5-2022

# Evaluation of Kappa Opioid Receptor Specific Compounds and Their Effect on PTSD-Like Behavioral Activity

Daniel Murnock

## Abstract

**Background:** Post-traumatic stress disorder (PTSD) is a complex and multifaceted neurological disorder that is characterized by intrusive thoughts, avoidance behaviors, alterations in cognition and mood, and alterations in arousal and reactivity. Current studies utilize stress paradigms to mimic behavioral symptoms and neurobiological changes associated with PTSD. The project aims to instead utilize the kappa opioid system and three distinct Kappa Opioid Receptor-specific compounds (Salvinorin A, U50,488, and nor-BNI) to induce these changes. Pharmacokinetic analysis of Salvinorin A and U50,488 revealed a time of peak plasma concentration (Tmax) of 0.25 hours in both groups. 3-Chamber Social Novelty testing revealed a significant decrease in sociability and social novelty preference in the Salvinorin A group. Elevated Plus Maze testing revealed a significant increase in anti-anxiety behavior in the U50,488 groups and a significant decrease in locomotion for both the Salvinorin A and U50,488 groups. Fear Conditioning showed a significant deficit in contextual fear memory for the Salvinorin A and U50,488 groups and no significant deficit in cued memory for any group.

**Importance:** Current PTSD paradigms require weeks of training and conditioning to elicit a PTSD-like phenotype. This comes at a cost of time and money to the researcher, and conditioning requires chronically subjecting the test subject to trauma. The development of a more acute and less invasive PTSD model would be beneficial as it would allow more research to be done in a shorter amount of time while providing a more humane experience for the animal. Furthermore, the development of a drug-induced model would provide better information on the biological mechanisms in play, and provide new pharmaceutical targets for treatment.

**Key Terms:** PTSD, Kappa opioid receptor, U50,488, Salvinorin A, nor-BNI, EPM, Fear Conditioning, 3-Chamber Social Interaction

MONTCLAIR STATE UNIVERSITY

# Evaluation of Kappa Opioid Receptor Specific Compounds and Their Effect on PTSD-Like Behavioral Activity

By

Daniel Murnock

A Master's Thesis Submitted to the Faculty of

Montclair State University

In Partial Fulfillment of the Requirements

For the Degree of

Master of Science

May 2022

College of Science and Mathematics

Department of Biology

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# EVALUATION OF KAPPA OPIOID RECEPTOR SPECIFIC COMPOUNDS AND THEIR EFFECT ON PTSD-LIKE BEHAVIORAL ACTIVITY

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Montclair, NJ

2022

# Acknowledgments

Dr. Elena Petroff, MSU for advisement and guidance

Dr. Vladislav Snitsarev for advisement and joining my thesis committee

Dr. Herman Fernandes, Psychogenics for advisement and joining my thesis committee

The Bonnie Lustigman Foundation for their generous donation

Biology Department, MSU, for providing funding and support

Psychogenic and Emer Leahy for their generous donation of mice, as well as for use of their vivarium

Psychogenics Animal Care Staff for providing husbandry to my mice

Dr. David Budac and Rebecca Peltz of Psychogenics for conducting bioanalysis

Christina Leahy and Dr. Leslie Diaz for help with IACUC writing and approval

Courtney Luing for assistance with preparing and organizing documentation for use of Psychogenics as a location for thesis research

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# Tables and Figures

# Kappa Opioid Receptor-Specific Compounds and Their Effect on PTSD-Like Behavioral Activity

## Background

The cause of most mental disorders is largely unknown; however, PTSD is an exception. PTSD is understood to manifest following exposure to a life-threatening, horrific, or as is its namesake – traumatic event. The DSM-V is the diagnostic and statistical manual of mental disorders and classifies PTSD as a trauma and stressor-related disorder (American Psychiatric Association 2013). Even with its recognition in the medical and scientific communities and the ever-expanding number of studies surrounding the disease, the cellular and molecular mechanisms behind its pathology need elucidation. There are four defined categories of symptoms for PTSD: intrusion, avoidance, alterations in cognition and mood, and alterations in arousal and reactivity. Human symptoms such as intrusive thoughts, dreams, and flashbacks cannot currently be induced in animal models however there do exist assays for quantifying symptoms such as fear memory, anxiety, and social avoidance. Most current animal projects involving PTSD focus on neurobiological stress pathways and the effect stress can have on the brain (Pitman et al. 2012). These models rely on using manipulations that researchers deem traumatic to induce a PTSD-like phenotype, such as predator exposure (PredEx), single prolonged stress (SPS), and foot shock tests (FS) (Verbitsky et al. 2020). This approach is lacking since it relies on the notion that the stress caused by these models is analogous to the stress that would cause PTSD in humans. While it is true that these tests have helped reveal some mechanisms involved in the neurobiology of PTSD such as abnormal ventromedial prefrontal cortex (vmPFC) glutamate levels and N-methyl-D-aspartate (NMDA) receptor levels in response to SPS as well as changes

in serotonin (5-HT) receptor expression levels in the amygdala in “stress-restress” models, they have not led to any practical pharmaceutical targets for the treatment of PTSD proper (Pitman et al. 2012). Therefore, it may be beneficial to search for a biological commonality between these typical PTSD symptoms, instead of looking broadly at stress when searching for PTSD biomarkers. Previous and current publications indicate that the kappa opioid receptor may be one of these commonalities and have shown it to have a relevant role in the molecular mechanisms underlying these symptoms (McLaughlin 2013; Roberto and Gilpin 2014; Verbitsky et al. 2020).

The kappa opioid receptor (KOR) is distinct from the other receptors in the opioid system. The endogenous ligand enkephalin which attenuates substance P in the spinal cord to inhibit afferent pain fibers (McLaughlin 2013) and endorphins which activate opiate receptors to produce analgesia (Endorphins….2021) both have a poor affinity for KORs, whereas the endogenous opioid dynorphin, which is generally associated with negative emotional states (Roberto and Gilpin 2014) has a high affinity for KORs and a low affinity for the other opioid receptors. It is widely thought to play an important role in the regulation and modulation of both reward and mood processes. Since PTSD is associated with negative changes in thinking and mood, this statement qualifies the KOR system as a good place to start when studying PTSD (Verbitsky et al. 2020).

The KOR is a G-protein coupled receptor (GPCR). Upon activation of the KOR by an endogenous ligand, the G-protein dissociates into two distinct subunits known as G-alpha and G-beta-gamma. These two subunits have been shown to modulate signaling through various pathways. One pathway is mediated by G-alpha-i, a further subunit of the G-alpha protein. In this case, the G-alpha-i dissociates from the G-beta-gamma subunit and directly interacts with the G-protein gated inwardly rectifying potassium (K+) channel (GIRK) Kir3. Activation of GPCRs

results in the hyperpolarization of neurons via GIRKs leading to self-inhibition of the neuron and the slowing of synaptic potentials (Lüscher and Slesinger 2010). GTP hydrolysis followed by the removal of the G-beta-gamma subunit from the channel results in channel deactivation. Further conductance-related pathways include calcium ($Ca^{2+}$) channel binding, where the G-beta-gamma subunit binds directly to the $Ca^{2+}$ channel. This leads to a reduction in $Ca^{2+}$ currents in the cell (Al-Hasani and Bruchas 2011). Furthermore, these G-proteins, namely the G-alpha-i and G-alpha-o subunits, have been shown to be responsible for inhibiting adenylyl cyclase as well as for activating other pathways downstream such as the beta-arrestin and ERK-MAPK pathways (Butelman and Kreek 2015). The analgesic response from KOR activation is thought to be mediated by this inhibition of adenylyl cyclase, and the more negative effects such as dysphoria are thought to stem from activation of MAPK and beta-arrestin pathways (Al-Hasani and Bruchas 2011). P38-MAPK activity following arrestin recruitment has also been shown to play a role in this dysphoric effect (White et al. 2014). It is important to note that activation of the KOR can lead to a phenomenon known as receptor internalization where the receptor is endocytosed. This mechanism is used to import ligands into the cell (Kaufman and Popolo 2018). This happens via phosphorylation of signal-regulated and receptor kinases (Appleyard et al. 1999; McLaughlin et al. 2003; McLaughlin et al. 2004; Al-Hasani and Bruchas 2011). Extracellular signal-regulated protein kinase 1 and 2 (ERK1 and ERK2) are phosphorylated during both an early (15 minutes) and late period (2 hours). The early phase is arrestin-independent and is mediated by G-beta-gamma, whereas the late phase is arrestin-dependent and arrestin-3 is required (Al-Hasani and Bruchas 2011). Other pathways of KOR-related ERK1 and 2 activations involve Phosphoinositide 3-kinases, protein kinase 3-zeta, as well as intracellular $Ca^{2+}$ (Al-Hasani and Bruchas 2011). In relation to the MAPK pathway, there is also KOR-related

manipulation of c-Jun N-terminal kinase (JNK) which is part of the MAPK pathway and is involved in stress-related signaling (Yarza et al. 2016). When the beta-arrestin-dependent p38 MAPK pathway is activated it has been shown to cause aversion, but when JNK is activated without activating arrestin then long-lasting inactivation of KOR signaling occurs (Mores et al. 2019). In summation, there are two distinct pathways. One has been shown to inhibit adenylyl cyclase and phosphorylate ERK1 and 2 during the early phase of agonism. The other has been shown to activate MAPK pathways and recruit arrestins. Both pathways are mediated by G-protein-related signaling. This type of signaling can change based on the ligand, leading to different results following activation of the same receptor by different ligands. This encompasses the current understanding of KOR signaling pathways in general.

