Title

Probing Mechanisms Driving Opioid Use Disorder Comorbidity with Post-Traumatic Stress Disorder and Chronic Pain

Author

Mondello, Jamie Elizabeth

Publication Date

2024

Peer reviewed|Thesis/dissertation

UNIVERSITY OF CALIFORNIA

Los Angeles

Probing Mechanisms Driving Opioid Use Disorder Comorbidity with Post-Traumatic Stress Disorder and Chronic Pain

A dissertation submitted in partial satisfaction of the

requirements for the degree Doctor of Philosophy

in Psychology

by

Jamie Elizabeth Mondello

2024

ABSTRACT OF THE DISSERTATION

Probing Mechanisms Driving Opioid Use Disorder Comorbidity

with Post-Traumatic Stress Disorder and Chronic Pain

By

Jamie Elizabeth Mondello

Doctor of Philosophy in Psychology

University of California, Los Angeles, 2024

Professor Catherine Marie Cahill, Co-Chair

Professor Christopher J. Evans, Co-Chair

Opioid Use Disorder (OUD) is highly comorbid with post-traumatic stress disorder (PTSD) and chronic pain, resulting in exacerbated symptoms and poorer treatment outcomes. Studying each of these disorders in isolation fails to capture the complexity of the interrelationships between OUD and PTSD/chronic pain. OUD, PTSD and chronic pain share common symptomology, risk factors, and impacted neurocircuitry, suggesting that these disorders share common mechanistic pathways that cross-sensitize and influence each other. For instance, OUD, PTSD and chronic pain are all marked by altered learning mechanisms that impact the pathophysiology of these disorders. Additionally, alterations in the dynorphin/kappa opioid receptor (KOR) system caused by stress and chronic pain are an emerging target for OUD comorbidities, due to involvement of this system in negative affect and stress-induced relapse of drug seeking. The goal of this presented dissertation

is to shed light on the stress- and pain-induced neuroadaptations that may promote vulnerability for enhanced opioid learning and reward. In Chapter 2, a rodent model of PTSD was utilized to test the impact of unpredictable stress on opioid-induced locomotion and opioid-context learning. We found that while unpredictable stress had no impact on subsequent morphine reward learning, it sensitized the locomotor response to low dose morphine. Interestingly, unpredictable stress also induced preference to contexts previously paired with low dose naltrexone, an opioid receptor antagonist that is typically considered aversive. In Chapter 3, a chronic neuropathic pain model was utilized to test the impact of neuropathic pain on KOR agonist-induced reinstatement of oxycodone place preference. Here, we found that KOR agonism-induced reinstatement in neuropathic pain females, but not neuropathic pain males, supporting previous findings that chronic pain-induced changes in the dynorphin/KOR system are sexually dimorphic. We additionally found a relationship between the magnitude of the reinstatement and mechanical withdrawal thresholds in neuropathic pain females. Specifically, females with greater mechanical allodynia had greater subsequent reinstatement of oxycodone place preference. Overall, this work underscores the need for integrated approaches to address the intricate interplay between OUD, PTSD, and chronic pain comorbidities.

The dissertation of Jamie Elizabeth Mondello is approved.

Laura Anne Wilke

Kate Wassum

Christopher J. Evans, Committee Co-Chair

Catherine Marie Cahill, Committee Co-Chair

University of California, Los Angeles

2024

Dedication

To my Grandma Mary - my guiding light during this journey.

Table of Contents

List of Figures

Chapter 1

Chapter 2

Chapter 3

List of Tables

Chapter 2

Chapter 3

List of Abbreviations

aCSF – Artificial cerebral spinal fluid

ACTH – Adrenocorticotropic hormone

ANOVA – Analysis of variance

BLA – Basolateral amygdala

CCI – Chronic constriction injury

CNO – Clozapine-N-oxide

CPA – Conditioned place aversion

CPP – Conditioned place preference

CRH – Corticotrophin-release hormone

CS – Conditioned stimulus

Dox – Doxycycline

GPCR – G protein coupled receptor

HPA – Hypothalamic-pituitary-adrenal (axis)

i.p. – Intraperitoneal

IL – Interleukin

KOR – Kappa opioid receptors

LDN – Low dose naltrexone

LTP – Long-term potentiation

mEPSC – miniature excitatory postsynaptic current

NAc – Nucleus accumbens

OUD – Opioid use disorder

PTSD – Post-traumatic stress disorder

PVN – paraventricular nucleus

s.c. – Subcutaneous

SEFL – Stress enhanced fear learning

SEM – Standard error of the mean

SUD – Substance use disorder

TLR4 – Toll-like receptor 4

tTA – Tetracycline transactivator

US – Unconditional stimulus

VTA – Ventral tegmental Area

WPRE – Woodchuck hepatitis virus post-transcriptional regulatory element

Acknowledgements

I must express my gratitude for all the people who helped me get to this point, as it truly takes a village. My thanks go to Dr. Cathy Cahill, who welcomed me into her lab with open arms and gave me the unwavering support to cross the finish line. To the rest of my committee, Drs. Chris Evans, Kate Wassum and Laura DeNardo who offered their expertise and guidance – it has been a privilege to learn from you. I want to thank the members of the Cahill lab who helped not just in the lab but also with their friendship, as well as Drs. Jeremy Trott and Chia-Wei Chang for their collaboration, counsel and companionship. I give thanks to all my undergraduate research assistants who aided in the execution of the experiments. I derived a lot of joy from the mentoring relationships, and it was a pleasure to learn from you all. I express my gratitude to the rats and mice, without whom this research would not exist. I also thank the DLAM and veterinary staff, whose tireless efforts are essential to making rodent research possible at UCLA.

To all the friends I have made while in Los Angeles, at UCLA and beyond, that got me through the ups and downs – thank you for your friendship and support. To the friends back home that made sure to stay in touch, no matter how far apart we were. To my parents, who always encouraged creativity, deep thinking and being well informed. To the thoughtful and caring women I am honored to call my sisters. To my goofy and loud extended family – our family zooms during lockdown meant so much to me during that isolating time.

To my partner, Peter– you were my rock during this journey. Thank you for your patience, love and sacrifice. Thank you for never making me apologize for giving this PhD journey my all. To my aunt Susie, Suz, SusieQ – I miss waking up to our yoga/qigong every morning. I wish you could've seen this moment and hope you are proud of what I accomplished. And finally, to my

Grandma Mary, Dr. Mary Mondello – the reason I first considered pursing a PhD in the first place. Everyday I strive to be as wise and kind as you are. It is an honor to walk in your footsteps.

This work was financially support by the following grants: NIDA T32 DA024635 (Jamie E. Mondello), NIDA F31 DA054792 (Jamie E Mondello), NIDA grant P50 DA005010 (Michael S. Fanselow) and NIDA R01-DA053752 (Catherine M. Cahill).

Curriculum Vitae

Education

2018-Present	**University of California, Los Angeles, California** Ph.D. program in Psychology Major area of study: Behavioral Neuroscience
2019	**University of California, Los Angeles, California** M.A. in Psychology
2017	**Binghamton University-State University of New York (SUNY), Harpur College of Arts and Sciences, Vestal, New York** B.S. with honors in Integrative Neuroscience, *summa cum laude*

Awards and Honors

2022	Society for Neuroscience Trainee Professional Development Award (TPDA)
2021-2024	Ruth L. Kirschstein National Research Service Award (NRSA) F31 NIH Predoctoral Fellowship (NIDA)
2021	Graduate Summer Research Mentorship Program, UCLA
2020-2021	T32 Training Program in the Translational Neuroscience of Drug Abuse: Pre-doctoral Traineeship (*competitive renewal)*
2020-2021	Graduate Research Mentorship Program, UCLA
2020	NSF Graduate Research Fellowship – Honorable Mention
2019-2020	T32 Training Program in the Translational Neuroscience of Drug Abuse: Pre-doctoral Traineeship (NIDA)
2019	Graduate Summer Research Mentorship Program, UCLA (*declined*)
2017	Undergraduate Award to Support Research and Creative Work, Binghamton University
2016	Undergraduate Conference Travel Fund, Binghamton University
2016	Annette Van Howe Award for Highest Academic Achievement by a Junior Woman, Binghamton University

Publications

Gano, A., Barney, T. M., Vore, A. S., **Mondello, J. E.**, Varlinskaya, E. I., Pautassi, R. M., & Deak, T. (2024). Cues associated with a single ethanol exposure elicit conditioned corticosterone responses in adolescent male but not female Sprague–Dawley rats. *Developmental Psychobiology*, 66(1), e22442.

Mondello, J.E., Gano, A., Vore, A.S., Deak, T. (2023). Cues associated with repeated ethanol exposure facilitate the corticosterone response to ethanol and immunological challenges in adult male Sprague Dawley rats: implications for neuroimmune regulation. *Am J Drug Alcohol Abuse.*

Mondello, J.E., Pak, J.E., Lovelock, D., Deak, T. (2019). Novel pharmacotherapeutics for stress-related disorders. In K. L. Harkness & E. P. Hayden (Eds.), *The Oxford Handbook of Stress and Mental Health*.

Gano, A., **Mondello, J.E.,** Doremus-Fitzwater, T.L., Deak, T. (2019). Rapid alterations in neuroimmune gene expression after acute ethanol: timecourse, sex differences and sensitivity to surgical manipulation. *Journal of Neuroimmunology***.**

Hennessy, M. B., Deak, T., Sensenbaugh, J. D., Gallimore, D. M., Garybush, A. M., **Mondello, J. E**., & Schiml, P. A. (2019). Central neuroimmune activity and depressive-like behavior in response to repeated maternal separation and injection of LPS. *Physiology & behavior*, *199*, 366-374.

Barney, T. M., Vore, A. S., Gano, A., **Mondello, J. E.,** & Deak, T. (2018). Assessment of Interleukin-6 Signaling Effects on Behavioral Changes Associated with Acute Alcohol Intoxication in adult male rats. *Alcohol*, *79,* 37-45.

Scientific Presentations *(abbreviated)*

Mondello, J.E., Tran, L., Chamoun, L., Rojas, A., Liu, S., Cahill, C.M. (Jan. 2024). Neuropathic pain increases Basolateral Amygdala kappa opioid receptor expression and function in male but not female mice. Presented at Winter Conference on Brain Research, Breckenridge, CO.

Mondello, J.E., Chang, C.W., Trott, J.M., Anaya, A., Solorio, S., Tran, L., Fanselow, M.S. (Nov. 2023). Stress Enhanced Fear Learning enhances excitatory synaptic transmission in Basolateral Amygdala neurons. Presented at Society for Neuroscience Conference, San Diego. *Awarded the Society for Neuroscience Trainee Professional Development Award.*

Mondello, J.E., Trott, J.M., Fanselow, M.S. (Oct. 2019). The effects of stress on morphine-induced conditioned place preference. Presented at Pavlovian Society Conference, Vancouver, CA.

Related teaching experience and community outreach

2019-2022 Brain Awareness Week - Lead coordinator and content creator
University of California, Los Angeles

2019-2021 Graduate Teaching Assistant
University of California, Los Angeles
Behavioral Neuroscience
Foundations of Learning
Introductory Psychology

Chapter 1: General Introduction

There is an acute need to innovate research approaches for studying opioid use disorder (OUD), which has been on the rise at an alarming rate in recent years[1]. OUD is highly co-morbid with other mental health and neurological disorders, compounding the burden on affected individuals and complicating treatment[2–4]. Individuals with OUD have the highest rates of comorbidity (33%) with post-traumatic stress disorder (PTSD), compared to those with other substance use disorders (SUDs)[3,5]. Additionally, a significant proportion of individuals with OUD have chronic pain (50-64%)[6,7]. While much research is devoted to studying these three disorders separately, more research is needed to understand the causal mechanisms that explain the substantial comorbidity between them. PTSD and chronic pain are more likely to precede than to follow SUDs, suggesting that traumatic events may predispose individuals to develop an OUD[2,8]. Furthermore, the prevalence of OUD is higher in individuals with comorbid PTSD/chronic pain compared to PTSD alone, suggesting the etiology of OUD, PTSD and chronic pain are functionally related[9]. In order to advance our understanding of these comorbidities, the goal of this dissertation is to elucidate mechanisms that may promote vulnerability to developing an OUD following a traumatic stressor or injury.

1.1 Overview of hypotheses for OUD comorbidities

Multiple mechanistic pathways have been proposed for explaining OUD, PTSD and chronic pain comorbidities. These hypotheses are not necessarily mutually exclusive but can be understood as different frameworks to understand drivers for these comorbidities. It should be noted that historically most of the focus has been on explaining SUD comorbidities with PTSD and chronic pain separately. The most popular explanation of the link between OUD-PTSD and OUD-chronic

pain is the "self-medication" hypothesis, in which individuals use opioids to relieve their PTSD or pain symptoms[10,11]. Studies of individuals with OUD have confirmed that relief from chronic pain and emotional distress are some of the major motivators for continued opioid use[12,13]. Additionally, increased severity of PTSD symptoms is associated with greater same-day non-medical opioid-use[14]. For those with comorbid OUD-chronic pain, pain-related catastrophizing and distress intolerance is more closely associated with opioid craving than pain severity alone [15,16]. As such, it's generally thought that chronic pain opioid misusers primarily turn to opioids to attenuate negative affect associated with chronic pain.

While the self-medication hypothesis is compelling, treatment strategies grounded in its principles have not shown to be more efficacious than other treatment approaches. A central tenant of the self-medication hypothesis is that individuals misuse substances to cope with their PTSD/chronic pain symptoms. However, integrated SUD/PTSD treatments targeted at improving coping skills are comparable in reducing SUD symptoms and *less* effective in reducing PTSD outcomes when compared to integrated trauma-focused treatments[17,18]. On the other hand, one study of coping-focused treatment found that improvements in PTSD symptoms was predictive of improvements in SUD symptoms, while the reciprocal relationship was not found[19]. This finding suggests that treating the underlying condition (PTSD/trauma) helped alleviate the SUD symptoms and has been touted as further support for the self-medication hypothesis[20]. Notably, integrated trauma-focused interventions in comorbid patients have not consistently shown to be more efficacious in treating SUDs symptoms beyond improvements seen from SUD treatment alone[18].

Furthermore, the self-medication hypothesis does not sufficiently explain the bidirectional relationship between OUD and PTSD/chronic pain. A basic assumption of the self-medication hypothesis is that a SUD should develop after manifestation of PTSD or chronic pain symptoms.

Indeed, around two thirds of individuals with these comorbidities experienced chronic pain or trauma prior to developing a SUD[2,3,7]. However, there is a significant proportion of individuals (15-34%) who develop a SUD *before* PTSD or chronic pain[3,7]. Additionally, Cottler et al., found that *onset* of substance use, particularly opioids and cocaine, tends to precede onset of PTSD symptoms[21]. Similar findings were found with chronic pain[22]. This evidence and more have led some to suggest a "susceptibility" hypothesis for SUD comorbidities: prolonged substance use produces long-lasting functional and structural changes in regions controlling reward, affect, impulse control, and the stress response, which may in turn make individuals more vulnerable to developing PTSD or chronic pain[23–25]. For instance, opioid-induced hyperalgesia can emerge after just one opioid exposure such as that with remifentanil due in part to acute opioid receptor desensitization, but also after prolonged opioid use in a dose- and duration-dependent manner. Opioid-induced hyperalgesia is often mistaken for disease progression and can increase the susceptibility to future chronic pain[25]. Despite having less evidence, the related "high-risk" hypothesis also proposes that individuals who misuse substances are more likely to engage in high-risk activities that could lead to trauma and consequently PTSD/chronic pain[26]. Overall, a broader theoretical framework that recognizes the bidirectional relationship between SUDs and PTSD/chronic pain would better address the complexities of their co-occurrence.

Much of the evidence used to support these presented comorbidity hypotheses may also support the "common pathways" hypothesis, or that overlapping neuroadaptations to trauma/pain and substance use predispose individuals to develop comorbidities[27–30]. For instance, PTSD symptoms correlating with same-day opioid-use might suggest not only self-medication in these individuals but also parallel reward and stress systems dysregulated by a history of trauma. Traumatic stress, chronic pain and excessive substance misuse are all known to induce allostatic

changes that shift reward/anti-reward systems towards a dysphoric state[29,31,32]. Additionally, PTSD, chronic pain, and OUD share overlap in risk factors, symptomology, and impacted neuralcircuitry[27,29]. Patients tend to believe their PTSD/SUD are functionally related and prefer concurrent, integrated treatment, further suggesting that shared neural mechanisms may underly their co-morbidity[33].

Another feature that links OUD, chronic pain and PTSD is that they each have separately been conceptualized as learning disorders[34–38]. For instance, Pavlovian conditioning of cues associated with opioid use contributes to the difficulty of maintaining abstinence, as opioid-related cues can trigger cravings and opioid-taking behaviors[39–41]. Individuals with PTSD show exaggerated fear responses to cues reminiscent of the original trauma and generalized fear responses to novel or intense stimuli[38]. Moreover, individuals with comorbid PTSD-SUD typically display more severe symptomology and enhanced drug cue reactivity compared to those with only a SUD diagnosis [42,43]. In the context of chronic pain, it has been proposed that pain is a particularly salient unconditioned stimulus (US) that is continually paired with surrounding conditioned stimuli (CS's), such as cues associated with the home environment. Re-exposure to the CS's may produce persistent conditioned responses that amplify pain signaling and are difficult to fully extinguish. Operant conditioning processes have shown to additionally be involved in all of these disorders (reviewed in [44–47]). Most relevant here is that OUD is thought to be largely driven by negative reinforcement or "learned association of relief of aversive states" [48,49].

Lastly, it is important to note the role of non-associative learning processes in development of these comorbidities, mainly sensitization and cross-sensitization. The incentive sensitization theory of addiction posits that compulsive drug-seeking and -taking arises from a sensitized mesolimbic dopaminergic system that is hyperreactive to drug-related cues and the behavioral

effects of drugs[50]. The incentive sensitization theory is largely driven by findings from the psychostimulant field but holds some relevance to OUD[51]. Although opioid administration fails to elicit a robust dopamine response in individuals with OUD, they show a sensitized dopaminergic and mesolimbic response to drug-related cues[52–54]. There is also evidence that OUD is driven by a sensitized hypothalamic-pituitary-adrenal (HPA) axis, the major stress response system[55]. Likewise, PTSD and chronic pain have been described as sensitization of the fear- and pain-response systems, respectively[30,56]. Cross-sensitization of these systems, i.e. experience with one stimulus (e.g. trauma or pain) increasing subsequent behavioral or neural responses to another stimulus (e.g. opioids), may further explain shared mechanisms that lead to and exacerbate comorbidities [31,57].

Preclinical rodent models have been particularly useful for elucidating common pathophysiological mechanisms that underly comorbidities, through both a learning theory and cross-sensitization lens. The following sections will first outline rodent models used in this dissertation for modeling aspects of OUD, PTSD and chronic pain, and then review preclinical evidence of how prior stress- and pain-induced neuroadaptations can cross-sensitize to impact the response to opioids.

1.2 Animal models of OUD, PTSD and chronic pain

Opioid Conditioned Place Preference as a measure of opioid cue learning:

Opioid-induced conditioned place preference (CPP) is a behavioral procedure used to assess appetitive associative learning of opioids by pairing a distinct context (i.e. CS) with opioid administration (i.e. US)[58]. One may also pair the aversive components of opioids (e.g. opioid

withdrawal, opioid antagonist administration) to a distinct context, in what is known as conditioned place aversion (CPA).

During CPP, rodents first typically undergo a preconditioning test in which they are exposed to the behavioral box to determine baseline preference for each distinct chamber (Figure 1.1). The chambers are distinguished by a mixture of visual, tactile and/or odor cues. Next rodents undergo conditioning training in which rodents receive opioid administration prior to confinement to a distinct chamber. Opioid training trials are intermixed with equal pairings of vehicle administration to the alternative context. Following training is a postconditioning test, in which rodents are given free access to all chambers in a drug-free state to measure whether preference for the opioid-paired side is above and beyond baseline preference. One may next also conduct a state-dependent test, in which rodents are tested under the influence of the drug (i.e. presence of US) to determine how reestablishing the internal state from conditioning training drives preference behavior. As with any conditioning paradigm, CPP memories are subject to extinction and reinstatement. During extinction training, rodents are exposed to the CS (distinct context) in the absence of the US (opioid administration) until preference scores return to baseline. This may be conducted through either repeated postconditioning testing or more explicit extinction training, in which vehicle administration is now paired to both distinct contexts. During a reinstatement test, one may attempt to reinstate preference for the opioid-paired context in a number of ways,

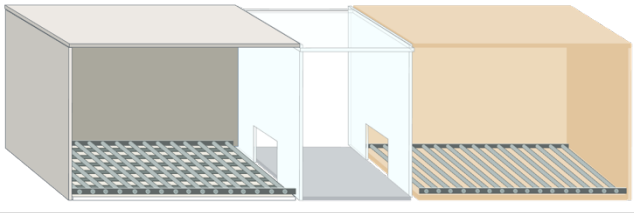


Figure 1.1. Conditioned place preference (CPP) apparatus. During opioid CPP training, rodents receive opioid administration just prior confinement to a distinct context (i.e. tan or grey compartment above). Interspersed with opioid-context pairings are vehicle-context training trials in the alternate distinct context. During CPP testing, rodents are placed in the central neutral chamber and allowed access to all chambers to determine if rodents spend greater time on the opioid-paired side as compared to preconditioning testing. Created with Biorender.com

including injecting a subthreshold priming dose of the training drug or administering a stressor (e.g. footshock or a highly aversive drug).

OUD is the product of repeated drug use and dependence that drives a compulsive pattern of opioid-seeking and -taking behaviors[59]. Although opioid CPP cannot directly investigate the manifestation of those behaviors, it is still useful for studying the initial rewarding effect of an opioid. Importantly, the degree of liking opioids after the first use predicts the development of OUD[60]. CPP is also valuable for examining the encoding of opioid-cue associations[58]. Understanding how cues become associated with opioid use is critical for the advancement of OUD research. Such knowledge allows for the disentanglement of how cues influence opioid-seeking, opioid-taking, relapse likelihood and treatment outcomes. While opioid self-administration can also be used to study drug-cue learning, including in a drug-free state, it requires repeated self-infusions and learning trials to produce reliable behavior. This precludes the possibility of isolating the early rewarding effects of morphine during the initial CS-US pairings. Therefore, CPP is a more appropriate approach for delineating the strength of opioid associative learning in the early stages of opioid use.

Stress Enhanced Fear Learning as a rodent stressor model:

Fear and defensive behaviors are adaptive responses to environmental threats. However, in order for fear to be effective, it must be limited to situations when a danger is truly present. Following an extremely traumatic experience, fear responses may switch from being largely adaptive to maladaptive in some individuals, resulting in the development of PTSD. Diagnostic criteria for PTSD include a host of symptoms that must last more than one month, including re-experiencing the traumatic event, avoidance of cues associated with the trauma, and heightened

arousal[61]. Moreover, PTSD patients have dysregulated *de novo* fear learning, and this symptom severely disrupts normal daily functioning[62–64].

The rodent stressor model Stress Enhanced Fear Learning (SEFL) was first developed by Rau and Fanselow and recapitulates some of the symptomology of PTSD[65] (Figure 1.2). SEFL involves administration of an unpredictable stressor that results in a sensitization by which subsequent minor stressors produce fear behaviors more appropriate for the original stressor. On Day 1, rats receive an unpredictable stressor in one context, Context A, consisting of 15, 1mA, 1 sec foot-shocks pseudo-randomly administered over 90min (Unpredictable Stressor). On Day 2, rats are returned to Context A for 8min to assess fear of that context (Unpredictable Stressor Test). On Day 3, rats are placed in a novel context, Context B, and after 3min, receive a single, 1 mA, 1 sec foot-shock (Minor Stressor) and are removed 30s later. On Day 4, they are placed back into Context B to assess fear to context associated with the minor stressor (SEFL Test). During the SEFL Test, rats that previously received the unpredictable stress respond with exaggerated fear responses (i.e., increased freezing) as compared to animals that did not receive the initial unpredictable stress in context A.

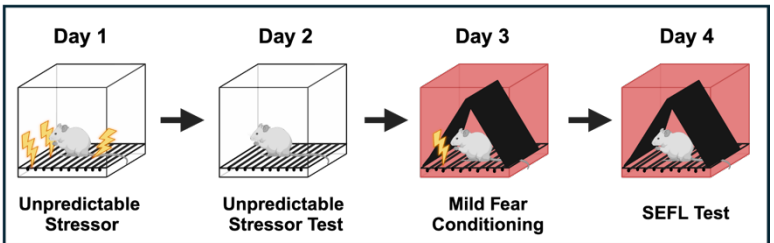


Figure 1.2. General schematic for the Stress Enhanced Fear Learning (SEFL) procedure. The SEFL rodent stressor model recapitulates some of the symptomology of post-traumatic stress disorder. Administration of an unpredictable stressor, i.e. 15 footshocks in rats, results in enhanced fear learning of subsequent mild fear conditioning. Created with Biorender.com

Extinction of the fear of the unpredictable stressor has no impact on the subsequent enhanced fear learning, indicating that SEFL is not merely due to fear generalization[65]. Moreover, the severe stressor must precede the minor stressor, demonstrating that SEFL is not due to summation of fear expression[65]. Rather, the unpredictable stressor causes long-lasting alternations in fear circuitry

that results in sensitization of future fear learning[66–68]. The enhanced fear learning lasts at least 90 days following the unpredictable stress[69] and accompanies a host of other behavioral changes, including decreased exploratory behavior in open fields, decreased time in the open arms while in elevated plus mazes and potentiated startle reactivity[67,68]. Overall, SEFL is a robust stressor model that allows us to examine the neural substrates that may drive the development of maladaptive opioid processing following stress.

Chronic constriction injury as a neuropathic pain model:

Much like fear, pain serves a protective and adaptive role for avoiding bodily injury. If acute pain extends beyond the normal recovery period it may shift into a pathological and persistent state. Chronic pain differs from acute pain not only in its prolonged duration but also in the underlying neural mechanisms. There are different types of chronic pain with varied but often overlapping etiologies including neuropathic, nociceptive, inflammatory and cancer-related pain[70,71]. Chronic neuropathic pain involves nervous tissue damage and is marked by a reorganization in the nociception system that drives exaggerated pain signaling, which can induce heightened sensitivity to painful stimuli (hyperalgesia), pain elicited by stimuli that are typically non-painful (allodynia), and spontaneous sensations of pain (dysesthesia)[72]. Beyond the sensory component, pain also has affective and cognitive appraisal components that are likely driven by distinct but converging neural pathways spanning the entire central nervous system but are well known to engage limbic structures[73,74]. Given the complicated nature of chronic pain, there has been great effort to develop animal models that capture many of these dimensions.

