BEHAVIOURAL EFFECTS OF NOVEL CLINICAL CANDIDATE DRUGS, l-TETRAHYDROPALMATINE (l-THP) AND Z944, ON MORPHINE WITHDRAWAL-INDUCED HYPERALGESIA

by

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submitted by Daria Oleinichenko in partial fulfilment of the requirements for

the degree of Master of Science

in Neuroscience

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Abstract

Opioid use disorder (OUD) is a major contributor to drug-related deaths worldwide. Opioid use cessation causes severe withdrawal symptoms, including prominent hyperalgesia – a contributor to the negative reinforcement of drug taking. Effective pain control is an underappreciated aspect of managing opioid withdrawal, and its absence presents a significant barrier to successful opioid detoxification. Exploring analgesic interventions for withdrawal-induced hyperalgesia may reveal novel OUD therapies. This thesis describes a model of hyperalgesia in both acute and extended withdrawal in morphine-dependent animals and the effect of two clinical candidate analgesic drugs on withdrawal-induced hyperalgesia. *l*-Tetrahydropalmatine (*l*-THP) is a tetrahydroprotoberberine compound and active ingredient of a botanical formulation used in Vietnam for OUD treatment with preclinical efficacy in neuropathic pain models. Z944 is a selective T-type calcium channel antagonist undergoing clinical trials as an anticonvulsive and analgesic. To establish drug dependence modelling intermittent access during abuse scenarios, morphine (15 mg/kg, i.p.) was given once a day, 5 days/week and Von Frey tests were conducted 2-3 times a week ~23 h after morphine injection. Animals subjected to three weeks of morphine treatment experienced a ~30% reduction in pain tolerance. To model hyperalgesia during detoxification, animals entered abstinence after 3 weeks of morphine treatment and with Von Frey testing showing that hyperalgesia was persistent for 14 days before spontaneous recovery. Both *l*-THP (5 or 7.5 mg/kg, p.o.) and Z944 (10 mg/kg, p.o.) were effective at attenuating hyperalgesia during acute withdrawal. Seven-day treatment with *l*-THP (5 mg/kg) or Z944 (10 mg/kg) in morphine-dependent animals undergoing extended withdrawal resulted in a significant increase in paw retraction thresholds compared to controls and this effect persisted after the completion of treatment. Importantly, the improvement in pain tolerance remained after
treatment completion, hastening pain tolerance recovery to baseline by 61% and 80% (l-THP and Z944, respectively). Neither candidate drug influenced mechanical sensitivity in morphine-naïve animals. Overall, these findings support the hypothesis that pain management during detoxification is necessary for improved treatment outcomes. l-THP and Z944, therefore, may be a valuable addition to the currently limited arsenal of opioid detoxification treatments.
Lay Summary

Opioid addiction is an epidemic that bears a tremendous impact on our socioeconomic and medical systems. Opioid users develop tolerance to the drug and undergo withdrawal if they quit abruptly. During withdrawal, patients become severely ill and have an increased sensitivity to pain. The negative experience of withdrawal is one of the driving forces behind the addiction cycle but achieving abstinence is necessary for remission. My thesis describes increased pain sensitivity present in a rat model of morphine-dependence and the effect of two clinical candidate drugs, $l$-tetrahydropalmatine ($l$-THP) and Z944 on pain during withdrawal. Both drugs are known to be effective against chronic pain but it is not known if they alleviate pain in drug withdrawal models. The results of the experiments confirm that both $l$-THP and Z944 improve pain tolerance during acute withdrawal and decrease the time to recovery of normal pain sensitivity in animals undergoing detoxification from morphine.
Preface

The experiments presented in this thesis were designed by Daria Oleinichenko with oversight by Drs Soyon Ahn, Terrance Snutch, and Anthony Phillips. Data collection and analysis were performed by myself with guidance from Dr Ahn. The manuscript and figures were created by Daria Oleinichenko and edited by Dr Phillips and Dr Snutch. Suggestions from the supervisory committee were also used to edit the manuscript. All experiments were carried out in accordance with the Canadian Council on Animal Care, with approval by the Animal Care Committee at the University of British Columbia under protocol number A21-0235.

As a result of this work, we published:


This paper described our model of withdrawal-induced hyperalgesia and the effect of l-tetrahydropalmatine on acute and extended withdrawal. For the purpose of this thesis, Chapter 2 presents an expanded version of model validation combined with further experiments. The data on the efficacy of l-THP will be presented in Chapter 3. Experiments 1-3 published in this paper and presented in this thesis were conceived and designed by myself, and I conducted the data collection and analysis. Ru Song designed and executed and described Experiment 4, not featured in this thesis. The manuscript was prepared through collective efforts of myself and Drs Soyon Ahn, and Anthony Phillips, edited by Dr Terrance Snutch.
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<table>
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>CFA</td>
<td>Complete Freund’s Adjuvant</td>
</tr>
<tr>
<td>CRF</td>
<td>Corticotrophin-releasing factor</td>
</tr>
<tr>
<td>DA</td>
<td>Dopamine</td>
</tr>
<tr>
<td>LC</td>
<td>Locus coeruleus</td>
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<tr>
<td>l-ICP</td>
<td>l-isocorypalmine</td>
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<tr>
<td>l-THP</td>
<td>l-tetrahydropalmatine</td>
</tr>
<tr>
<td>MOR</td>
<td>Morphine</td>
</tr>
<tr>
<td>NAc</td>
<td>Nucleus accumbens</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartate</td>
</tr>
<tr>
<td>OIH</td>
<td>Opioid-induced hyperalgesia</td>
</tr>
<tr>
<td>OUD</td>
<td>Opioid use disorder</td>
</tr>
<tr>
<td>OWS</td>
<td>Opioid withdrawal syndrome</td>
</tr>
<tr>
<td>PAG</td>
<td>Periaqueductal grey</td>
</tr>
<tr>
<td>RM ANOVA</td>
<td>Repeated measures analysis of variance</td>
</tr>
<tr>
<td>THPB</td>
<td>Tetrahydroprotoberberine</td>
</tr>
<tr>
<td>VEH</td>
<td>Vehicle</td>
</tr>
<tr>
<td>VTA</td>
<td>Ventral tegmental area</td>
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I extend a word of thanks to my friends in the Graduate Program of Neuroscience, Kelly Hrelja, Giulio Laino, and Melanie Lysenko-Martin, who were always there to guide me through uncertainty and share their perspectives.

I would like to thank my parents for their incessant support of my endeavours and the values they instilled in me. Finally, I have endless gratitude for the patient encouragement and love of my partner, Ratib Raif, who was with me through the highs and the lows of my graduate journey.
Dedication

I dedicate this thesis to my grandparents, who would have been immensely proud of my achievements and my parents, who dreamed of the best for me, and for whom no ambition of mine was too big.
Chapter 1: General Introduction

Increased access to potent pain-relieving opioids is linked to an unprecedented rise in opioid use disorder (OUD) and opioid-related deaths (Alexander et al., 2020; Dasgupta et al., 2018). Opioid detoxification, a process necessary to overcome physical dependence on opioids, is a critical first step in transitioning to long-term management of OUD. However, abrupt discontinuation triggers opioid withdrawal syndrome (OWS), a debilitating condition that includes severe pain, negative affect/dysphoria, and somatic signs that last for several days to many weeks (Kosten & Baxter, 2019). For individuals with a history of chronic opioid use, which often includes prior unsuccessful attempts to abstain from these drugs, the urge to avoid OWS is a primary cause of failure to complete detoxification and therefore is a powerful driving factor in maintaining opioid use (Koob, 2020; Kosten & Baxter, 2019). Current protocols for detoxification often involve substitution therapy with long-acting opioids (e.g., methadone, buprenorphine), which introduce problems related to side effects and diversion to illicit use. Accordingly, there is great interest in non-opioid compounds that may have efficacy in facilitating detoxification from opioids.

1.1 Withdrawal-induced Hyperalgesia in Opioid Use Disorder

The misuse of opioids commonly arises from the need to control pain, but ironically, their continued use leads to hypersensitivity to pain, a phenomenon known as opioid-induced hyperalgesia (OIH) or opioid tolerance (Mercadante et al., 2019). Clinically, OIH is evident in individuals with OUD currently on methadone maintenance and those prescribed opioids for perioperative pain (Angst & Clark, 2006; Higgins et al., 2019; Tompkins & Campbell, 2011). Adding insult to injury, we now know that opioid withdrawal, whether during voluntary detoxification, lack of access to an illicit drug supply, or naloxone-precipitated withdrawal, also
elicits a heightened state of pain perception, called “opioid withdrawal-induced hyperalgesia” (Carcoba et al., 2011; Ren et al., 2009). Both OIH and withdrawal-induced hyperalgesia are characterised by a disproportionate nociceptive response to a previously innocuous stimulant and can have a profound effect on quality of patients’ lives (Lee et al., 2011). Animal studies by Koob and others demonstrate that opioid withdrawal-induced hyperalgesia is a phenomenon that remains stable for several weeks, with the involvement of both central and peripheral neural substrates (Alvarez-Bagnarol et al., 2022; Dunbar et al., 2007; Marchette et al., 2021; McDevitt et al., 2021; Raghavendra et al., 2004). Thus, enhanced pain perception, which begins during acute withdrawal and persists into protracted abstinence (Carcoba et al., 2011; Ren et al., 2009), likely contributes to an aversive state that drives drug-seeking behaviour (Koob, 2021). Recent clinical work confirms a positive correlation between the severity of pain and the frequency at which patients report pain coping as their motivation for drug-seeking (McHugh et al., 2022). As yet, pain management remains an undertreated aspect of OUD, particularly following opioid detoxification (Koob, 2021; Laroche et al., 2012).