Salvinorin A, U50,488, and nor-BNI were chosen for this study due to the uniqueness of their mechanisms in relation to each other. Salvinorin A and U50,488 were chosen as agonists, however, they do not function in the same way. Salvinorin A is unique in that it is a non-nitrogenous neoclerodane, which is completely unlike other known opioid ligands (Butelman and Kreek 2015). Salvinorin A-related aversion and anhedonia have been shown to not be mediated by beta-arrestin 2, whereas U50,488-mediated aversion and dysphoria are attributed to arrestin recruitment followed by p38 MAPK pathway activation (White et al. 2014). U50,488 was chosen also because it has similar mechanisms to the endogenous dynorphin, in that they promote phosphorylation to the same extent (Allouche et al. 2014) as well as activate JNK in a G-alpha-i manner (Al-Hasani and Bruchas 2011). It is also worth noting that when evaluated in the peripheral nerve system, Salvinorin A increased JNK activation and U50,488 increased ERK activation. This was specific to each compound and the reverse was not true (Bedini et al. 2020). About nor-BNI, this compound was chosen due to its antagonistic effect. Nor-BNI was shown to

cause JNK phosphorylation and initiation of G-protein uncoupling which blocked G-alpha-i mediated transduction (Al-Hasani and Bruchas 2011) This compound was also shown to block inhibition of adenylyl cyclase in a JNK-dependent manner, however did not block activation of ERK (Jamshidi et al. 2016). A more granular description of the mechanisms at play have not been elucidated, however, the pathway is distinct, and the behavioral effects of the compound are well studied, both of which led to this compound being chosen for the study.

PTSD symptoms of interest for this study include fear and fear memory, anxiety, social avoidance, and preference for social novelty. Studies have provided evidence for the KOR system's role in despair responses in the presence of stressors as well as its ability to mediate the neurobiological effects of stress (Lalanne et al. 2014). These experiments established the activation of KORs as having an anti-rewarding effect, where dopamine (DA) is inhibited, and dysphoria is induced (Bruijnzeel 2009). These findings support the idea that KORs are involved in mood disorders, and more recent studies have helped to strengthen that idea. The pro-depressive effect of KOR activation in rodents and humans is widely known and although the current study does not focus on depression, this idea is important for establishing a role for KORs in mood disorders in general. Furthermore, changes in social behavior are a symptom of both depression and PTSD, and this study showed that chronic KOR activation by U50,488, a KOR agonist, caused a significant decrease in social behavior, and a combined treatment of U50,488 and nor-BNI, a long-acting non-competitive KOR antagonist, rescued the sociable phenotype (Dogra et al. 2016). A study on anxiety reported that the anxiogenic properties of stress are encoded by the endogenous KOR-specific peptide dynorphin acting in the basolateral amygdala. This was discovered by using a phospho-specific antibody KORp that identified where dynorphin was, which was possible because activated KORs are phosphorylated. This

experiment also used elevated plus maze to measure anxiety with pretreatments of corticotropin-releasing factor, U50,488, and nor-BNI. U50,488 caused a significant increase in anxiety-like behavior and a combined treatment of corticotropin-releasing factor (CRF) and nor-BNI rescued the non-anxious phenotype (Bruchas et al. 2009). KORs have also been shown to play a role in the formation and extinction of fear memories. Researchers were able to show that KOR signaling encodes the aversive emotional component of the stress-related event, and it does so by recalling stress-related memories. (Bruchas et al., 2007). In later and related studies, it was shown that dynorphin-mediated KOR activation brings about the aversive component of the stress situation (Land et al., 2009). Studies involving fear conditioning and the agonism of the KOR system seem to be scarce, but one study explored the involvement of prodynorphin (PDYN) and KOR antagonism. There, PDYN null mice showed a significant lack of extinction of the fear response when compared to the wild type. Furthermore, when wild-type mice were pretreated with nor-BNI after the first extinction session, there was a complete blockage of the extinction of fear memory in subsequent trials (Bilkei-Gorzo et al. 2012). Another showed when U50,488 was administered 15 minutes before the 1-day post-training test during fear conditioning, contextual and conditional stimulus-induced freezing were both significantly increased (Vunck et al. 2011). On the other hand, inhibition of the KOR system has been shown to significantly reduce aversion after stress tests. Researchers conducted a study where mice were taught to associate an odorant with stress in multiple paradigms and found that aversion was present in the no-stress group, but aversion to the odorant was not significant in the PDNY knock-out (KO) and nor-BNI (KOR antagonist) groups. They also showed that aversion was dose-dependent in the U50,488 (KOR agonist) group (Land et al. 2008). It is worth noting here that for the present study, U50,488 and nor-BNI were chosen due to their specificity and the

breadth of their use in research involving KORs. Salvinorin A was chosen as a second agonist group in this study due to its strong specificity for KORs and its novelty in the context of its research, its potency, and potential as a therapeutic for diseases involving perceptual distortions (Roth et al. 2002).

Now that a link between the KOR system and those behaviors has been established, it is necessary to link those behaviors to specific brain structures. The quantifiable PTSD behaviors of interest including fear memory, anxiety, and social avoidance have been widely studied in animal and human models, and the structures associated with them are important for research into PTSD. The fear response has been shown to begin in the amygdala which is responsible for the assessment of fear stimuli and regulation of anxiety (Ressler 2010). It has also been shown that social avoidance is a fear response, and therefore stems from the amygdala (Gellner et al. 2021). The prefrontal cortex (PFC) has been associated with fear extinction and implicated in the regulation of emotional behavior via top-down control of the basolateral amygdala (McGarry and Carter 2017). Furthermore, the hippocampus has been shown to be the memory center of the brain, and in this context is responsible for fear memory. The thalamus is also hypothesized to have a role in PTSD since it has been proposed that the thalamus plays an important role in dissociative-like states of altered consciousness that are seen in a subtype of PTSD known as D-PTSD (Krause-Utz et al. 2017). Previous research has shown that the mediodorsal thalamus is densely interconnected with the PFC (Bolkan et al. 2017), and since abnormalities in the PFC are commonly seen in patients with PTSD it would be wise to examine whether the thalamus is also involved. Neuroimaging studies of PTSD patients describe highly replicated abnormalities in the hippocampi and ventromedial prefrontal cortices (vmPFC) of PTSD patients as well as significantly heightened amygdalar activation during the acquisition of conditioned fear (Pitman

et al. 2012). In one study maltreated youth with PTSD, when compared to controls, showed significantly smaller left amygdalar and right hippocampal volumes as well as a trend for smaller vmPFC volume. In another larger meta-analysis of brain structure volumes of patients with PTSD, there were significant reductions in hippocampal volume. This same paper highlights positron emission tomography (PET) and single-photon emission tomography (SPECT) studies which showed alterations in cerebral blood flow at rest in the medial prefrontal, temporal, and dorsolateral prefrontal cortices, cerebellum, and amygdala. Other studies utilizing traumatic reminder exposure in PTSD patients showed a failure to activate the medial PFC as well as decreased function in the hippocampus and thalamus and increased function in the amygdala and parahippocampal gyrus (Bremner 2007).

With current data suggesting that the KOR modulates overlapping neuronal networks which link brainstem monoaminergic (serotonergic (5-HT+) and dopaminergic (TH+)) (Izzi and Charron 2013) nuclei with forebrain limbic structures, it stands to reason that modulation of limbic system function by 5-HT- and DA-KOR mediated signaling is a critical area of research (Lalanne et al. 2014). A study by Spanagel et. al. showed that infusion of a KOR agonist into the nucleus accumbens (NAc) decreased DA release in this region (Spanagel et. al. 1991). Another study by Bals-Kubik et. al. was able to show that local infusions of a KOR agonist into the PFC, lateral hypothalamus, and ventral tegmental area (VTA) induced a robust chronic place aversion which implies that KOR activation in these regions decreases DA release (Bals-Kubik et al. 1993). These two studies as well as the previously established relationship between KORs and DA provide enough evidence to warrant an investigation into DA release when studying the KOR system. It is also worth noting that the amygdala, thalamus, and hippocampus are limbic structures, which lends to their potential as players in this study. With the overlapping links

between PTSD, KORs, the behaviors of interest and their respective brain structures, and the role of DA properly established, there is now enough evidence to begin research into whether KOR-specific compounds are behaviorally active concerning PTSD-like symptoms.

