The Role of the PFC in Stress-Induced Relapse to Heroin Seeking Following Voluntary Abstinence in Rats

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Abstract

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The opioid crisis remains a critical public health concern, with relapse presenting a major challenge in the treatment of opioid use disorder. Stress during abstinence, such as hunger from caloric restriction, increases relapse vulnerability, yet the neural mechanisms underlying stress-induced relapse, particularly in the context of food deprivation, remain unclear. This thesis investigated behavioral and neural contributors to heroin use and relapse in rats, focusing on the orbitofrontal cortex (OFC) and prelimbic (PrL) cortex. First, we evaluated whether a 5-minute seek-take protocol effectively models intermittent access and compulsive heroin use in both male and female rats. After validating the model, we asked two key research questions: whether chemogenetic inhibition of (1) the OFC or (2) the PrL reduces stress-induced heroin seeking following punishment-imposed abstinence. Using an established intravenous heroin self-administration paradigm followed by punishment-imposed abstinence (via footshock), rats were tested for heroin seeking in either a sated or food-deprived state. Chemogenetic inhibition was achieved by expressing inhibitory Designer Receptors Exclusively Activated by Designer Drugs (DREADDs) in the OFC or PrL and activating them with systemic deschloroclozapine dihydrochloride (DCZ). We hypothesized that inhibiting these cortical regions would attenuate stress-induced relapse. Our results show that the 5-minute seek-take protocol successfully models intermittent and compulsive heroin use in both sexes. However, chemogenetic inhibition of the OFC or PrL cortex did not reduce heroin seeking during stress-induced relapse. These

findings suggest that the OFC and PrL might not be critically involved in stress-induced relapse to heroin seeking following punishment-imposed abstinence.

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Introduction

Substance Use Disorder

Substance use disorder (SUD) is a chronic and relapsing condition, marked by repeated cycles of drug use, abstinence, and relapse (Koob & Volkow, 2010). SUD is commonly conceptualized as progressing through three stages: binge/intoxication, characterized by the rewarding effects of the drug and compulsive use; withdrawal/negative affect, involving the emergence of negative emotional states in the absence of the drug; and preoccupation/anticipation, defined by cravings and persistent thoughts about obtaining and using the drug. Together, these stages illustrate the transition from active use to abstinence and, ultimately, to relapse, a cycle that characterizes the experience of many individuals struggling with opioid addiction. This pattern is particularly relevant in the context of the current opioid crisis.

The Opioid Crisis

Heroin, a powerful opioid, is used in the present study due to the urgent need to better understand its effects amid this ongoing public health emergency. Since the 1980s, opioid use has risen dramatically, largely driven by the overprescription of opioid pain medications such as oxycodone (Canadian Public Health Association, 2018). Although effective for managing pain, these medications have played a major role in the development of opioid misuse and addiction. It is estimated that 3–19% of individuals prescribed opioids go on to develop an addiction (American Medical Association, 2022), and nearly 45% of heroin users report that their opioid use initially began with prescription drugs.

In Canada, the opioid crisis has reached the level of a national public health emergency. In 2024 alone, the country recorded an average of 21 opioid-related deaths per day, over 7,600

deaths in total (Public Health Agency of Canada, 2025). The majority of these fatalities are attributed to fentanyl and its analogues, which are increasingly common in the unregulated drug supply due to their high potency and low production cost.

These alarming statistics highlight the devastating human toll of the opioid crisis and emphasize the urgent need to understand the neurobiological mechanisms that drive addiction and relapse.

Relapse and Its Primary Triggers

Relapse, the return to drug use following a period of abstinence, remains one of the greatest challenges in treating SUD. Despite the availability of various therapeutic approaches, sustaining long-term abstinence is notoriously difficult. This is especially true for opioid use disorder, where relapse rates have been reported as high as 91% (Smyth et al., 2010). Understanding the factors that contribute to relapse is therefore critical for developing more effective interventions and improving long-term recovery outcomes.

Research has identified three primary types of triggers that can provoke craving and reinstate drug-seeking behavior, even after prolonged periods of abstinence: exposure to drug-associated cues, re-exposure to the drug itself (priming), and stress. First, environmental cues, such as locations, paraphernalia, or people previously associated with drug use, can powerfully evoke craving and drug-seeking when encountered again. Through repeated pairings with drug use, these cues become conditioned stimuli that elicit physiological arousal and anticipation of the drug (Childress et al., 1993). Second, re-exposure to the drug itself (priming), even in small amounts, can trigger intense craving and rapidly precipitate a full relapse. This phenomenon emphasizes the pharmacological power of the drug to reactivate the addiction cycle, even after extended abstinence (de Wit, 1996). Third, both psychological and physiological forms of stress

are well-established relapse triggers. Stress activates overlapping neurobiological systems involved in negative affect and craving, including the hypothalamic-pituitary-adrenal (HPA) axis and corticolimbic circuits. This activation heightens emotional distress and impairs regulatory control, thereby increasing vulnerability to relapse (Sinha, 2001).

These findings emphasize the complexity of relapse and highlight the importance of addressing cue reactivity, drug exposure, and stress sensitivity in both clinical treatment and preclinical models of SUD.

The Role of Stress in Relapse in Humans

Among the primary triggers of relapse, stress stands out as one of the most potent and inescapable contributors, as demonstrated in clinical research. In humans, stress and negative emotional states significantly increase craving for opioids, even in the absence of drug-related cues, highlighting the powerful influence of internal states on relapse vulnerability (Childress et al., 1987, 1994). Similarly, exposure to personalized, stress-inducing scenarios has been shown to reliably increase cocaine craving, accompanied by heightened emotional distress and physiological indicators of stress, such as elevated cortisol levels (Sinha et al., 1999).

To develop more effective prevention and treatment strategies, researchers rely on animal models, which provide a controlled and ethical way to study the neural circuits and behaviors involved in opioid use disorder.

Using Animal Models to Study SUD

Animal models are essential for advancing our understanding of the neurobiological and behavioral mechanisms underlying SUD. These models allow researchers to systematically manipulate variables, such as drug availability, environmental conditions, and neural activity, that would be unethical or impractical to control in human studies (Lynch, 2010). Rodents,

particularly rats, are widely used due to their well-characterized physiology and ability to acquire operant behaviors that closely mimic voluntary drug use in humans.

Animal models have become increasingly sophisticated over the years to better capture the complexity of human substance use. Modern paradigms now include features such as extended drug access, voluntary abstinence, and relapse triggers like drug-associated cues or stress. However, concerns about how well these models translate to human addiction remain, prompting ongoing refinements to improve their relevance and accuracy.

Self-Administration Paradigms

Self-administration paradigms are a cornerstone of preclinical research aimed at modeling the compulsive drug use described in The Diagnostic and Statistical Manual of Mental Illnesses (DSM-5). In these paradigms, animals, typically rats, perform operant behaviors such as lever pressing to receive drug infusions, thereby mimicking voluntary drug use. These models are highly reliable: animals will consistently self-administer substances known to be addictive in humans, including opioids, psychostimulants, and alcohol (Lüscher et al., 2020). For opioids like heroin, drugs are usually delivered intravenously via surgically implanted catheters, allowing for rapid onset and consistent dosing.

Researchers can adjust various experimental parameters, such as drug dose, session duration, and access frequency, to model different patterns of drug use and evaluate how this influence intake and the development of compulsive behavior. Over time, this flexibility has allowed self-administration paradigms to evolve into highly informative tools for studying addiction-like behaviors.

One major breakthrough came from manipulating the duration of drug access. Ahmed and Koob (1998) demonstrated that rats given extended access to cocaine (6 hours per session)

showed a progressive escalation in intake over days, a pattern not seen in rats with limited access (1 hour per session). These extended-access rats also exhibited a "loading phase," characterized by high levels of consumption at the start of each session, suggesting a loss of control over intake. It was found that the loading phase was followed by a "maintenance" phase with more spaced-out infusions to maintain stable drug levels (Tsibulsky & Norman, 1999). However, this stable pattern does not reflect the typical drug-taking behavior of individuals with heroin use disorder, who often administer large doses of heroin followed by long abstinence periods (Dole, Nyswander & Kreek, 1966; McAuliffe & Gordon, 1974).

While extended-access models with continuous access have been widely used in the context of psychostimulant use, their validity has also been challenged, particularly in relation to escalation patterns (Allain & Samaha, 2019; Allain et al., 2018). Given the distinct pharmacological and behavioral profiles of opioids, early on it remained unclear whether heroin would produce similar escalation patterns under extended-access conditions. This uncertainty has since motivated refinements to opioid self-administration models aimed at better capturing core features of opioid addiction.

Modeling Heroin Use

Recent research has adapted extended-access self-administration models to better capture the dynamics of opioid use. For instance, Towers et al. (2019) demonstrated that mice given prolonged (6-hour) access to heroin exhibited escalating intake and more severe withdrawal symptoms compared to those with limited access, with these effects particularly pronounced in females. These results support the model’s validity in representing features of opioid use disorder, including escalation and dependence. However, despite their strengths, continuous extended-access models may not fully capture the intermittent, loading-phase consumption

patterns commonly observed in human heroin use. Consequently, their ability to model real-world opioid intake remains limited.

To better capture this human-like pattern, researchers developed an intermittent access self-administration procedure, in which drug availability is limited to short periods separated by abstinence intervals (Zimmer et al., 2012). Using this model, rats exhibit bursts of closely spaced cocaine infusions, resulting in spiking, rather than steady, brain drug concentrations, a pattern more consistent with human drug use dynamics (Zimmer et al., 2012; Allain et al., 2015). Heroin self-administration has rarely been studied using procedures other than continuous access, despite known differences between opioid and psychostimulant use disorders (Badiani et al., 2011).

To address this limitation, D'Ottavio et al. (2023) adapted the intermittent access self-administration procedure originally developed for cocaine studies (Zimmer et al., 2012), based on the rationale that repeated spikes in brain drug levels drive addiction-related behaviors. Using this approach, they directly compared intermittent and continuous heroin self-administration and found that intermittent access led to significantly higher total heroin intake and intake frequency in both male and female rats. Rats with intermittent access earned most infusions within the first minute of each drug-available period, and this early intake increased progressively across sessions, indicating a burst-like pattern of heroin use. Kinetic modeling showed that intermittent access produced sharp, transient spikes in brain heroin levels and higher peaks of 6-monoacetylmorphine (6-MAM), the active metabolite, compared to continuous access, which resulted in lower and more stable concentrations. Following abstinence, rats in the intermittent-access condition exhibited elevated heroin-seeking behavior on day 1, which

remained stable by day 21, suggesting a lack of incubation of craving, in contrast to the incubation observed in the continuous-access group.

Although this model successfully captured the loading pattern typical of human heroin use and demonstrated that continuous access is not required for escalation, its fixed and predictable access periods limit its ecological validity.

In contrast to the controlled and predictable drug access in traditional models, human drug use typically occurs under unpredictable and variable conditions. Robinson et al. (2023) argued that the predictability inherent in these models fails to capture the uncertainty surrounding human drug access. To address this, they developed the Unpredictable Intermittent Access (UIntA) model, which introduces variability in access timing, reward magnitude, and reinforcement schedules, initially tested using non-drug rewards such as sucrose. Rats trained under this model exhibited greater persistence during extinction and heightened cue-induced reinstatement, suggesting that unpredictability intensifies compulsive drug-seeking behavior.

These findings highlight the importance of incorporating both intermittent and unpredictable access into preclinical models to more accurately simulate human drug-taking patterns.

Building on these insights, our study employed an intermittent heroin access paradigm designed to better reflect human patterns of opioid use. Central to this approach was a seek-take chain procedure that introduced unpredictability into the seeking requirements necessary to access the drug-taking lever. This work extends on a model previously used in our lab (Borges et al., 2023), who also utilized a seek-take chain design. However, their protocol limited animals to a single heroin infusion per trial, which does not adequately capture the rapid loading behavior commonly observed in human opioid use. To better model this aspect, our design allowed rats to

self-administer multiple infusions during brief, 5-minute drug-available periods, supporting a more naturalistic pattern of drug intake.

By introducing unpredictability into the seeking requirements, we aimed to model the uncertainty and effort often involved in real-world drug procurement.

Seek-Take Chain

In humans, drug use is often preceded by a series of complex behaviors, such as acquiring money, locating a dealer, or navigating risky environments, that typically involve greater effort, risk, and exposure to negative consequences than the act of drug consumption itself (Roberts et al., 2013). Accurately modeling SUD therefore requires distinguishing between drug-seeking and drug-taking behaviors, as they reflect distinct phases of the addiction cycle. Rodent models can capture this distinction using the seek-take chain paradigm, in which animals must first complete a seeking response (e.g., pressing a "seek" lever) to gain access to a separate "take" lever that delivers the drug (Olmstead et al., 2000). By incorporating variable and unpredictable seeking requirements, this approach enhances ecological validity and better reflects the uncertainty and effort associated with real-world drug procurement.

Examining both seeking and taking behaviors is critical because each phase is governed by distinct neural and psychological mechanisms (Kalivas & Volkow, 2005; Peters et al., 2009). Seeking behaviors are typically goal-directed and involve motivational and cognitive processes such as decision-making and risk evaluation, whereas taking behaviors are more directly driven by the drug's acute reinforcing effects (Everitt & Robbins, 2016).

Importantly, targeting the seeking phase may be especially valuable for relapse prevention, as this phase is highly sensitive to triggers such as stress, environmental cues, or contextual changes (Venniro et al., 2017; Shaham et al., 2003). Moreover, analyzing both

components allows for a more nuanced understanding of how factors like drug dose, satiety, abstinence, or punishment differentially affect motivation versus consumption (Olmstead et al., 2000).

For example, Olmstead et al. (2000) trained rats under a heterogeneous chain schedule to self-administer intravenous cocaine, with separate levers designated for seeking and taking responses. They found that variations in cocaine dose did not significantly affect the number of seeking responses per cycle but did influence the latency to initiate seeking: higher doses increased this latency in the absence of a time-out (TO) period. This suggests that cocaine infusions induce a short-term satiety effect that temporarily suppresses further seeking. However, when TO periods were introduced between infusions and subsequent seeking opportunities, higher doses maintained elevated drug-seeking levels and reduced latencies, indicating that both the reinforcing and activating effects of cocaine re-emerge after satiety dissipates (Olmstead et al., 2000). These findings highlight the importance of differentiating drug-taking from drug-seeking behaviors, as they are modulated differently by drug dose, timing, and behavioral contingencies.

Moreover, because drug-seeking behaviors are often associated with negative consequences, abstinence models can introduce these consequences specifically to the seeking phase, for example, by pairing punishment with the seek lever.

Abstinence Paradigms in Animal Models

Three main approaches have been used to model abstinence in rodents: extinction, forced abstinence, and voluntary abstinence.

Extinction, the most widely used method since the 1970s, involves placing animals back into the drug-associated environment while withholding drug reinforcement (Shaham et al.,

2003; de Wit, & Stewart, 1981). Over repeated sessions, animals reduce their responding as they learn the drug is no longer available. While extinction has yielded valuable insights into learning and relapse mechanisms, it lacks a direct human analog, individuals attempting to quit drugs rarely encounter a scenario in which the drug is explicitly unavailable in familiar environments.

Forced abstinence, in contrast, removes animals from the drug-associated context altogether, typically by placing them in their home cage without access to the operant chamber (Grimm et al., 2001). This models externally imposed abstinence, such as incarceration or inpatient treatment. Although more ecologically valid than extinction, forced abstinence still does not reflect the experience of many individuals who attempt to quit in their everyday environments without being physically removed from drug-associated contexts.

Voluntary abstinence most closely parallels human attempts to quit or reduce drug use, particularly when those efforts are motivated by adverse consequences or the availability of alternative rewards (Panlilio et al., 2003). In this model, animals are given the opportunity to abstain from drug seeking, often in response to punishments (e.g., footshock) or the availability of more appealing options (e.g., palatable food or social interaction). This approach reflects how people often choose to reduce or stop drug use due to negative outcomes.

Although all three models ultimately result in abstinence, they differ markedly in the degree of relapse vulnerability they produce. For example, D'Ottavio et al. (2023) found that incubation of craving, a time-dependent intensification of drug seeking, emerged only in animals that underwent forced abstinence after continuous drug access. In contrast, this effect was absent following voluntary abstinence, which was achieved by providing access to a palatable food alternative. Similarly, Venniro et al. (2018) demonstrated that animals allowed to abstain voluntarily by choosing social interaction over methamphetamine did not develop

incubated craving, whereas those subjected to forced abstinence displayed a marked increase in drug seeking over time. These findings suggest that the absence of agency during abstinence may heighten relapse risk, while self-initiated abstinence offers a protective effect against the progressive intensification of craving.

Translating these results to human addiction, individuals who choose to abstain may be less vulnerable to escalating urges to use again, one of the strongest predictors of relapse. Conversely, abstinence imposed without a person's readiness, such as during incarceration or mandated treatment, may increase susceptibility to relapse upon re-entry into drug-accessible environments. Despite its strong translational relevance, voluntary abstinence remains underutilized in preclinical research.

To address this gap, the present experiment employed a voluntary abstinence model previously used in our lab, punishment-imposed abstinence (Borges et al., 2023), in which drug-seeking behavior is suppressed through punishment (footshock). This approach induces voluntary abstinence by applying negative consequences specifically to the seeking phase, thereby mimicking how adverse outcomes motivate cessation in human drug users.

Relapse in Animal Models

Despite efforts to maintain sustained abstinence, relapse frequently occurs and is thus widely studied in animal models. As mentioned earlier, stress is a well-established trigger that can provoke relapse. This is supported by preclinical studies demonstrating that acute stress robustly reinstates drug-seeking behavior across several drug classes. This phenomenon was first described by Shaham and Stewart (1995), who showed that intermittent footshock stress reinstated heroin seeking in rats following extinction. Subsequent work has extended this effect to other substances: Erb et al. (1996) found that footshock also reinstates cocaine seeking, Lê et

al. (1998) reported that yohimbine, a pharmacological stressor that increases noradrenergic activity, induces relapse to alcohol seeking, and Buczek et al. (1999) showed that footshock triggers reinstatement of nicotine seeking. These findings demonstrate that both physical (e.g., footshock) and pharmacological (e.g., yohimbine) stressors can reliably provoke relapse-like behavior, highlighting the generalizability of stress-induced reinstatement across drug classes.

More recent research has expanded the scope of relevant stressors to include physiological challenges such as food deprivation. Acute food deprivation has been shown to reinstate heroin seeking following both extinction-based abstinence (Shalev et al., 2000) and voluntary abstinence (Borges et al., 2023). These studies demonstrate stress's central role as a relapse trigger across different substances, species, and experimental paradigms.

Given its translational relevance, employing more naturalistic stressors, such as food deprivation, may offer greater ecological validity and insight into the human experience of relapse. Building on this rationale, the present research investigates the impact of food deprivation stress on heroin seeking following punishment-imposed abstinence.

Once this model was validated, it served to further examine the neural mechanisms underlying stress-induced relapse to heroin seeking, with particular attention to the role of the prefrontal cortex (PFC).

The Role of the PFC in Drug Addiction and Relapse

The PFC plays a central role in addiction-related behaviors. Chronic drug exposure disrupts PFC function, leading to impairments in decision-making, behavioral flexibility, and emotional regulation (Limpens, 2015). These deficits contribute to the compulsive drug seeking that defines SUDs. Because the PFC is anatomically and functionally heterogeneous, research

has increasingly focused on identifying the specific subregions and circuits that drive relapse vulnerability and maladaptive behavior in addiction.

Orbitofrontal Cortex (OFC)

The OFC, a subregion of the PFC, is primarily involved in evaluating reward value and predicting outcomes to guide decision-making based on changing environmental contingencies (Wallis, 2007). Its extensive reciprocal connections with reward, emotional, and motivational systems, including the amygdala and striatum, position it as a key hub for evaluating outcomes and regulating behavior (Moorman, 2018). Among these connections, dopaminergic input from the ventral tegmental area (VTA) plays a particularly important role. Dopamine release in the OFC, driven by stress or reward, modulates its activity and influences how it encodes and updates the value of expected outcomes (Moore & Bloom, 1979). While the VTA is primarily involved in reward processing and motivation, the OFC contributes directly to outcome evaluation and goal-directed decision-making. It assigns value to different outcomes, integrates information about reward type and probability, and helps guide behavior based on updated predictions of future rewards. These functions make the OFC particularly important for adjusting behavior when contingencies change and for supporting cognitive flexibility and emotional regulation.

Role of the OFC in Addiction and Relapse

These functions become especially relevant in the context of addiction, where maladaptive decision-making and inflexible behavior are core features. SUD's are characterized by maladaptive decision-making, wherein drug-associated cues gain excessive motivational salience, while the value of natural rewards is diminished (Volkow & Morales, 2015). Stress, which frequently triggers relapse, activates the mesolimbic dopamine system and enhances the

perceived value of drug cues, further dysregulating decision-making processes (Sinha, 2008). In this context, stress-induced dopamine release in the OFC may impair its capacity to support adaptive choices, thereby promoting compulsive drug seeking. Importantly, the OFC is not required for the primary reinforcing effects of drugs (Hutcheson & Everitt, 2003), but it plays a critical role in mediating the influence of drug-associated cues on behavior (Fuchs et al., 2004; Guillem & Ahmed, 2018). This distinction highlights the OFC's role in guiding behavior based on learned associations with drug use, rather than the drug's direct effects. Following relapse, OFC activity increases (Fanous et al., 2012; Koya et al., 2006), and inactivation of the OFC reduces drug seeking (Cruz et al., 2013), reinforcing its involvement in relapse expression.