1.2 Neural Changes during Opioid Withdrawal

The aversive effects of withdrawal from drugs of abuse are believed to arise as a result of neuroadaptations that occur following intoxication. The neural effects of withdrawal are explained by opponent process theory, where the counteradaptive opponent process is a counteraction to the temporary disruption of hedonic homeostasis caused by drug taking (Koob, 2020). For instance, opioid withdrawal is associated with hypodopaminergia in the ventral tegmental area (VTA) and the nucleus accumbens (NAc) (Rossetti et al., 1992) and downregulation of dopamine (DA) D2 receptors in the striatum (Zijlstra et al., 2008). Hedonic effects of opioids are produced by their inhibitory activity on GABAergic interneurons in the
VTA, causing disinhibition of dopamine signalling, while in withdrawal an increase in GABA release can explain the reduction in dopamine (Bonci & Williams, 1997). An increase in glutamatergic tone also contributes to withdrawal behaviours and MK-801, an N-Methyl-D-aspartate (NMDA) receptor antagonist, reduces hyperalgesia and somatic signs of withdrawal (Laulin et al., 1998; Tokuyama et al., 1996). An increase in corticotropin-releasing factor (CRF) in the extended amygdala produces aversive emotional aspects of withdrawal (Koob, 2015), and elevated cortisol levels in withdrawal positively correlated with the severity of somatic symptoms (Bearn et al., 2001). Dynorphin, an endogenous kappa-opioid receptor ligand, is upregulated in withdrawal and is known for its aversive and pronociceptive actions (Nylander et al., 1995; Z. Wang et al., 2001). Chronic opioid use causes neuroimmune activation that is strongly linked to withdrawal-induced hyperalgesia (Deleo et al., 2004; Watkins et al., 2009). This complex dysregulation of neural pathways involves a vast number of brain regions and the combined effect of neural adaptation described above likely contributes to pronounced hyperalgesia during withdrawal.

1.3 Neurocircuitry of Nociception and Pain Modulation

The prominent neural changes in withdrawal impact central pain modulation circuitry, though the specific mechanisms by which they induce hyperalgesia have not been described. Painful stimuli activate sensory afferents that synapse onto a spinothalamic projection neuron in the dorsal horn of the spinal cord. Collateral projections from these neurons synapse onto the periaqueductal grey (PAG), a region critical in descending pain modulation (Ossipov et al., 2010). Pain signals reaching the thalamus are relayed to the amygdala and the sensory cortex, where the noxious information is integrated to produce behaviour changes. To activate the descending pain modulation pathway, signals from corticolimbic projections are relayed to the
PAG and subsequently to locus coeruleus (LC). Descending noradrenergic projections from LC interfere with the relay of ascending signals from sensory neurons in the dorsal horn of the spinal cord (Ossipov et al., 2010). Opioid drugs and some endogenous opioids exert an analgesic effect through the activation of the descending pain modulatory pathway in the PAG (Bagley & Ingram, 2020; Yeung et al., 1977). Opponent processes in the PAG during withdrawal, involving neuroinflammation and dysregulation of neurotransmission likely disrupt descending pain modulation and contribute to hyperalgesia (Ferrari et al., 2021; Ouyang et al., 2012). Dopamine, one of the neurotransmitters disrupted by withdrawal, is known to participate in descending pain control. Hypothalamic dopaminergic neurons project to the spinal cord to induce antinociception but even more importantly, DA plays a role in regulating the emotional dimension of pain (C. Li et al., 2019; S. Liu et al., 2019). In humans, depletion of DA causes increased pain effects, but not necessarily increased nociception (Tiemann et al., 2014), consistent with the interdependency of pain perception and negative affect states of withdrawal (Carcoba et al., 2011).

1.4 Search for Non-opioid Therapeutics

Recent clinical work confirms a positive correlation between the severity of pain and the frequency at which patients report pain coping as their motivation for drug-seeking (McHugh et al., 2022). The current clinical approach is not sufficient to address this need—long-term opioid replacement therapies are associated with persistent pain and patients on methadone maintenance therapy continue to show increased pain sensitivity (Compton et al., 2000). As yet, pain management remains an undertreated aspect of OUD, particularly following opioid detoxification (Koob, 2021; Laroche et al., 2012).

The urgent need for improved OUD therapies calls for an alternative approach to drug screening that would consider pain as a contributing factor to remission failure. Two candidate
drugs, \textit{l}-tetrahydropalmatine \textit{(l-THP)} and Z944, were selected for evaluation of efficacy in withdrawal-induced hyperalgesia, both compounds showing favourable analgesic profiles and ability to interfere with conditioned place preference to morphine in animal models (Cunningham, 2016; Harding et al., 2021; W.-N. Jiang et al., 2020; Y.-Y. Liu et al., 2019).

1.5 Research Aims and Hypotheses

This research aimed to evaluate the effects of \textit{l}-THP and Z944 on morphine withdrawal-induced hyperalgesia during acute withdrawal and in a clinically relevant drug detoxification paradigm. The sub-aims include successful modelling of withdrawal-induced hyperalgesia in male rats and a behavioural assessment of \textit{l}-THP and Z944 in this model. We hypothesize that chronic morphine treatment will induce stable hyperalgesia that will persist after cessation of drug use. Moreover, we predict that both candidate drugs will attenuate withdrawal-induced hyperalgesia when administered acutely. Finally, we expect that when administered repeatedly, \textit{l}-THP and Z944 will improve hyperalgesia during protracted drug abstinence.
Chapter 2: Preclinical models of morphine withdrawal-induced hyperalgesia

2.1 Introduction

The urgent need for more effective pain management in opioid use disorder treatment highlights the need for a reliable model of opioid withdrawal-induced hyperalgesia. Several studies have been done in humans to confirm the presence of hyperalgesia in withdrawal. Compton et al. (2003) report that in non-users a single treatment with opioids was sufficient to induce hyperalgesia when withdrawal was precipitated with naloxone. Chronic heroin users exhibit a higher sensitivity to pain in withdrawal than non-users and this phenomenon persists after several months of abstinence (Carcoba et al., 2011). In the state of negative affect, heroin users in acute withdrawal experienced a decline in pain tolerance, indicating that withdrawal increases the salience of negative stimuli, which intensifies pain perception.

Several works have explored opioid withdrawal-induced hyperalgesia in preclinical models. Laulin et al. (1998) showed that a single heroin treatment resulted in mechanical hyperalgesia that persisted for 48 h post-treatment. Further, morphine administration for 5-7 days elicited significant hyperalgesia that could be attenuated with propentofylline, ketorolac, or ginsenosides (Raghavendra et al., 2004; Dunbar et al., 2007; P. Li et al., 2014). Hyperalgesia is also observed during withdrawal following repeated treatment with heroin and fentanyl (Marchette et al., 2021; Wei & Wei, 2012). While these studies describe the effects of drug intervention on hyperalgesia, they do not capture the chronic intermittent access that is evident in most abuse scenarios. Finally, they do not characterize persistent hyperalgesia during detoxification from long-term opioid use (Roeckel et al., 2017). This set of experiments aimed to model morphine-withdrawal induced hyperalgesia for testing of potential OUD therapies.
The present model was inspired by a recent report from the Koob lab that a single treatment with the κ-opioid receptor antagonists nor-binaltorphimine or 5’-guanidinonaltrindole resulted in the long-lasting reversal of heroin withdrawal-induced hyperalgesia in male and female rats (Marchette et al., 2021). These results were possible due to the establishment of a reliable heroin-withdrawal induced hyperalgesia protocol, where for 2 weeks animals underwent 5 days of opioid treatment followed by 2 drug-free days. The hyperalgesia response was assessed 4-6 hours after administration using a hand-held electronic von Frey device, capturing the pain sensitivity during spontaneous withdrawal. The work here uses the core feature of the protocol from Marchette et al. (2021) (5 days of opioid treatment followed by 2 drug-free days) to successfully induce robust morphine withdrawal-induced hyperalgesia in rats. This approach incorporates extended (72 h) withdrawal periods to model intermittent morphine access associated with increased morphine-evoked hyperdopaminergia in the NAc (Lefevre et al., 2020) and more pronounced withdrawal hyperalgesia (Harris & Aston-Jones, 1994), emulating the intoxication-withdrawal cycle common in substance use disorders. This chapter describes the model validation and general methodology employed to assess whether acute or repeated treatment with novel therapeutics, l-THP and Z944, would reverse withdrawal-induced hyperalgesia, as assessed by von Frey testing for mechanical algesia.

2.2 General methodology

2.2.1 Animals

Ninety-four male Sprague-Dawley rats (250-275 g) from Charles River Laboratories (St. Constant, Canada) were acclimated to the facility for a week prior to any manipulations. Animals were pair-housed in Optirat Cages (Animal Care Systems, Centennial, CO, USA) and maintained with ad libitum access to food and water at ~ 21 °C in a reverse 12-h light/dark cycle (lights on at
The body weight of the animals was monitored daily using a digital scale. All experimental procedures were conducted in accordance with the ethical standards set by the Canadian Council on Animal Care and approved by the University of British Columbia Animal Care Committee (AUP A21-0235).