The chronic constriction injury (CCI) procedure in rodents was first developed by Bennett and Xi to more appropriately model neuropathy that provided both face and construct validity to clinical symptomology[75]. The original CCI procedure was achieved by loosely tying 4 chromic gut

ligatures around the sciatic nerve with 1 mm spacing. An updated CCI model replaced ligature ties with polyethylene tubing cuffs surrounding around the sciatic nerve, resulting in a more standardized model and reduction of inter-animal variability[76] (Figure 1.3). It also removed the significant inflammatory component caused by the chromic gut that produces neuritis by touching the nerve sheath independent of a constriction-induced injury. Targeting the sciatic nerve has experimental advantages because it allows for discrete sensory pain measurement in the impacted ipsilateral hind paw with procedures that test mechanical and thermal threshold sensitivities. The CCI model captures many sensory components of chronic pain not found in earlier animal models, mainly hyperalgesia, allodynia and dysesthesia. CCI rodents additionally display distinct gait and posture outcomes, including protective stances for the affected limb and a limp due not just to limb weakness but also a reluctance to place weight on the impacted foot[75]. These motor changes do not induce hypolocomotion in the rodents, allowing for normal conduct of behavioral testing[77]. Importantly, the CCI model has also shown to model the affective components of pain [77–80]. The significance of studying both sensory and affective dimensions of pain are emphasized by negative affect being a better predictor of quality of life compared to its sensory component, and patients with co-morbid diagnosis of a mental health disorder (especially anxiety and depression) have greater pain intensity and are less responsive to typical analgesic management[81,82]. CCI mice display greater anxiety-like behaviors, as measured with tests such as the open field, elevated plus-maze and light/dark assays, and depressive-like behaviors, as measured with the forced swim and splash tests[77–80]. The mechanical sensitivity outcomes tend to peak 1-2 weeks after injury induction and ablate around 4-6 weeks, while the affective outcomes have a slower onset and longer

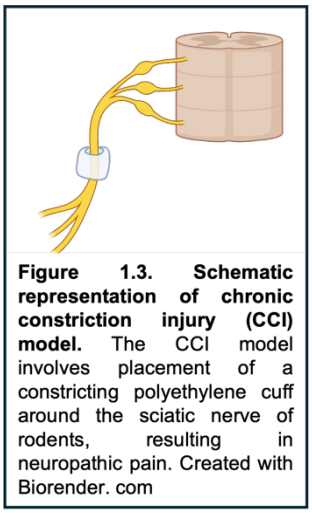


Figure 1.3. Schematic representation of chronic constriction injury (CCI) model. The CCI model involves placement of a constricting polyethylene cuff around the sciatic nerve of rodents, resulting in neuropathic pain. Created with Biorender. com

duration[76,80]. In summary, the CCI model offers a comprehensive approach to investigating multiple dimensions of chronic neuropathic pain, including the sensory and affective components, making it a valuable tool for studying chronic pain and OUD comorbidities.

1.3 Preclinical studies of stress x opioids: reward learning and behavioral sensitization

There are numerous stress models shown to sensitize opioid reward learning, as measured with opioid CPP. The impact of stress on opioid CPP depends on a variety of factors, including the timing, duration, controllability and predictability of the stressor. Acute shock generally tends to increase opioid CPP[83–87] *c.f.*[88]. Notably, inescapable tailshock enhances morphine CPP while escapable tailshock does not[83,85,86]. Given that the number of shocks administered in both conditions are the same, this implies that it's the stress compounded by lack of control that enhances subsequent opioid reward learning, and not exposure to tailshock itself. Others found that chronic restraint increased opioid CPP[89,90], while chronic unpredictable mild stress decreased opioid CPP[91], and chronic social isolation produced no change[92]. These disparate findings may indicate that the modality of stress matters, but it also may be a result of the timing of the stress: both the unpredictable stress and social isolation were administered *during* training and/or testing of opioid CPP while chronic restraint occurred *prior* to the beginning of CPP training. Likewise, forced swim stress administered either just prior to daily opioid CPP training or just prior to CPP testing decreased CPP scores compared to controls[93,94]. These studies overall reinforce that stress – when administered at least 1 day prior to CPP training – enhances opioid reward learning.

Alterations in the HPA axis as a result of traumatic stress exposure plays a major role in driving the potentiated opioid reward learning. In rats and mice, the major hormone in the HPA axis is corticosterone, a form of glucocorticoid that is released by the adrenal gland in response to stress and acts as a negative feedback signal to the HPA axis. Following inescapable stress, the

corticosterone response to subsequent morphine administration was found to be potentiated[95]. Inhibiting corticosterone levels during CPP morphine training blocked the stress-enhanced CPP while inhibiting corticosterone during the inescapable stressor had no effect. Alternatively, another group found that glucocorticoid receptor antagonism administered *during* chronic footshock blocked stress-enhanced opioid CPP[96]. The researchers additionally found that stress potentiated morphine-induced *FosB* mRNA in the striatum, and this potentiation was blocked by glucocorticoid receptor antagonism during the stress. As such, these studies provide direct evidence of stress's impact on the HPA axis cross-sensitizing to enhance future reward learning of opioids.

In addition to potentiating opioid reward learning, stress also sensitizes opioid-induced locomotion. There is a well-established literature that demonstrates the behavioral sensitization of psychostimulant-induced locomotion is tied to the reinforcing effects of stimulants[97]. Psychostimulant-induced locomotion and psychostimulant self-administration are both dopamine-dependent[98,99]. Stress enhances psychostimulant self-administration and sensitizes the locomotor and dopaminergic response to stimulants[97,100–102]. Moreover, inhibiting corticosterone blocks this stress-enhanced psychostimulant self-administration, locomotion, and dopaminergic responses[101,103,104]. Cross-sensitization of stress and opioid-induced locomotion has also been found, using the learned helpless model (inescapable shock), repeated days of footshock, repeated restraint stress and chronic variable stress[105–108]. Immediately after administration, opioids generally produce hyperlocomotion in mice, and hyperlocomotion at lower doses and *hypo*locomotion at higher doses in rats[109,110]. In the absence of stress, glucocorticoid receptor antagonists suppress morphine-induced hyperlocomotion and morphine-induced extracellular dopamine release in the nucleus accumbens (NAc)[111]. Likewise, restraint stress-induced

sensitization of the locomotor response to morphine can be blocked with adrenalectomy and D_1 and D_2 dopaminergic receptor antagonists administered just prior to stress[107,112]. Inescapable shock sensitizes opioid-induced dopamine release in the NAc shell[113]. Just as an intact HPA axis is required for stress-enhanced opioid CPP and behavioral sensitization, it is also required for stress-enhanced dopamine response to morphine[113]. In sum, converging pathways (the HPA axis and dopaminergic system) appear to drive cross-sensitization of stress to both opioid-induced reward and locomotion. In experiments contained within Chapter 2, I will assess how the unpredictable stressor used in the SEFL model can likewise sensitize opioid reward learning and opioid-induced locomotion.

1.4 Preclinical studies of pain x opioids: role of the dynorphin/kappa opioid receptor system

Substantial evidence indicates that the dynorphin/kappa system is highly involved in the dysphoric state produced by stress[114]. Kappa opioid receptors (KOR) are G protein coupled receptors (GPCRs), specifically coupled to the G protein G_i/G_o. KORs are activated by the endogenous opioid peptide dynorphin, resulting in decreased neuronal excitability. While KOR agonists produce antinociception (primarily due to spinal mechanisms or due to a stress-induced antinociception), they also produce aversion and hallucinations[115]. KOR agonism produces depressive- and anxiety-like behaviors, activates the HPA axis and can induce a CPA[116]. The dynorphin-KOR system has likewise been suggested to underly negative affective states driving OUD, given its involvement in negative emotional states induced by withdrawal and stress-induced reinstatement of drug seeking and place preference[114,117–119]. For instance, KOR antagonism blocks stress-induced reinstatement of heroin intravenous self-administration and morphine CPP[120–122]. Additionally, KOR agonism alone induces reinstatement of cocaine CPP,

cocaine self-administration and alcohol self-administration[123–125]. Whether KOR agonism could induce reinstatement of opioid CPP remains unclear.

The dynorphin/KOR system is likewise involved in the pathophysiology of chronic pain. In chronic pain states, the endogenous dynorphinergic tone is increased. This may serve as an adaptative response to counteract increased nociceptive input but could also drive the dysphoria found in chronic pain[114]. Chronic pain causes a functional upregulation of the dynorphin/KOR system in various regions, including the spinal cord and mesolimbic regions implicated in reward, pain and stress processing, such as the central amygdala, basolateral amygdala (BLA), ventral tegmental area (VTA) and NAc[126–130]. This upregulation in mesolimbic systems in particular may drive the negative affect state induced by chronic pain. Our laboratory has shown that CCI injury resulted in an upregulation of dynorphin/KOR gene transcripts and KOR agonist-stimulated [^{35}S]GTPγS binding in the VTA, NAc, and BLA[129,130]. Much of this upregulation of the dynorphin/KOR system in chronic pain states appear to be more prominent in males than females. CCI injury also induced a leftward shift in KOR agonist-induced CPA, specifically in chronic pain male mice but not female pain mice[129]. Antagonism of KOR was additionally able to recover depressive-like behaviors seen in both CCI male and female mice[129]. Of note, KOR antagonism had no impact on mechanical sensitivity in neuropathic pain mice, supporting that an upregulation of the dynorphin/KOR system in chronic pain drives more of the affective component of pain rather than the sensory component. The role of the dynorphin/KOR system in inducing dysphoria may in part be mediated by its interaction with the dopaminergic system; chronic pain induces a hypodopaminergic state that is recovered with KOR antagonism[129]. While there is a lot of evidence showing that chronic pain upregulates the dynorphin/KOR system, whether this upregulation cross-sensitizes to increase susceptibility to OUD is understudied and warrants further

investigation. In experiments contained within Chapter 3, I will assess how chronic neuropathic pain can promote susceptibility for stress-induced reinstatement of opioid place preference.

1.5 Outline of contained work:

There is a need to develop more animal models that can capture the symptomology of OUD comorbidities. The role of common pathways in the development of OUD comorbidities with PTSD and chronic pain will be investigated in the following chapters:

In Chapter 2, experiments are presented that examine the role of the unpredictable stressor from the SEFL model in promoting opioid reward learning and opioid-induced locomotion. The following questions were investigated: 1) The unpredictable stressor enhances future fear learning, but can it also enhance future opioid reward learning? 2) Likewise, does the unpredictable stress sensitize the locomotor response to opioid administration, as other stressors have shown to do? 3) Finally, does a history of stress impact aversive learning of opioid receptor antagonism?

In Chapter 3, experiments are presented that investigate how chronic neuropathic pain states impacts stress-induced reinstatement of opioid place preference, asking: 1) Are neuropathic pain mice, especially males, more susceptible to KOR agonist-induced reinstatement of opioid place preference? And 2) Are BLA KORs necessary for KOR agonist-induced reinstatement of opioid place preference in a chronic pain state?

Chapter 2: The impact of unpredictable stress on opioid reward learning and opioid-induced locomotion

2.1 Abstract

Opioid Use Disorder (OUD) and post-traumatic stress disorder (PTSD) are highly co-morbid disorders that likely share common mechanistic pathways. Stress Enhanced Fear Learning (SEFL) is a rodent stressor model that produces some of the PTSD symptomology, particularly enhanced future fear learning. Here we examined whether the stressor in SEFL could likewise alter subsequent opioid processing, specifically whether it would sensitize opioid conditioned place preference (CPP), opioid conditioned place aversion (CPA) and opioid-induced locomotion in adult male Long Evans rats. Unpredictable stress did not have a significant impact on morphine CPP. On the other hand, unpredictable stress consistently enhanced the locomotor response to the first experience with low dose morphine (2.5 mg/kg s.c.). Additionally, baseline locomotor activity was predictive of future opioid-related behaviors in a stress-dependent manner. Surprisingly, unpredictable stress additionally produced a CPP to low dose naltrexone (1 mg/kg s.c.) but not high dose naltrexone (10 mg/kg). Overall, these data reveal that while the SEFL model results in alterations in the response to low dose opioid agonists and antagonists, it may not be an appropriate model for studying stress-enhanced opioid associative learning.

2.2. Introduction

The Stress Enhanced Fear Learning (SEFL) model has been shown to recapitulate components of PTSD, but it is unknown if it is a useful model for studying PTSD-OUD comorbidities. Both PTSD and OUD involve maladaptive learning, in which cues associated with either fear- or opioid-related stimuli gain unparalleled control over behavior[38,50]. The unpredictable stressor in SEFL produces a long-term enhancement of future fear learning[65,67]. It is therefore

fitting to examine whether the stressor can likewise enhance future opioid reward learning. Opioid-induced CPP is a behavioral procedure that measures the rewarding properties of opioids and opioid-context learning. Experiment 1 tested whether unpredictable stress enhances 4 trial morphine CPP using both low dose morphine (2.5 mg/kg) and high dose morphine (10 mg/kg). Experiment 2 further tested the impact of unpredictable stress on subthreshold CPP (1 trial) with low dose morphine.

In addition to testing the impact of unpredictable stress on opioid reward, we also sought to test the impact of stress on opioid-induced locomotion. Numerous preclinical studies have demonstrated that stress can sensitize opioid-induced locomotion, including the learned helplessness model[105–108]. The intensity of the shock protocol used in the learned helplessness model is notably stronger than the shock protocol used in SEFL (100, 5s, 1mA tailshocks vs 15, 1s, 1mA footshocks, respectively). Therefore, it is unclear whether the stressor used in SEFL can likewise produce the same behavioral sensitization. The impact of stress on opioid reward and opioid-induced locomotion is thought to be both related to sensitization of the hypothalamic-pituitary-adrenal (HPA) axis and dopaminergic system[96,107,113,131]. However, it has not been tested before if the impacts of stress on opioid learning and opioid-induced locomotion are functionally related. Thus, another goal of these studies was to examine if there is a positive relationship between stress-enhanced opioid learning and opioid-induced locomotion.

To further test the effect of unpredictable stress on opioid processing, we lastly investigated its impact on opioid receptor antagonist-induced CPA. Naltrexone is a long-acting opioid receptor antagonist with high affinity binding to mu opioid receptors, but also has some minimal action on kappa and delta opioid receptors[132]. Opioid antagonist CPA is typically conducted in morphine-dependent rodents, as opioid-precipitated withdrawal is a highly aversive unconditioned stimulus.

However, large enough doses of opioid receptor antagonists can produce a CPA in opioid naïve rodents[133–136]. The final experiment tested whether unpredictable stress would produce a CPA to naltrexone using a subthreshold CPA procedure.

2.3 Methods

Subjects:

Subjects were 3 month old adult male Long Evans rats (Experiment 1, N = 25; Experiment 2, N = 32; Experiment 3, N = 30) purchased from Envigo. Rats were pair-housed in standard Plexiglas cages with paper twist tie enrichment and ad libitum access to food and water. The colony room was maintained at a 12:12 hr light/dark schedule and all behavioral testing was run during the light phase. Each animal had a cage mate assigned to the same experimental group. Subjects were assigned to experimental conditions in a randomized block design so that the running of subjects was counterbalanced across groups. Each experiment was completed using multiple cohorts, resulting in internal replications. Each cohort was balanced with respect to experimental groups. Chancellor's Animal Research Committee at UCLA approved all animal testing procedures.

All rats were handled for 7 days for 1-2 min per day prior to the start of experimentation. On the last 2 days of handling, rats were also habituated to transport from the colony vivarium housing room to the CPP behavioral testing room. Every experimenter involved in running behavioral experiments handled the rats at least once before experimentation. Typically, multiple 2-3 experimenters of both sexes were involved in each cohort of an experiment.

Footshock apparatus:

Administration of the unpredictable stressor occurred in standard Med Associate sound and light attenuating conditioning chambers (VFC-008; 30.5 x 24.1 x 21cm). The footshock

administration was controlled by Med Associates VideoFreeze software (Med Associates, St. Albans, VT). Med associate shock scramblers (ENV 414-S) delivered scrambled shocks to grid floors in the chambers. Sessions were recorded with infrared cameras, and motion was measured using the Med Associates Video Freeze Software.

Unpredictable Stressor:

Rats were administered the standard stressor used in the SEFL model, as described in previous publications [65,67], consisting of 15, 1mA, 1 second shocks, pseudo-randomly distributed over the course of 90 minutes. Rats were not habituated to the footshock room or apparatus prior to the stressor. Rats in the no stress control group spent an equal amount of time in the conditioning chamber with no shocks. The floor of the conditioning chamber contained evenly spaced metal grids at alternating heights. The conditioning chamber also contained a 50% Windex scent, red light and fan as background noise. The fan background noise and the sound attenuating chambers minimized vocalization of rats enough so that control rats did not appear to respond when other neighboring rats were being shocked. The experimenter handling the rats during the unpredictable stressor day was always different from the experimenter conducting CPP, to avoid aversive associations forming between the rats and the experimenters conducting CPP.

Conditioned Place Preference apparatus:

The CPP procedure was conducted in a 3-chamber behavioral apparatus. The apparatus had two larger opaque square conditioning chambers (34.3 x 40.6cm) connected by a smaller third intermediary ("neutral") rectangular chamber (12.5 x 25.0cm). The neutral chamber extended out of the behavioral chamber and contained three clear Plexiglas walls. The larger chambers had distinct contextual features: one chamber had vertical black and white stripes and a Plexiglas floor

insert with repeated circular holes, while the other chamber had horizontal black and white stripes and a floor insert with long parallel vertical inserts. Plastic doors could be inserted in order to block the exit of the chambers and contain animals to either distinct chamber. The CPP procedure was conducted in a room separate from the rat colony, with white noise in the background and white lighting overhead. Sessions was recorded with a camera arranged above the behavioral box and Ethovision software was used to track the activity of the rats. Activity was calculated as percentage of pixel change in a defined arena.

During the test days when rats were given access to all 3 chambers in the CPP apparatus, they were always placed in the neutral chamber first. Given the narrow size of the neutral chamber, animals had to be placed either facing one of the distinct chambers or the other. This raised the possibility that the rats would merely remain in the chamber they were facing, especially if they had a history of stress. Wherever possible, the direction of animal placement was counterbalanced during testing (i.e. across the habituation/preconditioning days and across the 2 postconditioning tests conducted per day).

Conditioned Place Preference procedure:

The typical experimental design for the CPP experiments was typically as follows: habituation, preconditioning, unpredictable stress, training, postconditioning tests, state-dependent test.

Habituation: The day after handling ended, animals were placed in the intermediary ("neutral") chamber within the CPP apparatus and allowed to explore all 3 chambers for 20 min (Experiments 1 & 2) or 30 min (Experiment 3).

Preconditioning: The following day, animals again were exposed to the CPP apparatus for 20 min (Experiments 1 & 2) or 30 min (Experiment 3). The time spent in each chamber during

this 2nd day of exposure was used to determine baseline preferences and calculate later postconditioning preference scores. The baseline preference score was used to determine which context the training drug was paired with. This pairing was assigned using an unbiased design to ensure that preconditioning preference for the drug-paired context was equalized across all experimental groups.

Training: On the first day of training (Training Day 1a), animals received either the training drug (see below for specific methods for each experiment) or saline injection and were confined to one context for 1 hr. The timing for conditioning was determined based on a meta-analysis on rat CPP studies that found training < 20 min or > 45 min resulted in greater effect sizes in CPP than 25-30 min [137]. On the 2nd day of training (Training Day 1b), animals were administered the alternate treatment (saline or drug) and were confined to the alternate context for 1 hr. This 2-day training schedule constituted "1 trial" of CPP training; the number of CPP trials varied across experiments. The order of the training drug vs. vehicle administration was counterbalanced across groups for each experiment.

Postconditioning Tests: Following CPP training, the next day rats were tested for preference of the drug-paired chamber in a drug-free state. Rats were placed in the neutral chamber and allowed to access to all 3 chambers for 20 min (Experiments 1 & 2) or 30 min (Experiment 3). Two postconditioning tests were conducted each day, to allow for counterbalancing of the chamber faced when that rat was placed in the neutral chamber (see above for further explanation). For the CPP post-conditioning tests, the preference scores across the 2 postconditioning test sessions per day were averaged. The post-conditioning preference score was calculated as: [Time during Postconditioning (Drug-paired side) – Time during Postconditioning (vehicle-paired side)] – [Time during Preconditioning (Drug) – Time during Preconditioning (vehicle)].

State Dependent Test: Because expression of preference for the drug-paired context may differ depending on whether the drug is present or not (i.e. presence of interoceptive cues), in some experiments the rats were next tested on their respective training drug/dose the day after postconditioning tested ended. Rats were administered their training drug, immediately placed in the neutral chamber and tested for 40 min.

Only the first 20 min of the test was used to calculate the preference score as to directly compare the data to preconditioning. The state dependent preference scores were calculated as: [Time during State Dependent (Drug-paired side) – Time during State Dependent (vehicle-paired side)] – [Time during Preconditioning (Drug) – Time during Preconditioning (vehicle)].

Specific methods for Experiment 1: Stress x 4 Trial morphine CPP

Rats first underwent CPP habituation and preconditioning (Figure 2.1A). The next day they were administered either the unpredictable stressor or acted as a no stress control. Next, the rats underwent 4 trials of CPP training with either 2.5 mg/kg or 10 mg/kg morphine used as the training dose. Following CPP training, the rats underwent 3 days of postconditioning testing (2 postconditioning tests per day) and a state-dependent test.

Specific methods for Experiment 2: Stress x 1 Trial morphine CPP

The experimental design for Experiment 2 (Figure 2.3A) remained nearly identical to Experiment 1 except for only 1 trial of CPP training using a single dose of 2.5 mg/kg morphine. Additionally, there was only 1 day of postconditioning testing (2 postconditioning tests total).

Specific methods for Experiment 3: Stress x 1 Trial naltrexone "CPA"

Following habituation, preconditioning and stress (or no stress) administration, rats underwent 1 trial CPA training with either 1 mg/kg or 10 mg/kg naltrexone s.c. (Figure 2.5A). Following training, rats underwent 1 day of postconditioning testing and no state dependent test.

Drug administration

For Experiments 1 & 2, all rats received alternating morphine and saline injections s.c. for the CPP training. The doses of morphine sulfate (Spectrum Chemical) were dissolved in sterile physiological saline (0.9%). For Experiment 3, all rats were administered their training dose of naltrexone hydrochloride (Sigma; dissolved in saline) and saline s.c. for conditioning training.

S*tatistical analysis and subject exclusions*

All statistical analysis was conducted on Graphpad Prism (v10). ANOVAs or unpaired T tests were used where relevant to determine group differences with the level of statistical significance set at $p < 0.05$. The Geisser and Greenhouse correction for ANOVAs was used when the assumption of sphericity was violated. For determining if there was place preference/aversion, one-sample t tests were conducted on the preference scores to test whether each group mean was significantly different from a hypothetical value of 0 (i.e. no preference). This test is equivalent to conducting a paired T test comparing the difference between the [postconditioning(time on drug-paired side) - postconditioning(time on vehicle-paired side)] vs. [preconditioning(time on drug-paired side) - preconditioning(time on vehicle-paired side)]. Post-hoc tests were conducted using Bonferroni's multiple comparisons test or Fisher's LSD. CPP scores and locomotor activity was correlated using Pearson's correlation tests, with level of statistical significance set at $p < 0.05$.

For Experiment 1, five rats (two from no stress/2.5 mg group, one from no stress/10 mg group, two from stress/10 mg group) were excluded from all analyses due to incorrect drug injection dose during one of the CPP training days. Two rats were excluded from Experiment 3 due to remaining in the chamber they were initially facing for both the habituation and preconditioning days (i.e. spending > 90% of time in chamber faced compared to opposite

chamber). The direction the rats were placed in the CPP apparatus did not appear to drive pre- or postconditioning preferences for any of the other rats tested.

2.4 Results

Experiment 1: Stress x 4 Trial morphine CPP

The impact of unpredictable stress was first assessed on 4 trial morphine CPP, utilizing training doses of either 2.5 mg/kg or 10 mg/kg (see Figure 2.1A for experimental design). During CPP training sessions, which was conducted 1 day after stress, we measured the locomotor activity response (i.e. pixel change) to morphine vs. vehicle administration. To control for individual differences in activity, morphine-induced activity scores were normalized, calculated as the within subject difference between the activity response to morphine and vehicle for each rat.

We first verified that there were no group differences in activity response to vehicle administration across the 4 training sessions (Figure 2.1B; see Table 2.1 for full statistical summary of Experiment 1). A 2-way ANOVA revealed no effect of Stress ($p=0.59$) or Dose ($p=0.87$) and no significant interactions. However, there was a trend for a main effect of Training Session ($p=0.06$), suggesting that over the training sessions rats slightly reduced locomotion in response to vehicle injections. Subsequent analysis of the (normalized) activity response to morphine detected a main effect of Dose ($p<0.01$) (Figure 2.1C). Specifically, rats receiving the 10 mg/kg morphine training dose exhibited lower morphine-induced activity compared to those receiving the 2.5 mg/kg dose. There was additionally a main effect of Training Session ($p<0.0001$), due to morphine-induced activity significantly increasing over training sessions. There was a slight trend for a main effect of Stress ($p=0.10$). No other differences between groups were observed.

To determine how stress impacted the *first* exposure to morphine, a two-way ANOVA was calculated on morphine-induced activity from just the first training session (Figure 2.1D). The ANOVA detected a Stress x Dose interaction ($p<0.05$) and corrected post-hoc comparisons revealed that stress increased morphine-induced activity in the 2.5 mg/kg group ($p<0.05$) but had no impact on activity scores in the 10 mg/kg group ($p=0.83$). To further clarify the impact of stress within the 2.5 mg/kg group, we compared unnormalized activity scores following morphine vs. vehicle administration within subjects during the first training session (Figure 2.1E). There was a trend for a Stress x Drug Treatment interaction ($p=0.07$) that was driven by a moderate difference between vehicle- vs. morphine-induced activity in the stress group.

Following CPP training, rats were tested for their preference for the morphine-paired context in a drug-free state across 3 days. There were no group differences in preference scores during the first day of testing (Figure 2.2A). While all groups generally increased their time on the morphine-paired side as compared to the preconditioning day (Supplemental Figure 2.1A), only the stress-2.5 mg dose group demonstrated a significant preference score during the first day of testing ($p<0.05$) (Figure 2.2A). For analysis of the preference scores across testing days, 2-way ANOVAs (Stress x Testing Day) were performed for each dose given that there was not a large enough sample size to run a complete 3-way ANOVA. CPP preference scores remained consistent over the subsequent testing days for both doses and did not differ among groups (Figures 2.2B/C). After postconditioning testing, rats underwent a state dependent test following administration of their respective morphine training dose (Figure 2.2D). All groups increased their time on the morphine-paired side as compared to the preconditioning day (Supplemental Figure 2.1B), but here only the 10mg/kg groups exhibited a significant preference for the morphine-paired side ($p<0.05$). Again, there were no group differences in their preference scores.

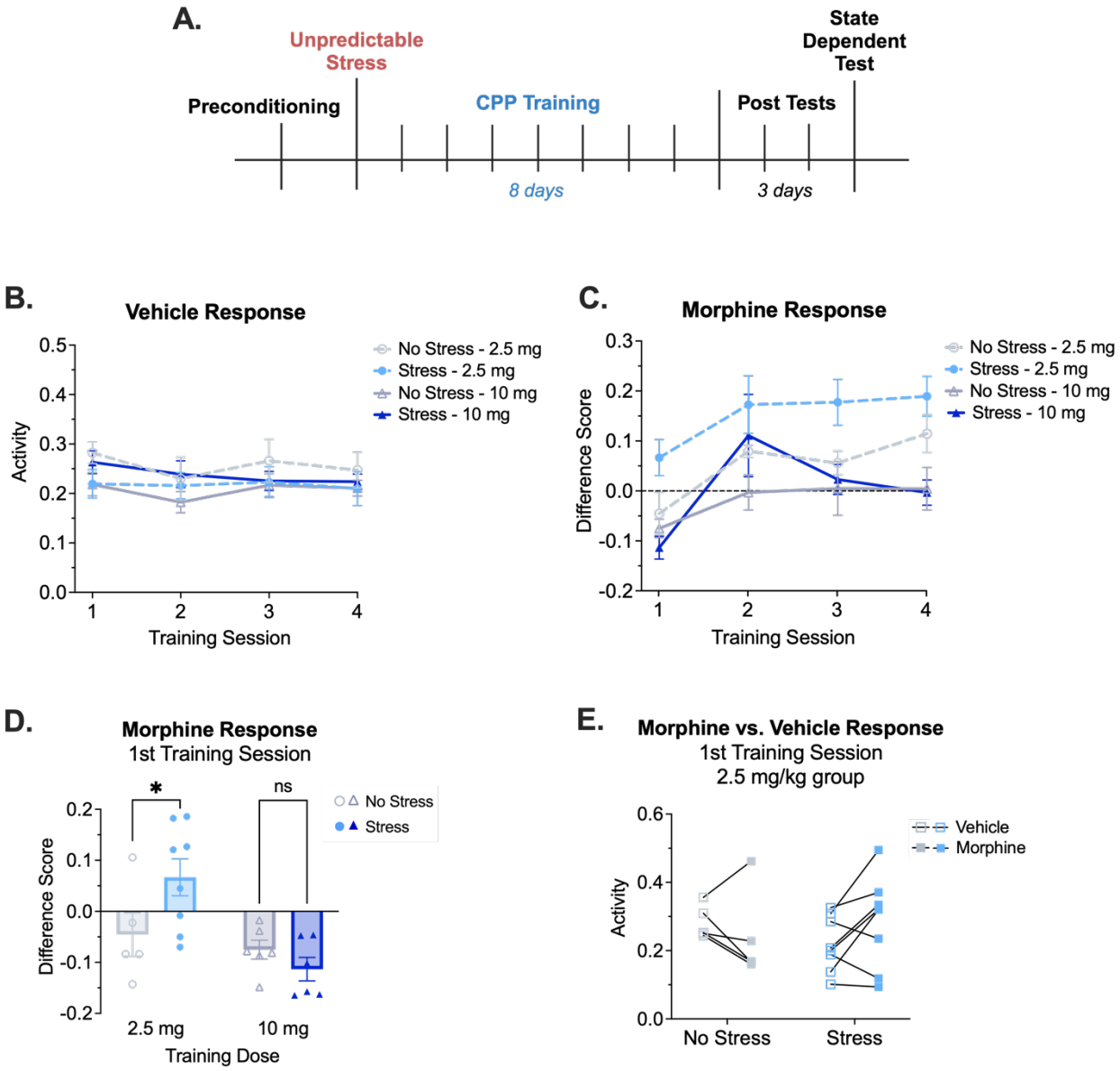


Figure 2.1. **Unpredictable stress increased morphine-induced activity during morphine CPP training. A.** Experiment 1 schematic. Rats underwent unpredictable stress or acted as a no stress control. The next day, rats began a 4 trial CPP training procedure performed across 8 days, with either 2.5 mg/kg or 10 mg/kg morphine, s.c. used as the training dose. After CPP training rats underwent 3 days of postconditioning testing and then a state dependent test in the presence of the morphine training dose. **B.** Vehicle (i.e. saline)-induced locomotor activity across CPP training sessions. **C.** Morphine-induced activity (normalized by subtracting vehicle-induced activity) across CPP training sessions. **D.** Morphine-induced activity during the first CPP training session only. **E.** Comparison of morphine- vs. vehicle-induced activity during the first CPP training session for just the 2.5 mg/kg groups. Data represent means ± s.e.m. ANOVA Post-hoc comparison denoted with * (**p**<0.05). Full statistical test analyses for these data are presented in Table 2.1.

