1. Title Page

Preliminary pharmacokinetics and in vivo studies indicate analgesic and stress mitigation effects of a novel NMDA receptor modulator

Blaise M. Costa[1,2&3*], Nakia Phillip[1], Seth C. Boehringer [1&4], McAlister Council-Troche[3], Jennifer Davis[3]

[1]Edward Via Virginia College of Osteopathic Medicine, [2]Center for One Health Research, Virginia-Maryland College of Veterinary Medicine, Virginia Tech, Blacksburg, VA. [3]Department of Biomedical Sciences and Pathobiology, Virginia-Maryland College of Veterinary Medicine, Virginia Tech, Blacksburg, VA, [4]Department of Biochemistry, Virginia Tech, Blacksburg, VA.

2. Running Title

a.

CNS4 improves fear memory and fear extinction in male mice

b.

Blaise M. Costa, PhD

Edward Via Virginia College of Osteopathic Medicine

Center for One Health Research, Virginia Tech

1410 Prices Fork Rd, Blacksburg, VA 24060.

Phone: (540) 231-1468

Fax: (540) 231-3426

Email: bcosta@vcom.vt.edu

c.

Number of text pages	:15
Tables	:1
Figures	:6+3 supplementary + 1 Graphical abstract
References	:81
Number of words in Abstract	:234
Introduction	: 671
Discussion	:1488

d.

List of nonstandard abbreviations:

Cmax: maximum concentration; CS: Conditioned stimuli; FC: Fear condition; GluN: Glutamate receptor NMDA subtype; LOQ: Limit of quantification; NMDAR: N-methyl D-aspartate receptor; Tmax: time taken to reach maximum concentration; US: Unconditioned stimuli. UPLC-MS: Ultra-performance liquid chromatography-mass spectrometry

e.

Drug Discovery and Translational Medicine

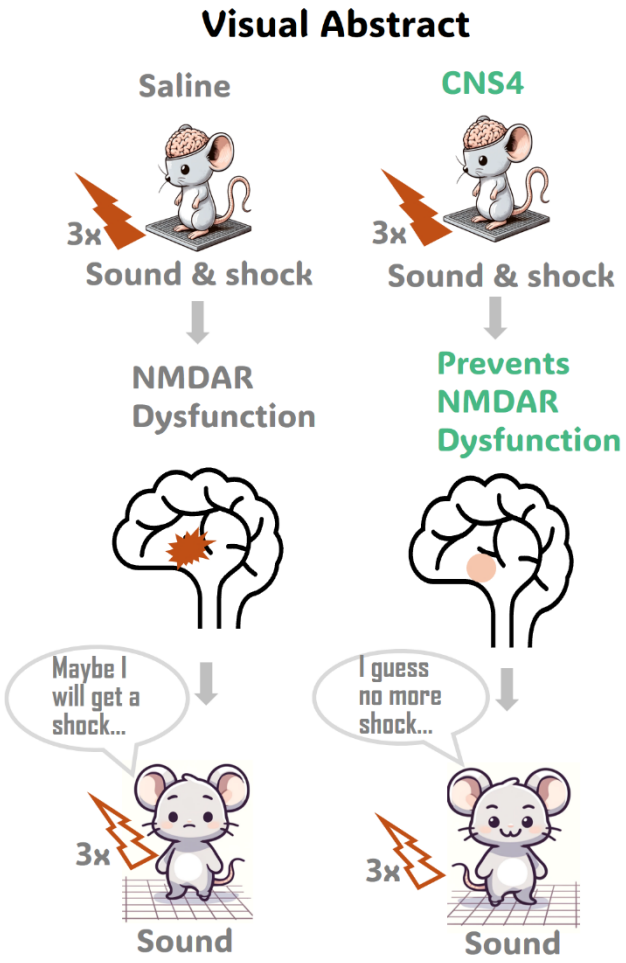
3. Abstract:

NMDA receptor (NMDAR) channel blockers, which produce analgesic and anti-depressant effects, preferentially block the GluN2D subtype of NMDAR at lower doses. Based on the knowledge of GluN2 subunit physiology, we hypothesized that compounds that concurrently modulate GluN2A and GluN2D subtypes of NMDARs to opposite directions can be useful analgesic and stress-mitigating agents. In this translational study, we explored in vivo activities of a recently discovered glutamate concentration-dependent NMDAR modulator (CNS4). Results from the pharmacokinetic study indicate that CNS4 reaches maximum plasma and brain concentration as quickly as 0.25 hours after intraperitoneal injection, and about 6% of the plasma concentration reaches brain tissue (54.5 vs 3.3 μg/ml). In preliminary in vivo studies, CNS4, a non-opioid compound, increased mice escape latency in a hotplate assay by 2.18-fold compared to saline and 1.78-fold compared to the positive control, meloxicam. Furthermore, in a fear conditioning (FC) experiment, CNS4 improved fear memory [decrease in freezing latency (11.78 vs 4.42s, p=0.0010)] and subsequent fear extinction [increase in freezing latency (3.56 vs 15.08s, p=0.049)] in male mice. CNS4 caused no changes in locomotion in 8 out of 9 parameters studied. About fifty hours after FC training, CNS4 increased water (5-fold) and sucrose intake (4.5-fold) in male mice. These results indicate that the glutamate concentration-biased modulatory effect of CNS4 could produce analgesia and stress-mitigating effects. Further studies in this direction will help develop clinically useful drugs for pain associated with stressful conditions.

4. Significance:

NMDA-subtype glutamate receptors are an attractive target for chronic pain and PTSD treatments as they play a critical role in forming emotional memories of stressful events. In this translational pharmacology work, we demonstrate the central analgesic and male sex-specific fear memory formation and fear extinction characteristics of a novel glutamate concentration-biased NMDA receptor modulator, CNS4.

5. **Visual Abstract:**



6. Introduction:

Functional glutamatergic N-methyl-D-aspartate receptors (NMDARs) consist of two identical glycine-binding GluN1 subunits and two glutamate-binding GluN2 subunits, which can be either similar or different. There are four subtypes of GluN2 subunits: GluN2A, GluN2B, GluN2C, and GluN2D. Given their widespread physiological role, dysfunction of NMDARs causes various neurological and psychiatric conditions (Hansen et al., 2017), one of which is pain. Pain affects more people than diabetes, heart disease, and cancer combined, and is the most common reason people seek medical attention (Wager, 2022). Pain restricts daily life activities and causes opioid addiction and depression (Dahlhamer et al., 2018; Gureje et al., 1998; Smith et al., 2001). A balanced interplay between the excitatory glutamatergic neurons and GABAergic inhibitory neurons is essential to initiating and maintaining central pain sensitization (Breitinger and Breitinger, 2023). NMDARs play a pivotal role in central pain sensitization, which evolves from activation of neuronal circuits in nociceptive pathways (Aiyer et al., 2018; De Kock and Lavand'homme, 2007; Petrenko et al., 2003). Chronic pain complaints are common in patients with a primary diagnosis of post-traumatic stress disorder (PTSD), with prevalence rates estimated to be as high as 80% (Lew et al., 2009; Otis et al., 2009). Both PTSD and chronic pain involve alterations in brain regions related to emotion, cognition, and sensory processing (Burris et al., 2009; Jenewein et al., 2009; Sharp and Harvey, 2001), where NMDARs are expressed. Therefore, these two pathogenically connected and co-existing conditions should be altered by pharmacological agents that modulate NMDAR function.

NMDAR serves as a major non-opioid analgesic drug target (Ghoreishi-Haack et al., 2018; Houck et al., 2019). Particularly, compounds that modulate the GluN2D subtype could be useful in treating neuropathic pain (Dedek and Hildebrand, 2022; Hildebrand et al., 2014; Jing et al., 2022). The potential utility of such compounds derives from two unique characteristics of GluN2D: (1) GluN2D expression in adults is largely confined to regions that are involved in neuropathic pain

perception, such as the thalamus, basal ganglia, and GABAergic interneurons across the brain and spinal cord (Dubois et al., 2016; Logan et al., 2007; Standaert et al., 1996; Tolle et al., 1993; Wenzel et al., 1996). (2) As the GluN1/2D channel is the slowest NMDAR subtype to deactivate, it can conduct a large number of cations into the neurons and trigger action potentials (Chen et al., 1999; Cull-Candy and Leszkiewicz, 2004; Erreger et al., 2005). Together, GluN2A and GluN2D subtypes of NMDARs play a critical role in excitatory and feed-forward inhibitory neurotransmission, as they predominantly express on primary neurons and GABAergic interneurons, respectively (Dubois et al., 2016; Hansen et al., 2021; Logan et al., 2007; Mohrmann et al., 2000; Petralia et al., 2010; Standaert et al., 1996; Thomas et al., 2006; Tolle et al., 1993; Wenzel et al., 1996). Of note, a highly effective, dose-dependent, anti-depressant activity of esketamine is believed to be mediated through inhibition of the GluN2D subtype of NMDARs (Hanson et al., 2024),demonstrating that direct inhibition and indirect disinhibition of primary neurons through GluN2D receptors have immediate and profound clinical effects (Hanson et al., 2023).

Therefore, we hypothesize that pain sensation-mediated glutamatergic dysfunction could be corrected by concomitantly modulating GluN2A and GluN2D subunits in opposite directions. This hypothesis fits with the pharmacology of a recently discovered glutamate dose-dependent NMDAR modulator, 4-fluoro-N-[[2-(3-pyridinyl)-1-piperidinyl]-sulfanylidenemethyl]benzamide, CNS4 (Boehringer et al., 2023; Costa, 2018; Costa et al., 2021). This compound preferentially potentiates neuroprotective GluN2A receptors by 22% and inhibits 35% of GluN2D when activated by 300μM glutamate. Conversely, CNS4 potentiates GluN2D currents by 8-fold when activated by 0.3μM glutamate. Similar glutamate dose-dependent activities of CNS4 on other GluN2 subtypes have recently been reported (Boehringer et al., 2023; Costa, 2018; Costa et al., 2021). In this work, we have initially performed preliminary pharmacokinetics and *in vivo* characterization to study the central analgesic effect of CNS4. Thereafter, we have continued to study the effect

of CNS4 on fear memory and fear extinction using an electric shock-induced fear conditioning (FC) model. Overall, pharmacokinetic profiling and in vivo assessments reveal central analgesic and male sex-specific stress-mitigating activities of CNS4.

