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(2*R*,6*R*)-hydroxynorketamine, a Ketamine Metabolite, is a Novel Pain
Therapeutic

by

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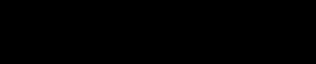
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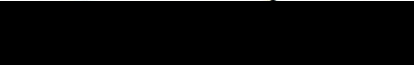
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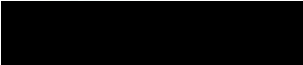
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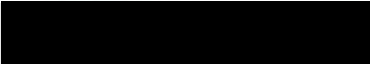
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DEDICATION

I dedicate this dissertation to my wife, Eliana, my children, Analis, Costen, Evelyn, Gabriela, Izabella, and Katerina, and my parents, Fred and Susan.

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ABSTRACT

(2*R*,6*R*)-hydroxynorketamine, a Ketamine Metabolite, is a Novel Pain Therapeutic

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Over 20% of adults in the U.S. experience pain most or every day that is sufficiently debilitating to contribute to limited work capability and decreased quality of life. Pain is also a problem among our military population. Over 40% of soldiers deployed to Iraq or Afghanistan reported a pain condition. Currently available analgesics have significant risk and frequently fall short of pain reduction goals. Identifying novel

and effective pharmaceutical pain treatments is an integral part of addressing the problem of pain and would promote health and fitness among the general population and increase readiness to deploy within the military.

Ketamine is an anesthetic agent in use since 1970 that is also used to treat pain. Front-line medics use ketamine to treat battlefield injuries because it is safer than opioids and still effective at reducing pain. However, the dissociation and motor impairment side effects of ketamine and concern for recreational abuse limit its use, making ketamine unavailable for patients to administer and treat their pain at home. (2*R*,6*R*)-HNK is a ketamine metabolite thought to share some of ketamine's beneficial treatment effects for depression. It lacks the psychotomimetic properties of its parent drug while retaining anti-stress effects in preclinical research. Because ketamine is recognized for pain reduction, it is natural to question whether (2*R*,6*R*)-HNK may also possess analgesic activity.

The overall goal of this thesis was to expand upon what little was known regarding the pain reduction-like effects of (2*R*,6*R*)-HNK. The results showed initially that (2*R*,6*R*)-HNK produced antinociception to a noxious thermal stimulus in healthy mice. The onset for (2*R*,6*R*)-HNK antinociception was delayed (> 10 hours) and showed an inverted U-shaped dose response. (2*R*,6*R*)-HNK also reversed mechanical allodynia in neuropathic and inflammatory pain models using C57BL/6J mice to model the conditions tested. The reversal of allodynia in neuropathic and inflammatory pain occurred rapidly (< 1 hour) and showed a uniphasic dose-response to 30 mg/kg. The antinociception and allodynia reversing effects on pain behaviors were shown to be mediated through AMPA receptors and did not involve opioid receptors. This differed from the mechanism for

ketamine antinociception, which was partially dependent on opioid receptors and independent of AMPA receptors. (2*R*,6*R*)-HNK reversed neuropathic and inflammatory pain with a similar magnitude compared to clinically available treatments for each condition.

The data presented in this study demonstrated that (2*R*,6*R*)-HNK can reduce pain in multiple models and should be considered for clinical investigations for its potential to treat neuropathic, inflammatory, and even other types of pain. Considering its beneficial safety profile, (2*R*,6*R*)-HNK would have an advantage compared to other analgesics because it would not be scheduled by the Drug Enforcement Agency and could be made widely available for patients to treat their pain at home. If developed successfully as a medication, (2*R*,6*R*)-HNK shows promise to better address current problems with treating pain in society. In military settings, (2*R*,6*R*)-HNK would also enhance combat readiness through more effective and safer treatment of acute and chronic pain in the Military Health System.

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CHAPTER 1: General Introduction

PAIN IS THE PROBLEM

Living with pain is a significant problem for many people around the world. In the U.S. alone, an estimated 55% of adults have experienced some pain within the past three months, and 10.5 million adults experience "a lot of pain" every day (97). Other survey results indicated that 20.4% of adults experience pain "most days" or "every day," and for over 36% of sufferers, their pain frequently limits their life and work activities (148). The impacts of pain on quality of life and economic consequences are considerable. Pain leads to an estimated four emergency room visits per year for patients with persistent pain conditions (97). The annual cost in the U.S. associated with pain conditions is estimated to be over \$260 billion annually to the health care system and another \$297 billion to the economy in the form of lost productivity (38).

Pain is a problem among U.S. veterans as well. Two-thirds of the veteran population report experiencing pain within the past three months, 44% report their pain to be persistent, and over 9% responded that their pain was severe (98). This problem is not limited to retirees. In 2015, a study surveying over 2,500 active-duty soldiers previously deployed to either Iraq or Afghanistan revealed that 44% had experienced pain recently, and most of those experienced pain daily (128). The continuous effects of pain on its members make maintaining an effective and ready-to-fight force challenging for the military.

It is no wonder that analgesic medications are the most widely consumed drugs in the U.S. (83). Despite availability, analgesia options frequently fall short of ideal pain reduction goals. Currently available therapeutics reduce pain by only 30% in patients

treated for persistent pain, and they provide only variable improvements to pain-limited function or activity (131). Historically, opioids have been considered the gold standard for pain treatment. However, the current understanding of the dangers and limitations associated with treating pain with opioids has produced medical recommendations that has caused their use in treating pain to be substantially limited [for a review of this topic, see ((3))].

Finding better ways to treat pain conditions is essential to addressing the problem of pain. Better pain treatment can improve the quality of life for people worldwide, decrease the financial impact of pain on health care systems and economies, and promote Force Health Protection within the military by making the current troops more fit to fight. Research investigating novel pharmaceutical analgesia drugs is essential for addressing the pain problem.

KETAMINE AS AN ANALGESIC DRUG

Ketamine has recognized utility for treating different types of pain, even pain refractory to standard analgesics. For acute pain, ketamine is used by first responders (80), emergency room personnel (109), anesthesia professionals, and even combat medics on the battlefield (95). Ketamine can be used as a sole analgesic in some cases and in combination with other analgesics as part of a multimodal approach. For the treatment of chronic pain (22), ketamine is used in hospital inpatient settings or in clinics where medical professionals can carefully monitor the ketamine administration. Nevertheless, ketamine is not approved for the treatment of pain by the Food and Drug Administration.

Ketamine has many targets for activity, including but not limited to; N-methyl-D-aspartate receptors (NMDAR) antagonism, opioid receptor activation, nicotinic receptor

antagonism, muscarinic receptor antagonism, activation of D2 dopamine receptors, and facilitation of GABA-A receptor activation (21). The precise mechanisms for ketamine's various therapeutic effects remain incompletely elucidated. Concerning analgesia, NMDAR antagonism is generally thought to mediate ketamine's effects. In pain conditions where there exists aberrant high activity to transmit pain signals, ketamine likely reduces pain by inhibiting the overactive neurotransmission signals through NMDAR antagonism, thereby resetting the abnormally high spontaneous nerve conduction (22).

Ketamine possesses an affinity to bind to and activate μ and κ opioid receptors (54). Opioid receptors have been implicated in ketamine's antidepressant activity in humans (134). For this reason, opioid receptors should be included in any mechanistic investigation for ketamine-mediated pain reduction. In preclinical studies of its antidepressant-like actions, α -amino-3-hydroxy-5-methylisoxazole-4-propionic acid receptor (AMPA) antagonism has prevented ketamine's beneficial effects (82; 145), indicating an AMPAR-dependent mechanism. Therefore, accounting for AMPAR activity is also important for any mechanistic investigation of ketamine-mediated analgesia.

Despite the clear benefits ketamine has for treating pain and other conditions, it is classified as a schedule III drug by the FDA. It is approved for use only in anesthesia. Its side effects include but are not limited to; dissociative sedation, motor incoordination (sometimes referred to as tonic-clonic movements), cardiovascular stimulation, hallucinations, and will produce a general anesthetic state at high doses. Ketamine is also self-administered repeatedly for recreational use. Because of its side effects, ketamine is

restricted by the FDA to administration by medical professionals with sufficient training. Although ketamine may be obtained through specialized clinics, these restrictions significantly reduce the clinical utility of ketamine as a pharmacological therapeutic for depression or pain.

THE KETAMINE METABOLITE (2*R*,6*R*)-HYDROXYNORKETAMINE

(2*R*,6*R*)-hydroxynorketamine (HNK) is a ketamine metabolite previously thought to be inactive due to its inability to induce anesthesia like its parent drug (73). New evidence demonstrates (2*R*,6*R*)-HNK is biologically active by enhancing glutamatergic signaling (111), altering AMPAR subunit protein expression (117; 145), and increasing mRNA transcript levels (51) among other molecular effects. Some evidence even supports the hypothesis that (2*R*,6*R*)-HNK may be responsible for some of ketamine's protracted therapeutic effects in the context of stress (137; 144; 145; 151). Ideas such as these encourage research into the potential biological properties of this metabolite independently of ketamine.

(2*R*,6*R*)-HNK is a natural byproduct of ketamine metabolism. Ketamine is comprised of a cyclohexanone ring with a methylated amine group at the C2 position and linked at the same position to a chlorophenyl ring. This composition makes the cyclohexanone C2 position a stereocenter allowing for *R* and *S* enantiomer conformations that combined form the racemic ketamine available in the U.S. as a therapeutic medication. Early reports outlined two primary ketamine metabolites: norketamine and hydroxyketamine (16). An abbreviated illustration of ketamine metabolism can be found in **Figure 1**. Norketamine is formed by demethylating the amine group at the cyclohexanone C2 position. Hydroxyketamine is formed by hydroxylation to one of three

cyclohexanone carbon positions; C4, C5, or C6. Next, norketamine is hydroxylated, or hydroxyketamine is demethylated to form HNK. Hydroxylation at one of the three available carbon positions creates an additional stereocenter for the molecule. All potential conformations of HNK with its two stereocenters yield 12 different HNK isomers. A more thorough description of ketamine metabolism and all of its metabolites can be found in previous reports (28; 48; 107; 145; 146).

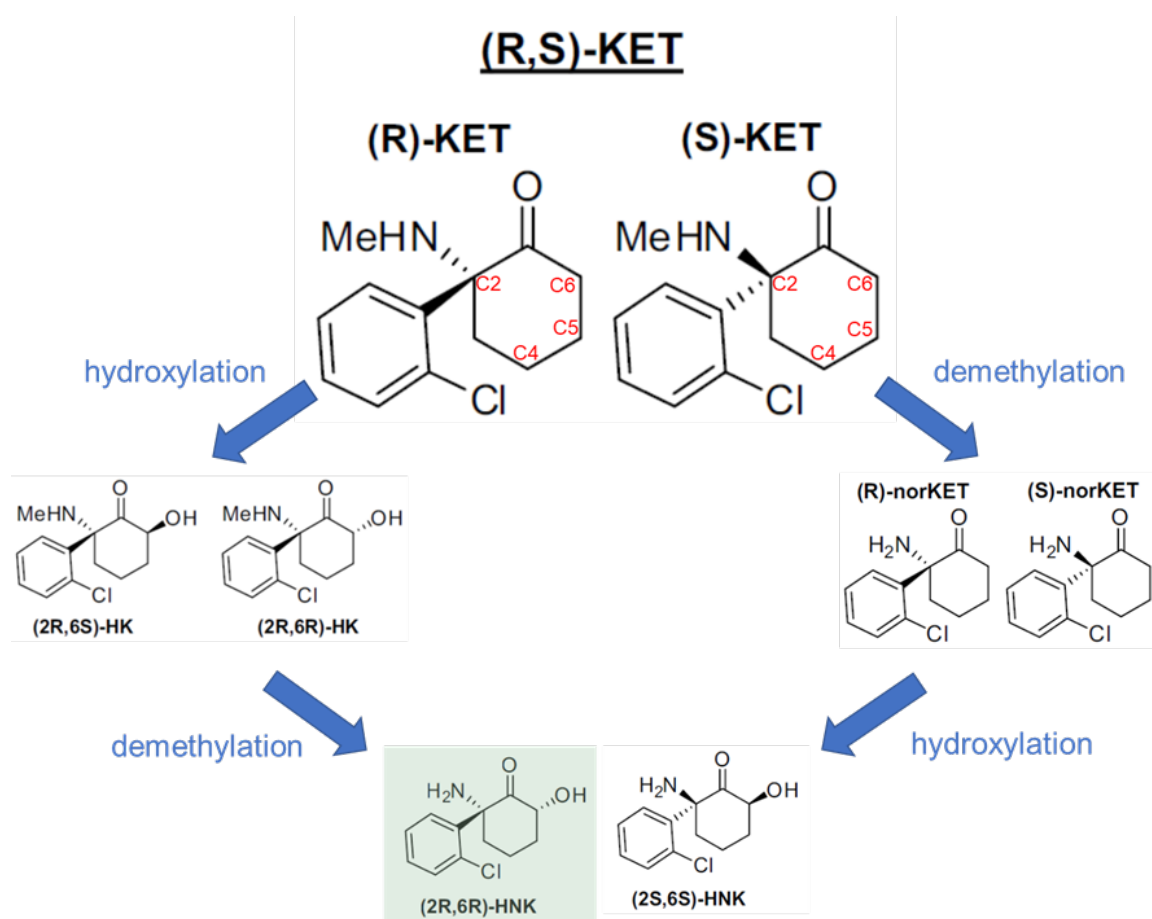


Figure 1. Metabolism of racemic ketamine to hydroxyketamine through hydroxylation, and to norketamine through demethylation. Further hydroxylation of norketamine or demethylation of hydroxyketamine forms hydroxynorketamine. Modified from (145).

The mechanisms for ketamine's many therapeutic effects are not fully understood, including its analgesic effects. Administration by slow intravenous infusion over time has been employed to minimize generating ketamine's dissociative side effects. And some evidence even supports the need for prolonged infusions of 10 hours or more to effectively reduce pain in some types of chronic conditions (59; 99). These data signify the potential need for ketamine exposure over time to initiate the desired effect and may indicate a mechanism more complex than simple NMDAR antagonism. Considering these ideas has led researchers to take another look at ketamine's metabolites to assess their potential role in ketamine's therapeutic activities.

Available pharmacokinetic data show that of the twelve isoforms, (2*R*,6*R*)- and (2*S*,6*S*)-HNK are the major hydroxynorketamine metabolites in the plasma of humans (146) and the brain and plasma of mice following ketamine administration (145). If ketamine metabolites were to share responsibility for ketamine's therapeutic effects, these metabolites should be considered among the leading candidates.

(2*R*,6*R*)-HNK, on its own, has demonstrated valuable potential within depression research. In preclinical assays and models that can be tied to mouse antidepressant-like behavior, (2*R*,6*R*)-HNK has been shown to reduce immobility times in the forced swim test (2; 36; 81; 104; 144; 145), reverse stress-induced deficits in sucrose preference (142; 145), reduce latency to eat in the novelty-suppressed feeding test (2; 81; 145), and reverse stress-induced deficits in female urine preference (145). For a complete review of preclinical data supporting the use of (2*R*,6*R*)-HNK in the context of depression, refer to Highland et al. (50).

Available evidence using ketamine deuterated at the cyclohexanone C6 position to prevent its metabolism into (2*R*,6*R*)-HNK supports a role for (2*R*,6*R*)-HNK in ketamine's preclinical antidepressant-like effects. The deuterated ketamine does not alter ketamine's pharmacological activity to bind and inhibit NMDARs or to cross the blood/brain barrier. The administration of deuterated ketamine failed to decrease immobility times or reduce escape attempts in a learned helplessness model 24 hours after administration in the same way that ketamine did (144; 145). These data support further investigation into the potential role of (2*R*,6*R*)-HNK in other therapeutic effects of ketamine.

The neuropharmacological mechanisms underlying (2*R*,6*R*)-HNK mediating the behaviors outlined above is currently being investigated. The available data show that (2*R*,6*R*)-HNK facilitated the presynaptic release of glutamate in brain regions linked to depression, such as the PFC and hippocampus (111). A clinical treatment scenario offered is that the drug potentially reverses a glutamatergic signaling imbalance. (2*R*,6*R*)-HNK's effects have been shown to be dependent on AMPAR signaling (145) and (2*R*,6*R*)-HNK alters AMPAR subunit levels (145), and their insertion into the postsynaptic membrane (117) in some brain regions.

THE AVAILABLE EVIDENCE FOR (2*R*,6*R*)-HNK ANALGESIA

Evidence for a preclinical antidepressant-like effect for (2*R*,6*R*)-HNK has been reported and even repeated by numerous laboratories, but data regarding a possible pain reduction effect for (2*R*,6*R*)-HNK is limited. Before this thesis, only one published report investigated (2*R*,6*R*)-HNK analgesia. Using mice to model postoperative and two types of neuropathic pain, the report demonstrated that (2*R*,6*R*)-HNK reversed mechanical

allodynia associated with each condition (66). They also found that (2*R*,6*R*)-HNK's reversal of incisional pain-induced allodynia was not blocked by pretreatment with a non-selective opioid receptor antagonist, although the mechanisms for the other analgesic-like effects were not examined. A limitation of the report is that the authors examined analgesic effects in female animals only and did not characterize the effect beyond demonstrating the time course. Therefore, sparse available evidence indicates that (2*R*,6*R*)-HNK's pain-reduction activity is not mediated through opioid receptors and no further mechanistic data are available.

APPROACHES USED IN THIS THESIS

Hot plate antinociception in healthy animals

Proposed in 1953 (31), the hot plate assay has a long history of investigating basic antinociception properties for novel analgesia candidates. Testing in healthy animals is beneficial to identify pharmacological effects in animals with normal physiology, unaltered by pathological conditions or by spontaneous pain. This assay requires the animal to respond by either licking a hind paw or jumping from the plate, incorporating higher-order pain and escape behaviors. Thus, the assay measures more than a simple reflexive action compared to a reflexive flick of the tail used in other antinociception assays. The hot plate is a simple assay and easy to use. It can be repeated with limited time between measurements, allowing for more frequent testing of parameters if needed. These beneficial characteristics made the inclusion of this assay ideal for examining the antinociception parameters of (2*R*,6*R*)-HNK analgesia.

Manual von Frey mechanosensitivity

Esthesiometry using von Frey monofilaments is a way to test changes in mechanical sensitivity. This assay is not intended to measure pain itself but hypersensitivity to typically innocuous stimuli resulting from a pathological condition. It can also be used to assess the effectiveness of treatment by measuring the reversal of hypersensitivity, and in this way, it is used to investigate novel analgesia candidates. The mechanosensitivity protocol used in the research of this thesis was developed initially by Dixon (29) and later modified (17; 30). Researchers now refer to it as the classic up-down method.

SNI model of neuropathic pain

When the work presented here was started, a report was published demonstrating the ability for (2*R*,6*R*)-HNK to reverse allodynia associated with two types of neuropathic pain and incisional pain in female mice (66). Encouraged by this work, the spared nerve injury (SNI) condition was selected to model neuropathy in this thesis. SNI is intended to model a neuropathic pain syndrome caused by a peripheral nerve injury such as would be seen in patients with diabetic neuropathy, phantom limb pain, postherpetic neuralgia, compression injury, or other neuropathies (26).

In rats, SNI surgery produced mechanical hypersensitivity for up to and beyond seven months and thermal allodynia for at least two months (26). In mice, SNI produced mechanical hypersensitivity within seven days and it persisted for at least four weeks (12). Still, it did not produce thermal hypersensitivity in the same way as was seen in rats (120). In this study, SNI surgery produced robust mechanical allodynia in most animals

by three days and in all animals by seven days after surgery. The allodynia persisted for at least 28 days (**Figure 2**).

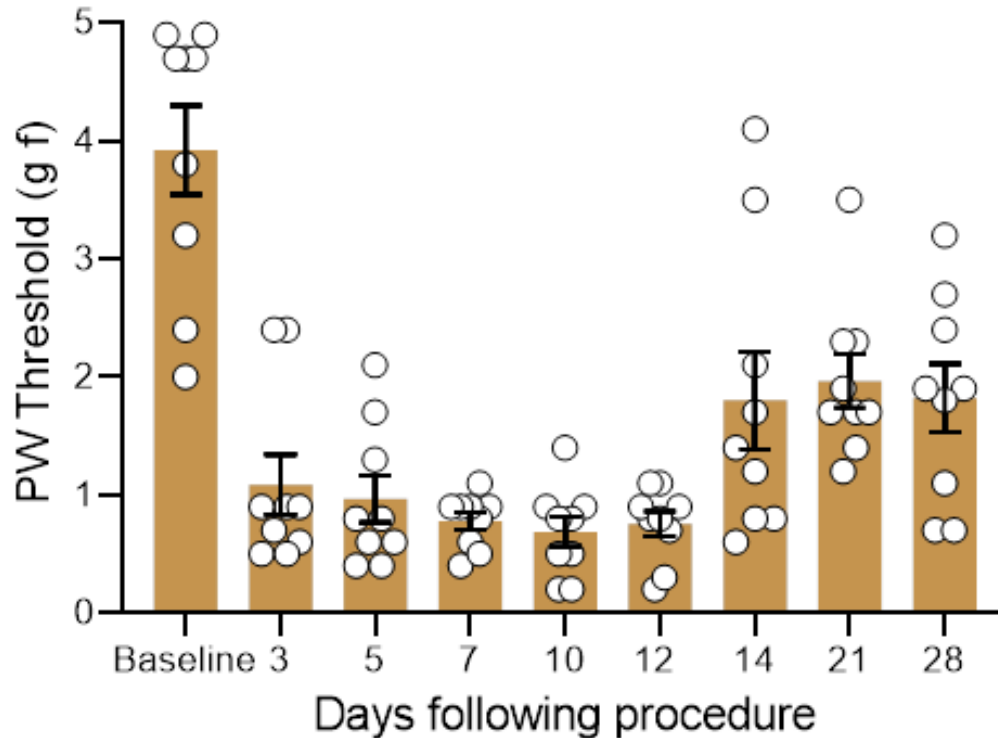


Figure 2. Time course for spared nerve injury (SNI) induced mechanical allodynia in male C57BL/6J mice measured using manual von Frey filaments and the classic up-down method. The methods for surgery and mechanical allodynia assessment are described in the methods section of Chapter 2. Within 3 days of surgery, all mice exhibited mechanical allodynia, which peaks between 7 and 12 days. Based on this pilot data, tested for treatment reversal of allodynia began on day 11. Vertical bars represent the group means $\pm$ SEM. N = 9.

The allodynia seen with the SNI condition represents a type of pain sensitization that can be debilitating for patients suffering from neuropathic pain. Sensitization is thought to be caused by unevoked signaling of C and A δ fibers and the incorporation of signals from larger myelinated fibers, ordinarily responsible for sending non-painful (light touch) signals only. For a current review of the mechanisms behind sensitization,

refer to Meacham et al. (85). Inducing neuropathy with SNI surgery and testing for changes in mechanosensitivity is a valuable model to investigate a potential treatment for this type of pain.