Preclinical studies in animals have established a causal role for the OFC in stress-induced relapse. In one study, pharmacological inactivation of the OFC blocked the reinstatement of cocaine-seeking behavior induced by intermittent footshock, demonstrating that the OFC is necessary for translating stress signals into relapse behavior (Capriles et al., 2003). Moreover, dopaminergic mechanisms within the OFC appear to contribute to this process, as the infusion of D1-like dopamine receptor antagonists into the OFC blocked the reinstatement of cocaine seeking following footshock (Capriles et al., 2003). These findings highlight the OFC's dopaminergic modulation as a key mechanism linking stress to relapse.

The OFC is also involved in cue- and context-induced reinstatement; its inactivation reduces the motivational value of drug-associated stimuli (Lasseter et al., 2014; Arguello et al., 2017). This aligns with the OFC's established role in encoding the value of conditioned stimuli and integrating motivationally relevant information to guide behavior.

OFC Contributions to Flexible Behavior and Drug-Seeking Under Conflict

Neuroimaging studies in humans validate the translational importance of the OFC. Activity in this region increases in response to heroin-related cues and correlates positively with self-reported craving (Sell et al., 2000; Huang et al., 2024). Moreover, the OFC supports cognitive flexibility, particularly in reversal learning, an ability disrupted by both chronic drug use and OFC lesions (Moorman, 2018; Schoenbaum, 2008). For example, rats exposed to chronic cocaine show persistent reversal learning deficits, paralleling impairments observed in humans with OFC damage (Calu et al., 2007). These impairments can persist for months after withdrawal and reflect a broader decline in behavioral flexibility and neural plasticity (Schoenbaum, 2008). Reversal learning has been a main paradigm for assessing OFC function, as it requires updating response strategies when reward contingencies change (Murray, 2007). Rats with OFC lesions or extended cocaine exposure show slower acquisition of reversal tasks and difficulty disengaging from previously rewarded responses (Chudasama & Robbins, 2003; Calu et al., 2007). Thus, the OFC is essential for flexible decision-making, enabling pursuit of rewards while avoiding harmful outcome, abilities that are impaired in addiction.

The OFC also contributes to decision-making under conflict, a process highly relevant to relapse. Individuals with SUD's frequently face choices involving risk or punishment, such as weighing the urge to use against potential negative consequences. Dysfunction in frontostriatal circuits, including the OFC, has been linked to compulsive drug use in such contexts. For instance, increased activity in the lateral OFC can drive drug seeking when inhibitory control regions like the prelimbic cortex are compromised (Hu et al., 2019). In punishment-based models of relapse, OFC activation promotes risky drug seeking and enhancing its activity increases drug use despite adverse outcomes (Ishikawa et al., 2020; Murphy et al., 2023). Although the OFC's role in stress, cue, and context-induced relapse is well established, its involvement in relapse

following punishment-imposed abstinence remains understudied. Recently, Murphy and colleagues (2023) found that activation of the OFC during punishment sessions heightened drug-seeking behavior, while inhibition had no effect. This suggests that the OFC enhances drug-seeking behavior under adverse conditions, but that its inhibition alone may be insufficient to suppress it. Because this study used cocaine, it remains unclear whether heroin produces the same effects.

Together, these findings highlight the OFC's central role in regulating adaptive behavior and the consequences of its dysfunction in addiction. As an integrator of reward and stress signals, its dysregulation may underlie the persistent vulnerability to relapse. Understanding how stress alters OFC function, particularly under ecologically relevant conditions like punishment-imposed abstinence, is essential for identifying relapse mechanisms and developing interventions that restore behavioral flexibility in addiction.

Medial Prefrontal Cortex (mPFC)

The mPFC, another key subdivision of the PFC, is crucial for integrating information about past experiences and current contexts to guide goal-directed actions, support behavioral flexibility, and enable adaptive decision-making in complex environments (Euston et al., 2012). By evaluating the consequences of different actions, the mPFC learns which responses are most appropriate in specific situations, allowing it to shape emotional reactions (such as fear or freezing when faced with a threat) or to promote reward-seeking behaviors. Rather than being defined by unique structural features, the distinct role of the mPFC arises from its specialized network of connections with other brain regions. Additionally, like other parts of the cortex, it is believed to store abstract, generalized knowledge or behavioral strategies derived from repeated experiences, rather than detailed memories of single events (Euston et al., 2012).

The Role of the mPFC in Addiction

When the mPFC's integrative and regulatory functions become compromised, these same mechanisms that normally support adaptive behavior can contribute to maladaptive outcomes. In the context of addiction, dysregulation of the mPFC impairs its ability to evaluate consequences and flexibly guide behavior, leading to maladaptive decision-making, compulsive drug seeking, and heightened vulnerability to relapse (Goldstein & Volkow, 2011; Koob & Volkow, 2016). For example, individuals have difficulty evaluating the long-term consequences of drug use, often continuing to use substances despite serious negative outcomes such as health deterioration, financial difficulties, or strained relationships (Goldstein & Volkow, 2011). This shift reflects a transition from flexible, goal-directed control to rigid, automatic drug-seeking behaviors driven by habitual responses (Koob & Volkow, 2016).

Exposure to drug-associated environments or internal states such as craving and stress can further overwhelm already weakened self-control mechanisms, increasing the likelihood of relapse (Goldstein & Volkow, 2011; Koob & Volkow, 2016). Dysregulation of the mPFC enhances sensitivity to drug-related cues and internal stress signals, thereby amplifying craving and facilitating compulsive drug use. This combination of impaired cognitive control and heightened motivational drive creates a neural environment highly conducive to relapse, even after prolonged abstinence (Goldstein & Volkow, 2011; Koob & Volkow, 2016). Disruptions in the mPFC's ability to integrate internal states (e.g., craving, stress) with external cues (e.g., drug-associated contexts) bias behavior toward overvaluing drug-related stimuli and diminish consideration of negative consequences (Kalivas & Volkow, 2005; Goldstein & Volkow, 2011).

Together, these changes contribute to the persistence of rigid, automatic responses to drug-predictive cues that can endure long after drug use has stopped (Koob & Volkow, 2016).

The mPFC's role in addiction extends notably to relapse triggered by stress-induced relapse. Under normal conditions, the mPFC exerts top-down control over subcortical structures such as the amygdala, nucleus accumbens, and ventral tegmental area (VTA), integrating emotional, motivational, and contextual information to suppress impulsive drug seeking and support adaptive, goal-directed behavior (George & Koob, 2010). However, chronic stress impairs mPFC function by increasing inhibitory tone on its output neurons, disrupting executive functions such as working memory, behavioral flexibility, and response inhibition (McKlveen et al., 2016). These stress-induced impairments weaken the mPFC's regulatory control and increase vulnerability to relapse. Successful abstinence requires intact mPFC-mediated executive functions to override conditioned responses to drug cues and internal urges, allowing voluntary suppression of drug seeking even when faced with strong triggers (Chen et al., 2013; Goldstein & Volkow, 2011). When mPFC activity is compromised, this capacity is undermined, elevating relapse risk and promoting compulsive drug use.

Preclinical studies further support the mPFC's critical role in stress-induced drug seeking. Dopaminergic input from the VTA to the mPFC increases during stress, highlighting its sensitivity to neurochemical modulation (Moore & Bloom, 1979). Additionally, selective inactivation of the mPFC reduces stress-induced and cocaine priming induced reinstatement of cocaine seeking, underscoring its specific involvement in stress-triggered relapse (Capriles et al., 2003).

Collectively, these findings establish the mPFC as a central regulator of relapse vulnerability, especially under stress. Its ability to integrate information from stress, reward, and decision-making circuits allows it to guide adaptive behavior and maintain abstinence. However, chronic drug use or repeated stress can disrupt this regulation, increasing relapse risk. Identifying

how specific mPFC subregions and pathways contribute to stress-induced relapse, particularly following punishment-imposed abstinence, could inform targeted interventions aimed at restoring prefrontal control and promoting long-term recovery.

Although the mPFC is often presented as a single structure, it actually consists of distinct subregions with specialized functions. Notably, the prelimbic (PrL) and infralimbic (IL) cortices exert complementary, and often opposing, influences on drug-seeking behavior. The PrL cortex facilitates goal-directed drug seeking, particularly in response to drug-associated cues and contexts and is linked to heightened relapse vulnerability. For example, increased CaMKII signaling in the PrL correlates with elevated cue reactivity (Hamel et al., 2022; Liu et al., 2024). In contrast, the IL cortex supports behavioral inhibition and extinction learning, helping to suppress drug-seeking behavior (Euston et al., 2022; Heidbreder & Groenewegen, 2003). Experimental evidence supports this functional dichotomy: inactivation of the PrL reduces cocaine seeking (Capriles et al., 2003), cue (McLaughlin & See, 2003), and context (Fuchs et al., 2005) induced reinstatement of cocaine seeking, while IL activation suppresses it and facilitates extinction (Peters et al., 2008). Disruption of the balance between PrL and IL activity by chronic drug use may therefore underlie the impaired behavioral control observed in addiction, contributing to maladaptive and compulsive drug-seeking behaviors.

Given the mPFC's central role in relapse, the PrL cortex emerges as a particularly strong candidate for investigation. The PrL is consistently implicated in promoting goal-directed drug seeking and is activated by both drug-related cues and stressors. In the punishment-imposed abstinence paradigm, where animals learn to suppress drug seeking to avoid aversive outcomes, relapse reflects a breakdown in this behavioral control. The PrL is well positioned to mediate this shift, as it integrates motivational and contextual information to guide action. The present

research aims to clarify the PrL's role in stress-induced relapse, following punishment-imposed abstinence.

Objectives and Research Questions

The initial objective of this study was to establish a reliable model of intermittent access to heroin self-administration using a 5-minute seek-take chain protocol. This model was designed to capture compulsive-like drug use while maintaining precise experimental control. Once this robust protocol was established, the focus shifted toward investigating the neural mechanisms underlying stress-induced relapse to heroin seeking following punishment-imposed abstinence.

This study addresses three central research questions. First, can a 5-minute seek-take protocol effectively model intermittent access and compulsive heroin use in rats? Second, does chemogenetic inhibition of the orbitofrontal cortex (OFC) reduce stress-induced relapse to heroin seeking after punishment-imposed abstinence? Third, does inhibition of the prelimbic (PrL) cortex similarly attenuate stress-induced relapse under the same conditions?

To answer these questions, we employed a model that more closely mirrors human patterns of drug use, abstinence, and relapse than traditional paradigms. Specifically, we used a seek-take chain procedure with an extended heroin-taking link, allowing rats to self-administer multiple infusions within each trial. This was followed by punishment-imposed abstinence, during which seeking responses were probabilistically punished with mild footshock to model real-world negative consequences of drug use. After abstinence was achieved, relapse was triggered by an acute stressor (food deprivation), a manipulation previously shown to reinstate heroin seeking (Borges et al., 2023). To examine the contributions of the OFC and PrL, we used

chemogenetic inhibition via DREADDs to selectively silence neural activity in these regions during relapse testing.

By integrating extended access, voluntary abstinence, and stress-induced relapse, this study provides a more ecologically valid framework for investigating the neural mechanisms of heroin seeking. We hypothesize that inhibition of the OFC and PrL will reduce stress-induced relapse.

Method

Subjects

Male (275–300 g; N = 50) and female (250–275 g; N = 10) Long-Evans rats (Charles River Laboratories, St. Constant, Quebec, Canada) were used. Rats were housed under a reverse 12-hour light/dark cycle (lights off at 9:30 AM) with ad libitum access to chow (Envigo) and water, except during periods of food deprivation. Prior to surgery, animals were pair-housed in standard cages within the animal care facility (ACF) at Concordia University (Montreal, Canada) and subsequently housed individually in operant chambers for the duration of behavioral testing. All procedures were approved by the Animal Research Ethics Committee at Concordia University and conformed to the guidelines of the Canadian Council on Animal Care.

Apparatus

Behavioral testing was conducted in standard operant conditioning chambers (29.0 × 29.0 × 25.5 cm; Med Associates, St. Albans, VT), enclosed in sound-attenuating boxes. Each chamber was equipped with a red house light, a 2.9 kHz tone generator, and two retractable levers located on the right wall. One lever (designated the "take" lever) was paired with heroin infusions, while the

second lever (designated the "seek" lever) allowed access to the take lever. A white cue light was positioned above each lever.

The chamber floor consisted of stainless-steel bars suspended over a metal tray containing bedding. Intravenous drug delivery was facilitated via a plastic Tygon housed within a protective metal spring, connected to a liquid swivel (Lomir Biomedical, QC, Canada) and an infusion pump. A 20 mL syringe mounted on the pump enabled heroin delivery.

Surgeries

Rats were weighed prior to surgery to ensure they met minimum surgical weight criteria (≥300 g for males, ≥230 g for females). Animals received preoperative subcutaneous injections of Depocillin (180 mg/kg), carprofen (5 mg/kg), and 0.9% saline (3 mL).

Intravenous catheters (made in-house) were surgically implanted into the right jugular vein under 2% isoflurane anesthesia. The external portion of the catheter was connected to a modified 22-gauge cannula (made in-house), which was secured to the skull using five screws and dental cement. Catheters were flushed daily with 0.2 mL of sterile saline containing heparin (7.5 IU) and gentamicin (0.8 mg). Postoperative care included daily carprofen administration for three days, dosed according to body weight.

Drugs

Heroin hydrochloride (provided by the National Institute on Drug Abuse, Research Triangle Park, NC, USA) was dissolved in 0.9% sterile saline and administered intravenously at doses of 0.1 mg/kg/infusion and 0.05 mg/kg/infusion, depending on the experimental phase. Each infusion was delivered in a volume of 0.13 mL. Deschloroclozapine (DCZ), a water-soluble DREADD ligand (Hello Bio), was dissolved in 0.9% sterile saline at a concentration of 0.1 mg/mL.

Procedure

Self-Administration

Habituation. Following post-surgical recovery, rats were individually housed in operant conditioning chambers and allowed to habituate for 24 hours before behavioral testing began. During this period, rats were not connected to the infusion apparatus, and no stimuli were presented (i.e., houselight, cue light, tone, or levers). Rats remained housed in the chambers for the entire duration of the experiment.

Unless otherwise specified, behavioral sessions were conducted daily for 6 hours beginning at 09:30 AM, during the dark phase of the reversed light/dark cycle. At the start of each session, the Tygon tubing (enclosed in a protective metal spring) was connected to the infusion system, and the houselight was activated to signal session onset.

Heroin Self-Administration Training with Only the Take Lever Available under Fixed-Ratio 1 (FR1). Only the take lever was available during this phase. At the start of each session, the houselight was turned on and the take lever was inserted. A single press on the take lever (FR1 schedule) resulted in a heroin infusion (0.1 mg/kg in 0.13 mL), retraction of the lever, activation of the cue light above the take lever and a tone for 12 seconds, and deactivation of the houselight. This was followed by a 30-second inter-trial interval (ITI), during which all cues were off, and the levers were retracted. The next trial began with the re-insertion of the take lever and reactivation of the houselight.

After two days of the take-lever-only protocol, rats progressed to the next phase, the seek-take chain under an FR1 schedule, provided they achieved at least 10 take lever presses in a single session.

Heroin Self-Administration with Seek-Take Chain under FR1 (2 Days). The seek lever was introduced during this stage. At the start of each session, only the seek lever was inserted, and the houselight was turned on. A single press on the seek lever (FR1 schedule) resulted in its retraction and the insertion of the take lever. A single press on the take lever delivered a heroin infusion (0.05 mg/kg in 0.13 mL), activated the cue light and tone for 12 seconds, and turned off the houselight. The take lever remained inserted for 5 minutes. Each trial was followed by a 30-second ITI, during which all cues were off, and both levers were retracted. At the end of the ITI, the seek lever was reinserted and the houselight reactivated to begin the next trial. Throughout all self-administration sessions involving the seek-take chain, if a rat failed to complete the seek-take sequence within 10 minutes, the trial was terminated, and the ITI began automatically. This rule was applied consistently across all days of seek-take training.

Self-Administration with Seek-Take Chain under Variable Intervals. Variable interval (VI) schedules were introduced to the seek lever at this stage. At the start of each session, only the seek lever was inserted, and the houselight was turned on. A single press on the seek lever initiated the VI schedule. For VI5, the system randomly selected an interval of 0.1 s, 5 s, or 10 s (mean = 5 s); for VI30, the possible intervals were 15 s, 30 s, or 45 s (mean = 30 s); and for VI60, the intervals were 45 s, 60 s, or 75 s (mean = 60 s). The first press on the seek lever after the selected interval resulted in the retraction of the seek lever and the insertion of the take lever. Pressing the take lever resulted in a heroin infusion (0.05 mg/kg in 0.13 mL), activation of the cue light and tone for 12 seconds, and deactivation of the houselight. The take lever remained inserted for 5 minutes following the infusion. After each trial, an ITI was implemented during

which all cues were off, and both levers were retracted. The ITI duration began at 30 seconds during the FR1 and VI5 schedules and was progressively increased by doubling each day, up to a maximum of 7 minutes during VI60.

Punishment-Imposed Abstinence

Punishment sessions were conducted daily until rats met the criterion of two or fewer heroin infusions across two consecutive 6-hour sessions. Each session began with the insertion of the seek lever and activation of the houselight. Animal health and welfare were closely monitored throughout this phase.

Under the VI60 schedule, successful completion of the seek component led to one of two outcomes: a 30% probability of receiving a mild footshock (0.5 s) or a 70% probability of take lever being inserted. Footshock intensity was increased incrementally across punishment days, beginning at either 0.2 mA or 0.4 mA depending on the experiment. Shock trials were immediately followed by a 7-minute ITI. The next trial began with seek lever insertion and houselight reactivation.

On non-shock trials, a single press on the take lever resulted in a heroin infusion (0.05 mg/kg in 0.13 mL), activation of the cue light and tone for 12 seconds, and deactivation of the houselight. The take lever remained inserted for 5 minutes, followed by a 7-minute ITI.

Break Day (1 Day)

After meeting the abstinence criterion, rats underwent a rest day during which no behavioral testing was conducted. Heroin syringes were removed from the pumps; although rats remained connected to the infusion lines, no stimuli were presented, and no drug was administered.

Acute Food Deprivation (24 Hours)

One day after the final punishment session, rats were divided into two groups, Food Deprived

(FD) and Sated, matched based on body weight and the average number of seek lever presses and heroin infusions during the last three days of self-administration training. At approximately 09:30 AM, food hoppers were removed for the FD group and maintained for the Sated group. All rats had ad libitum access to water and were closely monitored for signs of distress or health complications throughout the deprivation period.

Food Deprivation-Induced Heroin Seeking Test

After 24 hours, rats were tested for heroin seeking during a 3-hour session under the VI60 schedule with a 7-minute ITI. No heroin or shocks were delivered. Presses on the take lever activated the cue light and tone for 12 seconds but did not result in drug delivery.

After the session, food was returned to the FD group. The following day, the feeding conditions were reversed to counterbalance order effects.

Experiment 1a: The Effects of Increased Heroin Availability on Punishment-Imposed Abstinence and Stress-Induced Relapse in Male Rats

Subjects

Male Long Evans rats (N = 10; 275–300 g; Charles River, St. Constant, Quebec, Canada) were used in this experiment. All housing and handling conditions were as described in the general protocol.

Apparatus

As described in the general protocol, except that standard operant cages were from Coulbourn Instruments (Allentown, PA, USA; 29.0 cm × 29.0 cm × 25.5 cm).

Surgeries

Intravenous catheter implantation was performed as described in the general protocol.

Drugs

As described in the general protocol.

Procedure

As described in the general protocol, with the following exceptions:

Punishment-Imposed Abstinence

The footshock intensity began at 0.2 mA and increased by 0.1 mA every other day, up to 0.6 mA. In cases where no reduction in seeking behavior was observed, the intensity was increased further, up to a maximum of 1.0 mA.

Nociception Test

Following the completion of the relapse tests, rats were retrained to self-administer heroin without footshock for 5 days using the seek-take chain under an FR1 schedule, with the heroin dose set at 0.05 mg/kg.

To assess whether heroin-induced analgesia contributed to punishment resistance, given that punishment involved physical discomfort, tail-flick nociception tests were conducted before and after heroin exposure. The first test was conducted prior to heroin re-exposure to obtain baseline tail-flick latencies. Water was heated to 52 °C on a hot plate, and each rat's tail was marked 4.0 cm from the tip and submerged in the water up to that mark. A second nociception test was conducted after rats passively received five heroin infusions (0.05 mg/kg/infusion in 0.13 mL), spaced 40 seconds apart, each paired with cue presentation, followed by a 7-minute ITI.

All tests were video recorded. Tail-flick latency, used as an index of pain sensitivity, was measured from the video recordings.

Experiment 1b: The Effects of Increased Heroin Availability on Punishment-Imposed Abstinence and Stress-Induced Relapse in Female Rats

Subjects

Female Long Evans rats (250-275 g; N = 10; Charles River, St. Constant, Quebec, Canada) were used in this experiment. All housing and handling procedures were identical to those described in the general protocol.