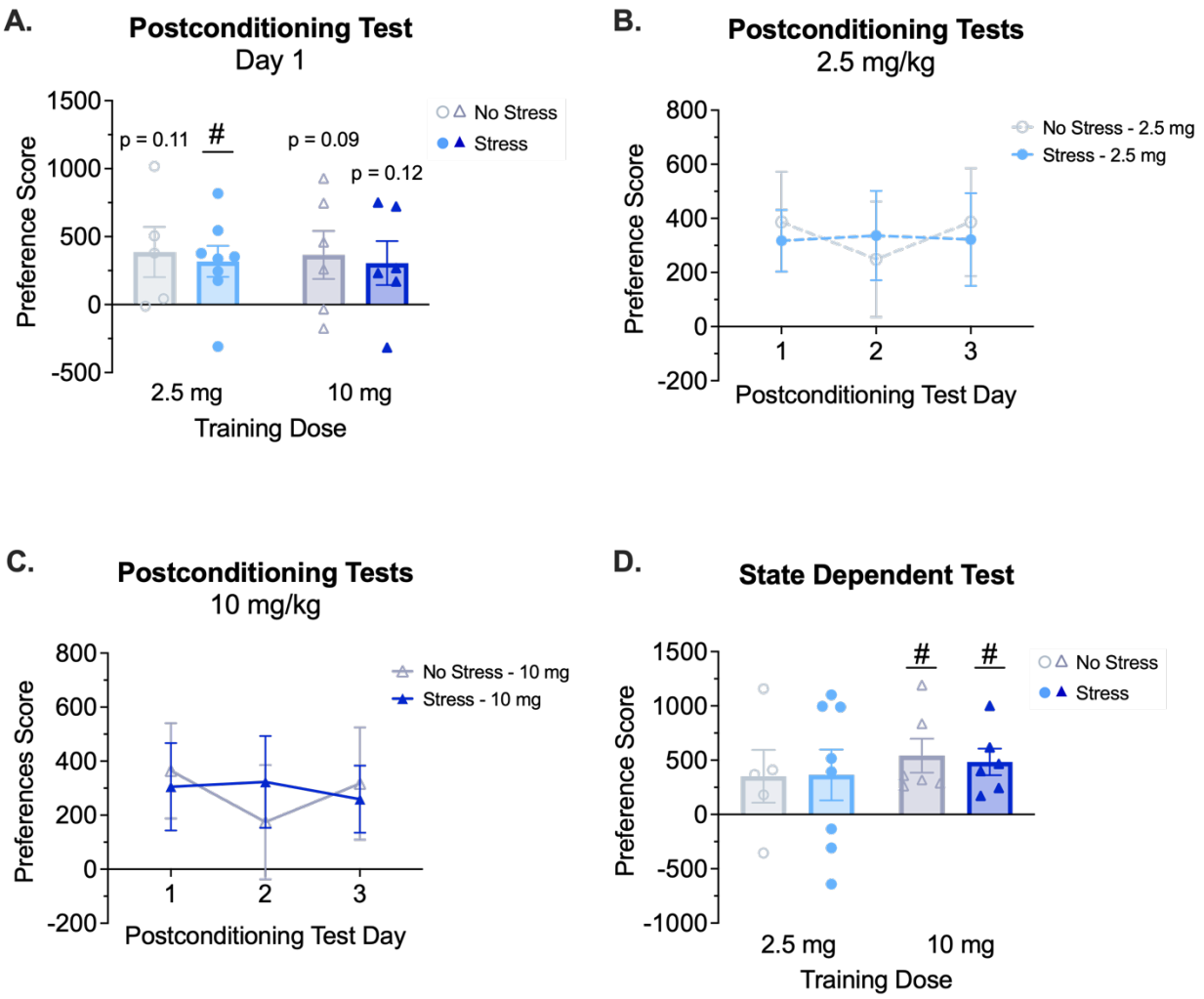


Figure 2.2. **Unpredictable stress had no impact on postconditioning or state dependent preference scores using a 4 trial morphine CPP training protocol. A.** Preference scores from only the first day of postconditioning testing. Postconditioning preference scores were averaged across the two tests/day and then calculated as a comparison to baseline preconditioning preference scores. **B.** Preference scores across postconditioning test days for the 2.5 mg/kg groups. **C.** Preference scores across postconditioning test days for the 10 mg/kg groups. **D.** Preference scores from the state dependent test. State dependent preference scores were calculated as a comparison to baseline preconditioning preference scores. Data represent means ± s.e.m. One-sample t test denoted with # (**p**<0.05). Full statistical test analyses for these data are presented in Table 2.1.

Experiment 2: Stress x 1 Trial morphine CPP

In Experiment 2, we next investigated whether stress would impact subthreshold (i.e. 1 trial) morphine CPP, using only 2.5 mg/kg morphine for the training dose (Figure 2.3A). Similar to Experiment 1, stressed rats exhibited higher morphine-induced activity during their first (and only) CPP training session ($p<0.05$) (Figure 2.3B; see Table 2.2 for full statistical summary of Experiment 2). A within subjects comparison between activity scores following morphine vs. vehicle administration revealed a Stress x Drug Treatment interaction ($p<0.05$) (Figure 2.3C). Post-hoc analysis revealed that while morphine administration reduced activity as compared to vehicle administration in the no stress group ($p<0.05$), morphine had no impact on activity in the stress group ($p=0.27$). Additionally, morphine-induced activity was higher in the stress group as compared to morphine-induced activity in the no stress group ($p<0.05$). During the postconditioning and state dependent tests (Figures 2.3D/E), non-stressed rats did not show a CPP in either test. On the other hand, stressed rats exhibited a trend for a preference ($p=0.08$) during the postconditioning test and a significant preference ($p<0.05$) during the state dependent test. The two groups did not significantly differ from each other during either test.

Correlation analyses were conducted to determine the relationship between locomotion and CPP scores (see Supplemental Figure 2.2 for full correlation matrices and statistics). No relationship was found between CPP scores and normalized morphine-induced locomotor activity in either group. In the no stress group only, there was a slight trend for a positive relationship between CPP scores and both vehicle-induced activity and unnormalized morphine-induced activity ($p=0.095$ and $p=0.097$, respectively) (Supplemental Figures 2.2A/C). When examining whether baseline activity (i.e. locomotion during the first time in the CPP chamber) was predictive of future behavior, an interesting pattern emerged between the stress and no stress groups. Within

the no stress group, baseline activity was positively correlated with CPP scores ($p<0.01$) and vehicle-induced activity ($p<0.05$) (Figures 2.4A/B; Supplemental Figures 2.2 A/C). On the other hand, within the stress group, baseline activity was only positively associated with morphine-induced activity (both normalized, $p<0.01$, and unnormalized, $p<0.001$) (Figures 2.4C/D; Supplemental Figures 2.2 B/D).

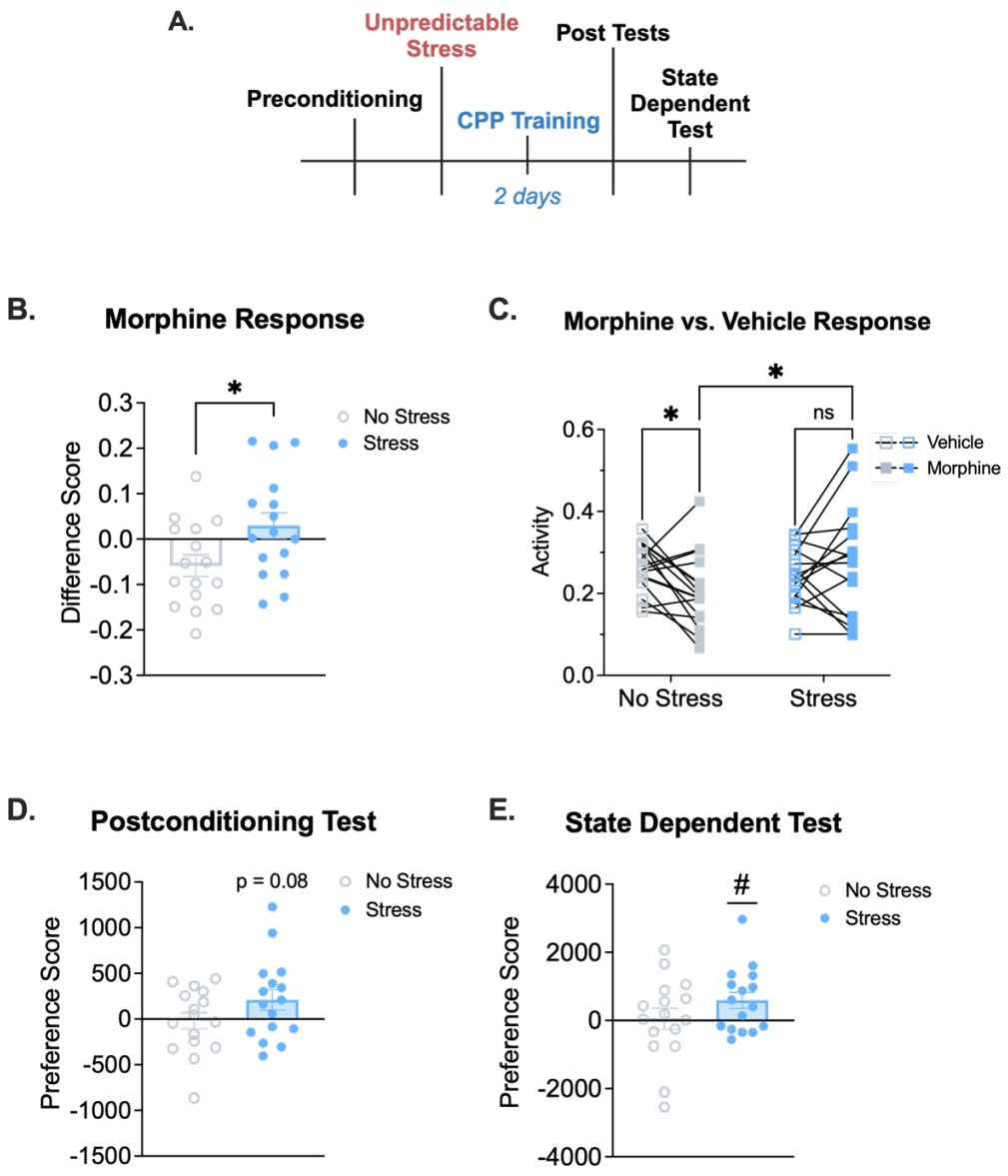


Figure 2.3. Unpredictable stress enhanced morphine-induced activity and had moderate effects on 1 trial morphine CPP. A. Experiment 2 schematic. Rats underwent unpredictable stress or acted as a no stress control. The next day, rats underwent 1 trial CPP training with 2.5 mg/kg morphine, s.c. used as the training dose. After CPP training rats underwent 1 day of postconditioning testing (2 tests within the day) and then a state dependent test. **B.** Morphine-induced locomotor activity (subtracting vehicle-induced activity) during CPP training. **C.** Comparison of morphine- vs. vehicle-induced activity during the CPP training. **D.** Preference scores from postconditioning testing. Postconditioning preference scores were averaged across the two tests in the day and then calculated as a comparison to baseline preconditioning preference scores **E.** Preference scores from the state dependent test. State dependent preference scores were calculated as a comparison to baseline preconditioning preference scores. Data represent means ± s.e.m. ANOVA Post-hoc comparison denoted with * (**p**<0.05). One-sample t test denoted with # (**p**<0.05). Statistical analyses can be found in Table 2.2.

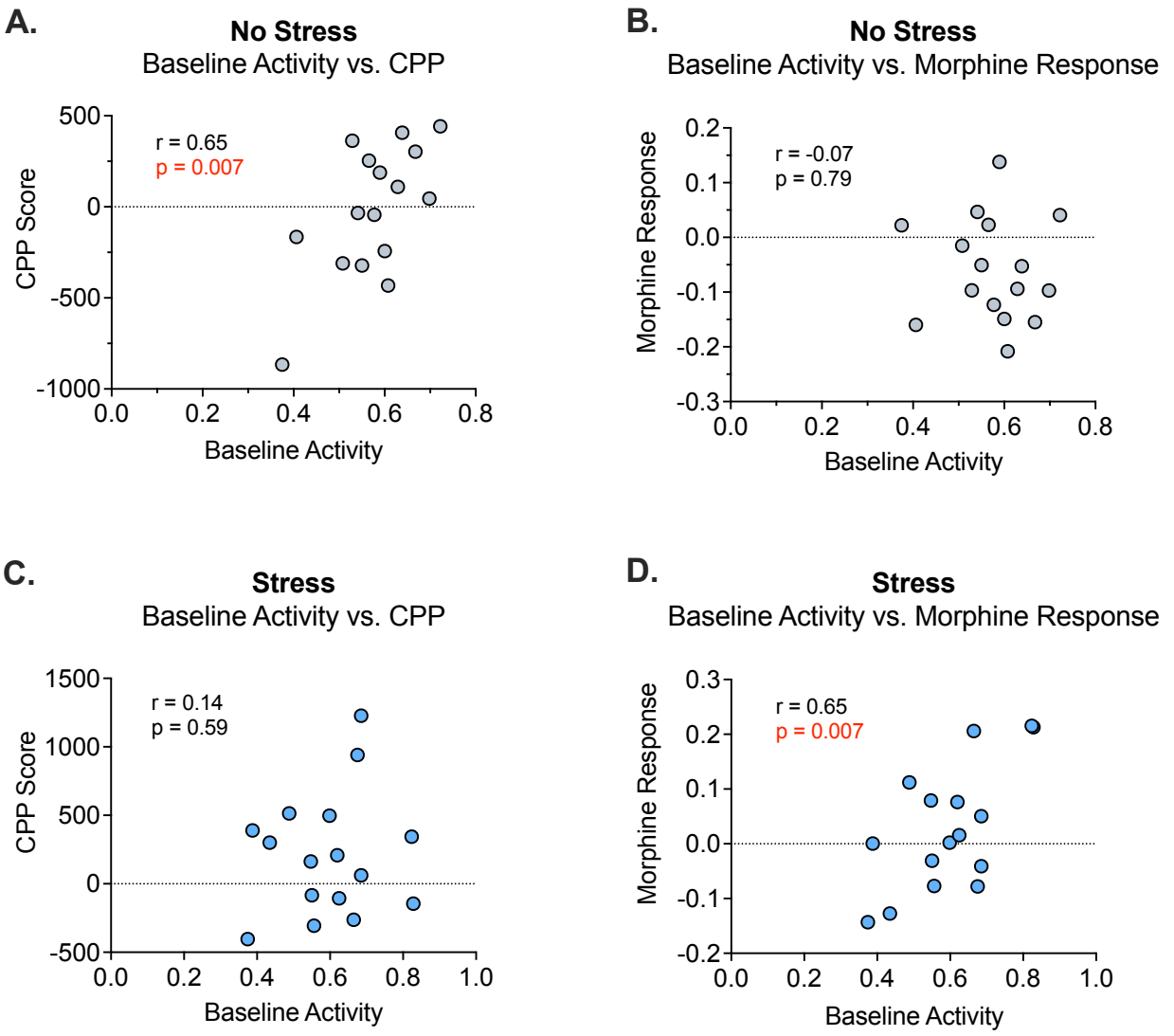


Figure 2.4. The relationship between baseline locomotor activity and CPP scores or morphine response depended on stress history following 1 trial CPP. A. Relationship between baseline activity (i.e. locomotor activity during the first time in the CPP chamber) and CPP scores in the no stress group. **B.** Relationship between baseline activity and morphine-induced activity (normalized by subtracting vehicle-induced activity) in the no stress group. **C.** Relationship between baseline activity and CPP scores in the stress group. **C.** Relationship between baseline activity and morphine-induced activity in the stress group.

Experiment 3: Stress x 1 trial naltrexone "CPA"

In Experiment 3, we investigated whether stress impacts the response to subthreshold (i.e. 1 trial) opioid antagonist naltrexone to produce a CPA (Figure 2.5A). Rats were trained with naltrexone at doses of either 1 mg/kg or 10 mg/kg. During training, naltrexone administration induced moderate hypolocomotion (compared to vehicle administration), but there were no group differences in activity scores (Figure 2.5B; see Table 2.3 for full statistical summary of Experiment 3). Unexpectedly, postconditioning testing revealed that the stress-1 mg group displayed a *preference* for the naltrexone-paired side ($p<0.05$) (Figure 2.5C). No other groups exhibited a preference or aversion for the naltrexone-paired side. A two-way ANOVA revealed a Dose x Stress interaction ($p<0.05$), indicating that the stress-1 mg/kg group exhibited significantly higher preference scores compared to both the no stress-1 mg/kg group ($p<0.05$) and the stress-10 mg/kg group ($p<0.05$).

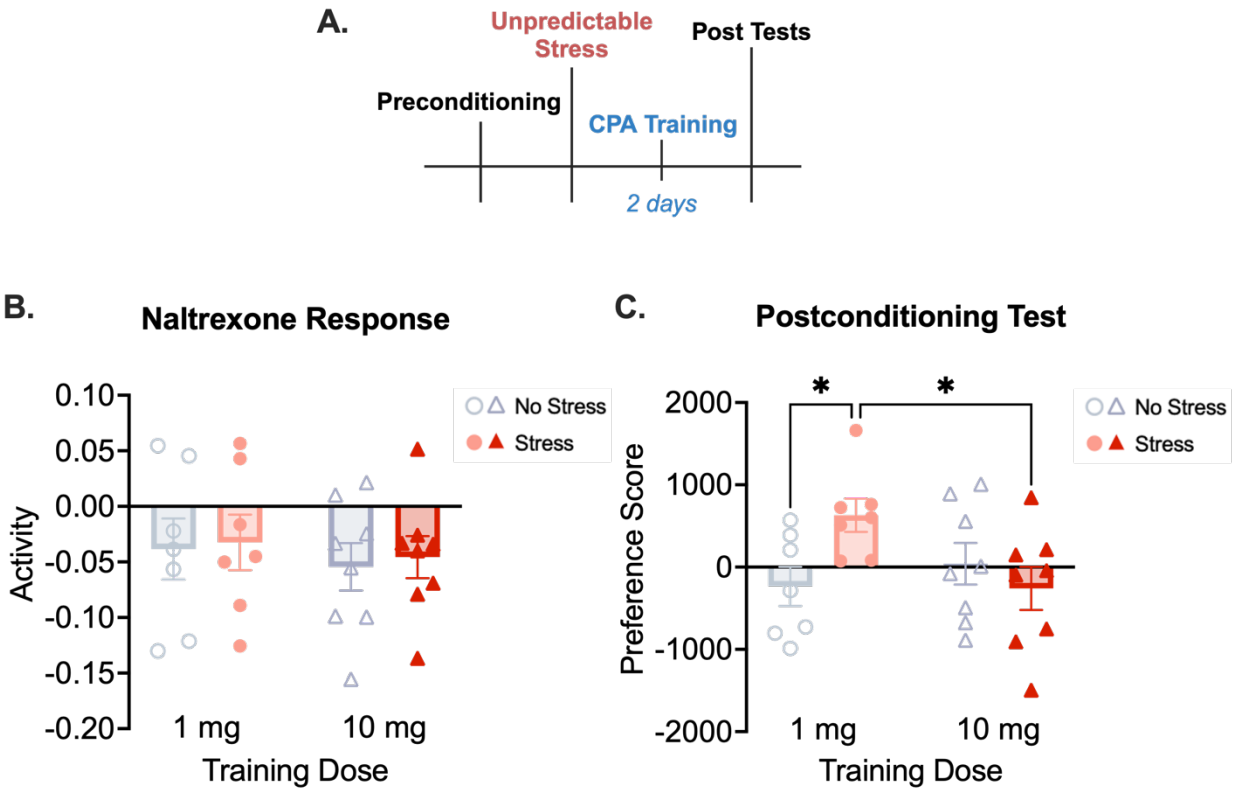


Figure 2.5. Unpredictable stress produces a CPP to low dose but not high dose naltrexone. A. Experiment 3 schematic. Rats underwent unpredictable stress or acted as a no stress control. The next day, rats underwent 1 trial "CPA" training with either 1 mg/kg or 10 mg/kg naltrexone, s.c. used as the training dose. After CPP training rats underwent 1 day of postconditioning testing (2 tests within the day). **B.** Naltrexone-induced activity (subtracting vehicle-induced activity) during CPP training. **C.** Preference scores from postconditioning testing. Postconditioning preference scores were averaged across the two tests in the day and then calculated as a comparison to baseline preconditioning preference scores. Data represent means ± s.e.m. ANOVA Post-hoc comparison denoted with * (**p**<0.05). Statistical analyses can be found in Table 2.3.

Table 2.1. Summary of statistical results from Experiment 1

Figure	Statistical Test	Results		
Figure 2.1B	Three-way repeated measures ANOVA	Training session	$F_{2.6,53.8}=15.83$	$p<0.0001$
		Dose	$F_{1,21}=9.13$	$p<0.01$
		Stress	$F_{1,21}=2.92$	$p=0.10$
		Session x Dose	$F_{3,63}=0.16$	$p=0.92$
		Session x Stress	$F_{3,63}=1.28$	$p=0.29$
		Dose x Stress	$F_{1,21}=1.87$	$p=0.19$
		Session x Dose x Stress	$F_{3,63}=0.76$	$p=0.52$
Figure 2.1C	Three-way repeated measures ANOVA	Training Dession	$F_{2.60,54.58}=2.71$	$p=0.06$
		Dose	$F_{1,21}=9.13$	$p=0.59$
		Stress	$F_{1,21}=0.03$	$p=0.87$
		Session x Dose	$F_{3,63}=1.16$	$p=0.33$
		Session x Stress	$F_{3,63}=0.17$	$p=0.33$
		Dose x Stress	$F_{1,21}=1.22$	$p=0.28$
		Session x Dose x Stress	$F_{3,63}=1.41$	$p=0.25$
Figure 2.1D	Two-way ANOVA	Dose	$F_{1,21}=10.41$	$p<0.01$
		Stress	$F_{1,21}=1.29$	$p=0.27$
		Stress x Dose	$F_{1,21}=5.35$	$p<0.05$
Figure 2.1E	Two-way repeated measures ANOVA	Drug Treatment	$F_{1,11}=0.14$	$p=0.72$
		Stress	$F_{1,11}=0.02$	$p=0.90$
		Drug x Stress	$F_{1,11}=3.92$	$p=0.07$
Figure 2.2A	Two-way ANOVA	Dose	$F_{1,21}=0.01$	$p=0.91$
		Stress	$F_{1,21}=0.17$	$p=0.69$
		Stress x Dose	$F_{1,21}=0.001$	$p=0.98$
	One-sample t tests	No Stress – 2.5 mg/kg	$t_4=2.09$	$p=0.11$
		Stress – 2.5 mg/kg	$t_7=2.79$	$p<0.05$
		No Stress – 10 mg/kg	$t_5=2.07$	$p=0.09$
		Stress – 10 mg/kg	$t_5=1.89$	$p=0.12$
Figure 2.2B	Two-way repeated measures ANOVA: 2.5 mg/kg	Testing Day	$F_{1.7,19}=0.71$	$p=0.49$
		Stress	$F_{1,11}=0.004$	$p=0.95$
		Testing Day x Dtress	$F_{2,22}=1.15$	$p=0.33$
Figure 2.2C	Two-way repeated measures ANOVA: 10 mg/kg	Testing Day	$F_{1.7,17.3}=0.92$	$p=0.41$
		Stress	$F_{1,10}=0.002$	$p=0.97$
		Testing Day x Stress	$F_{2,20}=1.76$	$p=0.20$
Figure 2.2D	Two-way ANOVA	Dose	$F_{1,21}=0.56$	$p=0.46$
		Stress	$F_{1,21}=0.01$	$p=0.91$
		Stress x Dose	$F_{1,21}=0.03$	$p=0.87$

Table 2.1 (cont.). Summary of statistical results from Experiment 1

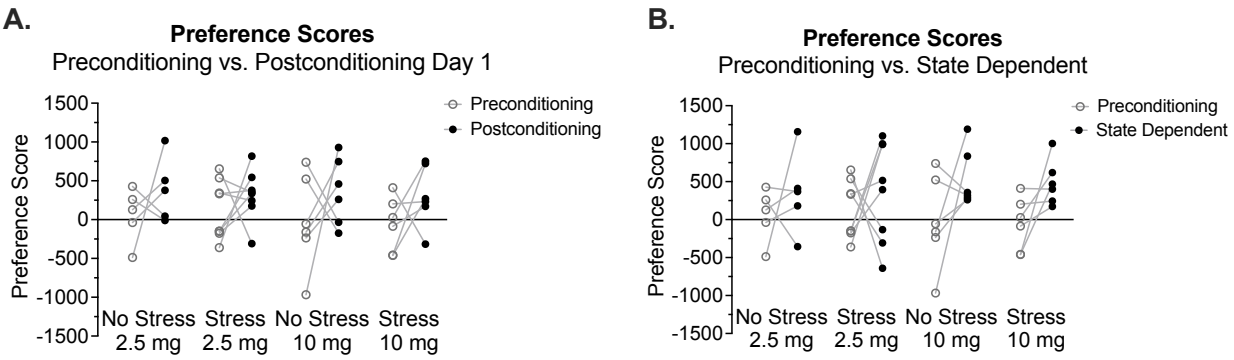
Figure 2.2D	One-sample t tests	No Stress – 2.5 mg/kg	t_4=1.45	p=0.22
		Stress – 2.5 mg/kg	t_7=1.56	p=0.16
		No Stress – 10 mg/kg	t_5=3.46	p<0.05
		Stress – 10 mg/kg	t_5=3.98	p<0.05

Table 2.2. Summary of statistical results from Experiment 2

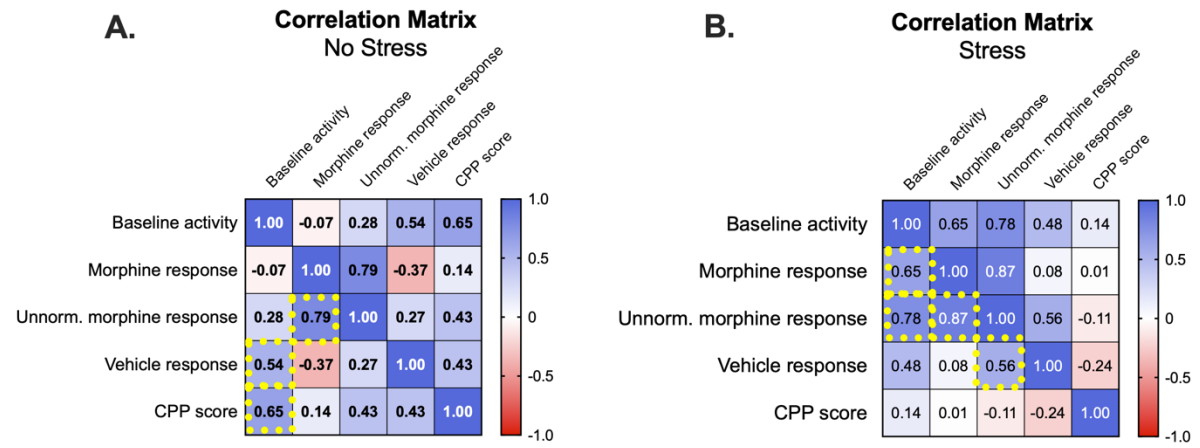
Figure	Statistical Test	Results		
Figure 2.3A	Unpaired t test	Stress x No Stress	t_{30}=2.36	p<0.05
Figure 2.3B	Two-way repeated measures ANOVA	Drug Treatment	$F_{1,30}$=0.60	p=0.45
		Stress	$F_{1,30}$=0.84	p=0.37
		Drug x Stress	$F_{1,30}$=5.57	p<0.05
Figure 2.3C	One-sample t tests	No Stress	t_{15}=0.21	p=0.83
		Stress	t_{15}=1.87	p=0.08
	Unpaired t test	Stress x No Stress	t_{30}=1.59	p=0.12
Figure 2.3D	One-sample t tests	No Stress	t_{15}=0.16	p=0.87
		Stress	t_{15}=2.50	p<0.05
	Unpaired t test	Stress x No Stress	t_{30}=1.41	p=0.17

Table 2.3. Summary of statistical results from Experiment 3

Figure	Statistical Test	Results		
Figure 2.5A	Two-way ANOVA	Dose	$F_{1,26}$=0.40	p=0.53
		Stress	$F_{1,26}$=0.10	p=0.75
		Dose x Stress	$F_{1,26}$=0.004	P=0.95
Figure 2.5B	Two-way ANOVA	Dose	$F_{1,26}$=1.34	p=0.26
		Stress	$F_{1,26}$=1.58	p=0.22
		Stress x dose	$F_{1,26}$=5.71	p<0.05
	One-sample t tests	No Stress – 1 mg/kg	t_6=0.99	p=0.36
		Stress – 1 mg/kg	t_6=3.12	p<0.05
		No Stress – 10 mg/kg	t_7=0.16	p=0.87
		Stress – 10 mg/kg	t_7=0.99	p=0.36



Supplemental Figure 2.1. Comparison of preconditioning vs preference scores for Experiment 1. A. Comparison of preconditioning preference scores vs. CPP scores from day 1 of postconditioning. **B.** Comparison of preconditioning preference scores vs. state dependent preference scores.



