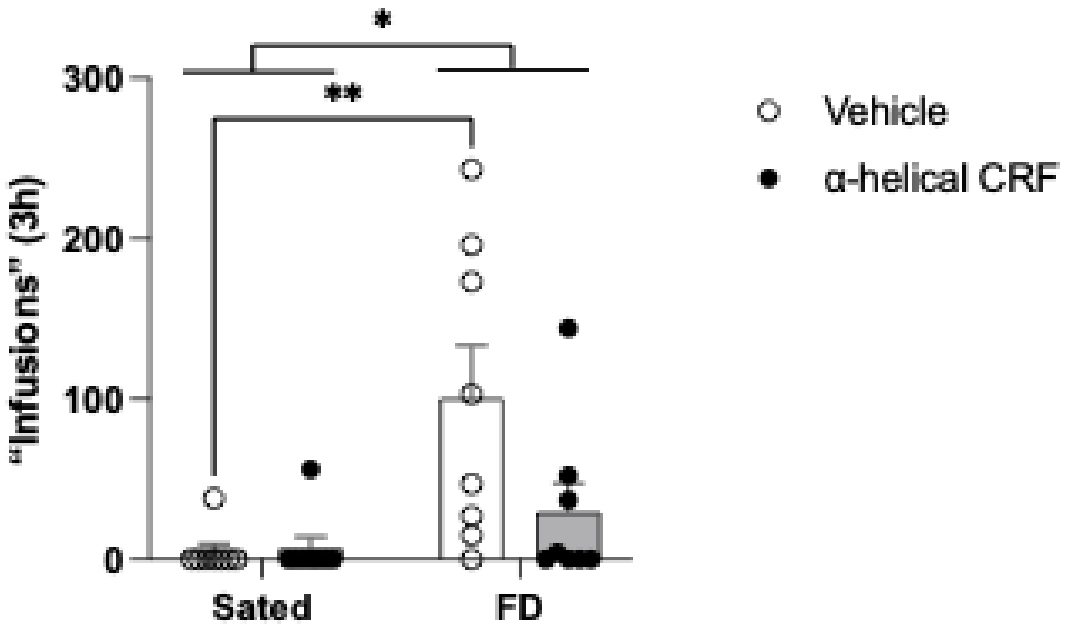


Figure 5. Number of "infusions" like responses during relapse test following punishment-imposed abstinence. Mean (+ SEM) number of heroin "infusions" during heroin-seeking tests under sated and food-deprived conditions for vehicle-treated (n = 8) and CRF antagonist-treated rats (n = 8). Asterisks indicate statistically significant differences ($p < 0.05$).

Locomotor: Rats treated with either vehicle or -helical CRF were assessed for total distance traveled. Due to technical limitations, locomotor testing was conducted only during one experimental run. An unpaired t-test revealed no significant difference in total distance traveled between vehicle-treated and α-helical CRF-treated rats, $t(7) = 1.17$, $p = .28$, 95% CI [−4189, 1419; Cohen's $d = 0.783$]. (Fig. 6). These results suggest that the CRF receptor antagonist did not alter baseline locomotor activity in this subset of animals.