Apparatus

As described in the general protocol.

Surgeries

Intravenous catheter implantation was performed as described in the general protocol.

Drugs

As described in the general protocol.

Procedure

Punishment-Imposed Abstinence

As described in the general protocol, with the following exception:

The footshock intensity began at 0.2 mA and increased by 0.1 mA every other day, up to 0.6 mA. In cases where no reduction in seeking behavior was observed, the intensity was increased further, up to a maximum of 1.0 mA.

Additionally, the probability of punishment was gradually increased from 30% to 40%, 50%, and up to 60%, as needed to meet the abstinence criteria.

Experiment 2a: The Role of the Orbitofrontal Cortex in Stress-Induced Relapse to Heroin Seeking Following Voluntary Abstinence in Male Rats

Subjects

Male Long Evans rats (275–300 g; N = 20, run 1: n = 10; run 2: n = 10; Charles River, St. Constant, Quebec, Canada) were used in this experiment. All other housing and handling conditions were identical to those described in the general protocol.

Apparatus

As described in the general protocol.

Surgeries

Intravenous catheter implantation was performed as described in the general protocol. In addition, during the same surgical procedure, rats received bilateral injections of 0.60 μL of viral vector AAV8-hSyn-hM4D(Gi)-mCherry (Canadian Neurophotonic Platform, Quebec City, QC) into the OFC (AP +3.2, ML ±2.5, DV −5.5 relative to Bregma) at a rate of 0.1 μL/min. The injector was left in place for an additional 10 minutes to ensure diffusion.

Drugs

As described in the general protocol. Additionally, Deschloroclozapine (DCZ), a water-soluble DREADD ligand (Hello Bio), was dissolved in 0.9% sterile saline at a concentration of 0.1 mg/mL.

Procedure

As described in the general protocol, except for the following:

Punishment-Imposed Abstinence

Run 1. Footshock intensity began at 0.2 mA and increased by 0.1 mA every two days, up to a maximum of 1.0 mA. The probability of punishment increased in stages from 30% to 40%, 50%, and 60%, until abstinence criteria were met.

Run 2. Footshock intensity began at 0.4 mA and increased by 0.1 mA daily, up to a maximum of 1.0 mA. The probability of punishment increased from 30% to 50% until abstinence criteria were met.

Food Deprivation-Induced Heroin Seeking Test

As described in the general protocol. In addition, following 24 hours of food deprivation, rats were randomly assigned to receive an intraperitoneal injection of either DCZ or saline, administered 20 minutes prior to the test session. The treatment conditions remained the same for both relapse tests.

Locomotor Activity Test

To assess baseline locomotor activity, rats were placed in the activity monitoring chamber (Coulbourn Instruments) for 30 minutes, and total distance traveled (in meters) was recorded. Thereafter, rats received an intraperitoneal injection of either DCZ or saline (vehicle) 20 minutes prior to a 1-hour session in the same activity chamber. Locomotor data were collected using TruScan software (Coulbourn Instruments).

Perfusions

Following completion of all behavioral testing, rats were injected with Euthanyl and transcardially perfused with phosphate-buffered saline (PBS) followed by 4% paraformaldehyde.

Histology

Following perfusion, brains were extracted and post-fixed in 4% paraformaldehyde for approximately 2 hours. The tissue was then transferred to a 30% sucrose solution and stored at 4°C until the brains sank, typically within 24 to 48 hours. Once fully equilibrated in sucrose, the brains were removed, gently dried using KimWipes, wrapped in aluminum foil, and stored at −80°C until sectioning. Coronal brain sections (40 µm thick) were subsequently cut using a

cryostat. To verify viral expression and accurate targeting of the OFC, mCherry fluorescence was examined (Appendix C, Figures 1 and 2).

Experiment 2b: The Role of the Prelimbic Cortex in Stress-Induced Relapse to Heroin Seeking Following Voluntary Abstinence in Male Rats

Subjects

Male Long Evans rats (275–300 g; N = 10; Charles River, St. Constant, Quebec, Canada) were used in this experiment. All other conditions were identical to those described in the general protocol.

Apparatus

As described in the general protocol.

Surgeries

As described in Experiment 2a. However, injections were targeted to the prelimbic cortex (AP +3.2, ML ±0.80, DV −3.5, relative to Bregma).

Drugs

As described in the general protocol and in Experiment 2a.

Procedure

As described in the general protocol, except for the following:

Punishment-Imposed Abstinence

Footshock intensity began at 0.4 mA and increased by 0.1 mA daily, up to 1.0 mA. Additionally, the probability of punishment was increased from 30% to 50% to achieve abstinence criteria.

Food Deprivation-Induced Heroin Seeking Test

As described in the general protocol and in Experiment 2a.

Locomotor Activity Test

As described in Experiment 2a.

Perfusions

As described in Experiment 2a.

Histology

As described in Experiment 2a; however, viral expression was verified in the PrL (Appendix C, Figure 3).

Statistical Analyses

All statistical analyses were conducted using GraphPad Prism version 10.4.2. An alpha level of 0.05 was used for all statistical tests. Data were assessed for normality and sphericity prior to analysis. When the assumption of sphericity was violated, the Greenhouse-Geisser correction was applied, and corrected degrees of freedom are reported. Effect sizes are reported as partial eta squared (η^2_p) for ANOVAs and Cohen's *d* for t-tests.

For self-administration data for Experiment 1a, Experiment 1b, Experiment 2b, repeated-measures one-way ANOVAs were used to assess changes in the number of heroin infusions, take lever presses, and seek lever presses across training days.

Due to technical constraints, the duration of the self-administration phases differed across subjects for Experiment 2a. Therefore, only sessions common to all subjects were included in the main analyses, with daily data presented in Appendix A.

Due to procedural differences across runs and variability in when rats reached the abstinence criterion, the main analyses of punishment-imposed abstinence were conducted using paired two-tailed *t*-tests. These tests compared baseline levels of heroin infusions, take lever

presses, and seek lever presses to corresponding values at abstinence, ensuring all rats were included in the analysis. Baseline was defined as the average of the final three self-administration sessions for each variable, and punishment-imposed abstinence was defined as the average of the last two punishment sessions.

For daily data, see Appendix B. In those analyses, only punishment sessions common to all rats were included; additional sessions required by some animals to reach the abstinence criterion (≤2 infusions on two consecutive days) were excluded. These daily data were analyzed using repeated-measures one-way ANOVAs comparing responding across punishment sessions to baseline levels. Dunnett's multiple comparisons test was used in these supplementary analyses to identify significant differences from baseline.

Relapse test data were analyzed using either two-way repeated-measures ANOVAs or paired-samples t-tests, depending on the experimental design. When only *feeding condition* (sated vs. food-deprived) was manipulated, paired-samples t-tests were used to compare responding between sated and food-deprived conditions (Experiment 1). When both *feeding condition* (within-subject; sated vs. food-deprived) and *treatment condition* (between-subject; vehicle vs. DCZ) were tested (Experiment 2), two-way repeated-measures ANOVAs were used. Dependent variables included the number of take and seek lever presses, as well as the number of "infusions." Notably, during relapse tests, rats did not receive actual heroin infusions; rather, the "infusion" measure refers to the number of times rats completed the response requirement on the take lever that would have resulted in a heroin infusion during training.

Changes in nociceptive sensitivity were analyzed using a paired-samples t-test comparing tail-flick latencies before (baseline) and after heroin infusions within each subject. This analysis was conducted only for animals in Experiment 1a.

Locomotor data were analyzed using an unpaired t-test comparing total distance traveled between DCZ and vehicle treated rats in a 1-hour session. Cohen's *d* was calculated to report the effect size.

All data are presented as mean ± SEM unless otherwise noted. Graphs and figures were generated using GraphPad Prism.

Results

Experiment 1a. The Effects of Increased Heroin Availability on Punishment-Imposed Abstinence and Stress-Induced Relapse in Male Rats

Data Integrity

One rat was excluded from the analyses for failing to meet the abstinence criterion. Therefore, data from 9 male rats were included in the final analyses.

Self-administration training

All male rats (n = 9) increased their responding on the seek lever as the variable interval (VI) increased across training days. Heroin intake remained stable throughout the training period, with rats self-administering a consistent number of infusions each day. Take lever presses increased from training Days 3 to 5 when the seek-take chain was introduced and then remained relatively stable for the remainder of the training period (Figure 1). A repeated-measures one-way ANOVA using a mixed-effects model revealed no significant effect of training day on the number of heroin infusions, $F(2.02, 15.64) = 2.34$, $p = .129$, $\eta^2_p = .13$, or on take lever

presses, $F(1.69, 13.03) = 2.38$, $p = .137$, $\eta^2_p = .19$, supporting the stability of drug consumption behavior. In contrast, seek lever responding significantly increased across training days, $F(2.79, 22.34) = 3.77$, $p = .027$, $\eta^2_p = .32$, as rats adapted to the progressive increase in the VI schedule.

Punishment-Imposed Abstinence

Punishment robustly suppressed heroin infusions as well as drug-taking and drug-seeking behaviors, evidenced by significant reductions in all three measures relative to baseline. Paired, two-tailed t-tests comparing baseline to punishment-imposed abstinence revealed a significant decrease in infusions, $t(8) = 3.22$, $p = .0122$, $d = 1.07$, (Figure 2). Take lever responses were also significantly reduced, $t(8) = 2.53$, $p = .0353$, $d = 0.84$, (Figure 3). Seek lever responding showed the most pronounced reduction, $t(8) = 4.34$, $p = .0025$, $d = 1.45$, (Figure 4).

Relapse Test

Food deprivation significantly increased measures of heroin "infusions" (Figure 5), taking (Figure 6) and seeking (Figure 7) compared to the sated condition, indicating a robust relapse response under conditions of stress. To quantify this effect, paired-samples t-tests were conducted comparing behavior under food-deprived and sated conditions. Rats received significantly more heroin "infusions" when food deprived than when sated, $t(8) = 4.95$, $p = .0011$, $d = 1.65$. Similarly, take lever responding was higher in the food-deprived condition compared to the sated condition, $t(8) = 4.12$, $p = .0034$, $d = 1.37$. Seek lever pressing followed the same pattern, with more responses when food deprived than when sated, $t(8) = 4.07$, $p = .0036$, $d = 1.36$.

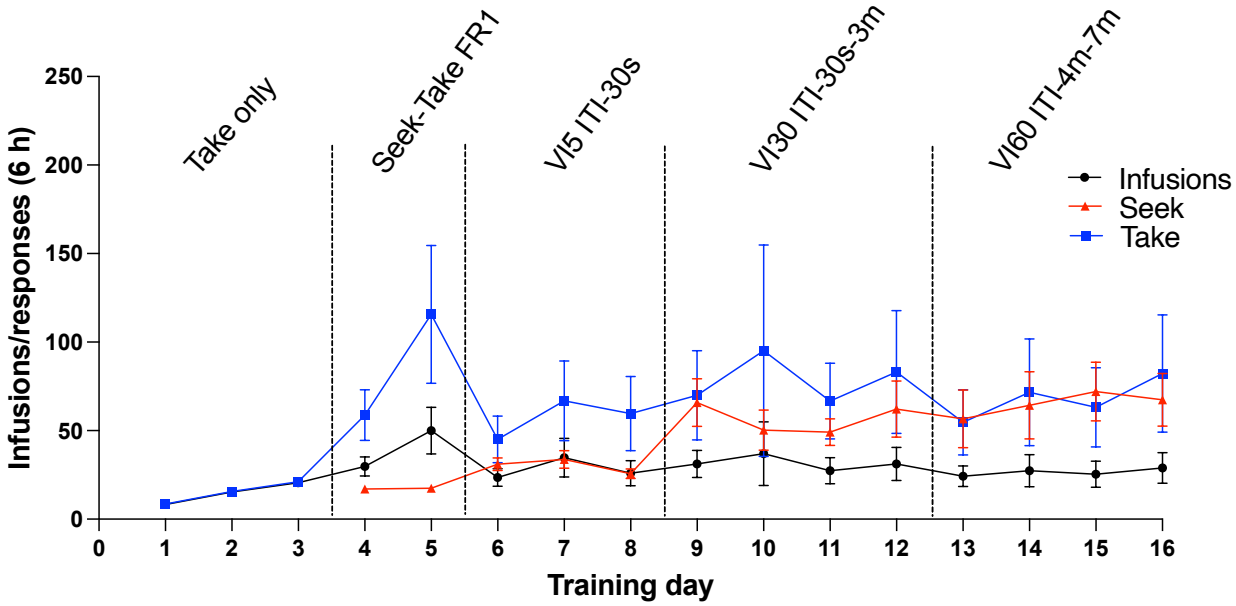
Nociception Test

Heroin administration produced a robust analgesic effect, as evidenced by increased tail flick latencies following drug administration (Figure 8). To assess this effect, tail flick tests were

conducted on 8 rats; one rat was excluded due to a blocked catheter. A paired-samples t-test comparing latencies before and after heroin administration revealed a significant increase in response time, $t(7) = 3.05$, $p = .0185$, $d = 1.08$, 95% CI [−8.43, −1.07], $\eta^2_p = .57$. Mean latency increased from 3.62 seconds (SD = 2.15) before heroin to 8.37 seconds (SD = 4.16) after heroin administration.

Figure 1

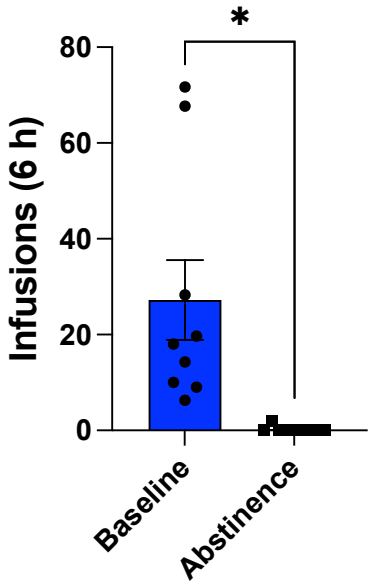
Changes in Heroin Self-Administration: Infusions, Seek Lever Presses, and Take Lever Presses Across Reinforcement Schedules in Male Rats



Note. Mean (± SEM) number of heroin infusions (black), seek lever presses (red), and take lever presses (blue) across self-administration training in male rats (n = 9), over the changing reinforcement schedules (FR1, VI5, VI30, VI60). "Take only" indicates initial access to a single lever delivering 0.1 mg/kg/infusion heroin. In "Seek-Take FR1," rats pressed a seek lever to access a take lever for 5 minutes, with the dose reduced to 0.05 mg/kg/infusion. "VI5 ITI-30s" introduced a 5-second variable interval (VI) and a 30-second intertrial interval (ITI). The VI and ITI were further increased during subsequent phases: "VI30 ITI-30s–3 min" (VI = 30 s, ITI = 30 s–3 min) and "VI60 ITI-4–7 min" (VI = 60 s, ITI = 4–7 min).

Figure 2

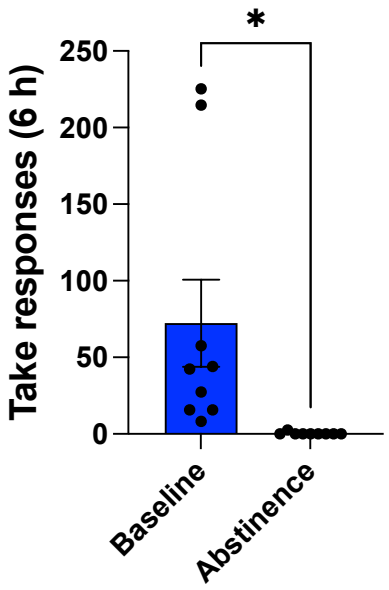
Reduction in Heroin Infusions During Punishment-Imposed Abstinence Compared to Baseline Self-Administration



Note. Mean (± SEM), with individual data points shown. Baseline values represent the average number of heroin infusions during the last three days of self-administration training. Abstinence values represent the average number of heroin infusions during the last two days of punishment sessions. * Denotes $p < .05$, n = 9.

Figure 3

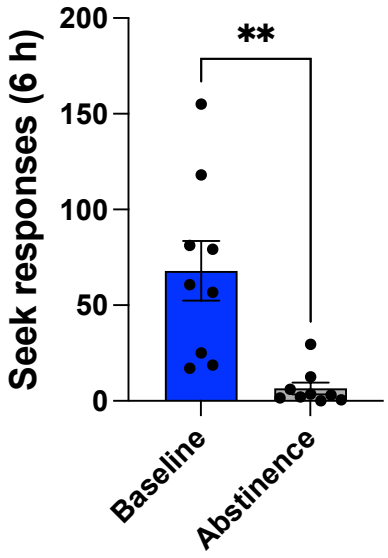
Reduction in Take Lever Presses During Punishment-Imposed Abstinence Compared to Baseline Self-Administration



Note. Mean (± SEM), with individual data points shown. Baseline values represent the average number of take lever presses during the last three days of self-administration training. Abstinence values represent the average number take lever presses during the last two days of punishment sessions. * Denotes $p < .05$, n = 9.

Figure 4

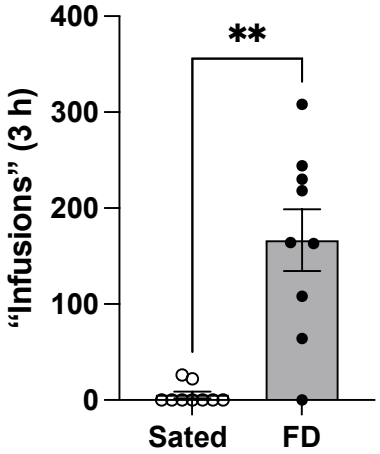
Reduction in Seek Lever Presses During Punishment-Imposed Abstinence Compared to Baseline Self-Administration



Note. Mean (± SEM), with individual data points shown. Baseline values represent the average number of seek lever presses during the last three days of self-administration training. Abstinence values represent the average number of seek lever presses during the last two days of punishment sessions. ** denotes $p < .01$, n = 9.

Figure 5

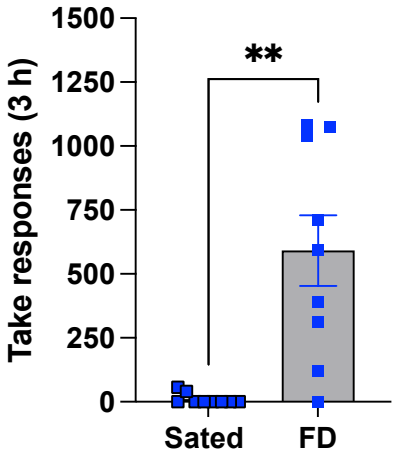
Number of "Infusions" During the Relapse Test as a Function of Feeding Condition (Sated vs. Food Deprived)



Note. Mean (±SEM) number of "infusions" for males during heroin-seeking tests under sated and food deprived (FD) conditions, following footshock-induced abstinence (n = 9). ** denotes $p <$ .01.

Figure 6

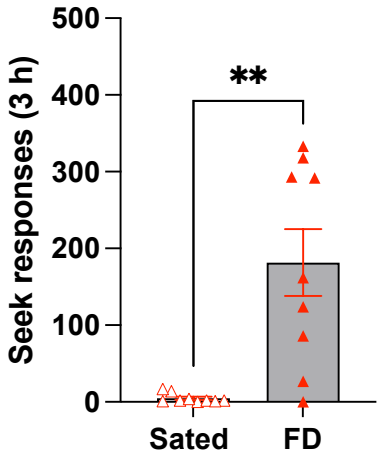
Take Lever Responses During the Relapse Test as a Function of Feeding Condition (Sated vs. Food Deprived)



Note. Mean (±SEM) number of take lever responses for males during heroin-seeking tests under sated and food deprived (FD) conditions, following footshock-induced abstinence (n = 9). ** denotes $p < .01$.

