TriService Nursing Research Program Final Report Cover Page

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Signatures

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Table of Contents

Abstract

Purpose:
Nearly 2/3rd of U.S. veterans report experiencing pain in the past three months. Over 9% claim their pain to be severe, resulting in significant pain-related limitations to work capability and social life. There is a considerable need to identify novel pain therapeutics to address this pain problem and increase operational readiness. (2*R*,6*R*)-hydroxynorketamine (HNK) is a ketamine metabolite that has been shown to share some of ketamine's therapeutic potential for depression while lacking its adverse side effects. Since ketamine has demonstrated utility in treating pain without the respiratory depression effects caused by opioid medications, it was natural to question whether (2*R*,6*R*)-HNK also produced analgesic effects.

Methods: (2*R*,6*R*)-HNK treatment was tested in different preclinical pain models using C57BL/J6 mice. Experiments characterizing the time course, comparison between sexes, and mechanism of action were conducted under the selected models and compared with reference analgesic drugs. The potential for (2*R*,6*R*)-HNK to reverse opioid tolerance and augment opioid analgesia was also investigated.

Findings: (2*R*,6*R*)-HNK produced antinociception in healthy mice and reversed hypersensitivity in animals with induced pain conditions. The antinociceptive effects of (2*R*,6*R*)-HNK was dependent on AMPA receptor activity and was independent of opioid receptors. (2*R*,6*R*)-HNK increased opioid responses in a model of opioid tolerance, potentially enhancing opioid-based analgesia during a critical treatment period. These results provide evidence that encourages future support of clinical research examining (2*R*,6*R*)-HNK as a novel analgesic and its AMPA receptor-dependent mechanism of analgesia.

Implications for Military Nursing:
As integral members of the health care team, military nurses share responsibility for addressing the critical need to treat pain within our veteran population. If made clinically available, (2*R*,6*R*)-HNK has the potential to make an immediate impact on the military nurse's capability to treat pain effectively and safely for past and present service members and their dependents.

Keywords: analgesia; (2*R*,6*R*)-hydroxynorketamine; ketamine metabolite; novel pain therapeutic; non-opioid pain therapeutic

TSNRP Research Priorities

Primary Priority (Select ONE)

Force Health Protection:	☒ Fit and ready force ☐ Deploy with and care for the warrior ☐ Care for all entrusted to our care
Nursing Competencies and Practice:	☐ Patient outcomes ☐ Quality and safety ☐ Translate research into practice/evidence-based practice ☐ Clinical excellence ☐ Knowledge management ☐ Education and training
Leadership, Ethics, and Mentoring:	☐ Health policy ☐ Recruitment and retention ☐ Preparing tomorrow's leaders ☐ Care of the caregiver
Other:	☐

Secondary Priority, if any (select as many as needed)

Force Health Protection:	☐ Fit and ready force ☐ Deploy with and care for the warrior ☒ Care for all entrusted to our care
Nursing Competencies and Practice:	☐ Patient outcomes ☐ Quality and safety ☐ Translate research into practice/evidence-based practice ☐ Clinical excellence ☐ Knowledge management ☐ Education and training
Leadership, Ethics, and Mentoring:	☐ Health policy ☐ Recruitment and retention ☐ Preparing tomorrow's leaders ☐ Care of the caregiver
Other:	☐

Project Results

This research investigated the pain reduction effects of the ketamine metabolite (2*R*,6*R*)-hydroxynorketamine (HNK). The hypothesis behind the study was that (2*R*,6*R*)-HNK provides pain reduction on its own, enhances the pain reduction effects of opioids, and reduces opioid tolerance. The following specific aims were utilized to guide the investigation of this hypothesis.

Specific Aim #1: To determine the pain reduction effects of (2*R*,6*R*)-HNK in healthy mice and mice with induced neuropathic and inflammatory pain.

Specific Aim #2: To examine the interaction between (2*R*,6*R*)-HNK and opioid-induced pain reduction in mice.

Specific Aim #3: To investigate the mechanism of action for (2*R*,6*R*)-HNK mediated pain reduction.

This study utilized C57BL/6J mice (Jackson Laboratory; Bar Harbor, ME), age 8-15 weeks, to investigate the research questions. All experiments were approved through the Uniformed Services University of the Health Sciences Institutional Animal Care and Use Committee and carried out following the National Institutes of Health (NIH) guidelines for the care and use of laboratory animals.

Specific Aim #1: To determine the pain reduction effects of (2*R*,6*R*)-HNK in healthy mice and mice with induced neuropathic and inflammatory pain.

The focus of this specific aim was to characterize (2*R*,6*R*)-HNK mediated pain reduction-like effects using the mouse models described below. This study used healthy animals to examine basic antinociception to a thermal stimulus, λ-carrageenan (CARR) hind paw injection to model localized inflammatory pain, and sciatic nerve injury (SNI) to model neuropathic pain.

Healthy animals were used to evaluate basic pain reduction properties because they had no pathophysiological condition that could alter normal pain signal processing and modulation. The healthy animals were placed on the hot plate, and the latency for them to respond to the hot plate stimulus was measured.

To test for alterations in sensitivity related to spontaneous pain conditions (inflammatory and neuropathic), the animals underwent von Frey mechanosensitivity testing using the classic up-down method (Chaplan et al., 1994; Dixon, 1980). To model inflammatory pain, the animal's hind paw was injected subcutaneously with CARR, a seaweed derivative that induces a localized inflammatory response (Adamson et al., 2010; Shepherd et al., 2018; Smeester et al., 2017). CARR (2.5%, 20 µl) injection produced redness and swelling of the paw within 1 hour, and these animals exhibited a dramatic increase in mechanical sensitivity within 2 hours of injection.

To model neuropathic pain, an SNI procedure was performed where the peroneal and tibial branches of the sciatic nerve were transected, leaving the sural nerve intact (Bourquin et al., 2006; Decosterd & Woolf, 2000; Shields et al., 2003). The surgery was performed under isoflurane anesthesia, and the animals were allowed ten days to recover from surgery before any further experimentation was performed. In the experiment evaluating (2*R*,6*R*)-HNK's capability to reverse SNI-induced mechanical hypersensitivity, gabapentin, a calcium channel blocker used in humans and animals to treat neuropathy type pain, was used as a reference control to allow for comparison of the magnitude of effect.