λ -Carrageenan paw injection model of inflammatory pain

λ -Carrageenan (CARR) is a complex polysaccharide derived from seaweed. It is used in preclinical research to induce inflammation in joints or a paw as a highly reproducible means to model joint inflammation, inflammation-mediated edema, and inflammatory pain. CARR interacts with toll-like receptor 4 (TLR-4) on the cell surface and initiates an inflammation-inducing cascade. TLR-4 activation leads to phosphorylation of B-cell lymphoma/leukemia 10, which activates nuclear factor-kappa B and interleukin-8 that recruit leukocytes and promote the release of cellular mediators leading to inflammation (11; 96).

CARR injection into a rodent paw induces swelling and hypersensitivity within hours of injection. It has been used for many years to test novel anti-inflammatory and analgesic compounds (135) and perform mechanistic examinations of pain modulation systems (55; 87). Edema produced by CARR injection is biphasic, producing early edema that peaks at approximately 6 hours and a later phase peaking at 72 hours and then declining 96 hours after injection (108). CARR paw injection induces mechanical hypersensitivity within 0.5 hours and begins to gradually subside 24 hours after injection over several days (150). Within the parameters of the studies used in this thesis, the CARR injection induced mechanical hyperalgesia that peaked at approximately 6 hours after injection. The magnitude of hypersensitivity was reduced by 48 hours following the CARR injection, albeit the animals still exhibited hyperalgesia compared to baseline.

DigiGait™ analysis of gait characteristics

The DigiGait™ apparatus and video acquisition software is a treadmill videography platform used as a simple and objective method to analyze gait characteristics in small laboratory animals. The animals are placed inside a well-lit enclosure onto a transparent treadmill that runs across clear plexiglass with a camera recording the animal from underneath. To minimize the impact stress may have on the animal during gait assessment, the animals are habituated to the testing room and allowed time to become familiar with the apparatus enclosure before testing. A video segment is captured of the animal walking at a consistent speed while the treadmill is on. The video is then analyzed to quantify spatiotemporal parameters for comparison, including over 30 components of the animal's stride and paw print characteristics.

The DigiGait system is reported to be sensitive enough to quantify gait characteristic changes in conditions expected to impact functional capability. DigiGait has been used to successfully identify functional gait alterations associated with joint inflammation in rats to model arthritic conditions (10) and in rat models of neuropathic and inflammatory pain (106; 140). DigiGait was used to capture gait characteristic changes associated with potential drug-induced motor incoordination or impairment in this thesis. Previously, DigiGait was used to successfully quantify motor incoordination caused by the influence of ethanol in mice (45). Analysis of motor impairment using this system was sensitive enough to quantify changes in a mouse model of multiple sclerosis before the condition became clinically significant (149).

GOAL AND AIMS OF THIS THESIS

Novel pharmacological pain therapeutics are needed to enable better treatment of pain conditions. Ketamine has demonstrated potential for treating acute and even chronic difficult-to-treat pain, but its unenviable side effect profile limits widespread use as an analgesic. (2*R*,6*R*)-HNK has shown potential to share, and perhaps even be responsible for, some of ketamine's therapeutic effects. When this thesis research began, there was and remains scant evidence available to indicate potential (2*R*,6*R*)-HNK analgesia. The available data were limited to only postoperative and neuropathic types of pain. Besides one experiment demonstrating (2*R*,6*R*)-HNK's postoperative pain reduction effect was independent of opioid receptors, no additional data was available about its mechanism for pain reduction effects.

The overall goal of this thesis was to expand upon what was known regarding the pain reduction-like effects of (2*R*,6*R*)-HNK. In addition to determining if (2*R*,6*R*)-HNK could mediate analgesic-like behavior in pain models yet unstudied, the studies went further to characterize important analgesic properties, including the time course, sex differences, and the mechanism underlying its activity. Ultimately, this thesis research intended to reveal essential data needed to determine if the clinical investigation of (2*R*,6*R*)-HNK as a novel analgesic was warranted.

The following aims were used to guide this thesis research.

- 1) Examine the antinociceptive effect of (2*R*,6*R*)-HNK in healthy animals and examine differences in mechanism, effect timing, and side effect profile compared to ketamine mediated antinociception.
- 2) Investigate the anti-allodynic effects of (2*R*,6*R*)-HNK in animals with induced pain conditions.

3) Interrogate a potential interaction between opioids (morphine) and (2*R*,6*R*)-HNK to determine if (2*R*,6*R*)-HNK can reverse opioid tolerance and augment opioid analgesia.

CHAPTER 2: Antinociceptive and analgesic effects of (2*R*,6*R*)-hydroxynorketamine

This chapter is a modified version of a manuscript that has been accepted for publication to the Journal of Pharmacology and Experimental Therapeutics.

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ABSTRACT

Commonly utilized pain therapeutics like opioid medications exert dangerous side effects and lack effectiveness in treating some types of pain. Ketamine is also used to treat pain, but side effects limit its widespread use. (2*R*,6*R*)-hydroxynorketamine (HNK) is a ketamine metabolite that potentially shares some beneficial behavioral effects of its parent drug without causing significant side effects. This study compared the profile and potential mechanisms mediating the antinociception activity of ketamine and (2*R*,6*R*)-HNK in C57BL/6J mice. Additionally, this study compared the reversal of mechanical allodynia by (2*R*,6*R*)-HNK with gabapentin in a model of neuropathic pain.

Unlike the near-immediate and short-lived antinociception caused by ketamine, (2*R*,6*R*)-HNK produced late-developing antinociception 24 hours following administration. Pharmacological blockade of AMPA receptors with NBQX prevented the initiation and expression of (2*R*,6*R*)-HNK antinociception, suggesting the involvement of AMPA receptor-dependent glutamatergic mechanisms in the pain reduction-like responses. Blockade of opioid receptors with naltrexone partially prevented the antinociceptive effect of ketamine but was ineffective against (2*R*,6*R*)-HNK. Furthermore, (2*R*,6*R*)-HNK did not produce dystaxia, even when tested at doses five times greater than those needed to produce antinociception, indicating a superior safety profile for (2*R*,6*R*)-HNK over ketamine. Additionally, (2*R*,6*R*)-HNK reversed mechanical allodynia in an SNI model of neuropathic pain with similar short-term efficacy to gabapentin (within 4 hours) while outperforming gabapentin 24 hours after administration. These findings support the further study of (2*R*,6*R*)-HNK as a potentially valuable agent for treating different types of pain and establish certain advantages of (2*R*,6*R*)-HNK treatment over ketamine and gabapentin in corresponding assays for pain.

INTRODUCTION

An estimated 20.4% of adults in the U.S. suffer from a pain condition. For many adults, pain is sufficient to cause significant limitations in their work capability, social life, or ability to provide self-care (24). Inadequately treating pain over time increases the risk of developing psychological disorders such as depression (34). Finding effective pain treatments has proven difficult for many suffering from pain conditions. Ketamine is an anesthetic medication in use since 1970 and known to effectively treat both acute pain (115) and pain conditions that are chronic and difficult to treat (21; 32; 68; 124). However, clinical treatment of pain with ketamine or its use by patients at home is limited because the drug causes a hallucinogenic state of dissociation, induces behavioral impairment through sedation, and is diverted for recreational abuse. Ketamine is not approved for treating pain by the Food and Drug Administration.

More recently, ketamine has demonstrated effectiveness in providing rapid onset and prolonged relief from treatment-resistant depression (9; 56; 147). Based on a compelling body of evidence, the FDA approved the use of the S enantiomer (esketamine) for the indication of treatment-resistant depression in 2019. This breakthrough in depression treatment helped drive a new investigation of ketamine metabolites. Available preclinical data suggest one of the ketamine metabolites, (2*R*,6*R*)-hydroxynorketamine (HNK), may share ketamine's therapeutic effects in rodent models of stress exposure (20; 145) while lacking the liabilities for abuse potential and dissociative cognition seen with the parent drug (81; 104; 145).

Given ketamine's efficacy in treating pain conditions, it was natural to question whether (2*R*,6*R*)-HNK shares analgesic properties with the parent compound. A study recently demonstrated that a single (2*R*,6*R*)-HNK dose (10 mg//kg) produced a reversal

of allodynia in models of postoperative pain, neuropathic pain, and Chronic Regional Pain Syndrome type pain for up to 24 hours following treatment in mice (66). These studies supported the supposition that (2*R*,6*R*)-HNK could be a putative analgesic agent. Still, the time course of treatment onset, ideal doses to treat acute and chronic pain conditions, mechanism of action, and the impact of sex on these outcomes are unknown. Moreover, the antinociceptive effects of (2*R*,6*R*)-HNK have not been examined in animals with no existing pain condition. To replicate the initial findings and provide more information on the antinociceptive effects of (2*R*,6*R*)-HNK, a series of studies were conducted in healthy mice to determine if (2*R*,6*R*)-HNK produces antinociception, to identify the optimal dose and temporal pattern for (2*R*,6*R*)-HNK mediated pain reduction, to evaluate sex differences, and to compare the side effect profile and mechanism of action between (2*R*,6*R*)-HNK and ketamine. Mechanism of action experiments targeted AMPA and opioid receptors due to the identified association between (2*R*,6*R*)-HNK and increases in AMPA receptor (AMPA) dependent glutamatergic signaling and receptor expression (104; 145) as well as the implication of opioid receptor dependency for some of ketamine's therapeutic effects. The effects of (2*R*,6*R*)-HNK were then examined in the SNI model of neuropathic pain, comparing the time course for reversing allodynia by two doses of (2*R*,6*R*)-HNK with two doses of the common neuropathic pain treatment gabapentin.

The results showed that (2*R*,6*R*)-HNK produced delayed antinociception lasting more than 24 hours in male and female mice. Unlike ketamine, (2*R*,6*R*)-HNK antinociception did not involve opioid receptor activation but was initiated and sustained by the activation of AMPAR. Additionally, (2*R*,6*R*)-HNK reversed mechanical allodynia

in SNI-treated mice with a similar short-term onset but lasted longer than gabapentin. Finally, (2*R*,6*R*)-HNK did not produce the dystaxic effects associated with ketamine treatment. These findings support the further development of (2*R*,6*R*)-HNK as a potential analgesic agent for treating different types of pain.

MATERIALS AND METHODS

Animals

Male and female C57BL/6J mice (Jackson Laboratory; Bar Harbor, ME), age 8-15 weeks, were housed with four to five animals per cage, maintained on a standard 12-hour light/dark cycle (lights on at 06:00), and provided with food and water ad libitum. Each experiment was conducted with separate cohorts of animals. The animals were randomly assigned to treatment groups for all experiments. Measurements from mice were excluded from experiments if their baseline response extended beyond mean group values by two standard deviations. All experiments were carried out following the National Institutes of Health (NIH) guidelines for the care and use of laboratory animals and with approval from the Uniformed Services University of the Health Sciences Institutional Animal Care and Use Committee.

Drugs

The (2*R*,6*R*)-HNK and (2*S*,6*S*)-HNK used for these experiments were obtained from the National Center for Advancing Translational Sciences (NCATS; Bethesda, MD), which was synthesized as previously described (94). (R/S) ketamine was purchased from Mylan Pharmaceuticals (Canonsburg, PA; #67457010810), while gabapentin was obtained from Acros Organics through Fisher Scientific (#458020010). The α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPA) antagonist NBQX

disodium salt (2,3-dioxo-6-nitro-7-sulfamoyl-benzo[f]quinoxaline, Tocris, #1044) and the opioid receptor antagonist naltrexone hydrochloride (Millipore Sigma, MO; #N3136) were used in the pharmacological mechanism of action studies. Physiological saline (0.9% NaCl; Quality Biological, MD; #114-055-721) was used as the control and to dilute medications. Doses were calculated according to the molecular weight of the base. All drugs were administered via the intraperitoneal route at a 10 ml/kg volume.

Antinociception in healthy animals

Antinociception was assessed in normal healthy mice by measuring paw withdrawal latencies on a Thermal Analgesia Meter (Ugo Basile, Stoelting Co.; Wood Dale, IL) set to 50°C, as described previously (58). After placement on the hot plate, the latency for the mouse to either jump or lick a hind paw was measured. The animal was immediately removed from the hot plate upon response.

The antinociceptive effects of ketamine 10 and 30 mg/kg, (2*R*,6*R*)-HNK 10 mg/kg, and (2*S*,6*S*)-HNK 10 mg/kg with saline control were examined in healthy male mice (N = 10-22, 10 for ketamine 30 mg/kg, 21 for ketamine 10 mg/kg, 20 for saline control, 22 for both HNK groups). Two animals (one from the control group and one from the ketamine 10 mg/kg group) were excluded for baseline values beyond two standard deviations from the group mean. The beneficial effect of (2*R*,6*R*)-HNK (10 mg/kg) on nociception was confirmed in a second experiment that used both males (N = 30) and females (N = 36). Dose-response curves were generated from latencies of male (N = 10-12 per dose) and female (N = 16-17 per dose) mice tested 24 hours following administration of (2*R*,6*R*)-HNK at quarter log doses beginning with 3 mg/kg (100.5) for male or 6 mg/kg (100.75) for female mice and ending with 100 mg/kg (102), comparing

each dose to saline control. Treatment groups in the male mice consisted of 12 animals for the saline group, 11 animals for the 6 and 18 mg/kg groups, and 10 animals for all other groups. Two female animals, one from the saline group and one from the 18 mg/kg group, were excluded for baseline values that exceeded two standard deviations from the group mean.

To test the contribution of opioid receptors in mediating the initiation of (2*R*,6*R*)-HNK's effect on nociception, the nonselective opioid receptor antagonist naltrexone (1 mg/kg) was administered 30 minutes before (2*R*,6*R*)-HNK treatment (N = 12) and the animals underwent testing 24 hours following treatment. The 1 mg/kg dose was selected because it blocked the antinociceptive effects of morphine in the hot plate test (data not shown). To test the contribution of opioid receptors in mediating the expression of (2*R*,6*R*)-HNK's behavioral effects, naltrexone 1 mg/kg was administered 24 hours following (2*R*,6*R*)-HNK treatment (N = 12) and 30 minutes before behavioral testing.

The contribution of AMPAR in the initiation of (2*R*,6*R*)-HNK's delayed antinociception effect was tested by administering the antagonist NBQX 10 mg/kg 30 minutes before (2*R*,6*R*)-HNK treatment (25; 61; 145) with the animals (N = 12) undergoing testing 24 hours following (2*R*,6*R*)-HNK. Testing for the contribution of AMPAR to the sustained expression of (2*R*,6*R*)-HNK's effect was done by administering NBQX 10 mg/kg 24 hours following (2*R*,6*R*)-HNK (N = 11-12) with hot plate testing 30 minutes later. One animal from the saline/saline group was excluded for a baseline measurement beyond two standard deviations from the group mean. NBQX 10 mg/kg, naltrexone 1 mg/kg, or saline pretreatment 30 minutes before ketamine 10 mg/kg or saline treatment were administered to test the contribution of both opioid and AMPAR in

mediating ketamine's antinociception effect (N = 10). In this case, the animals were tested on the hot plate 10 minutes following ketamine or saline treatment.

Spared nerve injury

Surgery:

Spared nerve injury (SNI) surgery was performed to model a neuropathic pain condition on male mice aged 8-12 weeks. The model was initially developed in rats (26) but is also used in mice (12; 120). Briefly, mice underwent axotomy of the tibial and peroneal branches of the sciatic nerve while carefully preserving the sural branch under aseptic conditions and 2-2.5% isoflurane anesthesia. The sciatic nerve branches were gently dissected to minimize stretching of the sural nerve. A 6-0 silk suture was tied tightly around the tibial and peroneal branches just distal to the bifurcation of the sural nerve from the sciatic nerve bundle. A second 6-0 silk suture was tightly applied 1.5 mm distal to the first, and a 1 mm section of the adjoined tibial and peroneal branches was removed. Following surgery, the incision was closed with sutures, and the mice were returned to their home cages after recovery from anesthesia. The total time required for the procedure was 15 minutes or less for each animal. The animals were given 11 days to recover from surgery before further experimentation. All animals exhibited normal food intake, grooming, and regular movements other than favoring the injured limb before post-surgery experimentation.

Mechanosensitivity (von Frey):

Paw withdrawal threshold (PWT) was measured in SNI treated mice by examining the responses to lateral-plantar stimulation with von Frey aesthesiometer monofilaments (Stoelting Co.; Wood Dale, IL; 58011) using the classic up-down method

(17; 30). The animals were placed in small plastic enclosures atop a wire mesh platform. Monofilaments (beginning with 1.0 g f) were gently applied with sufficient force only to bend the filament slightly. The monofilament was removed after one second of application. A positive response was defined as licking or rapid paw withdrawal upon stimulation. The subsequent monofilament applied was either the next higher or lower force dependent upon the previous response. The animals were given 3-4 minutes between each application. Once a change in response from the previous monofilament applications was recorded, four additional monofilaments were applied, moving up or down according to the prior response. The paw withdrawal threshold (PWT) was defined as the 50% threshold in grams force according to the following formula: 50% threshold $(g f) = \frac{10^{Xf+k\delta}}{10,000}$ where Xf was the value in log units of the final monofilament used, k was the tabular value for the response pattern [see Appendix 1 in (17)], and δ was the average log unit increment between monofilaments (0.224).

The mechanical allodynia reversing effects of (2*R*,6*R*)-HNK (10 and 30 mg/kg) and gabapentin (10 and 30 mg/kg) were compared to saline injection in the SNI operated male mice (N = 9-10, 9 for the saline and gabapentin 30 mg/kg groups and 10 for all other groups). Gabapentin is a calcium channel blocker commonly used to treat neuropathic type pain in humans. In mice, gabapentin has been shown to suppress inflammatory edema (27), reverse pain-depressed burrowing behavior (118), produce antinociception in healthy animals (62), and reverse SNI-induced allodynia (119) at doses as small as 1 mg/kg and up to 30 mg/kg depending upon the effect. In this study, the SNI mice received once-daily treatment for three days with mechanosensitivity testing

immediately preceding treatment on day two and at the same time on day four (24 hours following the third and final treatment, see **Figure 6A**).

Gait analysis

Gait analysis was used to determine if ketamine or (2*R*,6*R*)-HNK produced motor impairments at the doses effective in the pain assays ($N = 12$ for each group). Gait analysis was performed utilizing the DigiGait™ treadmill system and Video Imaging Acquisition software (Mouse Specifics Inc.; Framingham, MA) adapted from the original protocol (136) with speed set to 9 cm/s and data represented as fore and hind paw measurements for each animal (13). This system analyzes paw placement and measures spatiotemporal gait characteristics. Following 30 minutes of habituation to the testing room and five minutes of familiarization with the treadmill apparatus, the animals were treated with either ketamine (10 mg/kg or 30 mg/kg), (2*R*,6*R*)-HNK (10 mg/kg or 50 mg/kg), or saline. Video sequences of two to five seconds were captured for each animal walking on the treadmill ten minutes after injection. The images were then analyzed using the software. Eight animals were excluded from the study for refusal to walk on the treadmill, one from the ketamine 10 mg/kg, one from the ketamine 30 mg/kg, two from the (2*R*,6*R*)-HNK cohort saline control, two from the (2*R*,6*R*)-HNK 10 mg/kg, and two from the (2*R*,6*R*)-HNK 50 mg/kg groups.

Data analysis

The data are expressed as scatter plots showing group means $\pm$ S.D. A repeated measures two-way ANOVA with Dunnett multiple comparisons was used to determine significant differences between groups comparing ketamine, (2*R*,6*R*)-HNK, (2*S*,6*S*)-HNK, and vehicle control thermal pain sensitivity over time. A repeated measures two-

way ANOVA with Šidák multiple comparisons was used to compare (2*R*,6*R*)-HNK against saline control in the time course thermal antinociception experiments for male and female animals. In contrast, a repeated measures three-way ANOVA with Tukey multiple comparisons was used to analyze a sex effect in the combined data.

A one-way ANOVA with Dunnett multiple comparisons was used to compare thermal nociception responses to different doses of (2*R*,6*R*)-HNK 24 hours following treatment. Mechanism of action data were analyzed using a two-way ANOVA with Tukey multiple comparisons. Gait characteristics of groups following ketamine or (2*R*,6*R*)-HNK were compared to the respective saline control group using a one-way ANOVA. Repeated measures two-way ANOVA with Tukey multiple comparisons was used to compare two doses of (2*R*,6*R*)-HNK, two doses of gabapentin, and saline control in reversing mechanical allodynia in SNI operated mice. All statistical analyses were performed using GraphPad Prism 9.0.0 for Windows (GraphPad Software; San Diego, CA, www.graphpad.com). Statistical significance was defined as $P < 0.05$.

RESULTS

(2*R*,6*R*)-HNK produced antinociception: time course, sex differences and dose-response curve

The time course for the effects of ketamine, (2*R*,6*R*)-HNK, and (2*S*,6*S*)-HNK on the hot plate showed different patterns of antinociceptive response (**Figure 3**; time x treatment interaction $F_{(20, 450)} = 10.60$, $P < 0.0001$, data analyzed together and shown in separated graphs for clarity). Mice that received (2*R*,6*R*)-HNK at 10 mg/kg demonstrated increased latency to respond to the heat stimulus 24 hours following injection compared to saline (**Figure 3B**, $P = 0.0087$). Ketamine produced short-lived antinociception with response latencies increasing significantly only at 10 minutes (**Figure 3A**, $P = 0.0003$ for

10 mg/kg and $P = 0.0008$ for 30 mg/kg). In contrast, (2*S*,6*S*)-HNK did not increase the response latency at any time point (**Figure 3B**).

An additional experiment compared the time course for (2*R*,6*R*)-HNK in male and female mice. The results of this experiment confirmed that (2*R*,6*R*)-HNK increased latency to respond for both male (**Figure 4A**; $P = 0.0149$) and female (**Figure 4B**; $P = 0.0203$) mice 24 hours following treatment when compared to saline control (males: time x treatment interaction $F_{(4, 232)} = 4.28$, $P = 0.0023$; females: time x treatment interaction $F_{(4, 280)} = 3.884$, $P = 0.0044$). The time-course experiment results indicate the onset of (2*R*,6*R*)-HNK's delayed antinociception in healthy mice to be between 10- and 24-hours following administration. A three-way ANOVA of the male and female data, excluding the baseline measurement, revealed a sex effect ($F_{(1, 62)} = 5.216$, $P = 0.0258$). However, the post-hoc analysis revealed no significant sex differences at the same treatment and time.

The response on the hot plate was evaluated at quarter log scale doses of (2*R*,6*R*)-HNK 24 hours after administration and revealed an inverted U-shaped dose response curve for both males (**Figure 4C**; $F_{(7, 76)} = 4.986$, $P = 0.0001$) and females (**Figure 4D**; $F_{(6, 110)} = 4.971$, $P = 0.0002$). Specifically, males exhibited increased latencies to doses of 10 mg/kg ($P = 0.0004$) and 18 mg/kg ($P = 0.0010$). Females displayed increases in latency to (2*R*,6*R*)-HNK at doses of 10 mg/kg ($P = 0.0028$), 18 mg/kg ($P = 0.0262$) and 32 mg/kg ($P = 0.0049$). (2*R*,6*R*)-HNK at a dose of 30 mg/kg was also tested at 10 minutes, 1, and 4 hours (data not shown) but did not increase latency to respond to the hotplate at any measured time. Additional examination is required to determine if even higher doses of (2*R*,6*R*)-HNK can reduce the onset of its antinociception effect.