Figure 7

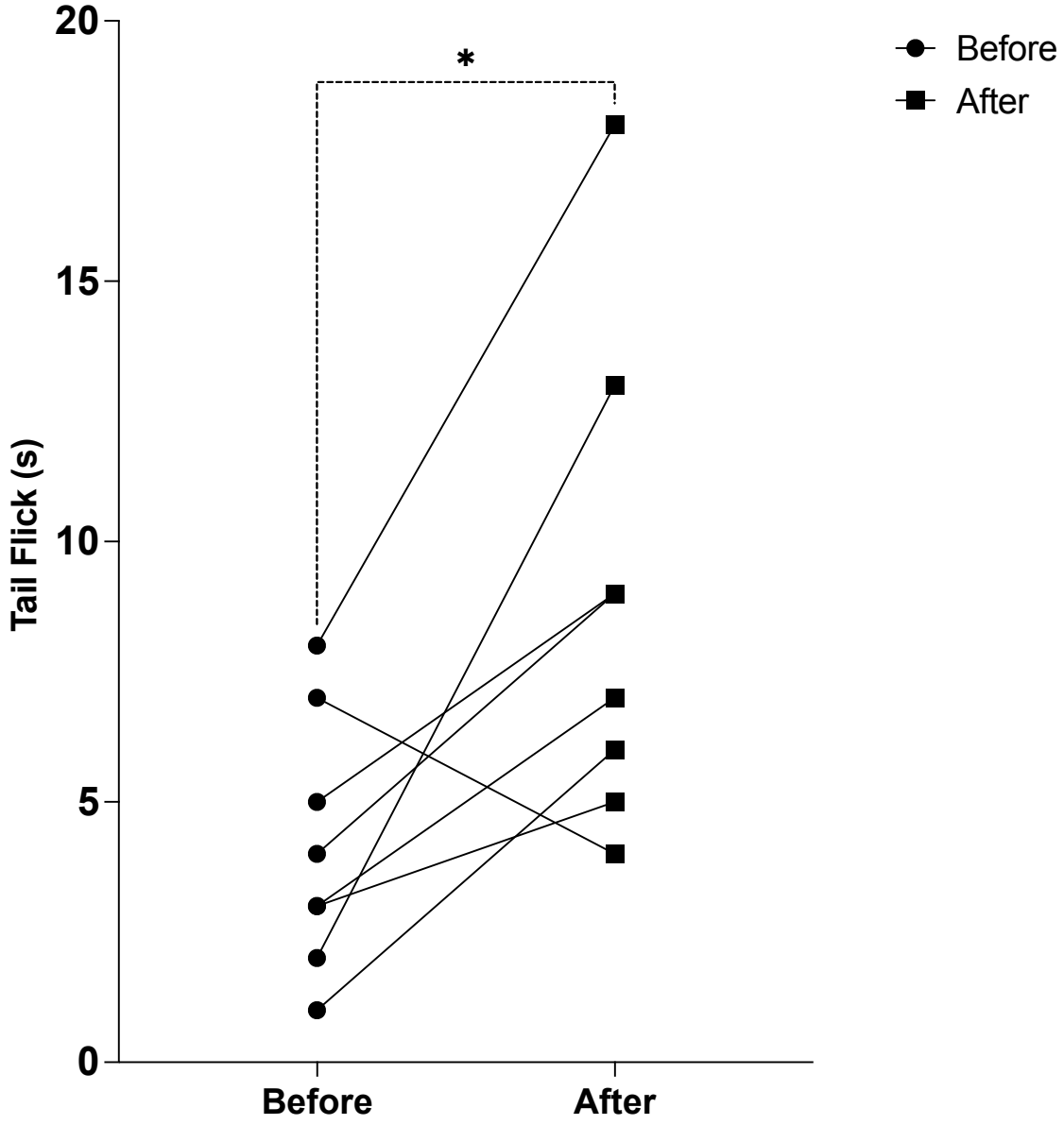
Seek Lever Responses During the Relapse Test as a Function of Feeding Condition (Sated Vs. Food Deprived)



Note. Mean (±SEM) number of seek lever responses for males during heroin-seeking tests under sated and food deprived (FD) conditions, following footshock-induced abstinence (n = 9). ** denotes $p < .01$.

Figure 8

Tail Flick Latency Before and After Heroin Infusion



Note. Data are presented as mean ± SEM. n = 8. "Before" refers to baseline measurements prior to heroin infusion; "After" refers to measurements following heroin infusion. * Denotes $p < .05$.

Experiment 1b. The Effects of Increased Heroin Availability on Punishment-Imposed Abstinence and Stress-Induced Relapse in Female Rats

Data Integrity

Two rats were excluded from the analyses for failing to meet the abstinence criterion. Therefore, data from 8 female rats were included in the final analyses.

Self-Administration

During heroin self-administration, all rats (n = 8) showed stable infusion rates across training. Take lever responses increased early in training but then gradually declined and stabilized, while seek lever responses progressively increased over time (Figure 9). A repeated-measures one-way ANOVA revealed no significant effect of training day on either heroin infusions, $F(2.78, 19.45) = 2.71$, $p = .077$, $\eta^2_p = .28$, or take lever responses, $F(2.12, 14.83) = 2.33$, $p = .130$, $\eta^2_p = .25$, indicating that both measures remained relatively consistent across sessions. In contrast, seek lever responding significantly increased over time, $F(2.84, 19.85) = 5.67$, $p = .006$, $\eta^2_p = .45$, reflecting behavioral adaptation to the gradually increasing variable interval schedule.

Punishment-Imposed Abstinence

Punishment significantly reduced heroin infusions and led to decreases in both seek and take lever responses, although only the reduction in seek responding reached statistical significance. These comparisons were conducted using paired, two-tailed t-tests between baseline performance and abstinence days. Heroin infusions were significantly reduced following punishment, $t(7) = 2.73$, p = .0295, $d = 0.96$ (Figure 10). While both seek and take lever responding declined, only seek responses were significantly reduced, $t(7) = 2.51$, $p = .0405$, $d = 0.89$ (Figure 11); take responses showed a non-significant decrease, $t(7) = 2.13$, $p = .0705$, d

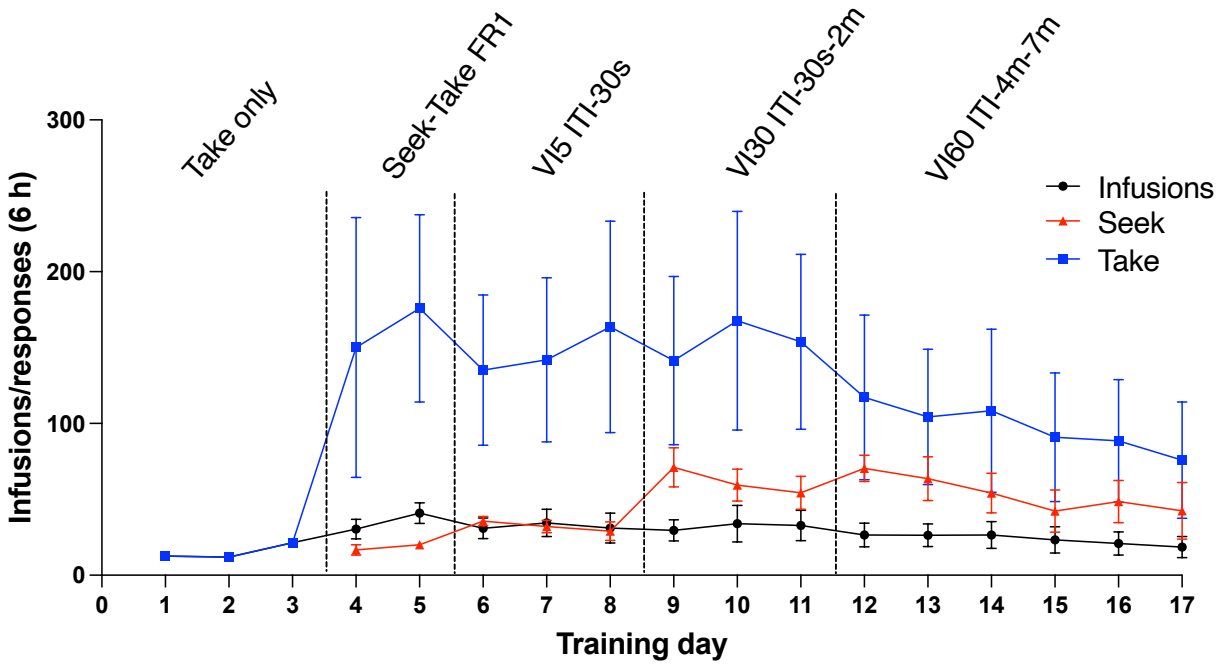
= 0.75 (Figure 12). Despite the lack of statistical significance, the take lever responses exhibited a moderate to large effect size, suggesting a meaningful behavioral reduction. The lack of statistical significance for take responses may reflect greater variability across subjects during this phase.

Relapse Test

Paired-samples t-tests revealed significant differences between sated and food-deprived conditions across all measures during the relapse test. Rats took significantly more heroin "infusions" when food-deprived compared to when sated, $t(7) = 3.64$, $p = .0083$, $d = 1.29$, (Figure 13). Similarly, take lever presses were significantly higher in the food-deprived condition than in the sated condition, $t(7) = 2.99$, $p = .0202$, $d = 1.06$, (Figure 14). Seek lever presses also increased significantly with food deprivation, relative to the sated condition, $t(7) = 3.36$, $p = .0121$, $d = 1.19$ (Figure 15).

Figure 9

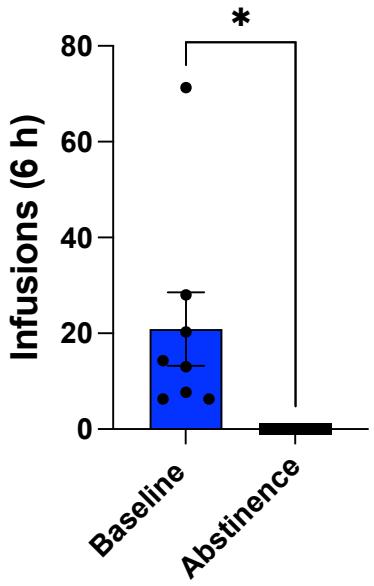
Changes in Heroin Self-Administration: Infusions, Seek Lever Presses, and Take Lever Presses Across Reinforcement Schedules in Female Rats



Note. Mean (± SEM) number of heroin infusions (black), seek lever presses (red), and take lever presses (blue) across self-administration training in male rats (n = 8), over the changing reinforcement schedules (FR1, VI5, VI30, VI60). "Take only" indicates initial access to a single lever delivering 0.1 mg/kg heroin. In "Seek-Take FR1," rats pressed a seek lever to access a take lever for 5 minutes, with the dose reduced to 0.05 mg/kg. "VI5 ITI-30s" introduced a 5-second variable interval (VI) and a 30-second intertrial interval (ITI). The VI and ITI were further increased during subsequent phases: "VI30 ITI-30s–3 min" (VI = 30 s, ITI = 30 s–3 min) and "VI60 ITI-4–7 min" (VI = 60 s, ITI = 4–7 min). Doses are expressed in mg/kg/infusion.

Figure 10

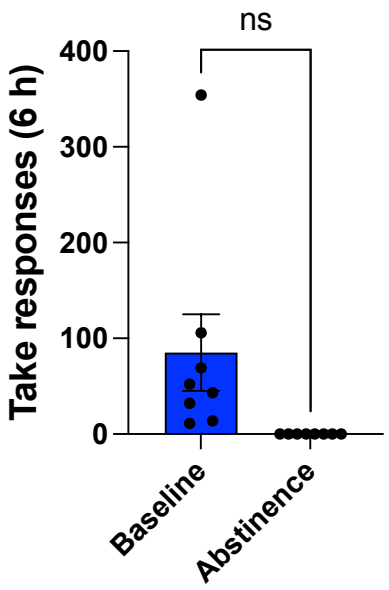
Reduction in Heroin Infusions During Punishment-Imposed Abstinence Compared to Baseline Self-Administration



Note. Mean (± SEM), with individual data points shown. Baseline values represent the average number of heroin infusions during the last three days of self-administration training. Abstinence values represent the average number of heroin infusions during the last two days of punishment sessions. * Denotes $p < .05$, n = 8.

Figure 11

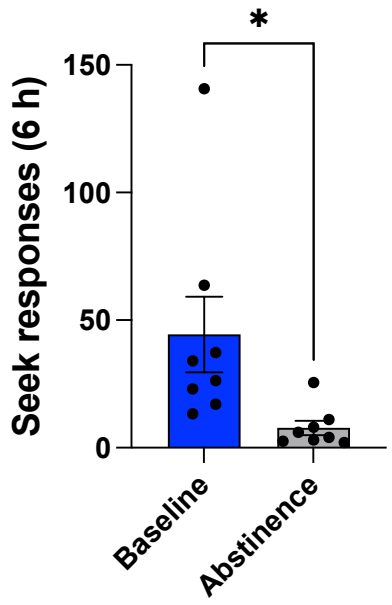
Reduction in Take Lever Presses During Punishment-Imposed Abstinence Compared to Baseline Self-Administration



Note. Mean (± SEM), with individual data points shown. Baseline values represent the average number of take lever presses during the last three days of self-administration training. Abstinence values represent the average number of take lever presses during the last two days of punishment sessions. "NS" denotes a non-significant difference ($p > 0.05$), $n = 8$.

Figure 12

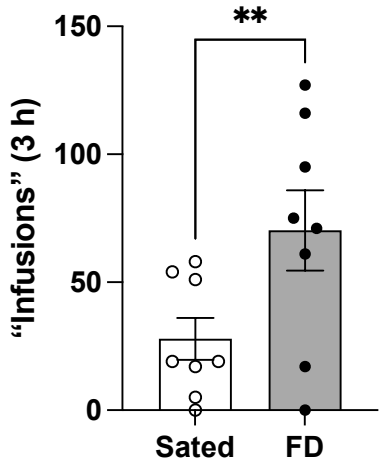
Reduction in Seek Lever Presses During Punishment-Imposed Abstinence Compared to Baseline Self-Administration



Note. Mean (± SEM), with individual data points shown. Baseline values represent the average number of seek lever presses during the last three days of self-administration training. Abstinence values represent the average number of seek lever presses during the last two days of punishment sessions. * Denotes $p < .05$, n = 8.

Figure 13

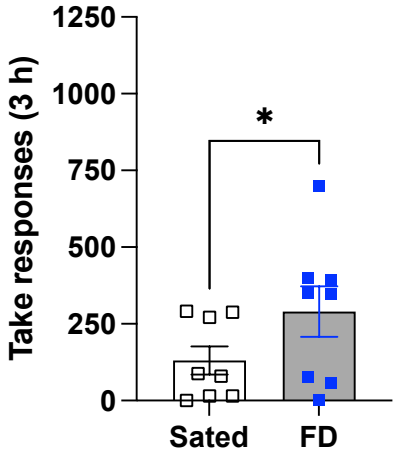
Number of "Infusions" During the Relapse Test as a Function of Feeding Condition (Sated vs. Food Deprived)



Note. Mean (±SEM) number of "infusions" for females during heroin-seeking tests under sated and food deprived (FD) conditions, following footshock-induced abstinence (n = 8). ** denotes $p < .01$.

Figure 14

Take Lever Responses During the Relapse Test as a Function of Feeding Condition (Sated vs. Food Deprived)

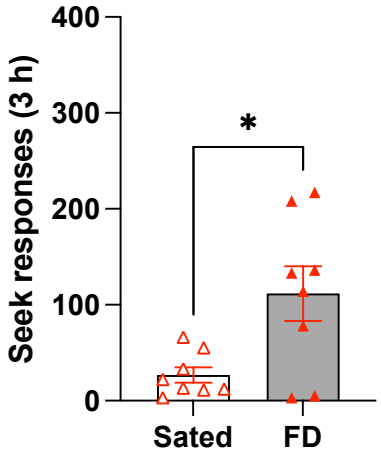


Note. Mean (±SEM) number of take lever responses for females during heroin-seeking tests under sated and food deprived (FD) conditions, following footshock-induced abstinence (n = 8).

* Denotes $p < .05$.

Figure 15

Seek Lever Responses During the Relapse Test as a Function of Feeding Condition (Sated vs. Food Deprived)



Note. Mean (±SEM) number of seek lever responses for females during heroin-seeking tests under sated and food deprived (FD) conditions, following footshock-induced abstinence (n = 8). * Denotes $p < .05$.

Experiment 2a: The Role of the Orbitofrontal Cortex in Stress-Induced Relapse to Heroin Seeking Following Voluntary Abstinence in Male Rats

Data Integrity

Four rats were excluded from the analyses: two for failing to meet the abstinence criterion, one due to a broken 5-up, and one for inadequate training. Therefore, data from 16 male rats were included in the final analyses.

Self-Administration

Throughout training, heroin infusions, as well as take and seek lever presses, showed overall increases across days (Figure 16). A repeated-measures one-way ANOVA revealed a significant effect of training day on heroin infusions, $F(2.88, 43.24) = 4.37$, $p = .010$, $\eta^2_p = .23$; on take lever presses, $F(2.38, 35.74) = 5.78$, $p = .004$, $\eta^2_p = .28$; and on seek lever presses, $F(3.63, 54.41) = 6.73$, $p < .001$, $\eta^2_p = .31$, as rats adjusted to the gradual increase in the variable interval (VI) schedule. These data represent combined results from both runs that are common to all subjects; for individual run data, please refer to Appendix A, Figures A1 and A2.

Punishment-Imposed Abstinence

Punishment robustly suppressed heroin infusions as well as drug-taking and drug-seeking behaviors, as evidenced by significant reductions in infusions, take lever responses, and seek lever responses relative to baseline. There was a significant reduction in the number of infusions during punishment compared to baseline, $t(15) = 6.13$, $p < .0001$, $d = 1.53$ (Figure 17). Similarly, taking responses were significantly reduced following punishment, $t(15) = 4.49$, $p = .0004$, $d = 1.12$ (Figure 18) Seeking responses were also significantly reduced during punishment, $t(15) = 7.50$, $p < .0001$, $d = 1.88$ (Figure 19).

Relapse Test

The relapse test results reported here combine data from both Run 1 and Run 2, with a total sample size of 16 rats (8 rats per run).

Rats exhibited significantly greater heroin-seeking behavior, reflected in increased "infusions", and take and seek lever presses, under food-deprived conditions. Chemogenetic inhibition of the OFC with DCZ had no significant effect. A series of two-way repeated-measures ANOVAs were conducted to examine the impact of feeding condition (sated vs. food-deprived) and treatment condition (vehicle vs. DCZ) on heroin-seeking behavior, including the number of "infusions," take lever presses, and seek lever presses.

For heroin infusions, there was a significant main effect of feeding condition, $F(1, 14) = 60.46$, $p < .0001$, $\eta^2_p = .81$, with rats receiving significantly more infusions when food deprived (M = 60.12, SD = 41.09) compared to when sated (M = 4.37, SD = 8.82; Figure 20). There was no significant main effect of treatment condition, $F(1, 14) = 0.21$, $p = .651$, $\eta^2_p = .02$, nor a significant interaction, $F(1, 14) = 1.09$, $p = .315$, $\eta^2_p = .07$.

Similarly, take lever presses showed a significant main effect of feeding condition, $F(1, 14) = 34.28$, $p < .0001$, $\eta^2_p = .71$, with rats pressing the take lever significantly more when food deprived (M = 283.20, SD = 193.08) than when sated (M = 12.75, SD = 29.35; Figure 21). The main effect of treatment condition was not significant, $F(1, 14) = 0.08$, $p = .785$, $\eta^2_p < .01$, nor was the interaction effect, $F(1, 14) = 0.36$, $p = .558$, $\eta^2_p = .03$.

Finally, seek lever presses were also significantly increased by food deprivation, $F(1, 14) = 18.85$, $p = .0007$, $\eta^2_p = .57$, with rats making more seek presses when food deprived (M = 95.84, SD = 99.80) than when sated (M = 5.75, SD = 11.54; Figure 22). No significant main

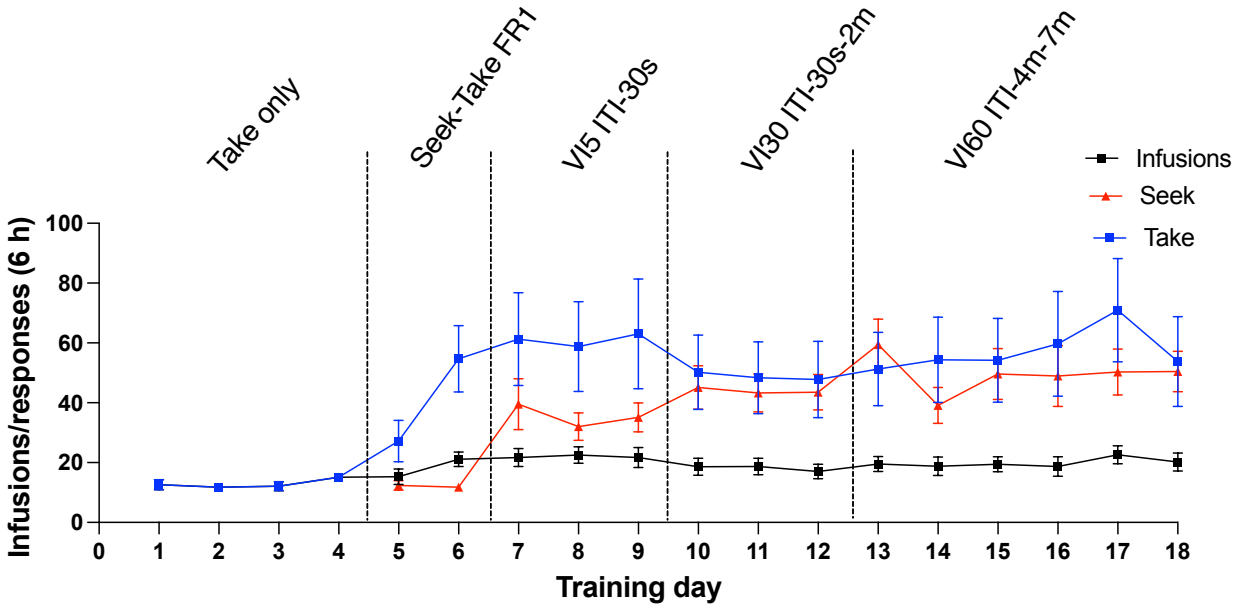
effect of treatment condition was found, $F(1, 14) = 1.25$, $p = .282$, $\eta^2_p = .08$, nor a significant interaction, $F(1, 14) = 1.04$, $p = .324$, $\eta^2_p = .07$.