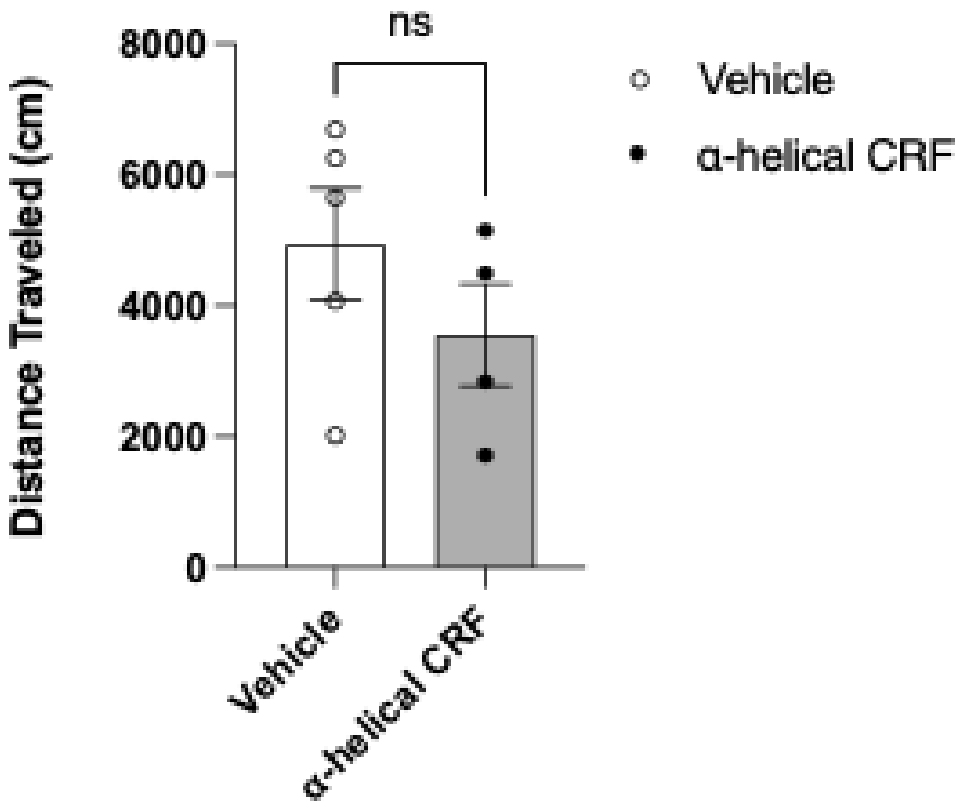


Figure 6. Total distance traveled (cm) during the locomotor activity test in vehicle-treated (n = 5) and α-helical CRF-treated rats (n = 4).

Experiment 2. The Role of the Paraventricular Nucleus of the Hypothalamus in Stress-Induced Relapse to Heroin Seeking

Data Integrity: During the experiment, three rats were removed due to health issues. Four rats were excluded for not meeting the training criterion defined as five or fewer heroin infusions as average of the final three training days during the self-administration phase. Additional three rats were excluded due to incorrect viral injection placement outside the paraventricular nucleus (PVN). Lastly, one rat was excluded from analysis due to behavioral data that were identified as a statistical outlier.

Self-Administration training: All rats (n = 19) demonstrated reliable heroin self-administration behavior. Across the 18 days of training, rats showed a significant increase in seek lever presses, take lever presses, and heroin infusions as the variable interval (VI) schedule progressed. One-way repeated measures ANOVAs revealed a significant effect of training day on seek responses ($F(4.68, 84.16) = 9.86$, $p < .001$, $\eta^2_p = 0.35$), take responses ($F(4.35, 78.25) = 7.63$, $p < .001$, $\eta^2_p = 0.30$), and heroin infusions ($F(4.60, 82.71) = 7.21$, $p < .001$, $\eta^2_p = 0.29$), (Fig. 7).

Punishment Phase: Over the 4-day punishment phase, rats (n = 19) were exposed to increasing footshock intensity while heroin remained available. A clear and statistically significant downward trend was observed in heroin-seeking behavior across punishment days, with seek lever presses decreasing significantly ($F(1.57, 28.20) = 13.00$, $p < .001$, $\eta^2_p = 0.42$). Similarly, reductions in take lever presses ($F(1.94, 34.86) = 11.33$, $p < .001$, $\eta^2_p = 0.39$) and heroin infusions ($F(2.59, 46.53) = 12.59$, $p < .001$, $\eta^2_p = 0.41$). These results indicate robust punishment-induced suppression of operant responding across all three behavioral measures, ultimately leading to abstinence (Fig. 8).