7. Methods:

Animals

All animal experimental procedures and housing were approved by the Institutional Animal Care and Use Committee (IACUC) of Virginia Tech, protocol number 23-151. Thirty adult male CF-1 mice were used for pharmacokinetics work and twenty C57BL/6J mice (10 female and 10 male) were used for analgesic, FC, open field, and sucrose preference assay.

Compound: CNS4 used in this study was pharmaceutical grade, with >98% purity as verified by HPLC, NMR and LCMS assays, and as previously published (Boehringer et al., 2023; Costa et al., 2021).

Pharmacokinetics (PK)

CNS4 was initially dissolved in 100% DMSO, then diluted in an appropriate volume of sterile normal saline and one equivalent of sodium hydroxide to get a fully solubilized clear solution. The final concentration of DMSO was 5%. CF-1 male mice were injected intraperitoneally with 100mg/kg CNS4 using a 27-gauge needle. Injection volume was between 100-200µl based on animal body weight. Animals were euthanized at 0, 0.25, 0.5, 1, 2, 4, 8, 12, 24, and 48 hours after CNS4 administration to collect blood and brain tissue samples. Three animals were used for each time point. Blood samples were collected in 0.4ml heparin EDTA vials and gently mixed before centrifuging at 2500 x g for 15 minutes to separate the plasma. The whole brain was excised and placed into vials containing RNA later solution (cat# AM7021) maintained at 4°C. One-half of the brain tissue samples were flash-frozen in liquid nitrogen and stored at -80°C. The other half was

homogenized in 1x phosphate buffered saline and spun at 3200 x g for 30 minutes at 4°C and the supernatant was used for determination of drug concentration. Plasma and brain samples were analyzed for the parent compound using UPLC-MS/MS with a limit of quantification (LOQ) of 0.003 µg/mL. Pharmacokinetic data was then generated using naïve averaged concentrations and noncompartmental analysis with Phoenix WinNonlin, version 8.4, Certara, Princeton, NJ.

The extracted samples were screened for the presence of metabolites, as predicted using Swiss ADME software (Daina et al., 2017). This program predicted CNS4 undergoing CYP450 3A4 mediated oxidation at the sulphonyl group (Metabolite-1) in the phase-1 metabolic reaction, and glucuronidation (Metabolite-2) at the nitrogen atom of the pyrimidine ring in the phase-2 reaction.

Hot plate assay

Three groups of adult C57BL mice were used for this experiment. About 15 minutes before the experiments, mice were injected with sterile normal saline (0.9% NaCl) to 7 mice (4 males and 3 females), or 100mg/kg CNS4 in normal saline to 6 mice (3 males and 3 females), or 5mg/kg of meloxicam in normal saline to 7 mice (3 males and 4 females), through intraperitoneal (IP) route. Ugo Basile Hot/Cold Plate equipment was used for this assay. The plate was digitally set to 55°C throughout the experiment. Although temperatures in the range of 45-50°C are generally used for this type of experiment, we used a higher temperature of 55°C to produce a pain stimulus that requires greater central nervous system integration and processing, making it better for evaluating centrally-acting analgesics (Deuis et al., 2017; Hijazi et al., 2017). Mice were individually placed on the hot plate and quickly removed after they licked the paw for the first time. The time interval between placement and removal was noted as paw-licking (escape) latency. Statistical analysis of this data was performed using one-way ANOVA and post hoc Tukey's multiple comparison tests. GraphPad Prism 10.2.3 software program was used for all statistical analysis. The same cohort of mice was used for the fear conditioning assay 5 days later.

Open field assay (OFA)

A rodent open field apparatus (Maze engineers, part# 3203) was used for this assay. The AnyMaze 7.4 video recording system (Stoelting Co. IL) was used to monitor animal behavior, data collection, analysis, and statistics. Each mouse was individually placed in the apparatus for three minutes, and its uninterrupted movement was recorded and analyzed for nine different parameters presented in the results section. Freezing and immobility were calculated from 3 seconds cutoff. Four groups of mice were used for the OFA, sucrose preference test, and FC experiments (control male, control female, CNS4 male & CNS4 female). Each group contained 5 mice. All parameters were initially analyzed using two-way ANOVA, then post hoc Tukey's multiple comparisons test, to find out differences between individual groups.

Sucrose preference test (SPT)

Two identical 50ml tubes, with drip nozzle, were filled with an equal volume of water or 1% weight per volume of sucrose in water solution. Regular water bottles were replaced, for about 16 hours (overnight), with tubes on the same side by inserting the nozzles into the cage where the original water bottle was present. Each tube was weighed before and after the experiment and the difference was calculated. Two sets of sucrose preference assays were performed, before and after the FC experiment. The first SPT (baseline) was performed on the male and female mice housed in six cages, three male cages and three female cages. The second SPT was performed on four groups of animals, control male, control female, CNS4 treated male and CNS4 treated female. Each of these groups had an equal (five) number of mice. Note: Since mice were caged together as a group, and received foot shock as a group, the second SPT was performed as a group instead of per individual animal, to avoid the influence of social isolation on fluid intake. The Wilcoxon matched-pairs signed rank test was used to identify the statistical significance in sucrose preference.

Fear conditioning (FC) assay

Maze Engineers' (part# 5801) FC apparatus was used for this study. Conditioned stimuli (CS) and unconditioned stimuli (US) were precisely delivered into the chamber in a sequential manner through a software program, ConductMaze, that comes with the apparatus. The FC apparatus contains an acrylic cage with dimensions 17 x 17 x 25cm (width x depth x height), with four walls and no top and no base, a circular door that opens in the middle, and a floor grid that delivers foot shock. The apparatus is equipped with dual speakers on the backside inside of the chamber that deliver white noise at a tone of frequency 100-20,000 Hz, with a volume between 1-130dB. Before each test, the acrylic walls and floors were cleaned with water, while the shock grids were wiped with 70% ethanol to prevent interference of olfactory cues from previous animals. Mice were housed in their regularly enriched home cages before being transferred to a separate room where the FC apparatus was placed. All five mice in a group underwent the day 1 FC protocol together and returned to their home cage before returning to their original housing room. However, mice were tested individually on day 2 for their fear response associated with FC. The FC training (day 1) and testing (day 2) protocol diagram is shown in the results section. Below are the details on duration and intensity of CS and US: Tone, volume=50, duration=26s, frequency=4kHz; white noise, volume=50, duration=2s, 20kHz; shock, 0.5mA, 2s. On FC training day, CS (tone & white noise) was paired with US (foot shock); and on FC testing day, CS was unpaired with US, as there was sound (tone & white noise) but no shock.

About 15 minutes before the FC training, mice were injected with sterile normal saline (0.9% NaCl) to control male and control female groups, and 100mg/kg CNS4 in normal saline to CNS4 male and CNS4 female groups, through intraperitoneal (IP) route. On testing day, animals underwent the CS (unpaired with US) protocol individually. Freezing episodes, duration, latency, hyperarousal (jumping behavior), and number of fecal pellets of individual animals were monitored during their time in the FC chamber, using the Any-Maze video recording program, and analyzed.

Since animals were allowed to acclimatize in the chamber for the first three minutes (180 seconds), freezing episode analysis was done for the 180-240s and 300-360s of the stimulus protocol. These time points cover the first and last 60 seconds after they began hearing the tone. Freezing was calculated from 3 seconds immobility cutoff. Freezing data sets were initially analyzed using two-way ANOVA, then Sidak's or Tukey's multiple comparisons post hoc tests, to determine differences between individual groups. Hyperarousal (jumping behavior after placing the animal inside the FC chamber) was monitored throughout the duration inside the chamber on testing day.

On testing day, a picture of the dirt collection tray, placed underneath the FC chamber, was taken for each animal after completion of the test protocol. The fecal pellets were counted and compared between saline and CNS4-treated groups using the Mann-Whitney test.

8. Results:

Distribution of CNS4 into plasma and brain compartments

To facilitate *in vivo* studies, we have carried out preliminary PK to determine the basic PK properties of CNS4 as presented in **Figure 1** and **Table 1**. CNS4 reaches maximum plasma and brain concentrations (T_{max}) as quickly as 0.25 hours and was above the LOQ (T_{min}) for up to 8 hours in the plasma and 12 hours in the brain tissue after a single intraperitoneal (IP) injection. Approximately 6% of the total (protein bound and unbound) plasma concentration is present in the brain tissue based on a comparison of the maximum concentrations (54.5 µg/mL vs 3.3 µg/ml) or 3.6% based on $AUC_{0-\infty}$ (58.9 hr*µg/mL vs 2.10 hr*µg/mL). Further, the plasma volume of distribution (Vd/F) of 1.78 L/kg suggests that CNS4 is well distributed outside of the plasma compartment. Mass spectrometry analysis of plasma and brain tissue samples identified ionization of molecules that match the molecular weight of metabolite-1 in both plasma and brain tissue and metabolite-2 only in plasma.

Analgesic effect produced by CNS4 is different from an NSAID

With the evidence that CNS4 reaches the expected site of action, we have started preclinical studies. To investigate the *in vivo* analgesic effect of CNS4, we first performed a hot plate analgesic assay. Results from this experiment revealed that CNS4 increased mice escape latency (paw-licking) by 218% compared to the saline control and 178% compared to the positive control, meloxicam (**Figure 2**). A 55ºC hotplate was used to increase the central nervous system heat response. Meloxicam is a non-steroidal anti-inflammatory drug that has no central analgesic effect (Engelhardt, 1996). Thus, meloxicam's effect was insignificant. This data also validates the PK assay finding that CNS4 crosses the BBB, and CNS4's effect is different from that of the peripherally acting non-steroidal anti-inflammatory drug meloxicam.

Effect of CNS4 on foot shock sensation and fear memory

Since CNS4 increased thermal escape latency, we hypothesized that it might also reduce the sensitivity to foot shock, and therefore influence the fear memory formation in the fear conditioning experiment. A flow chart diagram (**Figure 3A&B**) shows the FC training and testing protocol. FC assay results revealed that on paired CS and US (day 1) experiments, CNS4-treated animals (both males and females) experienced anecdotally less shock compared to the saline-treated group [supplementary figure 1 (video)].