Locomotor Activity

An unpaired two-tailed t-test revealed no significant difference in locomotor activity between the VEH and DCZ groups, $t(14) = 0.91$, $p = .379$, 95% CI [−626, 1547], Cohen's $d = 0.48$ (Figure 23), indicating that DCZ did not affect general movement.

Figure 16

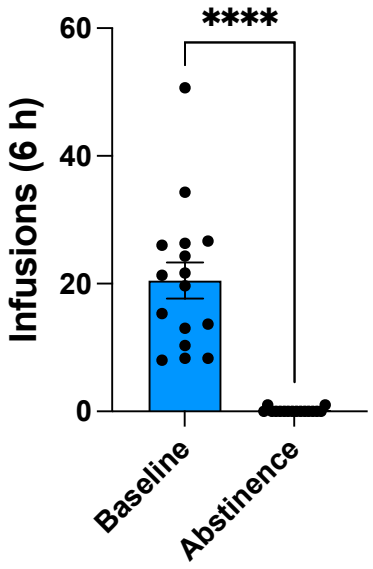
Changes in Heroin Self-Administration: Infusions, Seek Lever Presses, and Take Lever Presses Across Reinforcement Schedules in Male Rats



Note. Mean (± SEM) number of heroin infusions (black), seek lever presses (red), and take lever presses (blue) across self-administration training in male rats (n = 16), over the changing reinforcement schedules (FR1, VI5, VI30, VI60). "Take only" indicates initial access to a single lever delivering 0.1 mg/kg heroin. In "Seek-Take FR1," rats pressed a seek lever to access a take lever for 5 minutes, with the dose reduced to 0.05 mg/kg. "VI5 ITI-30s" introduced a 5-second variable interval (VI) and a 30-second intertrial interval (ITI). The VI and ITI were further increased during subsequent phases: "VI30 ITI-30s–3 min" (VI = 30 s, ITI = 30 s–3 min) and "VI60 ITI-4–7 min" (VI = 60 s, ITI = 4–7 min). Doses are expressed in mg/kg/infusion. This behavioral paradigm was used to characterize heroin self-administration patterns prior to orbitofrontal cortex focused analyses.

Figure 17

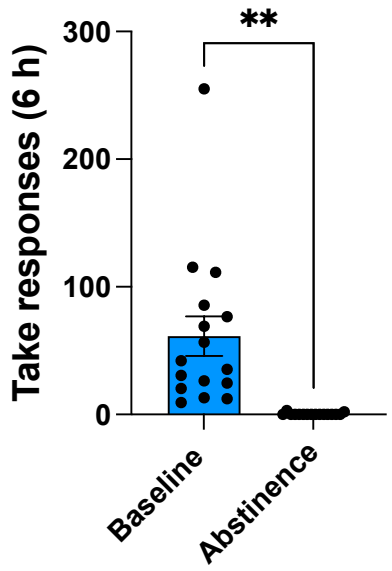
Reduction in Heroin Infusions During Punishment-Imposed Abstinence Compared to Baseline Self-Administration



Note. Mean (± SEM), with individual data points shown. Baseline values represent the average number of heroin infusions during the last three days of self-administration training. Abstinence values represent the average number of heroin infusions during the last two days of punishment sessions. **** denotes $p < .0001$, n = 16 rats.

Figure 18

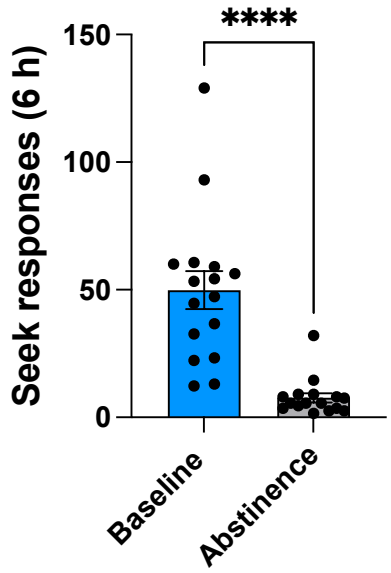
Reduction in Take Lever Presses During Punishment-Imposed Abstinence Compared to Baseline Self-Administration



Note. Mean (± SEM), with individual data points shown. Baseline values represent the average number of take lever presses during the last three days of self-administration training. Abstinence values represent the average number of take lever presses during the last two days of punishment sessions. ** denotes $p < .01$, n = 16 rats.

Figure 19

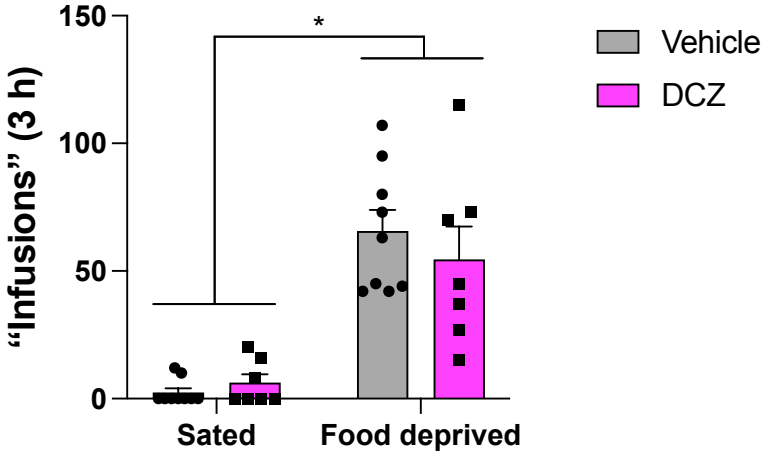
Reduction in Seek Lever Presses During Punishment-Imposed Abstinence Compared to Baseline Self-Administration



Note. Mean (± SEM), with individual data points shown. Baseline values represent the average number of seek lever presses during the last three days of self-administration training. Abstinence values represent the average number of seek lever presses during the last two days of punishment sessions. **** denotes p <.0001, n = 16 rats.

Figure 20

Number of Heroin "Infusions" During the Relapse Test as a Function of Feeding Condition (Sated vs. Food-Deprived) and Treatment (Vehicle vs. DCZ-Induced Orbitofrontal Cortex Inhibition)

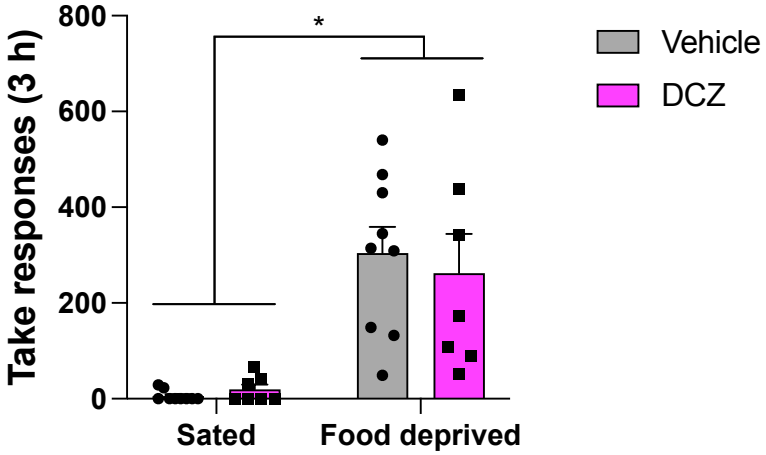


Note. Mean (+SEM) number of heroin "infusions" in male rats (n = 9 in the vehicle group, n = 7 in the DCZ group) during relapse tests conducted under sated and food-deprived conditions, following treatment with either vehicle or DCZ. VEH = vehicle; DCZ = deschloroclozapine.

* Denotes $p < .05$.

Figure 21

Number of Take Lever Presses During the Relapse Test as a Function of Feeding (Sated vs. Food-Deprived) and Treatment (Vehicle vs. DCZ-Induced Orbitofrontal Cortex Inhibition)

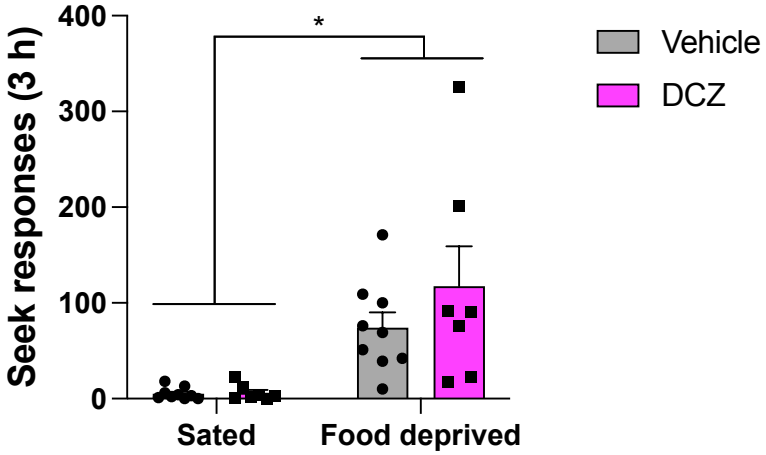


Note. Mean (+SEM) number of take lever presses in male rats (n = 9 in the vehicle group, n = 7 in the DCZ group) during relapse tests conducted under sated and food-deprived conditions, following treatment with either vehicle or DCZ. VEH = vehicle; DCZ = deschloroclozapine

* Denotes $p < .05$.

Figure 22

Number of Seek Lever Presses During the Relapse Test as a Function of Feeding (Sated vs. Food-Deprived) and Treatment (Vehicle vs. DCZ-Induced Orbitofrontal Cortex Inhibition)

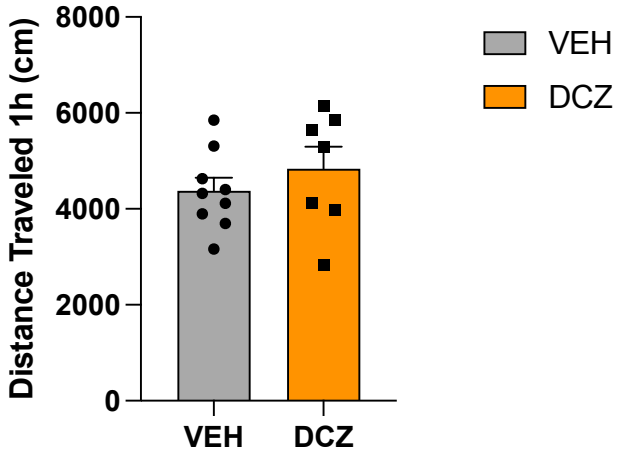


Note. Mean (+SEM) number of seek lever presses in male rats (n = 9 in the vehicle group, n = 7 in the DCZ group) during relapse tests conducted under sated and food-deprived conditions, following treatment with either vehicle or DCZ. VEH = vehicle; DCZ = deschloroclozapine

* Denotes $p < .05$.

Figure 23

Locomotor Activity During 1-Hour Test in VEH and DCZ-Treated Rats Following Orbitofrontal Cortex (OFC) Inhibition



Note. Bars represent mean (± SEM). VEH = vehicle; DCZ = deschloroclozapine. Total distance traveled (in centimeters) was measured using an automated activity monitoring system over a 1-hour locomotor activity test. Male rats were divided into VEH (n = 9) and DCZ (n = 7) groups.

Experiment 2b: The Role of the Prelimbic Cortex in Stress-Induced Relapse to Heroin Seeking Following Voluntary Abstinence in Male Rats

Data Integrity

Three rats were excluded from analyses for the following reasons: one due to catheter leakage that prevented successful training, one due to inadequate training, and one due to incorrect viral vector placement. Thus, seven rats were included in the final analyses.

Self-Administration

During training, heroin infusions remained stable, while take and seek lever presses both showed overall increases across sessions (Figure 24). A repeated-measures one-way ANOVA revealed no significant effect of training day, on the number of infusions $F(1.62, 9.69) = 3.21$, $p = .092$, $\eta^2_p = .35$ or on take lever presses $F(1.31, 7.88) = 3.48$, $p = .094$, $\eta^2_p = .37$, suggesting consistent drug-taking behavior across sessions. In contrast, seek lever presses increased significantly $F(2.04, 12.27) = 4.40$, $p = .036$, $\eta^2_p = .42$, as the rats adjusted to the gradual increase in the VI schedule.

Punishment-Imposed Abstinence

Punishment robustly suppressed heroin infusions as well as drug-taking and drug-seeking behaviors, as evidenced by significant reductions in infusions, take lever responses, and seek lever responses relative to baseline. There was a significant reduction in the number of infusions during punishment compared to baseline, $t(6) = 2.85$, $p = .0294$, $d = 1.08$, $\eta^2_p = .57$ (Figure 25). Similarly, take lever responses were significantly reduced following punishment, $t(6) = 2.66$, $p = .0375$, $d = 1.01$, $\eta^2_p = .54$ (Figure 26). Seek lever responding was also significantly reduced during punishment, $t(6) = 2.95$, $p = .0255$, $d = 1.12$, $\eta^2_p = .59$ (Figure 27).

Relapse Test

Rats exhibited significantly greater heroin-seeking behavior, reflected in increased "infusions", and take and seek lever presses, under food-deprived conditions. However, chemogenetic inhibition of the PrL with DCZ had no significant effect. To analyze these effects, a series of two-way repeated-measures ANOVAs were conducted to examine the impact of feeding condition (sated vs. food-deprived) and treatment condition (vehicle vs. DCZ) on heroin-seeking behavior, including the number of "infusions," take lever presses, and seek lever presses.

For heroin "infusions," there was a significant main effect of feeding condition, $F(1, 5) = 17.57$, $p = .0086$, $\eta^2_p = .78$, with rats self-administering significantly more heroin when food deprived (M = 93.50, SD = 74.03) compared to when sated (M = 10.63, SD = 15.80; Figure 28).There was no significant main effect of treatment condition, $F(1, 5) = 0.58$, $p = .48$, $\eta^2_p = .10$, nor a significant interaction, $F(1, 5) = 1.58$, $p = .26$, $\eta^2_p = .24$.

For take lever presses, there was a significant main effect of feeding condition, $F(1, 5) = 19.18$, $p = .0072$, $\eta^2_p = .79$, with rats pressing the take lever more when food deprived (M = 473.40, SD = 369.17) than when sated (M = 40.17, SD = 75.39; Figure 29).The main effect of treatment condition was not significant, $F(1, 5) = 2.09$, $p = .208$, $\eta^2_p = .29$, nor was the interaction, $F(1, 5) = 2.76$, $p = .158$, $\eta^2_p = .36$.

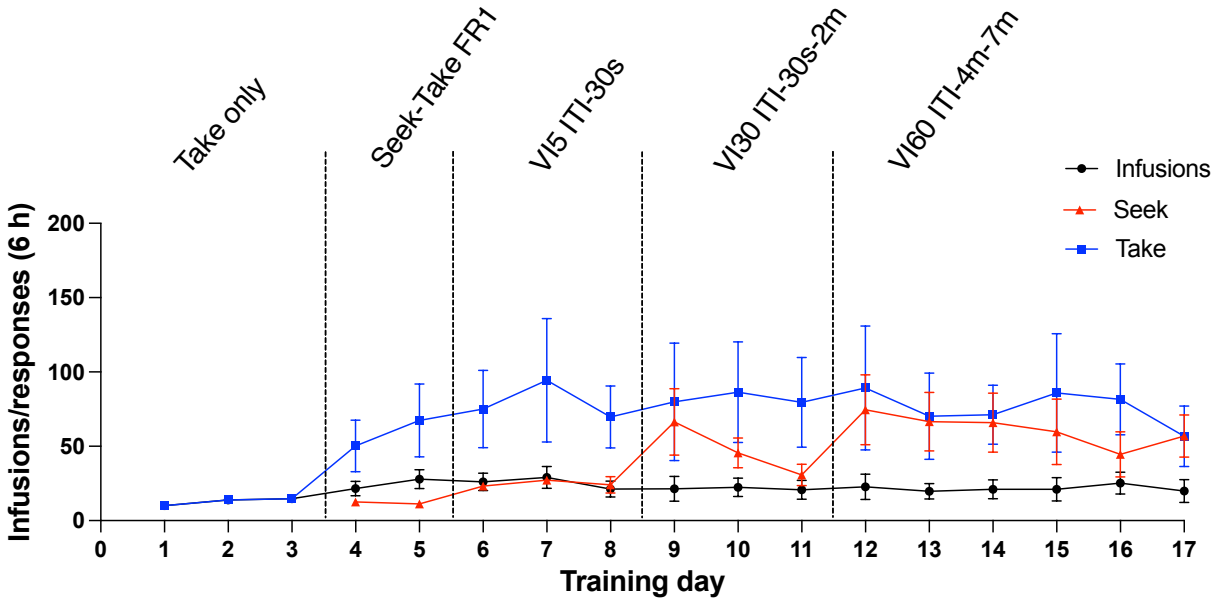
For seek lever presses, there was a significant main effect of feeding condition, $F(1, 5) = 18.93$, $p = .0073$, $\eta^2_p = .79$, with increased responding during food deprivation (M = 90.00, SD = 81.15) compared to sated conditions (M = 8.63, SD = 18.17; Figure 30). No significant main effect of treatment condition was found, $F(1, 5) = 0.22$, $p = .656$, $\eta^2_p = .04$, nor was there a significant interaction, $F(1, 5) = 0.01$, $p = .934$, $\eta^2_p < .01$.

Locomotor Activity

An unpaired-samples t-test revealed no significant difference in total locomotor activity between DCZ and vehicle conditions during the 1-hour locomotor test, $t(2) = 0.36$, $p = .75$, 95% CI [−7163, 6050], Cohen's $d = 0.21$ (Figure 31).

Figure 24

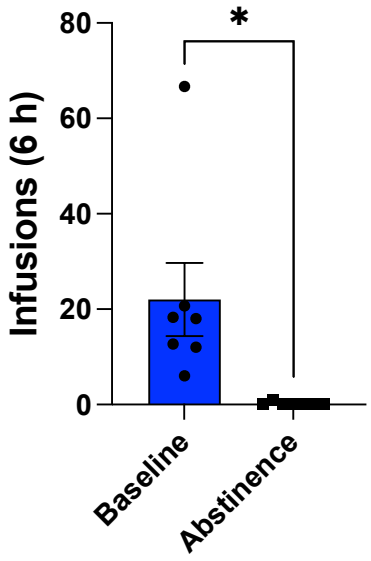
Changes in Heroin Self-Administration: Infusions, Seek Lever Presses, and Take Lever Presses Across Reinforcement Schedules in Male Rats



Note. Mean (± SEM) number of heroin infusions (black), seek lever presses (red), and take lever presses (blue) across self-administration training in male rats (n = 7), over the changing reinforcement schedules (FR1, VI5, VI30, VI60). "Take only" indicates initial access to a single lever delivering 0.1 mg/kg heroin. In "Seek-Take FR1," rats pressed a seek lever to access a take lever for 5 minutes, with the dose reduced to 0.05 mg/kg. "VI5 ITI-30s" introduced a 5-second variable interval (VI) and a 30-second intertrial interval (ITI). The VI and ITI were further increased during subsequent phases: "VI30 ITI-30s–3 min" (VI = 30 s, ITI = 30 s–3 min) and "VI60 ITI-4–7 min" (VI = 60 s, ITI = 4–7 min). Doses are expressed in mg/kg/infusion. This behavioral paradigm was used to characterize heroin self-administration patterns prior to prelimbic cortex focused analyses.

Figure 25

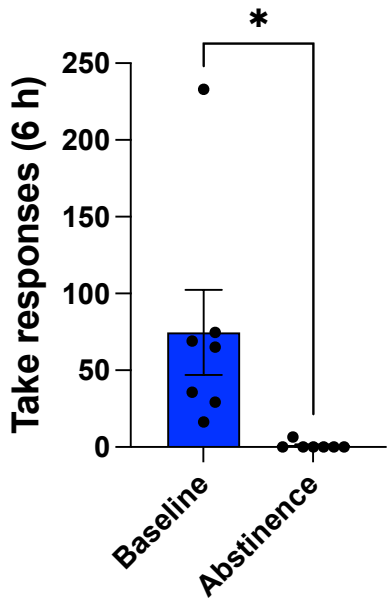
Reduction in Heroin Infusions During Punishment-Imposed Abstinence Compared to Baseline Self-Administration



Note. Mean (± SEM), with individual data points shown. Baseline values represent the average number of heroin infusions during the last three days of self-administration training. Abstinence values represent the average number of heroin infusions during the last two days of punishment sessions. * Denotes $p < .05$, n = 7 rats.

Figure 26

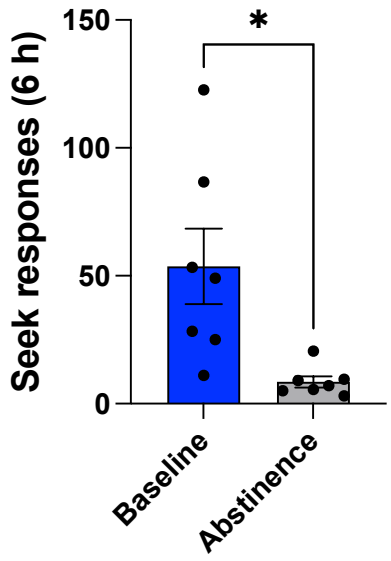
Reduction in Take Lever Presses During Punishment-Imposed Abstinence Compared to Baseline Self-Administration



Note. Mean (± SEM), with individual data points shown. Baseline values represent the average number of take lever presses during the last three days of self-administration training. Abstinence values represent the average number of take lever presses during the last two days of punishment sessions. * Denotes $p < .05$, n = 7 rats.