## Animals

Male C57BL/6J mice were ordered (The Jackson Laboratory, Bar Harbor, ME) and given food and water ad libitum on a 12-h light/dark (7 a.m./7 p.m.) cycle. All mice were mature adult mice aged 14 weeks old +/- 4 days at the duration of their testing to ensure full maturation of biological processes and structures. Colony rooms and cage supplies were provided by Psychogenics, and animal husbandry was conducted by the Animal Care Staff at Psychogenics. 80 of these mice were a gift from Psychogenics and were ordered in the same fashion. Four mice from each treatment group were group-housed in Opti-MICE cages and six mice from each treatment group were single-housed in Opti-MICE cages. Animals were habituated in the testing room for at least 1 hour before experimental procedures, except for the cued fear conditioning test where animals were habituated in an adjacent room for 1 hour before. The room temperature was maintained between 20 and 23 degrees Celsius with a relative humidity maintained at around 50%. Mice that were collected for pharmacokinetic analysis and brain samples were sacrificed with live decapitation via guillotine. Mice still alive at the end of the study were euthanized by CO2 asphyxiation followed by cervical dislocation. 24 mice were used for pharmacokinetic analysis, 120 mice for behavioral testing- 20 of which were collected immediately following the elevated plus maze (EPM) to look at DA levels in specific brain tissues, and 4 mice for social interaction/ social novelty testing, Pharmacokinetic subjects were subdivided into 8 subgroups, n=3, to establish the most efficacious pretreatment window for U50,488 and Salvinorin A which was determined by Tmax data. Behavioral subjects were

subdivided so that each animal underwent one behavioral assay; with N=40 C57BL/6J naïve mice were assigned to the EPM to assess anxiety, Fear Conditioning (FC) to assess fear memory, and three-chambered social interaction (SI) for changes in social interaction and social novelty. For the SI assay, an additional naïve N=4 CH3/HeJ male mice were enrolled as "stimulus mice." All animals were naïve for testing. Animals were dosed one at a time intraperitoneally (IP) at a 10mL/kg dose volume. All animals were euthanized within 24 hours upon testing completion. All experiments were carried out following Psychogenics' Institutional Animal Care and Use Committee (IACUC) protocols which were further approved by Montclair State University.

## Drugs

(−)-trans-(1S,2S)-U-50488 hydrochloride hydrate (U50,488), Salvinorin A, and nor-Binaltorphimine dihydrochloride (nor-BNI) were ordered from Sigma-Aldrich. All drugs were administered intraperitoneally at 10ml/kg. All vehicles were saline, except for Salvinorin A used DMSO, tween80, and saline in a 1:1:8 ratio. Pretreatment times were determined to be the following: Saline – 15 min., Salvinorin A (1mg/kg) – 15 min., U50,488 (2mg/kg) – 15 min., nor-BNI (10mg/kg) – 60 min.

## Methods

### Pharmacokinetics

N=24 mice were divided into 8 groups. 4 groups were dosed with U50,488 (2mg/kg) and collected at 15, 30, 45, and 60 minutes respectively, and 4 groups were dosed with Salvinorin A (1mg/kg) and collected in the same fashion. For terminal collection, mice were euthanized via live decapitation and trunk blood (~500ul) was collected in EDTA-K2 coated tubes and stored on wet ice for no more than 15 minutes. Blood was centrifuged at 10,000g for 10 minutes at 4degC.

At least 100ul of plasma was pipetted into a 1.5ml Eppendorf tube for analysis. Whole brains were collected, weighed, rinsed with PBS, and frozen immediately on a cold plate before placing in a specimen container on dry ice. Whole brains and plasma were placed on dry ice, then stored at -80degC before analysis. Pharmacokinetic procedures were conducted by Dr. David Budak and Rebecca Peltz of Psychogenics, and analysis was completed by Dr. Andrew Aschenbrenner of Psychogenics.

For analysis, frozen 50-100 µL plasma aliquots were thawed on wet ice over 30 minutes. Once thawed, a 25 µL volume of plasma was removed and dispensed into inserts containing 150 µL of acetonitrile with internal standard. The resultant solutions were centrifuged for 20 minutes at 2688 g while maintaining a temperature of 4degC. Three aliquots of the sample supernatant (30 µL) were then separately mixed with 70 µL of water (MilliQ) in fresh wells of a QuanRecovery plate (Waters Corp) and placed onto the UPLC-MS/MS system for analysis. In addition, standards, quality controls (QC), and blanks, all utilizing mouse plasma (C57BL/6 Mouse male plasma from BioIVT) were prepared in the same manner as the samples and added for quantification of the samples ensuring a robust analysis.

Pre-weighed whole brain tissue was homogenized (Omni Prep multichannel tissue homogenizer) using a 0.2% acetic acid water (MilliQ) mixture which was added to the brain using a 4:1 solution to brain ratio. Subsequently, the homogenate was centrifuged for 10 min at 4degC at a speed of 2688 g. Three 25µL aliquots out of the top layer of the centrifuged homogenate were crashed into three QuanRecovery wells, each containing 150 µL of acetonitrile/internal standard. The crash was then centrifuged for 20 minutes at 2688 g while maintaining a temperature of 4degC. Mixing of the resultant supernatant with water provided samples for triplicate analysis. Brain standards, QCs, and blanks were prepared using a male Mouse C57BL/6 brain acquired

from BioIVT using the same protocol outlined above. All standards, QCs, samples, and blanks were then loaded onto the UPLC-MS/MS for analysis.

**UPLC-MS/MS Conditions**

| | |
|---|---|
| **Instrument** | Waters Xevo TQ-XS coupled to an Acquity i-Class Plus UPLC setup |
| **Detection** | Positive Electrospray ionization with fragmentation and MRM scans |
| **Transition** | 369.3 Da to 72.98 and 266.97 Da |
| **Column** | ODS 3 Inertsil C18 column 2.1 x 50 mm, 2 µm (GL Sciences 5020-84652) |
| **LC Conditions** | Gradient 0.5 mL/min, 2 min run, injection volume 5 µL, column temp. 40degC, pre-column temp. 40degC, sample temp. 4degC |
| **Mobile Phase** | A: 15mM Ammonium Acetate/water, B: 15mM Ammonium Acetate/5% Water/Acetonitrile |
| **Software** | Masslynx V4.2 SCN1012 for instrument control and data acquisition |

The range of quantitation was 0.1 to 10,000 ng/mL for plasma and brain.

**Elevated Plus Maze (EPM)**

The automated maze consists of two closed arms (high x wide x length: 15 x 6 x 30 cm) and two open arms (high x wide x length: 1 x 6 x 30 cm) forming a cross, with a square center platform (6 x 6 cm). The open arms have a 1 cm rim along each side. All visible surfaces are made of black acrylic. Each arm of the maze is placed on a support column 50 cm above the floor. The EPM is surrounded by a black plastic curtain. Animals are brought to the experimental room at

least 1 hour before the test in the home cages (food and water available). Lighting in the testing arena was measured to be 15 lux. One animal at a time is evaluated on the EPM for a 5 min test, and placed in the center (Walf and Frye 2007).

**Fear Conditioning (FC)**

**Contextual Fear Conditioning and Testing:** To assess contextual and cued (tone) learning and memory, we use a standardized contextual fear conditioning task developed for the evaluation of memory in mice (Bourtchouladze, et al., 1994). For contextual conditioning, on day 1 mice were placed into the conditioning chambers to habituate to the context for 120 sec where they were exposed to three 20-second 80 dB tones (conditioned stimulus, CS) spaced 100 seconds apart. 15 seconds after each tone; mice received a foot shock (0.5mA for 1 sec), the unconditioned stimulus (US). The mouse remained in the conditioning chamber for another 60 sec and then was returned to its home cage. 24 hours after training the mice were evaluated for contextual memory where they were placed into the same chamber that they were trained in for 5 min without shock or any other interference. Contextual memory was evaluated by measuring the percent of the time the mouse spends emitting the conditioned response, in this case, freezing behavior. Freezing is defined as the complete lack of movement except that required for respiration (Phillips & LeDoux, 1992; Bourtchouladze, et al., 1994). After each experimental subject, the experimental apparatus was thoroughly cleaned with 70% ethanol.