(2*R*,6*R*)-HNK treatment increased latency to respond to hot thermal pain stimulus in healthy male and female mice and demonstrated a U-shaped dose response for this behavioral effect

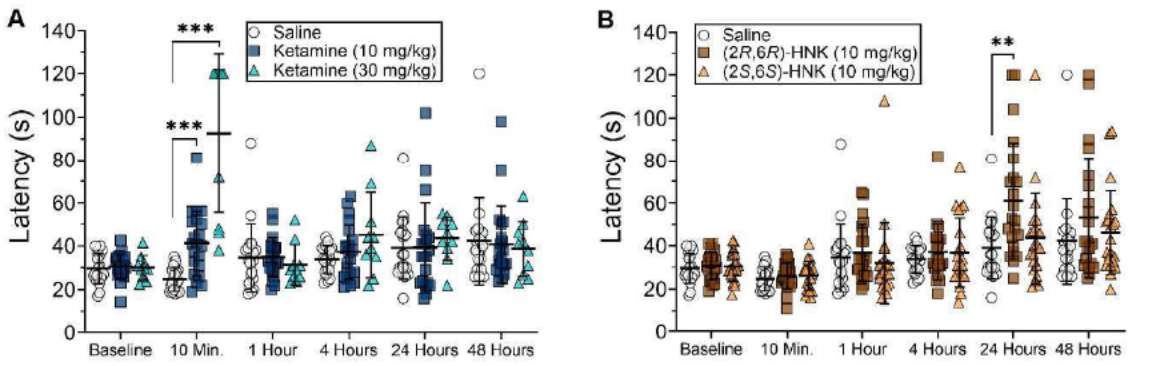

Figure 1: (2*R*,6*R*)-HNK increased latency to respond in a delayed yet persistent fashion (**B**), while ketamine produced a rapid and short-lived effect only (**A**). (2*S*,6*S*)-HNK did not produce antinociception at any time point (**B**). The error bars represent group means ± S.D. Comparisons are 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. (Yost et al., 2022)

The initial experiment compared (2*R*,6*R*)-HNK treatment in healthy male mice with its stereoisomer (2*S*,6*S*)-HNK and its parent compound, ketamine. (2*S*,6*S*)-HNK has also received interest in recent research for its potential role in some of ketamine's therapeutic effects (Yokoyama et al., 2020). The five treatment groups for this experiment were run together, and the data are shown in two separate graphs (**Figure 1**). Analysis of the data revealed a time x treatment interaction ($F_{(20, 450)} = 10.60$, $P < 0.0001$). As expected, animals treated with ketamine responded very rapidly and in a dose-dependent fashion. Within ten minutes of injection, ketamine-treated mice exhibited increased time to respond to the pain stimulus. However, the ketamine effect was short-lived. Within one hour of treatment, the animals' latency to respond to the stimulus returned to baseline levels. Conversely, (2*R*,6*R*)-HNK failed to produce a rapid treatment effect but did increase the animals' latency to respond 24 hours following treatment. (2*S*,6*S*)-HNK treatment did not significantly elevate the animals' latency to respond at any time measured.

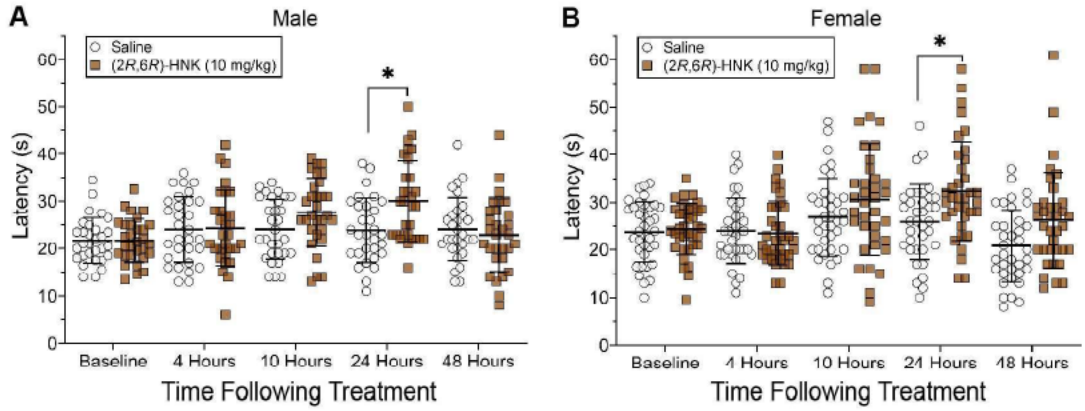

Figure 2: The time course for male (**A**) and female (**B**) mice latency to respond to hot plate stimulus following (2*R*,6*R*)-HNK 10 mg/kg treatment. Comparisons are made between the treatment and saline groups at the same time point: * $P < 0.05$. N = 30 for males and N = 36 for females. The error bars represent group means ± S.D. For the dose-response figures, the mean of the saline group is represented by the dashed line while the ± S.D. are represented by dotted lines. (Yost et al., 2022)

To further characterize the effect in healthy animals, an additional experiment examined (2*R*,6*R*)-HNK treatment in male and female mice with altered measurement time points. The subsequent experiment (**Figure 2**) confirmed the delayed antinociception effect (at 24 hours) in male animals ($F_{(4, 232)} = 4.28$, $P = 0.0023$) and revealed a similar effect in healthy female animals ($F_{(4, 280)} = 3.884$, $P = 0.0044$). Although the difference between the treated and saline control groups at 10 hours did not quite achieve statistical significance, the data here support an estimated onset for (2*R*,6*R*)-HNK antinociception in healthy animals to be approximately 10-12 hours. A three-way ANOVA analyzing the data together revealed a sex effect ($F_{(1, 62)} = 5.216$, $P = 0.0258$). However, further analysis revealed no significant sex differences at any time point.

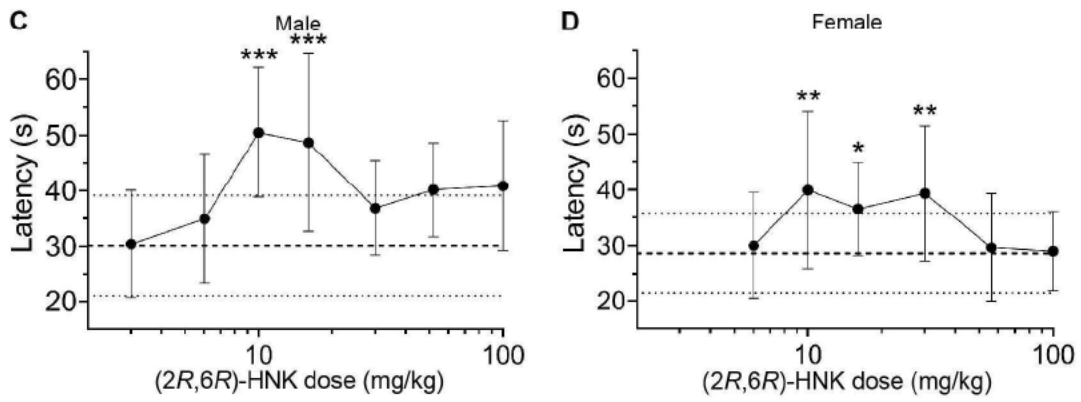

Figure 3: The dose response for (2*R*,6*R*)-HNK to mediate an increase in latency to respond to hot plate stimulus in male (**C**) and female (**D**) mice using a quarter log dosing scale and measured 24 hours following treatment. Asterisks indicate significant differences compared to the saline control. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. N = 10-12 for males, and N = 17 for females. The error bars represent group means ± S.D. For the dose-response figures, the mean of the saline group is represented by the dashed line while the ± S.D. are represented by dotted lines. (Yost et al., 2022)

The dose response relationship was determined for this behavioral antinociception effect occurring 24 hours following treatment using quarter log dosing. The results (**Figure 3**) revealed an inverted U-shaped dose-response curve for both males ($F_{(7, 76)} = 4.986$, P = 0.0001) and females ($F_{(6, 110)} = 4.971$, P = 0.0002). Specifically, males exhibited increased latencies to 10 and 18 mg/kg doses. Females displayed increases in latency at doses of 10, 18, and 32 mg/kg.