Figure 27

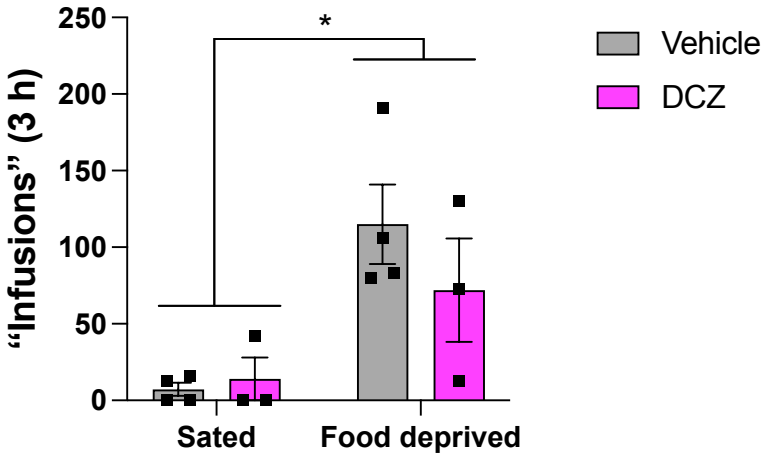
Reduction in Seek Lever Presses During Punishment-Imposed Abstinence Compared to Baseline Self-Administration



Note. Mean (± SEM), with individual data points shown. Baseline values represent the average number of seek lever presses during the last three days of self-administration training. Abstinence values represent the average number of seek lever presses during the last two days of punishment sessions. * Denotes $p < .05$, n = 7 rats.

Figure 28

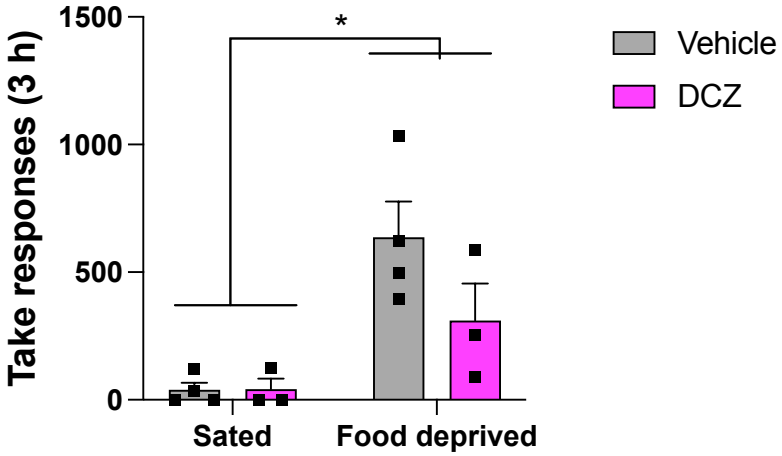
Number of Heroin "Infusions" During the Relapse Test as a Function of Feeding (Sated vs. Food-Deprived) and Treatment (Vehicle vs. DCZ-Induced Prelimbic Cortex Inhibition)



Note. Mean (± SEM) number of heroin "infusions" in male rats, with n = 4 rats in the vehicle group and n = 3 rats in the DCZ group, during relapse tests conducted under sated and food-deprived conditions following treatment with either vehicle or DCZ. VEH = vehicle; DCZ = deschloroclozapine. * Denotes $p < .05$.

Figure 29

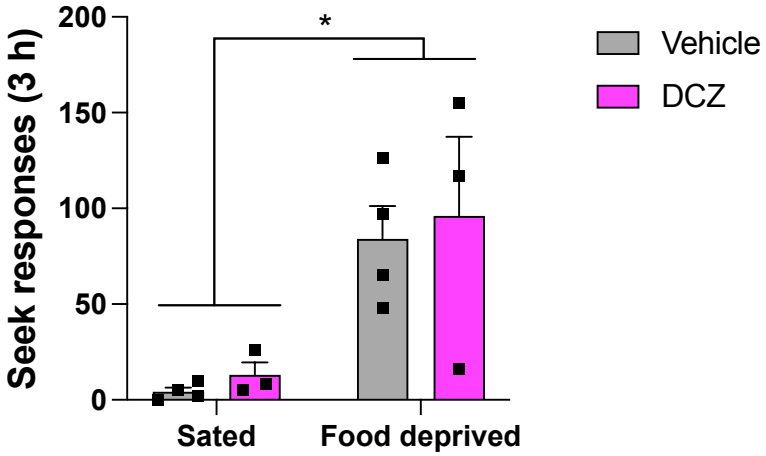
Number of Take Lever Presses During the Relapse Test as a Function of Feeding (Sated vs. Food-Deprived and Treatment (Vehicle vs. DCZ-Induced Prelimbic Cortex Inhibition)



Note. Mean (+SEM) number of take lever presses in male rats, with n = 4 rats in the vehicle group and n = 3 rats in the DCZ group during relapse tests conducted under sated and food-deprived conditions, following treatment with either vehicle or DCZ. VEH = vehicle; DCZ = deschloroclozapine. * Denotes $p < .05$.

Figure 30

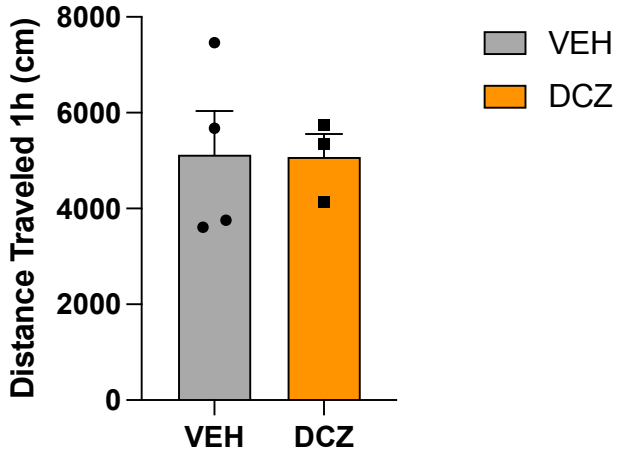
Number of Seek Lever Presses During the Relapse Test as a Function of Feeding (Sated vs. Food-Deprived) and Treatment (Vehicle vs. DCZ-Induced Prelimbic Cortex Inhibition)



Note. Mean (+SEM) number of seek lever presses in male rats, with n = 4 rats in the vehicle group and n = 3 rats in the DCZ group during relapse tests conducted under sated and food-deprived conditions, following treatment with either vehicle or DCZ. VEH = vehicle; DCZ = deschloroclozapine. * Denotes $p < .05$.

Figure 31

Locomotor Activity During 1-Hour Test in VEH and DCZ-Treated Rats Following Prelimbic Cortex Inhibition



Note. Bars represent mean (± SEM). VEH = vehicle; DCZ = deschloroclozapine. Total distance traveled (in centimeters) was measured using an automated activity monitoring system over a 1-hour locomotor activity test. Male rats were divided into VEH (n = 4) and DCZ (n = 3) groups (total n = 16).

Discussion

This thesis investigated three central research questions addressing the behavioral and neural mechanisms underlying heroin use and relapse in rats. Specifically, the study examined whether a 5-minute seek-take protocol could effectively model intermittent access and compulsive heroin use in both male and female rats. It also assessed whether chemogenetic inhibition of the OFC would reduce stress-induced relapse to heroin seeking following punishment-imposed abstinence, and whether inhibiting the PrL cortex would similarly attenuate stress-induced relapse under the same conditions. The corresponding hypotheses were that the 5-minute seek-take protocol would successfully capture features of intermittent access and compulsive heroin use across sexes, that chemogenetic inhibition of the OFC would attenuate stress-induced relapse to heroin seeking after punishment-imposed abstinence, and that chemogenetic inhibition of the PrL cortex would likewise reduce stress-induced relapse following punishment-imposed abstinence.

Experiment 1a: Effects of Increased Heroin Availability on Punishment-Imposed Abstinence and Stress-Induced Relapse in Male Rats

Experiment 1a demonstrated that male rats given extended intermittent access to heroin maintained stable overall intake while exhibiting progressively escalating drug-seeking behavior over time. These findings align with recent work by Rakowski et al. (2025), who reported a dissociation between heroin intake and incentive motivation in rats given intermittent versus continuous access. Specifically, rats with intermittent access showed lower overall intake but elevated heroin-seeking behavior and greater motivational drive, suggesting that repeated spikes in drug exposure may enhance the incentive value of heroin without necessarily increasing total

consumption.

Building on this dissociation, our study further showed that transitioning to a seek–take chain schedule with a halved heroin dose per infusion initially triggered compensatory increases in intake. This underscores the animals' drive to maintain stable brain heroin levels despite reduced per-infusion doses. Moreover, as seeking requirements increased (i.e., with longer variable intervals), rats adapted by intensifying their seeking responses while ultimately maintaining relatively constant overall heroin consumption. This adaptive regulation supports the model's validity in replicating persistent and motivated drug pursuit characteristic of human opioid addiction.

The intermittent access model may have contributed to the patterns of self-administration observed in the current study, particularly the dissociation between stable heroin intake and progressively increasing drug-seeking behavior. Prior work using intermittent access paradigms for heroin has revealed similar profiles. For example, D'Ottavio et al. (2022) found that rats with intermittent, compared to continuous, access to heroin exhibited burst-like intake patterns and significantly greater cue-induced seeking during early abstinence, suggesting that intermittent availability enhances the motivational salience of heroin-associated cues.

Although the intermittent access procedure was originally developed in cocaine research, where it promotes addiction-like behaviors independent of overall consumption (Allain & Samaha, 2019), recent findings suggest that the impact of drug-use pattern may reflect a broader principle conserved across substances. In both cocaine and heroin models, repeated spikes in brain drug concentrations appear to drive the emergence of compulsive drug-seeking, regardless of total intake. This aligns with the classic "loading and maintenance" framework described in stimulant studies (Tsibulsky & Norman, 1999; Gerber & Wise, 1989), wherein animals initially

engage in a rapid loading phase to elevate brain drug levels, followed by a maintenance phase to sustain those levels. The extension of this framework to opioids highlights the value of intermittent access models in capturing core features of addiction, particularly their relevance for understanding the dynamics of opioid use disorders.

In the present findings, the observation of stable heroin intake despite escalating drug-seeking suggests that rats are able to maintain a preferred brain heroin concentration, even as their motivation to obtain the drug intensifies over time. This dissociation between consumption and seeking effort reflects patterns observed in human opioid addiction, where individuals may expend increasing effort to obtain opioids despite stable intake levels.

Thus, the extended intermittent access model used here not only captures the regulation of heroin intake, but also effectively models the emergence of persistent, compulsive drug-seeking behavior.

During punishment-imposed abstinence, rats demonstrated remarkable persistence in heroin seeking, continuing to respond despite probabilistic footshocks (Appendix B, Figure B1). The initial surge in seeking responses likely reflects attempts to compensate for reduced drug availability due to punishment-imposed omission (Borges et al., 2023). Moreover, the analgesic properties of heroin may have contributed to this persistence, as lower-intensity shocks were initially insufficient to deter responding. Only at higher shock intensities did a reduction in seeking and taking responses emerge, ultimately leading to abstinence. These findings underscore the critical role of aversive stimulus intensity in overcoming compulsive drug use, consistent with models emphasizing punishment thresholds (Pelloux et al., 2007). Furthermore, as seen in cocaine models, extended drug-taking experience can strengthen resistance to punishment, with some animals continuing to seek the drug despite aversive consequences,

suggesting the emergence of a more compulsive phenotype (Pelloux et al., 2007).

Notably, individual differences in shock sensitivity revealed phenotypic variability in compulsivity, with some rats achieving abstinence at lower shock intensities, while others persisted even at higher levels. This variability aligns with the concept of punishment-sensitive versus punishment-resistant phenotypes (Pelloux et al., 2007). Compared to Borges et al. (2023), who observed behavioral suppression at 0.4 mA, the delayed suppression in our study may reflect higher cumulative heroin exposure during extended intermittent access, potentially enhancing analgesia or resistance to punishment.

Finally, the robust relapse induced by acute food deprivation following punishment-imposed abstinence supports prior evidence that metabolic stress is a potent trigger for opioid seeking (Borges et al., 2023; Shalev et al., 2000). The magnitude of this relapse effect highlights the utility of the extended intermittent access and seek-take paradigm in producing relapse-prone phenotypes, closely modeling key features of human opioid use disorder.

Experiment 1b: Effects of Increased Heroin Availability on Punishment-Imposed Abstinence and Stress-Induced Relapse in Female Rats

Experiment 1b demonstrated that female rats subjected to an extended intermittent-access heroin protocol progressively escalated their heroin intake, as indicated by increased heroin infusions over time. This pattern reflects a robust escalation trajectory similar to prior reports, emphasizing that females may be particularly vulnerable to developing compulsive opioid use (Becker & Koob, 2016). Such escalation is thought to be driven by neuroadaptations in reward circuits that enhance drug reinforcement, particularly in females, who often show heightened sensitivity to opioids and faster progression to dependence.

Interestingly, although take-lever responses initially increased during early sessions, they subsequently declined and stabilized even as heroin infusions continued to rise. This suggests that over time, females became more efficient in their consummatory behavior, refining their lever-pressing patterns to maintain or increase drug intake with reduced effort. This behavioral optimization may reflect an adaptive strategy to minimize effort while maintaining high reward intake.

In contrast, the significant rise in seek-lever responding indicates a parallel increase in motivational drive to obtain heroin. This heightened seeking behavior suggests stronger incentive salience attributed to drug-associated cues, consistent with findings that females exhibit greater cue-induced motivation and are more responsive to stressors that trigger drug seeking (Becker & Koob, 2016). These effects are often modulated by ovarian hormones such as estradiol, which enhance dopaminergic signaling in mesolimbic pathways (Becker & Hu, 2008; Becker & Koob, 2016). These results highlight sex-specific neurobehavioral adaptations that facilitate opioid use escalation in females and this combination of increased motivation and refined consummatory efficiency may underlie the elevated relapse rates and heightened vulnerability observed in women with opioid use disorder.

Following the observed escalation of heroin intake and enhanced motivational drive, the relative resistance to punishment suggests that the same neuroadaptations promoting escalation may also reduce sensitivity to negative consequences, further reinforcing compulsive use patterns in females.

During punishment-imposed abstinence, female rats exhibited less pronounced suppression of heroin-seeking and taking behaviors compared to males, despite receiving identical shock intensities and probabilities (Appendix B, Figure B2). Although not statistically

significant, a visual decrease in seeking behavior was observed when shock intensity increased to 0.3 mA, suggesting initial sensitivity to the aversive consequences. However, this suppression was not sustained across subsequent sessions. This pattern aligns with evidence that female rodents often show heightened initial behavioral inhibition in response to punishment or aversive cues (Truckenbrod et al., 2023) yet resume reward-seeking over time despite negative outcomes (Becker & Koob, 2016).

These findings suggest that while females transiently suppress drug-seeking when punishment is introduced, motivational drive, potentially modulated by ovarian hormones, quickly overrides this initial sensitivity (Becker & Hu, 2008). The brief reduction in seeking on the first punishment day may therefore represent an adaptive but short-lived response to immediate aversive stimuli, highlighting a potential sex-specific vulnerability to compulsive drug use.

Biological factors may further underlie females' relative resistance to punishment. For instance, sex differences in opioid sensitivity, nociception, and punishment learning could diminish the perceived aversiveness of footshock during heroin self-administration in females (Craft, 2008). Females altered opioid analgesia and reinforcement profiles may attenuate the impact of punishment, contributing to sustained drug-seeking despite negative consequences.

Despite these sex differences in punishment-imposed abstinence, female rats showed robust relapse to heroin seeking following acute food deprivation, mirroring male patterns. This underscores metabolic stress as a potent relapse trigger across sexes and reinforces the translational relevance of this model for human opioid use disorder.

Experiment 2a: The Role of the Orbitofrontal Cortex in Stress-Induced Relapse to Heroin Seeking Following Voluntary Abstinence in Male Rats

This experiment examined whether chemogenetic inhibition of the OFC would alter heroin-seeking and taking behaviors under metabolic stress (food deprivation) following punishment-imposed abstinence.

Rats readily acquired heroin self-administration through a seek-take chain schedule, progressively increasing seeking and taking responses over time despite stable infusion levels. The seek-take chain paradigm is particularly informative as it dissociates the motivational (seeking) phase from the consummatory (taking) phase, allowing for precise assessment of drug-directed motivation versus consumption. This escalation of operant responding, even without increased heroin infusions is consistent with evidence that intermittent access promotes compulsive drug seeking (Allain et al., 2015). Such a pattern mirrors clinical observations in humans with opioid use disorder, where individuals often escalate drug-seeking efforts and show persistent pursuit of drug-related cues despite stable or diminishing drug effects (American Psychiatric Association, 2013).

Punishment-imposed abstinence was ultimately effective, as all rats met the criteria for abstinence by the end of the phase. All dependent measures, including infusions, seek responses, and take responses, were significantly reduced relative to self-administration. However, an examination of the daily run data (Appendix B, Figures B3 and B4), rats demonstrated strong resistance to behavioral suppression, persisting in heroin seeking and taking even as footshock intensities increased. Only at the highest shock levels did heroin intake significantly decrease, underscoring the compulsive nature of drug use modeled in this study. This finding aligns with reports that extended drug histories diminish sensitivity to punishment, reducing the capacity to inhibit drug seeking in response to aversive consequences (Pelloux et al., 2007). The prolonged self-administration period in our study (minimum 19 days) likely contributed to this reduced

suppression capacity.

Following punishment-imposed abstinence, acute food deprivation robustly reinstated both heroin seeking and taking behaviors. This outcome reinforces the established role of metabolic stress as a potent trigger for relapse (Shalev et al., 2000; Borges et al., 2023).

Contrary to our hypothesis, chemogenetic inhibition of the OFC did not significantly alter relapse behaviors under food-deprivation stress. This result diverges from prior studies demonstrating a role for the OFC in reinstatement of drug seeking. For example, Capriles et al. (2003) demonstrated that inactivation of the OFC using tetrodotoxin reduced reinstatement of cocaine seeking caused by footshock stress but had no effect on reinstatement induced by cocaine priming. Similarly, Reiner et al. (2020) showed that pharmacological inactivation of the OFC suppressed context-induced reinstatement of fentanyl seeking following voluntary abstinence, implicating the OFC in mediating contextual motivational triggers for opioid relapse. Fuchs et al. (2004) also reported that pharmacological inactivation of the lateral OFC significantly reduced cue-induced reinstatement of cocaine seeking after extinction, highlighting this region's role in integrating conditioned cues that drive relapse.

Several methodological and mechanistic factors may help explain these discrepancies. Our study employed punishment-imposed abstinence, which more closely models human voluntary abstinence driven by adverse consequences than extinction-based paradigms. Unlike extinction, which relies on learning that the reward is no longer available and engages flexible, outcome-based control, punishment-imposed abstinence involves the active suppression of drug-seeking behavior due to the delivery of aversive consequences, such as footshock. This approach may engage distinct neural mechanisms associated with negative reinforcement and inhibitory control (Bossert et al., 2013). The current procedure also differs from the food choice–induced

abstinence model used in Reiner et al. (2020), in which drug-taking is voluntarily suppressed in favor of an alternative, non-aversive reward (e.g., palatable food). Whereas the food choice model reflects decision-making in the context of competing rewards, punishment-based abstinence reflects suppression due to negative outcomes, making it potentially more relevant to relapse triggered by stress or adversity in humans. Moreover, our relapse trigger was metabolic stress (food deprivation), consistent with past research (Shalev et al., 2000; Borges et al., 2023), rather than contextual or discrete conditioned cues. This distinction suggests that the OFC may play a more prominent role in relapse driven by learned cues and contexts but may be less critical in stress-induced relapse following punishment-imposed abstinence.

Considerable evidence suggests that the involvement of prefrontal subregions like the OFC is highly context dependent (Bossert et al., 2013). The OFC is critical for encoding expected outcomes (Moorman et al., 2015) and updating reward value, functions that are particularly engaged during cue- or context-induced reinstatement after extinction (Lucantonio et al., 2012). In contrast, relapse following punishment-imposed abstinence may rely more on habitual, stimulus–response processes mediated by regions such as the dorsal striatum (Corbit et al., 2012), as well as the central amygdala and insular cortex (Marchant et al., 2019). Our findings suggest that, under conditions emphasizing punishment and metabolic stress, relapse is driven more by inflexible, habitual mechanisms than by flexible, OFC-dependent decision-making processes.

Finally, these findings carry important clinical implications. They suggest that in individuals characterized by high resistance to adverse consequences and strong habitual drug-seeking tendencies, interventions aimed at reducing stress reactivity or targeting habitual

behavior circuits (e.g., the dorsal striatum) may be more effective than strategies focused solely on enhancing prefrontal executive control.

Experiment 2b: The Role of the Prelimbic Cortex in Stress-Induced Relapse to Heroin Seeking Following Voluntary Abstinence in Male Rats

This experiment investigated the involvement of the prelimbic cortex (PrL) in stress-induced relapse to heroin seeking after voluntary abstinence, using a seek-take self-administration model combined with chemogenetic inhibition. Rats maintained stable heroin infusions throughout training but exhibited significant escalation in seek lever responding, consistent with prior evidence, as mentioned above.