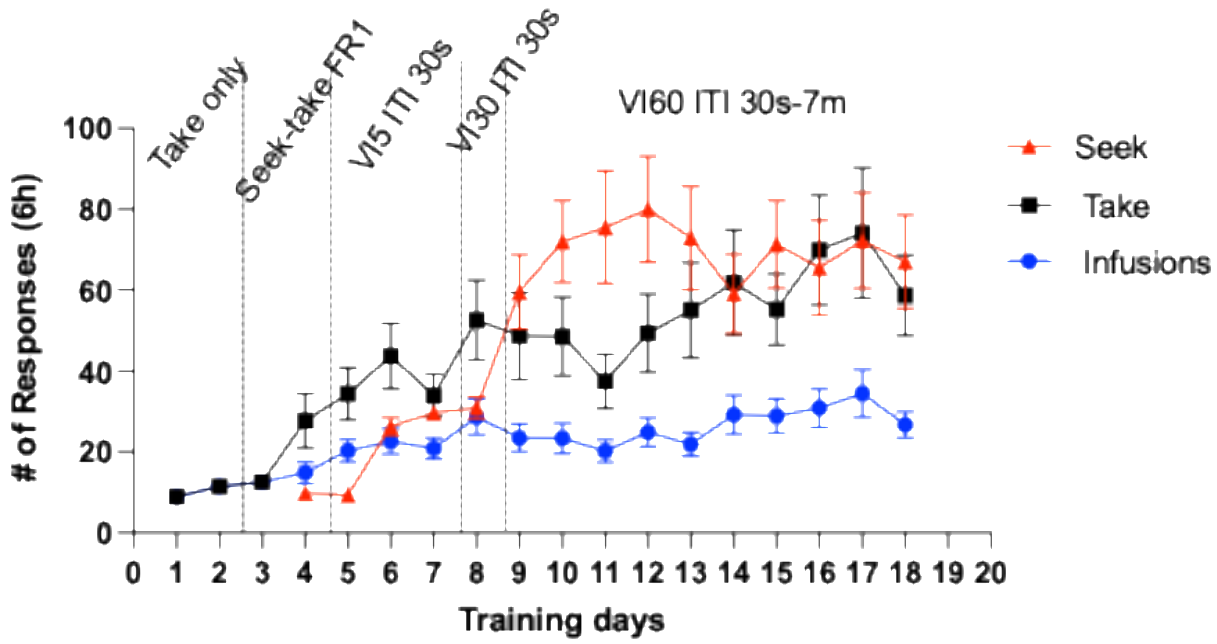


Figure 7. Self-administration training in male rats (N = 19). Mean (+ SEM) number of seek lever presses (red), take lever presses (black), and heroin infusions (blue) across 18 days of training. Data are presented across the progression of reinforcement schedules: Fixed Ratio 1 (FR1), Variable Interval 5 s (VI5), Variable Interval 30 s (VI30), and Variable Interval 60 s (VI60).

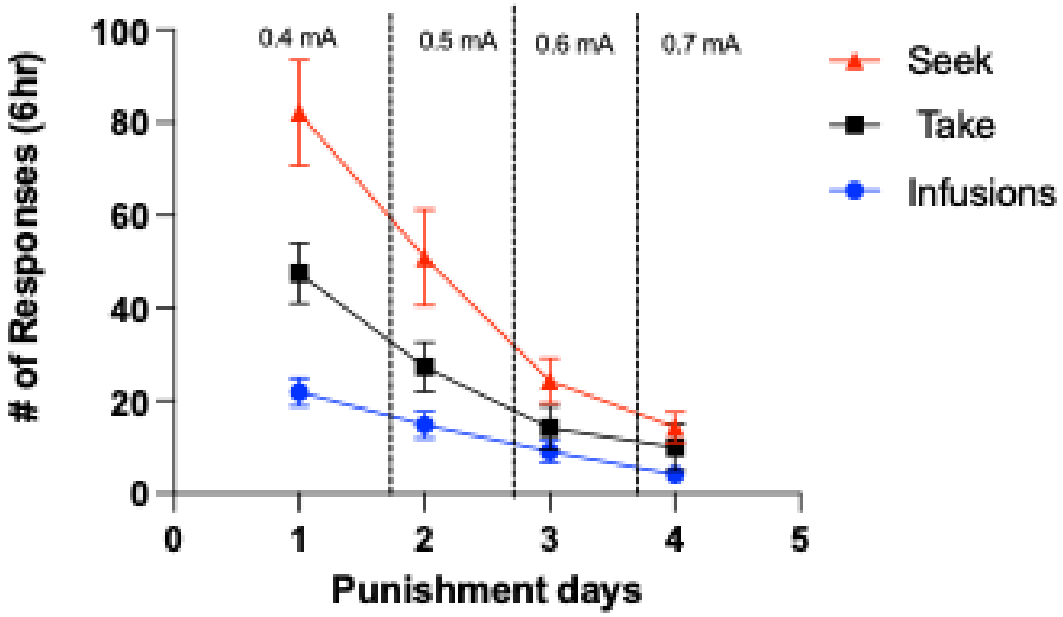


Figure 8. Punishment-imposed abstinence in male rats (N = 19). Mean (+ SEM) number of seek lever presses (red), take lever presses (black), and heroin infusions (blue) across 4 days of punishment. Footshock intensity increased each day to induce abstinence.

Heroin-seeking tests: A two-way repeated measures ANOVA was conducted to examine the effects of feeding condition (sated vs. food deprivation) and treatment (vehicle vs. DCZ) on seek responses. There was a significant main effect of feeding condition on seeking behavior ($F(1,17) = 7.67$, $p = 0.013$, $\eta p^2 = 0.31$), with food-deprived rats exhibiting higher seek responses ($M = 38.46$, SD = 10.24) compared to sated rats ($M = 2.17$, SD = 0.24). Neither the main effect of treatment ($F(1,17) = 0.30$, $p = 0.59$, $\eta p^2 = 0.02$) nor the feeding by treatment interaction ($F(1,17) = 0.32$, $p = 0.58$, $\eta p^2 = 0.02$) were statistically significant. (Fig. 9). In addition, close inspection of the data found no substantial attenuation of seeking responses in the DCZ-treated rats likely due to a rat being an outlier in this specific response. Bonferroni-corrected comparisons revealed no statistically significant effect of food deprivation in the vehicle-treated rats, $t(17) = 2.42$, $p = 0.05$, Cohen's $d = 0.59$, or in the DCZ-treated rats, $t(17) = 1.52$, $p = 0.29$, Cohen's $d = 0.37$. Similarly, a two-way repeated measures ANOVA examining the effects of feeding and treatment on **"**infusion" responses revealed a significant main effect of feeding condition ($F(1,17) = 21.33$, $p = 0.0002$, $\eta p^2 = 0.56$), with food-deprived rats receiving more infusions ($M = 42.32$, SD = 21.04) than sated rats ($M = 2.17$, SD = 1.03). The main effect of treatment ($F(1,17) = 2.22$, $p = 0.15$, $\eta p^2 = 0.16$) and the feeding by treatment interaction ($F(1,17) = 2.65$, $p = 0.12$, $\eta p^2 = 0.14$) were not statistically significant. (Fig. 10). However, close inspection of the data found a substantial attenuation of "infusion" responses in the DCZ-treated rats. Bonferroni-corrected comparisons revealed a statistically significant effect of food deprivation in vehicle-treated rats, $t(17) = 4.54$, $p < .001$, Cohen's $d = 1.10$, but not in DCZ-treated rats, $t(17) = 2.06$, $p = 0.11$, Cohen's $d = 0.50$.