(2*R*,6*R*)-HNK reverses hypersensitivity associated with inflammatory pain with a more rapid onset of action

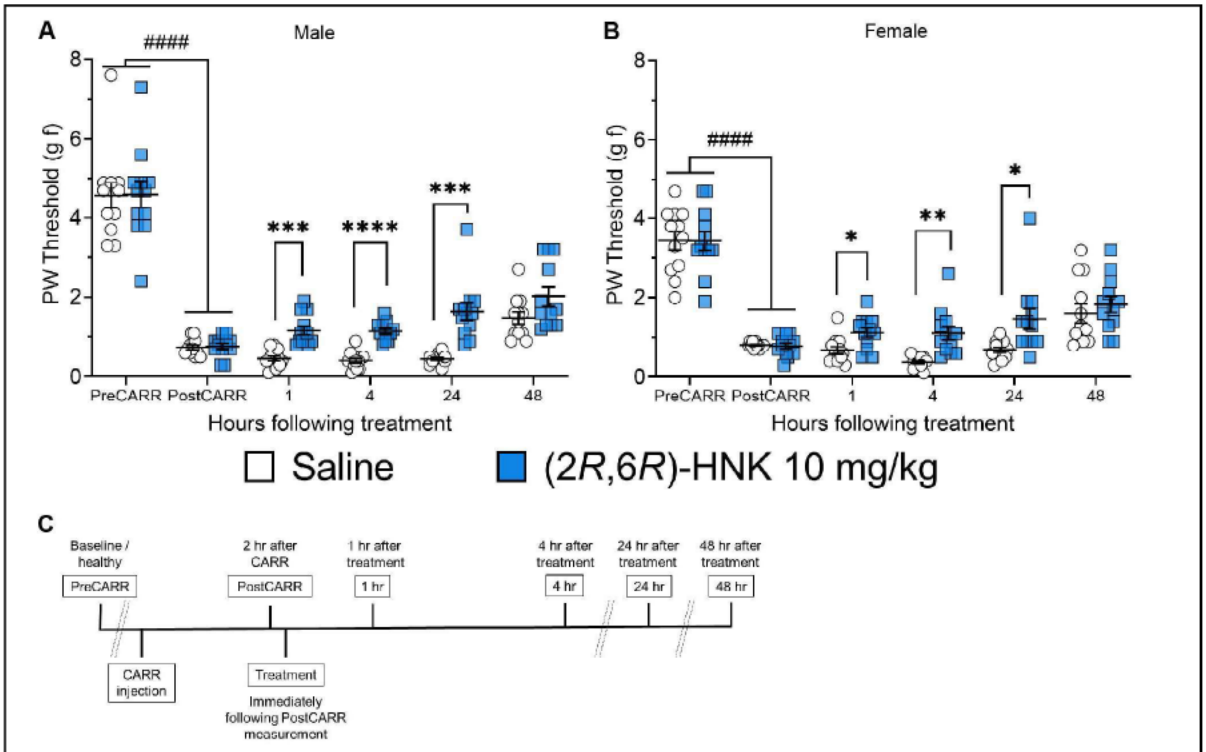

Figure 4: **Panel C** outlines the timeline used for this experiment. The inflamed animals were treated with (2*R*,6*R*)-HNK or saline control immediately following the PostCARR measurement (~ 2.3 hours following CARR injection). **Panel A & C**: CARR hind paw injection produced robust mechanical hypersensitivity within two hours of injection for male and female mice (#### P < 0.0001, Mann-Whitney test of combined saline & (2*R*,6*R*)-HNK groups before and after CARR injection, N = 24). Treatment with (2*R*,6*R*)-HNK reversed mechanical hypersensitivity associated with the inflammatory condition in male (**A**) and female (**B**) mice compared to saline control within one hour of treatment and persisted for greater than 24 hours following a single administration. The data shown are group means ± SEM. * P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001, N = 12 for all groups.

CARR hind paw injection induced mechanical hypersensitivity within 2 hours of CARR injection, an effect that lasted for more than 48 hours. In this model of localized inflammatory pain, (2*R*,6*R*)-HNK reversed the resultant mechanical allodynia within 1 hour of treatment, and the effect endured for greater than 24 hours following a single injection. (2*R*,6*R*)-HNK mediated reversed inflammation-induced hypersensitivity similarly in male and female animals (**Figure 4**). A dose-response experiment in this model revealed a

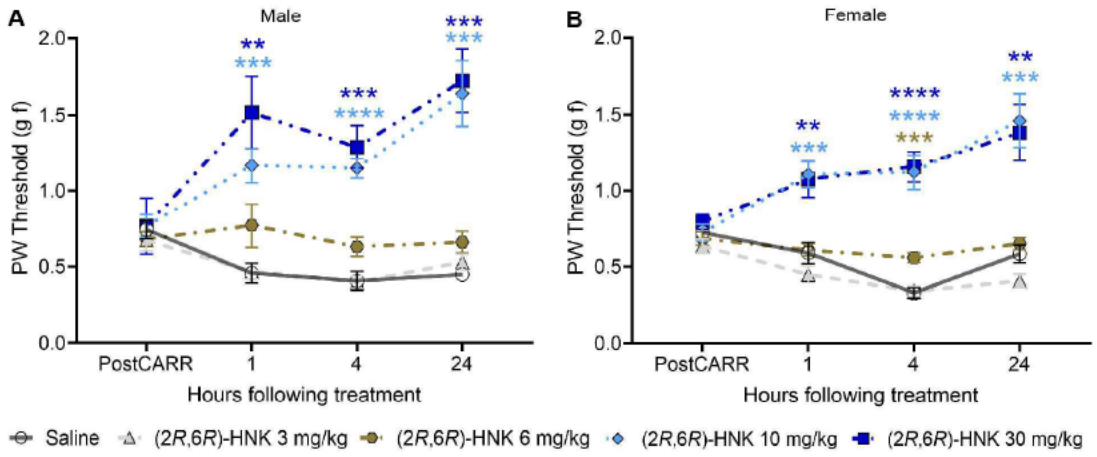

Figure 5: The line graphs represent the mechanosensitivity dose-response over time for (2*R*,6*R*)-HNK treatment in a localized inflammatory pain condition using a modified quarter log dosing scale. (2*R*,6*R*)-HNK similarly reversed mechanical hypersensitivity for male (A) and female (B) mice at doses of 10 and 30 mg/kg within one hour and with a duration greater than 24 hours. The error bars represent group means ± 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 male groups, and $N = 12$-18 for female groups.

minimum effective treatment dose of 10 mg/kg for (2*R*,6*R*)-HNK. The 30 mg/kg dose produced a similar treatment effect in both sexes (**Figure 5**).