2.2.2 Drug preparation and administration

Morphine sulfate from Unipharm Wholesale Drugs Limited (Richmond, Canada) was prepared in saline (15 mg/ml) 1 h before treatment. For 3-5 weeks, animals received a daily injection of 15 mg/kg (i.p.) for five days followed by no morphine for two days each week, an injection schedule adapted from a heroin withdrawal-induced hyperalgesia study by Marchette et al (Marchette et al., 2021).

\( l \)-THP, purchased from Santa Cruz Biotechnology (Dallas, Texas, US), was dissolved in 0.1 M sulphuric acid, and diluted with sterile water to a final concentration of 2.5 mg/ml in 5% acid (pH 4). \( l \)-THP (5 or 7.5 mg/kg, p.o.) or its vehicle (1% acid at pH 4) was administered by oral gavage 30 min before behavioural testing.

Z944, 4-\([(3\text{-Chloro-5-fluorobenzoyl}amino)methyl]-N-(1,1\text{-dimethylethyl})-1-piperidineacetamide, was synthesized at UBC’s Dept. of Chemistry according the methods described in Tringham et al. (2012) and dissolved in 0.1 M sulphuric acid, and diluted with sterile water to a final concentration of 5 mg/ml in 1% acid (pH 4). Z944 (10 mg/kg, p.o.) or its vehicle (1% acid at pH 4) was administered by oral gavage 30 min before behavioural testing. Before oral drug treatment, animals were habituated to the oral gavage method for 3 days.

2.2.3 Assessment of hyperalgesia with Von Frey device

Hyperalgesia during morphine withdrawal was evaluated using an electronic hand-held Von Frey device, version 3.4 (Ugo Basile, Gemonio, Italy). Before assessments began, animals
were transported to the testing room to acclimate in their home cages for 30 min a day for 3 days. The following week, baseline measurements of sensitivity to mechanical stimulation were assessed on three non-consecutive days (Figure 2.1A). Each day, animals were brought to the testing room and, after 30 min, placed into the testing apparatus which consisted of an elevated platform with 6 Plexiglas compartments (27 cm × 16 cm × 13 cm) and stainless-steel fine-mesh flooring. After 15 min, the Von Frey device was used to apply increasing grams of force to the mid-plantar area of the hind paw until paw retraction was elicited. Each day, six paw withdrawal thresholds were recorded for each subject (three trials per left and right paw) with at least 30 s elapsed between attempts. Any measurements concurrent with walking, jumping, and grooming were discarded, and the trials were repeated. Baseline von Frey scores were plotted against the animal weight to assess whether individual variability in body mass may be correlated to baseline pain tolerance.

2.2.4 Experimental design

2.2.4.1 Modelling morphine withdrawal-induced hyperalgesia.

Rats (n=12) were administered morphine injections (15 mg/kg, i.p.) as described above for five weeks. After starting morphine injections, Von Frey tests were conducted as described above on the 2nd and 4th day of each week, ~ 23 h after the previous morphine injection (Figure 2.1A). The timing of the test was chosen to capture the peak of withdrawal severity, which occurs at approximately 24 h post-morphine, as reported by Brewer et al (Brewer et al., 2023).

2.2.4.2 Hyperalgesia during extended withdrawal in drug abstinence.

Following the measurement of baseline values in drug-naïve rats (n=24), subjects were injected once daily with morphine (15 mg/kg, i.p.) during Weeks 1-3 as described above (Figure 2.2A). Von Frey thresholds were assessed bi-weekly throughout Weeks 1-3 on the 2nd and 4th
day of each week, after 23 h withdrawal from the previous morphine injection. After completing 3 weeks of morphine, subjects entered the abstinence period. On week 4 rats received a vehicle treatment for 7 days. Von Frey testing was conducted 30 min after the 1st, 4th, and 6th treatment, as well as 23 h after the 7th delivery of the vehicle. Finally, Von Frey assessments continued into Weeks 5-6 in absence of any further interventions.

2.2.4.3 Effect of l-THP and Z944 on pain thresholds of morphine-naïve animals

Rats (n=11) were administered saline (1 ml/kg, i.p.) 5 days per week for 2 weeks and assessed for Von Frey thresholds bi-weekly. After baseline measures of paw retraction thresholds in an initial test, subsequent assessments were preceded by the administration of either vehicle, 5 mg/kg l-THP, or 10 mg/kg Z944 30 min before the tests following a Latin square design.

2.2.5 Data presentation and statistical analysis

Paw withdrawal thresholds, expressed in grams of force (gf) required to elicit paw withdrawal, are presented as a mean of six measurements obtained during each Von Frey test day. Within-subject comparisons of paw retraction thresholds against baseline were conducted using one-way RM ANOVA followed by Dunnett’s multiple comparisons. The box plot method of outlier analysis was applied to mean paw withdrawal thresholds and one animal identified as an outlier was excluded from the analysis in Figure 2.4. The correlation of body weight with baseline pain tolerance was assessed by fitting simple linear regression.

GraphPad Prism version 9.5.0 for Windows was used for all statistical analyses and data visualization (GraphPad Software, San Diego, CA, USA, www.graphpad.com)
2.3 Results

2.3.1 Hyperalgesia during acute morphine withdrawal

This experiment established the time course for inducing withdrawal-induced hyperalgesia in rats receiving morphine for five weeks (Figure 2.1A). During the week prior to starting morphine (baseline week), Von Frey tests were conducted on three alternating days to determine the grams of force (gf) required to elicit the paw retraction reflex. There was no significant difference between the three measurements as indicated by a one-way RM ANOVA (F2, 22 = 0.593, P=0.56). This indicated that repeated testing did not lead to the development of sensitization or tolerance of the paw retraction reflex and, hence, the mean of the three threshold values (24.46±1.20 gf) served as the control value in subsequent within-subject analyses.

Starting Week 1, rats received injections of morphine (15 mg/kg, i.p.) 5 days each week, for 5 weeks. Bi-weekly Von Frey tests, conducted ~23 h after the previous day’s morphine treatment, confirmed a significant effect of morphine injections on paw retraction threshold (F10, 110 = 13.390, P<0.01). In comparison to baseline, the threshold was significantly lower on Test Day 2 (Dunnett’s test, P<0.01) (Figure 2.1B). From Test Day 4 (16.76±1.26 gf) forwards, thresholds persisted under 30% below baseline until the final assessment on Day 11 (15.80±1.43 gf). These results demonstrated that in our rat model of morphine dependence, a hyperalgesic response to Von Frey tests appears within three cycles of experiencing morphine and acute withdrawal states and that the severity of hyperalgesia was maintained at a consistent level with continued morphine exposure.
Figure 2.1. Intermittent morphine administration induces persistent hyperalgesia during acute withdrawal

**A)** Schedule of morphine (MOR) injections, vehicle treatments (tx), and Von Frey testing in a model of acute withdrawal-induced hyperalgesia. During Weeks 1-5, MOR (15 mg/kg, i.p., n=12) was administered 5 days per week. During Weeks 4 and 5, vehicle (VEH) was administered 30 min before Von Frey assessments.

**B)** Induction curve of hyperalgesia as indicated by changes in paw retraction threshold at 23 h post-MOR injection. Datapoints are the paw retraction threshold (mean+SEM) in grams of force (gf). The dotted line represents the BL threshold value. Dunnett’s test: **P<0.01 vs. baseline (BL).
2.3.2 Hyperalgesia during extended morphine withdrawal

This experiment established the time course for recovery from withdrawal-induced hyperalgesia in rats that entered morphine abstinence after 3 weeks of treatment (Figure 2.2B). The mean baseline paw retraction threshold (31.80±0.67 gf) served as the control value in subsequent within-subject analyses. Withdrawal-induced hyperalgesia was established with 3 weeks of morphine treatment, after which the drug administration was stopped and animals received 7 days of vehicle treatment (Figure 2.2A). One-way RM ANOVA confirmed a significant effect of time on paw retraction threshold (F8, 264 = 24.61, P<0.01). On Test Day 6, the paw retraction threshold was 36% below baseline at 20.32±0.74 gf (Dunnett’s test: P<0.01) (Figure 2.2B). On Test Days 7, 8, and 9 during vehicle treatment, the pain sensitivity was continually significantly below baseline (Dunnett’s test: P<0.01). This significant level of hyperalgesia was also observed 23 h after the last vehicle treatment (25.41±1.19 gf, Dunnett’s test: P<0.01). During the second week of morphine abstinence, the paw retraction thresholds begin to recover toward the baseline, reaching 28.82±0.92 gf on Test Day 12 (Dunnett’s test: P<0.03), and are no longer significantly different from baseline on Test Day 13 (P=0.99). These results demonstrated that, during extended withdrawal, a hyperalgesic response to Von Frey tests is maintained for two weeks before recovering to near-baseline levels.
Intermittent morphine administration induces stable hyperalgesia during extended withdrawal

A) Schedule of morphine (MOR) injections, vehicle treatments (tx), and Von Frey testing in a model of acute withdrawal-induced hyperalgesia. During Weeks 1-3, MOR (15 mg/kg, i.p., n=24) was administered 5 days per week. During Weeks 4 and 5, VEH was administered 30 min before Von Frey assessments. B) Recovery curve of hyperalgesia after morphine discontinuation as indicated by changes in paw retraction threshold. Datapoints are the paw retraction threshold (mean+SEM) in grams of force (gf). The dotted line represents the BL threshold value. Dunnett’s test: *P<0.05, **P<0.01 vs. baseline (BL).

2.3.3 Correlation of body weight to baseline von Frey test performance

To exclude the possibility that variability in body weight between animals could influence their baseline sensitivity to mechanical stimuli, body weight at baseline (g) was plotted
against paw retraction thresholds (gf). Simple linear regression resulted in a line of best fit with a slope of -0.7044, not significantly deviating from zero. This analysis suggests that there is no correlation between body weight and von Frey test performance.