Finally, for take responses, analysis showed a significant main effect of feeding condition ($F(1,17) = 13.72$, $p = 0.0018$, $\eta p^2 = 0.45$), with food-deprived rats demonstrating higher take

responses (M = 108.0, SD = 52.25) compared to sated rats (M = 8.64, SD = 4.59). There was no significant main effect of treatment ($F(1,17) = 1.07$, $p = 0.32$, $\eta p^2 = 0.09$) nor a significant feeding by treatment interaction ($F(1,17) = 2.25$, $p = 0.15$, $\eta p^2 = 0.12$). (Fig. 11). However, close inspection of the data found a substantial attenuation of take responses in the DCZ-treated rats. Bonferroni-corrected comparisons revealed a statistically significant effect of food deprivation in vehicle-treated rats, $t(17) = 3.78$, $p < .01$, Cohen's $d = 0.92$, but not in DCZ-treated rats, $t(17) = 1.52$, $p = 0.29$, Cohen's $d = 0.37$.

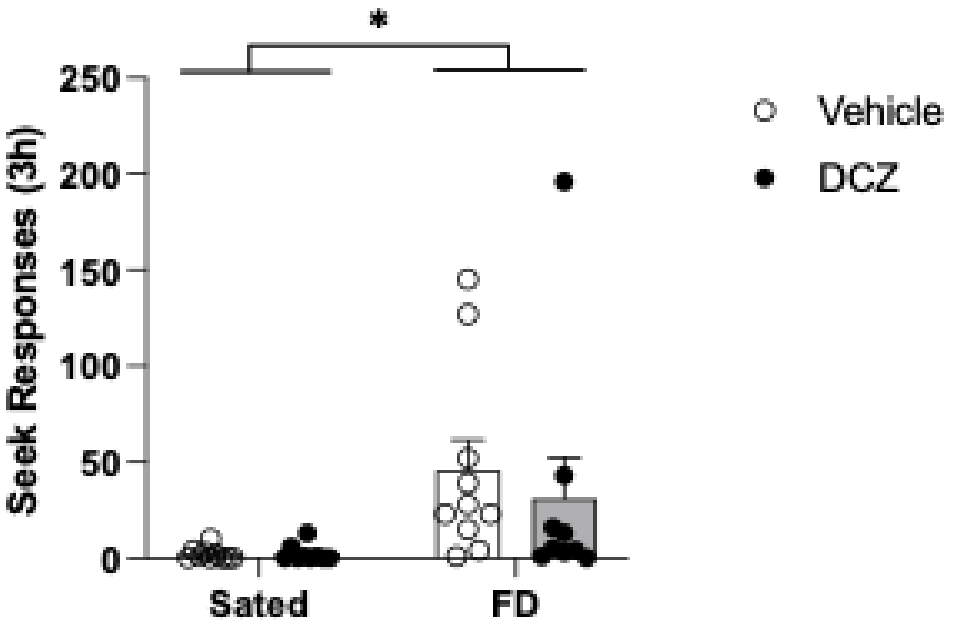


Figure 9. Seek lever response during relapse test following punishment-imposed abstinence. Mean (+ SEM) number of seek lever presses during heroin-seeking tests under sated and food-deprived conditions for vehicle-treated (n = 10) and DCZ-treated rats (n = 9). Asterisks indicate statistically significant differences ($p < 0.05$).