**Cued Conditioning and Testing:** 24 hours after contextual FC, animals are evaluated for cued memory. Mice are placed in a novel context for 2 min (Pre-Cue). Then the CS (80dB tone) will be presented for 3 minutes. To ensure the chamber was novel the flooring material was changed, as was the lighting and the background of the chamber. A novel odorant (McCormick orange extract) was used to distinguish the novel from the trained context. Instead of 70% ethanol,

Nolvasan was used to clean the chambers. Mice were scored for "freezing" with automated software as for contextual conditioning described above. The proceeding of each experiment was videotaped for analysis.

**Equipment and Maintenance:** The Coulbourne fear conditioning mouse chambers are 7" W x 7" D x 12" H and a camera is mounted on the ceiling of the chamber. The FC chambers are contained in sound-attenuating chambers. All the freezing response information is processed by automated software. Fear conditioning chambers are calibrated before each experiment and the shock and sound intensity are assessed to ensure that the maximum approved value is not exceeded. The experimental chambers are thoroughly cleaned with 70% ethanol or Nolvasan, dried, and ventilated for a few minutes between subjects.

**Social Interaction (SI) Test Three Chamber Method in Mice**

Procedures and apparatus are based on those described in Nalder et al. The apparatus is a clear Plexiglas chamber with 2 sliding doors (5x8 cm). Before testing, lux is measured to be between 13-15 in each chamber. The choice test has three 10-min phases: (1) Habituation – the test mouse is first placed in the middle chamber and allowed to explore, with the doorways into the two side chambers open. (2) Sociability – after the habituation period, the test mouse is enclosed in the center compartment of the social test box, and an unfamiliar mouse (stranger 1) is enclosed in a wire cage placed in a side chamber and an empty wire cage is placed on the opposite side. To ensure preference for one side of the chamber does not influence results, the location for stranger 1 alternates between the left and the right sides of the social test box across subjects. Following the placement of stranger 1, the doors are reopened, and the subject is allowed to explore the entire social test box. Measures are taken of the amount of time spent in each chamber and the number of entries into each chamber by the Noldus tracking system (Nadler et al. 2004).

**Preference for Social Novelty**

At the end of the sociability test, each mouse is further evaluated for preference to spend time with a new stranger. A new unfamiliar mouse is placed in the wire cage that had been empty during the previous session. The test mouse then has a choice between the first, already-investigated mouse (stranger 1) and the novel unfamiliar mouse (stranger 2). The same measures are taken as with the sociability test (Kaidanovich-Beilin et al. 2011).

**Behavioral Analysis**

Statistical analysis and graphs were completed using GraphPad Prism 9. For 3-Chamber testing of Sociability and Social Novelty, a two-way ANOVA was conducted to assess differences across treatment groups in interest (time spent sniffing), chamber preference (total time spent in each chamber), and chamber entries. To determine if mice presented with normal preference for sociability and social novelty, an unpaired two-tailed t-test of "time spent sniffing" was utilized. Locomotion was assessed with an ordinary one-way ANOVA. For Elevated Plus Maze, a one-way ANOVA was used to examine the percent time spent in and percent entries into the open arms, as well as total arm entries. A Brown-Forsythe test was used to examine the total distance traveled. A two-way ANOVA was used to look for a relationship between open/closed arms and treatment for distance and entries. For Fear Conditioning, all data underwent log transformation followed by a one-way ANOVA. All one-way or two-way ANOVAs were followed by an Uncorrected Fisher's LSD test for multiple comparisons. Brown-Forsythe tests were used when the standard deviations were significantly different, and the data failed the D'Agostino & Pearson test for normality indicating the data was non-Gaussian. All Brown-Forsythe tests were followed by an unpaired t-test with Welch's correction for multiple comparisons. Post-hoc tests compared each treatment group to the vehicle group.

# Results

## Pharmacokinetic Analysis

A non-compartmental analysis was performed on plasma and brain levels for the determination of pharmacokinetic parameters using gPKPDSim, a MATLAB-based graphical user interface (GUI) application (Dogra et al. 2016; Hosseini et al. 2020).

Pharmacokinetic Definitions:

| | |
|---|---|
| $AUC_{0-24}$ | The area under the concentration-time curve from 0-24 h |
| $AUC_{0-\infty}$ | The area under the concentration-time curve from 0 to infinity |
| Cmax | The maximum concentration over the duration of the study |
| Tmax | Time at which the maximum concentration occurs |
| t½ | The terminal half-life |

U50,488 Parameters:

| **Parameter** | **IP** |
|---|---|
| **Nominal dose (mg/kg)** | 2 mg/kg |
| **Cmax (µM)** | 0.7 |
| **Tmax (h)** | 0.25 |
| **t1/2 (h)** | 0.63 |
| **$AUC_{0-24}$ (min.µM)** | 34 |
| **$AUC_{0-\infty}$ (min.µM)** | 49 |

*Table 1: U50,488 parameter table indicating a Tmax of 0.25 hours.*

Salvinorin A Parameters:

| **Parameter** | **IP** |
|---|---|
| **Nominal dose (mg/kg)** | 1 mg/kg |
| **Cmax (µM)** | 0.4 |
| **Tmax (h)** | 0.25 |
| **t1/2 (h)** | 0.24 |
| **AUC $_{0-24}$ (min.µM)** | 38 |
| **AUC $_{0-\infty}$ (min.µM)** | 39 |

*Table 2: Salvinorin A parameter table indicating a Tmax of 0.25 hours.*

A B

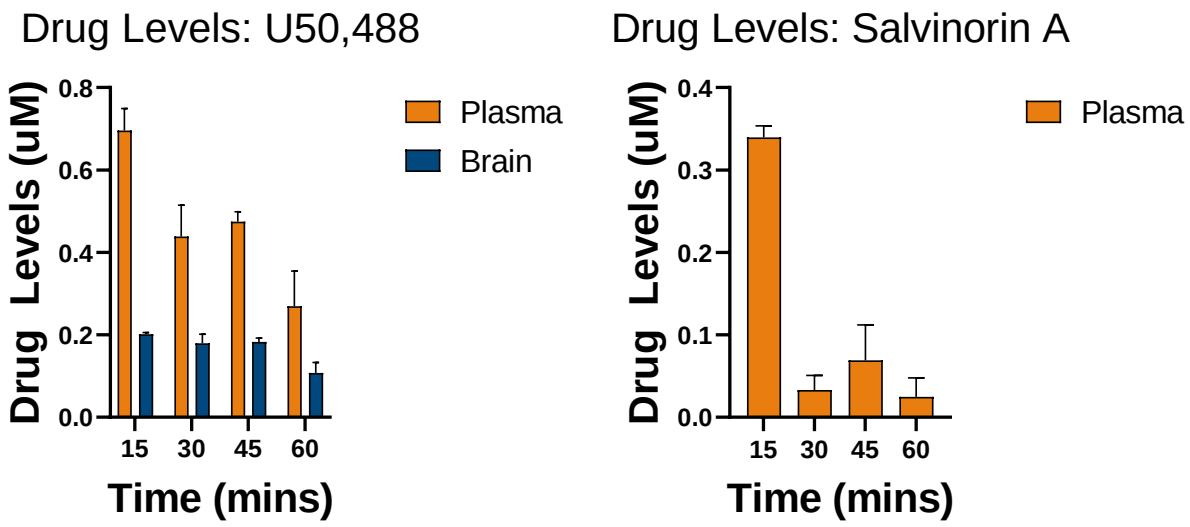


*Figure 1: Pharmacokinetic Drug Levels (1A and B: Drug levels over time for U50,488. N=3 for each time point. SEM plotted.*

Both U50,488 and Salvinorin A showed a Tmax of 0.25 as indicated by the analysis of drug in the plasma (and brain for U50,488) levels over time. Drug levels in the brains of the Salvinorin A treated mice were undetectable at all four time points.

**3-Chamber Social Interaction**

For the sociability aspect of this assay, we recorded time spent sniffing the stranger mouse (denoted as Mouse) as well as time spent sniffing the empty wire cup (denoted as Object). Time spent in as well as entries into each chamber (denoted as Mouse, Center, and Object) were also recorded. For the social novelty aspect, we recorded time spent sniffing the known mouse (denoted as Known) as well as time spent sniffing the novel mouse (denoted as Novel). Time spent in as well as entries into each chamber (denoted as Known, Center, Novel). For both tests, distance traveled was also recorded.