![Figure 2.3. Variability in body weight is not correlated with paw retraction threshold](image)

Mean baseline paw withdrawal threshold (gf) plotted against body weight (g) at baseline (n=94). The solid line represents the line of best-fit $y = -0.7044x + 456.9$.

**2.3.4 Experiment 3: l-THP or Z944 do not alter pain perception in morphine-naïve rats.**

Here we assessed the possibility that l-THP or Z944 may have general analgesic properties that could reduce basal sensitivity to mechanical stimulation. In rats (n=11) that received daily saline injections, oral treatment with 5 mg/kg l-THP (33.34±1.91 gf) (Figure 2.4A) or 10 mg/kg Z944 (35.29±1.398 gf) (Figure 2.4B) had no significant effect on paw retraction thresholds assessed by Von Frey tests conducted 30 min later, compared to the vehicle treatment (34.85±1.18 gf) (one-way RM ANOVA: $F_{3, 33} = 0.547$, $P=0.65$). These findings
indicate that in morphine-naïve rats, \textit{l}-THP does not have any effect on pain perception in the absence of hyperalgesia.

![Figure 2.4](image)

\textbf{Figure 2.4.} \textit{l}-THP or Z994 do not affect mechanical stimulus sensitivity in morphine-naïve rats

Rats (n=11) treated with repeated saline injections were administered \textit{l}-THP, Z944, or VEH 30 minutes before conducting Von Frey tests. \textbf{A}) Null effect of \textit{l}-THP (5 mg/kg, p.o.) on mechanical sensitivity. \textbf{B}) Null effect of Z944 (10 mg/kg, p.o.) on mechanical sensitivity. Data are paw retraction threshold (Mean+SEM) in grams of force (gf).

\subsection{2.4 Discussion}

This series of experiments established a preclinical model of opioid withdrawal-induced hyperalgesia in morphine-dependent rats using a 5-day-per-week opioid treatment protocol adapted from Marchette et al. (2021). In rats exposed to daily morphine injections, there was a rapid induction and stable expression of mechanical hyperalgesia, similar to that reported by Marchette et al. in heroin-dependent rats (2021). Moreover, this work describes the novel finding that animals continued to exhibit hyperalgesia following morphine discontinuation. Indeed, hyperalgesia persisted for more than 10 days, with a gradual recovery to baseline levels of pain.
tolerance in the final days of the abstinence period. These findings were consistent with clinical observations that former opioid users continue to exhibit heightened pain sensitivity long after completing detoxification, despite experiencing improvement relative to the degree of hyperalgesia measured during the acute withdrawal period (12-72 h) (Carcoba et al., 2011). The replicability and face validity of this preclinical model of hyperalgesia demonstrates its utility in identifying non-opioid treatment options for the effective management of pain during opioid detoxification. Importantly, this chapter addresses experiments that control for the confounding effects of inter-subject variability and non-model-specific effects of the candidate drugs. Our results show that performance on the von Frey test for mechanical algesia is not affected by increased body weight or administration of l-THP/Z994 in the absence of opiate dependence. This further validates our model of preclinical investigation of morphine withdrawal-induced hyperalgesia.

Human and animal studies confirm the importance of biological sex in OUD, with estrogen-DA interactions enhancing the vulnerability of females to addiction (Becker & Koob, 2016; Kokane & Perrotti, 2020). Although there is limited preclinical research specifically focused on sex differences (Becker & Koob, 2016), available data show that female rats are more sensitive to the reinforcing effects of opioids. They acquire higher levels of heroin self-administration and demonstrate greater responsiveness during extinction testing (Bakhti-Suroosh et al., 2021; Becker & Koob, 2016). Female rats also display more pronounced and protracted somatic withdrawal signs during abstinence (Bobzean et al., 2019; Gipson et al., 2021). Furthermore, female rats require higher doses of heroin to achieve similar levels of analgesia and experience withdrawal-induced hyperalgesia (Marchette et al., 2021). Moreover, sex differences in the metabolism of morphine may contribute to the attenuated analgesia observed in females.
(Doyle & Murphy, 2018). Soley employing male subjects is a notable limitation of this study and we recognize the importance of expanding the model presented here to evaluate the efficacy of $l$-THP and Z944 in attenuating hyperalgesia in female rats. This is particularly important in light of the finding by Marchette et al. (2021) that there are sex differences in the efficacy of k-opioid receptor antagonists in attenuating heroin withdrawal-induced hyperalgesia. In future studies investigating the effect of $l$-THP and Z944 on withdrawal-induced hyperalgesia, careful attention must be paid to determining the dose of morphine required to induce dependence in female rats. Additionally, potential sex differences in the dose range of $l$-THP and Z944 required to ameliorate this condition should be anticipated and taken into consideration.
Chapter 3: L-Tetrahydropalmatine attenuates hyperalgesia during acute and extended withdrawal in a rat model of morphine dependence.

3.1 Introduction

3.1.1 Corydalis Yanhusuo in Treatment of Substance Use Disorders

Medicinal plant research has been gaining prominence as many herbs used in traditional Asian medical practice have proven to be efficacious against a spectrum of nervous system disorders. A recent review by Jaffal and Abazid highlights more than a dozen medicinal plants that have demonstrated potential as remedies against substance misuse (Jaffal & Abazid, 2021). A notable candidate, Corydalis Yanhusuo, is well recognized in China for its analgesic properties and its efficacy in an array of preclinical assays for allodynia (L. Wang et al., 2016). A database analysis of traditional Chinese medicine used for the treatment of substance use disorders highlights Corydalis Yanhusuo among the most frequently used medicinal herbs (Min et al., 2007). Over 100 compounds, approximately 60 of which are pharmacologically active alkaloids, have been isolated from Corydalis Yanhusuo (Sun et al., 2014). Corydalis Yanhusuo is a tetrahydropyrotoberberine (THPB)-rich plant, which is one class of compounds that hold promise as novel non-opioid treatments for OUD (Nesbit & Phillips, 2020). It is widely recognized that L-tetrahydropalmatine (L-THP), a THPB alkaloid also found in several medicinal plants of the Stephania genus (Desgrouas et al., 2014; Y. Jiang et al., 2020; Semwal & Semwal, 2015; Xiao et al., 2018), is one of the most prominent active constituents of Corydalis Yanhusuo. Indeed, the analgesic and substance misuse remediying properties of the extracts are primarily attributed to L-THP (Tian et al., 2020; Zhu et al., 2017), as well as its active metabolite, L-isocorypalmine (L-
ICP), which is another THBP constituent of Corydalis Yanhusuo with a similar pharmacodynamic profile (J. Liu et al., 2021).

### 3.1.2 *l*-Tetrahydropalmatine in Substance Use Disorders

In China, *l*-THP is one of the most extensively investigated phytochemicals for the treatment of brain disorders, including schizophrenia, addiction, and pain (Q. Du et al., 2022; Nesbit & Phillips, 2020). Both Corydalis Yanhusuo extract and *l*-THP show efficacy in treating addiction in animal models. *l*-THP inhibits self-administration and relapse in animal models of cocaine misuse (Figueroa-Guzman et al., 2011; Mantsch et al., 2007) when administered alone, and it exhibits even greater efficacy when used in combination with naltrexone, an opioid antagonist (Sushchyk et al., 2016). Similar effects of *l*-THP are seen in an animal model of methamphetamine dependence (Gong et al., 2016). *l*-ICP, the active metabolite of *l*-THP, is also effective in attenuating cocaine-induced conditioned place preference (Xu et al., 2021). *l*-THP has been evaluated for efficacy in several models of OUD and is shown to reduce self-administration and relapse rates in a rat heroin abuse model (Yue et al., 2012) and prevent expression of morphine-induced conditioned place preference (W.-N. Jiang et al., 2020). Similar effects are seen with opioids, known for their high abuse potential. *l*-THP prevents the development of locomotor sensitization to oxycodone (Y. Liu et al., 2005) and blocks fentanyl-induced conditioned place preference (K. Du et al., 2021). Recently, Alhassen et al. (2021) have demonstrated in preclinical studies that Corydalis Yanhusuo extract improves morphine analgesia while preventing the development of morphine tolerance. The extract also attenuates the rewarding properties of morphine and reduces the presentation of somatic signs of withdrawal, indicating its potential for preventing the development of dependence. Clinically, *l*-THP also reduces craving and improves treatment outcomes in heroin users undergoing
detoxification (Yang et al., 2008). Orally administered l-THP in cocaine-dependent patients and drug-free volunteers is bioavailable, safe, and well-tolerated (Chao-Wu et al., 2011; Hassan et al., 2017).