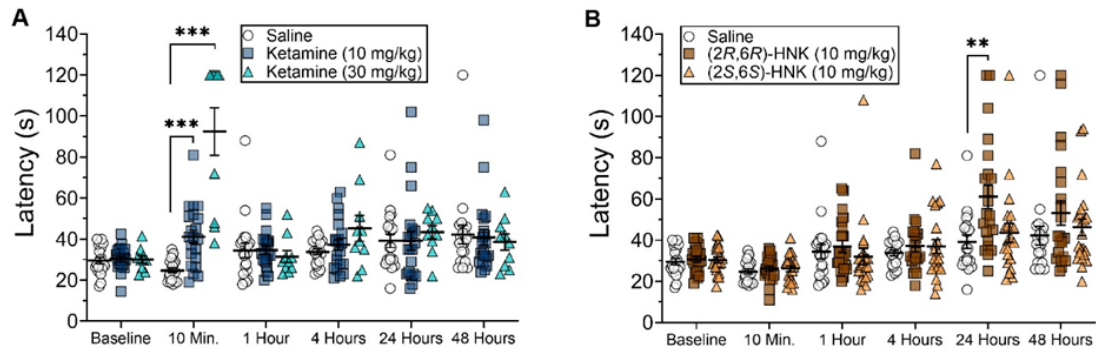


Figure 3. (2*R*,6*R*)-HNK produced delayed antinociception (**Panel B**), while ketamine produced rapid and short-lived antinociception (**Panel A**). (2*S*,6*S*)-HNK did not produce antinociception at any time point (**Panel B**). The data were analyzed together using a repeated measures two-way ANOVA but are shown in two separate graphs for clarity. The error bars represent group means $\pm$ SEM. Comparisons are made between drug/dose and saline at the same time point: ** $P < 0.01$, *** $P < 0.001$. $N = 20$ for saline control, $N = 21$ for ketamine 10 mg/kg, $N = 10$ for ketamine 30 mg/kg, $N = 22$ for both HNK groups.

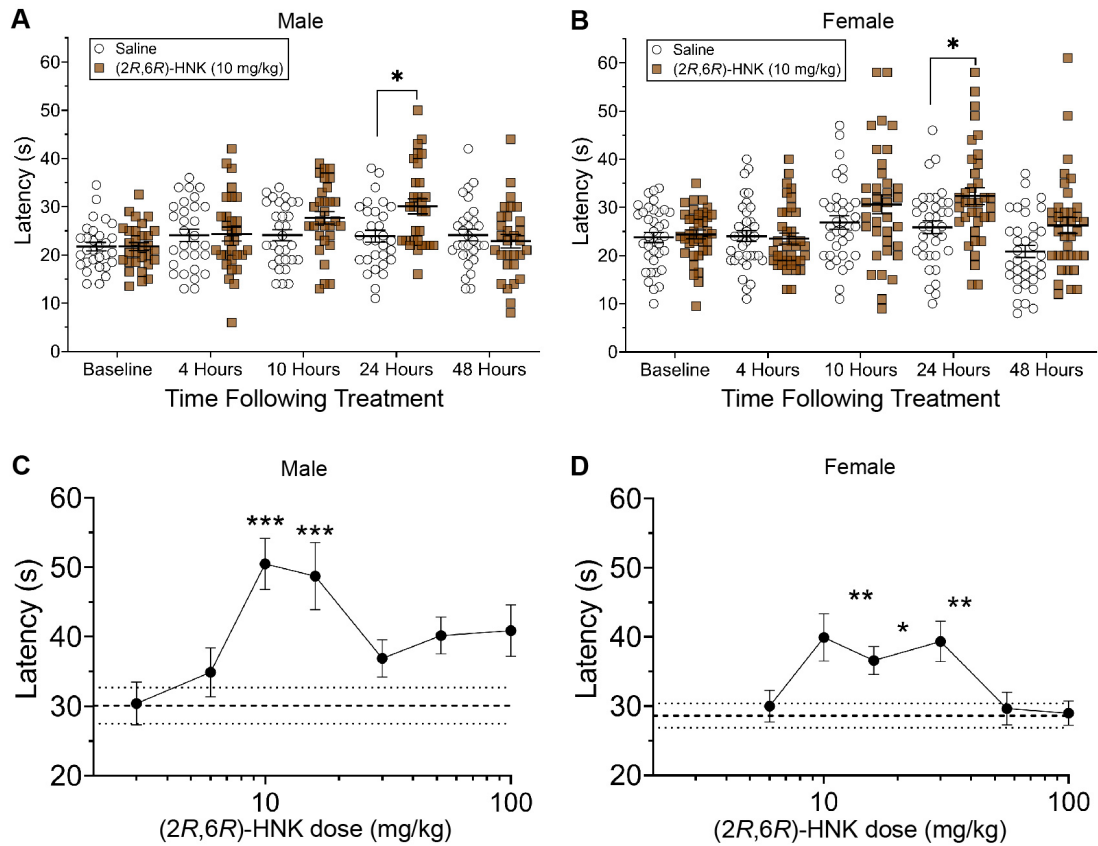


Figure 4. Male (**Panel A**) and female (**Panel B**) time course for latency to respond to hot plate stimulus following (2R,6R)-HNK 10 mg/kg treatment. Comparisons are made between the treatment and saline groups at the same time point: * $P < 0.05$. $N = 30$ for males and $N = 36$ for females. **Panel C** (males) & **Panel D** (females) represent the quarter log dose-response for (2R,6R)-HNK mediated antinociception measured 24 hours after treatment. (2R,6R)-HNK produced antinociception at doses of 10 and 18 mg/kg in both sexes, while the 32 mg/kg dose produced antinociception in female mice only. Asterisks indicate significant differences compared to the saline control. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. $N = 10-12$ for males, and $N = 17$ for females. The error bars represent group means $\pm$ SEM. For the dose-response figures, the mean of the saline group is represented by the dashed line while the $\pm$ SEM are represented by dotted lines.

AMPA but not opioid receptors are involved in the initiation and expression of the antinociceptive effects of (2*R*,6*R*)-HNK

The effect of AMPAR blockade on the initiation of (2*R*,6*R*)-HNK antinociception was tested by administering NBQX 30 minutes before injection. Pretreatment with NBQX (10 mg/kg) blocked the (2*R*,6*R*)-HNK generated hot plate effect when tested 24 hours post-injection (**Figure 5A**; pretreatment x treatment interaction, $F_{(1, 58)} = 4.592$, $P = 0.0363$, saline/saline vs. saline/HNK $P = 0.0015$, saline/HNK vs. NBQX/HNK $P = 0.0491$). The effect of AMPAR blockade on the expression of (2*R*,6*R*)-HNK antinociception was tested by administering NBQX 24 hours following treatment and 30 minutes before testing. NBQX given at this time also blocked the (2*R*,6*R*)-HNK mediated increase in latency to respond to the hot plate (**Figure 5C**; treatment x post-treatment interaction, $F_{(1, 43)} = 4.972$, $P = 0.0310$, saline/saline vs. HNK/saline $P = 0.0077$, HNK/saline vs. HNK/NBQX $P = 0.0496$). Altogether, these data suggest both the initiation and expression of the delayed antinociception effect of (2*R*,6*R*)-HNK is dependent on the interaction between (2*R*,6*R*)-HNK and AMPAR.

The nonselective opioid receptor antagonist naltrexone (1 mg/kg) was used to test the involvement of opioid receptors in the initiation and expression of (2*R*,6*R*)-HNK (10 mg/kg) antinociception. Administration of the naltrexone 30 minutes before (2*R*,6*R*)-HNK treatment had no impact on the initiation of antinociception produced by (2*R*,6*R*)-HNK when measured 24 hours after treatment (**Figure 5B**; HNK/saline treatment effect $F_{(1, 44)} = 9.364$, $P = 0.0038$, pretreatment x treatment interaction $F_{(1, 44)} = 0.01805$, $P = 0.8937$). Similarly, the administration of naltrexone 24 hours after (2*R*,6*R*)-HNK treatment and 30 minutes before hot plate testing failed to alter the expression of (2*R*,6*R*)-HNK antinociception (**Figure 5D**; HNK treatment effect, $F_{(1, 44)} = 13.93$, $P = 0.0005$,

HNK treatment x naltrexone administration interaction $F_{(1, 44)} = 0.9235$, $P = 0.3418$).

These data suggest that any potential interaction (2*R*,6*R*)-HNK may have with opioid receptors had no impact on its mediation of antinociception at the initiation of the delayed effect or its expression 24 hours later.

Pretreatment with either saline, NBQX, or naltrexone 30 minutes before ketamine injection was used to test the involvement of AMPA and opioid receptors in the antinociceptive effect of ketamine (10 mg/kg) (**Figure 5E**; pretreatment x ketamine treatment interaction, $F_{(2, 54)} = 4.634$, $P = 0.0139$). AMPAR antagonism with NBQX had no impact on ketamine-mediated antinociception ($P = 0.9763$). In contrast, naltrexone pretreatment reversed ketamine mediated antinociception (saline/ketamine vs naltrexone/ketamine $P = 0.0072$). These results suggests that the mechanism for ketamine's antinociception differs from that of (2*R*,6*R*)-HNK.

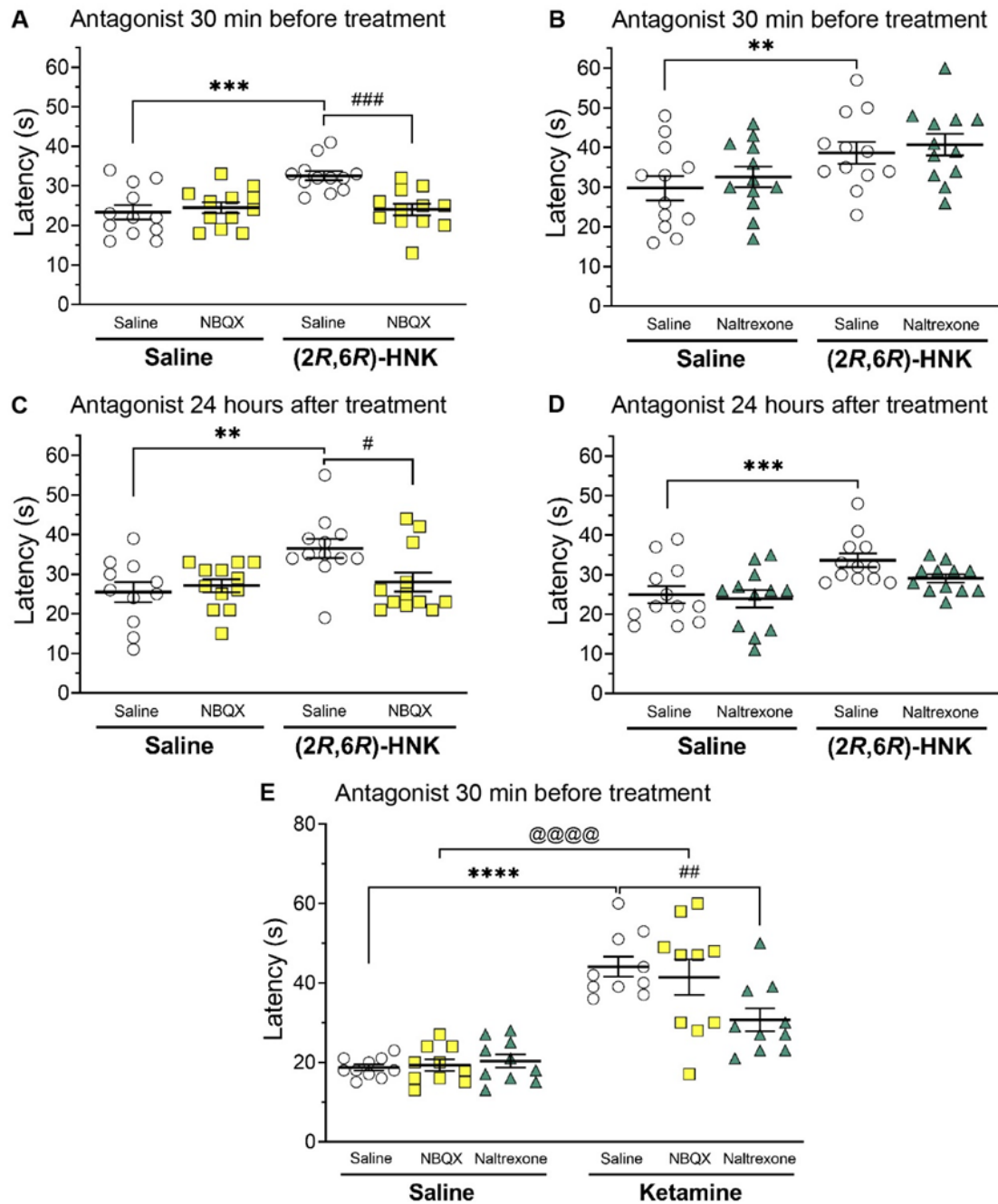


Figure 5. The effects of AMPA receptor antagonism with NBQX (10 mg/kg) or opioid receptor antagonism with naltrexone (1 mg/kg) on the initiation or expression of antinociception by (2R,6R)-HNK. The effects of (2R,6R)-HNK (10 mg/kg) were measured 24 hours after injection. **Panel A:** The NBQX given 30 minutes before (2R,6R)-HNK treatment blocked the initiation of (2R,6R)-HNK antinociception. **Panel B:** Naltrexone given 30 minutes before (2R,6R)-HNK treatment did not block the initiation of (2R,6R)-HNK antinociception. **Panel C:** NBQX given 24

hours following (2*R*,6*R*)-HNK treatment blocked the expression of (2*R*,6*R*)-HNK mediated antinociception. **Panel D:** Naltrexone given 24 hours following (2*R*,6*R*)-HNK treatment did not alter (2*R*,6*R*)-HNK antinociception. **Panel E:** Ketamine (10 mg/kg) mediated antinociception measured 10 minutes following treatment was unaffected by pretreatment with NBQX (10 mg/kg given 30 minutes prior) while naltrexone (1 mg/kg given 30 minutes prior) blocked ketamine antinociception. The error bars represent group means $\pm$ SEM. Group comparisons are indicated with horizontal lines. Asterisks represent treatment/saline to saline/saline comparisons: ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$. The # symbol represents treatment/NBQX to treatment/saline comparisons: # $P < 0.05$, ## $P < 0.01$, and ### $P < 0.001$. The @ symbol represents NBQX/saline compared with NBQX/ketamine: @@@@, $P < 0.0001$. $N = 11-12$ for the (2*R*,6*R*)-HNK experiments, $N = 10$ for the ketamine experiment.

(2*R*,6*R*)-HNK reversed mechanical allodynia in SNI treated mice with a longer duration of effect than gabapentin

Two doses of (2*R*,6*R*)-HNK (10 and 30 mg/kg) were compared with two doses of gabapentin (10 and 30 mg/kg) for reversal of mechanical allodynia in an SNI model of neuropathic pain. All of the animals exhibited a significantly lower threshold to respond to mechanical stimulation on day 11 after SNI surgery compared to their corresponding baseline values (data not shown, average baseline before surgery for all groups was 4.3 ± 1.10 g f), indicating the presence of mechanical allodynia. There was no significant difference between groups at time 0 (day 11) before treatment. The mice were treated once daily for three days. Mechanosensitivity testing was repeated 4 and 24 hours following the first treatment and again 24 hours following the third treatment (**Figure 6A**). Analysis of the results revealed a time x treatment interaction ($F_{(12, 129)} = 6.211$, $P < 0.0001$). (2*R*,6*R*)-HNK reversed hypersensitivity associated with the SNI condition 4 hours after administration (**Figure 6B**, $P = 0.0003$ for 10 mg/kg and $P < 0.0001$ for 30 mg/kg). (2*R*,6*R*)-HNK's allodynia-reversing effect persisted for greater than 24 hours after a single dose ($P < 0.0001$ for 10 mg/kg and $P < 0.0001$ for 30 mg/kg) and was still

present 24 hours following the third treatment ($P = 0.0023$ for 10 mg/kg and $P < 0.0001$ for 30 mg/kg). Both doses of gabapentin reversed the hypersensitivity when tested 4 hours after treatment ($P = 0.0005$ for 10 mg/kg and $P < 0.0001$ for 30 mg/kg). However, gabapentin failed to maintain a reversal of mechanical allodynia 24 hours following the first or even third drug treatment.

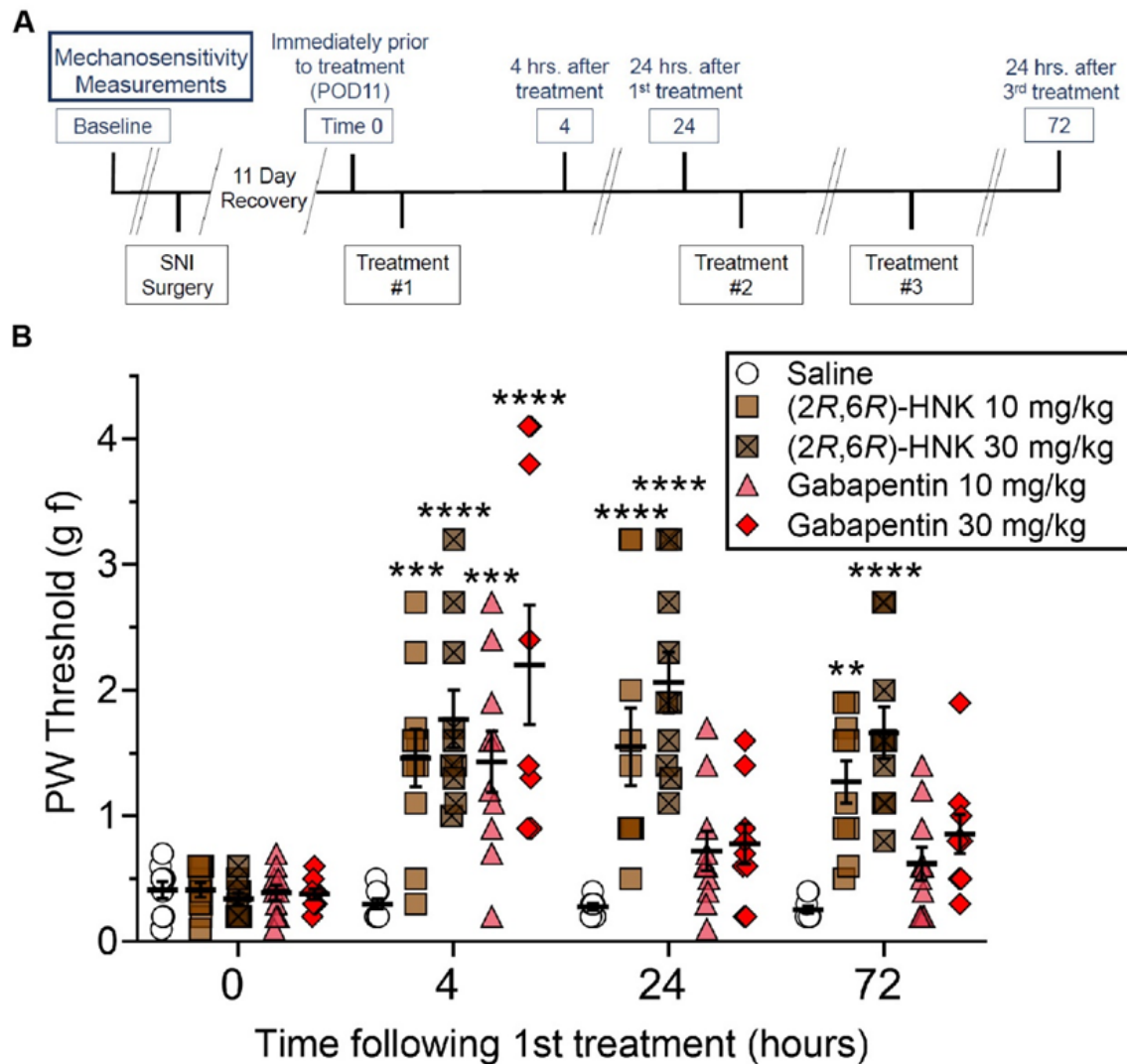


Figure 6. The SNI condition produced mechanical allodynia 11 days after surgery. Time 0 represents mechanosensitivity results on day 11 before treatment. Results of a baseline measurement before surgery demonstrated no significant difference between groups (data not shown, average baseline before surgery for all groups was 4.3 ± 1.10 g f). **Panel A:** The experimental paradigm comprised once-daily treatment for three days and mechanosensitivity testing before daily treatment. **Panel B:** (2R,6R)-HNK reversed mechanical allodynia associated with the neuropathic pain condition within 4 hours of treatment, and the effect persisted for at least 24 hours. Repeat dosing did not appear to lessen the pain reduction-like effect. Gabapentin reversed mechanical allodynia 4 hours following treatment but did not produce a significant effect 24 hours following the first or third treatment. Vertical bars represent the group means $\pm$ S.D. Drug doses are compared to saline at the same time point, ** P < 0.01, *** P < 0.001, **** P < 0.0001, N= 9-10 (N = 9 for saline and gabapentin 30 mg/kg groups, N = 10 for all other groups).

(2*R*,6*R*)-HNK did not impair gait or induce loss of motor coordination, unlike ketamine

Male mice underwent gait analysis 10 minutes following treatment with either ketamine at doses of 10 mg/kg and 30 mg/kg or (2*R*,6*R*)-HNK at doses of 10 mg/kg and 50 mg/kg. The animals' gait characteristics were compared to animals that received saline injections. Animals receiving ketamine exhibited dose-dependent alterations in fore and hind paw gait parameters, including brake/stride ratio, propel/stride ratio, paw area variability at peak stance, propel duration, and brake duration (**Table 1**). These gait alterations, taken altogether, reflect a reduction in motor coordination. In contrast, animals receiving (2*R*,6*R*)-HNK did not exhibit significant changes in any of these parameters even at 50 mg/kg, which is five times greater than the dose needed to produce antinociception or analgesia.