(2*R*,6*R*)-HNK reverses hypersensitivity associated with neuropathic pain in male mice with a similar acute magnitude of effect but with a longer duration of action than gabapentin

Previously, (2*R*,6*R*)-HNK had been shown to reverse hypersensitivity associated with SNI-induced neuropathic pain in female mice only at a dose of 10 mg/kg (Kroin et al., 2019). The results in **Figure 6** confirmed and expanded upon what was previously known by showing that (2*R*,6*R*)-HNK produced a similar effect in male animals at doses of 10 and 30 mg/kg. Additionally, this experiment compared (2*R*,6*R*)-HNK to gabapentin, a putative treatment for neuropathic pain in humans and animals. Analysis of the results revealed a time x treatment interaction ($F_{(12,129)} = 6.211$, $P < 0.0001$). Acutely, (2*R*,6*R*)-HNK treatment produced a similar reversal of hypersensitivity compared to gabapentin. However, gabapentin treatment had an effect duration of less than 24 hours even after three once-daily treatments. On the other hand, the duration of (2*R*,6*R*)-HNK treatment exceeded 24 hours following a single treatment with no apparent tachyphylaxis following the third once-daily dose.

To summarize the results of this specific aim, (2*R*,6*R*)-HNK produces antinociception in healthy mice in a delayed yet persistent manner (> 24 hours) and reverses hypersensitivity associated with inflammatory and neuropathic pain in a more rapid yet similarly persistent manner. The data available in this study greatly expand upon the previously known characteristics of (2*R*,6*R*)-HNK pain reduction. It is now known that (2*R*,6*R*)-HNK mediates antinociception in healthy animals. Identifying a treatment effect that persists 24 hours after administration is very significant because the effect greatly outlasts the physical presence of the drug in circulation (half-life of (2*R*,6*R*)-HNK in mice is 30 minutes to 1 hour).

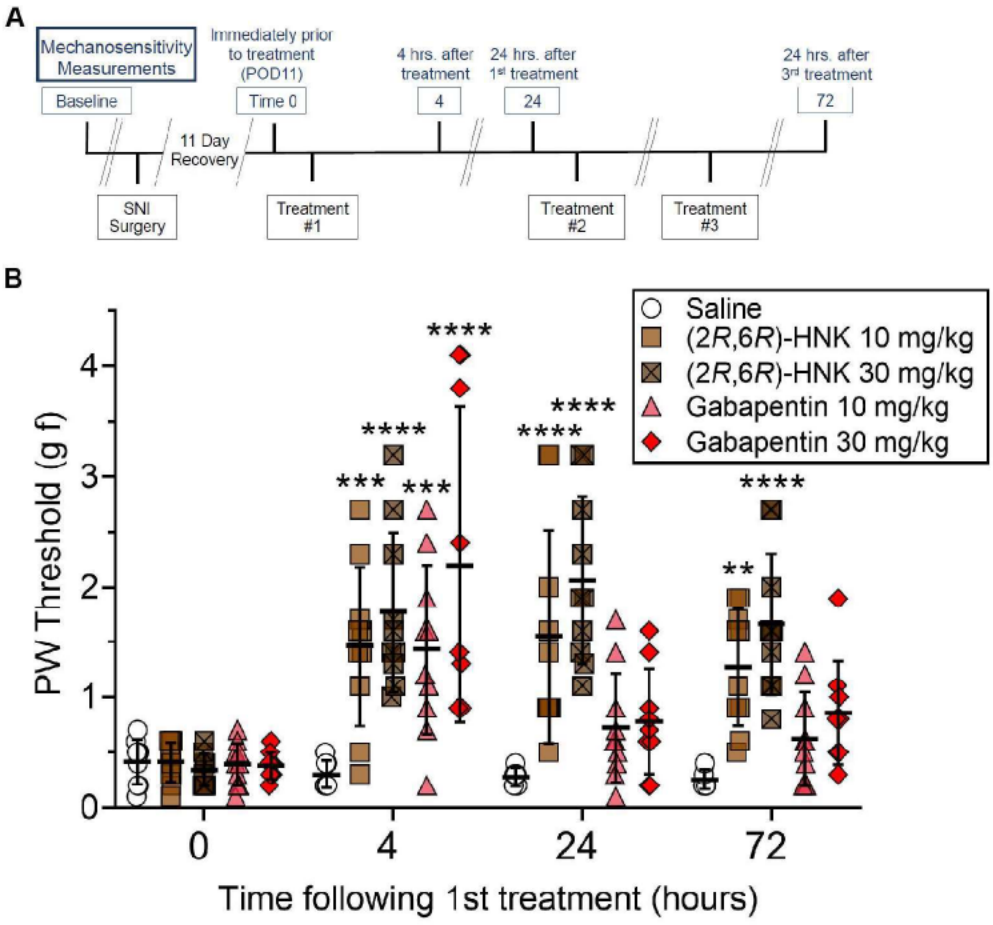

Figure 6: The SNI condition produced mechanical allodynia 11 days after surgery (Time 0, decrease from PW Threshold of 4.3 ± 1.10 g f before surgery). **Panel A**: The experimental paradigm comprised once-daily treatment for three days and mechanosensitivity testing at 4, 24, and 72 hours following the first treatment dose. **Panel B**: (2*R*,6*R*)-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 ± 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). (Yost et al., 2022)

Additionally, this data provides an important timeline for investigating potential (2*R*,6*R*)-HNK mechanisms.

(2*R*,6*R*)-HNK also reverses hypersensitivity in animals with inflammatory pain and male animals with neuropathic pain more rapidly (< 1 hour) yet equally persistent duration (> 24 hours). These results highlight significant differences in treatment effect between healthy animals and animals with induced spontaneous pain conditions that will also assist with investigating (2*R*,6*R*)-HNK mechanisms of action. Additionally, these data add two dose-response relationships that may help with dosing parameters when considering future clinical studies. Moreover, it is now known that (2*R*,6*R*)-HNK can reverse neuropathic pain-induced hypersensitivity with a similar magnitude of effect to gabapentin, a treatment used for human

and animal conditions of neuropathic pain. These data provide considerable supporting evidence to drive future clinical studies into the pain reduction effects of (2*R*,6*R*)-HNK in humans.