Punishment-imposed abstinence was ultimately effective, as all rats met the criteria for abstinence by the end of the phase. All dependent measures, including infusions, seek responses, and take responses, were significantly reduced relative to self-administration. However, an examination of the daily data (Appendix B, Figure B5) reveals that some rats required a longer period of punishment to reach abstinence criteria, resulting in a prolonged abstinence period for certain subjects. Specifically, heroin infusions declined significantly as shock intensity increased, but robust reductions in seek and take lever responses were observed only after extended punishment exposure. These findings suggest that while punishment effectively reduced drug consumption, the motivation to seek heroin persisted in some animals, supporting the idea that aversive consequences can suppress intake without fully extinguishing drug-seeking behavior, a key feature of compulsive drug use. Additionally, individual differences in shock sensitivity observed here parallel prior findings demonstrating phenotypic variability in punishment sensitivity and relapse vulnerability (Vanderschuren et al., 2017).

Following abstinence, acute food deprivation robustly reinstated heroin-seeking behavior across all measures, reaffirming metabolic stress as a potent trigger for relapse (Shalev et al., 2000; Borges et al., 2023).

Although chemogenetic inhibition of the PrL using DCZ did not produce statistically significant effects, visual observations suggest trends for reduced heroin infusions and take responses in food-deprived rats receiving DCZ compared to vehicle controls. This pattern indicates a potential modulatory role of the PrL in stress-induced relapse, which may have been masked by limited statistical power due to sample size.

If these trends are confirmed, they align with prior extinction-based studies demonstrating that PrL inactivation attenuates reinstatement of drug seeking. For example, McFarland et al. (2003) showed that pharmacological inactivation of the dorsal medial prefrontal cortex, including the PrL, blocked cocaine-induced reinstatement by inhibiting prefrontal glutamate release into the nucleus accumbens core. Similarly, Capriles et al. (2003) found that TTX inactivation of the prelimbic/anterior cingulate cortex blocked reinstatement of cocaine seeking induced by both foot-shock stress and cocaine priming. Additionally, McLaughlin and See (2003) reported that reversible pharmacological inactivation of the dorsomedial prefrontal cortex (encompassing the prelimbic and anterior cingulate cortices) attenuated responding to a cocaine-paired stimulus in an extinction/reinstatement model. Consistent with these results, Stefanik et al. (2013) demonstrated that optogenetic inhibition of PrL neurons suppressed context-induced reinstatement of cocaine seeking. Similarly, the ventral prelimbic cortex has been implicated in context-induced reinstatement, as reversible inactivation of this area reduces drug-seeking during relapse tests (Bossert et al., 2011).

Notably, no differences in locomotor activity between DCZ- and vehicle-treated rats indicate that behavioral changes were unlikely due to nonspecific motor impairments but rather reflect targeted modulation of relapse circuits.

Limitations

This study has several limitations. The relatively small sample size may have reduced statistical power to detect subtle effects. Additionally, as with all studies using viral approaches, variability in viral expression and potential off-target effects may have limited the extent of regional silencing, potentially confounding interpretation. Moreover, only male rats were used in Experiment 2, limiting the generalizability of the findings to females. Given known sex differences in stress responsivity and relapse vulnerability, future studies should include both sexes to assess potential sex-specific effects.

Methodological Considerations

Several methodological factors should be carefully considered when interpreting the findings of these experiments. First, while DCZ maintains sufficient brain concentrations for at least 2 hours in non-human primates, its pharmacokinetic profile in rodents is markedly different (Nagai et al., 2020). In mice, DCZ concentrations in brain tissue and cerebrospinal fluid decline rapidly and become undetectable by 2 hours post-injection, suggesting a shorter duration of action. Given that our relapse test in rats lasted 3 hours, it is possible that the effects of DCZ diminished before the end of the session, potentially reducing the efficacy of PrL and OFC inhibition. Future studies may benefit from using a shorter relapse test session (e.g., 1–2 hours) to better match the temporal window of DCZ activity in rodents and to ensure more consistent chemogenetic modulation. Additionally, an analysis focusing on just the first 1–2 hours of the test could provide more precise insights into the immediate effects of chemogenetic manipulation

during this critical period.

Additionally, the choice of a punishment-imposed abstinence model, while clinically relevant, fundamentally differs from extinction- or forced abstinence-based models commonly used in relapse research. This approach likely engages distinct neural circuits and cognitive processes, emphasizing habitual control and punishment resistance.

Lastly, the current study primarily relied on measures of lever presses and "infusion" counts. Including additional behavioral metrics, such as response latency, progressive ratio breakpoints, or real-time neural activity recordings (e.g., fiber photometry or in vivo electrophysiology), could enrich future investigations and provide a more comprehensive understanding of relapse mechanisms.

Future Directions

Future studies should incorporate larger cohorts to increase statistical power and enable detection of subtle or context-dependent effects of orbitofrontal and prelimbic cortex inhibition on relapse behavior. Systematically quantifying cumulative drug exposure and comparing individual shock thresholds will help clarify differences in punishment sensitivity and compulsivity across subjects.

Complementary techniques, such as in vivo electrophysiology or calcium imaging, could provide direct insight into neural activity underlying stress-induced relapse and help define the conditions under which prelimbic cortex inhibition modulates behavior. Expanding circuit-level investigations to additional brain regions implicated in stress- and reward-related relapse, such as the insula, basolateral amygdala, and infralimbic cortex, using chemogenetic or optogenetic approaches, will further refine our understanding of the network interactions that govern relapse vulnerability.

Finally, examining hormonal fluctuations and sex-specific differences in heroin seeking, punishment sensitivity, and relapse propensity is critical for developing more personalized and effective treatment strategies, especially in female subjects.

Conclusion

In summary, these studies demonstrate that extended intermittent access to heroin, combined with a seek-take chain and punishment-imposed abstinence, effectively models key features of opioid addiction, including escalation of drug seeking, punishment resistance, and robust relapse triggered by metabolic stress. Both male and female rats exhibited strong relapse responses under food deprivation, underscoring the translational relevance of this model for capturing clinically significant aspects of opioid use disorder.

Contrary to our initial hypotheses, chemogenetic inhibition of the OFC and PrL did not significantly attenuate stress-induced relapse following punishment-imposed abstinence. These findings suggest that the neural substrates of relapse are highly context dependent, with OFC and PrL contributions potentially more critical in cue- or context-driven models than in those emphasizing punishment or physiological stress. This highlights the importance of considering both the nature of abstinence and the relapse trigger when evaluating prefrontal cortical involvement, as stress-based or habitual relapse may rely less on flexible, outcome-guided decision-making mechanisms typically mediated by prefrontal circuits.

Overall, the seek-take punishment model offers a powerful platform for dissecting the complex behavioral and neurobiological mechanisms underlying opioid relapse. By integrating advanced circuit-specific manipulations and directly comparing different relapse triggers across abstinence models, future research can delineate when and how prefrontal involvement shifts and

develop more targeted, personalized interventions to improve treatment outcomes for opioid use disorder.

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Appendix A

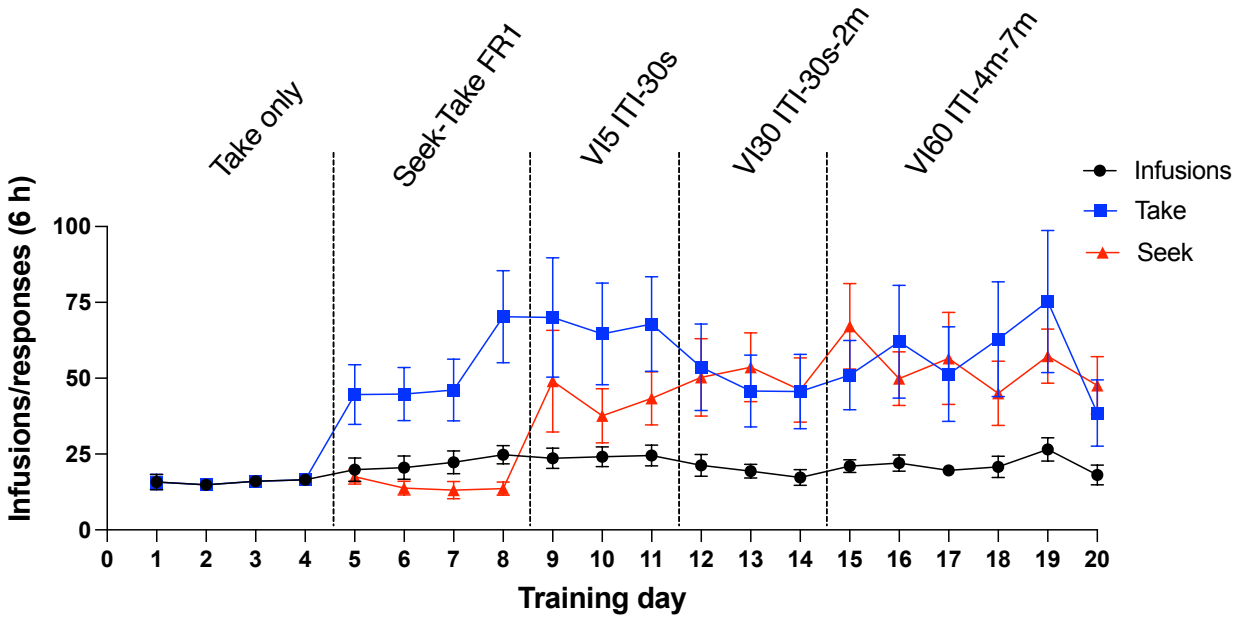
Self-Administration Data: Experiment 2a

Experiment 2a: The Role of the Orbitofrontal Cortex in Stress-Induced Relapse to Heroin Seeking Following Voluntary Abstinence in Male Rats (RUN 1)

During training, heroin infusions remained relatively stable, while both take and seek lever presses increased across days (Figure A1). A repeated-measures one-way ANOVA revealed a non-significant increase in heroin infusions, $F(3.65, 25.55) = 1.96$, $p = .136$, $\eta^2_p = .22$. In contrast, take lever presses, $F(3.00, 21.01) = 3.10$, $p = .049$, $\eta^2_p = .31$, and seek lever presses, $F(2.68, 18.73) = 4.67$, $p = .016$, $\eta^2_p = .40$, increased significantly across sessions. These findings indicate that although heroin intake remained stable, motivation to seek and take the drug increased progressively during training.

Figure A1

Changes in Heroin Self-Administration: Infusions, Seek Lever Presses, and Take Lever Presses Across Reinforcement Schedules in Male Rats (Run 1)



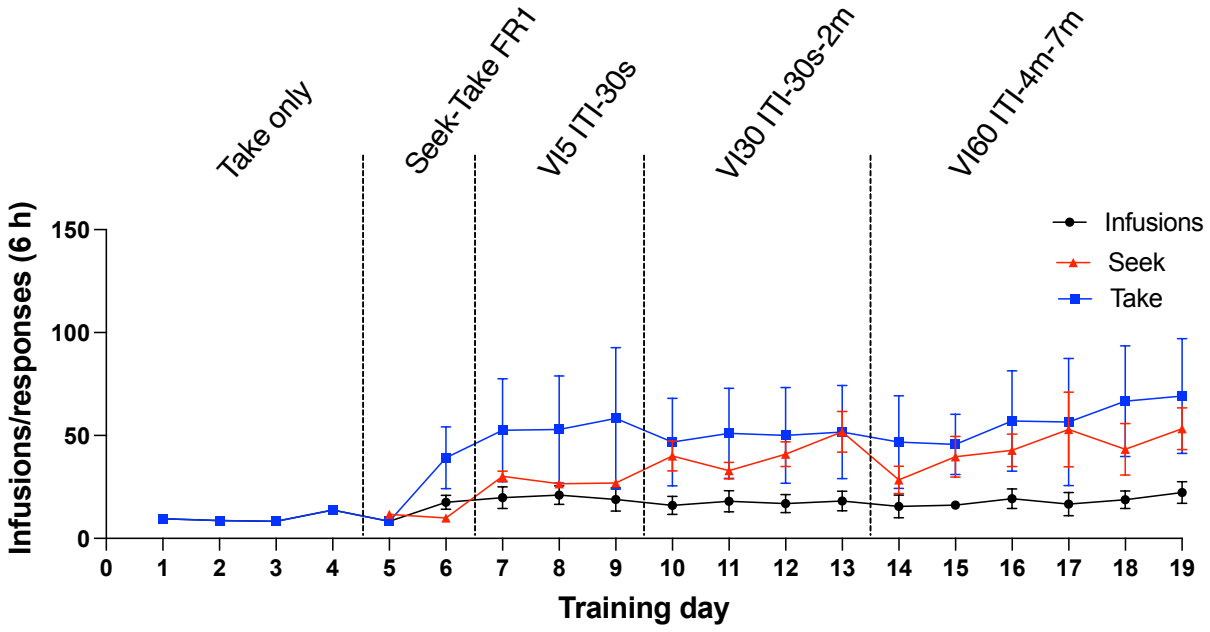
Note. Mean (± SEM) number of heroin infusions (black), seek lever presses (red), and take lever presses (blue) across self-administration training in male rats (n = 8), over the changing reinforcement schedules (FR1, VI5, VI30, VI60). "Take only" indicates initial access to a single lever delivering 0.1 mg/kg heroin. In "Seek-Take FR1," rats pressed a seek lever to access a take lever for 5 minutes, with the dose reduced to 0.05 mg/kg. "VI5 ITI-30s" introduced a 5-second variable interval (VI) and a 30-second intertrial interval (ITI). The VI and ITI were further increased during subsequent phases: "VI30 ITI-30s–3 min" (VI = 30 s, ITI = 30 s–3 min) and "VI60 ITI-4–7 min" (VI = 60 s, ITI = 4–7 min). Doses are expressed in mg/kg/infusion.

Experiment 2a: The Role of the Orbitofrontal Cortex in Stress-Induced Relapse to Heroin Seeking Following Voluntary Abstinence in Male Rats (RUN 2)

Throughout training, heroin infusions remained relatively stable, while both take and seek lever presses showed overall increases across days (Figure A2). A repeated-measures one-way ANOVA revealed no significant effect of training day on heroin infusions, $F(1.44, 10.09) = 3.43$, $p = .084$, $\eta^2_p = .33$. Similarly, take lever responding did not change significantly across days, $F(1.16, 8.09) = 3.00$, $p = .119$, $\eta^2_p = .30$. In contrast, seek lever presses increased significantly over time, $F(2.54, 17.81) = 3.63$, $p = .039$, $\eta^2_p = .34$, indicating progressive escalation in drug-seeking behavior during training.

Figure A2

Changes in Heroin Self-Administration: Infusions, Seek Lever Presses, and Take Lever Presses Across Reinforcement Schedules in Male Rats (Run 2)



Note. Mean (± SEM) number of heroin infusions (black), seek lever presses (red), and take lever presses (blue) across self-administration training in male rats (n = 8), Over the changing reinforcement schedules (FR1, VI5, VI30, VI60). "Take only" indicates initial access to a single lever delivering 0.1 mg/kg heroin. In "Seek-Take FR1," rats pressed a seek lever to access a take lever for 5 minutes, with the dose reduced to 0.05 mg/kg. "VI5 ITI-30s" introduced a 5-second variable interval (VI) and a 30-second intertrial interval (ITI). The VI and ITI were further increased during subsequent phases: "VI30 ITI-30s–3 min" (VI = 30 s, ITI = 30 s–3 min) and "VI60 ITI-4–7 min" (VI = 60 s, ITI = 4–7 min). Doses are expressed in mg/kg/infusion.

Appendix B
Punishment-Imposed Abstinence Data

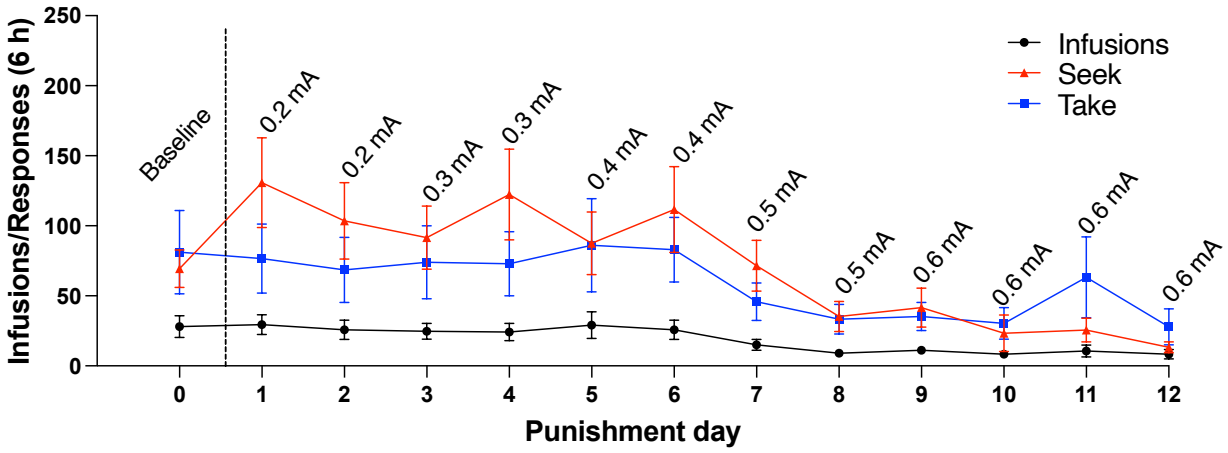
Experiment 1a. The Effects of Increased Heroin Availability on Punishment-Imposed Abstinence and Stress-Induced Relapse in Male Rats

During the punishment phase, the number of infusions, take lever responses, and seek lever responses decreased; however, not all measures reached statistical significance (Figure B1). Responses during punishment training were compared to baseline levels (calculated as the mean of the final three days of self-administration) using repeated-measures one-way ANOVAs with Dunnett's multiple comparisons. Only punishment sessions with shock intensities between 0.2 and 0.6 mA were included in the analysis, as these were common to all subjects. Sessions at higher intensities (up to 1.0 mA), which were required by some rats to reach the abstinence criterion (≤ 2 infusions on two consecutive days), were excluded.

A significant decrease in heroin infusions was observed across punishment days, $F(2.59, 23.32) = 6.22$, $p = .004$, $\eta^2_p = .41$. Dunnett's tests revealed that the final two days at 0.6 mA were significantly lower than baseline ($p = .025$ and .016). While take lever responding remained relatively stable until 0.5 mA, it then decreased sharply; however, this change did not reach statistical significance, $F(2.75, 24.78) = 2.53$, $p = .084$, $\eta^2_p = .22$. In contrast, seek lever responding initially increased with the introduction of punishment and remained elevated through 0.4 mA, before declining rapidly at higher shock intensities. This pattern was supported by a significant main effect of punishment day, $F(2.54, 22.83) = 7.03$, $p = .002$, $\eta^2_p = .44$. Dunnett's tests indicated significant reductions in seek lever responding on the final three days at 0.6 mA ($p = .049$, .011, and .018).

Figure B1

Punishment-Imposed Abstinence: Infusions, Seek Lever Presses, and Take Lever Presses Across Increasing Footshock Intensities in Male Rats



Note. Mean (±SEM) number of heroin infusions (in black), seek lever presses (in red) and take lever presses (in blue) on the last three days on VI60 schedule (Baseline) followed by punishment-imposed abstinence (Footshock). The footshock intensity increased by 0.1 mA per two training days until 0.6 mA (n = 10).

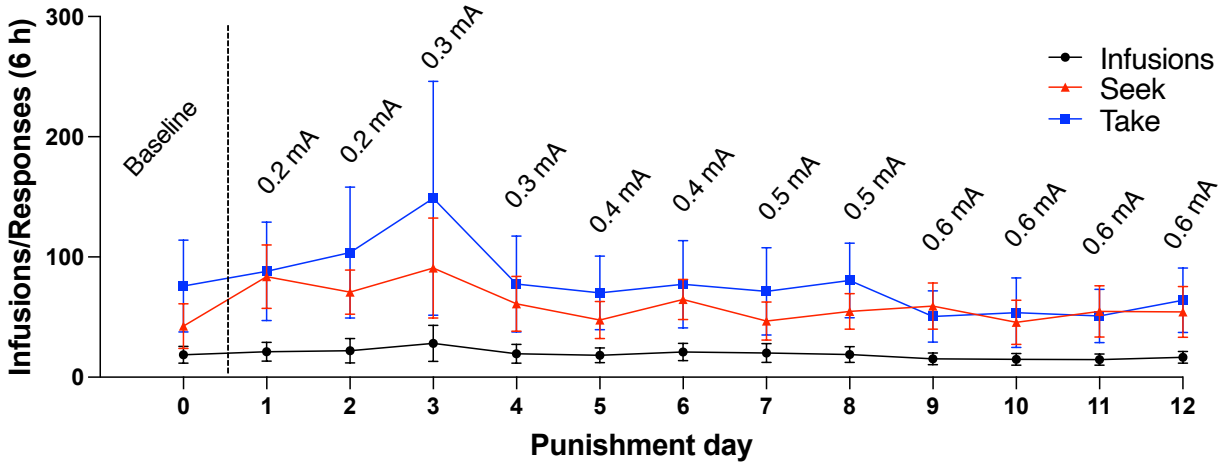
Experiment 1b. The Effects of Increased Heroin Availability on Punishment-Imposed Abstinence and Stress-Induced Relapse in Female Rats

During the punishment phase, reductions in heroin infusions, take lever responses, and seek lever responses did not reach statistical significance (Figure B2). Responses during punishment were compared to baseline levels (calculated as the mean of the final three days of self-administration) using repeated-measures one-way ANOVAs with Dunnett's multiple comparisons. Only sessions with shock intensities between 0.2 and 0.6 mA were included in the analysis, as these were common across all subjects; sessions at higher intensities (up to 1.0 mA), used for some rats to reach the abstinence criterion (≤2 infusions on two consecutive days), were excluded. As a result, not all rats had reached abstinence within the analyzed range, which contributed to the lack of statistically significant effects.