C. **Correlation Matrix - p values**
No Stress

	Baseline activity	Morphine response	Unnorm. morphine response	Vehicle response
Baseline activity				
Morphine response	0.788			
Unnorm. morphine response	0.298	0.0002		
Vehicle response	0.030	0.162	0.307	
CPP score	0.007	0.599	0.097	0.095

D. **Correlation Matrix - p values**
Stress

	Baseline activity	Morphine response	Unnorm. morphine response	Vehicle response
Baseline activity				
Morphine response	0.007			
Unnorm. morphine response	0.0004	0.00001		
Vehicle response	0.059	0.775	0.024	
CPP score	0.594	0.969	0.687	0.375

Supplemental Figure 2.2. Correlation matrices from Experiment 2. Pearson r values from correlation matrix analysis in the no stress group (**A.**) and stress group (**B.**) examining the relationship between baseline activity (i.e. locomotor activity during the first time in the CPP chamber), morphine response (normalized by subtracting vehicle-induced activity from morphine-induced activity), unnormalized morphine response, vehicle response, and CPP score. Correlations that reached statistical significance ($p<0.05$) are denoted in a yellow dotted-line box. **C.** p values from correlation matrix for the no stress group. **D.** p values from correlation matrix for the stress group. Correlations that reached statistical significance are denoted with red lettering.

2.5 Discussion

In these presented set of experiments, we investigated whether unpredictable stress would sensitize opioid agonist reward learning, opioid antagonist aversive learning and opioid-induced locomotion. In Experiments 1 and 2, we tested the impact of unpredictable stress on 4 trial and 1 trial morphine CPP, respectively. In Experiment 3, we tested the impact of unpredictable stress on 1 trial opioid antagonist naltrexone-induced CPA.

The first major finding was that unpredictable stress sensitized the locomotor response to the first experience with a 2.5 mg/kg dose of morphine. This result was replicated across 2 studies, highlighting the robustness of this finding. While 2.5 mg/kg morphine produced slight depressive effects in unstressed rats, stress either failed to produce this hypolocomotion (Experiment 2) or produced a hyperlocomotor response (Experiment 1). This sensitized locomotor response in the stressed rats was specific to the first experience with morphine, although there was a trend for an effect of stress across training trials. Morphine-induced activity additionally significantly increased across training sessions, irrespective of group or training dose. Of note, morphine-induced activity was normalized to vehicle-induced activity for each training trial, and this increase in the morphine response is likely driven by a reduction in vehicle-induced activity across days. One interpretation of these data is that while rats habituated their locomotion to vehicle injections across training trials, they failed to habituate their locomotor response to morphine injections. Brice-Tutt et al., reported similar locomotor results during oxycodone CPP training, with rats demonstrating habituation to saline-induced locomotion while oxycodone-induced locomotion remained the same across trials[138]. Behavioral sensitization to repeated opioid administration has been consistently shown in rats across a large range of doses and is blocked by co-administration of naltrexone, suggesting that mu opioid receptors are involved in this sensitization[139–141].

This evidence of stress potentiating the locomotor response to morphine adds to the literature of stress cross-sensitizing with opioid-induced locomotion. Other shock models have found sensitized opioid-induced locomotion but these studies involved a greater duration of days and/or cumulative number of shocks[105,106]. This present finding confirms that a less intense stressor (15 footshocks in 90 min) is sufficient to produce this locomotor sensitization. These results also parallel previous findings that chronic variable stress sensitizes the locomotor response to low dose (1.5 mg/kg i.p.) morphine but not higher doses (5 mg/kg and 10 mg/kg) of morphine[108]. Multiple mechanisms may drive this stress-induced locomotor sensitization, including sensitization of the HPA axis and the dopaminergic system, as outlined in Chapter 1[111,113,131]. Opioid-induced locomotion has also shown to have dopamine independent mechanisms[142,143]. For instance, there is evidence that muscarinic input to ventral tegmental area (VTA) dopaminergic neurons mediate opioid-induced locomotion and dopamine release in mice[144]. The cholinergic system likewise has also been shown to be involved in opioid reward[145]. Another proposed dopamine-independent mechanism of opioid-induced hyperlocomotion is mu receptor-D_1 receptor heterodimers formed in the striatum[146]. Finally, the orexinergic receptors in the VTA have also shown to be involved in morphine behavioral sensitization, including stress-induced sensitization[147,148].

While unpredictable stress sensitized opioid-induced locomotion, it did not have a meaningful impact on opioid CPP. In Experiment 1, only the stress-2.5 mg group displayed a significant preference for the opioid-paired side after 4 trials of CPP training. However, this was likely due to a smaller sample size in the other groups (n=5-6) rather than a true effect of stress; the rest of the groups' CPP scores were positive and there were no group differences. It is possible that unpredictable stress did not impact morphine CPP in Experiment 1 due to a ceiling effect in preference. Experiment 2 consequently aimed to see if stress would impact a subthreshold CPP (1

trial). As expected, unstressed rats did not show preference for the opioid-paired side during the postconditioning or state dependent tests. There were some indications that unpredictable stress impacted opioid reward learning, as demonstrated with a trend for preference during the postconditioning test and significant preference during the state dependent test. Given the substantial sample size (n=16/group) and lack of differences between the stress and no stress groups, the overall impact of stress on opioid reward can only be considered moderate. Similar null findings using the SEFL model and opioid CPP were found in mice[149]. While the unpredictable stressor used in SEFL is useful for capturing stress-induced behavioral sensitization to morphine, it does not seem like it is an appropriate stressor to induce stress-enhanced reward learning, as shown with other acute stressor models (e.g. learned helplessness[83]). Notably, the learned helplessness model inversely does not enhance future contextual fear learning, demonstrating that greater shock volume doesn't necessarily translate to greater impacts on associative learning[150].

Though stress has been shown to separately enhance both opioid reward and opioid-induced locomotion, less is known about whether there is a relationship between the two. The larger sample size in Experiment 2 allowed for correlation analyses between opioid CPP and locomotor activity scores. There was no relationship between CPP scores and normalized morphine-induced activity, although there was a slight relationship between CPP scores and unnormalized morphine-induced activity in the no stress group. On the other hand, baseline activity (i.e. locomotion in a novel environment) was predictive of subsequent morphine-related behavior depending on the stress history. Higher baseline activity was associated with higher CPP scores in the no stress group, while higher baseline activity was associated with higher morphine-induced activity in the stress group. The High/Low responder model of addiction proposes that novelty-induced locomotion predicts individual vulnerability to substance use disorders (SUDs)[151].

Novelty-induced locomotion is most commonly thought to model sensation-seeking in humans, which has been implicated with SUDs[152]. Evidence for the High/Low responder model is substantial with psychostimulants, with more mixed results in the opioid field[153]. There is some evidence that (delayed) novelty-induced locomotion is predictive of opioid CPP[154]. Others have shown that it may be *novelty-seeking* specifically, and less so spontaneous locomotor activity in a novel environment, that is predictive of opioid CPP[155,156]. An association between novelty-induced and morphine-induced locomotion has likewise been shown[157] but it was previously unknown how stress may impact these relationships. It is unclear why a history of stress decoupled the relationship between baseline activity and opioid CPP, while also producing a relationship between baseline activity and morphine-induced activity. One clue is alterations in the HPA axis, as it has been previously shown that the relationship between novelty-induced locomotion and morphine-induced locomotion is dependent on glucocorticoid signaling[157]. Stress did not largely impact opioid CPP using the SEFL model. It is possible that using a more effective model of stress-enhanced opioid reward learning would reveal a stronger relationship between the stress-induced sensitization of opioid-induced locomotion and opioid reward.

The most surprising finding was from Experiment 3, in which the impact of stress on naltrexone-induced place conditioning was investigated. Similar to Experiment 2, the goal of Experiment 3 was to administer a subthreshold conditioning procedure to test if unpredictable stress would exacerbate the avoidance learning to an opioid receptor antagonist, given that naltrexone is typically considered aversive. While stress had no impact on high dose (10 mg/kg) naltrexone place conditioning, stress induced a CPP in the low dose (1 mg/kg) naltrexone group. Although this was an unexpected result, there is some evidence in humans that low dose naltrexone, at 1-5 mg per day, can be therapeutic in disease states. Low dose naltrexone (LDN) is increasingly

being used as an off-label treatment for chronic pain-associated conditions, such as Multiple Sclerosis, Crohn's disease and fibromyalgia[158]. Beyond reducing pain symptoms, LDN has shown to increase quality of life and reduce anxiety in individuals with Multiple Sclerosis[159,160]. While it is not fully understood why LDN has therapeutic effects, it may be due to the inhibitory effect of naltrexone on Toll-like receptor 4 (TLR4) signaling[161–163]. Activation of TLR4 results in activation of NF-κB transcription factor and subsequent production of pro-inflammatory cytokines such as interleukin (IL)-1, IL-6, and tumor necrosis factor-α. Thus, LDN may be reducing the inflammatory state in these patients, as confirmed in a small single-blind trial of LDN treatment in individuals with Fibromyalgia[164]. Interestingly, IL-1 has shown to be implicated in the SEFL rodent model and blockade of IL-1 signaling prevents induction of SEFL[165,166].

An important limitation of these studies is that they were all conducted in male rodents, and it is unknown if these results generalize to females. This was an oversight and particularly unfortunate given that most previous studies investigating stress cross-sensitization with opioids have been conducted in males[167]. Women are more likely to have prescription opioid use problems and more severe PTSD symptoms[168]. Moreover, women are more likely than men to have comorbid OUD/opioid use problems and PTSD[168,169]. There is evidence that women have a greater degree of opioid regulation over the HPA axis, perhaps explaining the stronger link between OUD and PTSD in women[170]. Preclinically, one group found that chronic immobilization stress increased oxycodone CPP in females as compared to males, likely due to sexually dimorphic impacts of chronic stress on hippocampal functioning[171,172]. Ultimately, more research needs to be done to understand why females are more susceptible to OUD-PTSD comorbidities.

Overall, this body of work suggests that the stress induced by the SEFL model is useful for investigating some aspects of cross-sensitization with opioids, specifically opioid-induced

locomotion. We found that novelty-induced baseline locomotion may be used to determine individual vulnerability for future opioid preference and behavioral sensitization. Additionally, we have provided the first preclinical evidence supporting the positive mental health outcomes seen from off-label low dose naltrexone treatment in clinical settings. Nevertheless, the SEFL model ultimately did not extend to enhance future opioid reward learning, limiting its ability to investigate OUD-PTSD comorbidities.

2.6 Future Directions

Mechanisms driving stress-enhanced morphine behavioral sensitization:

It is unknown whether the observed stress-enhanced opioid behavioral sensitization is driven by dopamine dependent or dopamine independent mechanisms. Stress has previously been shown to sensitize the dopaminergic response to opioids and there is evidence that blocking D_1 and D_2 receptors blocks restraint stress-induced sensitization of the opioid locomotor response[113,131]. On the other hand, the cholinergic and orexinergic systems have been shown to be dopamine independent mechanisms driving opioid behavioral sensitization[144,148]. A first step to investigating the mechanisms driving stress-enhanced behavioral sensitization in the SEFL model is to first determine the impact of stress on opioid-induced dopamine responses in the NAc. If unpredictable stress does in fact sensitize the dopaminergic response to opioids, it would be valuable to next test the necessity of D_1 and D_2 receptors on stress-enhanced opioid behavioral sensitization.

Further characterization of the SEFL model using the "RAM" virus:

It would additionally be useful to further characterize how the SEFL model induces sensitization of future fear learning, an effect that is likely largely driven by stress-induced neural

plasticity the basolateral amygdala (BLA). More specifically, the unpredictable stressor in SEFL causes a long-lasting upregulation of GluA1 protein levels within the BLA[67,68]. SEFL is dependent on BLA glucocorticoid receptor activation[68], and both SEFL and associated increases of BLA GluA1 are prevented by inhibition of glucocorticoid synthesis[67]. Therefore, stress-induced neuroadaptations in BLA neurons likely drive the SEFL model.

In order to visualize BLA neurons that are activated by unpredictable stress, I designed a novel AAV (AAV9-RAM-d2TTA:TRE-hM4Di-mCherry-WPREgamma, "RAM" virus) from an existing backbone[173]. The RAM virus contains a synthetic promoter that is sensitive to c-fos and Npas4, immediate early genes that are associated with neuronal activation. The RAM virus is under the repressive control of the antibiotic Doxycycline (Dox), such that when animals are placed on a Dox diet, tagging of neurons is inhibited. Replacement of the Dox diet with regular chow diet allows for activity-dependent expression of tetracycline transactivator (tTA), which in turn binds to the tTA-responsive element promotor, driving the effector gene cassette.

Tagging of neuronal activity has largely been conducted in genetic mouse lines[174–179]. The RAM virus allows for viral tagging in rats, a model species that has far less been used for *in vivo* activity-dependent labelling of neurons[173,180]. The advantage of the RAM virus as compared to another available activity-dependent viral vector ("ESARE"), is that it allows for robust expression of labelled cells relatively quickly (within 24 hours) and more selectively. When compared to the ESARE virus, the RAM virus had higher fold induction due to lower basal activity[173]. While tagging of cells can only be achieved once animals have cleared Dox completely (i.e., 4 days in rats), the RAM virus has been expertly designed to ensure low background signaling. This included incorporation of d2tTA, a destabilized version of tTA that leads to less accumulation and therefore less background expression outside the designated tagging window.

I used an existing empty AAV-RAM plasmid with a multiple cloning site (Addgene Plasmid #63931) to design the new virus that includes hM4Di-mCherry as the effector gene. Additionally, the woodchuck hepatitis virus post-transcriptional regulatory element (WPRE) was reduced to only its gamma subunit. This was because inclusion of the entire WPRE sequence would have made the genome size of the AAV too large to effectively package, and the gamma subunit alone aids in expression of the effector gene[181]. Demonstrating feasibility, the virus was used to successfully tag BLA neurons with a fluorescent mCherry label in both rats and mice (Figure 2.7). Notably, even though the tagging window was relatively long in rats (4 days), there was minimal neuronal tagging in the home cage control.

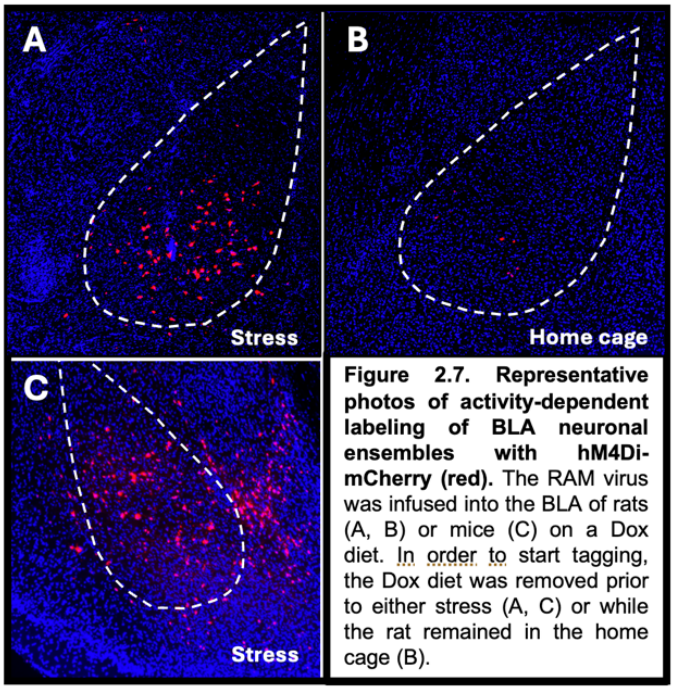
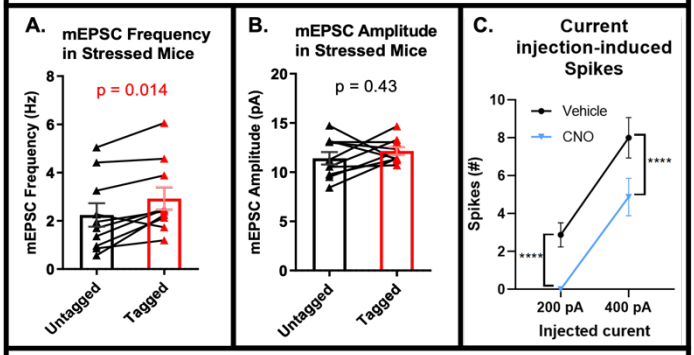


Figure 2.7. Representative photos of activity-dependent labeling of BLA neuronal ensembles with hM4Di-mCherry (red). The RAM virus was infused into the BLA of rats (A, B) or mice (C) on a Dox diet. In order to start tagging, the Dox diet was removed prior to either stress (A, C) or while the rat remained in the home cage (B).

In order to further validate this novel virus and test the functional impact of unpredictable stress, the RAM virus was next used to tag BLA neurons responsive to unpredictable stress to measure the electrophysical properties of those neurons. Whole-cell voltage clamp recordings were conducted on the tagged neurons to measure miniature excitatory postsynaptic currents (mEPSCs). We found increased mEPSC frequency (Figure 2.8A), but not amplitude (Figure 2.8B), in tagged vs. untagged BLA neurons. We next demonstrated that clozapine-N-oxide (CNO) can successfully inhibit neuronal excitability in tagged BLA cells (Figure 2.8C) while having no effect on untagged cells (not shown). Taken together, these data suggest that unpredictable stress

promotes excitatory input onto BLA neurons, likely through either increased presynaptic neurotransmitter release and/or number of synapses synaptic transmission. Additionally, our findings suggest we can use the RAM virus to dissect the functional difference between different neuronal populations based on their prior history of activity. It has been previously shown that neurons with higher excitability are more likely to be incorporated into activity-dependent labeling[182]. Future work will include an important control involving administering an aversive stimulus that is not enough to induce SEFL but produces similar BLA neuronal activation as the unpredictable stressor. Tagging of these aversive-responsive cells and comparing their excitability will be useful to confirm the enhanced excitatory input on tagged stress-responsive neurons is above and beyond excitability generally found in tagged aversive-responsive neurons. Ultimately, this virus is a useful tool for characterizing stress-responsive neurons and testing the role of these neurons in expression of SEFL. Such work will shed light on how alterations in BLA function as a result of traumatic stress can induce susceptibility to developing PTSD.

Figure 2.8. Whole-cell voltage clamp recordings in the BLA neurons of mice that experienced activity-dependent labeling during unpredictable stress 1 day prior



A. mEPSCs frequency was higher in tagged vs. untagged neurons. B. mEPSC amplitude did not differ between tagged vs. untagged neurons. C. CNO successfully reduced the number of current-induced spikes in tagged neurons

Chapter 3: The impact of chronic neuropathic pain on kappa opioid receptor agonist-induced reinstatement

3.1 Abstract

Chronic neuropathic pain is a multidimensional condition that is highly co-morbid with Opioid Use Disorder (OUD) and manifests differently between males and females. The dynorphin/kappa opioid receptor (KOR) system is an emerging target for treating anhedonia associated with chronic pain and stress-induced relapse of drug seeking. Recent findings suggest males are more susceptible than females to the functional upregulation of the dynorphin/KOR system induced by chronic pain, specifically in the nucleus accumbens (NAc), ventral tegmental area (VTA) and basolateral amygdala (BLA). The dynorphin/KOR system has been shown to regulate stress-induced reinstatement of drug self-administration and conditioned place preference (CPP). Given that males appear to have a greater upregulation of the dynorphin/KOR system in mesolimbic regions, we hypothesized that males would be more susceptible to KOR agonist-induced reinstatement. All studies used a peripheral nerve injury neuropathic pain model in C57BL/6J adult male and female mice. Using an oxycodone CPP assay, we found no difference in postconditioning CPP scores or extinction rates as a function of sex or pain. Unexpectedly we found that the KOR agonist U50,488 (5 mg/kg, i.p.) induced reinstatement of oxycodone CPP in chronic pain *female* but not male mice. Reinstatement of oxycodone CPP was not evident in the sham mice of either sex. A preliminary follow-up study replicated these findings and indicated that this female-specific susceptibility of KOR-induced reinstatement is not driven by BLA KORs. Overall, these data provide mechanistic evidence of how chronic neuropathic pain-induced changes in the dynorphin/KOR system may drive susceptibility for future opioid misuse in a sex-dependent manner.

3.2 Introduction

Chronic pain and OUD are highly comorbid, with estimates that 50-64% of individuals with OUD have chronic pain[6,7]. Chronic pain has multiple dimensions, including sensory, affective and cognitive appraisal components that are likely driven by distinct but converging neural pathways[59]. Pain-induced changes in the dynorphin/KOR system is increasingly recognized as a potential mechanism driving susceptibility for comorbid OUD/chronic pain[114,117–119]. Dynorphin is an endogenous opioid that when bound to its receptor, KOR, induces dysphoria, and depressive- and anxiety-like behaviors[116]. Additionally, KORs mediates stress-induced reinstatement of opioid seeking and place preference, and KOR agonism alone can induce reinstatement of drug (i.e. cocaine and alcohol) seeking[120–125]. However, it is unknown whether KOR agonism can reinstate opioid CPP, and what role chronic pain may play in stress-induced reinstatement of opioid CPP.

Recent findings indicate that chronic neuropathic pain induces an upregulation of the dynorphin/KOR system in mesolimbic regions, such as the VTA, NAc and BLA, that are especially apparent in males[129,130]. It is yet to be determined whether this sexual dimorphism is due to inherent sex differences in the dynorphin/KOR system or divergent adaptation pathways in response to neuropathic pain. In healthy control humans, one study found that males have more available KOR than females[183]. On the other hand, KOR agonist-stimulated [^{35}S]GTPγS binding was found to not be different between rodent males and females in a number of brain regions, including the NAc and amygdala[184]. Multiple preclincial findings indicate female rodents are more resistant to the depressive effects of KOR agonism with U50,488[185–188]. Females additionally have been found to be less impacted by the antinociceptive effects of KOR agonism although this sex-difference is not consistently observed, especially in humans[186,188–196]. Finally, females are more resistant to KOR-mediated stress-induced analgesia[197]. While the literature on the dynorphin/KOR system is

complex, there are numerous studies suggesting that male are more sensitive to the effects of KOR agonism.

Given the current knowledge on sexual dimorphism in the dynorphin/KOR system, we hypothesized that chronic neuropathic pain males would exhibit greater susceptibility than females to reinstatement of oxycodone place preference induced by KOR activation. Following induction of CCI injury, mice were subjected to 3 trial oxycodone CPP training, extinction and reinstatement testing with systemic administration of the KOR agonist, U50,488. The BLA is necessary for CPP acquisition, extinction and stress-induced reinstatement of drug seeking[198–200]. Additionally, BLA KORs have shown to mediate stress-induced nicotine CPP reinstatement[201]. We consequently further tested whether BLA KORs are necessary for systemic KOR agonist-induced reinstatement.

3.3 Methods

Subjects

Subjects were 8-10 weeks adult male and female c57BL/6J mice (Experiment 1, N = 43; Experiment 2, N = 15) purchased from Jackson Labs. Mice were group-housed with 2-4 mice per cage in standard Plexiglas cages with nesting enrichment and ad libitum access to food and water. The colony room was maintained at a 12:12 hr light/dark reverse schedule and all behavioral testing was run during the dark phase. Each animal had a cage mate assigned to the same pain experimental group. Subjects were assigned to experimental conditions in a randomized block design so that the running of subjects was counterbalanced across groups. Each experiment was completed using multiple cohorts, resulting in internal replications. Each cohort was balanced with respect to experimental groups. Chancellor's Animal Research Committee at UCLA approved all animal testing procedures.

All mice were handled for 3-5 days, for 1-2 min per day, prior to start of experimentation. All mice were allowed to acclimate to behavioral testing rooms for at least 15 min prior to any behavioral testing or apparatus habituation. Males were always tested separately before females and returned to the vivarium housing room before bringing females to behavioral testing room. All testing equipment was cleaned with a 20% Vimoba solution between testing different sexes.

Chronic Constriction Injury (CCI)

Prior to surgery, mice received ~30 mg/kg of acetaminophen orally and then were anesthetized with gaseous isoflurane (3% induction and ~2.0 – 2.5% maintenance in O2). After removing hair from the area and swabbing the surgical site with alcohol and iodine, an ~1 cm incision was made to the upper left hind leg of each mouse. For the CCI groups, the muscle was carefully torn using scissors and a 2 mm cuff (polyethylene tubing, PE20) was wrapped around the sciatic nerve to constrict the nerve as previously described[129]. Mice in the sham group received similar anesthesia and pharmaceutical treatment but following the skin incision, they were immediately sutured (no muscle tearing or cuff insertion). Each cage received ~12 mg of acetaminophen diluted in 5 mL Ensure and an enrichment dome was provided to each cage post-operatively. Following surgery mice remained group housed.