All four groups showed significant sociability ($p$=<0.001) for all four treatment groups (Fig. 2A-D), indicating significant and robust interest in interacting with the mouse over the object. For all three analyses, there was a significant interaction between Treatment x Chamber ($p$=<0.0001 for sniffing and chamber preference, $p$= 0.0415 for entries) indicating the treatment and the chamber both influenced sniffing, chamber preference, and entries. For time spent sniffing (Fig. 3), post-hoc analysis shows a significant decrease in interest for the mouse ($p$=0.0029) for the Salvinorin A group as well as a significant decrease in interest for the object ($p$= 0.0028) for the nor-BNI group. For chamber preference (Fig. 4), posthoc analysis indicates that there was a significant increase ($p$=0.0034) in the U50,488 group for time spent in the mouse chamber, as well as a significant decrease ($p$=0.0185) in time spent with the object. For chamber entries (Fig. 5), there was as a significant decrease in entries to Mouse, Center, and Object chambers for the Salvinorin A (Mouse $p$=0.0030, Center $p$=<0.0001, Object $p$=< 0.0067) and U50,488 (Mouse $p$=0.0006,

Center $p$=<0.0001, Object $p$=< 0.0005) groups. Locomotion was also assessed (Fig. 6) and results showed locomotion was significantly decreased ($p$= <0.0001, $p$= <0.0001) compared to the control in both Salvinorin A and U50,488 groups.

For Social Novelty testing, there was a significant preference for social novelty in the vehicle ($p$=0.0012) and the Salvinorin A ($p$=0.0401) groups. (Fig. 7A-D), For time spent sniffing (Fig. 8), there was no significant interaction effect, however, there were significant chamber ($p$=0.0027) and treatment ($p$=0.0182) effects. Post-hoc analysis showed a significant decrease ($p$=0.0025) for the Salvinorin A group in preference for the novel mouse as well as a significant decrease in time spent sniffing overall in both the Salvinorin A ($p$=0.0030) and nor-BNI (0.0213) groups. There was no significant interaction or treatment effect when analyzing chamber preference (Fig. 9), indicating that no group differed in their chamber preference, and all similarly avoided the center chamber. There was a significant interaction between Treatment x Chamber for Entries (Fig. 10) ($p$=0.0115) indicating the differences between treatments were inconsistent between chambers. This test showed that U50,488 and nor-BNI groups completed significantly fewer entries into the Known chamber ($p$=0.0073, $p$=0.0052 respectively), while Salvinorin A and U50,488 completed significantly fewer entries into the Center and Novel chambers (Center $p$=0.0006, $p$=0.0010; Novel $p$=0.0039, $p$=<0.0001 respectively). Locomotion was also assessed, and results showed locomotion (Fig. 11) was significantly decreased ($p$= <0.0001, $p$= <0.0001) compared to the control in both Salvinorin A and U50,488 groups.

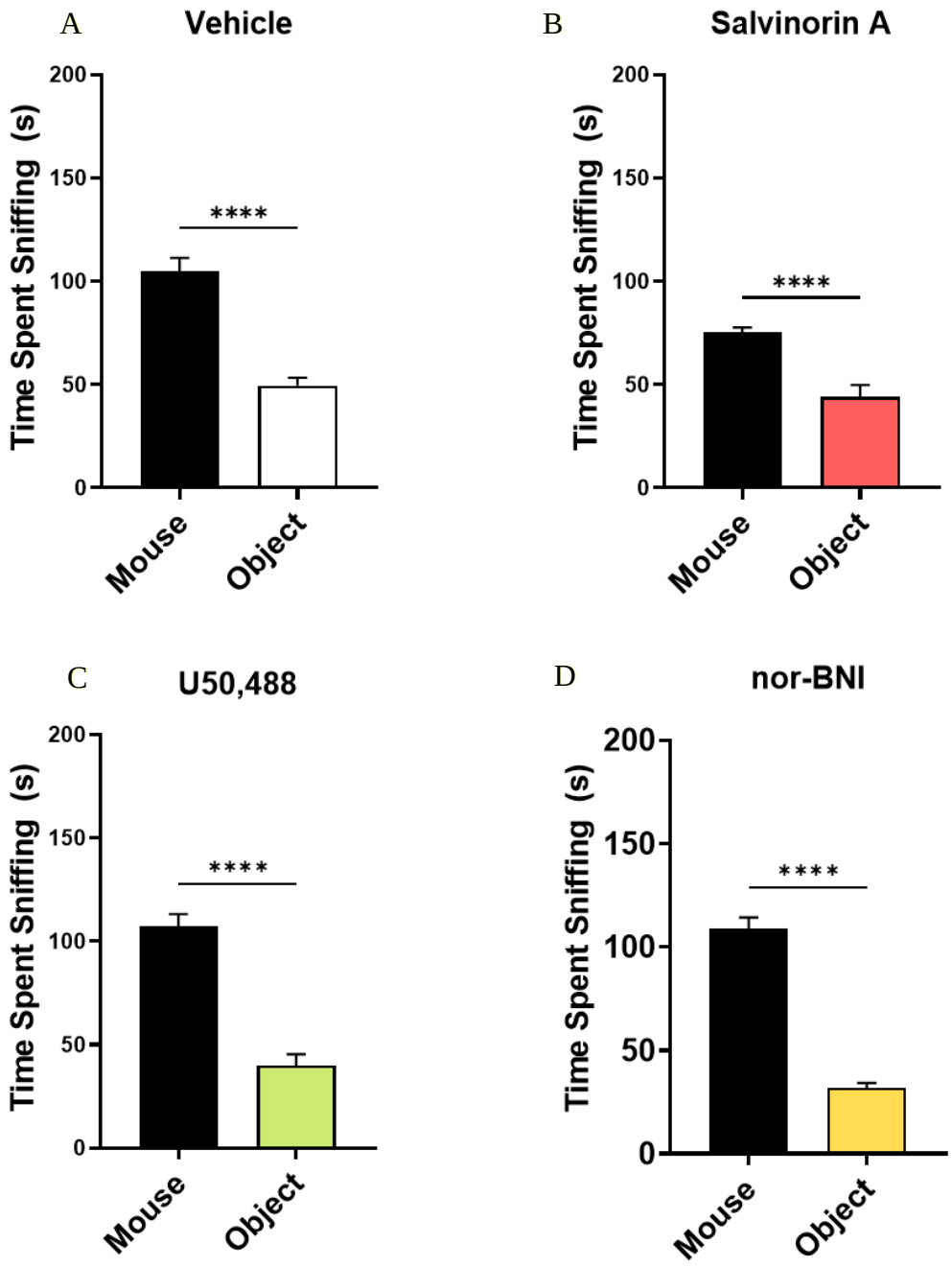


*Figure 2: 2A, Sociability: Vehicle sociability p=<0.0001; 2B, Salvinorin A sociability p=<0.0001; 2C, U50,488 sociability p=<0.0001, nor-BNI sociability p=<0.0001*

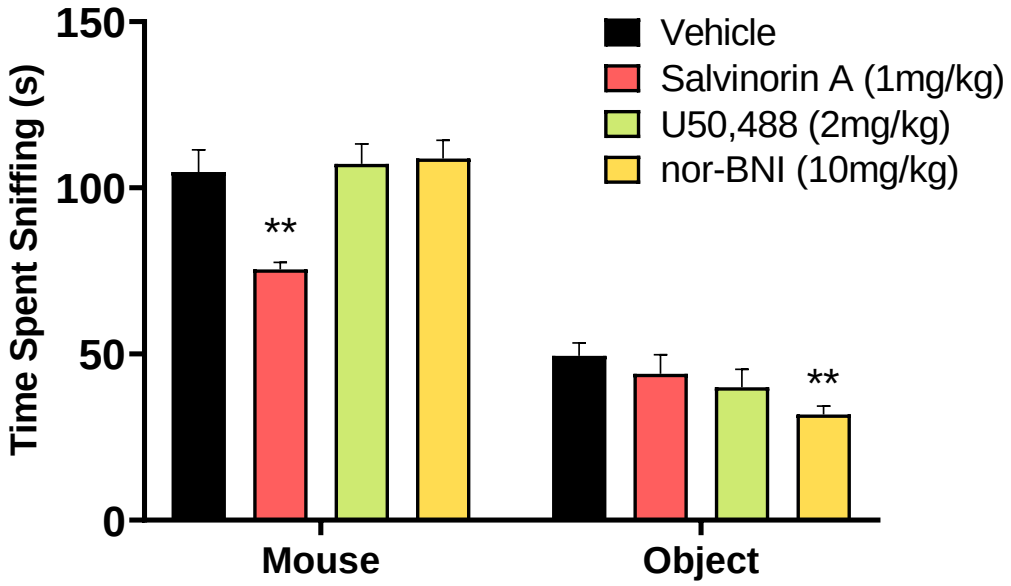


*Figure 3: Time Spent Sniffing (Sociability): Mouse; Salvinorin A p<0.005, Object; nor-BNI p<0.005 compared to vehicle-treated animals*