No significant decrease in heroin infusions was observed across punishment days, $F(1.44, 10.09) = 0.52$, $p = .553$, $\eta^2_p = .07$. Similarly, take lever responding did not change, $F(1.27, 8.88) = 0.74$, $p = .443$, $\eta^2_p = .10$. Seek lever responding also showed no significant change, $F(1.72, 12.02) = 1.09$, $p = .357$, $\eta^2_p = .13$. Moreover, Dunnett's multiple comparisons revealed no individual session that significantly differed from baseline for any of the three behavioral measures.

Figure B2

Punishment-Imposed Abstinence: Infusions, Seek Lever Presses, and Take Lever Presses Across Increasing Footshock Intensities in Female Rats



Note. Mean (±SEM) number of infusions (in black), seek lever presses (in red) and take lever presses (in blue) on the last three days on VI60 schedule (Baseline) followed by punishment-imposed abstinence (Footshock). The footshock intensity increased by 0.1 mA per two training days until 0.6mA (n = 8).

Experiment 2a: The Role of the Orbitofrontal Cortex in Stress-Induced Relapse to Heroin Seeking Following Voluntary Abstinence in Male Rats (RUN 1)

During the punishment phase, reductions in the number of infusions, take lever responses, and seek lever responses did not reach statistical significance (Figure B3). Responses during punishment training were compared to baseline (mean of the last three training days) using one-way repeated measures ANOVAs with Dunnett's multiple comparisons.

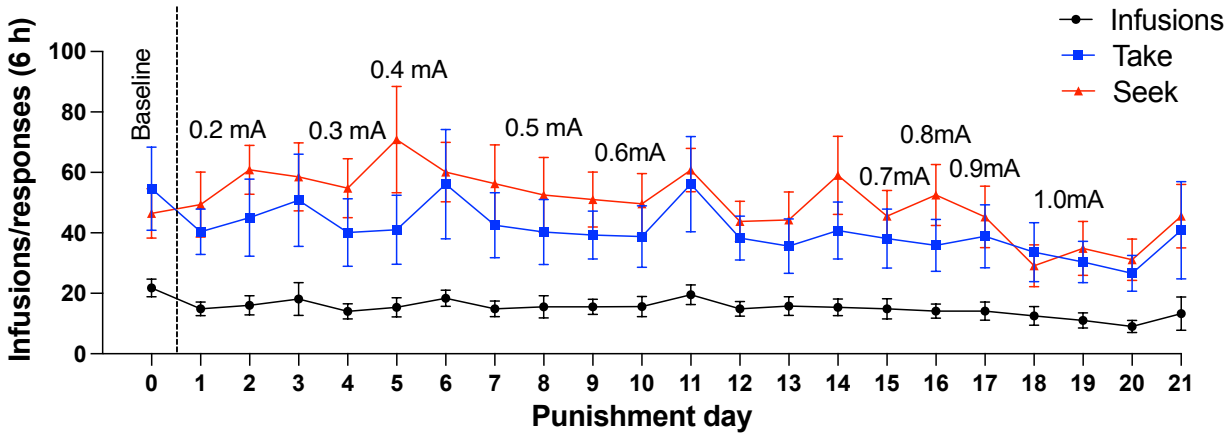
Only punishment days with shock intensities from 0.2 mA up to four days at 1.0 mA were included, as these were common across all subjects. Some rats required an increased shock probability (50%) to meet the abstinence criterion (≤ 2 infusions on two consecutive days); these additional days were excluded from the analysis.

There was no significant overall decrease in heroin infusions across punishment days, $F(3.85, 26.93) = 2.67$, $p = .056$, $\eta^2_p = .28$. However, Dunnett's multiple comparisons revealed significant reductions in infusions relative to baseline at several shock intensities, including 0.2 mA ($p = .029$), 0.3 mA ($p = .021$), multiple comparisons at 0.6 mA ($ps < .044$), as well as stronger reductions at higher intensities: 0.8 mA ($p < .001$), 0.9 mA ($p = .026$), and 1.0 mA ($ps \leq .0067$). Take lever presses, $F(2.89, 20.21) = 1.47$, $p = .253$, $\eta^2_p = .17$, and seek lever presses, $F(3.51, 24.59) = 1.90$, $p = .149$, $\eta^2_p = .21$, did not show a significant overall change across punishment days. However, Dunnett's tests revealed that 1.0 mA produced significant reductions in seek lever presses compared to baseline ($p = .0048$ and $p = .036$).

This lack of consistent overall response suppression was expected, as only one rat reached the abstinence criterion (≤2 infusions on two consecutive days) during the 21-day punishment phase; however, by the end of the punishment period, all rats ultimately achieved abstinence.

Figure B3

Punishment-Imposed Abstinence: Infusions, Seek Lever Presses, and Take Lever Presses Across Increasing Footshock Intensities in Male Rats (Run 1)



Note. Mean (±SEM) number of infusions (in black), seek lever presses (in red) and take lever presses (in blue) on the last three days on VI60 schedule (Baseline) followed by punishment-imposed abstinence (Footshock). The footshock intensity increased by 0.1mA per two training days until 0.6mA, in which it stayed at 0.6mA for 6 days and then increased by 0.1mA per training day until 1.0mA, which was the highest (n = 8).

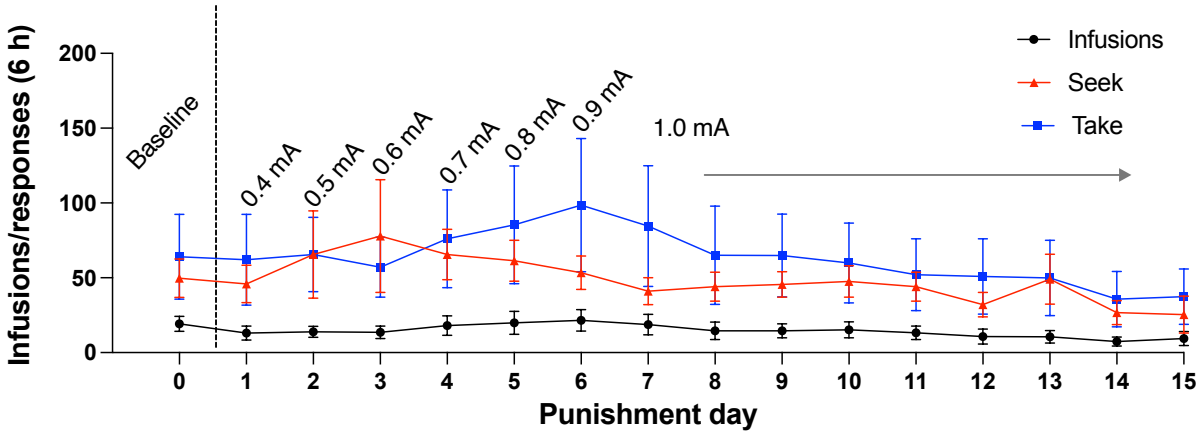
Experiment 2a: The Role of the Orbitofrontal Cortex in Stress-Induced Relapse to Heroin Seeking Following Voluntary Abstinence in Male Rats (RUN 2)

During the punishment phase, heroin infusions decreased significantly, whereas take and seek lever presses did not show significant reductions (Figure B4). Responses during punishment were compared to baseline (mean of the last three self-administration days) using one-way repeated-measures ANOVAs with Dunnett's multiple comparisons. The analysis included punishment days with shock intensities from 0.2 mA up to nine days at 1.0 mA, including the 50% shock probability phase, which were consistent across all subjects. Additional punishment days required for some rats to meet the abstinence criterion (≤ 2 infusions on two consecutive days) were excluded.

The ANOVA revealed a significant overall decrease in infusions across punishment days, $F(2.65, 18.54) = 3.37$, $p = .045$, $\eta^2_p = .33$. However, take lever presses, $F(2.18, 15.26) = 2.21$, $p = .141$, $\eta^2_p = .24$, and seek lever presses, $F(1.77, 12.38) = 1.47$, $p = .265$, $\eta^2_p = .17$, did not significantly change across punishment days.

Figure B4

Punishment-Imposed Abstinence: Infusions, Seek Lever Presses, and Take Lever Presses Across Increasing Footshock Intensities in Male Rats (Run 2)



Note. Mean (+SEM) number of infusions (black), seek lever presses (red), and take lever presses (blue) across the last three days of VI60 training (Baseline), followed by punishment-imposed abstinence (Footshock). Footshock intensity increased by 0.1 mA per training day until reaching 1.0 mA, where it remained constant (n = 8).

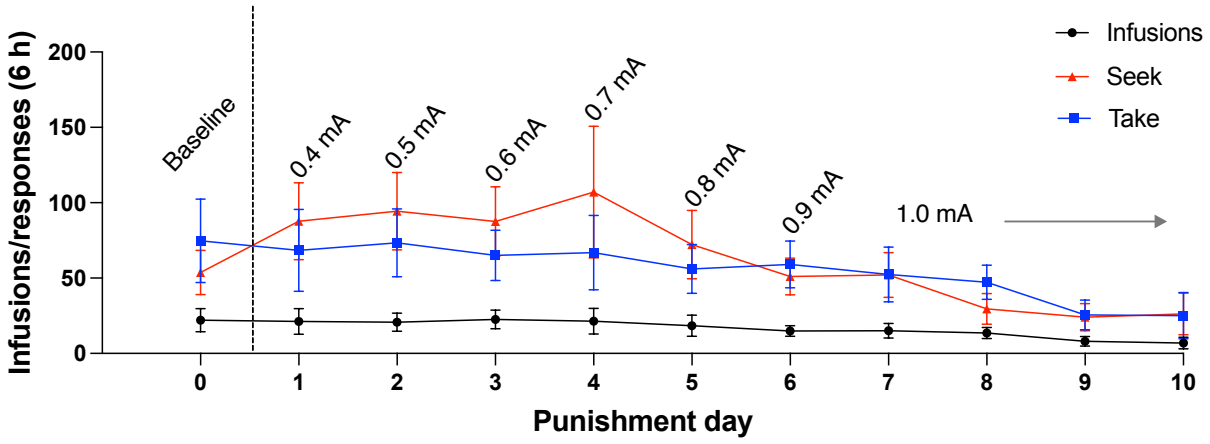
Experiment 2b: The Role of the Prelimbic Cortex in Stress-Induced Relapse to Heroin Seeking Following Voluntary Abstinence in Male Rats

During the punishment phase, heroin infusions decreased significantly, whereas take and seek lever presses did not show significant reductions (Figure B5). Responses during punishment were compared to baseline (the mean of the last three self-administration days) using one-way repeated-measures ANOVAs with Dunnett's multiple comparisons. The analysis included punishment days with shock intensities ranging from 0.2 mA up to nine days at 1.0 mA, including the 50% shock probability phase, which were consistent across all subjects. Additional punishment days required by some rats to meet the abstinence criterion (≤ 2 infusions on two consecutive days) were excluded.

A repeated-measures one-way ANOVA revealed a significant overall decrease in heroin infusions across punishment days, $F(1.56, 9.36) = 5.55$, $p = .031$, $\eta^2_p = .48$, indicating that heroin intake declined significantly during punishment. Dunnett's post hoc test identified a significant reduction in infusions on the day when shock intensity increased to 0.8 mA ($p = .036$, 95% CI [0.26, 6.98]), showing lower heroin intake at this higher shock level compared to baseline. In contrast, take lever presses, $F(2.23, 13.38) = 3.09$, $p = .075$, $\eta^2_p = .34$, and seek lever presses, $F(1.82, 10.94) = 3.17$, $p = .085$, $\eta^2_p = .35$, did not significantly change throughout the punishment phase.

Figure B5

Punishment-Imposed Abstinence: Infusions, Seek Lever Presses, and Take Lever Presses Across Increasing Footshock Intensities in Male Rats



Note. Mean (+SEM) number of infusions (black), seek lever presses (red), and take lever presses (blue) across the last three days of VI60 training (Baseline), followed by punishment-imposed abstinence (Footshock). Footshock intensity increased by 0.1 mA per training day until reaching 1.0 mA, where it remained constant (n = 7).

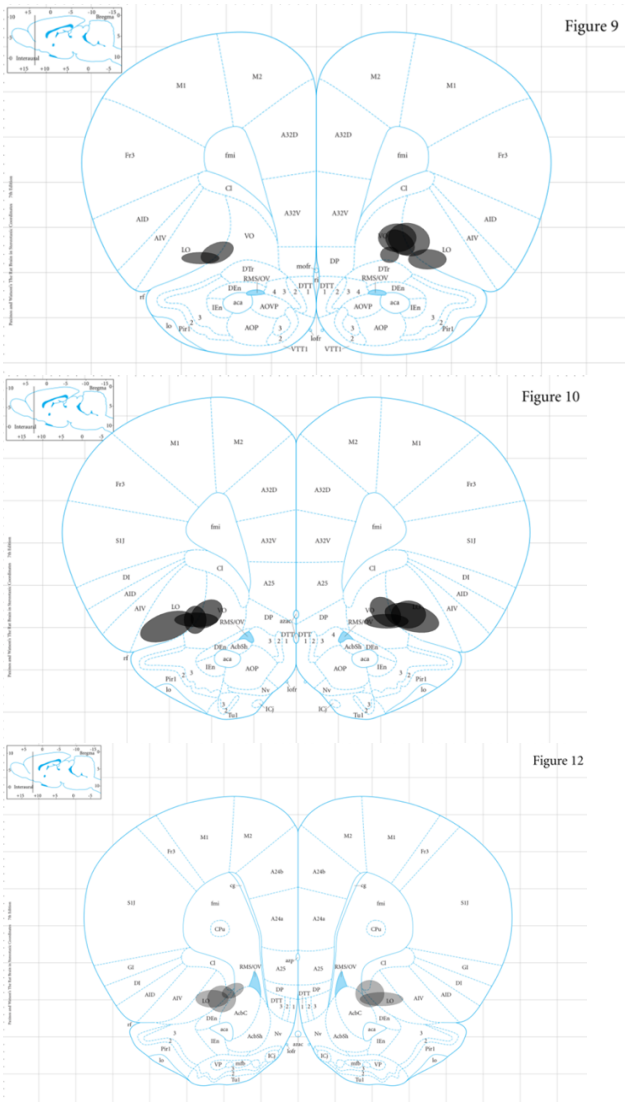
Appendix C

mCherry-Labeled DREADD Expression

Experiment 2a: The Role of the Orbitofrontal Cortex in Stress-Induced Relapse to Heroin Seeking Following Voluntary Abstinence in Male Rats (RUN 1)

Figure C1

mCherry-Labeled DREADD Expression in the Orbitofrontal Cortex (Run 1)



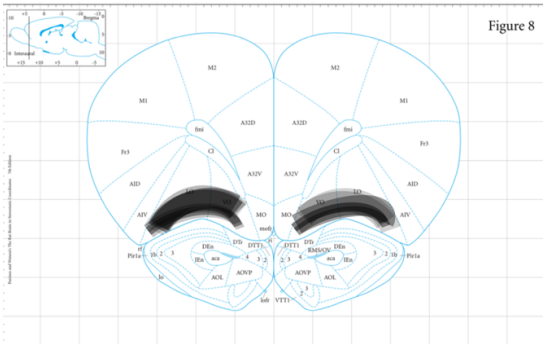
Note. Representative coronal sections showing mCherry fluorescence indicating DREADDs expression in the orbitofrontal cortex (OFC). Images correspond to anterior-posterior coordinates +3.72, +3.24, and +2.76 mm relative to bregma, based on the Paxinos and Watson rat brain atlas.

Black shaded areas indicate viral expression overlays, confirming accurate targeting of the OFC region.

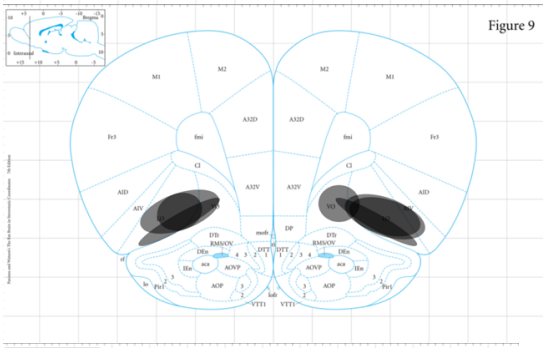
Experiment 2a: The Role of the Orbitofrontal Cortex in Stress-Induced Relapse to Heroin Seeking Following Voluntary Abstinence in Male Rats (RUN 1)

Figure C2

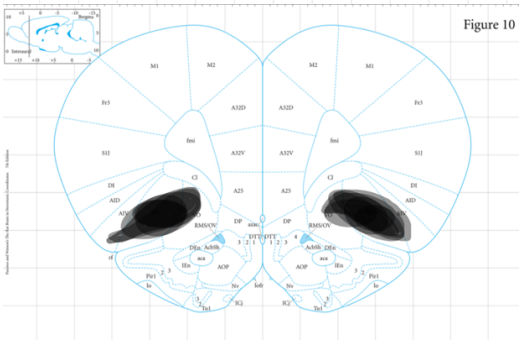
mCherry-Labeled DREADD Expression in the Orbitofrontal Cortex (Run 2)



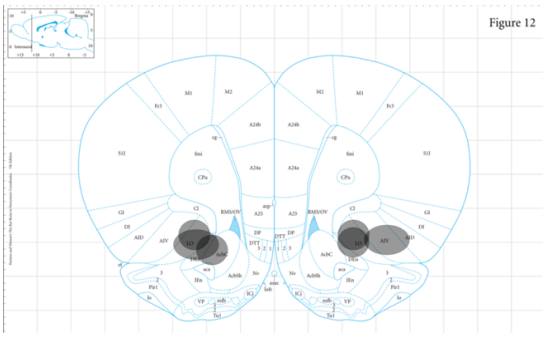
4.20



3.72



3.24



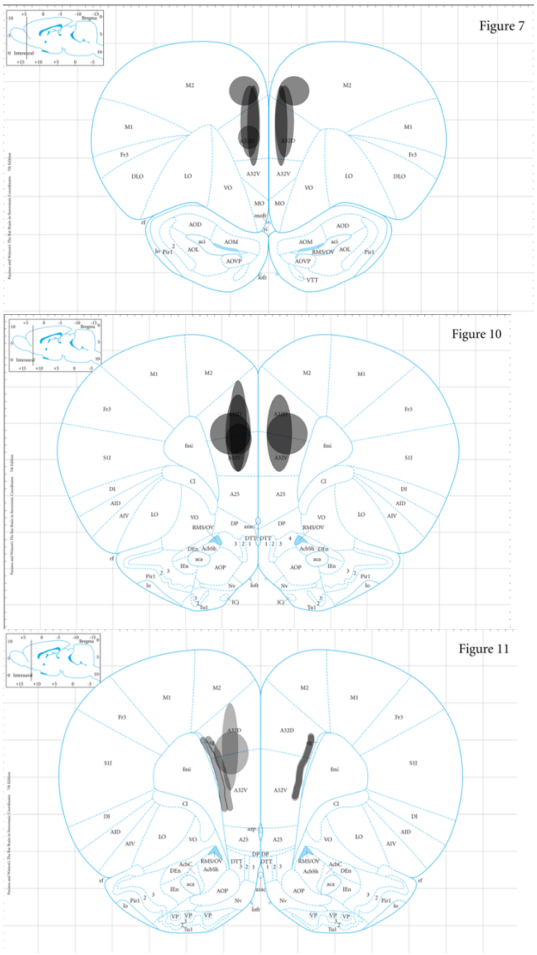
2.76

Note. Representative coronal sections showing mCherry fluorescence indicating DREADDs expression in the orbitofrontal cortex (OFC). Images correspond to anterior-posterior coordinates AP +4.20mm, +3.72 mm, +3.24 mm, and +2.76 mm relative to bregma, based on the Paxinos and Watson rat brain atlas. Black shaded areas indicate viral expression overlays, confirming accurate targeting of the OFC region.

Experiment 2b: The Role of the Prelimbic Cortex in Stress-Induced Relapse to Heroin Seeking Following Voluntary Abstinence in Male Rats

Figure C3

mCherry-Labeled DREADD Expression in the Prelimbic Cortex



Note. Representative coronal sections showing mCherry fluorescence indicating DREADDs expression in the prelimbic cortex (PrL). Images correspond to anterior-posterior coordinates +4.68, +3.24, and +3.00 mm relative to bregma, based on the Paxinos and Watson rat brain atlas. Black shaded areas indicate viral expression overlays, confirming accurate targeting of the PrL region.