Von Frey Testing

To measure the mechanical withdrawal thresholds, Von Frey stimulation was conducted in which monofilaments of various forces were applied to the plantar surface of the left (ipsilateral) hind paw. The Von Frey apparatus consisted of individual Plexiglas compartments placed over a raised mesh platform. Before the first test, mice underwent a 3-day habituation period, involving

a 10 min exposure to the Von Frey apparatus each day. On the last day of habituation, all mice received 1 poke with a 0.4 g strength filament to left hind paw to further acclimate them.

On days of Von Frey testing, mice were placed in the Von Frey apparatus to habituate for 10+ minutes. Mice were tested using the "up-down" Von Frey method [202,203], which determines the mechanical force used to cause a withdrawal response in 50% of the animals. First, mice were tested on the left hind paw with a 0.4g filament. If there was no paw withdrawal response, a filament with a stronger force was applied; if there was a withdrawal response, a weaker filament was applied. The filament strengths used were as follows: 0.008 g, 0.04 g, 0.07 g, 0.16 g, 0.4 g, 0.6 g, 1 g and 2 g. Testing for each mouse concluded when either: 4 responses in the same direction occurred in a row, or 2 responses in opposite directions and 4 more responses after that in either direction were recorded. Mice were tested for their baseline mechanical threshold sensitivity prior to injury induction and then 7-9 days, 14 days and 21 days after injury.

Conditioned Place Preference Apparatus

The CPP procedure was conducted in a 3-chamber behavioral apparatus. The apparatus had two larger opaque square chambers (17.75 x 13.25 cm) connected by a smaller third intermediary ("neutral") rectangular chamber (12.25 x 10.5 cm). The neutral chamber extended out of the behavioral chamber and contained three clear Plexiglas walls. The larger chambers had distinct contextual features: one chamber had vertical black and white stripes and a squared wire floor insert, while the other chamber had black circles on a white background and a wire mesh floor insert. Plastic doors could be inserted in order to block the exit of the chambers and confine animals to either distinct chamber. The CPP procedure was conducted in a room separate from the mouse colony with red lighting. Locomotor activity was recorded with cameras arranged above the behavioral box that was measured with Anymaze software.

Conditioned Place Preference procedure:

The typical experimental design for the CPP experiments was typically as follows: habituation, preconditioning, CCI injury induction, training, postconditioning test, state-dependent test, extinction training, reinstatement test.

Habituation: The day after handling ended, animals were placed in the neutral chamber within the CPP apparatus and allowed to explore all 3 chambers for 15 min.

Preconditioning: The following day, animals again were exposed to the CPP apparatus for 30 min. The time spent in each chamber during this 2nd day of exposure was used to calculated baseline preferences and later postconditioning preference scores. The baseline preference score was used to determine which context the training drug was paired with. This pairing was assigned using an unbiased design to ensure that preconditioning preference for the drug-paired context was equalized across all experimental groups.

CCI surgery: Within 1 week of conducting preconditioning, mice next underwent CCI surgery or acted as shams (see above for further description).

Training: CPP training always began 7 days after CCI/cannulation surgery, when animals showed lower mechanical thresholds in the CCI groups. On the first day of training (training day 1a), mice were injected with either 3mg/kg oxycodone HCl i.p. or vehicle [0.1 M Phosphate Buffered Saline (PBS)]. Mice were then immediately confined to one context for 30 min. On the 2nd day of training (training day 1b), animals were administered the alternate treatment and were confined to the alternate context for 30 min. This 2-day training schedule constituted "1 trial" of CPP training; the number of CPP trials varied across experiments. The order of the training drug vs. vehicle administration was counterbalanced across groups for each experiment. During CPP training, average speed was measured, calculated as: (distance travelled/time). To measure the

hyperlocomotor effects of morphine and control for individual differences in activity, oxycodone-induced activity was normalized, calculated as the within subject difference between the activity response to oxycodone and saline for each mouse. Unnormalized oxycodone-induced average speeds and saline-induced average speeds were also presented.

Postconditioning Test: The day following training, mice were next tested for preference for the drug-paired chamber in a drug-free state. Mice were placed in the neutral chamber and allowed to access to all 3 chambers for 30 min. For the CPP postconditioning tests, the preference score was calculated as: [Time during Postconditioning (Drug-paired side) – Time during Postconditioning (vehicle-paired side)] – [Time during Preconditioning (Drug) – Time during Preconditioning (vehicle)]. For the CPP state dependent test and extinction, the preference score was calculated similarly (compared to preconditioning preference score).

Extinction training: Mice were injected with PBS (vehicle) and then placed in the neutral chamber and allowed access to all 3 chambers for 30 min. Extinction training ceased when none of the groups displayed preference for the drug-paired context. For Experiments 1 and 2 extinction training took 2 days.

Reinstatement: Reinstatement testing was conducted 3-5 days after extinction training ended. All mice were injected with KOR agonist trans-(±)-3,4-dichloro-N-methyl-N-(2-[1-pyrrolidinyl]-cyclohexyl) benzeneacetamide methanesulfonate (i.e. U50,488) at a dose of 5 mg/kg i.p, immediately prior to being placed in the neutral chamber and allowed access to all 3 chambers for 30 min (see below for specific methods for each experiment).

The reinstatement preference score was calculated as a comparison to the last day of extinction preference scores: [Time during Reinstatement (Drug-paired side) – Time during Reinstatement (vehicle-paired side)] – [Time during Last Day of Extinction (Drug) – Time during

Last Day of Extinction (vehicle)]. Given that preconditioning occurred 21 days+ prior to the reinstatement test, it was determined that the last day of extinction was a better representation of baseline preferences at this point of this experiment. Additionally, comparing preference scores to the last extinction day ensured that preference scores during the reinstatement were truly an increase above their extinction day preference scores. Nevertheless, reinstatement preference scores compared to preconditioning scores were also calculated to ensure that the pattern of behavior remained similar between the two calculations. During reinstatement testing, locomotor measures were also measured and presented as distance travelled.

Stereotaxic microinjection of atrans-(3R,4R)-dimethyl-4-(3-hydroxyphenyl) piperidine (JDtic)

For Experiment 2, JDtic was bilaterally infused directly into the BLA 1-2 days after extinction ended and 3 days prior to the reinstatement test. JDtic is a KOR antagonist with long lasting action (14+ days[204]), however the effects produced by JDTic are shorter lasting in chronic pain states[129]. Therefore, reinstatement testing occurred no later than 3 days following microinjection of JDtic. Post-operative mice were monitored closely to ensure they had recovered enough following surgery to engage in behavioral testing. Prior to microinjection of JDtic, mice received 5 mg/kg i.p. Carpofen (no acetaminophen). Mice were injected bilaterally (BLA coordinates: AP -1.3mm, ML ± 3.4, DV -5.3mm from Bregma) with JDtic (3 μg/0.2 μL per hemisphere) or vehicle (aCSF) at a rate of 0.1 μL/1 min. The glass pipette was left in place for 5 min after the injection to allow for drug diffusion into tissue. Each cage received ~12 mg of acetaminophen diluted in 5 mL Ensure post-operatively. Following surgery, mice remained group housed. Infusion placements were verified and are shown in Supplementary Figure 3.4.

Specific methods for Experiment 1: The impact of chronic neuropathic pain on oxycodone CPP and systemic KOR agonist-induced reinstatement

Following handling, mice underwent CPP habituation and preconditioning (Figure 3.1A). Mice next underwent a CCI injury (or acted as a sham). Following 7 days of recovery, mice underwent 3 trials of CPP training, a post-conditioning test, 2 days of extinction training and finally a reinstatement test. On reinstatement day, all mice were administered 5 mg/kg U50,488 i.p. in the colony room. Mice were transported to the behavioral room 15 min after KOR agonist treatment for CPP testing. After an additional 15 min in the CPP testing room (and 30 min after injection), mice were placed in the neutral chamber and allowed access to all 3 chambers for 30 min. Von Frey testing was conducted to ensure CCI mice experienced mechanical sensitivity throughout the duration of the experiment.

Specific methods for Experiment 2: Role of BLA KORs in chronic pain-enhanced CPP reinstatement

Following handling, mice underwent CPP habituation and preconditioning (Figure 3.3A). All mice next underwent CCI injury (no sham controls). Following 7 days of recovery, mice underwent 3 trials of CPP training, a post-conditioning test, and 2 days of extinction training. 1-2 days after extinction training ended, mice were microinjected with JDtic or aCSF in the BLA. No more than 3 days after JDtic BLA microinjections, reinstatement testing was conducted. Reinstatement testing occurred exactly as executed in Experiment 1 (i.e. all mice received 5 mg/kg U50,488 i.p. and 30 min later were tested for CPP reinstatement).

Drug administration

For CPP training, all mice received alternating Oxycodone HCl (Spectrum Chemical) dissolved in 0.1 M PBS or PBS i.p. injections. U50,488 (Tocris, dissolved in 0.9% saline) was

injected i.p. in Experiments 1 and 2. For Experiment 2, either aCSF or JDtic dissolved in aCSF was infused intracranially.

Statistical analysis and subject exclusions

All statistical analysis was conducted on Graphpad Prism (v10). ANOVAs were used to determine group differences with the level of statistical significance set at $p < 0.05$. The Geisser and Greenhouse correction for ANOVAs was used when the assumption of sphericity was violated. Occasionally during testing there were missed values due to procedural or video-tracking errors. Given that the repeated measures ANOVA is unable to accommodate missing values, a "mixed-effects analysis" was used in place of a 3-way ANOVA if there were any missing values in the analysis. For determining if there was place preference/aversion, one-sample t tests were conducted on the preference scores to test whether each group mean was significantly different from a hypothetical value of 0 (i.e. no preference). Post-hoc tests were conducted using Tukeys multiple comparisons test or Fisher's LSD. Mechanical withdrawal thresholds and CPP reinstatement preference scores were correlated using Pearson's correlation tests, with level of statistical significance set at $p < 0.05$.

One mouse was excluded from all results in Experiment 1 (CCI-female) due to being a statistical outlier during the reinstatement test. Additionally, in Experiment 1, a missed injection during the first day of extinction resulted in an accidental death in one of the mice (sham-male)– that mouse's cagemate was singly housed as a result and the data from both mice were excluded onwards in the experiment. Finally, two mice (CCI-females) were placed in the wrong box during reinstatement in Experiment 1– their data from that day only was excluded. For Experiment 2, two female mice were dropped after the postconditioning test due to their failure to show a CPP, thereby

rendering their inclusion in extinction training and reinstatement testing unnecessary. One mouse from the CCI-male JDtic group was removed due to infusion misplacement.

3.4 Results

Experiment 1: The impact of chronic neuropathic pain on oxycodone CPP and systemic KOR agonist-induced reinstatement

In this study, we set out to determine the impact of chronic neuropathic pain on oxycodone CPP and KOR agonist-induced reinstatement (see Figure 3.1A for experimental design and Table 3.1 for full statistical summary of Experiment 1). To verify that CCI decreased mechanical withdrawal thresholds, Von Frey testing was conducted throughout the experiment. Indeed, there was a Pain x Day interaction ($p<0.0001$) for withdrawal thresholds, driven by lower withdrawal thresholds in the CCI mice but not the sham mice in the weeks after surgery (Figure 3.1B). There was no difference between sexes in the magnitude of pain-induced withdrawal thresholds.

During CPP training, males exhibited greater hyperlocomotion to oxycodone administration than females ($p<0.05$), with no impact of chronic pain (Supplemental Figure 3.1). Following oxycodone CPP training, mice underwent a postconditioning test. All groups except for the sham-female group ($p=0.11$) exhibited a significant preference for the oxycodone-paired side ($p<0.05$ for the other groups) (Figure 3.1C). There were no group differences in preference scores during the postconditioning test. Following the establishment of an oxycodone CPP, mice underwent extinction training, comprising a vehicle injection followed by open access to all CPP chambers for 30 min (Figures 3.1D/E). Although no significant preference was observed in any of the groups during the first day of extinction, the CCI-female group exhibited a trend towards preference ($p=0.08$). By the second day of extinction, no groups displayed any preference. There were no group differences on either of the extinction days.

Following extinction, mice underwent a reinstatement test in which the KOR agonist, U50,488, was administered 30 min prior to open access to all CPP chambers for 30 min. Strikingly, only the CCI-females exhibited a reinstated preference for the oxycodone-paired side ($p<0.05$; Figure 3.2A). There additionally was a trend for a main effect of Pain for reinstatement preference scores ($p=0.07$). Here, reinstatement preference scores were calculated with respect to preference on the last extinction day rather than to the preconditioning day, given the considerate time elapsed since preconditioning testing (see methods for further explanation). Nevertheless, whether the reinstatement preference score was calculated relative to the preconditioning day or as a standalone measure without baseline comparison, the pattern of heightened preference in CCI-females persisted (Supplemental Figures 3.2A/B).

U50,488 can induce hypolocomotion in a sex-dependent fashion[185,186]. During reinstatement testing, females exhibited higher locomotion during the reinstatement test than males, irrespective of pain ($p<0001$; Supplemental Figure 3.1C). This higher locomotion in the females was present throughout the time course of the 30 min test ($p<0001$; Supplemental Figure 3.1D). The higher locomotion wasn't simply a result of overall increased activity in females; calculating locomotion scores during the reinstatement test as a within subjects comparison to the last day of extinction locomotion revealed the females were instead less resistant to the hypolocomotor effects of U50,488 ($p<0.05$; Figure 3.2B). Notably, it does not appear that males were so incapacitated by the U50,488 that they failed to move through the chambers, as shown by representative track plots of movement (Supplemental Figure 3.1E).

Finally, in order to determine whether mechanical threshold sensitivity induced by neuropathic pain was related to propensity for reinstatement, correlations between mechanical withdrawal thresholds and CPP reinstatement preference scores were conducted in the CCI groups.

In CCI-females, but not CCI-males, there was a significant negative correlation ($p<0.05$) between reinstatement preference scores and withdrawal threshold scores 7/9 days post-CCI injury (Figures 3.2C/D; see Supplemental Figure 3.3 for full correlation statistics). Of note, in CCI-males there was a slight trend ($p=0.095$) for a negative correlation between reinstatement preference scores and withdrawal threshold scores 14 days post-injury (Figure 3.1C). There was no relationship between postconditioning CPP scores and withdrawal threshold scores or postconditioning CPP scores and reinstatement preference scores (data not shown).

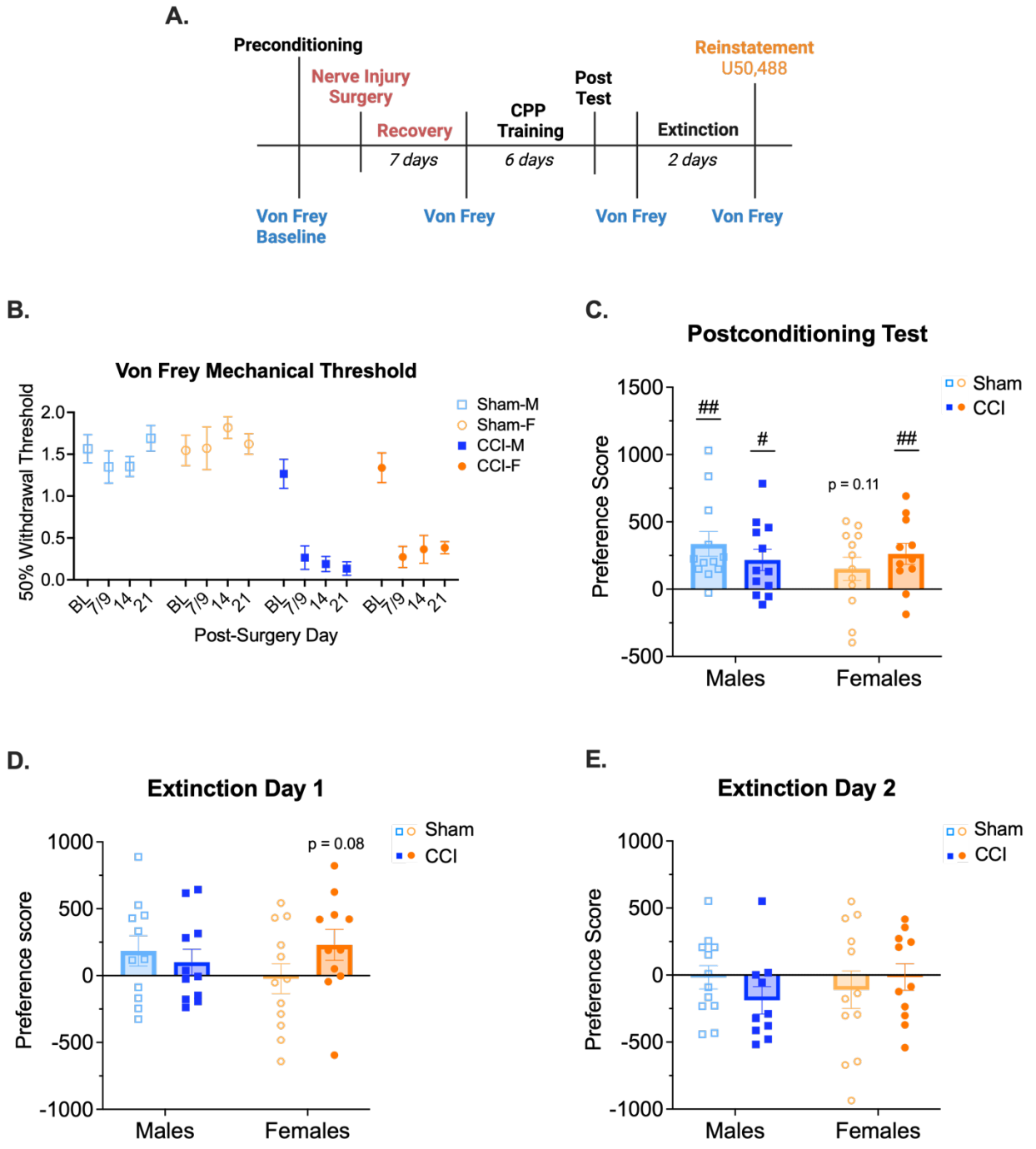


Figure 3.1. **Neuropathic pain had no impact on oxycodone CPP or extinction. A.** Experiment 1 schematic. Mice underwent CCI injury or acted as a sham control. After a 7 day recovery period, mice underwent 3 trial CPP training performed across 6 days, with 3 mg/kg oxycodone, i.p. used as the training dose. After CPP training, mice underwent postconditioning testing and extinction training. Reinstatement testing was conducted 3-5 days after extinction training ended, involving all mice receiving a KOR agonist U50,488 (5 mg/kg i.p.) challenge. **B.** Mechanical withdrawal thresholds at baseline (BL), and 7-9 days (D7/9), 14 days (D14) and 21 days (D21) post-CCI injury. **C.** Preference scores from postconditioning testing. Preference scores were calculated as a

comparison to baseline preconditioning preference scores. **D.** Preference scores from the 1st day of extinction training. Preference scores were calculated as a comparison to baseline preconditioning preference scores. **E.** Preference scores from the 2nd day of extinction training. Preference scores were calculated as a comparison to baseline preconditioning preference scores. Data represent means ± s.e.m. One-sample t test denoted with # (**p**<0.05) and ## (**p**<0.01). Full statistical test analyses for these data are presented in Table 3.1.

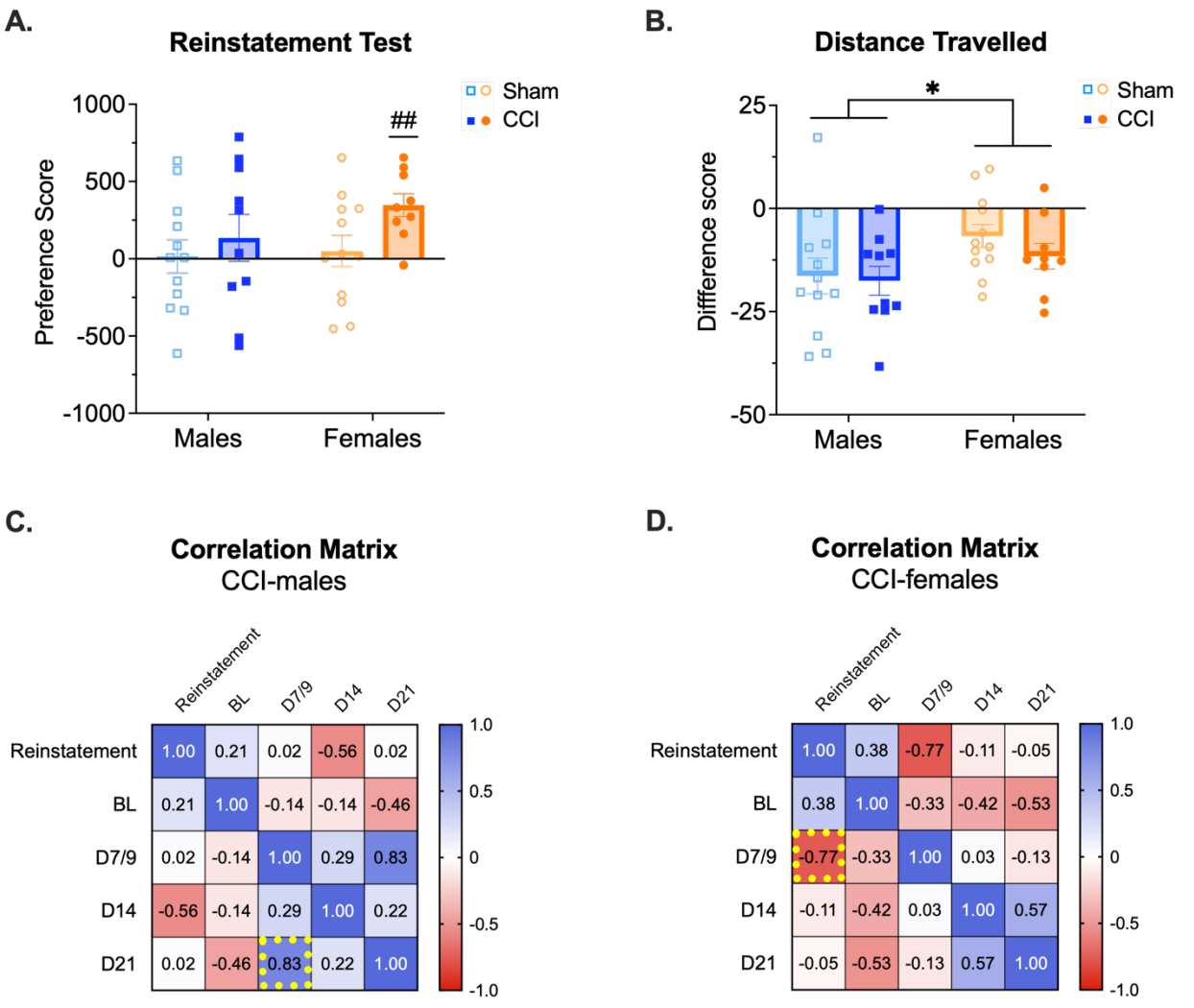


Figure 3.2. KOR agonism induced reinstatement of oxycodone CPP only in CCI-females. **A.** Preference scores from reinstatement testing following a systemic U50,488 KOR agonist challenge were calculated as a comparison to the last day of extinction training preference scores. **B.** Distance travelled during reinstatement testing. Difference scores were calculated as: (distance travelled during reinstatement) – (distanced travelled during last day of extinction). **C.** Pearson r values from correlation matrix analysis in the CCI-male group (or **D.** in the CCI-female group) examining the relationship between reinstatement preference scores and mechanical withdrawal threshold scores at baseline (BL), and 7-9 days (D7/9), 14 days (D14) and 21 days (D21) post-CCI injury. Data in **A.** and **B.** represent means ± s.e.m. One-sample t test denoted with ## (**p**<0.01). Significant main effect of Sex denoted with * (**p**<0.05). Full statistical test analyses for **A.** and **B.** are presented in Table 3.1. Correlations that reached statistical significance (p<0.05) in **C.** and **D.** are denoted in a yellow dotted-line box. p values from correlation matrices in **C.** and **D.** are listed in Supplemental Figure 3.3.

Experiment 2: Role of BLA KORs in chronic neuropathic pain-enhanced CPP reinstatement

The observation that chronic neuropathic pain females are more prone to KOR-agonist induced CPP reinstatement prompted further investigation into the necessity of BLA KORs for this effect. In Experiment 2, the CPP procedure from Experiment 1 was repeated, with the addition of bilateral BLA infusions of the long-acting KOR antagonist JDtic or vehicle 3 days before a systemic KOR agonist reinstatement test. Experiment 2 used a 2 (Sex) x 2 (BLA Drug Treatment) design, with all mice receiving a CCI injury and systemic U50,488 administration on reinstatement day (see Figure 3.3A for experimental design and Table 3.2 for full statistical summary of Experiment 2).

The data presented in Experiment 2 are from a small sample size (n=15) and are preliminary in nature. To increase power, data from JDtic/vehicle groups were combined within each sex until the reinstatement day when the JDtic manipulation finally occurred (Figures 3.3B-D vs. Figures 3.3E/F). While there were no significant differences in postconditioning CPP scores, only the CCI-males demonstrated a significant preference for the oxycodone-paired side ($p<0.05$) (Figure 3.3B). The lack of preference in the CCI-females group was driven by two mice that demonstrated aversion to the oxycodone-paired side. Given a lack of CPP in those two mice, they were excluded from further behavioral testing. Preference scores lowered to below significance across the two extinction days and did not differ between groups (Figures 3.3C/D). During reinstatement testing with a systemic U50,488 challenge, there was a main effect of Sex ($p<0.05$) with CCI-females displaying higher preference scores irrespective of BLA drug treatment (Figure 3.3E). This pattern of higher preference scores in the females was the same regardless of whether the reinstatement scores were calculated compared to preconditioning scores or as a standalone

measure without baseline comparison (Supplemental Figures 3.5A/B). No groups showed a significant preference during reinstatement testing, but this is likely driven by small group sizes.

Regarding distance travelled during reinstatement testing (calculated as a within-subjects comparison to the last day of extinction training), a two-way ANOVA revealed a nearly significant Sex x (BLA) Drug Treatment interaction ($p=0.051$) (Figure 3.3F). This was largely driven by the male-CCI-vehicle group showing greater hypolocomotion than the female-CCI-vehicle group ($p<0.05$). Additionally, BLA JDtic treatment in the male-CCI group appeared to recover hypolocomotion produced by systemic U50,488 treatment as compared to the male-CCI-vehicle ($p<0.05$). Of note, these locomotor differences were less apparent when examining unnormalized distance travelled (without comparison to extinction day locomotion), although again, BLA JDTic induced a similar pattern of increased locomotion only in the males (Supplemental Figure 3.5C).