### 3.1.3 Pharmacodynamic Effects of l-Tetrahydropalmatine

l-THP shows efficacy in neuropathic and inflammatory models of neuropathic pain, likely through a combination of spinal (Kang et al., 2016) and supraspinal mechanisms (Hu & Jin, 1999; Y.-Y. Liu et al., 2019; Zhou et al., 2016). The wide-ranging medicinal properties of l-THP (e.g., analgesic, anxiolytic, anti-inflammatory, and relief of gastrointestinal illness) (Chu et al., 2008; Q. Du et al., 2022; Ingram, 2014; Nesbit & Phillips, 2020; Xu et al., 2021) are attributed in part to DA D2 receptor antagonism (Ahn et al., 2020; Jin, 1987; Y.-Y. Liu et al., 2019; J. B. Wang & Mantsch, 2012; Xu et al., 2021). Liu et al. (2019) showed in a mouse model of neuropathic pain that the antinociceptive effects of l-THP are abolished with the administration of DA D2 agonists. Similar to other D2 antagonists, l-THP has sedative effects at high doses, but the therapeutic effects mentioned above are evident at much lower doses (e.g., <5 mg/kg in rat models of dependence) (Faison et al., 2016; Figueroa-Guzman et al., 2011; Gong et al., 2016; Hu & Jin, 1999; Kim et al., 2013; Leung et al., 2003; Mantsch et al., 2007, 2010; Sushchyk et al., 2016; Yue et al., 2012). Recent studies from the Phillips lab were the first to demonstrate in vivo that l-THP modulates DA release in the NAc via antagonism of the DA D2 autoreceptors (Ahn et al., 2020). This is particularly intriguing as it provides a plausible mechanism of action for our observation that l-THP reverses withdrawal-associated hypodopaminergia in morphine-dependent rats (Ahn et al., 2020). These experiments also confirmed the presence of l-THP in blood plasma and brain cerebrospinal fluid following treatment with Heantos-4, a botanical formulation used in Vietnam to facilitate opioid
detoxification (Ahn et al., 2020). These findings are part of a growing body of work, both preclinical and clinical, that supports the potential therapeutic utility of l-THP in pain and substance use disorders (Chu et al., 2008; Q. Du et al., 2022; Nesbit & Phillips, 2020). However, it has yet to be demonstrated whether l-THP is effective in relieving withdrawal-induced pain sensitivity. The main purpose of this series of experiments was to assess the efficacy of l-THP in improving pain tolerance during withdrawal.

3.2 Methods

All experiments were performed following the methodology described in detail in Chapter 2. Briefly, for 3-5 weeks, animals received a daily injection of morphine (15 mg/kg, i.p.) for five days each week. l-THP or its vehicle was administered by oral gavage 30 min before behavioural testing. Hyperalgesia during morphine withdrawal was evaluated using an electronic hand-held Von Frey device.

3.2.1 Experiment 1: Effect of l-THP on hyperalgesia during acute withdrawal

Rats (n=12) were administered morphine injections (15 mg/kg, i.p.) for five weeks and their mechanical sensitivity was tested ~ 23 h after the previous morphine injection as described in Figure 3.1A. The effect of l-THP on hyperalgesia was evaluated on 2nd day of Week 4, with half the animals receiving 5 mg/kg and the other half receiving 7.5 mg/kg 30 min before Von Frey testing. During Week 5, rats received the alternate dose of l-THP. On the 4th day of Weeks 4 and 5, all animals received vehicle treatment.

3.2.2 Experiment 2: Effect of l-THP on hyperalgesia during extended withdrawal

Following the measurement of baseline values in drug-naïve rats (n=24), during Weeks 1-3 subjects were injected once daily with morphine (15 mg/kg, i.p.) and underwent bi-weekly Von Frey test in 23 h withdrawal as described in Figure 3.2A. After completing 3 weeks of
morphine, subjects entered the abstinence period. On week 4, rats received either $l$-THP (5 mg/kg, p.o.) or vehicle for 7 days. Von Frey testing was conducted 30 min after the 1st, 4th, and 6th treatment, as well as 23 h after the 7th delivery of either $l$-THP or vehicle. Finally, Von Frey assessments continued into Weeks 5-6 in the absence of any further drug interventions.

3.2.3 Data presentation and statistical analysis

Paw withdrawal thresholds, expressed in grams of force (gf) required to elicit paw withdrawal, are presented as a mean of six measurements obtained during each Von Frey test day. Within-subject comparisons of paw retraction thresholds against baseline were conducted using one-way RM ANOVA followed by Dunnett’s multiple comparisons. Tukey’s post-hoc test was used for within-group comparisons of treatment conditions (Figure 3.1C). Between-group comparisons were performed using two-way RM ANOVA followed by Holm-Sidak multiple comparisons. The box plot method of outlier analysis was applied to mean paw withdrawal thresholds and no outliers were identified.

Days to return to 95% of BL were determined as the first instance since entering morphine abstinence on which an individual animal displayed a paw retraction response equal to or greater than 95% of baseline thresholds. Animals that failed to reach baseline by the last Test Day were given a score of 19 days, one above the number of morphine-free days in this experiment. Between-group comparison of days to recover to baseline was conducted using an unpaired t-test with Welch’s correction.
3.3 Results

3.3.1 Experiment 1: Hyperalgesia during acute withdrawal from morphine is attenuated by l-THP

3.3.1.1 Hyperalgesia during acute morphine withdrawal.

This experiment confirmed the successful induction of hyperalgesia in rats receiving morphine for five weeks (Figure 3.1A). During the week prior to starting morphine (Baseline week), Von Frey tests were conducted on three alternating days to determine the grams of force (gf) required to elicit the paw retraction reflex. There was no significant difference between the three measurements as indicated by a one-way repeated measures analysis of variance (RM ANOVA; $F_{2, 22} = 1.180, P=0.326$). The mean of the three threshold values (27.06±1.28 gf) served as the control value in subsequent within-subject analyses. Bi-weekly Von Frey tests, conducted ~23 h after the previous day’s morphine treatment, confirmed a significant effect of morphine injections on paw retraction threshold ($F_{8, 88} = 29.75, P<0.001$). In comparison to the baseline, the threshold was significantly lower on Test Day 2 (Dunnett’s test, $P<0.01$) (Figure 1B). From Test Day 4 (16.93±1.41 gf) forwards, thresholds persisted at ~40% below baseline until the final assessment on Day 10 (15.21±1.01 gf).
Figure 3.1. l-THP attenuates hyperalgesia during acute withdrawal from morphine

A) Schedule of morphine (MOR) injections, l-tetrahydropalmatine (l-THP) treatments (tx), and Von Frey testing in Experiment 1. B) Induction curve of hyperalgesia as indicated by changes in paw retraction threshold at 23 h post-MOR injection. C) Dose-dependent alleviation of MOR withdrawal-induced hyperalgesia by l-THP (5 and 7.5 mg/kg, p.o.). VEH condition is the average of the thresholds obtained after VEH treatment on Test Days 8 and 10 in panel B. Datapoints are the paw retraction threshold (mean+SEM) in grams of force (gf). The dotted line represents the BL threshold value. Dunnett’s test: *P<0.05 and **P<0.01 vs. baseline (BL). Tukey’s test: ###P<0.01, l-THP vs VEH.
3.3.1.2 Effect of \( \text{l-THP} \) on hyperalgesia presentation during acute withdrawal from morphine.

Following three weeks of morphine administration, we assessed the effects of \( \text{l-THP} \) on the paw retraction reflex, with either 5 or 7.5 mg/kg (p.o.) administered 30 min before Von Frey assessments on Test Days 7 and 9 in a counterbalanced order (Figure 3.1A). A one-way ANOVA revealed a significant effect of treatment on paw retraction thresholds (\( F_{2,22} = 24.74, P<0.001 \)). In comparison to the vehicle, thresholds were significantly higher following treatment with both 5 and 7.5 mg/kg of \( \text{l-THP} \) (Figure 3.1C) (Tukey’s, \( P<0.01 \)), representing a 37% and 47% efficacy in attenuation of hyperalgesia, respectively. While the improvement was greater following the 7.5 mg/kg than the 5 mg/kg dose, there were no statistical differences between the two doses (Tukey’s, \( P=0.36 \)). Notably, the therapeutic effects of both doses of \( \text{l-THP} \) were limited to the day of treatment, as Von Frey tests conducted 48h later under vehicle treatment (Test Days 8 and 10, Figure 3.1B) indicated hyperalgesia levels comparable to pre-treatment levels (Test Day 6, Figure 3.1B).

3.3.2 Experiment 2: Repeated \( \text{l-THP} \) facilitates recovery of hyperalgesia during extended withdrawal from morphine.

To test the potential for the use of \( \text{l-THP} \) in the treatment of OUD, the second experiment was designed to model detoxification, a critical window of time following the discontinuation of morphine. Following the model described in Chapter 2, rats received weekly morphine injections in Weeks 1-3 (Figure 3.2A). During the baseline week, there was no effect of repeated Von Frey testing on paw retraction thresholds (one-way RM ANOVA: \( F_{2,46} = 0.68, P=0.511, n=24 \)). During Weeks 1-3, there was a significant effect of MOR injections (15 mg/kg, i.p.) on paw retraction thresholds (one-way RM ANOVA, \( F_{6, 138} = 28.21, P<0.001 \)).
During the first week of morphine detoxification (i.e., the treatment period), rats were treated daily with either \( l \)-THP (5 mg/kg, p.o.; \( n=12 \)) or vehicle (\( n=12 \)). Morphine discontinuation did not alter the continued expression of hyperalgesia in the vehicle group (\( F_{13, 143} = 14.00, P<0.001 \)). The paw retraction thresholds at 24, 96, and 144 h of withdrawal from morphine (21-31% below baseline on Days 7, 8, and 9, respectively) were statistically comparable to that observed following 23 h of withdrawal (30% below baseline on Test Day 6) (Figure 3.2B). As morphine withdrawal extended into the third week, thresholds in the vehicle-treated group began to approach pre-treatment baseline sensitivity to mechanical stimulation, demonstrating that in our rat model of morphine dependence, pain tolerance spontaneously recovers over the course of three weeks.