Specific Aim #2: To examine the interaction between (2*R*,6*R*)-HNK and opioid-induced pain reduction in mice.

One of the problems with opioid analgesia is that tolerance develops rapidly with repeated administration. To investigate the interaction between (2*R*,6*R*)-HNK and opioid analgesia, this experiment induced opioid tolerance by administering morphine (IP) twice-daily for 7 days at the doses shown in the experimental paradigm outlined in **Figure 7A**. Changes to morphine sensitivity were assessed by measuring the animal's latency to respond to the hot plate after receiving cumulative morphine dosing (MCD) according to the schedule outlined in **Figure 7B**. Treatment with either (2*R*,6*R*)-HNK or saline began at the morning injection on Day 5 and continued once daily for three days. Morphine tolerance was confirmed on Days 6 & 8 (**Figure 7C**) and can be identified by the rightward shift of the morphine dose-response curve on Days 6 & 8 relative to Day 1. Morphine naïve animals received saline injections instead of morphine for each MCD sensitivity test and the twice-daily injections. 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, $F_{(1, 108)} = 15.99$, $P = 0.0001$). These data further confirm the antinociception effect provided by (2*R*,6*R*)-HNK treatment. %MPE was defined as: [(current latency – baseline latency) / (60 seconds – baseline latency)] x 100.

Treatment with (2*R*,6*R*)-HNK did not attenuate preexisting opioid tolerance. Treatment with 10 mg/kg (2*R*,6*R*)-HNK did not significantly reverse the rightward shift in the morphine dose-response curve compared to saline-treated animals on Day 6 (**Figure 7D**). 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 was still effective at producing analgesia in opioid-tolerant animals. (2*R*,6*R*)-HNK treatment nearly achieved treatment effect significance on Day 6 ($F_{(1, 108)} = 3.358$, $P = 0.0696$), and repeat (2*R*,6*R*)-HNK treatment was significant ($F_{(1, 108)} = 9.541$, $P = 0.0026$) on Day 8 when compared to saline-treated morphine-exposed animals. On day 8, the lowest effective morphine dose (producing a 25% increase in antinociception) in (2*R*,6*R*)-HNK treated morphine exposed animals was 6 mg/kg. In contrast, this dose was ineffective in the non-treated morphine-exposed animals. These data demonstrate a significant augmentation effect for (2*R*,6*R*)-HNK treatment when used in conjunction with lower doses of morphine in opioid-tolerant animals.

In animals, ketamine has demonstrated the potential to inhibit the development of opioid tolerance (Trujillo & Akil, 1994), attenuate preexisting opioid tolerance (Kissin et al., 2000; Lilius et al., 2018; Shimoyama et al., 1996), and augment opioid analgesia (Campos et al., 2006; Lilius et al., 2018; Shikanai et al., 2014). Thus, it was only natural to question whether its metabolite (2*R*,6*R*)-HNK could produce similar effects. While the results of this study did not support the hypothesis that (2*R*,6*R*)-HNK is capable of reversing morphine tolerance, the identified morphine augmentation effect is significant. These results support the theory that

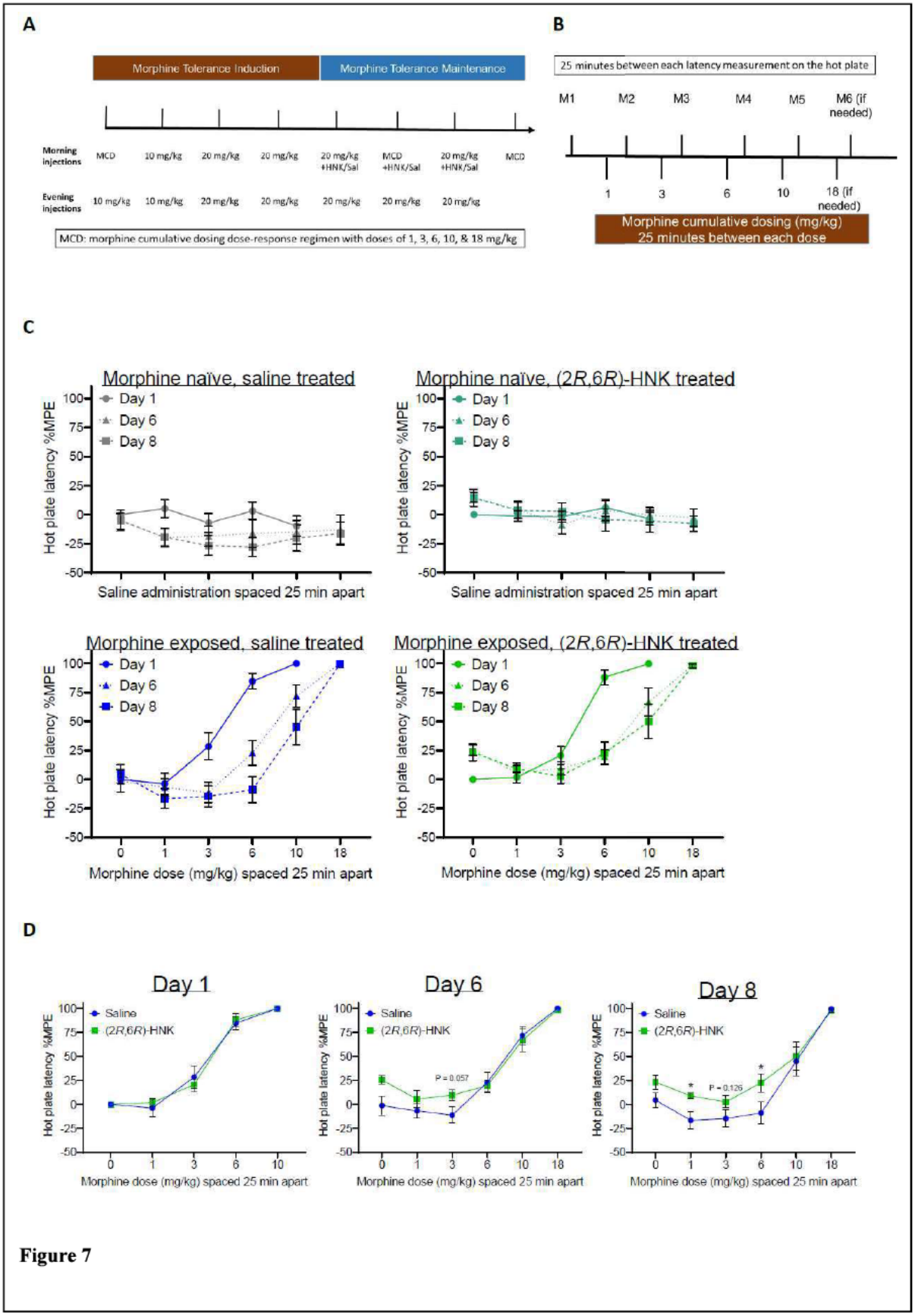
A
Morphine Tolerance Induction
Morphine Tolerance Maintenance
Morning injections
MCD
10 mg/kg
20 mg/kg
20 mg/kg
20 mg/kg +HNK/Sal
MCD +HNK/Sal
20 mg/kg +HNK/Sal
MCD
Evening injections
10 mg/kg
10 mg/kg
20 mg/kg
20 mg/kg
20 mg/kg
20 mg/kg
20 mg/kg
MCD: morphine cumulative dosing dose-response regimen with doses of 1, 3, 6, 10, & 18 mg/kg
B
25 minutes between each latency measurement on the hot plate
M1
M2
M3
M4
M5
M6 (if needed)
1
3
6
10
18 (if needed)
Morphine cumulative dosing (mg/kg) 25 minutes between each dose
C
Morphine naïve, saline treated
Morphine naïve, (2R,6R)-HNK treated
Morphine exposed, saline treated
Morphine exposed, (2R,6R)-HNK treated
Day 1
Day 6
Day 8
Hot plate latency %MPE
Saline administration spaced 25 min apart
Morphine dose (mg/kg) spaced 25 min apart
D
Day 1
Day 6
Day 8
Saline
(2R,6R)-HNK
P = 0.057
P = 0.126
Hot plate latency %MPE
Morphine dose (mg/kg) spaced 25 min apart