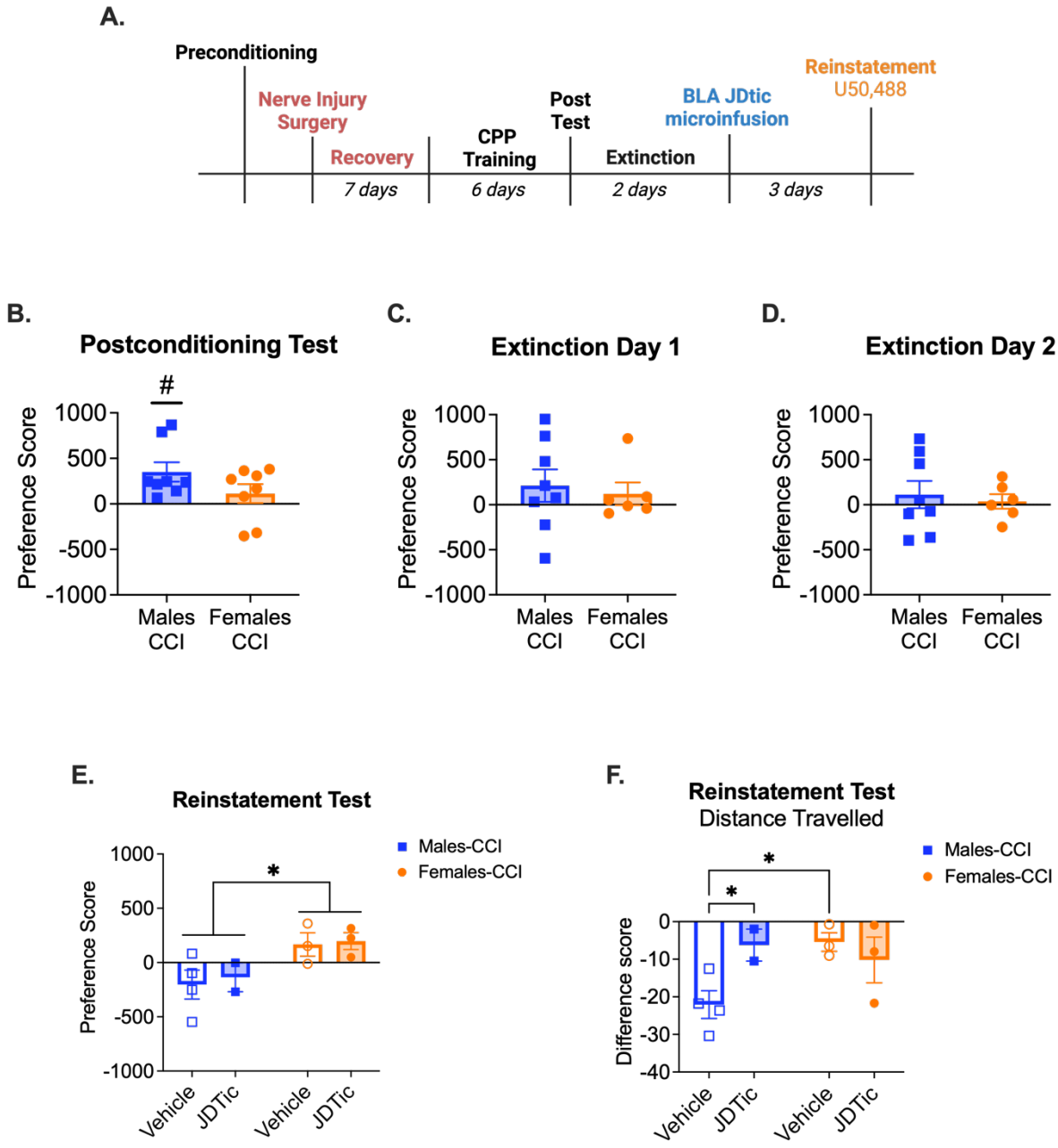


Figure 3.3. Preliminary evidence that BLA KORs are not necessary for chronic pain-enhanced CPP reinstatement. A. Experiment 2 schematic. All mice underwent CCI injury. After a 7 day recovery period, mice underwent 3 trial CPP training performed across 6 days, with 3 mg/kg oxycodone, i.p. used as the training dose. After CPP training mice underwent postconditioning testing and extinction training. JDtic or aCSF was bilaterally infused in the BLA 1-2 days after extinction training ended. After a 3 day recovery period, all mice were challenged with U50,488 (5 mg/kg i.p.) and underwent reinstatement testing. **B.** Preference scores from postconditioning testing. Preferences scores were calculated as a comparison to baseline preconditioning preference scores. **C.** Preference scores from the 1st day of extinction training. Preferences scores were calculated as a comparison to baseline preconditioning preference scores.

D. Preference scores from the 2nd day of extinction training. Preferences scores were calculated as a comparison to baseline preconditioning preference scores. **E.** Preference scores from reinstatement testing following a systemic U50,488 KOR agonist challenge. Reinstatement preference scores calculated as a comparison to the last day of extinction training preference scores. **F.** Distance travelled during reinstatement testing. Difference scores were calculated as a comparison to distanced travelled during the last day of extinction training. Data represent means ± s.e.m. One-sample t test denoted with # (**p**<0.05). Significant main effect of Sex denoted with * (**p**<0.05) in **E.** ANOVA Post-hoc comparison denoted with * (**p**<0.05). in **F.** Full statistical test analyses for these data are presented in Table 3.2.

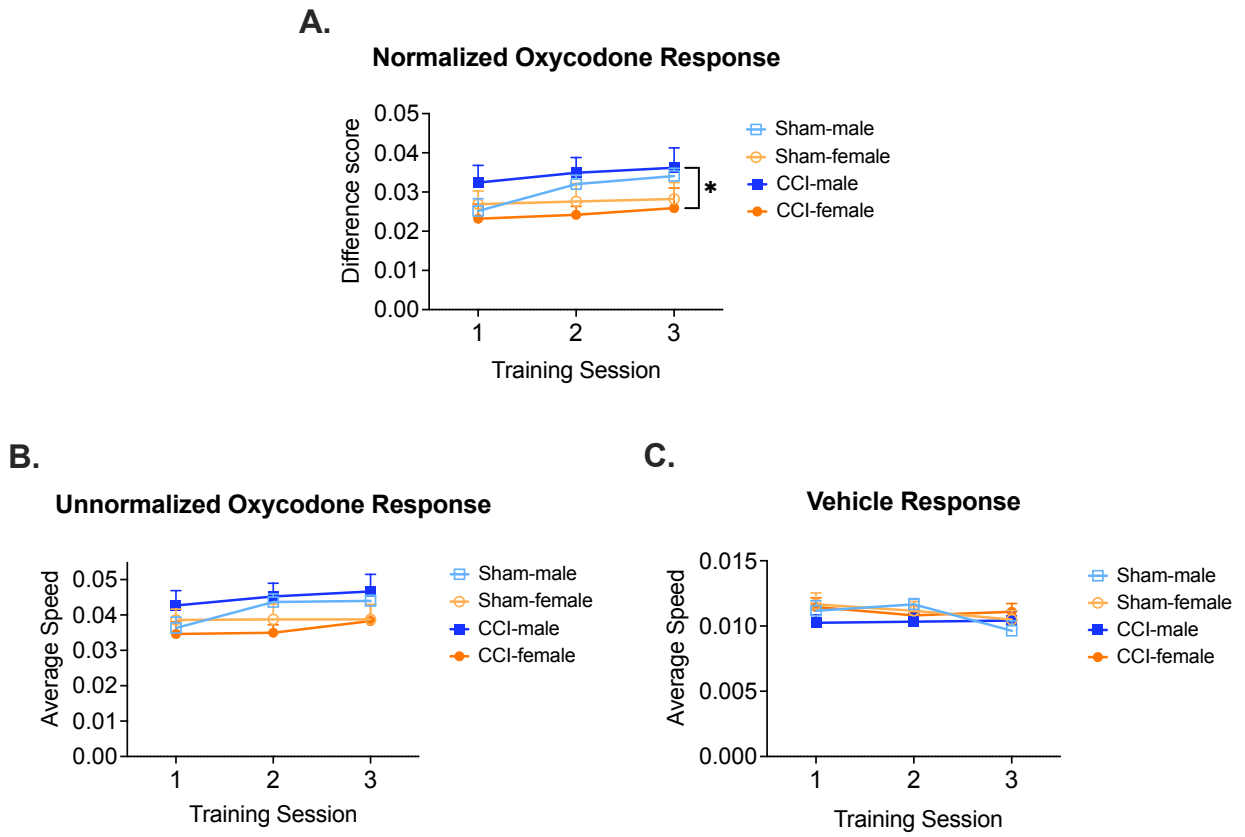
Table 3.1. Summary of statistical results from Experiment 1

Figure	Statistical Test	Results		
Figure 3.1B	Mixed-effects analysis	Day	$F_{2.347,131.4}=11.14$	$p<0.0001$
		Pain	$F_{1,168}=175.8$	$p<0.0001$
		Sex	$F_{1,168}=3.12$	$p=0.08$
		Day x Pain	$F_{3,168}=11.56$	$p<0.0001$
		Day x Sex	$F_{3,168}=0.67$	$p=0.57$
		Pain x Sex	$F_{1,168}=0.02$	$p=0.88$
		Day x Pain x Sex	$F_{3,168}=0.79$	$p=0.50$
Figure 3.1C	Two-way ANOVA	Sex	$F_{1,43}=0.68$	$p=0.41$
		Pain	$F_{1,43}=0.002$	$p=0.96$
		Sex x Pain	$F_{1,43}=1.86$	$p=0.18$
	One-sample t tests	Male – Sham	$t_{11}=3.67$	$p<0.01$
		Female – Sham	$t_{11}=1.74$	$p=0.11$
		Male – CCI	$t_{11}=2.73$	$p<0.05$
		Female – CCI	$t_{10}=3.35$	$p<0.01$
Figure 3.1D	Two-way ANOVA	Sex	$F_{1,41}=0.13$	$p=0.72$
		Pain	$F_{1,41}=0.59$	$p=0.45$
		Sex x Pain	$F_{1,41}=2.37$	$p=0.13$
	One-sample t tests	Male – Sham	$t_{10}=1.64$	$p=0.13$
		Female – Sham	$t_{11}=0.21$	$p=0.84$
		Male – CCI	$t_{10}=1.05$	$p=0.32$
		Female – CCI	$t_{10}=1.99$	$p=0.07$
Figure 3.1E	Two-way ANOVA	Sex	$F_{1,41}=0.14$	$p=0.71$
		Pain	$F_{1,41}=0.12$	$p=0.73$
		Sex x Pain	$F_{1,41}=1.44$	$p=0.24$
	One-sample t tests	Male – Sham	$t_{11}=0.20$	$p=0.85$
		Female – Sham	$t_{11}=0.78$	$p=0.45$
		Male – CCI	$t_{9}=1.83$	$p=0.10$
		Female – CCI	$t_{10}=0.15$	$p=0.89$
Figure 3.2A	Two-way ANOVA	Sex	$F_{1,39}=1.17$	$p=0.29$
		Pain	$F_{1,39}=3.36$	$p=0.07$
		Sex x Pain	$F_{1,39}=0.61$	$p=0.44$
	One-sample t tests	Male – Sham	$t_{11}=0.14$	$p=0.89$
		Female – Sham	$t_{11}=0.49$	$p=0.64$
		Male – CCI	$t_{9}=0.89$	$p=0.40$
		Female – CCI	$t_{8}=4.69$	$p<0.01$
Figure 3.2B	Two-way ANOVA	Sex	$F_{1,39}=4.74$	$p<0.05$
		Pain	$F_{1,39}=0.72$	$p=0.40$
		Sex x Pain	$F_{1,39}=0.27$	$p=0.61$

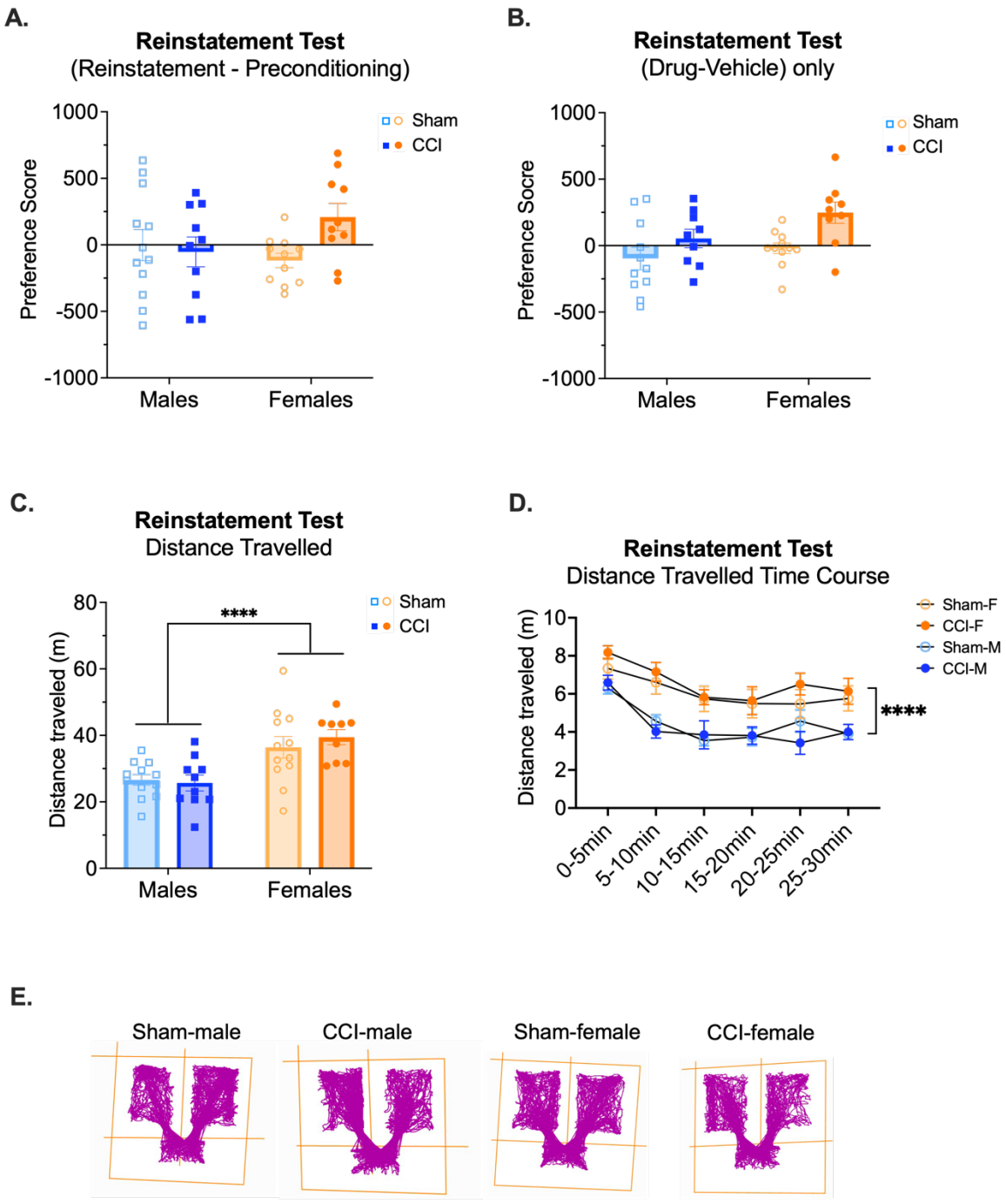
Suppl. Figure 3.1A	Mixed-effects analysis	Time	$F_{1.6,67.5}=2.74$	p=0.08
		Pain	$F_{1,43}=0.01$	p=0.91
		Sex	$F_{1,43}=4.42$	p<0.05
		Time x Pain	$F_{2,83}=0.22$	p=0.81
		Time x Sex	$F_{2,83}=0.91$	p=0.41
		Pain x Sex	$F_{1,43}=1.33$	p=0.26
		Time x Pain x Sex	$F_{2,83}=0.45$	p=0.64
Suppl. Figure 3.1B	Mixed-effects analysis	Time	$F_{1.9,81.5}=2.21$	p=0.12
		Pain	$F_{1,43}=0.15$	p=0.70
		Sex	$F_{1,43}=0.91$	p=0.35
		Time x Pain	$F_{2,84}=3.04$	p=0.053
		Time x Sex	$F_{2,84}=1.05$	p=0.35
		Pain x Sex	$F_{1,43}=0.23$	p=0.63
		Time x Pain x Sex	$F_{2,84}=0.28$	p=0.75
Suppl. Figure 3.1C	Mixed-effects analysis	Time	$F_{1.6,69.8}=2.4$	p=0.11
		Pain	$F_{1,43}=0.02$	p=0.90
		Sex	$F_{1,43}=3.79$	p=0.058
		Time x Pain	$F_{2,85}=0.26$	p=0.77
		Time x Sex	$F_{2,85}=1.00$	p=0.37
		Pain x Sex	$F_{1,43}=1.11$	p=0.30
		Time x Pain x Sex	$F_{2,85}=0.54$	p=0.58
Suppl. Figure 3.2C	Two-way ANOVA	Sex	$F_{1,39}=21.9$	p<0.0001
		Pain	$F_{1,39}=0.18$	p=0.67
		Sex x Pain	$F_{1,39}=0.62$	p=0.44
Suppl. Figure 3.2D	Three-way repeated measures ANOVA	Time	$F_{5,195}=22.96$	p<0.0001
		Pain	$F_{1,39}=0.18$	p=0.67
		Sex	$F_{1,39}=21.86$	p<0.0001
		Time x Pain	$F_{5,195}=0.33$	p=0.90
		Time x Sex	$F_{5,195}=1.16$	p=0.33
		Pain x Sex	$F_{1,39}=0.62$	p=0.44
		Time x Pain x Sex	$F_{5,195}=1.28$	p=0.27

Table 3.2. Summary of statistical results from Experiment 2

Figure	Statistical Test	Results		
Figure 3.3B	One-sample t tests	Male	$t_7=3.29$	$p<0.05$
		Female	$t_7=1.09$	$p=0.31$
	Unpaired t test	Male x Female	$t_{14}=1.60$	$p=0.13$
Figure 3.3C	One-sample t tests	Male	$t_7=1.18$	$p=0.28$
		Female	$t_5=0.96$	$p=0.40$
	Unpaired t test	Male x Female	$t_{12}=0.39$	$p=0.71$
Figure 3.3D	One-sample t tests	CCI-male	$t_7=0.74$	$p=0.48$
		CCI-female	$t_5=0.45$	$p=0.67$
	Unpaired t test	Male x Female	$t_{12}=0.40$	$p=0.70$
Figure 3.3E	Two-way ANOVA	Sex	$F_{1,8}=7.88$	$p<0.05$
		Drug Treatment	$F_{1,8}=0.15$	$p=0.71$
		Sex x Drug	$F_{1,8}=0.02$	$p=0.89$
	One-sample t tests	Male – Vehicle	$t_3=1.53$	$p=0.22$
		Male – JDtic	$t_1=1.02$	$p=0.49$
		Female – Vehicle	$t_2=1.56$	$p=0.26$
		Female – JDtic	$t_2=2.5$	$p=0.13$
Figure 3.3F	Two-way ANOVA	Sex	$F_{1,8}=1.99$	$p=0.20$
		Drug Treatment	$F_{1,8}=1.51$	$p=0.25$
		Sex x Drug	$F_{1,8}=5.25$	$p=0.051$



Supplemental Figure 3.1. Oxycodone- and vehicle-induced locomotion from Experiment 1. **A.** Oxycodone-induced locomotor activity (normalized by subtracting vehicle-induced activity) across CPP training sessions. **B.** Unnormalized oxycodone-induced locomotor activity. **C.** Vehicle (i.e. PBS)-induced locomotor activity across CPP training sessions. Data represent means ± s.e.m. Significant main effect of Sex denoted with * (**p**<0.05). Full statistical test analyses for these data are presented in Table 3.1



Supplemental Figure 3.2. Reinstatement test preference scores and distance travelled from Experiment 1. A. Reinstatement preference scores calculated as comparison to baseline preconditioning preference scores. **B.** Reinstatement preference scores calculated as a standalone measure, i.e. [(time on oxycodone-paired side) – (time on vehicle-paired side)] **C.** Distanced travelled during reinstatement testing. **D.** Time course of distance travelled during reinstatement testing, split into 5 min bins. **E.** Representative track plots of locomotion during reinstatement testing. Data represent means ± s.e.m. Significant main effect of Sex denoted with * (**p**<0.05) and **** (**p**<0.0001). Full statistical test analyses for these data are presented in Table 3.1.

A.

Correlation Matrix - p values
CCI-males

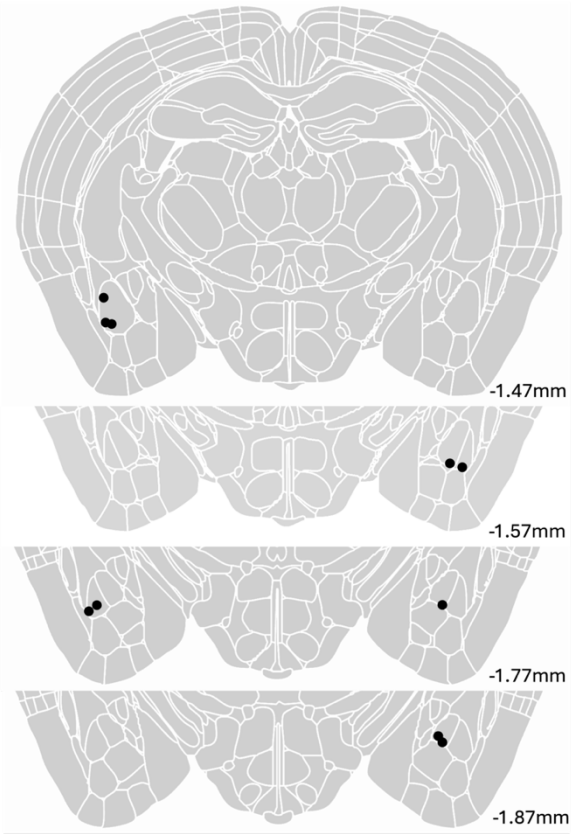
	Reinstatement preference score	Baseline	Day 7/9	Day 14	Day 21
Reinstatement preference score					
Baseline	0.57				
Day 7/9	0.95	0.68			
Day 14	0.095	0.68	0.39		
Day 21	0.96	0.19	0.003	0.55	

B.

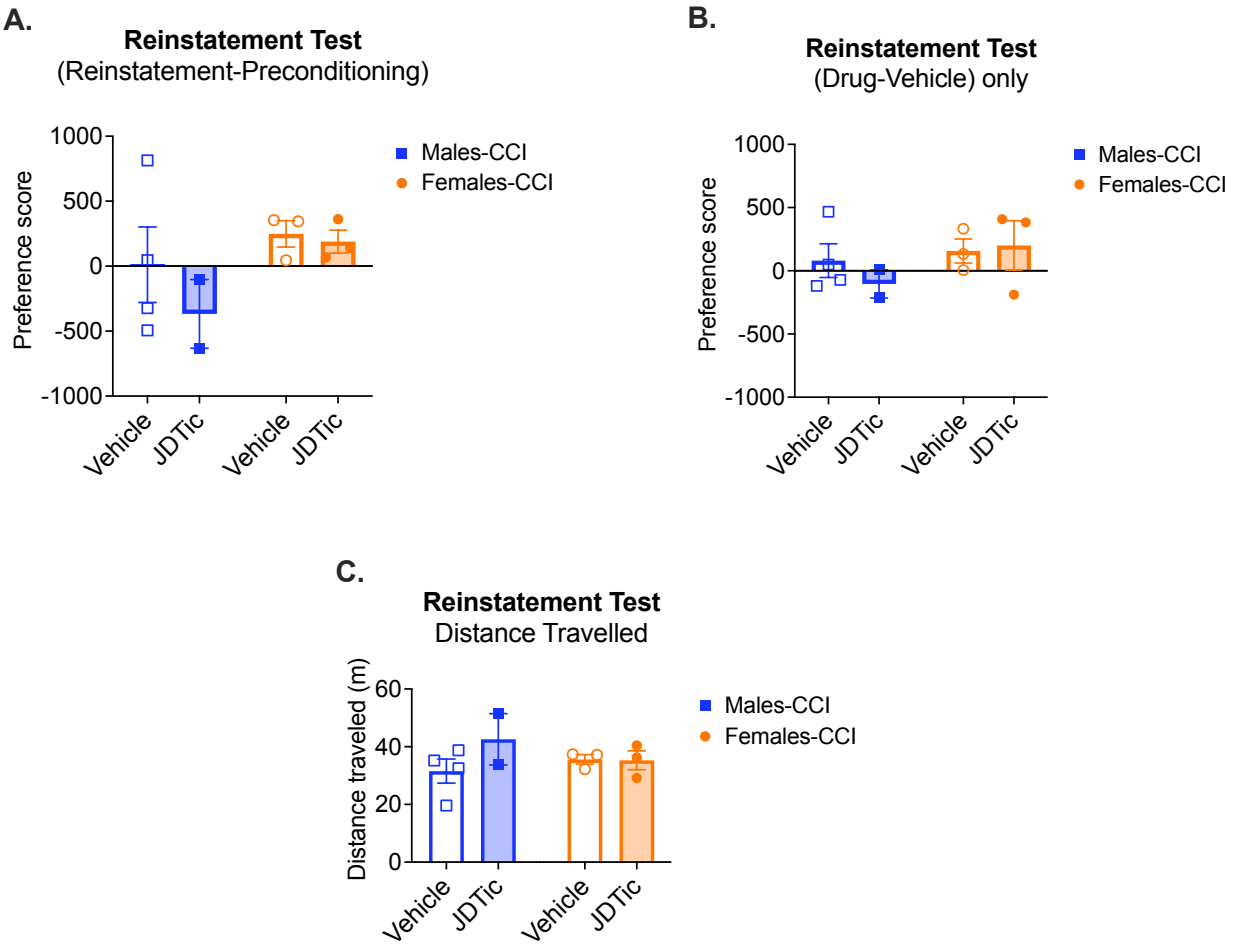
Correlation Matrix - p values
CCI-females

	Reinstatement preference score	Baseline	Day 7/9	Day 14	Day 21
Reinstatement preference score					
Baseline	0.31				
Day 7/9	0.016	0.32			
Day 14	0.78	0.20	0.93		
Day 21	0.9	0.11	0.71	0.09	

Supplemental Figure 3.3. Correlation Matrix p values from Experiment 1. A. p values from the correlation matrix for CCI-males (from Figure 3.2C) and **B.** p values from the correlation matrix for CCI-females (from Figure 3.2D) examining the relationship between reinstatement preference scores and mechanical withdrawal thresholds at baseline, and 7-9, 14, and 21 days after CCI-injury. Correlations that reached statistical significance are denoted with red lettering.



Supplemental Figure 3.4. Histological verification of BLA microinfusion placements from Experiment 2. Schematic representation of microinfusion injector tip placement in male-JDtic and female-JDtic groups. Diagrams of each section adapted from the Scalable Brain Atlas[205,206] (-1.47) – (-1.87) from bregma.



Supplemental Figure 3.5. Reinstatement test preference scores and distance travelled from Experiment 2. A. Reinstatement preference scores calculated as comparison to baseline preconditioning preference scores. **B.** Reinstatement preference scores calculated as a standalone measure, i.e. [(time on oxycodone-paired side) – (time on vehicle-paired side)] **C.** Distanced travelled during reinstatement testing. Data represent means ± s.e.m.

3.5 Discussion

Given that recent findings suggest that males in chronic neuropathic pain states display more of a functional upregulation of the mesolimbic dynorphin/KOR system than females[129,130], we hypothesized that neuropathic pain males would be more susceptible to systemic KOR agonist-induced oxycodone CPP reinstatement. Instead, only females with neuropathic pain demonstrated KOR agonist-induced opioid CPP reinstatement. Reinstatement of oxycodone place preference was not seen in sham mice using these CPP parameters, indicating that sexually dimorphic neuropathic-induced adaptations drove reinstatement in the females. In the CCI model, mechanical threshold sensitivity typically peaks 1-2 weeks after injury induction[76]. In the present study, neuropathic pain-induced alterations in mechanical allodynia did not differ between sexes. However, we found that in the neuropathic pain females, but not males, lower mechanical withdrawal thresholds ~1 week after injury induction was predictive of greater subsequent CPP reinstatement. These findings are the first to demonstrate that females experiencing chronic neuropathic pain are more susceptible to KOR agonist-induced opioid reinstatement, and this susceptibility may be directly related to mechanical sensitivity alterations. This association is particularly interesting because previous findings indicated that the dynorphin/KOR system is less involved in the sensory component of chronic pain[128,129].

Stress-induced reinstatement (of nicotine place preference) has been shown to be regulated by BLA KORs in a non-pain state[201]. Therefore, in a preliminary follow-up study we tested whether BLA KORs are necessary for the sex-specific KOR agonist-induced reinstatement. Neuropathic pain females but not males, again, displayed a similar pattern of reinstatement in response to KOR agonism. Blockage of BLA KORs during the systemic KOR agonist challenge did not seem to impact this reinstatement. It is possible that neuropathic pain activates divergent

pathways for reinstatement of compared to non-pain conditions. However, additional cohorts are necessary to fully determine whether BLA KORs are involved.