Analysis of mice behavior data from the first 60 seconds (180-240s) after hearing the tone on FC testing reveals that CNS4 had no impact on the freezing episode (m, 3 vs 3.2, p=0.99; f, 4.4 vs 3.4, p= 0.79) and freezing duration (m, 15.44 vs 25.82s, p=0.83; f, 37.24 vs 37.06s, p=0.99) in both male and female mice (**Figure 3C-D**). However, there was a significant increase in freezing duration in females compared to males (m, 20.63 vs f, 37.15s, p= 0.01). Remarkably, CNS4-treated male mice exhibited a highly significant reduction in escape latency compared to the saline-treated males (11.78 vs 4.42s, p=0.001); however, a similar reduction was not observed

with the female mice (3.56 vs 5.76, p= 0.67), **Figure 3E**. Moreover, there was a highly significant difference in escape latency between saline-treated males and females (m, 11.78 vs f,3.56, p=0.0004). However, CNS4-treated males were indifferent from their counterpart females (m, 4.42 vs 5.76, p=0.95), **Figure 3E**. In the last 60 seconds (300-360s) of freezing data, none of the three freezing parameters (episodes, duration & latency) were different in males or females when compared using two-way ANOVA and post hoc Tukey's multiple comparison tests as shown in supplementary **Figure 2**.

CNS4-treated male mice dissociate CS and US sooner

To examine the differences in freezing within the first (180-240) and last 60 (300-360) seconds after hearing the tone on FC testing day, we have compared these two data sets, **Figure 4A-C**. During the first 60 seconds, mice received two unpaired (tone and noise but no shock) CS. During the last 60 seconds, which covered the time (2 seconds) after the third unpaired CS, animals received no CS. We hypothesized that if animals learned, from their three consecutive unpaired CS experiences, that the CS was no longer paired with the US, they should not freeze immediately. Thus, there should be an increase in freezing latency as the fear extinction begins. Results from this analysis revealed that there were changes in freezing episodes (row factor, % of total 15.58, p=0.016) and freezing duration (row factor, % of total 14.14, p=0.014) based on sex (row factor). However, there was no difference between the groups when compared using two-way ANOVA and post hoc Tukey's multiple comparison tests. In contrast to the freezing episodes and duration, freezing latency was not different based on sex (row factor, % of total 0.64, p=0.56). Instead, there was a significant difference between control and CNS4-treated male mice (column factor, % of total 21.63, p=0.019), and interaction between sex and treatment was significant (% of variation 24.50, p=0.011), revealing the effect of the treatment differs between the sexes. Further, post hoc Tukey's multiple comparisons, which were made to identify differences in escape latency between the first (180-240s) and last 60 seconds (300-360s) within

the same group, revealed a significant difference between the first and last 60 seconds only in CNS4-treated male mice (3.56 vs 15.08s, p=0.049). Remarkably, CNS4-treated male mice are the only group in which escape latency increased, while this was reduced or unchanged in the other three groups (con-m, 11.78 vs 7.00s, p= 0.81; con-f, 3.56 vs 3.70s, p=0.99; CNS4-f, 5.76 vs 3.52s, p=0.99), **Figure 4**.

Phenotypes associated with fear improved in CNS4-treated mice

Hyperarousal data reveals that CNS4-treated males made fewer jumps compared to the saline-treated males (2.60 vs 0.80, p=0.03), **Figure 3F**. Further, both saline- and CNS4-treated female mice made no jumps during their entire time in the FC chamber on day 2 (0.0 vs 0.0, p>0.99). Consequently, the difference in hyperarousal behavior between males and females was highly significant (m, 1.70 vs f,0.00, p=0.0009). However, CNS4-treated males were indifferent from the CNS4-treated female counterparts (n, 0.80 vs 0.00, p=0.54). Next, we studied the number of fecal pellets for each animal after their time in the FC chamber on testing day. Analysis of the number of fecal pellets indicates that CNS4-treated mice defecated a significantly higher number of fecal pellets compared to the saline-treated mice (3.1 vs 4.5 pellets, p=0.02, Mann Whitney test), **Figure 3G**. No difference was identified when comparing all four groups (con-m, con-f, CNS4-m, and CNS4-f) using the two-way ANOVA test (p=0.41). All animal fecal pellet pictures are provided as supplementary **Figure 3**.

Locomotion is not reduced in CNS4 treated male or female mice

To compare the individual animals' fear response with their baseline activity, an open field test was performed before (baseline) and after the FC experiment. Locomotion data collected from the open field experiment revealed that there was a significant difference between pre- and post-FC assessments (p<0.05, two-way ANOVA) in all parameters related to locomotion and anxiety, except for time spent in the outer zone (p=0.19), **Figure 5A-I**. Therefore, these differences are

not labeled on the **Figure 5** histograms. However, individual group comparisons made by post hoc Tukey's multiple comparison tests are labeled.

Analysis of distance traveled data indicates that FC reduced locomotion in all animals except for the CNS4-treated females (before 24.15 vs 15.38 meters, p= 0.09), **Figure 5A**. None of the four groups exhibited differences in time spent in the outer zone, time investigating the center zone, and total mobile time, **Figure 5B-D**. However, male animals exhibited higher mobile episodes after the FC experiment in both saline- and CNS4-treated groups (saline, 5.00 vs 12.75, p=0.01; CNS4, 3.60 vs 12.00, p= 0.003), and females did not show similar differences (saline, 6.75 vs 11.5, p=0.27; CNS4, 6.25 vs 12.25, p=0.08), **Figure 5E**.

Next, we analyzed the parameters related to anxiety or fear response, including immobility and freezing inside the open field apparatus. Both saline- and CNS4-treated male mice exhibited increases in total immobile time (saline, 20.22 vs 66.32s, p=0.01; CNS4, 6.67 vs 56.40s, p= 0.002) and total immobile episodes (saline, 4.50 vs 11.75, p=0.006; CNS4, 2.80 vs 10.00, p=0.002) after the FC experiment, **Figure 5F-G**. Further, the CNS4-treated female group, which rarely showed differences, also exhibited an increased number of immobile episodes after the FC experiment (5.50 vs 11.25, p=0.04). All four groups of mice showed comparable increments in freezing episodes (saline, m,19.80 vs 32.82, p= 0.01; saline, f, 20.00 vs 33.00, p=0.01; CNS4, m,16.40 vs 29.00, p= 0.01; CNS4, f, 15.60 vs 28.80, p=0.01) and a highly significant increase in freezing time (saline,m,45.10 vs 93.56s, p= 0.0007; saline,f, 37.00 vs 93.90s, p<0.0001; CNS4, m,31.84 vs 94.22s, p<0.0001; CNS4, f, 38.62 vs 85.68s, p=0.001), **Figure 5H-I**. Overall, in none of the nine different parameters studied, did CNS4 alter a phenotype that was unaltered in the saline-treated male or female mice. These findings indicate that CNS4 does not cause drowsiness or have anxiolytic effects.

CNS4 increased fluid intake in male mice

Further, we studied CNS4-induced changes in sucrose preference in male and female mice. Baseline SPT revealed a significant increase in 1% w/v sucrose in water solution consumption compared to plain water (3.0 ± 0.9 vs 7.55 ± 0.5, n=6 cages, p=0.03, Wilcoxon matched-pairs signed rank test), **Figure 6A**. Both male and female mice preferred sucrose over water 2.1x and 2.8x, respectively, **Figure 6B-C**. Remarkably, 2 days after the FC training, saline-treated (control) male mice consumed 5x less water and 4.5x less sucrose compared to the CNS4-treated male mice. Interestingly, control female mice consumed both water and sucrose 3.8x and 4.3x more than their male counterparts, respectively. However, control females consumed only 1.32x less sucrose than the CNS4-treated females, **Figure 6D-E**.

9. Discussion

Based on the predicted physicochemical properties and unique pharmacodynamic characteristics of CNS4 (Boehringer et al., 2023; Costa, 2018; Costa et al., 2021), we have carried out a translational pharmacology study. The preliminary PK work revealed that CNS4 reaches maximum concentration in plasma and brain tissue as rapidly as fifteen minutes after a single intraperitoneal injection (**Table 1**). The rapid T_{max} in the brain tissue suggests that CNS4 distributes simultaneously to both compartments following a concentration gradient. The highly lipophilic nature of CNS4 ($LogP_{o/w}$ =2.42) supports rapid passive diffusion across the BBB; however, an active transport mechanism may also be present. This study reports total drug concentrations and not unbound (active) drug concentrations, which requires further study, as does further quantification of the metabolites. However, these initial findings confirm distribution of the drug from the plasma into the CNS, the presumed site of action. These results motivated us to carry out a subsequent hot plate assay to study the analgesic effect of CNS4.

Results from the hot plate assay revealed that CNS4 increases escape latency, as an indicator of analgesic effect. Further, this effect differs from that of a peripherally acting non-steroidal anti-

inflammatory drug, meloxicam, **Figure 2**. Collectively, data obtained from the PK and hotplate assays suggest that CNS4 is crossing the BBB, reaching brain interstitial fluid, and producing a central analgesic effect. While this data does not necessarily confirm that the observed effect is an outcome of CNS4 binding with the neuronal NMDARs, it is known from previous *in vitro* experiments that CNS4 can directly potentiate or inhibit the NMDAR subtypes based on agonist concentration (Costa et al., 2021). Since there is a potential for target engagement, we then studied the effect of CNS4 on contextual FC as there is a compelling but enigmatic role of NMDAR subtypes in various components of post-traumatic stress disorders (Dalton et al., 2012; Dubois et al., 2016; Dubois and Liu, 2021; Gao et al., 2010; Leaderbrand et al., 2014; Rasmussen, 2015; Salimando et al., 2020).