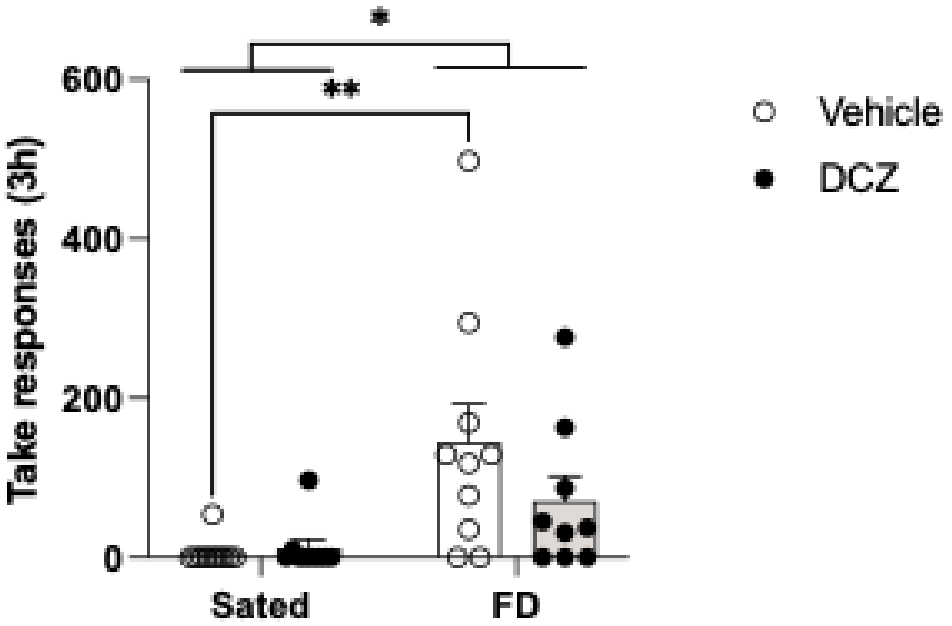


Figure 10. Take lever response during relapse test following punishment-imposed abstinence. Mean (+ SEM) number of take lever presses during heroin-seeking tests under sated and food-deprived conditions for vehicle-treated (n = 10) and DCZ-treated rats (n = 9). Asterisks indicate statistically significant differences ($p < 0.05$).

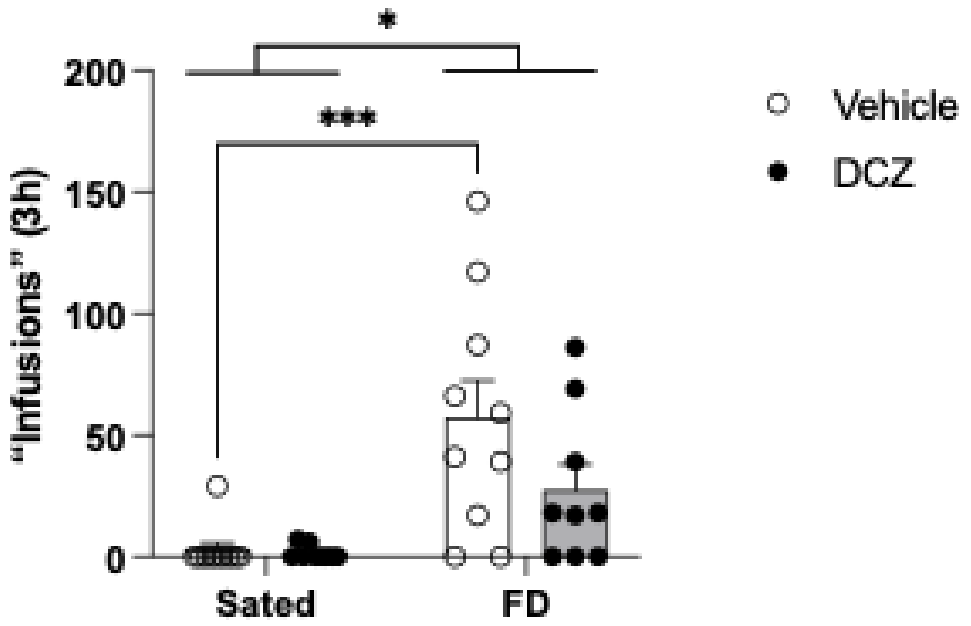


Figure 11. Number of "infusions" like responses during relapse test following punishment-imposed abstinence. Mean (+ SEM) number of heroin "infusions" during heroin-seeking tests under sated and food-deprived conditions for vehicle-treated (n = 10) and DCZ treated rats (n = 9). Asterisks indicate statistically significant differences ($p < 0.05$).

Locomotor: Rats treated with either vehicle or DCZ were assessed for total distance traveled. An unpaired *t*-test revealed no significant difference in locomotor activity between vehicle-treated rats and DCZ-treated rats, $t(17) = 0.22$, $p = .83$, 95% *CI* [−1389, 1128]; Cohen's $d = 0.101$. (Fig. 12).