It is not clear why neuropathic pain females were more susceptible to KOR agonist-induced oxycodone reinstatement, even though they are largely resistant to the KOR mesolimbic functional upregulation seen in neuropathic pain males. The greater susceptibility to reinstatement in females may be driven by sex differences in the stress response, particularly in pain states. KOR agonism acts as a stressor, due in part to its activation of the hypothalamo-pituitary–adrenocortical (HPA)-axis, the major stress response system[116]. Within the HPA axis, corticotrophin-release hormone (CRH) released from the hypothalamus stimulates the release of adrenocorticotropic hormone (ACTH) from the pituitary gland, which in turn results in the release of glucocorticoids from the adrenal gland. It is well established that adult female rodents produce a more robust HPA axis response to acute stress[207]. In response to U50,488 administration, females show a greater c-Fos response than males in the paraventricular nucleus (PVN), a region highly involved in HPA axis function[187,208]. While U50,488 administration did not produce a sex difference in ACTH and cortisol in non-human healthy primates, in humans with non-inflammatory chronic pain syndrome, females exhibited greater diurnal cortisol levels than males[209,210]. More research is needed on HPA axis function in chronic pain states, with particularly relation to mediation of the dynorphin/KOR system.

We found several sex-specific effects of U50,488 on locomotion that warrant discussion. It is well established that KOR agonism produces hypolocomotion and males are more susceptible to this depressive effect[185,186,188]. Consequently, reinstatement testing commenced 30 min after administration of U50,488 to mitigate the impact of hypolocomotion on behavior. In Experiment 1, we confirmed previous findings that females are less resistant to the hypolocomotor effects of

U50,488 administration. This reduced hypolocomotion manifested in females irrespective of pain history, indicating that the susceptibility of reinstatement in neuropathic pain females is not due to sex-specific motor responses to U50,488. It is possible that neuropathic pain males did not demonstrate KOR-agonist induced reinstatement because of excessive hypolocomotion, although movement tracking plots indicated males were not entirely immobile and did traverse all CPP chambers. KOR agonism-induced hypolocomotion is likely due to its suppressive effects on NAc dopamine release[211,212]. Reduced sensitivity to KOR agonist-induced dopamine suppression in females likely underlies their lower hypolocomotion in response KOR agonists[211]. Experiment 2 likewise found that males had greater hypolocomotion to KOR agonism than females. Interestingly, in neuropathic pain males, but not females, BLA KOR antagonism with JDtic recovered hypolocomotor effects produced by systemic U50,488 administration. It is important to note that there might have been a floor effect in the females; neuropathic pain females did not experience much hypolocomotion at this dose of U50,488 and it's possible that challenge with a higher dose would reveal an impact of BLA KORs in the females. Given that Experiment 2 only included mice with CCI injury, it is unknown if BLA KORs are necessary for U50,488-induced hypolocomotion in the absence of neuropathic pain. BLA KORs were not previously known to influence KOR agonist-induced hypolocomotion, and additional cohorts are needed to confirm this result.

In conclusion, these set of studies are the first to demonstrate females in a chronic neuropathic pain state are more susceptible to KOR agonist-induced reinstatement of opioid place preference. Overall, these data support previous findings that chronic pain-mediated changes in the KOR system are sexually dimorphic and provide important implications for stress-induced drug-seeking in chronic pain patients with a history of opioid use. These findings also reinforce

that multiple endpoints (behavioral, molecular, etc.) between sexes are necessary when studying animal disease models.

3.6 Future Directions

We have preliminary evidence that this pain-enhanced susceptibility to reinstatement is not driven by BLA KORs. Future work will add more cohorts to thoroughly establish whether KOR activation in the BLA is necessary for opioid CPP reinstatement in chronic neuropathic pain female mice. If indeed BLA KORs do not drive this effect, other brain regions will be tested for their involvement. The PVN is a promising potential target, given that U50,488 administration produces more c-Fos expression in the PVN in females than in males[187]. Additionally, it would be useful to determine whether this susceptibility to reinstatement in neuropathic pain females extends to other stressors. A future experiment will repeat the oxycodone CPP reinstatement paradigm but use a footshock stressor instead of KOR agonism just prior to the reinstatement test. The results from this study will be essential for delineating whether females with neuropathic pain are more susceptible to KOR agonism-induced reinstatement or rather stress-induced reinstatement in general.

Concluding Remarks

There is a well-established link between Opioid Use Disorder (OUD), post-traumatic stress disorder (PTSD) and chronic pain, but the mechanisms driving these complex relationships have yet to be fully elucidated. The popular self-medication hypothesis is a useful perspective for understanding motivated behaviors for opioid use but fails to fully explain the multidirectional relationship between these three disorders. This is an issue because comprehensive models are essential for driving better research-driven questions in preclinical and clinical fields. In Chapter 1, an alternative framework, i.e. the "common pathways" hypothesis, was emphasized for explaining the substantial comorbidities between OUD, PTSD and chronic pain. Cross-sensitization between the hypothalamic-pituitary-adrenal (HPA) axis, mesolimbic dopaminergic system, and the dynorphin/kappa opioid receptor (KOR) systems were highlighted as potential mechanistic drivers for these comorbidities. Ultimately, focusing on common pathophysiological mechanisms that drive the co-development of these disorders could lead to better tailored and importantly, more *integrated*, treatment for these comorbidities.

The goal of this presented experimental work was to examine how trauma, whether induced by an unpredictable stressor or neuropathic injury, influences associative learning of opioids and locomotor behaviors in rodents. Chapter 2 utilized the Stress Enhanced Fear Learning (SEFL) PTSD model in rats to examine how unpredictable stress affects opioid-induced behavioral sensitization and opioid learning. While unpredictable stress didn't alter morphine reward learning, it heightened the locomotor response to low dose morphine and unexpectedly led to a preference for contexts associated to low dose naltrexone, an opioid receptor antagonist typically thought of as aversive. The incentive sensitization theory suggests that sensitization of drug reward and drug-

induced locomotion are driven by the same mechanistic pathways (e.g. sensitized mesolimbic dopaminergic system). These findings highlight that the impact of stress on opioid reward and on opioid-induced behavioral sensitization are likely differentially regulated. Additionally, this work is the first preclinical evidence of the rewarding effects of low dose naltrexone in stressed states. This is particularly exciting given that off-label use of low dose naltrexone has been shown to help mental health outcomes in clinical settings. Finally, these findings suggest that although the SEFL model successfully recapitulates aspects of PTSD, it may not be appropriate for studying PTSD/OUD comorbidity.

Chapter 3 utilized the chronic constriction injury (CCI) model in mice to test the impact of chronic neuropathic pain on reinstatement of oxycodone place preference. This work is the first to demonstrate that KOR activation can reinstate opioid place preference, and in a pain- and sex-dependent manner. More specifically, only females with neuropathic pain, but not males, were susceptible to KOR agonist-induced oxycodone reinstatement. This finding is surprising given that males had previously shown more of a functional upregulation of the dynorphin/KOR system in neuropathic pain states. Additionally, mechanical withdrawal thresholds in neuropathic pain females were shown to be predictive of subsequent reinstatement, indicating that severity of sensory pain was directly related to opioid-related behaviors. Overall, these findings cement that the CCI model is useful for studying OUD/chronic pain comorbidities.

Continued development of more comprehensive preclinical models is necessary for studying the mechanisms underlying the relationship between OUD, PTSD and chronic pain. The work contained here focused on the relationship between OUD-PTSD and OUD-chronic pain separately, but it would also be useful to further examine the interrelations between these disorders. Chronic pain and PTSD comorbidity is the last connecting link not yet addressed, but equally as

important public health concern. Co-occurrence of PTSD and chronic pain is highly prevalent, with the estimated incidence ranging from 0.69-46.7% in the general public with chronic pain and as high as 50.1% in veterans with chronic pain[213]. Likewise, the development of comorbid PTSD and chronic pain has shown to have bidirectional relationships. For instance, greater severity of pain within a month of a road accident was found to predictive of greater post-traumatic stress symptoms at a 6-month follow-up[214]. On the other hand, the same group found that higher initial post-traumatic stress reactions after a road accident were predictors of future disability from chronic pain[214,215]. Related to the common pathways hypothesis, it has been proposed that PTSD and chronic pain are linked because of multiple processes that contribute to the "mutual maintenance" of both disorders[216]. For instance, avoidant coping strategies driven by the desire to minimize pain and fear leads to greater disability and maintenance of symptoms. Finally, it would be useful to develop better models that investigate OUD, PTSD and chronic pain comorbidities all together. In general, the preclinical field is currently focused on vulnerability for OUD, given a history of stress or chronic pain. It would be advantageous to devote more of an integrated approach for studying these three disorders together. Such attention would hopefully lead to more targeted interventions aimed at mitigating the complex nature of OUD, PTSD and chronic pain comorbidities.

References Cited

1. SAMHSA. National Survey on Drug Use and Health 2018. https://datafiles.samhsa.gov/ (2019).

2. Kessler, R. C., Sonnega, A., Bromet, E., Hughes, M. & Nelson, C. B. Posttraumatic stress disorder in the National Comorbidity Survey. *Arch Gen Psychiatry* **52**, 1048–1060 (1995).

3. Mills, K. L., Teesson, M., Ross, J. & Peters, L. Trauma, PTSD, and substance use disorders: Findings from the Australian National Survey of Mental Health and Well-Being. *American Journal of Psychiatry* **163**, 652–658 (2006).

4. Dennis, B. B. *et al.* The impact of chronic pain on opioid addiction treatment: a systematic review protocol. *SpringerBB Dennis, M Bawor, J Paul, M Varenbut, J Daiter, C Plater, G Pare, DC Marsh, A WorsterSystematic reviews, 2015•Springer* **4**, (2015).

5. Fareed, A. *et al.* Comorbid posttraumatic stress disorder and opiate addiction: A literature review. *Journal of Addictive Diseases* vol. 32 168–179 Preprint at https://doi.org/10.1080/10550887.2013.795467 (2013).

6. Delorme, J. *et al.* Systematic Review and Meta-Analysis of the Prevalence of Chronic Pain Among Patients With Opioid Use Disorder and Receiving Opioid Substitution Therapy. *J Pain* **24**, 192–203 (2023).

7. Hser, Y. I. *et al.* Chronic pain among patients with opioid use disorder: Results from electronic health records data. *J Subst Abuse Treat* **77**, 26–30 (2017).

8. Dufort, A. & Samaan, Z. Problematic Opioid Use Among Older Adults: Epidemiology, Adverse Outcomes and Treatment Considerations. *Drugs Aging* **38**, 1043 (2021).

9. Bilevicius, E., Sommer, J. L., Asmundson, G. J. G. & El-Gabalawy, R. Posttraumatic stress disorder and chronic pain are associated with opioid use disorder: Results from a 2012-2013 American nationally representative survey. *Drug Alcohol Depend* **188**, 119–125 (2018).

10. Khantzian, E. J. The self-medication hypothesis of addictive disorders: focus on heroin and cocaine dependence. *Am J Psychiatry* **142**, 1259–1264 (1985).

11. Khantzian, E. J. The self-medication hypothesis of substance use disorders: A reconsideration and recent applications. *Harv Rev Psychiatry* **4**, 231–244 (1997).

12. Barth, K. S. *et al.* Pain and Motives for Use among Non-Treatment Seeking Individuals with Prescription Opioid Dependence. *The American journal on addictions / American Academy of Psychiatrists in Alcoholism and Addictions* **22**, 486 (2013).

13. Stumbo, S. P., Yarborough, B. J. H., McCarty, D., Weisner, C. & Green, C. A. Patient-reported pathways to opioid use disorders and pain-related barriers to treatment engagement. *J Subst Abuse Treat* **73**, 47 (2017).

14. Badour, C. L. *et al.* Concurrent and Proximal Associations Among PTSD Symptoms, Prescription Opioid Use, and Co-Use of Other Substances: Results from a Daily Monitoring Study. *Psychol Trauma* **15**, 367 (2023).

15. MacLean, R. R., Spinola, S., Manhapra, A. & Sofuoglu, M. Systematic Review of Pain Severity and Opioid Craving in Chronic Pain and Opioid Use Disorder. *Pain Medicine* **21**, e146–e163 (2020).

16. Martel, M. O., Jamison, R. N., Wasan, A. D. & Edwards, R. R. The Association Between Catastrophizing and Craving in Patients with Chronic Pain Prescribed Opioid Therapy: A Preliminary Analysis. *Pain Medicine* **15**, 1757–1764 (2014).

17. Norman, S. B. *et al.* Efficacy of Integrated Exposure Therapy vs Integrated Coping Skills Therapy for Comorbid Posttraumatic Stress Disorder and Alcohol Use Disorder: A Randomized Clinical Trial. *JAMA Psychiatry* **76**, 791–799 (2019).

18. Simpson, T. L. *et al.* Efficacy and acceptability of interventions for co-occurring PTSD and SUD: A meta-analysis. *J Anxiety Disord* **84**, 102490 (2021).

19. Hien, D. A. *et al.* Do treatment improvements in PTSD severity affect substance use outcomes? A secondary analysis from a randomized clinical trial in NIDA's Clinical Trials Network. *Am J Psychiatry* **167**, 95–101 (2010).

20. Back, S. E. Toward an Improved Model of Treating Co-Occurring PTSD and Substance Use Disorders. *Am J Psychiatry* **167**, 11 (2010).

21. Cottler, L. B., Compton, W. M., Mager, D., Spitznagel, E. L. & Janca, A. Posttraumatic stress disorder among substance users from the general population. *Am J Psychiatry* **149**, 664–670 (1992).

22. Heimer, R. *et al.* Chronic pain, Addiction severity, and misuse of opioids in Cumberland County, Maine. *Addictive Behaviors* **37**, 346–349 (2012).

23. Younger, J. W. *et al.* Prescription opioid analgesics rapidly change the human brain. *Pain* **152**, 1803 (2011).

24. Upadhyay, J. *et al.* Alterations in brain structure and functional connectivity in prescription opioid-dependent patients. *Brain* **133**, 2098 (2010).

25. Huxtable, C. A., Roberts, L. J., Somogyi, A. A. & Macintyre, P. E. Acute pain management in opioid-tolerant patients: A growing challenge. *Anaesth Intensive Care* **39**, 804–823 (2011).

26. Windle, M. Substance use, risky behaviors, and victimization among a US national adolescent sample. *Addiction* **89**, 175–182 (1994).

27. Gisquet-Verrier, P., Tolédano, D. & Le Dorze, C. Bases physiologiques communes pour les troubles de stress post-traumatique et la dépendance aux drogues d'abus : conséquences pour de nouvelles approches thérapeutiques. *Therapies* **72**, 357–366 (2017).

28. María-Ríos, C. E. & Morrow, J. D. Mechanisms of Shared Vulnerability to Post-traumatic Stress Disorder and Substance Use Disorders. *Front Behav Neurosci* **14**, (2020).

29. Elman, I. & Borsook, D. Common Brain Mechanisms of Chronic Pain and Addiction. *Neuron* **89**, 11–36 (2016).

30. Miller, L. Neurosensitization: A model for persistent disability in chronic pain, depression, and posttraumatic stress disorder following injury. *NeuroRehabilitation* **14**, 25–32 (2000).
31. Elman, I. & Borsook, D. The failing cascade: Comorbid post traumatic stress- and opioid use disorders. *Neurosci Biobehav Rev* **103**, 374–383 (2019).

32. Koob, G. F. & Le Moal, M. Addiction and the brain antireward system. *Annu Rev Psychol* **59**, 29–53 (2008).

33. Brown, P. J., Stout, R. L. & Gannon-Rowley, J. Substance use disorder-PTSD comorbidity: Patients' perceptions of symptom interplay and treatment issues. *J Subst Abuse Treat* **15**, 445–448 (1998).

34. Milton, A. L. & Everitt, B. J. The persistence of maladaptive memory: Addiction, drug memories and anti-relapse treatments. *Neurosci Biobehav Rev* **36**, 1119–1139 (2012).

35. Everitt, B. J., Dickinson, A. & Robbins, T. W. The neuropsychological basis of addictive behaviour. *Brain Res Rev* **36**, 129–138 (2001).

36. Vlaeyen, J. W. S. Learning to predict and control harmful events: Chronic pain and conditioning. *Pain* **156**, S86–S93 (2015).

37. Hollander, M. Den *et al.* Fear reduction in patients with chronic pain: a learning theory perspective. *Expert Rev Neurother* **10**, 1733–1745 (2010).

38. Lissek, S. & van Meurs, B. Learning models of PTSD: Theoretical accounts and psychobiological evidence. *International Journal of Psychophysiology* vol. 98 594–605 Preprint at https://doi.org/10.1016/j.ijpsycho.2014.11.006 (2015).

39. Li, Q. *et al.* Predicting subsequent relapse by drug-related cue-induced brain activation in heroin addiction: an event-related functional magnetic resonance imaging study. *Addiction Biology* **20**, 968–978 (2015).

40. Franken, I. H. A., De Haan, H. A., Van Der Meer, C. W., Haffmans, P. M. J. & Hendriks, V. M. Cue reactivity and effects of cue exposure in abstinent posttreatment drug users. *J Subst Abuse Treat* **16**, 81–85 (1999).

41. Bossert, J. M. *et al.* Role of mu, but not delta or kappa, opioid receptors in context-induced reinstatement of oxycodone seeking. *European Journal of Neuroscience* **50**, 2075–2085 (2019).

42. Ouimette, P. C., Ahrens, C., Moos, R. H. & Finney, J. W. During treatment changes in substance abuse patients with posttraumatic stress disorder: The influence of specific interventions and program environments. *J Subst Abuse Treat* **15**, 555–564 (1998).

43. Sokhadze, E. *et al.* Attentional Bias to drug- and Stress-related pictorial cues in cocaine addiction comorbid with posttraumatic stress disorder. *J Neurother* **12**, 205–225 (2008).

44. Fordyce, W. E. & Company, M. C. V. Behavioral methods for chronic pain and illness. *Pain* **3**, 291–292 (1977).

45. Gatzounis, R., Schrooten, M. G. S., Crombez, G. & Vlaeyen, J. W. S. Operant learning theory in pain and chronic pain rehabilitation. *Curr Pain Headache Rep* **16**, 117–126 (2012).

46. Bigelow, G. E., Brooner, R. K. & Silverman, K. Competing motivations: Drug reinforcement vs non-drug reinforcement. *Journal of Psychopharmacology* **12**, 8–14 (1998).

47. Monson, C. M., Friedman, M. J. & La Bash, H. A. J. *Handbook of PTSD, First Edition: Science and Practice - Google Books*. (The Guilford Press, New York, 2010).

48. Koob, G. F. Negative reinforcement in drug addiction: The darkness within. *Current Opinion in Neurobiology* vol. 23 559–563 Preprint at https://doi.org/10.1016/j.conb.2013.03.011 (2013).

49. Evans, C. J. & Cahill, C. M. Neurobiology of opioid dependence in creating addiction vulnerability. *F1000Res* **5**, (2016).

50. Robinson, T. E. & Berridge, K. C. The neural basis of drug craving: An incentive-sensitization theory of addiction. *Brain Res Rev* **18**, 247–291 (1993).

51. Lueptow, L. M., Shashkova, E. C., Miller, M. G., Evans, C. J. & Cahill, C. M. Insights into the Neurobiology of Craving in Opioid Use Disorder. *Curr Anesthesiol Rep* **10**, 378–387 (2020).

52. Watson, B. J. *et al.* Investigating expectation and reward in human opioid addiction with [11C]raclopride PET. *Addiction Biology* **19**, 1032–1040 (2014).

53. Daglish, M. R. C. *et al.* Brain dopamine response in human opioid addiction. *The British Journal of Psychiatry* **193**, 65–72 (2008).

54. Zijlstra, F., Booij, J., van den Brink, W. & Franken, I. H. A. Striatal dopamine D2 receptor binding and dopamine release during cue-elicited craving in recently abstinent opiate-dependent males. *European Neuropsychopharmacology* **18**, 262–270 (2008).

55. Carmack, S. A. *et al.* Corticosteroid sensitization drives opioid addiction. *Molecular Psychiatry 2022 27:5* **27**, 2492–2501 (2022).

56. Friedman, M. J. Neurobiological Sensitization Models of Posttraumatic Stress Disorder: Their Possible Relevance to Multiple Chemical Sensitivity Syndrome. *Toxicol Ind Health* **10**, 449–462 (1994).

57. Burke, A. R. & Miczek, K. A. Stress in adolescence and drugs of abuse in rodent models: Role of dopamine, CRF, and HPA axis. *Psychopharmacology (Berl)* **231**, 1557 (2014).

58. Bardo, M. T. & Bevins, R. A. Conditioned place preference: What does it add to our preclinical understanding of drug reward? *Psychopharmacology* vol. 153 31–43 Preprint at https://doi.org/10.1007/s002130000569 (2000).

59. Koob, G. F. & Le Moal, M. Plasticity of reward neurocircuitry and the 'dark side' of drug addiction. *Nature Neuroscience* vol. 8 1442–1444 Preprint at https://doi.org/10.1038/nn1105-1442 (2005).

60. Haertzen, C. A., Kocher, T. R. & Miyasato, K. Reinforcements from the first drug experience can predict later drug habits and/or addiction: Results with coffee, cigarettes, alcohol, barbiturates, minor and major tranquilizers, stimulants, marijuana, hallucinogens, heroin, opiates and cocaine. *Drug Alcohol Depend* **11**, 147–165 (1983).

61. American Psychiatric Association. *Diagnostic and Statistical Manual of Mental Disorders : DSM-5.* (2013).

62. Wessa, M. & Flor, H. Failure of extinction of fear responses in posttraumatic stress disorder: Evidence from second-order conditioning. *American Journal of Psychiatry* **164**, 1684–1692 (2007).

63. Orr, S. P. *et al.* De novo conditioning in trauma-exposed individuals with and without posttraumatic stress disorder. *J Abnorm Psychol* **109**, 290–298 (2000).

64. Jovanovic, T. & Ressler, K. J. How the neurocircuitry and genetics of fear inhibition may inform our understanding of PTSD. *American Journal of Psychiatry* vol. 167 648–662 Preprint at https://doi.org/10.1176/appi.ajp.2009.09071074 (2010).

65. Rau, V., DeCola, J. P. & Fanselow, M. S. Stress-induced enhancement of fear learning: An animal model of posttraumatic stress disorder. in *Neuroscience and Biobehavioral Reviews* vol. 29 1207–1223 (2005).

66. Ponomarev, I., Rau, V., Eger, E. I., Harris, R. A. & Fanselow, M. S. Amygdala transcriptome and cellular mechanisms underlying stress-enhanced fear learning in a rat model of posttraumatic stress disorder. *Neuropsychopharmacology* **35**, 1402–1411 (2010).

67. Perusini, J. N. *et al.* Induction and Expression of Fear Sensitization Caused by Acute Traumatic Stress. *Neuropsychopharmacology Reviews* **41**, 45–57 (2015).

68. Poulos, A. M. *et al.* Amnesia for early life stress does not preclude the adult development of posttraumatic stress disorder symptoms in rats. *Biol Psychiatry* **76**, 306–314 (2014).

69. Rau, V. & Fanselow, M. S. Stress The International Journal on the Biology of Stress Exposure to a stressor produces a long lasting enhancement of fear learning in rats. *Stress* **12**, 125–133 (2009).

70. Cohen, S. P., Vase, L. & Hooten, W. M. Chronic pain: an update on burden, best practices, and new advances. *The Lancet* **397**, 2082–2097 (2021).

71. Berge, O. G. Predictive validity of behavioural animal models for chronic pain. *Br J Pharmacol* **164**, 1195–1206 (2011).

72. Jensen, T. S. & Finnerup, N. B. Allodynia and hyperalgesia in neuropathic pain: clinical manifestations and mechanisms. *Lancet Neurol* **13**, 924–935 (2014).

73. Price, D. D. Psychological and Neural Mechanisms of the Affective Dimension of Pain. *Science (1979)* **288**, (2000).

74. Elman, I., Borsook, D. & Volkow, N. D. Pain and suicidality: Insights from reward and addiction neuroscience. *Prog Neurobiol* **109**, 1–27 (2013).

75. Bennett, G. J. & Xie, Y.-K. A peripheral mononeuropathy in rat that produces disorders of pain sensation like those seen in man. *Pain* **33**, 87–107 (1988).

76. Mosconi, T. & Kruger, L. Fixed-diameter polyethylene cuffs applied to the rat sciatic nerve induce a painful neuropathy: ultrastructural morphometric analysis of axonal alterations. *Pain* **64**, 37–57 (1996).

77. Suzuki, T. *et al.* Experimental neuropathy in mice is associated with delayed behavioral changes related to anxiety and depression. *Anesth Analg* **104**, 1570–1577 (2007).

78. Matsuzawa-Yanagida, K. *et al.* Usefulness of Antidepressants for Improving the Neuropathic Pain-Like State and Pain-Induced Anxiety through Actions at Different Brain Sites. *Neuropsychopharmacology 2008 33:8* **33**, 1952–1965 (2007).

79. Gonçalves, L. *et al.* Neuropathic pain is associated with depressive behaviour and induces neuroplasticity in the amygdala of the rat. *Exp Neurol* **213**, 48–56 (2008).

80. Yalcin, I. *et al.* A time-dependent history of mood disorders in a murine model of neuropathic pain. *Biol Psychiatry* **70**, 946–953 (2011).

81. Martel, M. O., Dolman, A. J., Edwards, R. R., Jamison, R. N. & Wasan, A. D. The association between negative affect and prescription opioid misuse in patients with chronic pain: The mediating role of opioid craving. *The journal of pain : official journal of the American Pain Society* **15**, 90–100 (2014).

82. Jamison, R. N. & Edwards, R. R. Risk Factor Assessment for Problematic Use of Opioids for Chronic Pain. *Clin Neuropsychol* **27**, 60–80 (2013).

83. Will, M. J., Watkins, L. R. & Maier, S. F. Uncontrollable Stress Potentiates Morphine's Rewarding Properties. *Pharmacol Biochem Behav* **60**, 655–664 (1998).

84. Der-Avakian, A. *et al.* Surgical and pharmacological suppression of glucocorticoids prevents the enhancement of morphine conditioned place preference by uncontrollable stress in rats. *Psychopharmacology (Berl)* **179**, 409–417 (2005).

85. Der-Avakian, A. *et al.* The effects of a single exposure to uncontrollable stress on the subsequent conditioned place preference responses to oxycodone, cocaine, and ethanol in rats. *Psychopharmacology (Berl)* **191**, 909–917 (2007).

86. Rozeske, R. R. *et al.* The Medial Prefrontal Cortex Regulates the Differential Expression of Morphine-Conditioned Place Preference Following a Single Exposure to Controllable or Uncontrollable Stress. *Neuropsychopharmacology 2009 34:4* **34**, 834–843 (2008).

87. Szklarczyk, K., Korostynski, M., Golda, S., Solecki, W. & Przewlocki, R. Genotype-dependent consequences of traumatic stress in four inbred mouse strains. *Genes Brain Behav* **11**, 977–985 (2012).

88. Li, Y. *et al.* Subsequently Enhanced CPP to Morphine Following Chronic but Not Acute Footshock Stress Associated with Corticosterone Mechanism In Rats. *International Journal of Neuroscience* **117**, 1237–1255 (2007).

89. Meng, S., Quan, W., Qi, X., Su, Z. & Yang, S. Effect of baclofen on morphine-induced conditioned place preference, extinction, and stress-induced reinstatement in chronically stressed mice. *Psychopharmacology (Berl)* **231**, 27–36 (2014).

90. Reich, B. *et al.* Chronic immobilization stress primes the hippocampal opioid system for oxycodone-associated learning in female but not male rats. *Synapse* **73**, e22088 (2019).

91. Papp, M., Lappas, S., Muscat, R. & Willner, P. Attenuation of place preference conditioning but not place aversion conditioning by chronic mild stress. *Journal of Psychopharmacology* **6**, 352–356 (1992).