The contextual fear memory formation requires animals to remember the context in which they receive adverse stimuli, and this is dependent on the hippocampus, where GluN1/2AB tri heteromeric NMDAR are predominately expressed (Biedenkapp and Rudy, 2007; Davis, 1986; Kim and Jung, 2006; Lafon-Cazal et al., 2002; Phillips and LeDoux, 1992; Soares and Lee, 2013; Stroebel et al., 2018; Tovar et al., 2013). A reduction in freezing latency in CNS4-treated male mice compared to control male mice (4.4 vs 11.7s) within the first 60 seconds after they started hearing the tone on testing day (day 2) indicates improved fear memory consolidation, **Figure 3**. Also, the absence of any difference in freezing episodes or freezing duration suggests that CNS4-treated male mice did not continue freezing for a longer period or initiate new freezing episodes after realizing that the sound (CS) was unpaired with the shock (US). Therefore, CNS4 might have enhanced the fear memory formation and memory consolidation in male mice, as these events are connected with NMDAR activity. CNS4-treated male mice freezing latency (4.4s) data is comparable to that of both CNS4-treated and control female mice (5.7s and 3.5s). Decreased escape latency or quick freezing indicates NMDAR-mediated improved synaptic strength and stronger fear memory formation about the aversive stimuli that they experienced during FC

training. These findings indicate that from FC training, animals quickly learned to associate the CS with the US except for the control male, **Figure 3E**. It was previously reported that CNS4 increases glutamate potency in GluN1/2AB receptors, and activation of this subtype of receptor promotes neuronal survival (Costa et al., 2021; Lafon-Cazal et al., 2002; Papadia et al., 2005). Therefore, improved fear memory formation observed in CNS4-treated male mice might be associated with the modulation of GluN1/2AB receptors in vivo.

An increased freezing latency or a delayed start in freezing behavior during subsequent presentations of the CS alone (unpaired with US) might indicate that the animal is beginning to learn that the sound is no longer an indicator of upcoming foot shock (Chang et al., 2009; Pavlov and Anrep, 1927). This reduced response is a hallmark of fear extinction, where the association between the CS and the US is weakened (Davis, 2011). The increase in freezing latency in CNS4-treated male mice (3.56 vs 15.08s, **Figure 4Ciii**), when compared to the first and last 60 seconds after hearing the tone on FC testing day, indicates these animals already started dissociating the sound from the foot shock. This extinction learning was observed only in the CNS4-treated male mice, while the other three groups, instead, showed a numerical reduction in escape latency. Although fear extinction phenotypes are generally studied later than day 2, Davis (Davis, 2011) posits that extinction cannot be fully explained by forgetting because it requires exposure to the CS in the absence of the aversive event as opposed to the simple passage of time (Delamater, 2004). Therefore, we propose that the three consecutive experiences of unpaired CS, although they occurred a day after FC training, might have helped CNS4-treated males to start dissociating CS and US.

Previous studies demonstrated that blockade of NMDAR in the basolateral amygdala prevents fear extinction since NMDAR-mediated long-term potentiation in the basolateral amygdala and thalamus is critical for extinction learning (Blair et al., 2001; Johansen et al., 2011; Maren, 1999). Further, a growing body of evidence suggests that impairment in acquiring fear extinction is a key

mechanism of PTSD, and mice lacking the GluN2D subunit of NMDAR were unable to extinguish fear memory (Dubois and Liu, 2021; Ogden et al., 2014; Salimando et al., 2020). In contrast, low-dose NMDAR blockers reduced hyperarousal and depressive symptoms of PTSD in humans (Albott et al., 2018; Battista et al., 2007; Feder et al., 2014). Therefore, we speculate that CNS4-induced partial negative allosteric modulation of GluN1/2D subtype of NMDAR (Costa et al., 2021), particularly when activated by high concentrations of glutamate in the amygdala and thalamus, which occurs with heightened fear (Simic et al., 2021; Walker and Davis, 2002), might play a role in fear extinction learning.

It was reported that females exhibit reduced contextual fear responses compared to males, as represented by different behavioral phenotypes (Barker and Galea, 2010; Bethancourt et al., 2011; Clark et al., 2019). In this study, both control and CNS4-treated females performed positively, or at least better (positive or better being interpreted as expected normal mice behavior) than males in most FC parameters studied. This could be because CNS4 might have prevented shock sensation more in females than males [a less intense experience of shock, as seen in the supplementary (video) figure 2] and/or 0.5mA current shock is insufficient to cause fear in female C57BL mice. This evolves from the data that both control and CNS4-treated female mice performed positively in hyperarousal, open field, and sucrose preference assays, **Figure 3-6**. Therefore, stress-mitigating effects of CNS4 on female mice could not be identified from these parameters.

The increased number of fecal pellets in CNS4-treated mice (median of 3 vs 5, p=0.02, **Figure 3G**), indicating normal food intake, also aligns with the overall normal fluid intake (**Figure 6**), further suggesting that foot shock was less stressful for both male and female CNS4-treated mice. Unlike fluid intake, there was no difference between the male and female fecal pellet count (m, 3.50 vs f, 4.10, p=0.41). While connecting the number of fecal pellets directly with food intake, we also recognize that CNS4 might have increased gastrointestinal motility or improved fear memory

formation and that could have resulted in a psychological increase in gastrointestinal motility. This needs to be addressed in future studies.

Aberrant changes in synaptic glutamate concentrations are implicated in the etiology of clinical symptoms of depression and many other neuropsychiatric conditions (McGrath et al., 2022; Moussawi et al., 2011; O'Donovan et al., 2017). The GluN2D subtype of NMDAR plays a critical role in the pathogenesis of these conditions due to its peculiar characteristics, including slow deactivation, extrasynaptic localization, expression in both excitatory and inhibitory neurons, and developmental regulation (Hansen et al., 2017; Paoletti et al., 2013; Wyllie et al., 2013). Therefore, drugs that modulate GluN2D are of major clinical and pharmaceutical interest (Paoletti et al., 2013). In the past fifteen years, numerous compounds have been identified to selectively modify the function of GluN2D receptors with the goal of translating them into clinically useful drugs (Callahan et al., 2020; Costa et al., 2009; Costa et al., 2010; Mullasseril et al., 2010; Perszyk et al., 2020; Yi et al., 2020). However, to the best of our knowledge, none of the compounds that are primarily acting on GluN2D reached clinical trials. One of the reasons is lack of translational efforts. In the present study, we have successfully translated an in vitro based NMDAR modulator to rodents and proved its pharmacological effects, which are potentially associated with the modulation of native NMDA receptors. Future preclinical investigations of CNS4 and its analogs will help develop clinically useful drugs for pain associated with stressful conditions.

10. Acknowledgments:

Authors thank Virginia Tech animal house staff for taking care of our animals.

11. Data availability statement:

All data that supports the findings of this work is provided in the manuscript and in the supplementary documents. Raw data can be obtained from the corresponding author with a reasonable request.

12. Authorship Contribution:

Conceptualization and participated in research design: Costa

Conducted experiments: Costa, Phillip, Boehringer, Council-Troche

Performed data analysis: Costa and Davis

Wrote or contributed to writing the manuscript: Costa and Davis

13. References:

Aiyer R, Mehta N, Gungor S and Gulati A (2018) A Systematic Review of NMDA Receptor Antagonists for Treatment of Neuropathic Pain in Clinical Practice. *Clin J Pain* **34**:450-467.

Albott CS, Lim KO, Forbes MK, Erbes C, Tye SJ, Grabowski JG, Thuras P, Batres YCTM, Wels J and Shiroma PR (2018) Efficacy, Safety, and Durability of Repeated Ketamine Infusions for Comorbid Posttraumatic Stress Disorder and Treatment-Resistant Depression. *J Clin Psychiatry* **79**.

Barker JM and Galea LA (2010) Males show stronger contextual fear conditioning than females after context pre-exposure. *Physiol Behav* **99**:82-90.

Battista MA, Hierholzer R, Khouzam HR, Barlow A and O'Toole S (2007) Pilot trial of memantine in the treatment of posttraumatic stress disorder. *Psychiatry* **70**:167-174.

Bethancourt JA, Vasquez CE and Britton GB (2011) Sex-dependent effects of long-term oral methylphenidate treatment on spontaneous and learned fear behaviors. *Neurosci Lett* **496**:30-34.

Biedenkapp JC and Rudy JW (2007) Context preexposure prevents forgetting of a contextual fear memory: implication for regional changes in brain activation patterns associated with recent and remote memory tests. *Learn Mem* **14**:200-203.

Blair HT, Schafe GE, Bauer EP, Rodrigues SM and LeDoux JE (2001) Synaptic plasticity in the lateral amygdala: a cellular hypothesis of fear conditioning. *Learn Mem* **8**:229-242.

Boehringer SC, Johnston TV, Kwapisz LC, VandeVord PJ and Costa BM (2023) CNS4 causes subtype-specific changes in agonist efficacy and reversal potential of permeant cations in NMDA receptors. *Pharmacol Res Perspect* **11**:e01107.

Breitinger U and Breitinger HG (2023) Excitatory and inhibitory neuronal signaling in inflammatory and diabetic neuropathic pain. *Mol Med* **29**:53.

Burris JL, Cyders MA, de Leeuw R, Smith GT and Carlson CR (2009) Posttraumatic stress disorder symptoms and chronic orofacial pain: an empirical examination of the mutual maintenance model. *J Orofac Pain* **23**:243-252.

Callahan PM, Terry AV, Jr., Nelson FR, Volkmann RA, Vinod AB, Zainuddin M and Menniti FS (2020) Modulating inhibitory response control through potentiation of GluN2D subunit-containing NMDA receptors. *Neuropharmacology* **173**:107994.

Chang CH, Knapska E, Orsini CA, Rabinak CA, Zimmerman JM and Maren S (2009) Fear extinction in rodents. *Curr Protoc Neurosci* **Chapter 8**:Unit8 23.

Chen N, Luo T and Raymond LA (1999) Subtype-dependence of NMDA receptor channel open probability. *J Neurosci* **19**:6844-6854.

Clark JW, Drummond SPA, Hoyer D and Jacobson LH (2019) Sex differences in mouse models of fear inhibition: Fear extinction, safety learning, and fear-safety discrimination. *Br J Pharmacol* **176**:4149-4158.

Costa BM (2018) Biased nmda receptor modulators and uses thereof, in *PubChem [Internet] Bethesda (MD): National Library of Medicine (US), National Center for Biotechnology Information; 2004- PubChem Substance Record for SID 427554947, SID 427554947, Source: PATENTSCOPE (WIPO); [cited 2022 Feb 15] Available from: https://pubchemncbinlmnihgov/substance/427554947* (Medicine EVCoO ed), USA.