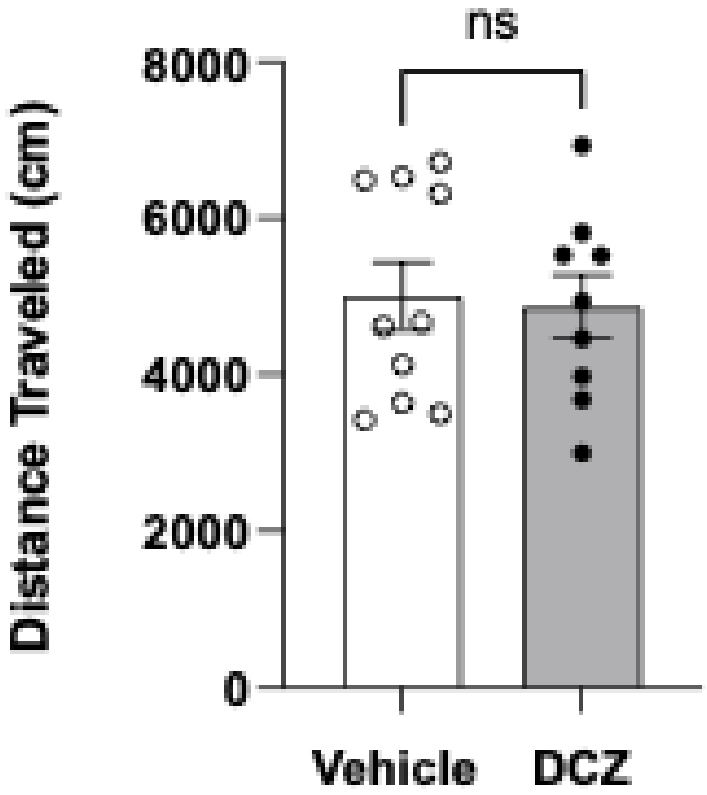


Figure 12. Total distance traveled (cm) during the locomotor activity test in vehicle (n = 10) and DCZ treated rats (n = 9).

Placements: Placement of viral injections was validated in 19 rats. A representative example of DREADDs expression within the paraventricular nucleus (PVN) is shown in the cropped image (Fig. 13).

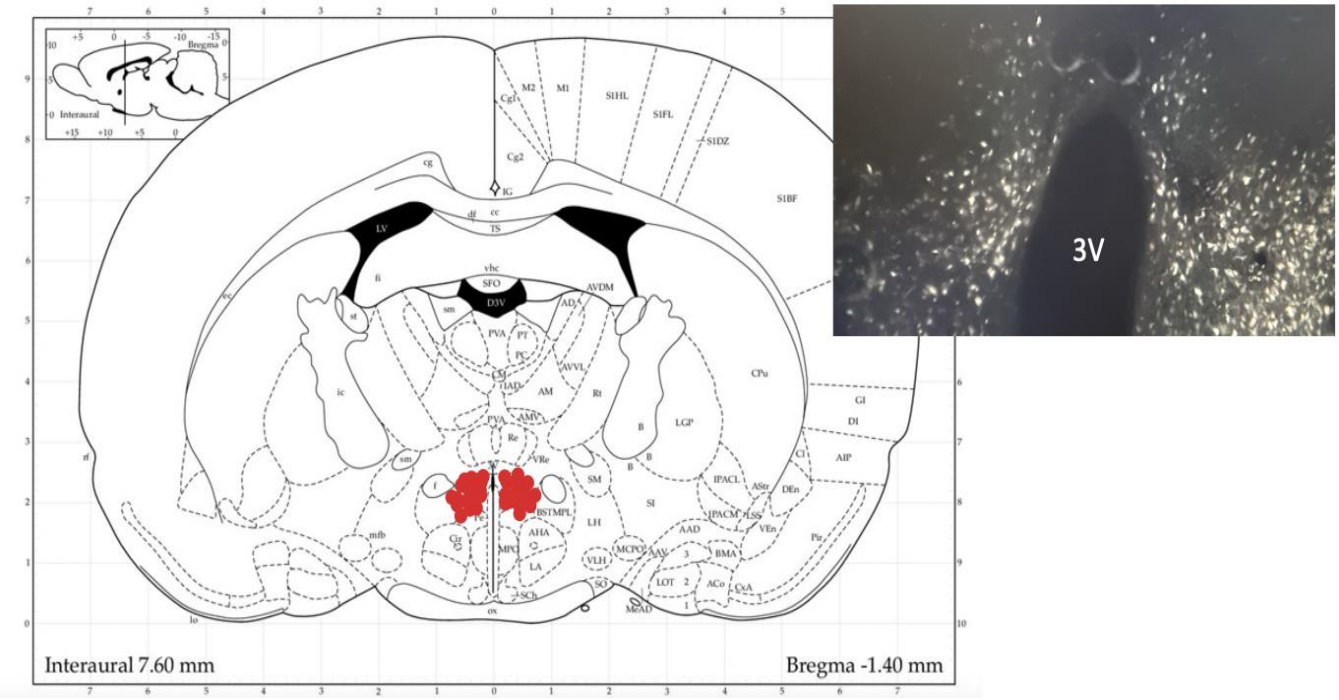


Figure 13. Viral injection placements and DREADDs expression in the paraventricular nucleus (PVN). Left: Coronal schematic of a rat brain indicating the viral injection sites (red dots) in the 19 rats. Right: Representative coronal microscope image showing the expression of DREADDs within the PVN.