92. Schenk, S., Ellison, F., Hunt, T. & Amit, Z. An examination of heroin conditioning in preferred and nonpreferred environments and in differentially housed mature and immature rats. *Pharmacol Biochem Behav* **22**, 215–220 (1985).

93. Attarzadeh-Yazdi, G. *et al.* Inhibitory effects of forced swim stress and corticosterone on the acquisition but not expression of morphine-induced conditioned place preference: Involvement of glucocorticoid receptor in the basolateral amygdala. *Behavioural Brain Research* **252**, 339–346 (2013).

94. Haghparast, A. *et al.* Changes in the Levels of p-ERK, p-CREB, and c-fos in Rat Mesocorticolimbic Dopaminergic System After Morphine-Induced Conditioned Place Preference: The Role of Acute and Subchronic Stress. doi:10.1007/s10571-013-0011-z.

95. Der-Avakian, A. *et al.* Surgical and pharmacological suppression of glucocorticoids prevents the enhancement of morphine conditioned place preference by uncontrollable stress in rats. *Psychopharmacology (Berl)* **179**, 409–417 (2005).

96. Chen, M., Zhang, X. & Hao, W. H3K4 dimethylation at FosB promoter in the striatum of chronic stressed rats promotes morphine-induced conditioned place preference. *PLoS One* **14**, e0221506 (2019).

97. Marinelli, M. & Piazza, P. V. Interaction between glucocorticoid hormones, stress and psychostimulant drugs*. *European Journal of Neuroscience* **16**, 387–394 (2002).

98. Vezina, P. & Stewart, J. Conditioning and place-specific sensitization of increases in activity induced by morphine in the VTA. *Pharmacol Biochem Behav* **20**, 925–934 (1984).

99. Delfs, J. M., Schreiber, L. & Kelley, A. E. Microinjection of cocaine into the nucleus accumbens elicits locomotor activation in the rat. *Journal of Neuroscience* **10**, 303–310 (1990).

100. Goeders, N. E. & Guerin, G. F. Non-contingent electric footshock facilitates the acquisition of intravenous cocaine self-administration in rats. *Psychopharmacology (Berl)* **114**, 63–70 (1994).

101. Campbell, U. C. & Carroll, M. E. Effects of ketoconazole on the acquisition of intravenous cocaine self-administration under different feeding conditions in rats. *Psychopharmacology (Berl)* **154**, 311–318 (2001).

102. Kalivas, P. W. & Stewart, J. Dopamine transmission in the initiation and expression of drug- and stress-induced sensitization of motor activity. *Brain Res Rev* **16**, 223–244 (1991).

103. Deroche, V. *et al.* Stress-induced sensitization and glucocorticoids. I. Sensitization of dopamine-dependent locomotor effects of amphetamine and morphine depends on stress-induced corticosterone secretion. *Journal of Neuroscience* **15**, 7181–7188 (1995).

104. Rougé-Pont, F., Marinelli, M., Le Moal, M., Simon, H. & Piazza, P. V. Stress-induced sensitization and glucocorticoids. II. Sensitization of the increase in extracellular dopamine induced by cocaine depends on stress-induced corticosterone secretion. *Journal of Neuroscience* **15**, 7189–7195 (1995).

105. Will, M. J. *et al.* Modulation of the locomotor properties of morphine and amphetamine by uncontrollable stress. *Pharmacol Biochem Behav* **71**, 345–351 (2002).

106. Leyton, M. & Stewart, J. Preexposure to foot-shock sensitizes the locomotor response to subsequent systemic morphine and intra-nucleus accumbens amphetamine. *Pharmacol Biochem Behav* **37**, 303–310 (1990).

107. Deroche, V. *et al.* Stress-induced sensitization to amphetamine and morphine psychomotor effects depend on stress-induced corticosterone secretion. *Brain Res* **598**, 343–348 (1992).

108. Molina, V. A., Heyser, C. J. & Spear, L. P. Chronic variable stress enhances the stimulatory action of a low dose of morphine: reversal by desipramine. *Eur J Pharmacol* **260**, 57–64 (1994).

109. Grung, M. *et al.* Morphine-6-glucuronide-Induced Locomotor Stimulation in Mice: Role of Opioid Receptors. *Pharmacol Toxicol* **82**, 3–10 (1998).

110. Craft, R. M., Clark, J. L., Hart, S. P. & Pinckney, M. K. Sex differences in locomotor effects of morphine in the rat. *Pharmacol Biochem Behav* **85**, 850–858 (2006).

111. Marinelli, M., Aouizerate, B., Barrot, M., Le Moal, M. & Piazza, P. V. Dopamine-dependent responses to morphine depend on glucocorticoid receptors. *Proc Natl Acad Sci U S A* **95**, 7742–7747 (1998).

112. Del Rosario, C. N., Pacchioni, A. M. & Cancela, L. M. Influence of acute or repeated restraint stress on morphine-induced locomotion: involvement of dopamine, opioid and glutamate receptors. *Behavioural Brain Research* **134**, 229–238 (2002).

113. Der-Avakian, A. *et al.* The role of glucocorticoids in the uncontrollable stress-induced potentiation of nucleus accumbens shell dopamine and conditioned place preference responses to morphine. *Psychoneuroendocrinology* **31**, 653–663 (2006).

114. Cahill, C. M. *et al.* Kappa Opioid Signaling at the Crossroads of Chronic Pain and Opioid Addiction. *Handb Exp Pharmacol* **271**, 315 (2022).

115. White, K. L. & Roth, B. L. Psychotomimetic effects of kappa opioid receptor agonists. *Biol Psychiatry* **72**, 797–798 (2012).

116. Van'T Veer, A. & Carlezon, W. A. Role of kappa-opioid receptors in stress and anxiety-related behavior. *Psychopharmacology 2013 229:3* **229**, 435–452 (2013).

117. Chavkin, C. & Koob, G. F. Dynorphin, Dysphoria, and Dependence: the Stress of Addiction. *Neuropsychopharmacology* **41**, 373 (2016).

118. Wee, S. & Koob, G. F. The role of the dynorphin–κ opioid system in the reinforcing effects of drugs of abuse. *Psychopharmacology (Berl)* **210**, 121 (2010).

119. Bruchas, M. R., Land, B. B. & Chavkin, C. The dynorphin/kappa opioid system as a modulator of stress-induced and pro-addictive behaviors. *Brain Res* **1314**, 44–55 (2010).

120. Sedki, F. *et al.* A role for kappa-, but not mu-opioid, receptor activation in acute food deprivation-induced reinstatement of heroin seeking in rats. *Addiction biology* **20**, 423–432 (2015).

121. Zhou, Y., Leri, F., Grella, S. L., Aldrich, J. V. & Kreek, M. J. Involvement of dynorphin and kappa opioid receptor in yohimbine-induced reinstatement of heroin seeking in rats. *Synapse* **67**, 358 (2013).

122. Brice-Tutt, A. C. *et al.* Multifunctional opioid receptor agonism and antagonism by a novel macrocyclic tetrapeptide prevents reinstatement of morphine-seeking behaviour. *Br J Pharmacol* **177**, 4209–4222 (2020).

123. Redila, V. A. & Chavkin, C. Stress-induced reinstatement of cocaine seeking is mediated by the kappa opioid system. *Psychopharmacology (Berl)* **200**, 59–70 (2008).

124. Valdez, G. R., Platt, D. M., Rowlett, J. K., Rüedi-Bettschen, D. & Spealman, R. D. Kappa agonist-induced reinstatement of cocaine seeking in squirrel monkeys: a role for opioid and stress-related mechanisms. *J Pharmacol Exp Ther* **323**, 525–533 (2007).

125. Funk, D., Coen, K. & Lê, A. D. The role of kappa opioid receptors in stress-induced reinstatement of alcohol seeking in rats. *Brain Behav* **4**, 356–367 (2014).

126. Millan, M. J. *et al.* A model of chronic pain in the rat: functional correlates of alterations in the activity of opioid systems. *The Journal of Neuroscience* **7**, 77 (1987).

127. Faden, A. I., Molioneaux, C. J., Rosenberger, J. G., Jacobs, T. P. & Cox, B. M. Endogenous opioid immunoreactivity in rat spinal cord following traumatic injury. *Ann Neurol* **17**, 386–390 (1985).

128. Navratilova, E. *et al.* Kappa opioid signaling in the central nucleus of the amygdala promotes disinhibition and aversiveness of chronic neuropathic pain. *Pain* **160**, 824–832 (2019).

129. Liu, S. S. *et al.* Kappa Opioid Receptors Drive a Tonic Aversive Component of Chronic Pain. *J Neurosci* **39**, 4162–4178 (2019).

130. Liu, S. Investigating Neuroinflammation and Opioid Receptor Signaling Mechanisms in Chronic Pain Pathology. (University of California, Irvine, 2018).

131. Del Rosario, C. N., Pacchioni, A. M. & Cancela, L. M. Influence of acute or repeated restraint stress on morphine-induced locomotion: involvement of dopamine, opioid and glutamate receptors. *Behavioural Brain Research* **134**, 229–238 (2002).

132. Wang, D., Sun, X. & Sadee, W. Different Effects of Opioid Antagonists on μ-, δ-, and κ-Opioid Receptors with and without Agonist Pretreatment. *Journal of Pharmacology and Experimental Therapeutics* **321**, 544–552 (2007).

133. Mucha, R. F. & Iversen, S. D. Reinforcing properties of morphine and naloxone revealed by conditioned place preferences: a procedural examination. *Psychopharmacology (Berl)* **82**, 241–247 (1984).

134. Mucha, R. F. & Herz, A. Motivational properties of kappa and mu opioid receptor agonists studied with place and taste preference conditioning. *Psychopharmacology (Berl)* **86**, 274–280 (1985).

135. Mucha, R. F., Millan, M. J. & Herz, A. Aversive properties of naloxone in non-dependent (naive) rats may involve blockade of central β-endorphin. *Psychopharmacology (Berl)* **86**, 281–285 (1985).

136. Azar, M. R., Jones, B. C. & Schulteis, G. Conditioned place aversion is a highly sensitive index of acute opioid dependence and withdrawal. *Psychopharmacology (Berl)* **170**, 42–50 (2003).

137. Bardo, M. T., Rowlett, J. K. & Harris, M. J. Conditioned place preference using opiate and stimulant drugs: a meta-analysis. *Neurosci Biobehav Rev* **19**, 39–51 (1995).

138. Brice-Tutt, A. C. *et al.* An ethogram analysis of cutaneous thermal pain sensitivity and oxycodone reward-related behaviors in rats. *Scientific Reports 2023 13:1* **13**, 1–14 (2023).

139. Powell, K. R. & Holtzman, S. G. Parametric evaluation of the development of sensitization to the effects of morphine on locomotor activity. *Drug Alcohol Depend* **62**, 83–90 (2001).

140. Smith, M. A., Greene-Naples, J. L., Lyle, M. A., Iordanou, J. C. & Felder, J. N. The Effects of Repeated Opioid Administration on Locomotor Activity: I. Opposing Actions of μ and κ Receptors. *Journal of Pharmacology and Experimental Therapeutics* **330**, 468–475 (2009).

141. Babbini, M. & Davis, W. M. Time-dose relationships for locomotor activity effects of morphine after acute or repeated treatment. *Br J Pharmacol* **46**, 213 (1972).

142. Vaccarino, F. J., Amalric, M., Swerdlow, N. R. & Koob, G. F. Blockade of amphetamine but not opiate-induced locomotion following antagonism of dopamine function in the rat. *Pharmacol Biochem Behav* **24**, 61–65 (1986).

143. Teitelbaum, H., Giammatteo, P. & Mickley, G. A. Differential effects of localized lesions of n. accumbens on morphine- and amphetamine-induced locomotor hyperactivity in the C57BL/6J mouse. *J Comp Physiol Psychol* **93**, 745–751 (1979).

144. Hagino, Y. *et al.* Involvement of Cholinergic System in Hyperactivity in Dopamine-Deficient Mice. *Neuropsychopharmacology 2015 40:5* **40**, 1141–1150 (2014).

145. Rezayof, A., Nazari-Serenjeh, F., Zarrindast, M. R., Sepehri, H. & Delphi, L. Morphine-induced place preference: involvement of cholinergic receptors of the ventral tegmental area. *Eur J Pharmacol* **562**, 92–102 (2007).

146. Tao, Y. M. *et al.* Heteromers of μ opioid and dopamine D1 receptors modulate opioid-induced locomotor sensitization in a dopamine-independent manner. *Br J Pharmacol* **174**, 2842–2861 (2017).

147. Haghparast, A. & Rashvand, M. Role of the Orexinergic System Within the Ventral Tegmental Area in the Development of Sensitization to Morphine Induced by Lateral Hypothalamus Stimulation. *Basic Clin Neurosci* **13**, 97 (2022).

148. Mazaheri, S., Zendehdel, M. & Haghparast, A. Role of orexinergic receptors within the ventral tegmental area in the development of morphine sensitization induced by forced swim stress in the rat. *Prog Neuropsychopharmacol Biol Psychiatry* **116**, 110539 (2022).

149. Pennington, Z. Dissecting Comorbidity Between Opioid Use/Dependence and Post-Traumatic Stress Disorder - ProQuest. (University of California, Los Angles, 2018).

150. Conoscenti, M. A., Smith, N. J. & Fanselow, M. S. Dissociable consequences of moderate and high volume stress are mediated by the differential energetic demands of stress. *PLoS One* **17**, (2022).

151. Piazza, P. V., Deminière, J. M., Le Moal, M. & Simon, H. Factors That Predict Individual Vulnerability to Amphetamine Self-Administration. *Science (1979)* **245**, 1511–1513 (1989).

152. Wingo, T., Nesil, T., Choi, J. S. & Li, M. D. Novelty Seeking and Drug Addiction in Humans and Animals: From Behavior to Molecules. *Journal of Neuroimmune Pharmacology 2015 11:3* **11**, 456–470 (2015).

153. Kabbaj, M. Individual Differences in Vulnerability to Drug Abuse: The High Responders/Low Responders Model. *CNS Neurol Disord Drug Targets* **5**, 513–520 (2008).

154. Nadal, R., Rotllant, D., Márquez, C. & Armario, A. Perseverance of exploration in novel environments predicts morphine place conditioning in rats. *Behavioural Brain Research* **165**, 72–79 (2005).

155. Zheng, X. *et al.* Susceptibility to morphine place conditioning: relationship with stress-induced locomotion and novelty-seeking behavior in juvenile and adult rats. *Pharmacol Biochem Behav* **75**, 929–935 (2003).

156. Pelloux, Y., Costentin, J. & Duterte-Boucher, D. Novelty preference predicts place preference conditioning to morphine and its oral consumption in rats. *Pharmacol Biochem Behav* **84**, 43–50 (2006).

157. Deroche, V., Piazza, P. V., Le Moal, M. & Simon, H. Individual differences in the psychomotor effects of morphine are predicted by reactivity to novelty and influenced by corticosterone secretion. *Brain Res* **623**, 341–344 (1993).

158. Chiang, T.-H., Schmitt, K. & Nelson, A. Management of Patients on Low-Dose Naltrexone: A Clinical Review for Urgent Care Providers. *Journal of Urgent Care Medicine* **17**, 11–16 (2023).

159. Cree, B. A. C., Kornyeyeva, E. & Goodin, D. S. Pilot trial of low-dose naltrexone and quality of life in multiple sclerosis. *Ann Neurol* **68**, 145–150 (2010).

160. McLaughlin, P. J. *et al.* Low-dose naltrexone reduced anxiety in persons with multiple sclerosis during the COVID-19 pandemic. *Int Immunopharmacol* **113**, 109438 (2022).

161. Hutchinson, M. R. *et al.* Non-stereoselective reversal of neuropathic pain by naloxone and naltrexone: involvement of toll-like receptor 4 (TLR4). *European Journal of Neuroscience* **28**, 20–29 (2008).

162. Cant, R., Dalgleish, A. G. & Allen, R. L. Naltrexone inhibits IL-6 and TNFα production in human immune cell subsets following stimulation with ligands for intracellular toll-like receptors. *Front Immunol* **8**, 809 (2017).

163. Patten, D. K., Schultz, B. G. & Berlau, D. J. The Safety and Efficacy of Low-Dose Naltrexone in the Management of Chronic Pain and Inflammation in Multiple Sclerosis, Fibromyalgia, Crohn's Disease, and Other Chronic Pain Disorders. *Pharmacotherapy* **38**, 382–389 (2018).

164. Parkitny, L. & Younger, J. Reduced Pro-Inflammatory Cytokines after Eight Weeks of Low-Dose Naltrexone for Fibromyalgia. *Biomedicines 2017, Vol. 5, Page 16* **5**, 16 (2017).

165. Jones, M. E., Lebonville, C. L., Barrus, D. & Lysle, D. T. The Role of Brain Interleukin-1 in Stress-Enhanced Fear Learning. *Neuropsychopharmacology 2015 40:5* **40**, 1289–1296 (2014).

166. Jones, M. E. *et al.* Hippocampal interleukin-1 mediates stress-enhanced fear learning: A potential role for astrocyte-derived interleukin-1β. *Brain Behav Immun* **67**, 355–363 (2018).

167. Kamens, H., Flarend, G., … A. W.-E. and & 2023, undefined. The effect of stress on opioid addiction-related behaviors: A review of preclinical literature. *psycnet.apa.org*.

168. Meier, A. *et al.* Co-occurring prescription opioid use problems and posttraumatic stress disorder symptom severity. *American Journal of Drug and Alcohol Abuse* **40**, 304–311 (2014).

169. Rhee, T. G., Peltier, M. R., Sofuoglu, M. & Rosenheck, R. A. Do Sex Differences Among Adults With Opioid Use Disorder Reflect Sex-specific Vulnerabilities? A Study of Behavioral Health Comorbidities, Pain, and Quality of Life. *J Addict Med* **14**, 502 (2020).

170. Lovallo, W. R. *et al.* Cortisol Stress Response in Men and Women Modulated Differentially by the Mu-Opioid Receptor Gene Polymorphism OPRM1 A118G. *Neuropsychopharmacology 2015 40:11* **40**, 2546–2554 (2015).

171. Reich, B. *et al.* Chronic immobilization stress primes the hippocampal opioid system for oxycodone-associated learning in female but not male rats. *Synapse* **73**, e22088 (2019).

172. Chalangal, J., Mazid, S., Windisch, K. & Milner, T. A. Sex differences in the rodent hippocampal opioid system following stress and oxycodone associated learning processes. *Pharmacol Biochem Behav* **212**, 173294 (2022).

173. Sørensen, A. T. *et al.* A robust activity marking system for exploring active neuronal ensembles. *Elife* **5**, (2016).

174. Guenthner, C. J., Miyamichi, K., Yang, H. H., Heller, H. C. & Luo, L. Permanent genetic access to transiently active neurons via TRAP: Targeted recombination in active populations. *Neuron* **78**, 773–784 (2013).

175. DeNardo, L. A. *et al.* Temporal evolution of cortical ensembles promoting remote memory retrieval. *Nat Neurosci* **22**, 460–469 (2019).

176. Redondo, R. L. *et al.* Bidirectional switch of the valence associated with a hippocampal contextual memory engram. *Nature* **513**, 426–430 (2014).

177. Liu, X. *et al.* Optogenetic stimulation of a hippocampal engram activates fear memory recall. *Nature* **484**, 381–385 (2012).

178. Reijmers, L. G., Perkins, B. L., Matsuo, N. & Mayford, M. Localization of a stable neural correlate of associative memory. *Science (1979)* **317**, 1230–1233 (2007).

179. Denardo, L. & Luo, L. Genetic strategies to access activated neurons. *Curr Opin Neurobiol* **45**, 121–128 (2017).

180. Giannotti, G., Heinsbroek, J. A., Yue, A. J., Deisseroth, K. & Peters, J. Prefrontal cortex neuronal ensembles encoding fear drive fear expression during long-term memory retrieval. *Sci Rep* **9**, (2019).

181. Higashimoto, T. *et al.* The woodchuck hepatitis virus post-transcriptional regulatory element reduces readthrough transcription from retroviral vectors. *Gene Ther* **14**, 1298–1304 (2007).

182. Rao-Ruiz, P., Yu, J., Kushner, S. A. & Josselyn, S. A. Neuronal competition: microcircuit mechanisms define the sparsity of the engram. *Curr Opin Neurobiol* **54**, 163 (2019).

183. Vijay, A. *et al.* PET imaging reveals sex differences in kappa opioid receptor availability in humans, in vivo. *Am J Nucl Med Mol Imaging* **6**, 205 (2016).

184. Huang, P., Chen, C., Cao, D., Huang, M. & Liu-Chen, L. Y. Agonist-promoted kappa opioid receptor (KOR) phosphorylation has behavioral endpoint-dependent and sex-specific effects. *Neuropharmacology* **202**, 108860 (2022).

185. Craft, R. M. & Bernal, S. A. Sex differences in opioid antinociception: κ and 'mixed action' agonists. *Drug Alcohol Depend* **63**, 215–228 (2001).

186. Kavaliers, M. & Innes, D. G. L. Sex and day-night differences in opiate-induced responses of insular wild deer mice, Peromyscus maniculatus triangularis. *Pharmacol Biochem Behav* **27**, 477–482 (1987).

187. Russell, S. E. *et al.* Sex Differences in Sensitivity to the Depressive-like Effects of the Kappa Opioid Receptor Agonist U-50488 in Rats. *Biol Psychiatry* **76**, 213–222 (2014).

188. Wang, Y. J. *et al.* Sex Difference in κ-Opioid Receptor (KOPR)-Mediated Behaviors, Brain Region KOPR Level and KOPR-Mediated Guanosine 5′-O-(3-[35S]Thiotriphosphate) Binding in the Guinea Pig. *Journal of Pharmacology and Experimental Therapeutics* **339**, 438–450 (2011).

189. Barrett, A. C., Smith, E. S. & Picker, M. J. Sex-related differences in mechanical nociception and antinociception produced by μ- and κ-opioid receptor agonists in rats. *Eur J Pharmacol* **452**, 163–173 (2002).

190. Barrett, A. C. *et al.* Sex and rat strain determine sensitivity to κ opioid-induced antinociception. *Psychopharmacology (Berl)* **160**, 170–181 (2002).

191. Gordon, N. C. *et al.* Enhancement of morphine analgesia by the GABAB agonist baclofen. *Neuroscience* **69**, 345–349 (1995).

192. Gear, R. W. *et al.* Gender difference in analgesic response to the kappa-opioid pentazocine. *Neurosci Lett* **205**, 207–209 (1996).

193. Gear, R. W. *et al.* Kappa–opioids produce significantly greater analgesia in women than in men. *Nature Medicine 1996 2:11* **2**, 1248–1250 (1996).

194. Taylor, A. M. W. *et al.* Sex differences in kappa opioid receptor antinociception is influenced by the number of X chromosomes in mouse. *J Neurosci Res* **100**, 183–190 (2022).

195. Atkinson, LE. *et al.* Circulating Levels of Steroids and Chorionic Gonadotropin During Pregnancy in the Rhesus Monkey, With Special Attention to the Rescue of the Corpus Luteum in Early Pregnancy. *Biol Reprod* **12**, 335–345 (1975).

196. Stoffel, E. C., Ulibarri, C. M., Folk, J. E., Rice, K. C. & Craft, R. M. Gonadal hormone modulation of mu, kappa, and delta opioid antinociception in male and female rats. *J Pain* **6**, 261–274 (2005).

197. Menendez, L., Andrés-Trelles, F., Hidalgo, A. & Baamonde, A. Gender and test dependence of a type of kappa mediated stress induced analgesia in mice. *General Pharmacology: The Vascular System* **25**, 903–908 (1994).

198. Sun, N. & Laviolette, S. R. Inactivation of the basolateral amygdala during opiate reward learning disinhibits prelimbic cortical neurons and modulates associative memory extinction. *Psychopharmacology (Berl)* **222**, 645–661 (2012).

199. Karimi, S. *et al.* Forced swim stress but not exogenous corticosterone could induce the reinstatement of extinguished morphine conditioned place preference in rats: Involvement of glucocorticoid receptors in the basolateral amygdala. *Behavioural Brain Research* **264**, 43–50 (2014).

200. Gholizadeh, S. *et al.* Early versus Late-Phase Consolidation of Opiate Reward Memories Requires Distinct Molecular and Temporal Mechanisms in the Amygdala-Prefrontal Cortical Pathway. *PLoS One* **8**, (2013).

201. Nygard, S. K., Hourguettes, N. J., Sobczak, G. G., Carlezon, W. A. & Bruchas, M. R. Stress-Induced Reinstatement of Nicotine Preference Requires Dynorphin/Kappa Opioid Activity in the Basolateral Amygdala. *Journal of Neuroscience* **36**, 9937–9948 (2016).

202. Chaplan, S. R., Bach, F. W., Pogrel, J. W., Chung, J. M. & Yaksh, T. L. Quantitative assessment of tactile allodynia in the rat paw. *J Neurosci Methods* **53**, 55–63 (1994).

203. Taylor, A. M. W. *et al.* Microglia Disrupt Mesolimbic Reward Circuitry in Chronic Pain. *The Journal of Neuroscience* **35**, 8442 (2015).

204. Bruchas, M. R. *et al.* Long-acting κ Opioid Antagonists Disrupt Receptor Signaling and Produce Noncompetitive Effects by Activating c-Jun N-terminal Kinase. *J Biol Chem* **282**, 29803 (2007).

205. Bakker, R., Tiesinga, P. & Kötter, R. The Scalable Brain Atlas: instant web-based access to public brain atlases and related content. *Neuroinformatics* (2013).

206. Lein, E. S. *et al.* Genome-wide atlas of gene expression in the adult mouse brain. *Nature 2006 445:7124* **445**, 168–176 (2006).

207. Heck, A. L. & Handa, R. J. Sex differences in the hypothalamic–pituitary–adrenal axis' response to stress: an important role for gonadal hormones. *Neuropsychopharmacology 2018 44:1* **44**, 45–58 (2018).

208. Herman, J. P., Cullinan, W. E., Ziegler, D. R. & Tasker, J. G. Role of the paraventricular nucleus microenvironment in stress integration*. *European Journal of Neuroscience* **16**, 381–385 (2002).

209. Pascoe, J. E. *et al.* Effects of mu, kappa, and delta opioid receptor agonists on the function of hypothalamic–pituitary–adrenal axis in monkeys. *Psychoneuroendocrinology* **33**, 478–486 (2008).

210. Turner-Cobb, J. M., Osborn, M., Da Silva, L., Keogh, E. & Jessop, D. S. Sex differences in hypothalamic–pituitary–adrenal axis function in patients with chronic pain syndrome. *Stress* **13**, 293–301 (2010).

211. Conway, S. M. *et al.* Females are less sensitive than males to the motivational- and dopamine-suppressing effects of kappa opioid receptor activation. *Neuropharmacology* **146**, 231–241 (2019).

212. Wallace, C. W., Holleran, K. M., Slinkard, C. Y., Centanni, S. W. & Jones, S. R. Kappa Opioid Receptors Negatively Regulate Real Time Spontaneous Dopamine Signals by Reducing Release and Increasing Uptake. *bioRxiv* 2024.02.05.578840 (2024) doi:10.1101/2024.02.05.578840.

213. Fishbain, D. A., Pulikal, A., Lewis, J. E. & Gao, J. Chronic Pain Types Differ in Their Reported Prevalence of Post -Traumatic Stress Disorder (PTSD) and There Is Consistent Evidence That Chronic Pain Is Associated with PTSD: An Evidence-Based Structured Systematic Review. *Pain Medicine* **18**, 711–735 (2017).

214. Sterling, M. & Kenardy, J. The relationship between sensory and sympathetic nervous system changes and posttraumatic stress reaction following whiplash injury--a prospective study. *J Psychosom Res* **60**, 387–393 (2006).

215. Sterling, M., Jull, G., Vicenzino, B., Kenardy, J. & Darnell, R. Physical and psychological factors predict outcome following whiplash injury. *Pain* **114**, 141–148 (2005).

216. Sharp, T. J. & Harvey, A. G. Chronic pain and posttraumatic stress disorder: mutual maintenance? *Clin Psychol Rev* **21**, 857–877 (2001).