Costa BM, Feng B, Tsintsadze TS, Morley RM, Irvine MW, Tsintsadze V, Lozovaya NA, Jane DE and Monaghan DT (2009) N-methyl-D-aspartate (NMDA) receptor NR2 subunit selectivity of a series of novel piperazine-2,3-dicarboxylate derivatives: preferential blockade of

extrasynaptic NMDA receptors in the rat hippocampal CA3-CA1 synapse. *The Journal of pharmacology and experimental therapeutics* **331**:618-626.
Costa BM, Irvine MW, Fang G, Eaves RJ, Mayo-Martin MB, Skifter DA, Jane DE and Monaghan DT (2010) A novel family of negative and positive allosteric modulators of NMDA receptors. *The Journal of pharmacology and experimental therapeutics* **335**:614-621.
Costa BM, Kwapisz LC, Mehrkens B, Bledsoe DN, Vacca BN, Johnston TV, Razzaq R, Manickam D and Klein BG (2021) A glutamate concentration-biased allosteric modulator potentiates NMDA-induced ion influx in neurons. *Pharmacol Res Perspect* **9**:e00859.
Cull-Candy SG and Leszkiewicz DN (2004) Role of distinct NMDA receptor subtypes at central synapses. *Sci STKE* **2004**:re16.
Dahlhamer J, Lucas J, Zelaya C, Nahin R, Mackey S, DeBar L, Kerns R, Von Korff M, Porter L and Helmick C (2018) Prevalence of Chronic Pain and High-Impact Chronic Pain Among Adults - United States, 2016. *MMWR Morb Mortal Wkly Rep* **67**:1001-1006.
Daina A, Michielin O and Zoete V (2017) SwissADME: a free web tool to evaluate pharmacokinetics, drug-likeness and medicinal chemistry friendliness of small molecules. *Sci Rep* **7**:42717.
Dalton GL, Wu DC, Wang YT, Floresco SB and Phillips AG (2012) NMDA GluN2A and GluN2B receptors play separate roles in the induction of LTP and LTD in the amygdala and in the acquisition and extinction of conditioned fear. *Neuropharmacology* **62**:797-806.
Davis M (1986) Pharmacological and anatomical analysis of fear conditioning using the fear-potentiated startle paradigm. *Behav Neurosci* **100**:814-824.
Davis M (2011) NMDA receptors and fear extinction: implications for cognitive behavioral therapy. *Dialogues Clin Neurosci* **13**:463-474.
De Kock MF and Lavand'homme PM (2007) The clinical role of NMDA receptor antagonists for the treatment of postoperative pain. *Best Pract Res Clin Anaesthesiol* **21**:85-98.
Dedek A and Hildebrand ME (2022) Advances and Barriers in Understanding Presynaptic N-Methyl-D-Aspartate Receptors in Spinal Pain Processing. *Front Mol Neurosci* **15**:864502.
Delamater AR (2004) Experimental extinction in Pavlovian conditioning: behavioural and neuroscience perspectives. *Q J Exp Psychol B* **57**:97-132.
Deuis JR, Dvorakova LS and Vetter I (2017) Methods Used to Evaluate Pain Behaviors in Rodents. *Front Mol Neurosci* **10**:284.
Dubois CJ, Lachamp PM, Sun L, Mishina M and Liu SJ (2016) Presynaptic GluN2D receptors detect glutamate spillover and regulate cerebellar GABA release. *J Neurophysiol* **115**:271-285.
Dubois CJ and Liu SJ (2021) GluN2D NMDA Receptors Gate Fear Extinction Learning and Interneuron Plasticity. *Front Synaptic Neurosci* **13**:681068.
Engelhardt G (1996) Pharmacology of meloxicam, a new non-steroidal anti-inflammatory drug with an improved safety profile through preferential inhibition of COX-2. *Br J Rheumatol* **35 Suppl 1**:4-12.
Erreger K, Dravid SM, Banke TG, Wyllie DJ and Traynelis SF (2005) Subunit-specific gating controls rat NR1/NR2A and NR1/NR2B NMDA channel kinetics and synaptic signalling profiles. *J Physiol* **563**:345-358.
Feder A, Parides MK, Murrough JW, Perez AM, Morgan JE, Saxena S, Kirkwood K, Aan Het Rot M, Lapidus KA, Wan LB, Iosifescu D and Charney DS (2014) Efficacy of intravenous ketamine for treatment of chronic posttraumatic stress disorder: a randomized clinical trial. *JAMA Psychiatry* **71**:681-688.
Gao C, Gill MB, Tronson NC, Guedea AL, Guzman YF, Huh KH, Corcoran KA, Swanson GT and Radulovic J (2010) Hippocampal NMDA receptor subunits differentially regulate fear memory formation and neuronal signal propagation. *Hippocampus* **20**:1072-1082.

Ghoreishi-Haack N, Priebe JM, Aguado JD, Colechio EM, Burgdorf JS, Bowers MS, Cearley CN, Khan MA and Moskal JR (2018) NYX-2925 Is a Novel N-Methyl-d-Aspartate Receptor Modulator that Induces Rapid and Long-Lasting Analgesia in Rat Models of Neuropathic Pain. *The Journal of pharmacology and experimental therapeutics* **366**:485-497.

Gureje O, Von Korff M, Simon GE and Gater R (1998) Persistent pain and well-being: a World Health Organization Study in Primary Care. *JAMA* **280**:147-151.

Hansen KB, Wollmuth LP, Bowie D, Furukawa H, Menniti FS, Sobolevsky AI, Swanson GT, Swanger SA, Greger IH, Nakagawa T, McBain CJ, Jayaraman V, Low CM, Dell'Acqua ML, Diamond JS, Camp CR, Perszyk RE, Yuan H and Traynelis SF (2021) Structure, Function, and Pharmacology of Glutamate Receptor Ion Channels. *Pharmacol Rev* **73**:298-487.

Hansen KB, Yi F, Perszyk RE, Menniti FS and Traynelis SF (2017) NMDA Receptors in the Central Nervous System. *Methods Mol Biol* **1677**:1-80.

Hanson JE, Yuan H, Perszyk RE, Banke TG, Xing H, Tsai MC, Menniti FS and Traynelis SF (2023) Therapeutic potential of N-methyl-D-aspartate receptor modulators in psychiatry. *Neuropsychopharmacology*.

Hanson JE, Yuan H, Perszyk RE, Banke TG, Xing H, Tsai MC, Menniti FS and Traynelis SF (2024) Therapeutic potential of N-methyl-D-aspartate receptor modulators in psychiatry. *Neuropsychopharmacology* **49**:51-66.

Hijazi MA, El-Mallah A, Aboul-Ela M and Ellakany A (2017) Evaluation of Analgesic Activity of Papaver libanoticum Extract in Mice: Involvement of Opioids Receptors. *Evid Based Complement Alternat Med* **2017**:8935085.

Hildebrand ME, Pitcher GM, Harding EK, Li H, Beggs S and Salter MW (2014) GluN2B and GluN2D NMDARs dominate synaptic responses in the adult spinal cord. *Sci Rep* **4**:4094.

Houck DR, Sindelar L, Sanabria CR, Stanworth SH, Krueger M, Suh M and Madsen TM (2019) NYX-2925, A Novel N-methyl-D-aspartate Receptor Modulator: A First-in-Human, Randomized, Double-blind Study of Safety and Pharmacokinetics in Adults. *Clin Transl Sci* **12**:164-171.

Jenewein J, Wittmann L, Moergeli H, Creutzig J and Schnyder U (2009) Mutual influence of posttraumatic stress disorder symptoms and chronic pain among injured accident survivors: a longitudinal study. *J Trauma Stress* **22**:540-548.

Jing PB, Chen XH, Lu HJ, Gao YJ and Wu XB (2022) Enhanced function of NR2C/2D-containing NMDA receptor in the nucleus accumbens contributes to peripheral nerve injury-induced neuropathic pain and depression in mice. *Mol Pain* **18**:17448069211053255.

Johansen JP, Cain CK, Ostroff LE and LeDoux JE (2011) Molecular mechanisms of fear learning and memory. *Cell* **147**:509-524.

Kim JJ and Jung MW (2006) Neural circuits and mechanisms involved in Pavlovian fear conditioning: a critical review. *Neurosci Biobehav Rev* **30**:188-202.

Lafon-Cazal M, Perez V, Bockaert J and Marin P (2002) Akt mediates the anti-apoptotic effect of NMDA but not that induced by potassium depolarization in cultured cerebellar granule cells. *Eur J Neurosci* **16**:575-583.

Leaderbrand K, Corcoran KA and Radulovic J (2014) Co-activation of NR2A and NR2B subunits induces resistance to fear extinction. *Neurobiol Learn Mem* **113**:35-40.

Lew HL, Otis JD, Tun C, Kerns RD, Clark ME and Cifu DX (2009) Prevalence of chronic pain, posttraumatic stress disorder, and persistent postconcussive symptoms in OIF/OEF veterans: polytrauma clinical triad. *J Rehabil Res Dev* **46**:697-702.

Logan SM, Partridge JG, Matta JA, Buonanno A and Vicini S (2007) Long-lasting NMDA receptor-mediated EPSCs in mouse striatal medium spiny neurons. *J Neurophysiol* **98**:2693-2704.

Maren S (1999) Long-term potentiation in the amygdala: a mechanism for emotional learning and memory. *Trends Neurosci* **22**:561-567.