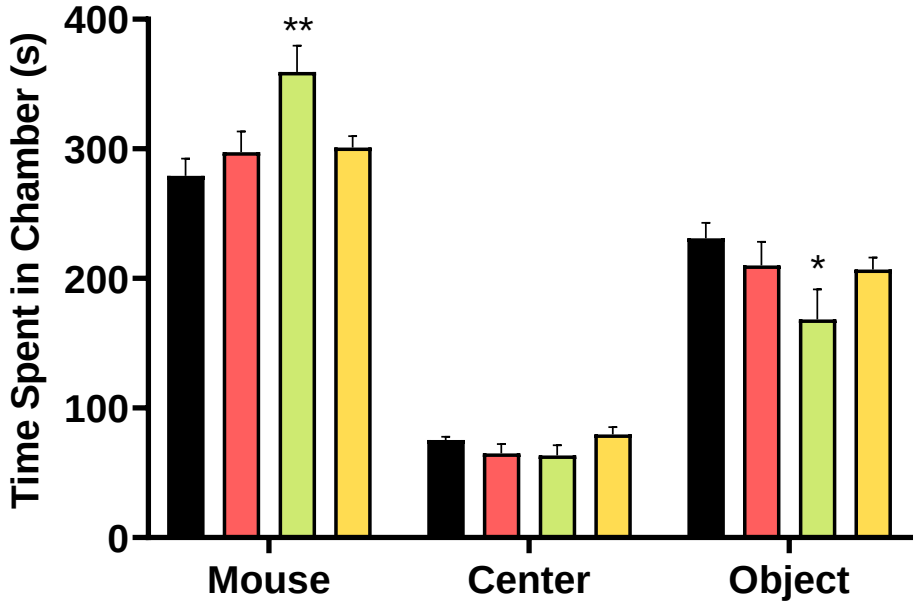


*Figure 4: Time Spent in Chamber (Sociability): Mouse; U50,488 p<0.005, Object; U50,488 p<0.05 compared to vehicle treated animals*

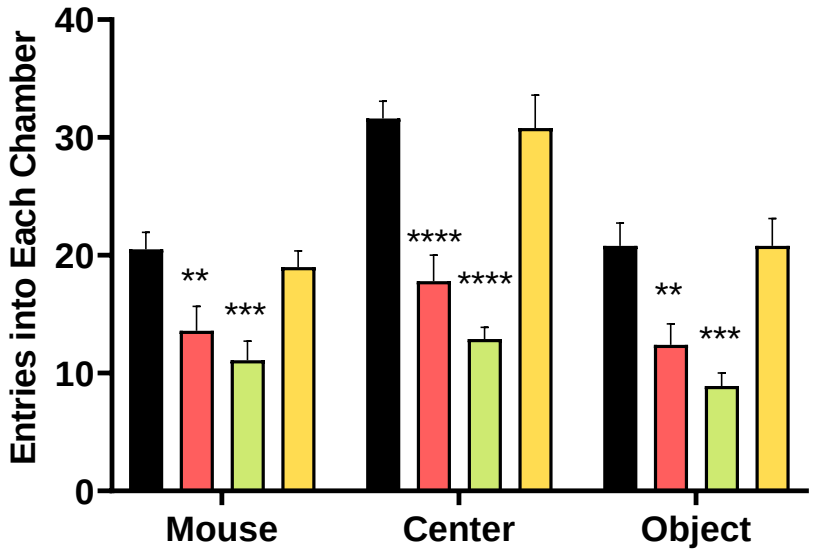


*Figure 5: Entries Into Each Chamber (Sociability): Mouse; Salvinorin A p<0.005, U50,488 p<0.0005; Center; Salvinorin A p<0.0001, U50,488 p<0.0001; Object; Salvinorin A p<0.005, U50,488 p<0.0005 compared to vehicle treated animals*

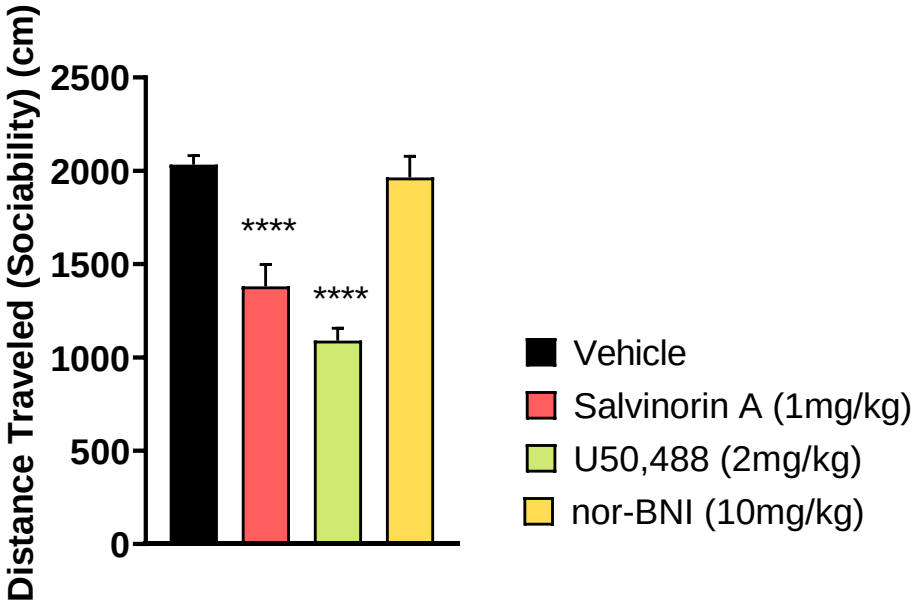


*Figure 6: Distance Traveled (Sociability): Salvinorin A p<0.0001, U50,488 p<0.0001 when compared to vehicle-treated animals*

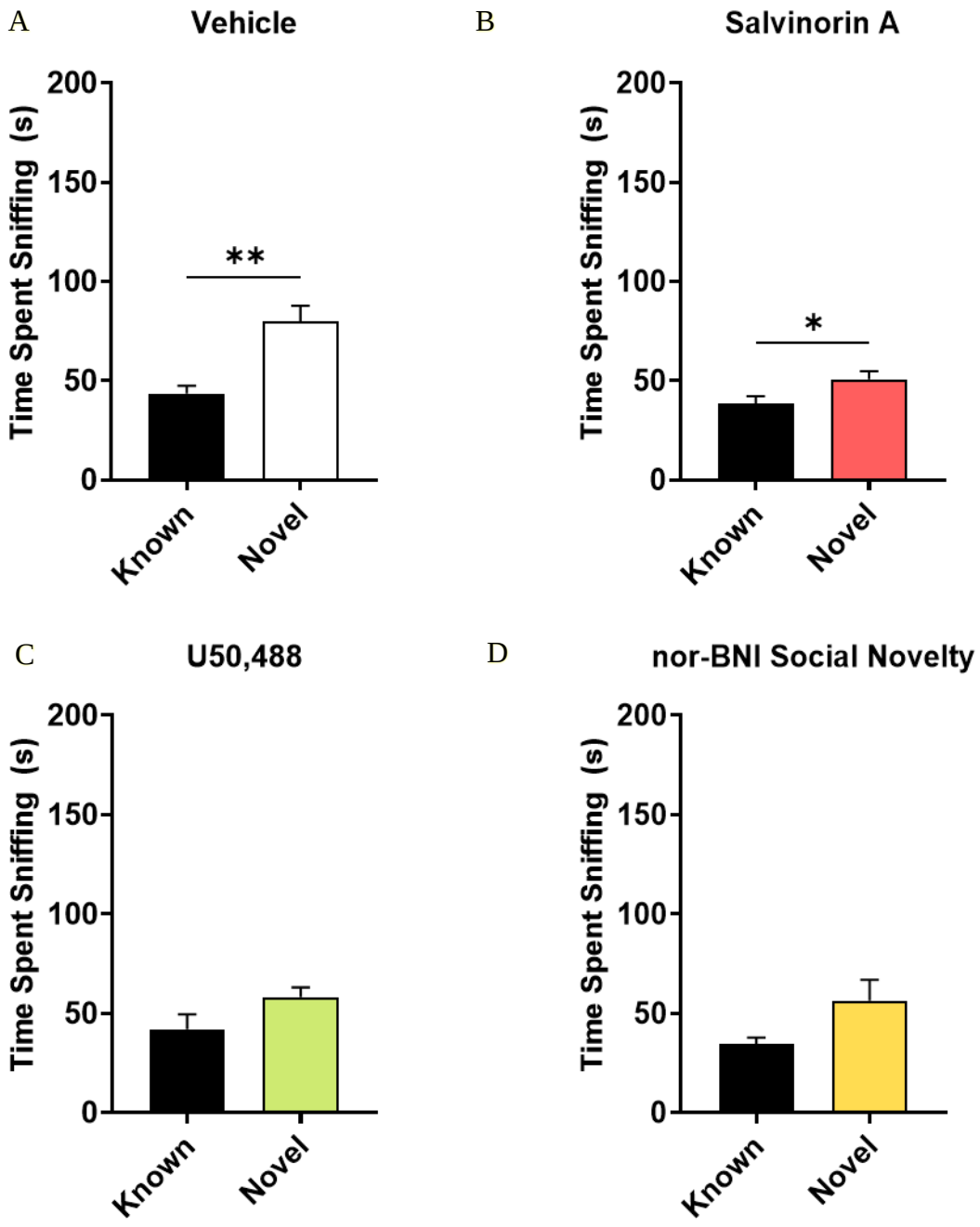


*Figure 7: 7A; Social Novelty: Vehicle Social Novelty, $p<0.005$, 7B; Salvinorin A Social Novelty, $p<0.05$*