In contrast to the vehicle treatment, 7 days of \( l \)-THP (5 mg/kg, p.o.) administration concurrently with morphine discontinuation had a significant effect on paw retraction thresholds during both the treatment (Treatment Group x Test Day, two-way RM ANOVA: \( F_{3, 66} = 4.142, P=0.009 \)) and post-treatment periods (Treatment Group x Test Day, two-way RM ANOVA: \( F_{4, 88} = 2.92, P=0.026 \)). On Test Days 7-9, \( l \)-THP administration 30 min before Von Frey tests resulted in significantly higher paw retraction thresholds compared to the vehicle-treated group (Holm-Sidak: \( P=0.03, P<0.05, P<0.05 \), respectively) (Figure 3.2B). Importantly, during the post-treatment period, thresholds on Test Days 10-12 were significantly higher in the \( l \)-THP-treated group than in the vehicle-treated group (Holm-Sidak: \( P<0.01 \)), suggesting that the pain-alleviating effect of \( l \)-THP extended significantly beyond its half-life of 4.5 h (W. Wang et al., 2017).
Repeated l-THP expedites recovery of hyperalgesia during extended withdrawal from morphine

A) Schematic of morphine (MOR) injection schedule, l-tetrahydropalmatine (l-THP) treatments, and Von Frey testing in Experiment 2 (n=24). B) The alleviation of hyperalgesia during extended withdrawal from MOR following repeated l-THP. Datapoints are the paw retraction threshold (mean+SEM) in grams of force (gf). Holm-Sidak: #P<0.05 and ##P<0.01, l-THP vs VEH. C) l-THP facilitates the rate of recovery from hyperalgesia during extended withdrawal from MOR. Datapoints represent the number of days to return to 95% of the baseline paw retraction threshold following the final MOR injection. Welch’s t-test: ##P<0.01, l-THP vs VEH.

We also calculated the number of days required for paw retraction values to return to 95% of baseline values in the morphine-naïve state (i.e., Test Day BL, Figure 3.2B). There was a significant effect of treatment on days to return to baseline sensitivity to mechanical
stimulation (Welch’s t-Test: t_{18.42}=4.20, P<0.01), with l-THP treatment significantly reducing the number of days (5.75±1.14) compared to the vehicle group (14.83±1.83) (Figure 3.2C). These findings were consistent with a faster rate of recovery to pre-morphine pain sensitivity in the l-THP-treated group compared to the rate of spontaneous recovery observed in the vehicle-treated group.

3.4 Discussion

The botanical compound l-THP, initially derived from *Stephania Glabra* and *Corydalis Yanhusuo*, is used extensively in traditional herbal medicines throughout China and Southeast Asia (Ingram, 2014). As noted in the introduction, both l-THP and its parent extracts have been used successfully to treat many forms of acute pain (J. Liu et al., 2021), along with the alleviation of hyperalgesia in mouse models of chronic neuropathic and inflammatory pain (Zhou et al., 2016). Here, we report that l-THP (5 and 7.5 mg/kg) given during morphine withdrawal significantly attenuated hyperalgesia (Figure 3.1). Even at the higher 7.5 mg/kg dose, animals did not show complete recovery to pre-morphine baseline levels, which may reflect the moderate doses used to avoid the possible confounding effect of sedation. Notably, higher doses (10–15 mg/kg) attenuate the behavioural effects of methamphetamine (Yun, 2014). The increase in paw retraction thresholds observed in our study with doses of 5.0 and 7.5 mg/kg is unlikely to be attributed to the locomotor effects of l-THP since prior studies report that acute or chronic doses of over 9 mg/kg were required to affect open field test performance (Y. Liu et al., 2005; Yun, 2014). Overall, our findings provide further confirmation of the analgesic potential of l-THP, previously demonstrated in animal models of neuropathic pain (Kang et al., 2016; Y.-Y. Liu et al., 2019; L. Wang et al., 2016; Zhou et al., 2016). Furthermore, our study extends this effect to
opioid withdrawal-induced hyperalgesia, which is clinically relevant to the effective management of opioid detoxification.

Importantly, our findings indicate that repeated dosing of \textit{l}-THP may facilitate detoxification by significantly improving the time to recovery of normal pain perception during abstinence. In animals subjected to a 7-day course of \textit{l}-THP treatment concurrently with morphine discontinuation (\textbf{Figure 3.2}), hyperalgesia is significantly attenuated during the treatment week. Furthermore, this improvement persists for the duration of the study, with a significant reduction in the number of days to recover to baseline pain threshold scores. Our results provide evidence of the analgesic properties of \textit{l}-THP in OUD, distinct from a recent study by Alhassen et al. (2021) confirming the efficacy of \textit{l}-THP in preventing morphine tolerance or OIH, another critical dimension of OUD. Together, these findings add to a growing body of work, both preclinical and clinical, that supports the therapeutic potential of \textit{l}-THP in substance use disorders (Chu et al., 2008; Q. Du et al., 2022; Nesbit & Phillips, 2020). Future studies should include lower doses of \textit{l}-THP to establish a more complete dose-response curve of its analgesic effect on morphine withdrawal-induced hyperalgesia.

\textit{l}-THP itself does not possess intrinsic rewarding or aversive properties, as it does not produce conditioned place preference or conditioned place aversion, respectively. However, it has been shown to successfully blocks drug reward, while inhibiting the acquisition and expression of morphine-induced conditioned place preference (W.-N. Jiang et al., 2020). Additionally, \textit{l}-THP has demonstrated the ability to reduce craving and drug-seeking behaviours associated with substances such as heroin, cocaine, ethanol, and nicotine (Faison et al., 2016; Kim et al., 2013; Sushchyk et al., 2016; Yang et al., 2008; Yue et al., 2012). Interestingly, very low doses of \textit{l}-THP, in combination with the opioid antagonist naltrexone, show a synergistic
effect in reducing cocaine-seeking in rats (Sushchyk et al., 2016). The current study elaborates on the behavioural substrates that may be involved in the relapse-preventing properties of l-THP (Figueroa-Guzman et al., 2011; Gong et al., 2016; Mantsch et al., 2007; Sushchyk et al., 2016; Yue et al., 2012). However, future inquiries into the ability of l-THP to mitigate the motivating properties of morphine and aversive states during withdrawal will further our understanding of its effects in OUD treatment. Adding to the established body of literature (Alhassen et al., 2021; K. Du et al., 2021; W.-N. Jiang et al., 2020; J. Liu et al., 2021; Y. Liu et al., 2005; Yue et al., 2012), the present findings suggest that l-THP could be a valuable non-opioid pain management option during and after opioid detoxification. These findings suggest that l-THP could potentially contribute to the successful long-term management of OUD in clinical populations.

The present findings that l-THP can attenuate morphine withdrawal-induced hyperalgesia are also relevant to the clinical use of Heantos-4, a herbal formulation developed in Vietnam as a supplement to facilitate opioid detoxification. This botanical formulation registered in Canada under the name TGIR has been awarded a Natural Product Licence and is therefore approved for human clinical studies. A series of preclinical studies confirm that Heantos-4 has significant effects on DA function in the NAc in morphine-dependent rats while also attenuating somatic signs associated with naloxone-precipitated withdrawal (Ahn et al., 2020; Dias et al., 2016). Importantly, l-THP is present in both plasma and brain cerebrospinal fluid following oral administration of Heantos-4 (Ahn et al., 2020). Treatment of morphine-dependent rats with either Heantos-4 or l-THP reverses the hypodopaminergia observed in the NAc after naloxone-precipitated withdrawal, along with behavioural signs of withdrawal (Ahn et al., 2020). It is also of interest that both l-THP and Heantos-4 reverse hypodopaminergia induced in the NAc by the DA D2 agonist quinpirole, implicating the DA D2 autoreceptor as their target (Ahn et al., 2020).
This is particularly intriguing, as it suggests a plausible mechanism of action for the reversal of withdrawal-associated hypodopaminergia by \( l \)-THP (Ahn et al., 2020).

These data also take on added significance given recent evidence that DA D1 and D2 receptors are involved in the analgesic effects of \( l \)-THP in a preclinical model of neuropathic pain. Liu et al. (2019) found that \( l \)-THP has antinociceptive effects in a mouse model of neuropathic pain, an effect that is blocked following the administration of a DA D1 receptor antagonist or DA D2 receptor agonist. Additionally, \( l \)-THP inhibited overexpression of immediate early genes in the cingulate cortex and PAG, which are brain regions involved in physical and emotional pain perception, highlighting these areas as the likely effectors of the antinociceptive effects of \( l \)-THP (Y.-Y. Liu et al., 2019). The involvement of dopaminergic signalling in pain perception is not limited to the areas identified by Liu et al. Recent optogenetic studies demonstrated that activation of dopaminergic inputs from the VTA into the medial prefrontal cortex reduces pain behaviours, further highlighting the role of DA signalling in hyperalgesia (Huang et al., 2020). While our study presents a novel behavioural pharmacological evaluation of \( l \)-THP, further studies on the relation between neural substrates of withdrawal-induced hyperalgesia and the mechanisms of the antinociceptive effects of \( l \)-THP are warranted.
Chapter 4: Z944, a selective T-type calcium channel inhibitor, reverses hyperalgesia in acute and extended morphine withdrawal

4.1 Introduction

4.1.1 Role of T-type calcium channels in pain and addiction

Out of the five classes of voltage-gated calcium channels referred to as L-, N-, P/Q-, R-, and T-types, the latter uniquely open at hyperpolarized membrane potentials and are known as low-voltage activated calcium channels (Cain & Snutch, 2011). In addition to activating at near-resting membrane potentials (-80 mV to -60 mV), T-type calcium channels exhibit fast inactivation and slow reactivation time constants (McRory et al., 2001). Compared to other calcium channel types, the T-types uniquely possess properties relevant to the regulation of neuronal excitability, including setting the threshold and frequency of action potential firing.