McGrath T, Baskerville R, Rogero M and Castell L (2022) Emerging Evidence for the Widespread Role of Glutamatergic Dysfunction in Neuropsychiatric Diseases. *Nutrients* **14**.
Mohrmann R, Hatt H and Gottmann K (2000) Developmental regulation of subunit composition of extrasynaptic NMDA receptors in neocortical neurones. *Neuroreport* **11**:1203-1208.
Moussawi K, Riegel A, Nair S and Kalivas PW (2011) Extracellular glutamate: functional compartments operate in different concentration ranges. *Front Syst Neurosci* **5**:94.
Mullasseril P, Hansen KB, Vance KM, Ogden KK, Yuan H, Kurtkaya NL, Santangelo R, Orr AG, Le P, Vellano KM, Liotta DC and Traynelis SF (2010) A subunit-selective potentiator of NR2C- and NR2D-containing NMDA receptors. *Nat Commun* **1**:90.
O'Donovan SM, Sullivan CR and McCullumsmith RE (2017) The role of glutamate transporters in the pathophysiology of neuropsychiatric disorders. *NPJ Schizophr* **3**:32.
Ogden KK, Khatri A, Traynelis SF and Heldt SA (2014) Potentiation of GluN2C/D NMDA receptor subtypes in the amygdala facilitates the retention of fear and extinction learning in mice. *Neuropsychopharmacology* **39**:625-637.
Otis JD, Keane TM, Kerns RD, Monson C and Scioli E (2009) The development of an integrated treatment for veterans with comorbid chronic pain and posttraumatic stress disorder. *Pain Med* **10**:1300-1311.
Paoletti P, Bellone C and Zhou Q (2013) NMDA receptor subunit diversity: impact on receptor properties, synaptic plasticity and disease. *Nat Rev Neurosci* **14**:383-400.
Papadia S, Stevenson P, Hardingham NR, Bading H and Hardingham GE (2005) Nuclear Ca2+ and the cAMP response element-binding protein family mediate a late phase of activity-dependent neuroprotection. *J Neurosci* **25**:4279-4287.
Pavlov IP and Anrep GV (1927) *Conditioned reflexes; an investigation of the physiological activity of the cerebral cortex*, Oxford University Press: Humphrey Milford, London.
Perszyk RE, Swanger SA, Shelley C, Khatri A, Fernandez-Cuervo G, Epplin MP, Zhang J, Le P, Bulow P, Garnier-Amblard E, Gangireddy PKR, Bassell GJ, Yuan H, Menaldino DS, Liotta DC, Liebeskind LS and Traynelis SF (2020) Biased modulators of NMDA receptors control channel opening and ion selectivity. *Nat Chem Biol* **16**:188-196.
Petralia RS, Wang YX, Hua F, Yi Z, Zhou A, Ge L, Stephenson FA and Wenthold RJ (2010) Organization of NMDA receptors at extrasynaptic locations. *Neuroscience* **167**:68-87.
Petrenko AB, Yamakura T, Baba H and Shimoji K (2003) The role of N-methyl-D-aspartate (NMDA) receptors in pain: a review. *Anesth Analg* **97**:1108-1116.
Phillips RG and LeDoux JE (1992) Differential contribution of amygdala and hippocampus to cued and contextual fear conditioning. *Behav Neurosci* **106**:274-285.
Rasmussen KG (2015) Ketamine for posttraumatic stress disorder. *JAMA Psychiatry* **72**:94-95.
Salimando GJ, Hyun M, Boyt KM and Winder DG (2020) BNST GluN2D-Containing NMDA Receptors Influence Anxiety- and Depressive-like Behaviors and ModulateCell-Specific Excitatory/Inhibitory Synaptic Balance. *J Neurosci* **40**:3949-3968.
Sharp TJ and Harvey AG (2001) Chronic pain and posttraumatic stress disorder: mutual maintenance? *Clin Psychol Rev* **21**:857-877.
Simic G, Tkalcic M, Vukic V, Mulc D, Spanic E, Sagud M, Olucha-Bordonau FE, Vuksic M and P RH (2021) Understanding Emotions: Origins and Roles of the Amygdala. *Biomolecules* **11**.
Smith BH, Elliott AM, Chambers WA, Smith WC, Hannaford PC and Penny K (2001) The impact of chronic pain in the community. *Fam Pract* **18**:292-299.
Soares C and Lee KF (2013) A prominent role for triheteromeric GluN1/GluN2A/GluN2B NMDARs at central synapses. *J Neurosci* **33**:14975-14977.

Standaert DG, Landwehrmeyer GB, Kerner JA, Penney JB, Jr. and Young AB (1996) Expression of NMDAR2D glutamate receptor subunit mRNA in neurochemically identified interneurons in the rat neostriatum, neocortex and hippocampus. *Brain Res Mol Brain Res* **42**:89-102.

Stroebel D, Casado M and Paoletti P (2018) Triheteromeric NMDA receptors: from structure to synaptic physiology. *Curr Opin Physiol* **2**:1-12.

Thomas CG, Miller AJ and Westbrook GL (2006) Synaptic and extrasynaptic NMDA receptor NR2 subunits in cultured hippocampal neurons. *J Neurophysiol* **95**:1727-1734.

Tolle TR, Berthele A, Zieglgansberger W, Seeburg PH and Wisden W (1993) The differential expression of 16 NMDA and non-NMDA receptor subunits in the rat spinal cord and in periaqueductal gray. *J Neurosci* **13**:5009-5028.

Tovar KR, McGinley MJ and Westbrook GL (2013) Triheteromeric NMDA receptors at hippocampal synapses. *J Neurosci* **33**:9150-9160.

Wager TD (2022) Managing Pain. *Cerebrum* **2022**.

Walker DL and Davis M (2002) The role of amygdala glutamate receptors in fear learning, fear-potentiated startle, and extinction. *Pharmacol Biochem Behav* **71**:379-392.

Wenzel A, Villa M, Mohler H and Benke D (1996) Developmental and regional expression of NMDA receptor subtypes containing the NR2D subunit in rat brain. *J Neurochem* **66**:1240-1248.

Wyllie DJ, Livesey MR and Hardingham GE (2013) Influence of GluN2 subunit identity on NMDA receptor function. *Neuropharmacology* **74**:4-17.

Yi F, Rouzbeh N, Hansen KB, Xu Y, Fanger CM, Gordon E, Paschetto K, Menniti FS and Volkmann RA (2020) PTC-174, a positive allosteric modulator of NMDA receptors containing GluN2C or GluN2D subunits. *Neuropharmacology* **173**:107971.

14. Financial disclosure:

BMC is the founder and CEO of Clab LLC.

15. Footnotes:

This work was supported by the VCOM Research Eureka Accelerator Program (REAP) Grant funded to BMC.

16. Figure legends

Figure 1. CNS4 reaches plasma (A) and brain (B) tissue after intraperitoneal injection in male CF-1 mice. Mice were euthanized, after 100mg/kg CNS4 dissolved in normal saline, in 0, 0.25, 0.5, 1, 2,4,8,12,24 and 48 hours. Limit of detection (LOD) was 0.25 ng analyte/mL plasma or brain tissue for all analytes. Plasma and brain sample analytes were within LOD until 8 and 4 hours respectively, as plotted. n=3 male CF-1 mice for each time point. Inset, chemical structure of CNS4. Data presented as mean ± sem. **C**. Table shows the calculated pharmacokinetic parameters: λ_z = Terminal rate constant. T ½ = Terminal-phase half-life. C_{max} = Maximum concentration. T_{max} = Time to maximum concentration. C_{min} = Minimum concentration. T_{min} = Time to minimum concentration. $AUC_{0-\infty}$ = Area under the concentration-time curve from time zero extrapolated to infinity. AUC_{extrap} = Percentage of AUC that was extrapolated. VD/F = volume of distribution for an extravascular route; Cl/F = clearance for an extravascular route. Brain:plasma = percentage of drug measured in the brain versus the plasma based on $AUC_{0-\infty}$ or C_{max}.

Figure 2. Delayed paw-licking response in CNS4-treated mice during the hot plate test. Histograms show paw licking (escape) latency for saline (7.67±0.72s), CNS4 (16.68±2.38s), and meloxicam (9.39±0.76s) in 55ºC hotplate after IP administration of saline or 100mg/kg CNS4 in sterile normal saline or meloxicam (melox) 5mg/kg. Dots represent sample size [saline 7(4m, f3), CNS4 6(3m,3f), melox 7(3m,4f)] of C57BL male (m) or female (f) mice. One way ANOVA, Tukey's multiple comparisons test. ***p=0.0008, **p=0.005. Data presented as mean ± sem.

Figure 3. Effect of CNS4 on electric shock-induced fear conditioning (FC) on male and female mice. Flow chart for fear condition (FC) training (**A**) and testing (**B**) experiment. Tone, volume=50, duration=26s, frequency=4kHz; White noise, volume=50, duration=2s, 20kHz; Shock, 0.5mA, 2s. On testing (day 2), mice underwent the same protocol but no shock. Freezing episode (**C**), duration (**D**), latency (**E**), hyperarousal (**F**), and number of fecal pellets data,

collected on testing day, were compared between saline and CNS4 treated mice. Freezing and hyperarousal data sets were initially analyzed by Two-Way ANOVA, then Tukey's (**C**) or Sidak's (**D, E&F**) post hoc multiple comparison tests, to find out differences between individual groups. Post hoc tests are marked as downward pointing edges and the Two-Way ANOVA comparison is marked as a straight line on top of the bars. Data points represent the biological replicates, n=5 per group. Fecal pellets (**G**) were analyzed by the Whitney test between male and female mice, n=10 per group. Data presented as mean ± sem. ns= not significant, $p>0.05$; *=$p<0.05$; **=$p<0.01$; ***=$p<0.001$. **C, D&E** shows the freezing data from the first 60 seconds (180-240s) after hearing the tone on FC testing day. The last 60 seconds (300-360s) of freezing data is provided as supplementary Figure 2.

Figure 4. Delayed freezing in CNS4 treated male mice after experiencing three unpaired conditional stimuli. Arrow-marked, paired data points represent an individual animal's freezing episodes (**A**), freezing duration (**B**), and freezing latency (**C**) during the first (180-240) and last 60 seconds (300-360) after hearing the tone on FC testing day. There were four groups of animals [control male (Saline-M), CNS4 treated male (CNS4-M), control female (Saline-F) and CNS4 treated female (CNS4-F)] and each group had five (n=5) animals. Since one animal did not start freezing during the last 60 seconds in the Con-M, CNS4-M, and Con-F groups, there are only four data points in these groups. Data sets were analyzed by Two-Way ANOVA and Tukey's post hoc multiple comparison tests, to find differences between individual groups and between the first and last 60 seconds within the group, respectively. Asterisks represent statistical significance within (3.56 vs 15.08s, $p=0.049$, post hoc Tukey's multiple comparison test) and between the male control and CNS4 groups (column factor, % of total 21.63, $p=0.019$ by Two-Way ANOVA).

Figure 5. Effect of CNS4 on locomotion and anxiety before and after the FC experiment. Histogram plots (**A–I**) indicate various locomotion and anxiety-related behavior parameters (as labeled) analyzed from the open field assay. Mice did not receive saline or CNS4 before the FC experiment; they did receive saline or CNS4 15 minutes before the FC training day (day 1), as labeled on top of panel **A**. There were four groups of animals [control male (Saline-m), CNS4 treated male (CNS4-m), control female (Saline-f) and CNS4 treated female (CNS4-F)] and each group had five (n=5) animals. All parameters were initially analyzed by Two-Way ANOVA, then post hoc Tukey's multiple comparisons test, to find out differences between individual groups. Post hoc tests are marked on top of the histograms. Data points represent biological replicates. Data presented as mean ± sem. ns= not significant, $p>0.05$; *=$p<0.05$; **=$p<0.01$; ***=$p<0.001$, ****=$p<0.0001$.