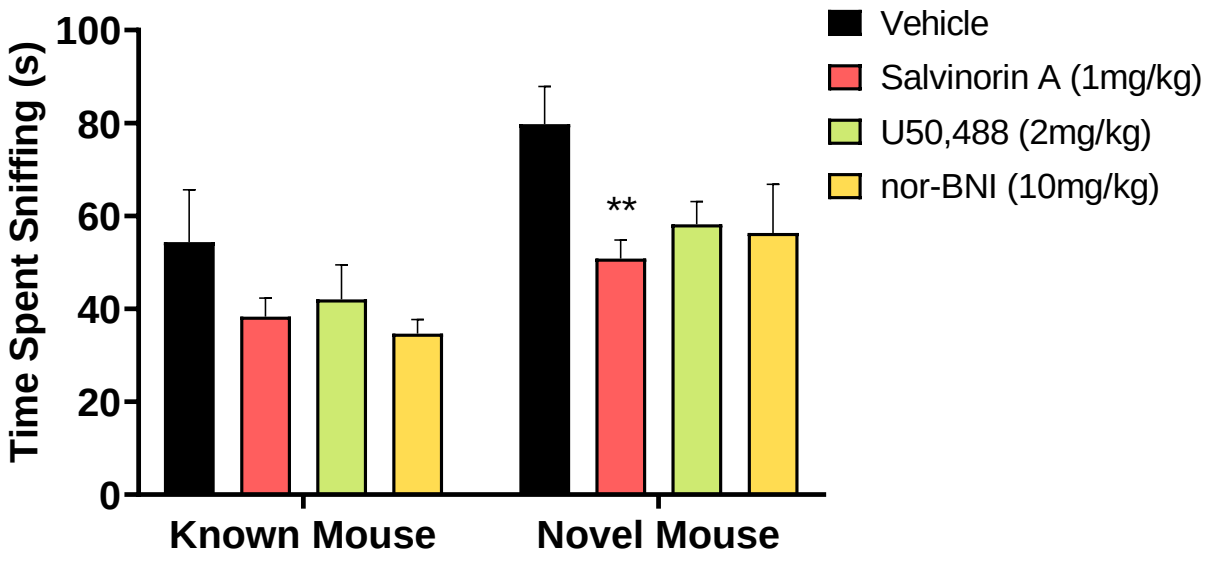


*Figure 8: Time Spent Sniffing (Novelty): Salvinorin A $p<0.005$ compared to vehicle-treated animals*

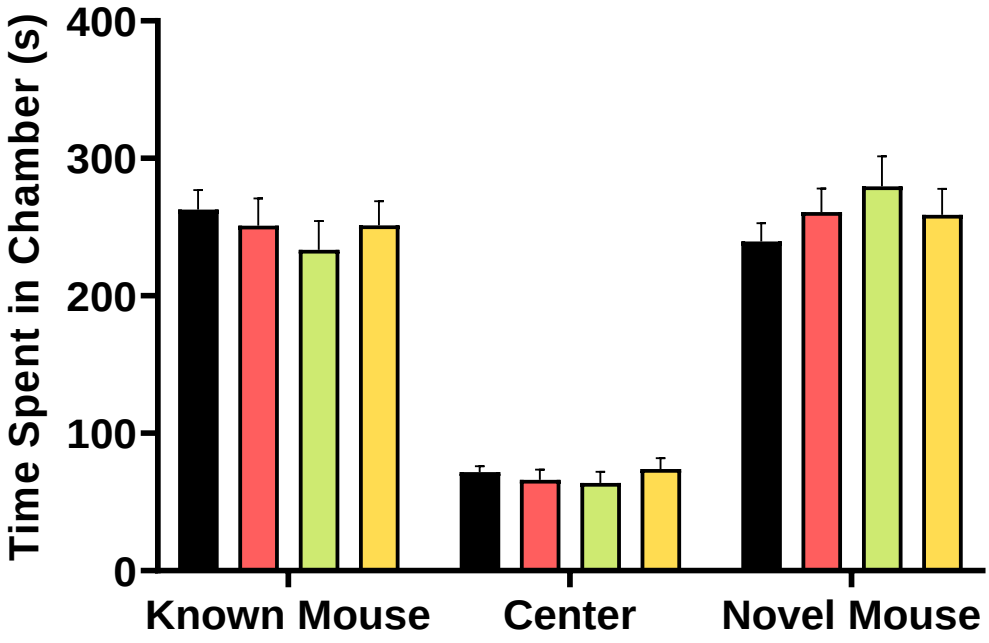


*Figure 9: Time Spent in Chamber (Novelty): no significance compared to vehicle-treated animals*

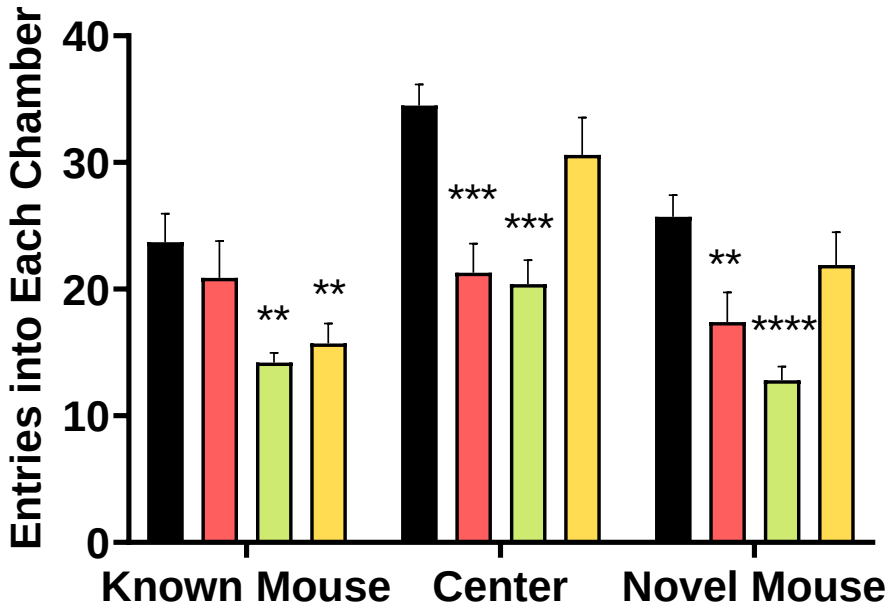


*Figure 10: Entries Into Each Chamber (Novelty): Mouse; U50,488 p<0.005, Nor-BNI p<0.005; Center; Salvinorin A p<0.0005, U50,488 p<0.0005; Object; Salvinorin A p<0.005, U50,488 p<0.0001 compared to vehicle treated animals*

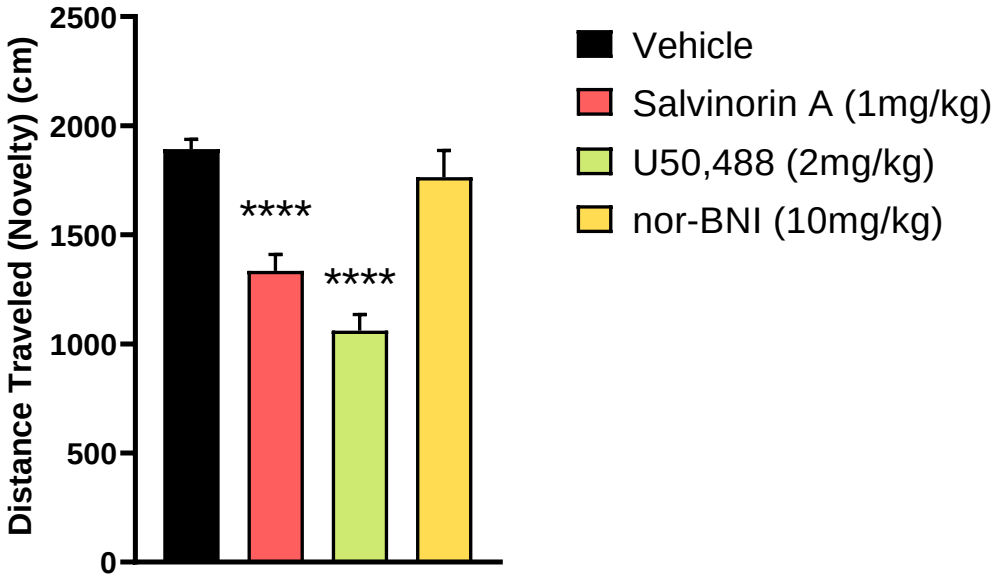


*Figure 11: Distance Traveled (Novelty): Salvinorin A p<0.0001, U50,488 p<0.0001 when compared to vehicle-treated animals*