DISCUSSION

In this study, the ketamine metabolite (2*R*,6*R*)-HNK produced an increase in the threshold for healthy mice to respond to a painful heat stimulus (**Figure 3 & 4**) and reversed mechanical allodynia in the SNI neuropathic pain model (**Figure 6**). The antinociception effect on the hot plate was produced in healthy animals with normal pain system physiology unaltered by a pre-existing pain condition and is a standard screen for analgesic drugs such as opioids (105). Ketamine showed some key differences from its metabolite (2*R*,6*R*)-HNK. Ketamine-mediated antinociception was rapid (onset < 10 minutes) and lasted less than 1 hour. In contrast, (2*R*,6*R*)-HNK produced delayed antinociception measured 24 hours after injection when the drug has already been eliminated from the body (145). Moreover, the pain-reducing effects of ketamine were

Parameter	Treatment		Saline	10 mg/kg (for Ket) 10 mg/kg (for HNK)	30 mg/kg (for Ket) 50 mg/kg (for HNK)	Treatment effect
Brake/Stride Ratio (%)	Ketamine	Forepaw	26.15 ± 7.35	29.06 ± 6.52	34.89 ± 9.27	$F_{(2, 65)} = 7.433, P = 0.0012$
		Hindpaw	13.35 ± 5.41	17.81 ± 6.56	19.69 ± 7.43	$F_{(2, 65)} = 5.849, P = 0.0046$
	(2 <i>R</i> ,6 <i>R</i>)-HNK	Forepaw	33.44 ± 6.91	32.92 ± 6.17	30.36 ± 7.68	n.s.
		Hindpaw	15.36 ± 6.35	16.50 ± 7.59	16.29 ± 5.99	n.s.
Propel/Stride Ratio (%)	Ketamine	Forepaw	43.55 ± 7.13	39.62 ± 7.16	37.09 ± 9.91	$F_{(2, 65)} = 3.689, P = 0.0304$
		Hindpaw	62.92 ± 7.32	60.46 ± 6.61	55.47 ± 8.83	$F_{(2, 65)} = 5.612, P = 0.0056$
	(2 <i>R</i> ,6 <i>R</i>)-HNK	Forepaw	37.88 ± 5.65	36.41 ± 6.36	38.89 ± 5.87	n.s.
		Hindpaw	62.00 ± 7.98	60.99 ± 8.42	61.59 ± 6.95	n.s.
Paw Area Variability at Peak Stance (cm ²)	Ketamine	Forepaw	0.026 ± 0.008	0.036 ± 0.017	0.048 ± 0.022	$F_{(2, 65)} = 9.973, P = 0.0002$
		Hindpaw	0.054 ± 0.026	0.057 ± 0.025	0.095 ± 0.045	$F_{(2, 65)} = 10.89, P < 0.0001$
	(2 <i>R</i> ,6 <i>R</i>)-HNK	Forepaw	0.036 ± 0.021	0.034 ± 0.016	0.031 ± 0.018	n.s.
		Hindpaw	0.050 ± 0.017	0.048 ± 0.023	0.039 ± 0.016	n.s.
Propel Duration (seconds)	Ketamine	Forepaw	0.177 ± 0.040	0.175 ± 0.046	0.142 ± 0.048	$F_{(2, 65)} = 4.240, P = 0.0186$
		Hindpaw	0.250 ± 0.037	0.259 ± 0.051	0.224 ± 0.052	$F_{(2, 65)} = 3.235, P = 0.0458$
	(2 <i>R</i> ,6 <i>R</i>)-HNK	Forepaw	0.147 ± 0.030	0.144 ± 0.042	0.151 ± 0.025	n.s.
		Hindpaw	0.250 ± 0.052	0.254 ± 0.050	0.253 ± 0.051	n.s.
Brake Duration (seconds)	Ketamine	Forepaw	0.105 ± 0.029	0.127 ± 0.031	0.132 ± 0.038	$F_{(2, 65)} = 4.577, P = 0.0138$
		Hindpaw	0.053 ± 0.024	0.076 ± 0.030	0.077 ± 0.028	$F_{(2, 65)} = 5.602, P = 0.0057$
	(2 <i>R</i> ,6 <i>R</i>)-HNK	Forepaw	0.130 ± 0.033	0.127 ± 0.026	0.120 ± 0.040	n.s.
		Hindpaw	0.061 ± 0.022	0.067 ± 0.029	0.065 ± 0.022	n.s.

Table 1. Gait analysis following the administration of ketamine and (2*R*,6*R*)-HNK. Computer analysis of gait form was performed 10 minutes following ketamine or (2*R*,6*R*)-HNK injection and assessed a variety of motor parameters using the DigiGait™ apparatus. The data shown are group means ± SD. Two separate cohorts, one for each medication, testing two doses against saline control were analyzed using a one-way ANOVA. N = 11-12 for the ketamine cohort and N = 10 for the (2*R*,6*R*)-HNK cohort.

mediated partially through opioid receptors, while those of (2*R*,6*R*)-HNK were AMPAR dependent and opioid-receptor independent (**Figure 5**). The persistent duration of (2*R*,6*R*)-HNK analgesia was also seen in effects measured with the SNI model. (2*R*,6*R*)-HNK produced an acute and prolonged increase in threshold to respond to mechanical stimulation with a duration of action exceeding that of gabapentin, a drug used to treat neuropathic pain, even after repeated dosing.

An earlier publication demonstrated that (2*R*,6*R*)-HNK could reverse mechanical allodynia associated with acute and chronic pain conditions in mice (66). The reduction in mechanical allodynia was evident as early as within 1 hour, persisted for 24 hours after a single administration, and continued even up to 72 hours after once-daily repeated administration. The onset of the antinociception effect of (2*R*,6*R*)-HNK described in this study occurred considerably later than that of ketamine. Still, it was active beyond the expected physical presence of the drug in the brain or plasma. The half-life of (2*R*,6*R*)-HNK in the brain is approximately 1 hour (145), and plasma half-life is 0.2-0.8 hours (49) in mice. These data suggest that persistent changes in neuroplasticity, presently unidentified, induced by (2*R*,6*R*)-HNK may underlie the protracted antinociception effect.

(2*S*,6*S*)-HNK is another ketamine metabolite receiving attention in the literature for its potential role in ketamine's antidepressant behavioral effects. This metabolite would be derived in racemic form from *RS*-ketamine or selectively from *S*-ketamine, an antidepressant approved by the FDA in the form of a nasal spray (Spravato®). Several studies have found that (2*R*,6*R*)-HNK was more potent and effective than (2*S*,6*S*)-HNK on tests in rodents for antidepressant activity (50; 145). However, a recent study demonstrated that (2*S*,6*S*)-HNK, and not (2*R*,6*R*)-HNK, effectively reversed depression-like behavior in a corticosterone-induced mouse model of depression (142). In the experiments described herein, (2*S*,6*S*)-HNK at doses of 10 mg/kg (**Figure 3B**) and 30 mg/kg (data not shown) did not provide antinociception at any time point. Although additional doses could be tested, (2*S*,6*S*)-HNK did not appear to produce the analgesia-

like effects produced by (2*R*,6*R*)-HNK and racemic ketamine under the conditions defined herein.

This study is the first to investigate a dose-response relationship for (2*R*,6*R*)-HNK mediated reduction of pain behavior. The inverted U-shaped dose response for antinociception was consistent between male and female mice. Interestingly, some of ketamine's behavioral and cellular therapeutic effects have also shown an inverted U-shaped dose response. Ketamine induced mTOR activation in the rat prefrontal cortex at moderate doses, while low or high doses produced no effect (76). (2*R*,6*R*)-HNK was reported to possess an inverted U-shaped dose-response to reduce helpless behavior in an inescapable shock paradigm of depression-like behavior when injected intracerebroventricularly in mice (144). The loss of antinociception as the (2*R*,6*R*)-HNK dose increased to and above 30 mg/kg in the model used in this study does not likely reflect behavioral competition, because (2*R*,6*R*)-HNK does not alter locomotor activity (145). Instead, the descending limb may reflect competing downstream effects of (2*R*,6*R*)-HNK at higher doses that are yet unidentified.

The delayed onset for antinociception indicates (2*R*,6*R*)-HNK does not directly activate ion channels to induce its effect since its effects are expressed long after the compound has been cleared from the system. Instead, synaptic adaptations proposed for (2*R*,6*R*)-HNK that persist at 24 hours post-injection, such as increased AMPAR in the hippocampus (145), may be responsible for sustained changes in transmission if they were also produced within pain processing or modulation pathways.

The mechanism underlying the initiation of the delayed antinociception effect of (2*R*,6*R*)-HNK was investigated using antagonists for two types of receptors thought to be

involved in ketamine's antidepressant and pain reduction effects. (2*R*,6*R*)-HNK has been reported to increase AMPAR dependent synaptic transmission by increasing glutamate release and AMPAR expression (104; 145). AMPAR antagonism with NBQX has been shown to prevent the sustained antidepressant-like effects in mice by ketamine and (2*R*,6*R*)-HNK (65; 145) 24 hours after injection, demonstrating that some persistent behavioral effects of these drugs appear to be mediated by the AMPAR. AMPAR glutamatergic signaling also plays a vital role in pain transmission. Correlations have been identified between increased AMPAR subunit composition within the anterior cingulate cortex and the facilitation of pain signal transmission (139) as well as AMPAR subunit expression in the spinal cord and a stress-induced transition from acute to chronic pain (74). Interestingly, AMPARs have also been implicated in pain-reduction, rather than pain-induction, effects in rodents. A subset of AMPAR positive allosteric modulators (AMPAkines) have been shown to reduce mechanical allodynia in rats with induced neuropathic and inflammatory type pains (72). The ability of NBQX to block the behavioral expression of (2*R*,6*R*)-HNK antinociception 24 hours after injection is consistent with (2*R*,6*R*)-HNK producing a persistent increase in AMPAR transmission (104; 145) in pain pathways. Further studies will focus on this hypothetical mechanism.

Opioid receptor involvement has also been implicated in ketamine's antidepressant effects. Pretreatment with the nonselective opioid receptor antagonist naltrexone blocked improvements in depression scores associated with ketamine infusion therapy in humans (134), signifying the acute antidepressant effects of ketamine are at least partially dependent upon opioid receptor activation. Opioid receptor antagonism has

also been shown to block the antinociceptive effects of ketamine on the tail-flick response to radiant heat in mice (23).

The results of this study suggest that (2*R*,6*R*)-HNK induced antinociception requires AMPA and not opioid receptor activation in both the initiation and expression of the delayed antinociceptive effect. These results align with previous data where naltrexone did not block (2*R*,6*R*)-HNK mediated reversal of mechanical allodynia in a model of postoperative pain (66). The dependence on AMPAR activity for this (2*R*,6*R*)-HNK mediated effect also aligns with previous data where AMPAR blockade inhibited (2*R*,6*R*)-HNK mediated behavioral effects in rodent models of stress and (2*R*,6*R*)-HNK mediated increases in gamma power measured via quantitative electroencephalography (145). Because (2*R*,6*R*)-HNK's parent compound ketamine has been suggested to work as an NMDA receptor antagonist to provide its therapeutic effects (93), the NMDA receptor may also be implicated in the mechanism for (2*R*,6*R*)-HNK mediated antinociception. However, a previous publication reported (2*R*,6*R*)-HNK does not block NMDA receptor activity at the doses used in this study. Indeed, the ED50 concentration of (2*R*,6*R*)-HNK required to prevent NMDA-induced lethality in hippocampal cells was 227.8 mg/kg relative to ketamine's ED50 of 6.4 mg/kg (81). These data suggest that NMDA receptor antagonism is unlikely to account for (2*R*,6*R*)-HNK mediated antinociception.

Kroin et al. (66) demonstrated that (2*R*,6*R*)-HNK 10 mg/kg reversed mechanical allodynia associated with a model of neuropathic pain in female mice. (2*R*,6*R*)-HNK's allodynia-reversing effect was confirmed in male SNI treated mice using multiple doses and compared the results to gabapentin treatment (**Figure 6**). Acutely, (2*R*,6*R*)-HNK reversed the allodynia 4 hours after administration at 10 and 30 mg/kg. The onset of

analgesia for (2*R*,6*R*)-HNK in the SNI model was more rapid than the antinociceptive effects in healthy animals and may reflect changes in behavioral sensitivity caused by testing in the presence of spontaneous pain. Nevertheless, (2*R*,6*R*)-HNK demonstrated a sustained analgesia-like effect 24 hours after treatment, whereas gabapentin did not. Although similar to gabapentin when tested at 4 hours, the effects of (2*R*,6*R*)-HNK persisted longer than gabapentin following cessation of a once-daily for three days dosing regimen, indicating that (2*R*,6*R*)-HNK possesses a longer duration of effect compared to gabapentin and is not subject to tolerance under these conditions. Additional research is needed to examine whether (2*R*,6*R*)-HNK's allodynia reversing effect in this model of neuropathic pain, or other pain models, is dependent on AMPAR.

Identifying novel analgesics that lack the side effects common with opioids and ketamine is critical to identifying more effective long-term pain treatments. Ketamine induces transient alterations in motor coordination (57; 110), produces dissociative cognition, and demonstrates considerable abuse potential in relevant behavioral assays. Careful assessment of motor impairment can differentiate between actual analgesia behavior and motor dysfunction masking as analgesia-like behavior in preclinical research. Here treatment-induced motor impairment following ketamine or (2*R*,6*R*)-HNK administration was evaluated by analyzing a sensitive computerized assay of gait characteristics using the DigiGait apparatus. DigiGait videography has been used preclinically to measure spatiotemporal gait alterations resulting from pathological conditions (10; 69; 149) and may be sensitive enough to identify gait alterations even before the impairment becomes clinically significant. Subsequent analysis identified dystaxia induced by ketamine with several alterations in gait parameters pertaining to the

animal's stance and placement of the paw onto the walking surface (**Table 1**). (2*R*,6*R*)-HNK did not produce these changes in gait, even at doses considerably higher than required to achieve a pain reduction-like effect. Based on the present and published data, (2*R*,6*R*)-HNK is unlikely to induce motor incoordination, impair sensorimotor gaiting, or possess the propensity for increased self-administration on demand at doses examined for a potential therapeutic effect (79; 145).

In conclusion, these data indicate the ketamine metabolite (2*R*,6*R*)-HNK can produce pain reduction-like effects in animals, both healthy and with induced pain conditions, with a duration of action that exceeds its physical presence. (2*R*,6*R*)-HNK likely initiates and sustains this effect through persistent activation of AMPAR, a different mechanism than most other common pain therapeutics. In addition, (2*R*,6*R*)-HNK does not produce the side effects that limit the clinical utility of strong analgesic medications like ketamine or opioids. Further investigation into the mechanism underlying (2*R*,6*R*)-HNK's antinociceptive qualities and exploration of its efficacy in different pain conditions may prove valuable towards the goal of developing novel therapeutics for treating pain conditions and discerning pathophysiological alterations that may be normalized by (2*R*,6*R*)-HNK treatment in spontaneous pain conditions.

CHAPTER 3: (2*R*,6*R*)-hydroxynorketamine (HNK) reverses mechanical allodynia in a model of localized inflammatory pain

This chapter is a modified version of a manuscript that has been submitted for publication to a peer reviewed journal.

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Conducted experiments: Yost, Browne

Performed data analysis: Yost, Browne, Lucki

Wrote or contributed to the writing of the manuscript: Yost, Browne, Lucki

ABSTRACT

The ketamine metabolite (2*R*,6*R*)-hydroxynorketamine was recently reported to evoke antinociception in response to a noxious thermal stimulus in healthy mice and reverse allodynia in a murine model of neuropathic pain. This study reports the behavioral effect of (2*R*,6*R*)-HNK in C57BL/6J mice exposed to a localized inflammatory pain condition and the broad pharmacological mechanism underlying this effect. Intraplantar injection of λ -carrageenan (CARR) in the mouse hind paw caused inflammation and mechanical allodynia within 2 hours of injection that lasted for at least 48 hours. Administration of (2*R*,6*R*)-HNK (10-30 mg/kg) 2 hours following CARR injection reversed the mechanical allodynia within 1 hour of treatment in male and female mice, and the effect persisted for 24 hours following a single dose. The anti-allodynic effect of (2*R*,6*R*)-HNK was blocked 4 and 24 hours after administration by pretreatment with the AMPA receptor antagonist NBQX. The magnitude and timing of the analgesic effect of (2*R*,6*R*)-HNK were comparable to the non-steroidal anti-inflammatory drug carprofen. These findings reinforce the growing evidence supporting (2*R*,6*R*)-HNK as a novel analgesic in multiple pain models and further support an AMPAR-dependent pain reduction mechanism.

INTRODUCTION

Inflammation is the body's response to the presence of a foreign substance or tissue damage, eliciting a cascade of molecular mediators to protect the body and promote healing. Inflammation that continues beyond eliminating an infectious organism or injury recovery serves no physiological function and leads to long-term sequelae such

as tissue degradation, pain, and hypersensitivity (116). In some cases, inflammation may even result in pain-related disability.

Among U.S. adults reporting a disability, inflammatory joint disease was the most common cause (19%), while back problems were the second most common (16.8%) (46). An estimated 5-6% of adults suffer from chronic inflammatory back pain alone (71). Mechanical pathologies do not cause these inflammation-induced pain conditions, so the pain improves with movement and exercise. Therefore, safe and effective pain relief will facilitate activity and improve the condition's activity-related impact on the patient, thereby reducing the severity of the disability.

Effective treatment of inflammatory pain conditions can be challenging. Initial therapy includes NSAID administration, typically at maximum doses (71), but may be limited by potentially severe gastrointestinal and cardiovascular side effects (70; 84; 114). More potent analgesics like opioids may be considered for persistent severe pain, but they also possess hazardous side effects (33) and are ineffective for long-term treatment. Alternative therapeutics are needed to improve treatment for people suffering from inflammatory pain.

The ketamine metabolite (2*R*,6*R*)-hydroxynorketamine (HNK) has recently demonstrated the potential to reverse hypersensitivity in mice with postoperative, Chronic Regional Pain Syndrome, and neuropathic types of pain (66) as well as confer antinociception in response to a noxious heat stimulus in healthy animals (143). (2*R*,6*R*)-HNK alters AMPAR-dependent glutamatergic signaling (51; 111; 117; 145), and AMPAR activation is necessary for its antinociception behavioral effect (143).

This study examined the pain reduction-like effects of (2*R*,6*R*)-HNK in a model of peripheral inflammation. λ -Carrageenan (CARR) is a plant-based sulfated polysaccharide that, when injected, initiates a robust inflammatory response. It is used in preclinical research to model acute dermal and joint inflammatory conditions (96). Subcutaneous injection of CARR into a hind paw of the mouse was used to model a localized inflammation condition that produces robust edema (100; 108) and mechanical hypersensitivity (8; 39; 41; 112). (2*R*,6*R*)-HNK reversed mechanical allodynia associated with CARR-induced inflammation within one hour of treatment, and the effect persisted for at least 24 hours following a single administration. Compared to the NSAID carprofen, (2*R*,6*R*)-HNK reversed allodynia in this model with a similar magnitude and time course. This analgesia-like effect was dependent upon the activation of AMPA receptors, demonstrating an important role of AMPARs in pain reduction-like behavior.

METHODS

Animals

Male and female C57BL/6J mice (Jackson Lab; Bar Harbor, ME), age 9-11 weeks, were housed four animals per cage, maintained on a standard 12-hour light/dark cycle (lights on at 06:00), and provided with food and water ad libitum. A separate cohort of animals was used for each experiment. Additional cohorts were used to augment the previous time course data for the dose-response experiments. The animals were assigned to treatment groups after paw withdrawal threshold measurements, either at baseline (before inflammation) in experiments using a healthy control group or following induction of inflammation in experiments without a healthy control group, to ensure equal representation of mechanosensitivity in each group. All experiments were carried

out in accordance with the National Institutes of Health (NIH) guidelines for the care and use of laboratory animals and with approval from the Uniformed Services University Institutional Animal Care and Use Committee.

Drugs

(2*R*,6*R*)-HNK was obtained from the National Center for Advancing Translational Sciences (NCATS; Bethesda, MD), synthesized as previously described (94). Doses were chosen based on published work (143). Subcutaneous injection with low viscosity λ -carrageenan (TCI America, C28711G) induced localized inflammatory pain. The NSAID carprofen (Rimadyl®) obtained from Zoetis Inc. (Kalamazoo, MI; 4019449, 5 mg/kg) was used as a positive control for inflammatory pain treatment. The dose was chosen based on previously published work (1; 119; 125). The AMPAR antagonist NBQX disodium salt (2,3-dioxo-6-nitro-7-sulfamoyl-benzo[f]quinoxaline, Tocris, #1044, 10 m/kg) was used in the pharmacological mechanism of action experiment. The dose was chosen based on the previously published work from our group and others (25; 61; 145). Physiological saline (0.9% NaCl) was used as the control and to dilute all medications. Doses were calculated according to the molecular weight of the base. All drugs (except λ -carrageenan) were administered via the intraperitoneal route at 10 ml/kg volume.

Localized paw inflammation

λ -Carrageenan (CARR) was injected subcutaneously (2.5% CARR, 20 μ l injection volume, via a 26g needle) into the plantar surface of the animal's hind paw inducing paw swelling and increased sensitivity to mechanical pressure within 2 hours of injection and persisted for at least 48 hours.

Mechanosensitivity

Manual von Frey esthesiometer monofilaments (Stoelting Co.; Wood Dale, IL; 58011) were used to measure responses to mid-plantar stimulation using the classic up-down method (17; 30) as previously described (143). The results are reported as a 50% paw withdrawal threshold (PWT). Briefly, the animals were placed in small plastic enclosures atop a wire mesh platform. A monofilament (beginning with 1.0 g) was gently applied for one second to the plantar surface of the animal's hind paw with sufficient force to bend the filament. A rapid withdrawal or lick of the affected paw indicated a positive response. The subsequent monofilament used was the next higher or lower force depending upon the previous reaction with 3-5 minutes between each application. Once a change in response was recorded, four additional monofilaments were applied, moving up or down each application according to the previous response. The 50% PWT was defined according to the following formula: $50\% \text{ threshold (g f)} = \frac{10^{(X_f + k\delta)}}{10,000}$ where X_f is the value in log units of the final monofilament used, k is the tabular value for the response pattern [see Appendix 1 in (17)], and δ is the average increment (in log units) between monofilaments (used 0.224). Pre-CARR measurements were obtained at least one day before induction of the localized inflammation. Post-inflammation measurements (labeled PostCARR) were taken two hours following CARR injection, and the animals were treated immediately following that measurement.

Study design

The time course for (2*R*,6*R*)-HNK reversal of mechanical allodynia (**Figure 7C**) was examined in male (N = 12) and female mice (N = 12). Dose-response data were generated from the %PWT of male (N = 10-16 per dose, N = 16 for the saline group, N =

12 for HNK doses of 10 and 30 mg/kg, and N = 10 for HNK doses of 3 and 6 mg/kg) and female (N = 12-18 per dose, N = 18 for the saline and HNK 10 mg/kg groups, and N = 12 for all other HNK doses) mice at 1, 4, and 24 hours following administration of (2*R*,6*R*)-HNK using a modified quarter log dosing scale; 0, 3, 6, 10, and 30 mg/kg. The comparison between (2*R*,6*R*)-HNK 30 mg/kg and the NSAID carprofen 5 mg/kg was examined in male mice with a sample size of N = 11-12 (N = 11 for the saline group and N = 12 for the HNK and carprofen groups). To test the contribution of AMPAR in mediating the anti-allodynic effect of (2*R*,6*R*)-HNK in the inflammatory pain model, the AMPAR antagonist NBQX 10 mg/kg was administered 30 minutes before (2*R*,6*R*)-HNK treatment and the animals underwent mechanosensitivity testing 4 and 24 hours following treatment (N = 10). Time points represent hours following treatment.