Figure 6. Effect of CNS4 on fluid intake on male and female mice after FC experiment. Randomly grouped six cages (3 male, 3 female) of 4-5wk old C57BL mice preferred 1% sucrose over water 5 days before FC experiment (**A**), 3.0 ± 0.9, n=6; 7.55 ± 0.5, n=6. Wilcoxon matched-pairs signed rank test, *p=0.031. Raw (**B**) and normalized (**C**) data indicate that both male (2.1x) and female (2.8x) mice preferred sucrose in a control experiment performed 5 days before the FC experiment. Two days after the FC experiment, control male mice drank less water 3.8x and less sucrose 4.3x than the next least consumed group (**D**). Comparison of control vs. CNS4 treated male mice data reveals a remarkable increase in consumption of both water (5.0x) and sucrose (4.5x)

volume in CNS4-treated male mice; whereas CNS4 treated females consumed almost equal volume of water (1.00 vs 0.98) and slightly more sucrose (1.32x) (**E**). Data presented as mean ± sem in **A**.

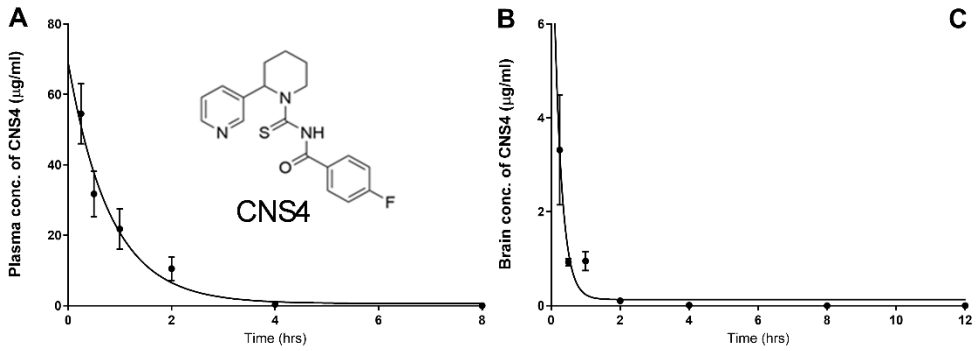
17. Tables

Table 1. Pharmacokinetics of CNS4

	Plasma	*Brain*
λ_z (1/hr)	0.95	0.56
T ½$_{\lambda z}$ (hr)	0.73	1.24
T_{max} (hr)	0.25	0.25
C_{max} (µg/mL)	54.5	3.32
T_{min} (hr)	8	12
C_{min} (µg/mL)	0.034	0.003
$AUC_{0-\infty}$ (hr*µg/mL)	58.9	2.10
$AUC_{\%extrap}$ (%)	0.06	0.24
Vd/F (L/kg)	1.78	---
Cl/F (mL/kg/min)	28.30	---
Brain:Plasma (AUC, %)	---	3.56
Brain:Plasma (C_{max}, %)	---	6.08

18. Figures

Figure 1.



C

Table 1. Pharmacokinetics of CNS4

	Plasma	*Brain*
λ_z (1/hr)	0.95	0.56
T $½_{\lambda z}$ (hr)	0.73	1.24
T_{max} (hr)	0.25	0.25
C_{max} (µg/mL)	54.5	3.32
T_{min} (hr)	8	12
C_{min} (µg/mL)	0.034	0.003
$AUC_{0-\infty}$ (hr*µg/mL)	58.9	2.10
$AUC_{\%extrap}$ (%)	0.06	0.24
Vd/F (L/kg)	1.78	---
Cl/F (mL/kg/min)	28.30	---
Brain:Plasma (AUC, %)	---	3.56
Brain:Plasma (C_{max}, %)	---	6.08

Figure 2.

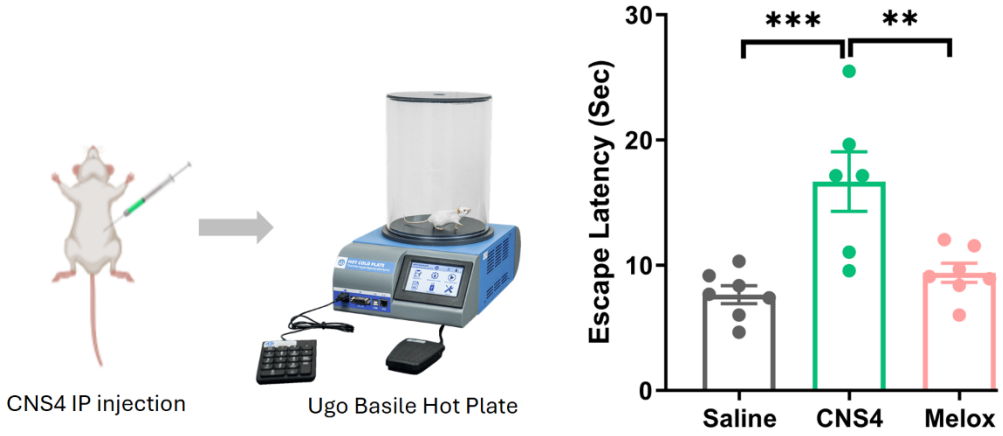

CNS4 IP injection
Ugo Basile Hot Plate
Escape Latency (Sec)
30
20
10
0
***
**
Saline
CNS4
Melox

Figure 3.

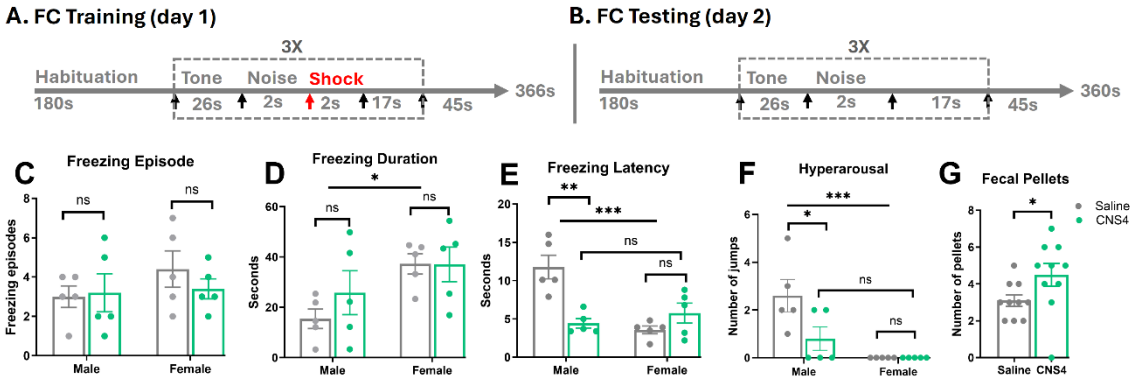

A. FC Training (day 1)
3X
Habituation
180s
Tone
26s
Noise
2s
Shock
2s
17s
45s
366s
B. FC Testing (day 2)
3X
Habituation
180s
Tone
26s
Noise
2s
17s
45s
360s
C
Freezing Episode
Freezing episodes
ns
ns
Male
Female
D
Freezing Duration
Seconds
*
ns
ns
Male
Female
E
Freezing Latency
Seconds
**
***
ns
ns
Male
Female
F
Hyperarousal
Number of jumps
***
*
ns
ns
Male
Female
G
Fecal Pellets
Number of pellets
*
Saline
CNS4
Saline
CNS4

Figure 4.

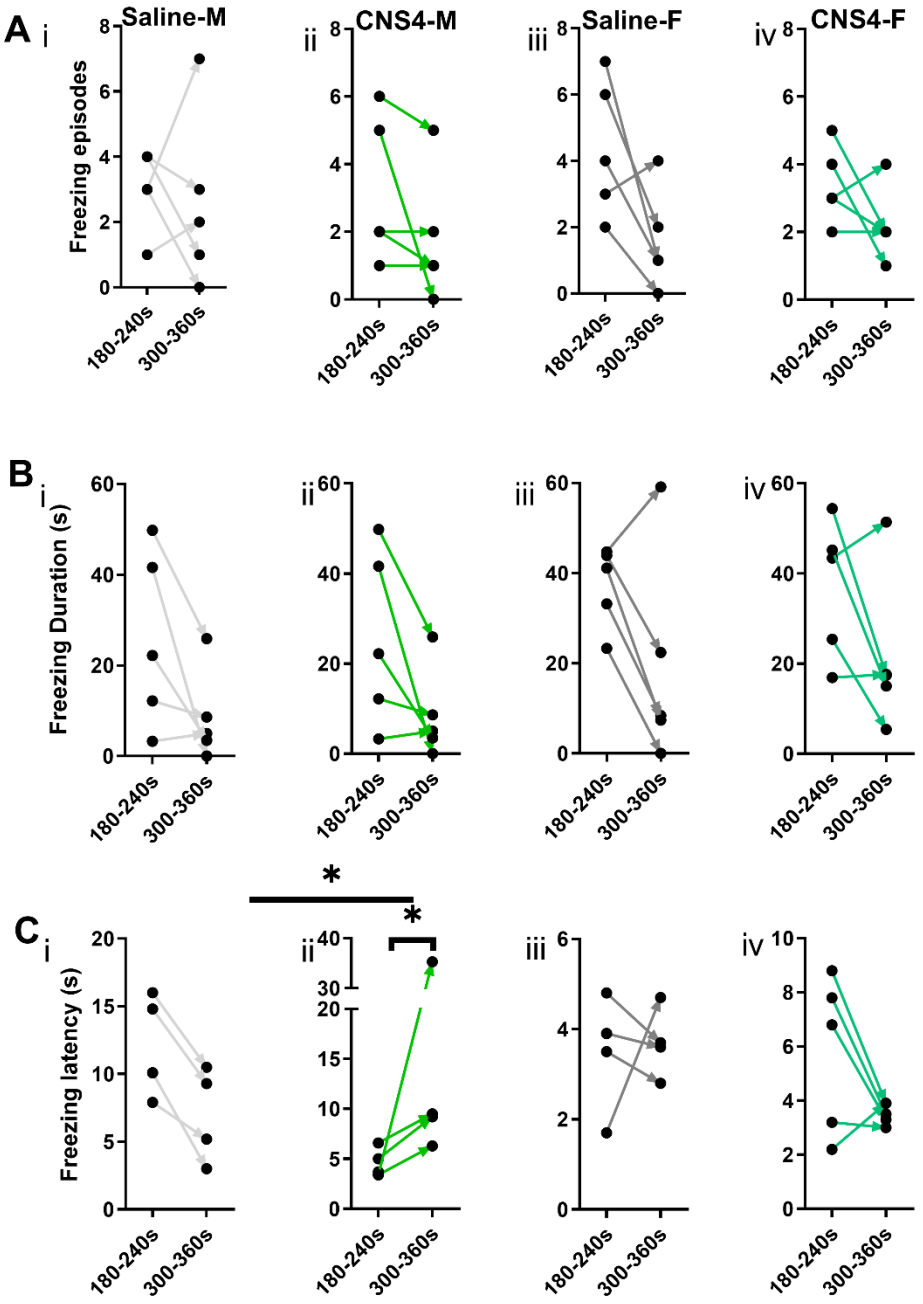

A
i
Saline-M
ii
CNS4-M
iii
Saline-F
iv
CNS4-F
Freezing episodes
180-240s
300-360s
B
Freezing Duration (s)
C
Freezing latency (s)
*
*

Figure 5.