Figure 7

Figure 7: (2*R*,6*R*)-HNK did not reverse morphine tolerance but did augment morphine antinociception at low doses. Following the twice-daily morphine dosing (IP) schedule shown in **panel A** and morphine sensitivity testing on the hot plate shown in panel B (hot plate measurements labeled as M1 through M6), animals receiving morphine demonstrated a significant rightward shift demonstrating tolerance (**panel C**). While (2*R*,6*R*)-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 (**panel C**), repeated (2*R*,6*R*)-HNK 10 mg/kg treatment did augment opioid antinociception on Day 8 at the lower morphine doses (**panel D**). Vertical bars represent the group means ± SEM. N = 10 for all groups.

(2*R*,6*R*)-HNK could be used in conjunction with opioid medications to reduce the amount of opioid required to control pain, thereby improving the safety profile of opioid pain treatment when (2*R*,6*R*)-HNK is used as an adjunct.

Specific Aim #3: To investigate the mechanism of action for (2*R*,6*R*)-HNK mediated pain reduction.

Two protein receptor classes were selected for interrogation to examine the broad mechanism of action for (2*R*,6*R*)-HNK mediated pain reduction. The α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid receptor (AMPAR) was selected based on previous reports demonstrating (2*R*,6*R*)-HNK altered AMPAR receptor subunit composition and concentration and (2*R*,6*R*)-HNK's effects may have been dependent on AMPAR glutamatergic signaling (Shaffer et al., 2019; Zanos et al., 2016). To examine whether AMPARs played a role in (2*R*,6*R*)-HNK mediated antinociception in healthy animals and mechanical hypersensitivity in animals with inflammatory pain, the AMPAR antagonist NBQX 10 mg/kg was administered 30 minutes before (2*R*,6*R*)-HNK treatment (Dalgaard et al., 1994; Karasawa et al., 2005; Zanos et al., 2016) and behavioral effects of the therapy were subsequently measured.

Opioid receptors have been implicated in ketamine's therapeutic effects (Williams et al., 2018), opening the door for a possible opioid mechanism for any therapeutic effect caused by a ketamine metabolite. To draw a direct comparison between ketamine and (2*R*,6*R*)-HNK induced antinociception and to determine if (2*R*,6*R*)-HNK is dependent on opioid receptors for its pain reduction effects, naltrexone (1 mg/kg), a non-selective opioid receptor antagonist, was selected to interrogate opioid receptor involvement.

Administration of NBQX before treatment blocked (2*R*,6*R*)-HNK antinociception 24 hours after treatment (**Figure 8A**; $F_{(1, 58)} = 4.592$, $P = 0.0363$). Similarly, NBQX given 24 hours following (2*R*,6*R*)-HNK treatment also blocked (2*R*,6*R*)-HNK mediated antinociception (**Figure 8C**; $F_{(1, 43)} = 4.972$, $P = 0.0310$). These two experiments demonstrated that the (2*R*,6*R*)-HNK mediated effect in healthy animals was dependent on AMPAR activity both at initiation and expression of the delayed effect. On the other hand, naltrexone administration before (**Figure 8B**) and 24 hours following (**Figure 8D**) treatment had no impact on (2*R*,6*R*)-HNK mediated antinociception. These results signify that (2*R*,6*R*)-HNK antinociception in healthy animals was not dependent on opioid receptor activity at either the initiation or expression of the therapeutic effect.