Data analysis

Error bars for all data represent group means $\pm$ SEM. A repeated measures two-way ANOVA with Tukey multiple comparisons was used to analyze time-course data for (2*R*,6*R*)-HNK at a dose of 10 mg/kg compared to saline only. A repeated measures three-way ANOVA was used to determine if there was a sex difference in the combined male and female time course data. A repeated measures two-way ANOVA with Dunnett multiple comparisons was used to analyze the dose-response data for each sex. To compare the anti-allodynic effects of (2*R*,6*R*)-HNK and carprofen, a two-way ANOVA with Tukey multiple comparisons was used. All statistical analyses were performed using GraphPad Prism 9.0.0 for Windows (GraphPad Software; San Diego, CA, www.graphpad.com). Statistical significance was defined as $P < 0.05$.

RESULTS

(2*R*,6*R*)-HNK reversed mechanical allodynia associated with localized inflammatory pain

Subcutaneous injection of CARR significantly reduced the %PWT of the affected paw within two hours of injection (PostCARR time point), indicating the development of mechanical allodynia. The hypersensitivity to stimulation lasted more than 48 hours for male and female mice (**Figure 7**). The animals were injected with (2*R*,6*R*)-HNK immediately following the PostCARR time point and repeated mechanosensitivity measurements were obtained 1, 4, 24, and 48 hours after treatment (**Figure 7C**). Compared to animals treated with saline, (2*R*,6*R*)-HNK 10 mg/kg significantly reversed mechanical allodynia associated with inflammation (**Figure 7A & B**) with an onset of < 1 hour and duration > 24 hours in male (time x treatment interaction, $F_{(8, 132)} = 5.856$, $P < 0.0001$) and female (time x treatment interaction, $F_{(8, 132)} = 3.254$, $P < 0.0001$) animals. Repeated-measures three-way ANOVA analyzing time, treatment, and sex while excluding the baseline measurement (PreCARR) revealed no significant sex effect ($F_{(1, 44)} = 0.04292$, $P = 0.8368$). There were significant effects of drug treatment ($F_{(1, 44)} = 40.24$, $P < 0.0001$), time ($F_{(2.82, 124.0)} = 43.04$, $P < 0.0001$), and a treatment x time interaction ($F_{(4, 176)} = 9.001$, $P < 0.0001$). Baseline %PWT (preCARR) was significantly higher in male ($M = 4.59$, $SD = 1.13$) than female ($M = 3.44$, $SD = 0.80$) mice (independent t-test, $t_{(46)} = 4.077$, $P = 0.0002$). The difference between sexes may reflect an average difference in body weight between the lighter females (19.3 g) and males (24.9 g).

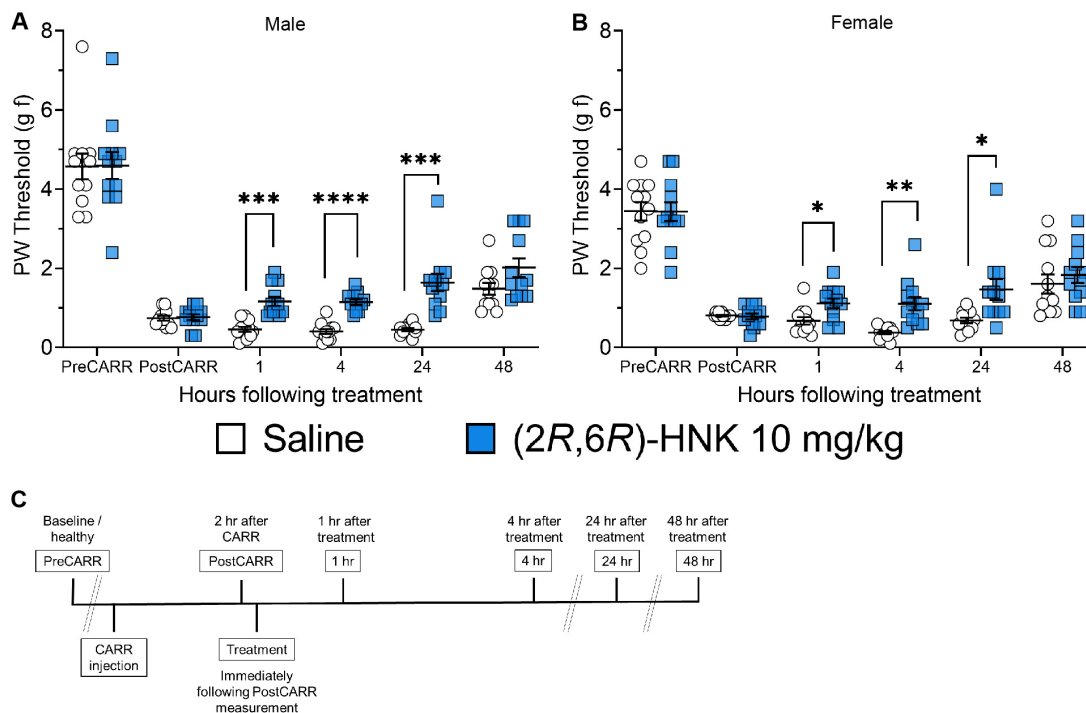


Figure 7. λ -Carrageenan (CARR) hind paw injection produced mechanical allodynia in the affected paw within 2 hours of injection and persisted for greater than 48 hours. **Panel C:** This timeline is used for all CARR inflammation experiments in this study. The inflamed animals were treated with (2R,6R)-HNK or saline control approximately 2.5 hours following CARR injection. **Panel A & C:** Treatment with (2R,6R)-HNK reversed mechanical allodynia associated with the inflammatory condition in male (A) and female (B) mice compared to saline control within 1 hour of treatment and the effect persisted for greater than 24 hours following a single administration. The data shown are group means $\pm$ SEM. Two separate cohorts, male and female, testing healthy/untreated animals and inflamed animals treated with either (2R,6R)-HNK 10 mg/kg or saline control, were analyzed using a two-way ANOVA. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$, $N = 12$ for all groups.

Dose-response for (2R,6R)-HNK's anti-allodynic effect in a localized inflammatory pain model

Additional (2R,6R)-HNK doses were tested following a modified quarter log scale under the same experimental paradigm. The dose-response data at 24 hours following treatment are shown in **Figure 8**. There was a similar dose-response relationship for male

(time x treatment interaction, $F_{(12, 153)} = 5.372$, $P < 0.0001$) and female (time x treatment interaction, $F_{(12, 201)} = 6.527$, $P < 0.0001$) mice. Male and female mice demonstrated a significant reduction of the allodynia after (2*R*,6*R*)-HNK at 10 and 30 mg/kg doses as early as one hour following treatment. Similar to the 10 mg/kg dose, (2*R*,6*R*)-HNK 30 mg/kg did not significantly reduce allodynia at 48 hours after administration compared to saline control (data not shown). The higher dose did not produce a longer duration under the conditions tested here.

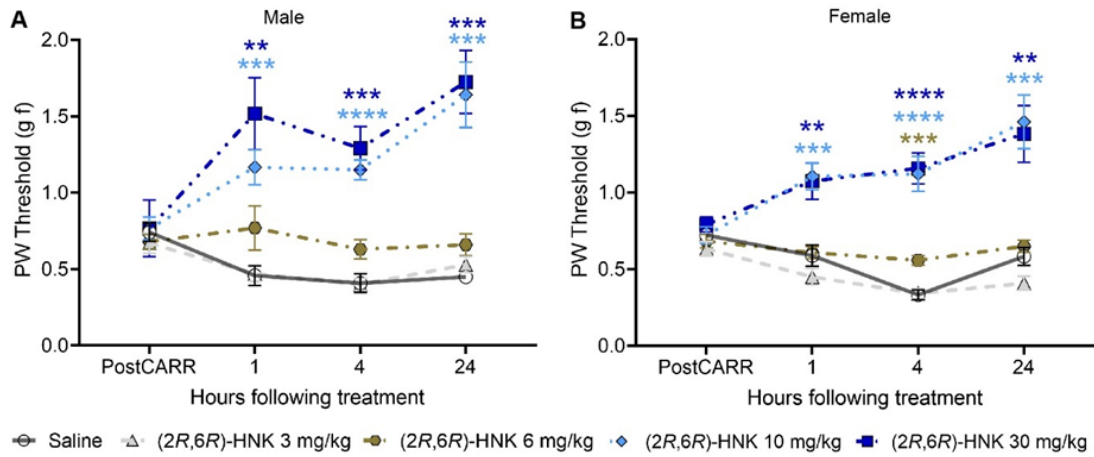


Figure 8. The lines represent the dose-response curve over time for mechanosensitivity in a localized inflammatory pain condition after (2*R*,6*R*)-HNK treatment using a modified quarter log dosing scale. (2*R*,6*R*)-HNK similarly reversed mechanical allodynia for male (A) and female (B) mice at doses of 10 and 30 mg/kg within one hour and with a duration greater than 24 hours. The error bars represent group means $\pm$ SEM. Asterisks indicate significant differences compared to the saline control at the same time point; ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$, $N = 10-16$ for males, and $N = 12-18$ for females.

(2*R*,6*R*)-HNK's anti-allodynia profile for localized inflammatory pain was similar to that of the NSAID carprofen

Carprofen is an NSAID recommended in veterinary medicine to treat pain in mice (35). This experiment compared the magnitude and time course of the anti-allodynic like effect of (2*R*,6*R*)-HNK to carprofen. (2*R*,6*R*)-HNK significantly reversed CARR-induced mechanical allodynia measured at 1, 4, and 24 hours following drug administration. Similarly, carprofen demonstrated reversal of the CARR-induced allodynia at 1, 4, and 24 hours following drug administration (**Figure 9**). The magnitude and time course of effects were similar between (2*R*,6*R*)-HNK and carprofen. Results of a repeated-measures ANOVA showed significant effects of time ($F_{(1.10, 63.92)} = 4.957$, $P = 0.0100$), treatment ($F_{(2, 32)} = 21.67$, $P < 0.0001$), and time x treatment interaction ($F_{(6, 96)} = 6.293$, $P < 0.0001$) when analyzed with the saline-treated animals.

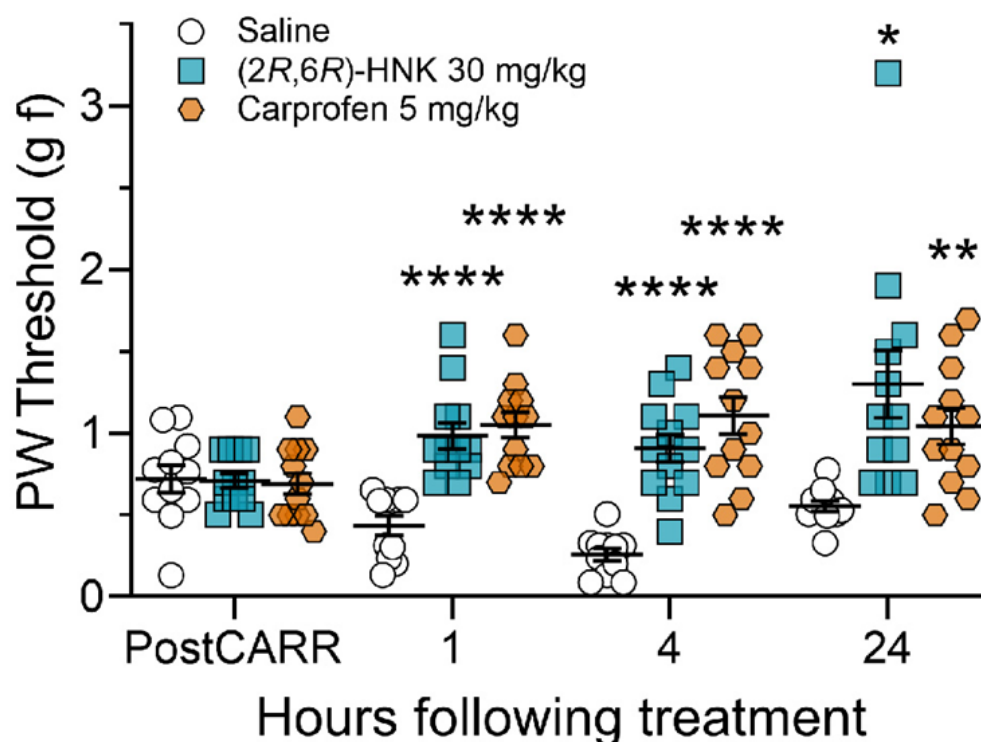


Figure 9. (2R,6R)-HNK at 30 mg/kg reversed mechanical allodynia associated with CARR-induced inflammation comparably with the NSAID carprofen (5 mg/kg) at 1, 4, and 24 hours following a single administration. The error bars represent group means $\pm$ SEM. The two medications were compared to saline control at the same time point, * $P < 0.05$, ** $P < 0.01$, **** $P < 0.0001$, $N = 11$ for the saline group, and $N = 12$ for the HNK and carprofen groups.

The anti-allodynic like effect of (2R,6R)-HNK is dependent on AMPA receptor activation

The final experiment examined the role of AMPAR in (2R,6R)-HNK's mediation of inflammation-induced mechanical allodynia. The AMPAR antagonist NBQX was administered 30 minutes prior to (2R,6R)-HNK or saline treatment and the effects on allodynia were measured at 4 and 24 hours after treatment. As shown in **Figure 10**, NBQX blocked (2R,6R)-HNK mediated reversal of mechanical allodynia in mice with localized inflammatory pain at 4 hours (treatment effect $F_{(1, 36)} = 10.16$, $P = 0.0030$;

pretreatment effect $F_{(1, 36)} = 10.16$, $P = 0.0030$; pretreatment x treatment interaction $F_{(1, 36)} = 11.20$, $P = 0.0019$) and 24 hours (treatment effect $F_{(1, 36)} = 21.01$, $P < 0.001$; pretreatment effect $F_{(1, 36)} = 2.21$, $P < 0.0001$; pretreatment x treatment interaction $F_{(1, 36)} = 12.43$, $P = 0.0012$) after treatment. This effect demonstrated that AMPAR activity is necessary to produce the acute effect of (2*R*,6*R*)-HNK and to maintain the persistent effect of (2*R*,6*R*)-HNK seen 24 hours after injection.

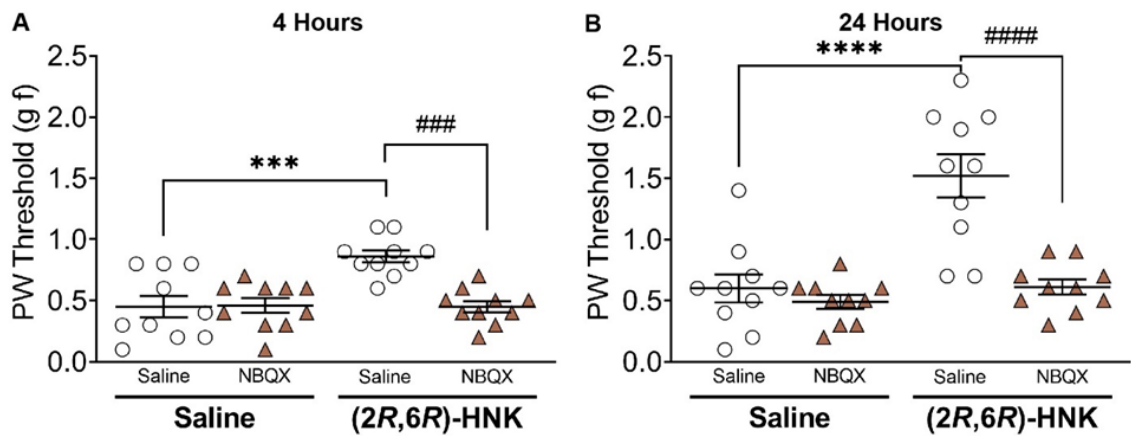


Figure 10. Pretreatment with the AMPA receptor antagonist NBQX 10 mg/kg (30 minutes prior) blocked reversal of mechanical allodynia associated with localized inflammation by (2*R*,6*R*)-HNK 30 mg/kg at 4 hours (**A**) and, in the same cohort, at 24 hours (**B**) following (2*R*,6*R*)-HNK treatment. The error bars represent group means $\pm$ SEM. Group comparisons are indicated with horizontal lines. Asterisks represent (2*R*,6*R*)-HNK/saline to saline/saline comparisons: *** $P < 0.001$, and **** $P < 0.0001$. The # symbol represents (2*R*,6*R*)-HNK/NBQX to (2*R*,6*R*)-HNK/saline comparisons: #### $P < 0.001$, and ##### $P < 0.0001$. N = 10 for this experiment.

DISCUSSION

The present study demonstrated that the ketamine metabolite (2*R*,6*R*)-HNK produced analgesic effects in a model of inflammatory pain induced by the localized injection of λ -carrageenan into the footpad in mice. (2*R*,6*R*)-HNK reversed the resulting

inflammation-induced mechanical allodynia within one hour of administration with a duration of effect lasting greater than 24-hours after a single dose. The anti-allodynic effect of (2*R*,6*R*)-HNK was similar between male and female mice. A dose-response analysis demonstrated a minimum dose of 6-10 mg/kg was required to achieve the effect in both sexes. In direct comparison, (2*R*,6*R*)-HNK mediated reversal of allodynia was similar in magnitude and duration to the NSAID carprofen in this model. The analgesic effect of (2*R*,6*R*)-HNK was blocked by pretreatment with the AMPA receptor (AMPA) antagonist NBQX, indicating that the (2*R*,6*R*)-HNK mediated analgesia was dependent on AMPARs.

Recently, (2*R*,6*R*)-HNK has emerged as a prospective novel analgesic compound because it reversed allodynia in models of postoperative, Complex Regional Pain Syndrome (CRPS) type 1, and neuropathic pain (66; 143) and decreased responses to hot thermal pain in healthy animals (143). The results of this study provide evidence that (2*R*,6*R*)-HNK can reverse hypersensitivity in a rodent model of localized inflammatory pain (**Figure 7**). The onset of the anti-allodynic effect was less than one hour, and the duration of the effect was greater than 24 hours following a single administration of (2*R*,6*R*)-HNK. Overall, the timing for (2*R*,6*R*)-HNK mediated behavioral changes has been shown to vary depending upon the examined behavior and modeled condition. The onset of (2*R*,6*R*)-HNK's analgesic effects (66; 143) and antidepressant-like effects [for review, see (50)] range from as early as 1 hour up to 24 hours after treatment, and the duration of antidepressant effects were shown to last for days (20). The (2*R*,6*R*)-HNK mediated analgesia-like effect outlined in this study was aligned with the rapid pain reduction in models of neuropathic pain and CRPS type 1 pain (66) but differs from the

delayed onset for (2*R*,6*R*)-HNK generated thermal antinociception in healthy mice (143) and mechanical allodynia reversal in a model of postoperative incisional pain (66). The difference in onset time may be explained by the presence or absence of spontaneous pain, inflammatory processes involved, and the persistent nature of the pain condition examined.

Without regard to the type of pain condition modeled, (2*R*,6*R*)-HNK produces a persistent pain reduction-like effect 24 hours following treatment that outlasts the presence of the drug in circulation. The half-life for (2*R*,6*R*)-HNK in the mouse brain is approximately 1 hour (145), while the plasma half-life is 0.2-0.8 hours (49). The duration of effect outlasting the presence of the drug in circulation may suggest (2*R*,6*R*)-HNK induces long-term plasticity changes in brain regions responsible for pain perception and modulation.

Qualitative sex differences exist in rodents for processing pain. Females are more sensitive than males to noxious stimuli and possess lower levels of stress-induced analgesia, while males demonstrate a more robust analgesic response [for reviews, see (90; 133)]. There was a significant difference in baseline mechanosensitivity between male and female C57BL/6J mice in the present study, which aligns with known data (75). There is also a significant difference in (2*R*,6*R*)-HNK pharmacokinetics between male and female mice (48). Despite the pain behavior and (2*R*,6*R*)-HNK pharmacokinetic sex differences, there were no sex differences in (2*R*,6*R*)-HNK's behavioral effect in this inflammatory pain model. (2*R*,6*R*)-HNK produced equivalent pain reduction effects after CARR-induced inflammation at doses of 10 and 30 mg/kg in male and female mice (**Figure 8**).

(2*R*,6*R*)-HNK reversed CARR-induced mechanical allodynia similarly to carprofen (**Figure 9**) both acutely at 4 hours and persistently at 24 hours after administration. Carprofen is an NSAID commonly used in veterinary medicine to treat inflammatory pain (35), but gastrointestinal and cardiac side effects can limit its use. Previously carprofen has been shown to reverse mechanical allodynia in a complete Freund's adjuvant (CFA) hind paw injection model of localized inflammation within 1.5 hours but did not reverse the condition 24 hours later (119). The variation in persistency for carprofen-mediated inflammatory pain reversal may result from using different inflammation-inducing agents (CFA vs. CARR). Additionally, giving the animals less time between measurements (≥ 5 seconds vs. 3 minutes) and differences in executing the classic up-down method to measure mechanosensitivity may have influenced the variation observed between studies. Other NSAIDs have demonstrated the capability to reverse CARR-induced peripheral inflammation to a percentage of baseline mechanosensitivity of 100% for celecoxib and 60% for ibuprofen at sufficiently high doses (41). The results this study demonstrate a percentage return to baseline mechanosensitivity of 22% at 4 hours and 14% at 24 hours for the carprofen treated animals and 17% at 4 hours and 21% at 24 hours for (2*R*,6*R*)-HNK. Compared to the previous report, the lower percentages to return to baseline seen in this study could signify a low magnitude of treatment effect. However, there are important differences between the studies. The CD1 mice used by Gris et al. (41) possessed far greater mechanosensitivity at baseline (1.5 g f) than the C57BL/6 mice used in this study. The difference in baseline sensitivity may be explained by using a different strain of mouse (CD1 vs. C57BL/6) and different timing between von Frey filament application (5-10

seconds vs. 3 minutes). The lower baseline paw withdrawal threshold would make any significant reversal of the mechanical allodynia state appear to have a greater magnitude of treatment. In this study, all groups' baseline mechanosensitivity (before CARR injection) was 4.1 ± 1.17 grams force (g f) in the male animals used in the test. The baseline mechanosensitivity data in this study correspond better with what has been reported previously using the C57BL/6 mouse strain used in this study (66; 91).