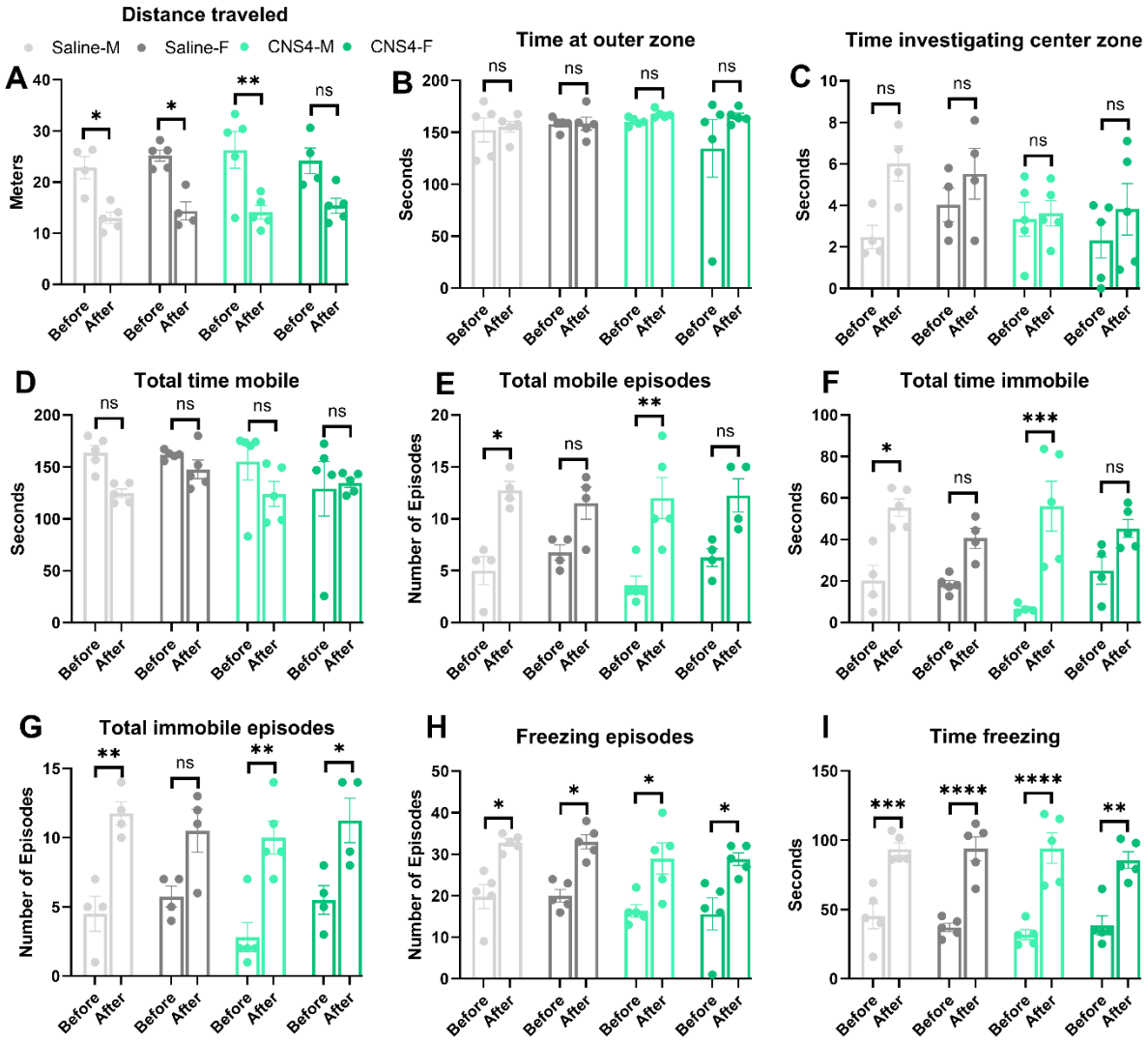
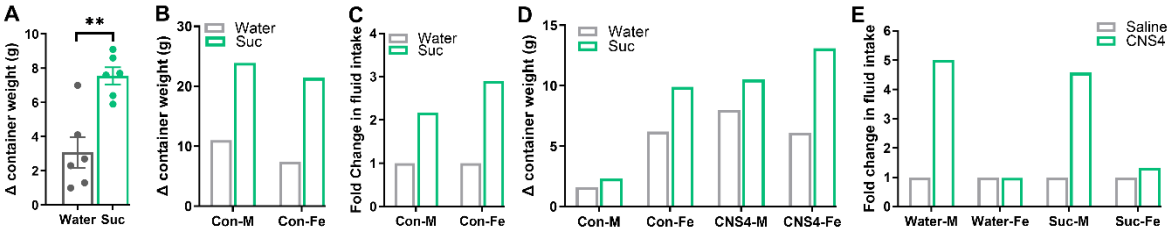
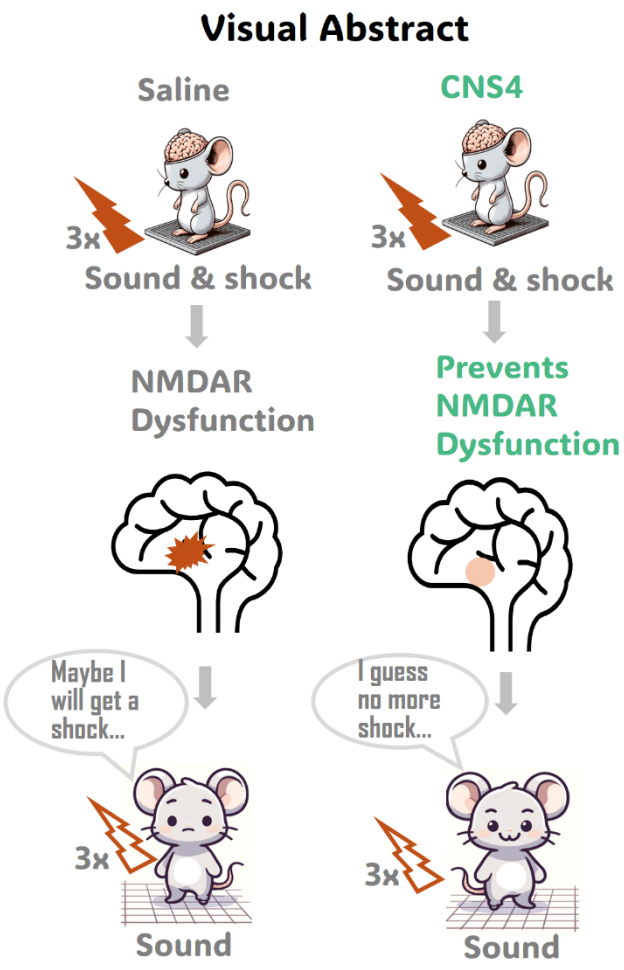

Distance traveled
Saline-M
Saline-F
CNS4-M
CNS4-F
A
Meters
Time at outer zone
B
Seconds
Time investigating center zone
C
Seconds
D
Total time mobile
Seconds
E
Total mobile episodes
Number of Episodes
F
Total time immobile
Seconds
G
Total immobile episodes
Number of Episodes
H
Freezing episodes
Number of Episodes
I
Time freezing
Seconds
Before
After
ns
*
**
***
****

Figure 6.


A
**
Δ container weight (g)
Water
Suc
B
Water
Suc
Δ container weight (g)
Con-M
Con-Fe
C
Water
Suc
Fold Change in fluid intake
Con-M
Con-Fe
D
Water
Suc
Δ container weight (g)
Con-M
Con-Fe
CNS4-M
CNS4-Fe
E
Saline
CNS4
Fold change in fluid intake
Water-M
Water-Fe
Suc-M
Suc-Fe

19. Cover Image



20. Supplemental Data

Supplementary Figure (video)1. CNS4 reduces electric shock sensation in male and female mice. This video file shows four groups of C57BL mice (control-male, CNS4-male, control-female, CNS4-female) undergoing 2s of 0.5mA current shock after the following condition stimuli: 3min of acclimatization → 26s of 4kHz, 50% volume tone → 2s of white noise → 2s 0.5mA shock →17s gap. This was repeated 3X. Response to the first shock is shown in this video. S=seconds. CNS4, 100mg/kg, IP, treated 15-30min before shock, n=5 mice per group. The video file is uploaded as .mp4 file.

Supplementary Figure 2. Effect of CNS4 on electric shock-induced fear conditioning (FC) on male and female mice. On testing (day 2), mice underwent the same protocol but no shock. Freezing episode (**A**), duration (**B**), and latency (**C**), were collected from 300-360s duration of FC on testing day and were compared between saline (control) and CNS4 treated mice. Post hoc tests are marked as downward pointing edges and the Two-Way ANOVA comparison is marked as a straight line on top of the bars. Data points represent biological replicates, n=5 per group. Data presented as mean ± sem. ns= not significant, $p>0.05$; *=$p<0.05$.

Supplementary Figure 3. Pictures show the fecal pellets observed on FC testing day (day 2). Pictures are arranged in the following order: Control male 1-5, control female 1-5, CNS4 male 1-5, and CNS4 female 1-5. Figure uploaded as a single .pdf file.