Discussion

This study had two primary goals. The first was to examine the role of corticotropin releasing factor, or CRF, in food deprivation induced relapse to heroin seeking using a punishment-imposed abstinence model. The second was to investigate whether the paraventricular nucleus of the hypothalamus, or PVN, is necessary for this form of relapse by chemogenetically inactivating it during the relapse test. Together, these two aims were designed to advance our understanding of how stress triggers relapse in a model that better reflects real world drug use patterns.

Role for Corticotropin-Releasing Factor in Acute Food Deprivation-Induced Relapse to Heroin Seeking after Punishment-Imposed Abstinence in Rats

In support of the first aim, we found that central administration of a CRF antagonist reduced heroin seeking following acute food deprivation. This finding confirms that CRF is a key component in stress induced relapse, extending a large body of previous work that has demonstrated the same in other contexts. Unlike most prior studies, however, this experiment was conducted in a punishment-imposed abstinence model rather than the traditional reinstatement of extinguished drug seeking procedure. In this model, rats learned to suppress drug seeking because it led to aversive consequences, more closely resembling the human experience of stopping drug use due to social, legal, or health related consequences. Because this model is more behaviorally complex and has greater face validity, verifying the role of CRF in this setting was an important step in establishing it for future research.

Although CRF has been strongly implicated in relapse across different drugs and stressors, this conclusion is not entirely free of contradictions. For example, earlier studies showed that blocking CRF with an intracerebroventricular infusion of alpha helical CRF reduced

food deprivation induced heroin seeking in animals that had undergone extinction (Shalev et al., 2006), but the same treatment had no effect on relapse in a model that used chronic food restriction and forced abstinence (Sedki et al., 2013). Given these inconsistencies, the present finding that CRF antagonism reduced relapse in the punishment model offers important validation and adds to the weight of evidence supporting CRF as a reliable mediator of acute stress-triggered drug seeking.

This result may also reflect long lasting changes in the CRF system that occur after chronic drug use. Repeated drug exposure can alter CRF signaling in a way that affects cognitive control, motivation, learning, and memory (Bangasser and Kawasumi, 2015; Bryce & Floresco, 2016; Tovar Díaz et al., 2018; Ritchie et al., 2021). These adaptations may make individuals more sensitive to stress and increase the likelihood of relapse (Mantsch, 2022). From this perspective, the effect of the CRF antagonist in our model may not only reflect stress responding, but also the enduring changes in the CRF system caused by previous heroin exposure.

Although stress induced relapse is a well-established phenomenon, the mechanisms behind it are still being studied. One central question is why animals, after reaching voluntary abstinence through repeated punishment, would return to drug seeking simply because they were food deprived. One explanation is that stress creates a negative internal state, and the pursuit of drugs becomes a way to relieve that discomfort, a form of negative reinforcement (Koob, 2003; Pecoraro et al., 2004). Another possibility is that stress heightens the incentive value of drug related cues (i.e., an increase in *incentive salience*), making them more attractive even when stress itself is not experienced as aversive (Pecina et al., 2006; Baumgartner et al., 2021; 2022). Thus, stress-related states can sensitize the dopamine system, amplifying the incentive salience

or “wanting” of drug-associated cues, even if those cues do not alleviate the stress itself (Berridge & Robinson, 2016).

CRF has been implicated in both of these motivational processes. It plays a role in enhancing the attractiveness of reward cues and in driving behavior to escape negative emotional states. While these mechanisms may be separate, they likely overlap. Different brain regions appear to be responsible for each. For example, CRF neurons in the central amygdala and nucleus accumbens have been shown to increase motivation for rewards like sucrose or cocaine by amplifying cue driven responding (Baumgartner et al., 2021; 2022). In contrast, CRF neurons in the central amygdala (possibly a different subpopulation from the one targeted by Baumgartner et al.) and the bed nucleus of the stria terminalis are thought to mediate aversive motivation, promoting drug seeking to escape withdrawal or discomfort (Pomrenze et al., 2021).