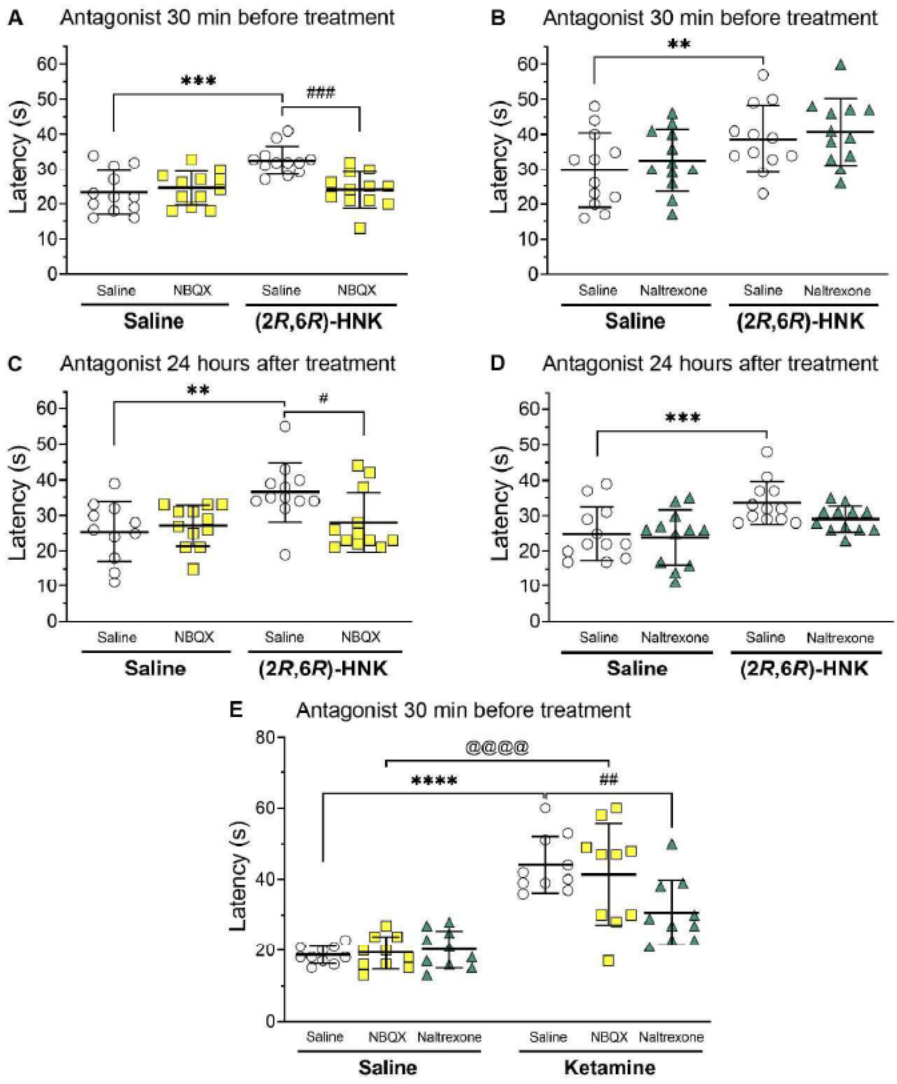

Figure 8: The effects of AMPA receptor antagonism with NBQX (10 mg/kg) or opioid receptor antagonism with naltrexone (1 mg/kg) on the initiation and expression of antinociception by (2*R*,6*R*)-HNK in healthy animals on the hot plate compared to ketamine antinociception. The effects of (2*R*,6*R*)-HNK (10 mg/kg) were measured 24 hours after treatment. **Panel A**: The NBQX given 30 minutes before (2*R*,6*R*)-HNK treatment blocked the initiation of (2*R*,6*R*)-HNK antinociception at 24 hours. **Panel B**: Naltrexone given 30 minutes before (2*R*,6*R*)-HNK treatment did not block the initiation of (2*R*,6*R*)-HNK antinociception at 24 hours. **Panel C**: NBQX given 24 hours following (2*R*,6*R*)-HNK treatment (30 minutes before hot plate measurement) blocked the expression of (2*R*,6*R*)-HNK mediated antinociception. **Panel D**: Naltrexone given 24 hours following (2*R*,6*R*)-HNK treatment (30 minutes before hot plate measurement) 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 ± S.D. 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. (Yost et al., 2022)

To allow for a comparison with ketamine, a similar experiment was executed but measured for a hot plate effect 10 minutes following treatment when ketamine achieves its peak effect. This time, NBQX administration before treatment did not block ketamine antinociception. But, naltrexone administration before treatment partially blocked ketamine antinociception (**Figure 8E**; $F_{(2, 54)} = 4.634$, $P = 0.0139$). These results signify that ketamine antinociception in healthy animals is at least partially dependent on opioid receptor function to produce the behavioral effect and independent of AMPAR function.

Next, an examination was made to determine if AMPAR activity was necessary for the hypersensitivity reversing effect of (2*R*,6*R*)-HNK in a model of inflammatory pain. NBQX administration before (2*R*,6*R*)-HNK treatment blocked the hypersensitivity reversing effect at 4 and 24 hours following treatment (**Figure 9**). These results signify that the (2*R*,6*R*)-HNK pain reduction effect in this model is also dependent on AMPAR glutamatergic signaling.

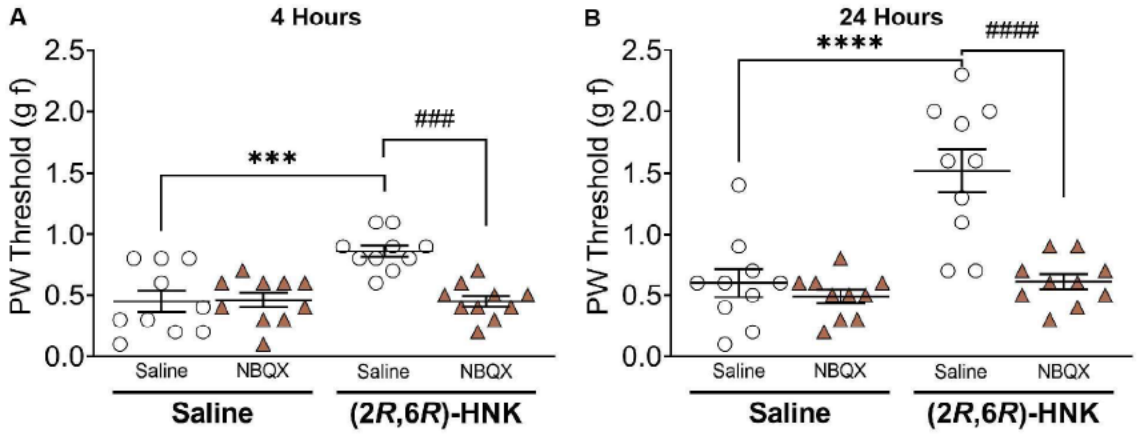

Figure 9: Pretreatment with the AMPA receptor antagonist NBQX 10 mg/kg (30 minutes prior) blocked (2*R*,6*R*)-HNK 30 mg/kg mediated reversal of mechanical hypersensitivity associated with localized inflammation at 4 hours (A) and in the same cohort at 24 hours (B) following (2*R*,6*R*)-HNK treatment. The error bars represent group means ± 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 all groups.

To summarize this aim, (2*R*,6*R*)-HNK requires AMPAR-dependent glutamatergic signaling to produce the observed behavioral effects in healthy animals and animals with localized inflammation. How AMPAR plays a role in this pain reduction effect remains unclear. Also, (2*R*,6*R*)-HNK mediates analgesia through a mechanism of action different from ketamine, its parent compound. A better understanding of how (2*R*,6*R*)-HNK works to provide these effects could open the door to a better understanding of pain conditions and analgesia in general.

Relationship of current findings to previous findings:

As described throughout this paper, only one previously published study reported a potential pain reduction effect of (2*R*,6*R*)-HNK. The previous research identified a hypersensitivity reversing effect of (2*R*,6*R*)-HNK in female mice with neuropathic and postoperative pain (Kroin et al., 2019). The findings in the present study align well with the previous data and greatly expand upon them.

Limitations:

All data gathered in this study describe a behavioral effect in animal research where the translation to an effect in humans has yet to be established. It is possible that the pain reduction behavior described here may not translate to effective pain reduction in human pain conditions. This is a significant limitation for all preclinical research. However, data such as those included in this study are important to generate interest for future clinical research and make it possible to examine mechanisms of effect that are very difficult to investigate in human research.