T-type channels are highly expressed in soma and dendrites of central neurons (McKay et al., 2006) as well as more than half of spinal lamina II neurons, which are the site of integration of afferent nociceptive signalling (Wu et al., 2018). They are also involved in regulating the firing of GABAergic interneurons in the PAG, the central site of pain modulation (Park et al., 2010). T-type calcium channels, therefore, have been a prominent target for antinociceptive treatments.

The T-type channel family is comprised of three isoforms, Cav3.1, Cav 3.2, and Cav 3.3, each encoded by a distinct gene in mammals. Although all three of these channels are implicated in pain signalling, the Cav3.2 T-type channel is a validated therapeutic target for chronic pain intervention (Zamponi, 2016). In dorsal root ganglia (DRG) neurons the current-inhibiting effect of TTA-P2, a T-type channel antagonist with analgesic properties, is abolished after Cav3.2 knockdown (Choe et al., 2011). The analgesia produced by NMP-7, a mixed T-type antagonist
and cannabinoid agonist, is also lost in Cav3.2 knockdown animals (Berger et al., 2014), whereas selective spinal knockdown of Cav3.2 results in pronounced antinociception in a chronic pain model (Bourinet et al., 2005). This evidence suggests that Cav3.2 mediates nociception and antinociceptive effects of T-type channel blocker act through Cav3.2 antagonism.

To date, T-type calcium channels have not been investigated extensively as a target for substance use disorder treatments, however, there is evidence suggesting their involvement in the expression of drug-seeking behaviour. It has been proposed that the Cav3.1 channel isoform is essential in regulating the excitatory outputs of the VTA (Tracy et al., 2018), which are known for their role in drug-related behaviours (Oliva & Wanat, 2016). Selective block of T-type calcium by TTA-A2 reduces nicotine self-administration and prevents relapse (Uslaner et al., 2010), while Z944 blocks reinstatement of conditioned place preference to morphine and amphetamine (Cunningham, 2016).

Moreover, Cain et al. (2016) demonstrated that Heantos-4, a botanical formulation used for OUD treatment in Vietnam and shown by the Phillips lab to contain l-THP (Ahn et al., 2020), suppresses Cav3.1 and Cav3.3 firing and reduces burst-firing in thalamocortical neurons. These findings suggest that antagonism of one or more T-type channel isoforms may in part contribute to the effect of Heantos-4 on opioid withdrawal (Dias et al., 2016) In the context of this thesis, the evidence demonstrates that targeting T-type calcium channels may be an effective approach for OUD treatment that addresses the need for effective pain management during the transition to abstinence.

4.1.2 Pharmacodynamic Effects of Z944

Z944 is a first-in-class orally available T-type calcium channel antagonist with submicromolar affinity to all three Cav3.1, Cav3.2, and Cav3.3 channel isoforms (Tringham et
It preferentially binds to channels in the inactivated state and reduces neuronal excitability in an activity-dependent manner. The efficacy of Z944 has been well characterized in preclinical models of epilepsy. It exerts anticonvulsive effects in amygdala kindling (Casillas-Espinosa et al., 2015) and kainic acid-induced temporal lobe epilepsy models (Casillas-Espinosa et al., 2019), and improves object recognition in Genetic Absence Epilepsy Rats from Strasbourg (Marks et al., 2016).

More recently, Z944 has gained prominence as a promising analgesic. Changes in thalamic burst firing associated with chronic pain are mediated at least in part by an increase in LVA calcium spikes (Lenz et al., 1989) and Z944 infusion into the thalamus reduces burst firing, leading to normalized cortical synchronicity in a chronic nerve constriction injury model of pain (LeBlanc et al., 2016). Z944 is effective in attenuating the behavioural presentation of pain in formalin and complete Freund’s adjuvant (CFA) models of inflammatory pain (Short et al., 2013). A recent report by Harding et al (2021) demonstrated that in vitro Z944 application to spinal slices reduces firing in laminae I/II neurons, while systemic Z944 is effective in rescuing mechanical hyperalgesia in a model of inflammatory pain. The above evidence suggests that Z944 may act at spinal and supraspinal sites to effectively alleviate pain perception.

Importantly, Z944 is currently undergoing clinical trials, making it a promising solution for the urgent need to facilitate opioid detoxification in the clinic. Human Phase 1 trials demonstrated that Z944 is safe, well-tolerated, and CNS penetrant (Lee, 2014). Of note, in Phase 1b trial, Z944 showed efficacy in models of neuropathic and inflammatory pain induced by topical capsaicin or UV irradiation, respectively (Lee, 2014). The profound involvement of T-type calcium channels in nociception and the efficacy of Z944 in preclinical and clinical models of pain suggest that Z944 may be an effective therapy against withdrawal-induced hyperalgesia.
This set of experiments aimed to characterize the effect of Z944 on morphine withdrawal-induced hyperalgesia in models of acute and extended withdrawal described in Chapter 2.

4.2 Methods

All experiments were performed following the methodology described in detail in Chapter 2. Briefly, for 3-5 weeks, animals received a daily injection of morphine (15 mg/kg, i.p.) for five days each week. Z944 or its vehicle was administered by oral gavage 30 min before behavioural testing. Hyperalgesia during morphine withdrawal was evaluated using an electronic hand-held Von Frey device.

4.2.1 Experiment 1: Effect of Z944 on hyperalgesia during acute withdrawal

Rats (n=12) were administered morphine injections (15 mg/kg, i.p.) for five weeks and Von Frey tests were conducted bi-weekly ~ 23 h after the previous morphine injection (Figure 4.1A). The effect of 10 mg/kg Z944 on hyperalgesia was evaluated on the 2\textsuperscript{nd} day of Weeks 4 and 5. On the 4\textsuperscript{th} day of Weeks 4 and 5, all animals received vehicle treatment.

4.2.2 Experiment 2: Effect of Z944 on hyperalgesia during extended withdrawal

Following the measurement of baseline values rats (n=22) were injected once daily with morphine (15 mg/kg, i.p.) and underwent Von Frey test bi-weekly during Weeks 1-3 as described in Figure 4.2A. After completing 3 weeks of morphine, subjects entered the abstinence period. On week 4, rats received either Z944 (10 mg/kg, p.o.) or vehicle for 7 days and Von Frey testing was conducted 30 min after the 1\textsuperscript{st}, 4\textsuperscript{th}, and 6\textsuperscript{th} treatment, as well as 23 h after the 7\textsuperscript{th} delivery of either Z944 or vehicle. Finally, Von Frey assessments continued into Weeks 5-6 in absence of any further drug interventions.
4.2.3 Data presentation and statistical analysis

Within-subject comparisons of paw retraction thresholds against baseline were conducted using one-way RM ANOVA followed by Dunnett’s multiple comparisons. Paired t-test was used for a within-group comparison of treatment conditions (Figure 4.1C). Between-group comparisons were performed using two-way RM ANOVA followed by Holm-Sidak multiple comparisons. The box plot method of outlier analysis was applied to mean paw withdrawal thresholds and no outliers were identified.

Days to return to 95% of BL were determined as the first instance since entering morphine abstinence on which an individual animal displayed a paw retraction response equal to or greater than 95% of baseline thresholds. Animals that failed to reach baseline by the last Test Day were given a score of 19 days, one above the number of morphine-free days in this experiment. Between-group comparison of days to recover to baseline was conducted using an unpaired t-test with Welch’s correction.

4.3 Results

4.3.1 Experiment 1: Acute morphine withdrawal-induced hyperalgesia is reversed by Z944

This experiment confirmed the successful induction of hyperalgesia in rats receiving morphine for five weeks (Figure 4.1A). During the baseline week, there was no significant difference between the three measurements (RM ANOVA; F_{2, 22}=0.335, P=0.72). The mean of the three threshold values (32.33±1.06 gf) was used in subsequent within-subject analyses. Von Frey tests confirmed a significant effect of morphine injections on paw retraction threshold (F_{8, 80}=11.64, P<0.001) (Figure 4.1B). From Test Day 3 (24.36±1.49 gf) forwards, thresholds persisted at ~25% below baseline until the final assessment on Test Day 10 (24.88±1.28 gf).
Figure 4.1. Z944 alleviates hyperalgesia during acute withdrawal from morphine

A) Schedule of morphine (MOR) injections, Z944 treatments (tx), and Von Frey testing in Experiment 1.

B) Induction curve of hyperalgesia as indicated by changes in paw retraction threshold at 23 h post-MOR injection. C) Attenuation of MOR withdrawal-induced hyperalgesia by Z944 (10 mg/kg, p.o.). VEH and Z944 conditions are the average of the thresholds obtained on Test Days 7-10 in panel B. Datapoints are the paw retraction threshold (mean+SEM) in grams of force (gf). The dotted line represents the BL threshold value.

Dunnett’s test: *P<0.05 and **P<0.01 vs. baseline (BL). Tukey’s test: ##P<0.01, Z944 vs VEH.

Following induction of stable hyperalgesia with 3 weeks of morphine treatment, we assessed the effects of 10 mg/kg Z944 administered 30 min before Von Frey assessments on Test Days 7 and 9 (Figure 4.1A). A paired t-test revealed a significant effect of treatment on paw retraction thresholds with pain tolerance approaching baseline (t_{10}=4.804, P<0.01) (Figure
4.1C). The therapeutic effect of Z944 did not have long-lasting effects, as Von Frey tests conducted 48h later under vehicle treatment (Test Days 8 and 10) indicated hyperalgesia levels comparable to pre-treatment levels on Test Day 6 (Figure 4.1B).