In our study, CRF receptor antagonism was administered directly into the ventricles, meaning that the drug could affect many brain regions at once. Because of this, we cannot determine which specific structures were responsible for the observed reduction in relapse. However, it is reasonable to speculate that some combination of the central amygdala, nucleus accumbens, and bed nucleus of the stria terminalis were involved. Future studies using more targeted methods, such as local microinjection or circuit specific manipulation, will be necessary to identify the precise brain circuits through which CRF exerts its effects in this model.
One promising direction was based on the recent study by Xu and colleagues (2024), which identified the new CRF related circuit involving the PVN and its projections to the VTA. The VTA known for its role in motivation and reward, and is a major source of dopaminergic signals to forebrain targets, including the nucleus accumbens. Xu and colleagues showed that activating CRF neurons in the PVN caused dopamine release in the nucleus accumbens and led animals to

seek out that stimulation, even in the absence of stress related hormones. This finding suggests that CRF is capable of directly promoting reward seeking behavior through positive reinforcement.

Although this PVN to VTA CRF pathway has not yet been studied in addiction models, other studies have shown that stress activates VTA to nucleus accumbens dopamine signaling, and that this increase is necessary for relapse (Cabib & Imperato, 1996; Kalivas & Stewart, 1991; Shaham & Stewart, 1995). Moreover, CRF signaling in the VTA has been shown to influence dopamine release and reinstatement of drug seeking (Ungless et al., 2003; Wang et al., 2005), although the source of CRF input to the VTA has not always been clear. In some cases, the bed nucleus of the stria terminalis has been identified as the origin (Rodaros et al., 2007), but the new evidence from Xu et al. (2024) pointed us to the PVN as a previously unrecognized contributor.

The Role of the Paraventricular Nucleus of the Hypothalamus in Stress-Induced Relapse to Heroin

The second aim of the study was to examine the effect of chemogenetic inhibition of the PVN on food deprivation stress induced relapse to heroin seeking following punishment imposed abstinence. Building on the rationale that CRF released from the PVN may contribute to stress induced relapse, we hypothesized that nonselectively inhibiting PVN activity in food deprived rats would reduce or prevent relapse, as indicated by a decrease in heroin seeking behavior during the relapse test.

Although initial analyses did not reveal a statistically significant group effect, post hoc testing with Bonferroni correction uncovered a statistically significant reduction in relapse behavior in food deprived rats following PVN inhibition. This finding suggests that PVN activity

does modulate stress induced heroin seeking, although the modest effect size and variability between subjects highlight the need for cautious interpretation. It is likely that the study was underpowered to detect a robust group effect, and additional replication with a larger sample size is necessary to confirm and extend these results.

Rather than a null outcome, these findings indicate that PVN inhibition influences relapse behavior, but that the effect may be subtle or subject to biological variability. This result points to a promising direction for future research, particularly considering the PVN's cellular complexity and its integration within broader stress related circuits. Because the PVN contains heterogeneous neuron populations, global silencing may have affected some subpopulations more than others.

The functional heterogeneity of the PVN complicates the interpretation of nonspecific chemogenetic inhibition. As Jiang and colleagues describe, the PVN integrates diverse internal and external inputs and contains a mixture of neuroendocrine, autonomic, and behavioral control neurons (Cullinan et al., 1996; Daviu, 2018; Jiang et al., 2019). While some PVN neurons may promote relapse related stress and motivation, others might contribute to stress buffering or behavioral suppression. Silencing these different populations simultaneously may have yielded opposing effects, dampening the overall behavioral outcome (Jiang et al., 2019).

Jiang and colleagues also describe how CRF neurons within the PVN are part of a broader intra PVN microcircuit involved in homeostatic regulation. This includes neurons that neither express CRF nor release hormones, suggesting that PVN function depends on interactions among distinct and finely tuned subpopulations (Justice et al., 2008; Ramot et al., 2017). Thus, it remains possible that a more selective manipulation targeting CRF specific PVN neurons may produce a stronger behavioral effect on stress induced relapse.

Importantly, the PVN does not operate in isolation. It is embedded within an extended stress related network that includes the brainstem, amygdala, and bed nucleus of the stria terminalis, coordinating behavioral, autonomic, and endocrine responses to stress (Ferguson et al., 2008; Füzesi et al., 2016; Jiang et al., 2019). Even with effective PVN inhibition, relapse behavior could be maintained by compensatory mechanisms in these other regions.

Together, these considerations suggest that PVN activity does play a modulatory role in stress induced relapse, but that the nonspecific inhibition used in this study may have limited our ability to isolate its precise contribution. Future work using cell type or projection specific tools, such as targeting CRF neurons projecting from the PVN to the VTA, may clarify the pathways through which the PVN contributes to stress related drug seeking.

In Summary

This study provides strong evidence that CRF signaling contributes to stress induced relapse to heroin seeking following punishment-imposed abstinence. The observed behavioral effect of PVN inhibition, although modest, suggests this region may be involved in relapse circuitry. These findings warrant follow up studies with greater statistical power and more refined targeting approaches. Collectively, this work supports the use of the seek take punishment model for investigating stress induced relapse and advances our understanding of the neural mechanisms underlying opioid addiction and relapse vulnerability.

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