The primary accepted mechanism for ketamine's anesthetic effects and other responses is by potent antagonism of the NMDA receptor. It is doubtful that NMDA antagonism is responsible for (2*R*,6*R*)-HNK's behavioral effects because (2*R*,6*R*)-HNK has such a low affinity for the NMDA receptor as evidenced by a $K_i > 100$ nM (88; 94) and a high ED₅₀ (227.8 mg/kg) for NMDA induced lethality in hippocampal cells (81). In addition, opioid receptors have been shown to mediate analgesic effects by ketamine but not by (2*R*,6*R*)-HNK (143). Instead, (2*R*,6*R*)-HNK provided its analgesic effect dependent on AMPAR activation (**Figure 10**). Pretreatment with the AMPAR antagonist NBQX blocked the (2*R*,6*R*)-HNK mediated reversal of allodynia. These data align with previous results demonstrating that (2*R*,6*R*)-HNK's antinociceptive effects in healthy animals were also AMPAR dependent (143). AMPAR activity has previously been shown to play a role in analgesia. Specifically, AMPAR positive allosteric modulators have been demonstrated to reverse mechanical allodynia induced by nerve injury in rats and the effect was blocked by NBQX (72; 127). Taken together, AMPAR potentiation appears to play an important role in analgesia and can facilitate the investigation of neural pathways important to the induction and treatment of pain conditions.

Although (2*R*,6*R*)-HNK requires AMPARs to evoke its analgesic action, understanding remains incomplete for how (2*R*,6*R*)-HNK works to produce behavioral changes via AMPARs. (2*R*,6*R*)-HNK does not directly bind to or activate AMPARs (111; 117). However, (2*R*,6*R*)-HNK enhances presynaptic release of glutamate (111) and alters AMPAR subunit expression in some brain regions (51; 117; 145). For example, systemically administered (2*R*,6*R*)-HNK rescued learned helplessness induced decreases in pGluR1/GluR1 ratio and decreases in cell surface GluA1 expression in the vIPAG (20). Moreover, (2*R*,6*R*)-HNK has been shown to increase cell signaling downstream of AMPARs, such as increasing BDNF signaling (36; 81; 145), activating receptor tyrosine kinase B (15), enhancing mTOR dependent protein synthesis (36; 81; 102), and increasing cAMP (137) and eEF2 (145) levels. These molecular alterations may explain (2*R*,6*R*)-HNK mediated analgesia if the changes occurred in brain regions responsible for pain signal transmission and modulation, such as the rostroventral medulla and periaqueductal gray (44; 132). This idea is also supported by data that demonstrated (2*R*,6*R*)-HNK targeted the periaqueductal gray to induce behavioral changes in rodents (19; 20; 141).

In conclusion, these results indicate that (2*R*,6*R*)-HNK reverses mechanical allodynia associated with inflammatory pain rapidly (within 1 hour) and persistently (> 24 hours) and works through an AMPAR-dependent glutamatergic signaling pathway. The analgesia-like effect was similar to that provided by the NSAID carprofen commonly used in veterinary medicine to treat inflammatory pain. These results support the further investigation into whether (2*R*,6*R*)-HNK can mediate analgesia in human inflammatory pain conditions and other types of pain. If made clinically available, the apparent safe

side effect profile of (2*R*,6*R*)-HNK could make it a primary treatment for inflammatory pain to help improve the quality of life for the many who suffer from debilitating inflammatory pain conditions.

CHAPTER 4: DISCUSSION

SUMMARY OF THESIS FINDINGS

The overall goal of this thesis research was to replicate and expand the investigation of the pain reduction-like effects of (2*R*,6*R*)-HNK using different models for studying pain in mice. The antinociceptive response of (2*R*,6*R*)-HNK to a noxious thermal stimulus on the hot plate was used to characterize the time course, sex differences, and pharmacological mechanism of action of (2*R*,6*R*)-HNK analgesia in healthy mice. (2*R*,6*R*)-HNK generated antinociception with a time course and mechanism that differed from its parent drug ketamine and revealed an inverted U-shaped dose-response. In the spared nerve injury (SNI) model of neuropathic pain, experimental results confirmed that (2*R*,6*R*)-HNK reversed allodynia. These studies showed that males were also sensitive to the analgesic effects of (2*R*,6*R*)-HNK, the opposite sex than previously studied. In a model of inflammatory pain, a type of pain that had not yet been employed to characterize (2*R*,6*R*)-HNK analgesia, experiments revealed a (2*R*,6*R*)-HNK analgesic effect by reversing allodynia. In the neuropathic pain and inflammatory pain studies, comparison of the effects of (2*R*,6*R*)-HNK to examples of established therapeutics for the type of pain condition treated revealed that (2*R*,6*R*)-HNK was equivalent (inflammatory pain) or superior (neuropathic pain) to analgesic standards. The antinociceptive and analgesic effects were dependent on AMPAR activation, suggesting AMPARs play a critical role in the pain reduction effects of (2*R*,6*R*)-HNK. These collective findings expand understanding of (2*R*,6*R*)-HNK's potential to treat pain and provide new information about the mechanism underlying its pain reduction-like effects.

(2*R*,6*R*)-HNK antinociception compared to ketamine and (2*S*,6*S*)-HNK

The experimental results presented in chapter 2 indicate (2*R*,6*R*)-HNK treatment mediated antinociception with delayed onset in healthy animals (**Figure 3 & 4**).

Ketamine, which has been shown to engender antinociception effects to thermal heat in healthy animals (23; 92; 101), produced rapid onset and short-lived antinociception only (**Figure 3**). In addition to differences in the time course of the effect, ketamine antinociception was independent of AMPAR activation while partially dependent on opioid receptors. On the other hand, (2*R*,6*R*)-HNK mediated antinociception was independent of opioid receptors and dependent on AMPARs for initiating the delayed effect and its expression 24 hours later (**Figure 5**). The time course and mechanistic data indicate (2*R*,6*R*)-HNK produces analgesic-like effects differently from its parent drug. While acute ketamine antinociception is likely mediated by blockade of the NMDAR and opioid receptor, (2*R*,6*R*)-HNK antinociception was dependent on the AMPAR. Additional research is needed to identify the underlying mechanisms for this AMPAR-dependent effect, a currently unknown mechanism for providing pain relief that could be used to develop even more pharmaceutical analgesic drugs in the future.

Like (2*R*,6*R*)-HNK, (2*S*,6*S*)-HNK has also been considered for its potential to share some of ketamine's therapeutic effects in treating depression (89; 102; 142). In this study, the isomer (2*S*,6*S*)-HNK (10-30 mg/kg) did not increase hot plate responses at any time point, signifying an inability to induce antinociception under the conditions and dosing used in this study (**Figure 3**). The contrast in capability for producing antinociception between these two ketamine metabolites is significant. If some of racemic ketamine's analgesic properties stem from activity by its metabolites, as has been proposed for ketamine's antidepressant effects, then any formulation of ketamine that

lacks the potential to be metabolized into (2*R*,6*R*)-HNK would not be expected to achieve the same level of analgesia seen with racemic ketamine treatment. Considering the FDA recently approved intranasal *S*-ketamine (Spravato[®]) to treat depression, these findings would be important to any researcher considering the potential for *S*-ketamine to treat pain conditions or pain conditions that may overlap with existing depression.

(2*R*,6*R*)-HNK reversal of mechanical allodynia and comparison with clinically used analgesics

The results of experiments presented in chapter two demonstrated that (2*R*,6*R*)-HNK is capable of reversing neuropathy-induced mechanical allodynia in male mice (**Figure 6**), with the time course and magnitude agreeing with results previously reported in female mice only (66). In this model, the allodynia reversing effects of (2*R*,6*R*)-HNK were contrasted with gabapentin. Gabapentin is a calcium channel blocker commonly used to treat neuropathic type pain in humans (113) and has been reported to reverse SNI induced allodynia in rodents (40; 67; 118; 121). The comparison revealed gabapentin to reverse mechanical allodynia acutely to a similar magnitude, but the effect was no longer apparent 24 hours after treatment. Repeated once-daily gabapentin treatment also failed to reverse allodynia 24 hours following the third administration. (2*R*,6*R*)-HNK acutely reversed allodynia with a similar magnitude to gabapentin. (2*R*,6*R*)-HNK's reversal of allodynia persisted for more than 24 hours following the first dose and was maintained following the third once-daily dose.

Gabapentin was previously reported to reverse neuropathy-induced mechanical allodynia in mice for approximately 3 to 6.5 hours after a single dose (67; 121), depending on the report. Twice daily administration effectively maintained the allodynia reversing effect for seven days (121). Additional experimentation evaluating once-daily

(2*R*,6*R*)-HNK with gabapentin administered more frequently would be needed to better compare the analgesic potential over time. Considering gabapentin's plasma half-life in a mouse was less than 2 hours (67), its allodynia reversing effects appear to depend on the availability of circulating drug, and this agrees with its recognized mechanism to inhibit Ca²⁺ activity. The data from this study support a longer duration of action for a single dose of (2*R*,6*R*)-HNK in this pain model compared to gabapentin because its effect on behavioral pain outlasts the presence of the drug. Currently, there are only four drug classifications recommended for neuropathic pain treatment, including tricyclic antidepressants, anticonvulsants like gabapentin, opioids, and local anesthetics (4). If the results of this study were to translate to a clinical effect in humans, (2*R*,6*R*)-HNK could introduce a new class of treatment for this difficult-to-treat pain condition.

The experiments presented in chapter 3 indicate (2*R*,6*R*)-HNK can also reverse mechanical hypersensitivity associated with inflammatory pain (**Figure 7**). This study was the first to investigate the pain-reducing effects of (2*R*,6*R*)-HNK in a model of inflammatory pain. The pain reduction effects of (2*R*,6*R*)-HNK in the CARR-induced inflammation model were compared to carprofen, an NSAID recommended for treatment in mice (35). In the time course comparison, (2*R*,6*R*)-HNK showed a similar pattern of allodynia reversal to carprofen (**Figure 9**). Both drugs significantly reversed mechanical allodynia within four hours (the earliest time point tested), and the effect persisted for 24 hours following a single dose. The onset of carprofen analgesia in these data corresponds well with another report where carprofen reversed allodynia in a complete Freund's adjuvant (CFA) hind paw injection model of inflammation within 1.5 hours in mice (119). Overall, these data broaden the potential clinical scope for (2*R*,6*R*)-HNK analgesia

to treat additional types of pain and demonstrate that its pain reduction-like effects are comparable to generally accepted treatments for neuropathic and inflammatory pain.

ONSET AND DURATION OF EFFECTS

The onset of antinociceptive and analgesic effects for (2*R*,6*R*)-HNK varied with different pain models. (2*R*,6*R*)-HNK produced significant antinociception in healthy animals with a delayed onset of effect (between 10 and 24 hours). A similar delay in onset (4 to 24 hours) was reported for (2*R*,6*R*)-HNK to reverse acute surgical paw incision-induced mechanical allodynia (66). In contrast, the onset for (2*R*,6*R*)-HNK's anti-allodynic effects for spontaneous neuropathic and inflammatory pain were more rapid (**Figure 6 & 7**) (66). The pathological differences underlying the causes of pain and the animal's overall health appear to impact how early (2*R*,6*R*)-HNK begins to provide analgesic-like effects.

Another aspect of the pharmacological effects of (2*R*,6*R*)-HNK regardless of the pain condition was that pain behavioral effects lasted for 24 hours following treatment or for even longer following repeated treatment. Considering that the half-life of (2*R*,6*R*)-HNK in the mouse brain is approximately 1 hour (145), and the plasma half-life is 0.2-0.8 hours in mice (49), (2*R*,6*R*)-HNK's duration of effect consistently outlasted the presence of the drug in circulation. Based on these data, it is hypothesized that changes in plasticity occurring in brain regions responsible for pain modulation, rather than direct effects of the drug, are responsible for inducing the lasting effects of (2*R*,6*R*)-HNK on pain behaviors. A complete discussion on the proposed plasticity changes can be found in the section titled "Proposed mechanism for (2*R*,6*R*)-HNK pain behavioral effects."

DOSE-RESPONSE DATA FOR (2*R*,6*R*)-HNK BEHAVIORAL CHANGES AND SEX DIFFERENCES

The initial examinations in this thesis used the (2*R*,6*R*)-HNK dose most commonly reported in the literature (10 mg/kg) to evaluate its potential analgesic effects. After establishing (2*R*,6*R*)-HNK mediated antinociception, the next step was to assess the dose-response function. The results of these experiments, presented in chapter 2, revealed an inverted U-shaped dose-response curve with doses of 10 and 18 mg/kg producing antinociception in male and female mice and 32 mg/kg effective in females only (**Figure 4C & D**). Doses exceeding 32 mg/kg failed to evoke antinociception in male or female mice. A previous report established a pharmacokinetic sex difference for (2*R*,6*R*)-HNK elimination in mice where male mice cleared (2*R*,6*R*)-HNK faster than females (48). The difference in drug clearance may have played a role in determining a wider effective dose range for (2*R*,6*R*)-HNK to evoke antinociception in female mice compared to males.

The inverted U-shape of (2*R*,6*R*)-HNK's dose-response curve for thermal antinociception is interesting and may indicate that (2*R*,6*R*)-HNK has competing mechanisms of action as plasma concentrations rise. But the U-shaped dose-response may also be explained by something as simple as the biphasic nature of glutamatergic activity involved in the initiation of pain modulation. Zhuo and Gebhart showed that glutamate administration directly into the rostroventral medulla (RVM), a brain region well recognized for initiating descending pain pathway signals, of naïve animals facilitated the activation of descending spinal signals to modulate pain at lower doses and inhibited the same signals as the dose increased (152).

In the CARR-induced model of inflammatory pain, an additional dose-response function for (2*R*,6*R*)-HNK analgesia effect was established (**Figure 8**). This time

(2*R*,6*R*)-HNK revealed a more typical dose-response curve to reverse allodynia.

However, since the highest dose tested was 30 mg/kg, testing doses higher than 30 mg/kg may offer a more complete picture of (2*R*,6*R*)-HNK's dose-response relationship for reversing allodynia. On the other hand, no significant difference was identified between doses of 10 and 30 mg/kg in both the inflammatory and neuropathic pain models signifying a potential maximum anti-allodynic effect in spontaneous pain conditions of these types.

Increased baseline mechanosensitivity was observed in female mice before CARR injection to induce inflammatory pain. A baseline sex difference in C57BL/6 mice has been described previously (75). Notwithstanding the disparity in baseline mechanosensitivity, there were no significant differences between male and female mice after (2*R*,6*R*)-HNK treatment at any time point measured in the inflammatory pain model. There does not appear to be a significant sex difference for (2*R*,6*R*)-HNK to reduce neuropathy-induced pain either when comparing the data from this study with those reported earlier (66). Generalized sex differences for processing pain in rodents have been described previously, where females were more sensitive to pain than males [for reviews, see (90; 133)]. Despite the reported sex differences, (2*R*,6*R*)-HNK showed similar potential for treating pain conditions for both sexes.

SIDE EFFECT PROFILE OF (2*R*,6*R*)-HNK COMPARED TO KETAMINE

When comparing ketamine and (2*R*,6*R*)-HNK mediated antinociception in healthy mice, ketamine treatment correlated with a greater increase in latency to respond to the stimulus. However, ketamine-treated animals exhibited swaying motions while they walked, demonstrating possible impairment. No visible impairment was detected in

(2*R*,6*R*)-HNK treated mice up to 50 mg/kg. One concern with analgesia testing in preclinical studies is that alterations in nocifensive behavior may be undermined by the impairing side effects of the tested drug rather than producing true analgesia. Ketamine has been shown to impair motor coordination (57; 110) which may undermine its analgesic effect.

A detailed examination of the animals' gait following treatment was made using the DigiGait™ treadmill system and software that allowed the quantification of gait characteristics between ketamine and (2*R*,6*R*)-HNK. The analysis revealed dose-dependent dystaxic-like gait alterations in the animals treated with ketamine at the doses that produced antinociception (**Table 1**). (2*R*,6*R*)-HNK did not induce the same gait changes even at 50 mg/kg, a dose that is five times the amount needed to induce antinociception and reverse allodynia.

There are considerable data available on (2*R*,6*R*)-HNK's side effect profile because its potential antidepressant properties have been evaluated for some time. The data indicate (2*R*,6*R*)-HNK does not alter memory performance (47), impair motor coordination in the rotarod or change locomotor activity (145), alter sensorimotor gating (145), or increase self-administration on demand (145), even when administered at doses up to 125 mg/kg for coordination and activity assays and 375 mg/kg to test sensorimotor gating. These data indicate a wide therapeutic window for (2*R*,6*R*)-HNK treatment considering only 10 mg/kg is needed to induce behavioral changes. Overall, there appear to be no overt adverse motor effects associated with (2*R*,6*R*)-HNK administration [for review, see (50)]. The evaluation of gait characteristics following treatment in this study adds to the mounting data demonstrating that (2*R*,6*R*)-HNK possesses a favorable side

effect profile compared to ketamine and other strong analgesic medications with motor impairing effects.

(2*R*,6*R*)-HNK'S OPIOID ENHANCEMENT AND IMPACT ON OPIOID TOLERANCE

Clinical evidence supports intraoperative ketamine administration to reduce analgesic requirements during recovery from surgery and decrease the overall intensity of surgical pain (7). The major theories for how ketamine may provide these effects and the preclinical data supporting them are as follows: 1) ketamine augments opioid analgesia (14; 122) and 2) ketamine suppresses the development of and even reverses opioid tolerance (63; 77; 78; 123; 129). Reducing the required opioid dose to produce analgesia is essential as patients develop tolerance and physical dependence and are at risk of dangerous side effects of opioids at higher doses, including respiratory depression.

Experiments were generated to test whether (2*R*,6*R*)-HNK might possess the same augmentation of opioid based analgesia as ketamine. This thesis research was the first to evaluate the potential for (2*R*,6*R*)-HNK alone to reverse opioid tolerance or enhance opioid antinociception. The methods for this examination are described in detail in Appendix 1. Briefly, (2*R*,6*R*)-HNK treatment was analyzed for its potential to influence opioid tolerance induced in mice by administering morphine repeatedly over four days. As expected, repeated morphine significantly increased the amount of morphine required to evoke antinociception (**Figure 12C**), similarly to previous reports (14; 63; 123). The results demonstrated that (2*R*,6*R*)-HNK did not significantly reverse morphine tolerance after a single dose or after three once-daily administrations, measuring for effect 24 hours after treatment (**Figure 12D**). However, (2*R*,6*R*)-HNK did produce a treatment effect compared to saline following repeated administration in the

morphine tolerant animals. At morphine doses of 1, 3, and 6 mg/kg, (2*R*,6*R*)-HNK treatment increased the antinociceptive effects of morphine, as evidenced by a difference in %MPE of 25%, 17.5%, and 31.4%, respectively, at the lower morphine doses which were ineffective when administered alone in morphine exposed animals (**Figure 12D**).

The mechanism by which (2*R*,6*R*)-HNK could enhance morphine antinociception may be similar to that of ketamine/opioid interactions, where ketamine was reported to inhibit the glucuronidation of morphine (28), effectively prolonging morphine's duration of action. In support of this hypothesis, one report described a 60% increase in morphine brain concentration following (2*R*,6*R*;2*S*,6*S*)-HNK (racemic) administration. Equally, morphine pretreatment was shown to prolong (2*R*,6*R*;2*S*,6*S*)-HNK levels (79). It remains unknown whether (2*R*,6*R*)-HNK can decrease morphine clearance on its own. Any clinical investigation of (2*R*,6*R*)-HNK's ability to enhance opioid analgesia would need to consider increased bioavailability of the opioid and monitor closely for respiratory depression. The results of this research indicate (2*R*,6*R*)-HNK treatment did not alter morphine tolerance under the conditions tested. The augmentation interaction between (2*R*,6*R*)-HNK and morphine probably does not involve direct actions at opioid receptors because pretreatment with the opioid antagonist naltrexone did not alter (2*R*,6*R*)-HNK antinociception (**Figure 5B & D**) or its mediated reversal of incision-induced allodynia as previously reported (66).

POTENTIAL TARGETS AND MECHANISMS INVOLVED IN (2*R*,6*R*)-HNK'S BEHAVIORAL PAIN EFFECTS

Potential sites for (2*R*,6*R*)-HNK mediated pain reduction activity

The rostral ventromedial medulla (RVM) is a brain region known for initiating adrenergic and serotonergic descending spinal signals that terminate in the dorsal horn of

the spinal cord and inhibit ascending nociceptive signaling (5; 152). Enhanced glutamatergic signaling in this region has been reported to inhibit hyperalgesia associated with inflammatory pain in rodents (44). Inflammatory pain has been shown to induce an upregulation of AMPAR subunit expression in the RVM (43) and increase phosphorylated GluA1 levels that enhance AMPAR Ca^{2+} conductance (42). The AMPAR subunit changes were blocked in animals pretreated with a local anesthetic before inducing the inflammatory pain condition, demonstrating that the changes were indeed associated with pain (42).

The periaqueductal gray (PAG) is another brain region well recognized for processing ascending pain signals and projecting to the RVM to initiate descending pain modulation pathways [for review, see (6)]. More current reports have identified specific circuits between the prefrontal cortex (PFC) and the ventrolateral PAG (vlPAG) that are impacted by neuropathic pain conditions in mice (18; 53). By way of retro labeling neuronal input to vlPAG neurons, Huang et al. identified PFC neurons projecting to the vlPAG with reduced activity that correlated with chronic sciatic nerve constriction, a model of neuropathic pain (53). The resulting decrease in PFC neuronal activity in this circuit led to suppressed activation of vlPAG neurons and thus inhibited signaling in the descending pain pathway.

In addition to the available data demonstrating how important the PAG is for modulating pain, several studies implicated the vlPAG as a target for (2*R*,6*R*)-HNK evoked electrophysiological and molecular changes. (2*R*,6*R*)-HNK microinjection into the vlPAG of mice reversed stress-induced decreases in frequency and amplitude of miniature excitatory post-synaptic currents (mEPSC)s of patch clamped vlPAG neurons,

sufficient to trigger action potentials (103). In healthy rats, direct vIPAG (2*R*,6*R*)-HNK injection enhanced glutamatergic synaptic transmission within the vIPAG (141). In brain slices from rats exhibiting learned helplessness, bath application of (2*R*,6*R*)-HNK increased both frequency and amplitude of mEPSCs in vIPAG neurons (20). Direct vIPAG administration in rats even ameliorated chronic stress-induced decreases in ratio of phosphorylated to nonphosphorylated GluA1 and cell surface expression of GluA1 in this region (20).

Several behavioral changes were also associated with (2*R*,6*R*)-HNK treatment localized to the vIPAG. (2*R*,6*R*)-HNK vIPAG microinjection in mice exposed to chronic restraint stress caused reduced forced swim test (FST) immobility time and increased sniffing time in the female urine sniffing test (103). In a learned helplessness model in rats, (2*R*,6*R*)-HNK given directly into the vIPAG reversed the increased FST immobility time and decreased sucrose preference associated with the stressed condition (20). Intra-vIPAG of (2*R*,6*R*)-HNK in mice also reversed the increased tail-suspension-test immobility time and decreased sucrose preference produced by a model of chronic inflammatory bowel disease (64). Localized (2*R*,6*R*)-HNK treatment into the vIPAG even reversed mechanical allodynia associated with chronic restraint stress in mice (103). Altogether, these data demonstrate significant promise for (2*R*,6*R*)-HNK targeted molecular and behavioral effects at the RVM and vIPAG. The effects of (2*R*,6*R*)-HNK given directly into the RVM and vIPAG have not been evaluated on pain endpoints. Nevertheless, because these regions are part of classical pain circuits, it is reasonable to hypothesize that molecular changes within these regions could induce its analgesic-like effects.