4.3.2 Experiment 2: Repeated Z944 dosing facilitates recovery of hyperalgesia during extended withdrawal from morphine.

To test the potential for the use of Z944 in the treatment of OUD, its efficacy was evaluated in a model of detoxification described in Chapter 2. To induce hyperalgesia rats received weekly morphine injections in Weeks 1-3 (Figure 4.2A). During the baseline week, there was no effect of repeated Von Frey testing on paw retraction thresholds (one-way RM ANOVA: F_{2, 42} = 1.034, P=0.36, n=24). During Weeks 1-3, there was a significant effect of MOR injections (15 mg/kg, i.p.) on paw retraction thresholds (one-way RM ANOVA, F_{6, 126} = 25.63, P<0.01).

During the first week of morphine detoxification, rats were treated daily with either Z944 (10 mg/kg, p.o.; n=12) or vehicle (n=12). In the vehicle group, the paw retraction thresholds at 96, and 144 h of withdrawal from morphine were 30-33% below baseline (Test Days 8 and 9, respectively), similar to those observed following 23 h of withdrawal on Test Day 6 (Figure 4.2B). As morphine withdrawal extended into the third week, thresholds in the vehicle-treated group began to approach pre-treatment baseline sensitivity to mechanical stimulation.

In contrast to vehicle treatment, 7 days of Z944 (10 mg/kg, p.o.) administration during morphine detoxification had a significant effect on paw retraction thresholds during both the treatment (Treatment Group x Test Day, two-way RM ANOVA: F_{3, 60} = 8.513, P<0.001) and post-treatment periods (Treatment Group x Test Day, two-way RM ANOVA: F_{4, 80} = 13.23, P<0.001). On Test Days 7-9, Z944 administration 30 min before Von Frey tests resulted in
significantly higher paw retraction thresholds compared to the vehicle-treated group (Holm-Sidak: P<0.01) (**Figure 4.2B**). Importantly, during the post-treatment period, thresholds on Test Days 10-11 were significantly higher in the Z944-treated group than in the vehicle-treated group (Holm-Sidak: P<0.01).

**Figure 4.2. Repeated Z944 facilitates recovery of hyperalgesia during extended withdrawal from morphine**

A) Schematic of morphine (MOR) injection schedule, Z944 treatments, and Von Frey testing in Experiment 2 (n=22). B) The alleviation of hyperalgesia during extended withdrawal from MOR following repeated Z944 (n=12), compared to vehicle (n=10). Datapoints are the paw retraction threshold (mean+SEM) in grams of force (gf). Holm-Sidak: #P<0.05 and ##P<0.01, Z944 vs VEH. C) Z944 accelerates the rate of recovery from hyperalgesia during extended withdrawal from MOR. Datapoints represent the number of days to return to 95% of the baseline paw retraction threshold following the final MOR injection. Welch’s t-test: ##P<0.01, Z944 vs VEH.
When comparing the number of days required for recovery to paw retraction thresholds at 95% of baseline values in the morphine-naïve state (i.e., Test Day BL, Figure 4.2B), there was a significant effect of treatment on days to return to baseline sensitivity to mechanical stimulation (Welch’s t-Test: \( t_{18.98} = 11.20, P<0.01 \)). Z944 treatment significantly reduced the number of days to return to baseline (3.41±1.00 gf) compared to the vehicle group (17.20±0.71 gf) (Figure 4.2C), suggesting that adequate analgesia during the critical window of detoxification facilitates recovery.

4.4 Discussion

Similar to many drugs with anticonvulsive properties (Dogrul et al., 2003; Maizels & Mccarberg, 2005), Z944 has shown efficacy in preclinical and clinical models of neuropathic and inflammatory pain (Harding et al., 2021; LeBlanc et al., 2016; Short et al., 2013) suggesting that Z944 may exhibit analgesic effects in models of withdrawal-induced hyperalgesia. The results described in this chapter demonstrate for the first time that Z944 (10 mg/kg) effectively eliminates hyperalgesia in a 23 h morphine withdrawal condition, with animals demonstrating near-baseline performance on the Von Frey test (Figure 4.1). Moreover, a repeated 7-day treatment with Z944 given to animals in extended withdrawal resulted in a remarkable improvement in pain tolerance that persisted after the treatment was completed (Figure 4.2). Overall, Z944 treatment resulted in an 80% faster rate of recovery to baseline compared to vehicle animals. These findings indicate that Z944 is a promising candidate for effective pain management in OUD patients undergoing detoxification, especially considering its progress in clinical trials (Lee, 2014).

A similar level of Z944-induced analgesia was recently demonstrated by Harding et al. (2021), their results showing that Z944 (10 mg/kg) completely attenuates inflammatory pain.
induced by intraplantar CFA. Moreover, both male and female animals showed a similar response to Z944 treatment, demonstrating a lack of a sex-specific effect, which is an important consideration for future studies of drug efficacy on withdrawal-induced hyperalgesia in females.

Z944 is not intrinsically motivating as it does not induce conditioned place preference (CPP) but it has been shown to reduce expression and reinstatement of morphine and amphetamine CPP, indicating that it interferes with positive motivating aspects of drug-taking (Cunningham, 2016). This, in combination with its antinociceptive effect during withdrawal, makes it a promising agent in OUD treatment. Further verification of this concept should include an investigation of Z944 in a conditioned place aversion to withdrawal and its ability to prevent relapse in a model of morphine self-administration.

It is known that Z944 acts on lamina I/II spinal neurons to reduce afferent pain signalling, which correlates with a recovery of pain tolerance in inflammatory pain (Harding et al., 2021). Since our study demonstrated similar efficacy of Z944 in withdrawal-induced hyperalgesia, Z944 likely acts through spinal mechanisms to produce antinociception during morphine withdrawal. However, the possibility that Z944 also modulates pain through central mechanisms cannot be excluded. T-type channels promote GABAergic tone in the PAG (Park et al., 2010), which reduces descending antinociceptive signalling from the PAG (Reichling et al., 1988). Therefore, antagonism at T-type channels by Z944 likely contributes to excitation in the pain-modulating PAG efferents. Finally, chronic pain models are associated with disruption of thalamic regulation of cortical synchronicity, resulting in increased cortical oscillations (Chen et al., 2023; LeBlanc et al., 2014, 2017). Cav3.1 knockout in a mouse model of neuropathic pain resulted in the normalization of thalamocortical synchronicity and reduced nociception (Choi et al., 2016), indicating that Z944 antagonism at Cav3.1 may act to reduce pro-nociceptive cortical
oscillations. Outlined here are the possible neural substrates of Z944-mediated analgesia, but it is not known whether they contribute significantly to the effects observed in our models of withdrawal-induced hyperalgesia. Future research should focus on resolving the neuronal effects of Z944 in the PAG and thalamic nuclei and further investigate the contribution of these pathways to morphine withdrawal-induced hyperalgesia.
Chapter 5: Conclusion

The present data demonstrate that both of the candidate molecules l-THP and Z944 are effective in attenuating hyperalgesia in acute and extended withdrawal from morphine. Most importantly, both drugs cause a lasting improvement in pain tolerance that persists after the completion of the treatment regimen and accelerate recovery of mechanical pain sensitivity to normal levels. These results confirm previous reports of the analgesic efficacy of these compounds (Harding et al., 2021; Kang et al., 2016; J. Liu et al., 2021; Zhou et al., 2016) and expand the understanding of their utility in OUD treatment.

l-THP is well characterized preclinically as a promising therapy for substance use disorders (Mantsch et al., 2010; Yang et al., 2008; Yun, 2014) and results described here indicate that it would also be effective in preventing negative aspects of withdrawal from reinforcing drug-taking. While Z944 is primarily studied for its anticonvulsive and analgesic properties (Casillas-Espinosa et al., 2019; Harding et al., 2021; Short et al., 2013), it also interferes with the reinstatement of conditioned place preference to morphine and amphetamine (Cunningham, 2016). This evidence makes Z944 another promising therapy that could prevent relapse to drug use by dampening hedonic motivations for drug-taking, and the aversive effects of withdrawal, as demonstrated by its effect on hyperalgesia.

The experiments described in detail in this thesis successfully addressed the research aims and confirmed the hypothesis that l-THP and Z944 have analgesic effects in models of withdrawal-induced hyperalgesia. The preclinical model design and validation are a considerable strength of this research project, namely, the induction of hyperalgesia and recovery during abstinence are replicable phenomena that enable the evaluation of candidate drug efficacy. Oral administration of the investigated therapeutics further strengthens the clinical applicability of
these findings. A major limitation of this work is the absence of female subjects, especially in light of recent evidence of sex differences in the induction of heroin withdrawal-induced hyperalgesia (Marchette et al., 2021). Future replication of this work with consideration of sex differences is of utmost importance. Please note that the present experiments are aimed at piloting the usability of the candidate drugs to alleviate hyperalgesia in withdrawal, which did not include the generation of a dose-response curve or target validation. Future works should complete a thorough characterization of the efficacy of L-THP and Z944 including the confirmation of the neural substrates and receptors involved in their antinociceptive effects. To further describe the attenuation of withdrawal behaviours by L-THP and Z944, their effect should be assessed on conditioned place aversion to withdrawal, relapse potential in models or self-administration, and somatic presentation of withdrawal.
Bibliography


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