Conclusion:

Based on the results of this TNSRP-funded research, it is now known that (2*R*,6*R*)-HNK causes antinociception in healthy animals. This pain reduction effect is interesting because of its potential generality to all types of pain. These data show a dose-response relationship for this analgesic behavioral effect that can aid in identifying treatment dosing parameters for future studies.
It is now known that (2*R*,6*R*)-HNK can reverse hypersensitivity in a model of inflammatory pain. Inflammation-based pain is a prevalent problem, and novel treatment modalities could have immediate and tremendous clinical impact. Additionally, it is now known that (2*R*,6*R*)-HNK treatment in a model of neuropathic pain possesses a magnitude of effect on par with carprofen, an anti-inflammatory treatment used in humans and animals.

The mechanistic data provided by this research demonstrated that (2*R*,6*R*)-HNK works in an AMPAR-dependent manner and represents a novel approach to treating pain. The effects of (2*R*,6*R*)-HNKwere independent of opioid receptors establishing (2*R*,6*R*)-HNK as a non-opioid treatment worthy of future research. Further research is warranted to better understand the locations and circuitry supporting this mechanism and how AMPAR may be recruited as a target for analgesia research. Overall, these data support the future investigation of (2*R*,6*R*)-HNK as a novel pain-reduction agent that could profoundly impact how pain conditions are treated in the military and beyond.

Significance to Military Nursing

Military nurses, along with all other military healthcare professionals, share responsibility for addressing the critical need to identify novel pain therapeutics for our veterans and service members. Pain and its consequences are a tremendous problem within the treatment populations seen by military nurses. While this research and the reported results may not immediately impact nursing clinical practice today, it is vital for our nurses at all levels to be aware of potential therapeutics that could be made available for use in the near future. Awareness of current research helps inspire new ideas that can have a more immediate impact on nursing care and drive the research needed tomorrow to address the challenges military nurses face today and will face in the future. Nurses need to see the results of research work done by fellow nurses to help them gain confidence in their abilities to get involved and make a difference in all types of research within the healthcare system we serve. A solid understanding of developing research will enable military nurses to be more involved in the research process to identify novel therapeutics, develop treatment protocols, and drive the translation of new research into clinical practice.

Resulting Changes

These results should not result in immediate changes in military nursing clinical practice. The research described in this report is preclinical. The information provided in this study cannot be implemented into practice until sufficient data are available to verify safety and a positive therapeutic effect in human patients. However, this research is important to drive future examination of this novel non-opioid pain treatment. (2*R*,6*R*)-HNK has cleared phase 1 safety trials and is used inadvertently by patients given ketamine chronically. Clinical trials will likely be launched soon to evaluate the effectiveness of (2*R*,6*R*)-HNK in various forms of chronic pain. Awareness of such research by military nurses is important to allow them to be involved in the clinical research and implementation of new therapies.

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Summary of Dissemination

Type of Dissemination	Citation	Date and Source of Approval for Public Release
Publications	Yost JG, Wulf HA, Browne CA, Lucki I. In Press. Antinociceptive effects of (2*R*,6*R*)-hydroxynorketamine in healthy mice are dependent on AMPA receptors. *Journal of Pharmacology and Experimental Therapeutics.* 2022;382:256-65, dx.doi.org/10.1124/jpet.122.001278	22 April 2022, USUHS PAO
Published Abstracts	Yost JG, Browne CA, Lucki I. Analgesic and Antinociceptive Effects of (2*R*,6*R*)-hydroxynorketamine (HNK) in Mice. Experimental Biology 2022, April 2-5, 2022, Philadelphia PA. DOI: https://doi.org/10.1096/fasebj.2022.36.S1.R2181	24 March 2022, USUHS PAO

Podium Presentations	Yost JG, Browne CA, Lucki I. Ketamine metabolite (2*R*,6*R*)-HNK provides pain reduction via an AMPA receptor-dependent mechanism. MHSRS 2022 Young Investigator Competition, September 12-15, 2022, Kissimmee FL	13 September 2022, MEDCoE PAO
	Yost JG, Browne CA, Lucki I. Analgesic and Antinociceptive Effects of (2*R*,6*R*)-hydroxynorketamine (HNK) in Mice. Experimental Biology 2022, April 2-5, 2022, Philadelphia PA	24 March 2022, USUHS PAO
	2021 USUHS Research Days Graduate Student Colloquium. May 2021, Bethesda MD	May 2021, USUHS PAO
	2021 TSNRP Research Dissemination Course. September 13-15, 2021	September 2021, USUHS PAO
	2020 USUHS Research Days Graduate Student Colloquium. May 2020, Bethesda MD	May 2020, USUHS PAO
Poster Presentations	Yost JG, Browne CA, Lucki I. Analgesic and Antinociceptive Effects of the Ketamine Metabolite (2*R*,6*R*)-hydroxynorketamine (HNK) in Mice. 2021 Annual meeting of the American College of Neuropsychopharmacology. December 5-9, 2021. San Juan, Puerto Rico.	15 November 2021, USUHS PAO

Reportable Outcomes

Reportable Outcome	Detailed Description
Applied for Patent	None
Issued a Patent	None
Developed a cell line	None
Developed a tissue or serum repository	None
Developed a data registry	None

Recruitment and Retention

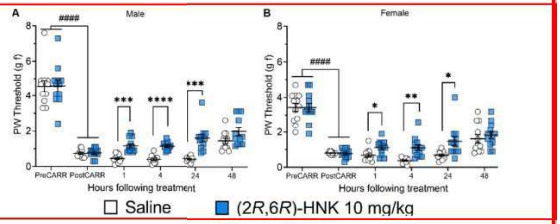
Recruitment and Retention Aspect	Number
Animals Projected in Grant Application	760
Animals Purchased	1,025
Model Development Animals	75
Research Animals	950
Animals With Complete Data	943
Animals with Incomplete Data	7

Project Overview (Quad Chart)

Pain reduction effects of a ketamine metabolite, (2*R*,6*R*)-HNK, using models of pain in mice (N20-A02GR)

PI:	Grantee Organization:	Award Amount:
LTC Jonathan G. Yost	Uniformed Services University	$54,997

- Aim #1: Determine pain reduction effects of (2*R*,6*R*)-HNK in healthy mice and mice with induced neuropathic and inflammatory pain
- Aim #2: Examine interaction between (2*R*,6*R*)-HNK and opioid-induced pain reduction
- Aim #3: Investigate mechanism of action for (2*R*,6*R*)-HNK mediated pain reduction



Key Findings

- (2*R*,6*R*)-HNK produces pain reduction-like effects in multiple pain models
- (2*R*,6*R*)-HNK augments opioid analgesia
- (2*R*,6*R*)-HNK works via an AMPA receptor dependent mechanism, differently than its parent compound ketamine

Recommendations

- (2*R*,6*R*)-HNK demonstrates significant potential as a novel, non-opioid analgesic medication
- Proceed with clinical investigation of this ketamine metabolite
- Proceed with future investigation of (2*R*,6*R*)-HNK mechanism of action to better understand AMPA receptor dependent analgesia