AMPA activity

Behavioral and molecular effects of (2*R*,6*R*)-HNK related to antidepressant endpoints have been shown to rely upon the activation of AMPARs. For example, AMPAR antagonism blocked (2*R*,6*R*)-HNK mediated decreases in FST immobility time in mice measured 24 hours after injection (65; 145). (2*R*,6*R*)-HNK reversed the increase in FST immobility and suppression of sucrose preference caused by a learned helplessness paradigm in rats, an effect that was blocked with AMPAR antagonism pretreatment (20). Furthermore, AMPAR antagonism prevented (2*R*,6*R*)-HNK's ability to mediate reversal of stress-induced downregulations of AMPAR subunits GluA1 and GluA2 and BDNF in the mouse hippocampus three days after treatment (60).

Experiments outlined in chapters 2 and 3 demonstrate that AMPAR activity is also necessary for (2*R*,6*R*)-HNK to cause pain reduction-like effects (**Figure 5 & 10**). AMPAR antagonism blocked both the initiation and expression of (2*R*,6*R*)-HNK's delayed antinociception effect in healthy mice. Furthermore, AMPAR activation was necessary for (2*R*,6*R*)-HNK to reverse mechanical allodynia caused by localized inflammatory pain in mice. AMPAR antagonist pretreatment blocked the allodynia reversal effect 4 and 24 hours after (2*R*,6*R*)-HNK treatment.

The impact of (2*R*,6*R*)-HNK on AMPAR subunit availability and cell surface expression may explain, at least in part, how (2*R*,6*R*)-HNK depends on AMPAR activity to produce delayed and persistent effects. This mechanism may commonly mediate the effects of (2*R*,6*R*)-HNK on antidepressant endpoints and pain behaviors, even if the effects on these behaviors involves distinct parts of the brain and more specific neural circuitry. At the molecular level, the AMPAR is a tetramer comprised of subunit proteins GluA1 through GluA4. The majority of AMPARs include GluA2 subunits within the

tetramer conformation. Most GluA2 subunits have undergone posttranscriptional modification that, when included in the AMPAR tetramer, makes the AMPAR impermeable to Ca^{2+} conductance. Any alteration to AMPAR subunit expression that would result in decreased availability of modified GluA2, such as increased levels of GluA1 or unmodified GluA2, would enhance AMPAR Ca^{2+} conductivity and create considerable calcium-related functional consequences. Available preclinical evidence indicates that (2*R*,6*R*)-HNK enhances GluA1 expression in cortical neurons (117), enhances GluA1, GluA2, and GluA4 subunit gene expression in cultured astrocyte progenitor cells (51), and increases GluA1 and GluA2 expression in the hippocampus but not the frontal cortex (145). These data demonstrate how (2*R*,6*R*)-HNK alters AMPAR subunit protein levels and that the changes are brain region selective. The involvement of (2*R*,6*R*)-HNK in a particular behavioral effect may also depend on conditions prior to behavioral testing. For example, exposure to chronic stress or pain may evoke changes in AMPAR expression in brain regions that may be necessary for (2*R*,6*R*)-HNK to produce its behavioral effects. For example, predisposing conditions could underlie the differences in response to (2*R*,6*R*)-HNK in models of pain versus antinociception.

Available evidence demonstrated (2*R*,6*R*)-HNK lacks significant binding affinity for AMPAR at concentrations likely to be achieved following a systemic 10 mg/kg dose (117). Despite (2*R*,6*R*)-HNK failing to bind to AMPAR directly, the evidence is clear that (2*R*,6*R*)-HNK mediated effects are dependent on AMPAR activation. The precise way in which (2*R*,6*R*)-HNK alters AMPAR activity remains unclear. Key evidence suggests (2*R*,6*R*)-HNK increases AMPAR activity by augmenting presynaptic glutamate release (111). (2*R*,6*R*)-HNK-mediated enhancement to glutamatergic signaling and

alteration to AMPAR subunit expression that increases its Ca^{2+} conductance could explain pain reduction effects if the alterations were to occur in brain regions responsible for pain perception and modulation.

Other (2*R*,6*R*)-HNK molecular targets

Despite not fully understanding how (2*R*,6*R*)-HNK initiates its activity, several molecular changes beyond those described above have been identified to correlate with its administration. Some lie directly downstream of glutamatergic signaling, while others may be tied indirectly. Below is a summary of the evidence supporting (2*R*,6*R*)-HNK mediated molecular effects and how they may impact analgesia.

BDNF is important for encouraging survival by promoting the differentiation of neurons and is involved in the development of synaptic plasticity. Among other activities, BDNF binds TrkB receptors, then activates ERK and AKT mediated mTOR pathways, ultimately inducing protein synthesis that could be responsible for altered AMPAR subunit expression and increased synthesis of other proteins important to the synaptic plasticity process. (2*R*,6*R*)-HNK has been shown to enhance the activity of this pathway in several ways. (2*R*,6*R*)-HNK treatment was shown to increase BDNF concentrations in the mouse hippocampus 24 hours after treatment (145). (2*R*,6*R*)-HNK treatment increased phosphorylated mTOR levels in the mouse prefrontal cortex 30 minutes after treatment (36). (2*R*,6*R*)-HNK increased BDNF and the ratio of phosphorylated to nonphosphorylated TrkB in the hippocampus of treated mice exposed to chronic stress (60). And lastly, (2*R*,6*R*)-HNK increased BDNF and the ratio of phosphorylated to nonphosphorylated mTOR 30 minutes after treatment in mice (81).

The (2*R*,6*R*)-HNK molecular effects outlined here could explain its ability to mediate persistent pain behavioral effects if the changes through mTOR mediated enhanced synaptic plasticity were to occur in brain regions such as the RVM and vlPAG. Available data supports the potential for (2*R*,6*R*)-HNK to evoke such changes in the PAG. Chou et al. demonstrated an increase in BDNF within the rat vlPAG two days following systemic (2*R*,6*R*)-HNK treatment (20).

NMDAR activity

Although the mechanisms underlying the antidepressant effects of ketamine are unknown, one group of investigators have suggested that ketamine induces its antidepressant effects by its NMDAR inhibition activity (93). Because (2*R*,6*R*)-HNK's parent compound works this way, it is reasonable to examine whether NMDAR blockade may also play a role in the mechanism for (2*R*,6*R*)-HNK's behavioral and molecular effects. (2*R*,6*R*)-HNK was reported to inhibit NMDAR currents at rest in cultured hippocampal neurons but only at high concentrations (50μM), above that likely required to induce behavioral antidepressant-like effects in rodents (126). On the other hand, results of an orthosteric binding assay demonstrated (2*R*,6*R*)-HNK does directly bind to the NMDAR (117). (2*R*,6*R*)-HNK showed a low affinity for the NMDA receptor with a reported $K_i > 100$ nM (88; 94) and a high ED₅₀ (227.8 mg/kg) for NMDA induced lethality in hippocampal cells (81). This suggests the need for levels of drug that are 200 times higher than those used to evoke a behavioral response, therefore it is unlikely that NMDARs play a role in mediating (2*R*,6*R*)-HNK's pain behavioral effects. This evidence collectively suggests that (2*R*,6*R*)-HNK generates behavioral and molecular alterations independent of NMDAR activity.

Opioid receptor activity

As briefly discussed earlier, opioid receptor activity influences some of ketamine's therapeutic effects. Opioid receptor antagonism with naltrexone blocked ketamine infusion therapy-induced improvements in depression scores (134) in humans. Ketamine's ability to decrease tail-suspension-test immobility times 30 minutes and 24 hours after administration in mice was blocked with naltrexone (150). Recently opioid receptor antagonism was shown to block (2*R*,6*R*)-HNK's behavioral effects. Treatment with (2*R*,6*R*)-HNK induced reductions in FST immobility times 24 hours after treatment in mice, and pretreatment with both the nonselective opioid receptor antagonist naltrexone and the selective kappa opioid receptor antagonist LY2444296 blocked the behavioral response (138).

A role for opioid receptors in (2*R*,6*R*)-HNK's pain behavioral effects have been examined only twice to the author's knowledge. Pretreatment with naltrexone failed to block (2*R*,6*R*)-HNK reversal of paw incision pain-induced mechanical allodynia in mice (66). The results presented in chapter 2 demonstrate that naltrexone failed to block the initiation or expression of (2*R*,6*R*)-HNK's delayed antinociception in healthy mice (**Figure 5B & D**). Overall, the available evidence does not support the involvement of opioid receptors in the antinociceptive or analgesic effects of (2*R*,6*R*)-HNK, and these data differ from comparable studies with naltrexone and ketamine on pain behaviors. However, considering that the involvement of opioid receptors in general and kappa opioid receptors specifically have been demonstrated in some (2*R*,6*R*)-HNK behavioral effects, opioid receptor activity needs to be evaluated in more detail in any future mechanistic studies.

PROPOSED MECHANISM FOR (2*R*,6*R*)-HNK PAIN BEHAVIORAL EFFECTS

Based on the data available in the literature and the results of experiments presented in this thesis, (2*R*,6*R*)-HNK is unlikely to induce its pain reduction-like effects through mechanisms involving opioid receptor activation or NMDA receptor inhibition. Instead, (2*R*,6*R*)-HNK depends on AMPAR activation to mediate its analgesic-like activity. How (2*R*,6*R*)-HNK mediates these AMPAR-dependent effects remains unclear, but an account of the following mechanism can be offered (**Figure 11**).

(2*R*,6*R*)-HNK enhances presynaptic glutamate release. Glutamate activates postsynaptic AMPARs resulting in a moderate depolarization of the membrane via Na⁺ conductance. AMPAR mediated depolarization is typically sufficient to remove the Mg²⁺ plug in the NMDAR, making it available for activation through agonism from available glutamate and glycine. However, (2*R*,6*R*)-HNK may not require NMDARs to mediate plasticity. AMPAR-mediated depolarization is sufficient to open voltage-dependent Ca²⁺ channels in the postsynaptic membrane, allowing for the influx of Ca²⁺ ions. Increased intracellular Ca²⁺ promotes the fusion of BDNF-containing vesicles to the cell membrane. Availability of BDNF in the synapse then activates TrkB receptors to initiate the MEK/ERK and AKT signaling cascade that enhances mTOR pathway activity. The activated mTOR pathway induces protein synthesis resulting in increased availability of proteins vital to promoting synaptic plasticity.

Therefore, as AMPAR subunits, GluA1 specifically, are among the available proteins increased by enhanced mTOR pathway activity, it is postulated that these receptors are a critical mediator of (2*R*,6*R*)-HNK driven molecular and behavioral endpoints. Increased levels of GluA1, made available in the postsynaptic density, increase the number of Ca²⁺ permeable AMPARs. When activated by glutamate, Ca²⁺

permeable AMPARs can generate a greater depolarization of the postsynaptic membrane by conducting both Na^+ and Ca^{2+} ions. This alteration greatly enhances the impact of activated AMPARs, and the process is no longer reliant on other channels to alter intracellular Ca^{2+} availability. Other proteins involved in this process, such as BDNF, may also be upregulated by mTOR within the insulin signaling pathway.

Globally enhancing glutamatergic signaling would not necessarily promote a pain reduction effect. Instead, AMPAR activity enhancement in some brain regions has been shown to facilitate pain signal transmission rather than reduce it, as demonstrated in the anterior cingulate cortex (139) and spinal cord (74). Instead, it is proposed that (2*R*,6*R*)-HNK targets specific brain regions responsible for pain perception and modulation, including the vIPAG and RVM. Furthermore, (2*R*,6*R*)-HNK evoked changes must be synapse-specific within these regions.

The difference in onset for (2*R*,6*R*)-HNK's pain reduction effects for healthy animals and those with an induced pain condition may lie with pain condition-mediated inhibition in glutamatergic signaling. Previously, researchers have identified reduced glutamate signaling in the vIPAG associated with a rat model of neuropathic pain (52). Specifically, there was a reduction in stimulation to EPSC amplitude and lower mEPSC frequency in patched vIPAG neurons compared to sham, indicating suppressed glutamate release. Furthermore, lower mEPSC amplitudes and decreased ratio of AMPAR to NMDAR EPSCs in vIPAG neurons in the same region demonstrated an impairment of AMPAR conductivity. The alterations in glutamatergic signaling were evident three days following the injury and even worse on day ten. The underlying mechanism for the impaired AMPAR conductivity was not elucidated, but levels of AMPAR subunits

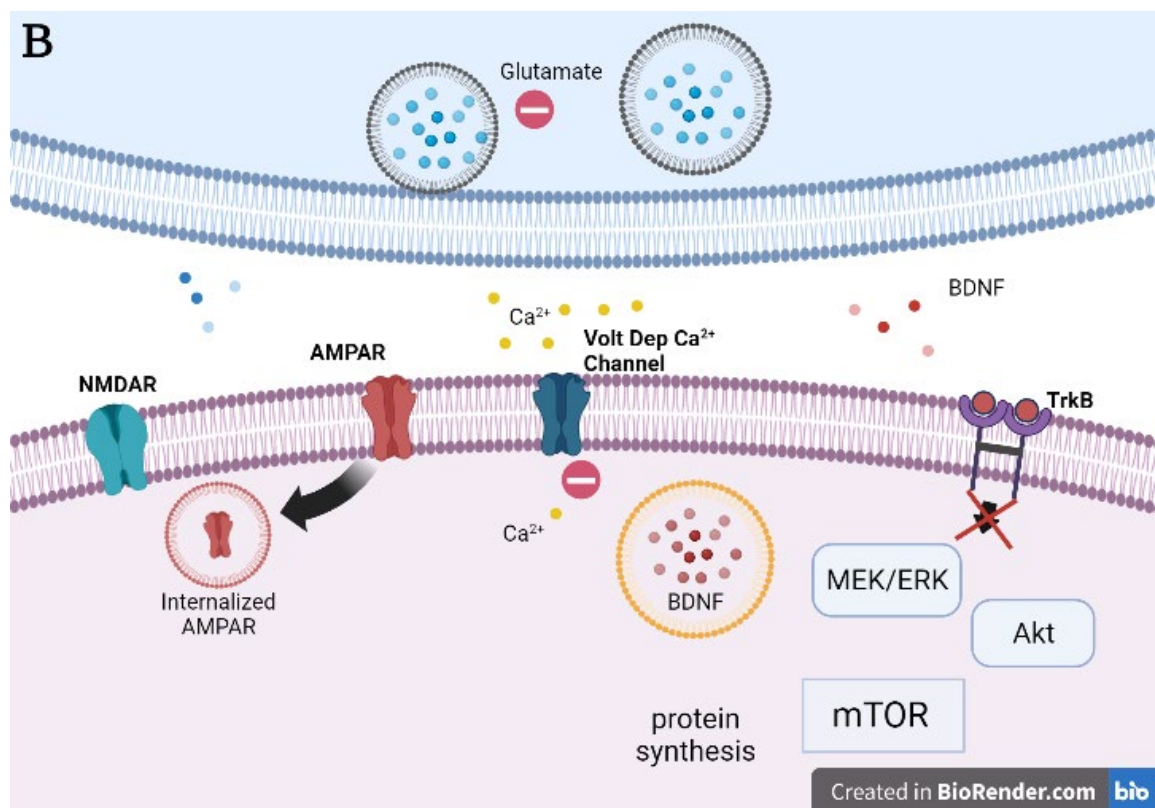
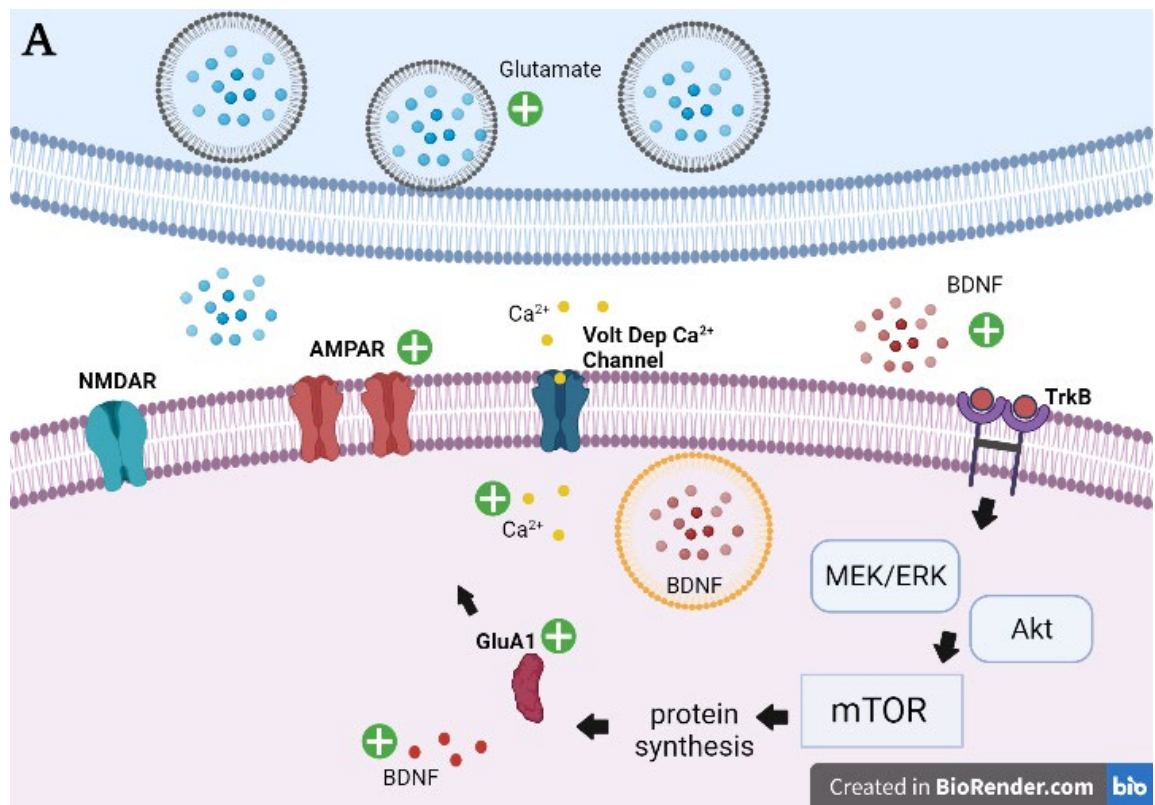


Figure 11. Graphical abstract illustrating the proposed mechanism of (2*R*,6*R*)-HNK analgesia. **Panel A:** (2*R*,6*R*)-HNK treatment enhances glutamate release which activates AMPARs. AMPAR activation depolarizes the membrane activating voltage dependent Ca²⁺ channels that stimulate BDNF vesicles to fuse to the membrane, releasing BDNF into the synapse. BDNF activates TrkB receptors, activating MEK/ERK and AKT signaling cascades to promote mTOR activity. Activated mTOR promotes synthesis of proteins important to enhancing synaptic plasticity including GluA1 and BDNF. These changes would be synapse-specific within the vIPAG and RVM that activate descending pain pathway signaling. **Panel B:** Persistent spontaneous pain results in decreased presynaptic glutamate release, internalization of AMPARs, and reduced availability of BDNF within the synapse. These changes would occur at specific synapses in the vIPAG and RVM, resulting in decreased descending pain pathway signaling. (2*R*,6*R*)-HNK treatment works acutely by enhancing presynaptic glutamate release and persistently through protein synthesis important to enhancing synaptic plasticity. GluA1 synthesis results in increased AMPAR Ca²⁺ conductivity, reversing AMPAR internalization and augmenting AMPAR glutamatergic signaling long-term.

GluA1 and GluA2 remained unchanged. Although not measured in that study, internalization of AMPAR subunits from the postsynaptic membrane could account for the impaired AMPAR activity while producing no changes in subunit concentrations.

Further research is needed to identify whether there is a shared reduction in vIPAG glutamatergic activity between neuropathic and inflammatory pain conditions and whether there exist similar changes within the RVM. Notwithstanding, it is suggested that mechanical allodynia produced by neuropathic and inflammatory pain conditions results from decreased glutamate release and decreased AMPAR availability in the vIPAG and RVM. In this case, (2*R*,6*R*)-HNK treatment corrects the suppressed glutamate release, producing an acute effect. The resultant mTOR mediated protein synthesis would then be responsible for rectifying the impaired AMPAR conductivity by increasing the amount of Ca²⁺ permeable AMPARs.

To summarize the larger picture, (2*R*,6*R*)-HNK produces acute pain reduction-like effects by enhancing glutamate release in animals with pain condition-induced

suppression of glutamatergic signaling in the vlPAG and possibly the RVM. The enhanced glutamatergic activity in these brain regions increases descending pain pathway signaling. (2*R*,6*R*)-HNK produces delayed and persistent pain reduction-like effects, including delayed effects in healthy animals, by escalating the availability of proteins such as GluA1, BDNF, and mTOR essential to enhance synaptic plasticity through AMPAR conductivity in the same brain regions that promote the activation of descending pain pathway signaling.

Based on this hypothesis and the data discussed here, it is recommended that future studies focus on enhanced glutamatergic signaling and AMPAR activation as the primary mechanism to investigate further (2*R*,6*R*)-HNK mediated analgesia. A focus on the vlPAG and RVM are justified as brain regions likely to be involved in pain regulation.

FUTURE DIRECTION

Given the data presented above, the following future experiments are proposed:

- 1.) To investigate whether (2*R*,6*R*)-HNK mediates pain reduction centrally or peripherally. Intracerebroventricular (2*R*,6*R*)-HNK administration followed by assessment of pain reduction-like effects would reveal whether (2*R*,6*R*)-HNK works centrally. Conversely, peripheral (2*R*,6*R*)-HNK administration into the region where the pain is localized (the affected hind paw for the models used in the studies of this thesis) followed by pain assessment would reveal whether (2*R*,6*R*)-HNK works peripherally.
- 2.) To examine the specific roles of the vlPAG and RVM in (2*R*,6*R*)-HNK mediated pain reduction-like effects. The primary focus would be on these

regions due to their prominent role in initiating descending pain pathway signals and how (2*R*,6*R*)-HNK has specifically induced molecular changes within them. The examination of pain reduction-like effects following (2*R*,6*R*)-HNK microinjection into these regions would help to reveal each region's involvement.

- 3.) If (2*R*,6*R*)-HNK microinjection into the vlPAG and RVM were to produce similar pain reduction-like effects as seen in this thesis, the subsequent step would be to interrogate the role of AMPAR involvement in those regions. This could be accomplished through a combination of systemic and region localized micro-infusions of an AMPAR antagonist in combination with systemic or region localized (2*R*,6*R*)-HNK treatment.
- 4.) To determine if (2*R*,6*R*)-HNK treatment increases descending pain pathway activation through monoamine pathways to induce its behavioral pain effects. This could be accomplished by measuring the impact of (2*R*,6*R*)-HNK administration on monoamine levels (5-HT and NE) in the spinal cord CSF in animals with an induced spontaneous pain condition. Additionally, prior depletion of 5-HT or NE could be used to determine the impact of monoamine levels on the behavioral effects of (2*R*,6*R*)-HNK.
- 5.) To investigate higher (2*R*,6*R*)-HNK doses to add to the dose-response data for its reversal of inflammatory and neuropathic pain-induced allodynia. Testing higher doses would ensure the ability to identify a maximum effect and whether there is an inverted U-shaped dose-response for (2*R*,6*R*)-HNK reversal of allodynia.

CONCLUSION

The identification of novel pharmacological pain therapeutics is integral for improving pain treatment options and addressing the pain problem in society. This thesis identified new pain reduction-like effects for (2*R*,6*R*)-HNK in mice. The characterized aspects of its analgesia-like effects, including time course, dose-response, and sex comparison studies, will help guide future research into the potential applications for this ketamine metabolite. In the models of pain used in this thesis research, the magnitude of the effects of (2*R*,6*R*)-HNK treatment was comparable with already utilized treatments, and (2*R*,6*R*)-HNK demonstrated the potential to produce effects that persist for a longer duration. This study's comparison of motor impairing side effects of (2*R*,6*R*)-HNK to ketamine contributed to the literature demonstrated that this metabolite possesses a side effect profile that is advantageous compared to other analgesic medications. This thesis also showed that AMPAR and not opioid receptor activity mediated (2*R*,6*R*)-HNK's pain behavioral effects using two pain models.

Should (2*R*,6*R*)-HNK be made clinically available, the evidence outlined here signifies ample potential for its use to improve upon currently available pain therapeutics. (2*R*,6*R*)-HNK could represent a valuable treatment option for patients suffering from different types of pain that are prevalent in medical treatment and cause an increase in economic burden by decreasing productivity and increasing health care needs. According to data in this thesis research, (2*R*,6*R*)-HNK could be employed to treat the following medical conditions. First, inflammatory pain could be treated independently or in combination with other analgesics to potentially reduce required doses and the risks associated with their use. That application alone could have an enormous impact on this common and debilitating type of pain. Second, its potential application to the

perioperative setting could also reduce postoperative opioid analgesic needs and thus improve the safety of opioid pain treatment in this setting. Third, (2*R*,6*R*)-HNK's demonstrated potential to reduce neuropathic pain is also considerable given this type of pain is chronic and challenging to treat effectively. Finally, exploration of additional treatment options would be encouraged and would be of great value to patients suffering from these types of pain.

Within the active-duty military population affected by pain, (2*R*,6*R*)-HNK as a potential novel pain treatment could make a significant impact right away. (2*R*,6*R*)-HNK represents a potential pain treatment that doesn't produce serious adverse side effects and can be utilized regularly by troops while remaining on active duty and in ready-to-fight status. Troops limited in activity due to their pain could be effectively treated with analgesics that do not impair their physical skills, thus improving military performance capability. Even considering some of (2*R*,6*R*)-HNK's analgesic-like effects possess a delayed onset, its extended duration of action would make its deployment worthwhile. In addition, early treatment of (2*R*,6*R*)-HNK could be implemented in combination with other analgesics as early as on the battlefield itself. Because of its safety profile, medics on the battlefield could be enabled to administer (2*R*,6*R*)-HNK just like the analgesics and antibiotics they already utilize immediately after injury, producing analgesia hours and days later that would greatly benefit the injured soldier.

The results of this thesis should encourage future clinical research into the potential human therapeutic pain-reducing potential of (2*R*,6*R*)-HNK. Future preclinical studies examining (2*R*,6*R*)-HNK pain reduction could enable researchers to understand the mechanism underlying AMPAR-dependent analgesia, which is currently unclear.

Understanding this mechanism could unlock the potential for an entirely new class of analgesic medications with promise for treating different types of pain, and especially those conditions that are difficult to treat.

APPENDIX 1: (2*R*,6*R*)-HNK's opioid augmentation and impact on opioid tolerance

INTRODUCTION

Ketamine reduces opioid consumption in humans (86). In animals, it inhibits the development of opioid tolerance (130), attenuates preexisting opioid tolerance (63; 79; 123), and augments opioid analgesia (14; 79; 122). Ketamine's metabolite, (2*R*,6*R*)-HNK, appears to share some of ketamine's analgesia producing effects (66; 143). In preclinical studies, (2*R*,6*R*)-HNK produces several antidepressant-like behaviors similar to ketamine's effect (104; 145). It is understandable then to question whether (2*R*,6*R*)-HNK interacts with opioids similarly to how ketamine enhances analgesia or reverses opioid tolerance.

Opioid tolerance increases the risk of overdose. Patients who use opioids to manage pain over time for chronic conditions will require higher and higher doses to achieve the same level of analgesia. In the face of analgesic tolerance, higher opioid doses increase the risk for respiratory depression and overdose. Because ketamine can decrease opioid tolerance and enhance opioid analgesia, concomitant prescriptive use of ketamine with opioids can reduce the need to escalate opioid dosing over time to achieve the same analgesic effect, thereby reducing the risk of overdose. However, ketamine's dissociative and general anesthetic properties limit use to administration only by medical professionals trained in anesthesia. (2*R*,6*R*)-HNK's use by patients could be more widespread, considering it lacks the dissociative and sedative effects of ketamine (145). If it were to achieve the same opioid enhancement or opioid tolerance reversal seen with ketamine, concomitant prescription of (2*R*,6*R*)-HNK with opioids could significantly reduce patients' risk of opioid overdose.

This study examines the effects of (2*R*,6*R*)-HNK administration in morphine tolerant mice to reduce response to hot plate pain stimulation. The experimental paradigm used here also allows assessment of (2*R*,6*R*)-HNK to enhance a morphine-induced reduction in nocifensive behaviors. The results revealed (2*R*,6*R*)-HNK did not reverse morphine tolerance under the conditions we tested. However, (2*R*,6*R*)-HNK demonstrated the potential to augment lower doses of morphine to mediate antinociception. These results are promising and support the further examination of (2*R*,6*R*)-HNK's potential to enhance opioid analgesia.

METHODS

Animals

Male C57BL/6J mice (Jackson Lab; Bar Harbor, ME), age 8-9 weeks, were housed four animals per cage, maintained on a standard 12-hour light/dark cycle (lights on at 06:00), and provided with food and water ad libitum. The animals were randomly assigned to treatment groups. All experiments were carried out in accordance with the National Institutes of Health (NIH) guidelines for the care and use of laboratory animals and with approval from the Uniformed Services University of the Health Sciences Institutional Animal Care and Use Committee.

Drugs

(2*R*,6*R*)-HNK used for these experiments was obtained from the National Center for Advancing Translational Sciences (NCATS; Bethesda, MD), synthesized as previously described (94). Morphine sulfate (Spectrum Chemical MFG Corp, New Brunswick, NJ) was used to induce morphine tolerance and test for morphine sensitivity to mediate antinociception. Physiological saline (0.9% NaCl) was used as the control and

to dilute all medications. All drugs were administered via the intraperitoneal route (alternating sides each injection) at 5 ml/kg volume.

Induction of morphine tolerance

Morphine tolerance was induced by administering morphine twice daily following the morphine regimen outlined below (**Figure 12A**). Morning doses were administered at 7 am. Morning morphine doses started at 10 mg/kg on Day 2 and increased to 20 mg/kg on Day 3. Mice continued to receive the 20 mg/kg dose on days 4, 5, and 7. Evening drug doses were administered between 4-6 pm, starting at 10 mg/kg on Days 1 and 2. The dose increased to 20 mg/kg on Day 3 and continued for Days 4, 5, and 7. Morphine was administered in the mornings of Days 1, 6, and 8 as part of the morphine sensitivity assessments (described below). The morphine naïve groups received saline in place of morphine each morning and night.

Morphine sensitivity by testing antinociception on the hot plate

Animals were tested in the morphine cumulative dosing sensitivity test (MCD) on Days 1, 6, and 8. Morphine sensitivity was measured by recording serial latencies for animals to respond to a noxious thermal stimulus (hot plate set to 50°C) during cumulative morphine dosing. The paradigm used is shown in **Figure 12B**. Increasing doses of morphine (1, 3, 6, 10, and 18 mg/kg) were administered 25 minutes apart. The 18 mg/kg dose was administered only if the previous dose did not produce a maximum effect in the majority of the animals, such as was the case for the Day 1 MCD only. Considering morphine's elimination half-life is approximately 30 minutes in the mouse strain used (37) and the frequency of morphine dosing, a slight cumulative effect was expected. The animal's latency to either jump or lick a hind paw was recorded on the

hotplate 15 minutes before the first morphine dose and 10 minutes following each subsequent dose. The latency for the mouse to either jump or lick a hind paw was recorded for each hot plate exposure. Male C57BL/6J mice were used with N = 10 per group. Four groups of animals were evaluated; two morphine naïve groups (controls for serial IP injections and repeat hot plate testing) receiving either (2*R*,6*R*)-HNK or saline treatment, and two morphine exposed groups receiving either (2*R*,6*R*)-HNK or saline treatment. The morphine naïve groups received saline injections instead of morphine during the MCD. (2*R*,6*R*)-HNK 10 mg/kg treatment (or saline control) began on day 5 (morning) and continued once daily on days 6 and 7.

The results are reported as a percentage of the maximum possible effect (%MPE). A cutoff for hot plate exposure was set to 60 seconds. The animals were immediately removed from the hot plate, and the latency was recorded as 60 seconds if they had not responded to the pain stimulus by that time. %MPE was calculated as: [(current latency – baseline latency) / (60 seconds – baseline latency)] x 100. The baseline latency was defined as the animal's latency to respond during its first exposure to the hot plate on the morning of Day 1 (M1 on Day 1).

Data analysis

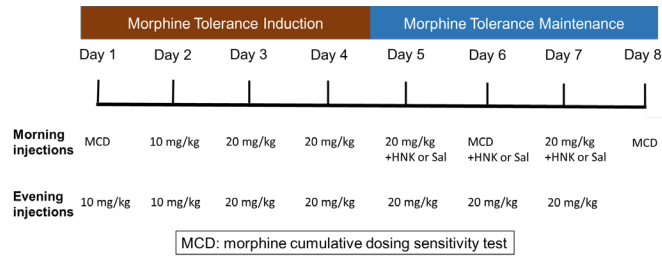
N = 10 per group. No animals were excluded. Error bars represent group means $\pm$ SEM. Differences in groups were analyzed using a two-way ANOVA and Šídák multiple comparisons for all parameters. All statistical analyses were performed using GraphPad Prism 9.0.0 for Windows (GraphPad Software; San Diego, CA, www.graphpad.com). Statistical significance was defined as $P < 0.05$.

RESULTS

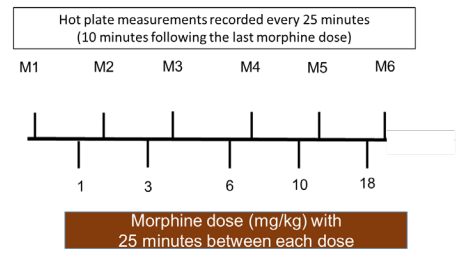
Sensitivity to morphine antinociception was measured on Days 1, 6, and 8 according to the MCD schedule shown in **Figure 12B**. Twice-daily morphine dosing (schedule shown in **Figure 12A**) successfully induced morphine tolerance in the morphine-exposed animals on Day 6 (**Figure 12C**), as evidenced by a rightward shift in the morphine sensitivity curve. Morphine naïve animals received saline injections instead of morphine (both for the MCD and twice-daily injection). In morphine naïve animals, (2*R*,6*R*)-HNK increased %MPE compared to saline control over repeated hot-plate exposure testing (same timing as that for the MCD) after a single treatment measured on Day 6 (treatment effect, $F_{(1, 108)} = 10.94$, $P = 0.0013$) and repeated treatment measured on Day 8 (treatment effect, $F_{(1, 108)} = 15.99$, $P = 0.0001$).

(2*R*,6*R*)-HNK 10 mg/kg did not significantly shift the morphine sensitivity curve to the left compared to saline-treated animals on Day 6 (**Figure 12D**). Once-daily repeated (2*R*,6*R*)-HNK treatment also failed to shift the morphine sensitivity curve to the left on Day 8. On the other hand, (2*R*,6*R*)-HNK treatment nearly achieved treatment effect significance on Day 6 (two-way ANOVA, $F_{(1, 108)} = 3.358$, $P = 0.0696$), and repeat (2*R*,6*R*)-HNK treatment did produce a treatment effect (two-way ANOVA, $F_{(1, 108)} = 9.541$, $P = 0.0026$) on Day 8 when compared to saline-treated morphine-exposed animals. On day 8, the lowest effective dose (producing a 25 % increase in antinociception) in (2*R*,6*R*)-HNK treated morphine exposed animals was 6 mg/kg. This dose was ineffective in saline-treated morphine-exposed animals.

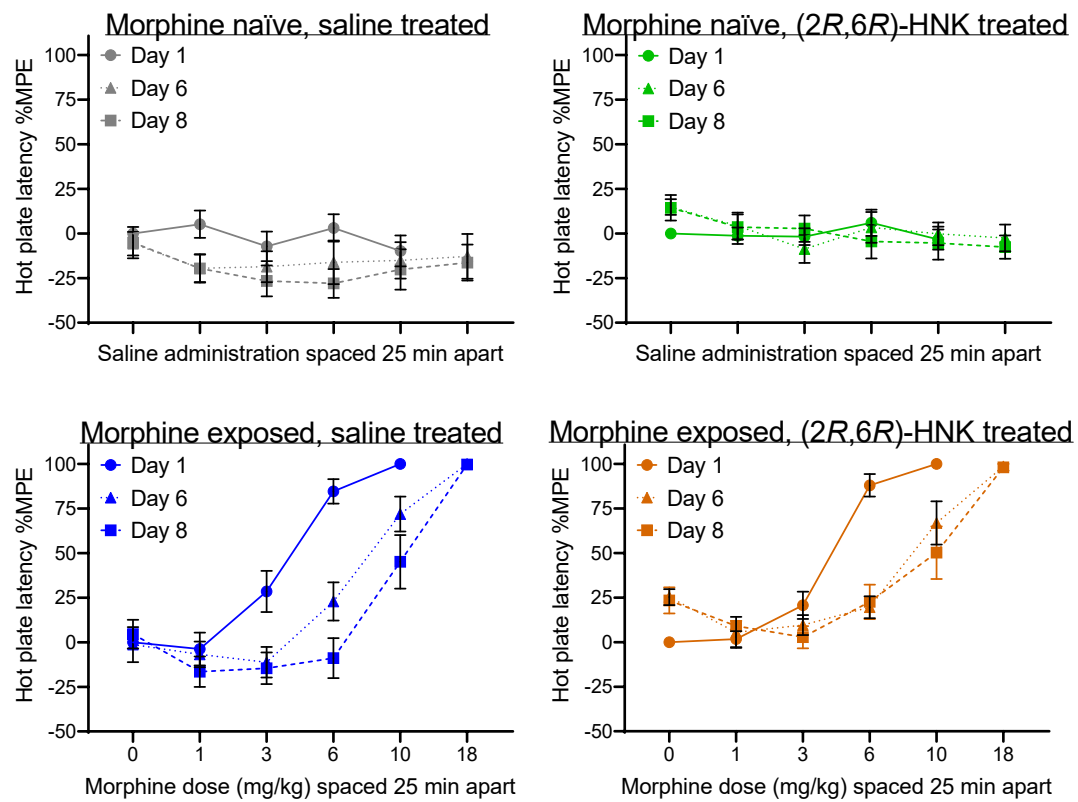
A



B



C



D

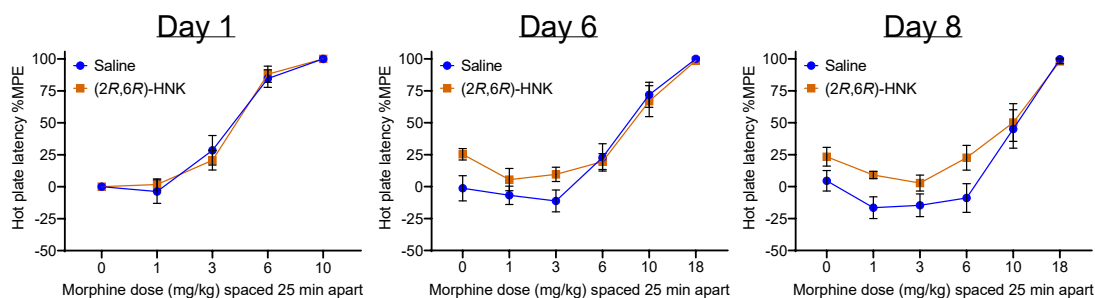


Figure 12. The capability for (2R,6R)-HNK to reverse morphine tolerance was tested in male mice and compared with saline-treated morphine-tolerant controls and morphine naïve animals. Morphine tolerance was induced using a twice-daily dosing schedule over five days shown in **panel A**. Treatment (either saline control or (2R,6R)-HNK) began on the morning of Day 5. The latency for the animals to respond on the hot plate was measured following the morphine cumulative dosing sensitivity test (MCD) dosing and measurement schedule shown in **panel B**. Morphine naïve animals received saline injections instead of morphine doses for each morphine sensitivity test and saline injections instead of morphine each morning and evening. **Panel C:** Morphine naïve animals (top left & right) demonstrated a significant (2R,6R)-HNK treatment effect that was not negatively impacted by repeat injections and hot plate exposure. Regardless of treatment in the morphine exposed groups (bottom left & right), the morphine sensitivity curve shifted to the right on Day 6, demonstrating morphine tolerance. **Panel D:** (2R,6R)-HNK 10 mg/kg did not reverse the rightward shift in the morphine sensitivity curve induced by morphine tolerance on Day 6 or after repeat treatment on Day 8. Repeated (2R,6R)-HNK 10 mg/kg treatment did augment opioid antinociception on Day 8 at the lower morphine doses. Vertical bars represent the group means $\pm$ SEM. N = 10 for all groups.

DISCUSSION

This study is the first to evaluate the potential for (2R,6R)-HNK on its own to reverse opioid tolerance or enhance opioid antinociception. Morphine sensitivity was assessed by testing serial hot plate responses while administering cumulative morphine doses (MCD outlined in **Figure 12B**). Morphine tolerance was successfully induced by administering morphine twice daily for four days (**Figure 12A**). (2R,6R)-HNK failed to

reverse morphine tolerance under the tested conditions. However, (2*R*,6*R*)-HNK did demonstrate the potential to enhance morphine antinociception (**Figure 12D**).

According to the outlined schedule, repeated morphine injection (IP) decreased morphine sensitivity on Day 6, producing a rightward shift in the morphine sensitivity curve (**Figure 12C**, bottom left & right). Morphine tolerance shown here aligns with previous reports (14; 63; 123) despite differences in methods used to induce it. (2*R*,6*R*)-HNK, compared to saline treatment, did not reverse the rightward shift of the curve after one (Day 6) or repeated treatments (Day 8), signifying a lack of ability to attenuate morphine tolerance under the conditions tested.

In contrast, one dose of (2*R*,6*R*)-HNK nearly produced a treatment effect on Day 6 compared to saline-treated animals. The treatment effect emerged on Day 8. It can be identified by the upward shift in the morphine sensitivity curve for (2*R*,6*R*)-HNK treated animals at the lower morphine doses (1, 3, & 6 mg/kg). Thus, (2*R*,6*R*)-HNK demonstrated promise for enhancing low-dose morphine antinociception. Repeated administration may be vital for producing this type of (2*R*,6*R*)-HNK interaction with opioid medication.

A previous report had tested the racemic mixture of (2*R*,6*R*;2*S*,6*S*)-HNK that showed the combination of the two did not attenuate morphine tolerance or enhance morphine antinociception (79). However, researchers in this study may have used suboptimal parameters to answer the question. Available data for (2*R*,6*R*)-HNK's pain behavioral effects demonstrate variation in the onset of its activity. While some (2*R*,6*R*)-HNK's effects are rapid (within 1 hour), others have a delayed onset. The onset for (2*R*,6*R*)-HNK to reverse allodynia associated with incisional pain was greater than 4

hours (66), and data from the Lucki lab has shown (2*R*,6*R*)-HNK needs greater than 10 hours to mediated antinociception in healthy mice. Considering the available data, Lilius et al. may have tested for (2*R*,6*R*)-HNK's ability to enhance morphine antinociception at time points too early to see an effect (79). Indeed, the effects reported in our study occurred 24 hours following (2*R*,6*R*)-HNK administration.

Likewise, Lilius et al. failed to examine the two ketamine metabolites they tested separately (79). Testing the metabolites together may have confounded their results, considering the differences in behavioral effects between the two metabolites (142; 145). Additionally, their study was underpowered, using a sample size of only 6-7. Ideally, researchers would use a larger sample size, test (2*R*,6*R*)-HNK separately, and measure for effect 24 hours after treatment to investigate potential (2*R*,6*R*)-HNK and morphine interaction effects.

Augmentation of opioid analgesia is one of ketamine's, the parent compound for (2*R*,6*R*)-HNK, previously reported therapeutic effects (14; 79; 122). Evidence is available that demonstrates that other NMDA receptor (NMDAR) antagonists have similar opioid enhancement and tolerance reversal capabilities when compared to ketamine [for review, see (129)]. These data have led researchers to conclude that NMDAR antagonism is the mechanism for these ketamine effects. Considering how (2*R*,6*R*)-HNK does not have a significant affinity to bind to or antagonize NMDAR, this would signify that (2*R*,6*R*)-HNK is unlikely to share these ketamine therapeutic attributes. However, ketamine interacts with many receptors, and the specific mechanisms for many of its therapeutic effects remain unclear. (2*R*,6*R*)-HNK's lack of

NMDAR antagonism should not exclude it from being examined for potential opioid therapy interaction.

The results of our study highlight the potential for (2*R*,6*R*)-HNK to enhance morphine antinociception at lower morphine doses. While it may have failed to reverse morphine tolerance under the conditions used in this study, (2*R*,6*R*)-HNK should be further tested to determine if it can produce other opioid therapy interactions that could improve the safety profile of opioid pain therapy. One such interaction to examine would be to assess (2*R*,6*R*)-HNK's ability to suppress the induction of opioid tolerance by administering it concomitantly with opioids at the beginning of treatment. Overall, these data identify the need for a more comprehensive evaluation of (2*R*,6*R*)-HNK interaction with opioids to identify the potential to generally enhance patient safety when treating pain conditions with opioid medications.

Considering the results of this study and its already identified analgesia-like effects, (2*R*,6*R*)-HNK could be indicated for use in place of opioids in some cases because its duration of action (>24 hours) is far greater than a typical opioid medication. Additionally, (2*R*,6*R*)-HNK could be used concomitantly with opioid treatment to achieve higher analgesic potential without requiring patients to take higher doses of opioids. Thus, (2*R*,6*R*)-HNK could improve the safety profile of opioid medications when still required while helping to reduce the need to consider opioid medications at all for types of pain (2*R*,6*R*)-HNK may help to manage alone.

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