**Elevated Plus Maze**

For this assay, closed arms are associated with anxiety and open arms are associated with anti-anxiety behavior. To assess anxiety, the percent time spent in the open arms (Fig. 12), percent entries into the open arms (Fig. 13) as well as counted entries into (Fig. 14, 15), and distances traveled in both the open and closed arms (Fig. 16, 17) were measured. The ANOVA was significant ($p$=0.0198) for the percent time spent in the open arms (Fig. 12) however when post-hoc tests compared the vehicle across treatments no significance was seen. U50,488 showed a significant increase in percent entries into the open arms ($p$=0.0067) (Fig. 13). To offer a more granular view of what occurred, distance traveled in the open vs. closed arms was compared and total distance was examined. There was a significant interaction effect for distance traveled in the open vs. the closed arms ($p$=0.0005), meaning the treatment and the arm type both attributed to the significant effect on distance traveled (Fig. 16). Post-hoc testing showed that Salvinorin A and U50,488 groups traveled significantly less distance ($p$=0.0019, $p$=<0.0001 respectively) in the closed arms. When examining total distance (Fig. 17), the Salvinorin A and the U50,488 groups had a significant reduction ($p$=0.0014, $p$=0.0262 respectively) in distance traveled. The number of entries into the open vs. closed arms (Fig. 15) was compared and a total number of entries (Fig. 14) was examined. There was a significant interaction effect ($p$=0.0064) and the posthoc test revealed that the Salvinorin A and U50,488 groups completed significantly fewer entries into the closed arms ($p$=0.0006, 0.0003 respectively) (Fig. 15). When total entries were examined the Salvinorin A and the U50,488 groups had a significant reduction ($p$=0.0006, $p$=0.0202 respectively) in total entries (Fig. 14).

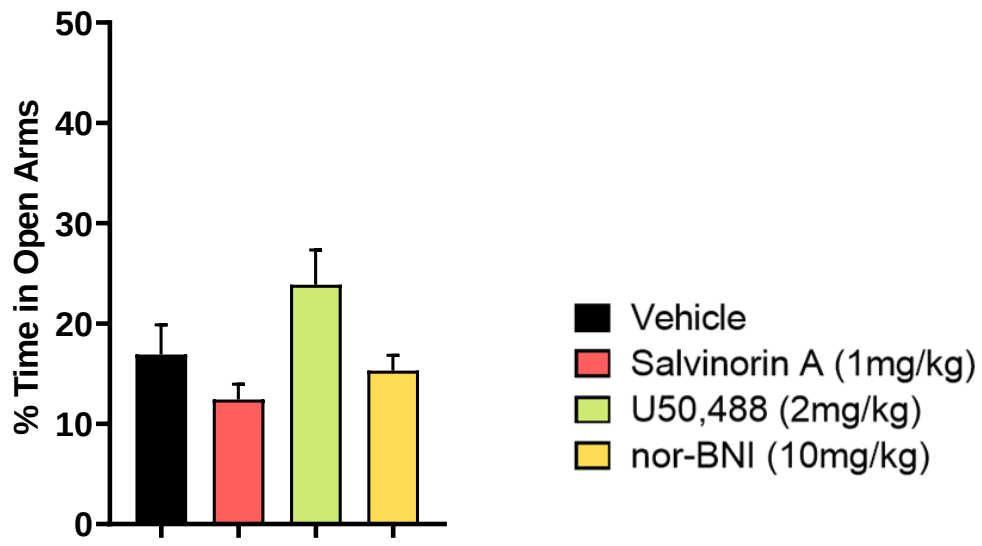


*Figure 12: % Time Spent in Open Arms: no significance in "% Time Spent in Open Arms" when compared to vehicle-treated animals*

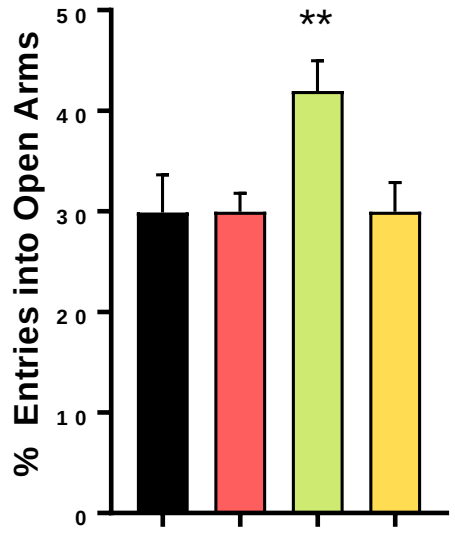


*Figure 13: % Entries into Open Arms: U50,488 $p<0.005$ when compared to vehicle-treated animals*

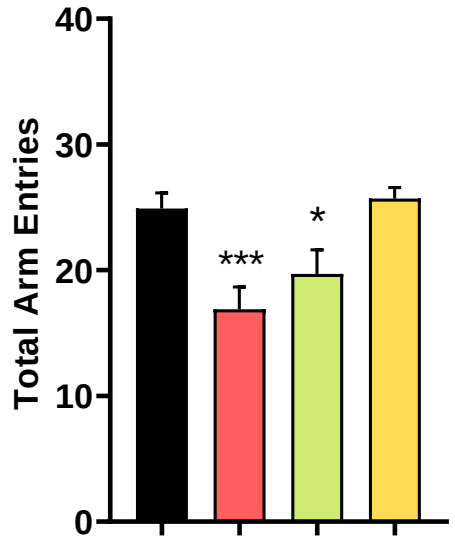


*Figure 14: Total Arm Entries: Salvinorin A p<0.0005, U50,488 p<0.05 when compared to vehicle-treated animals*

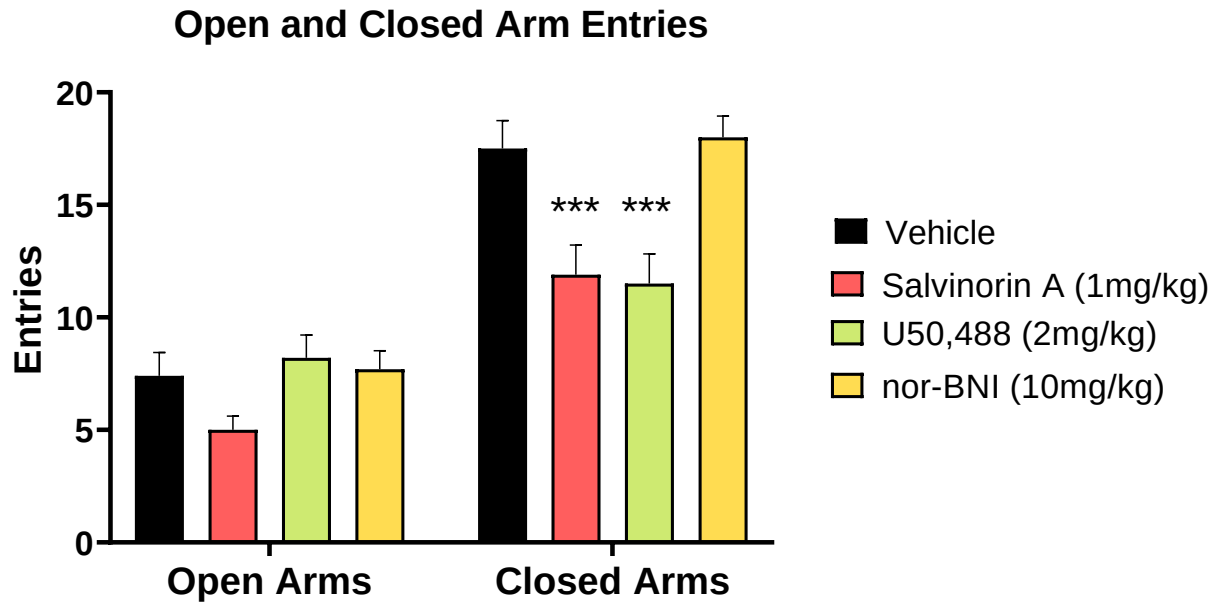


*Figure 15: Entries Open vs. Closed Arms: Closed Arms; Salvinorin A p<0.0005, U50,488 p<0.0005 when compared to vehicle-treated animals*

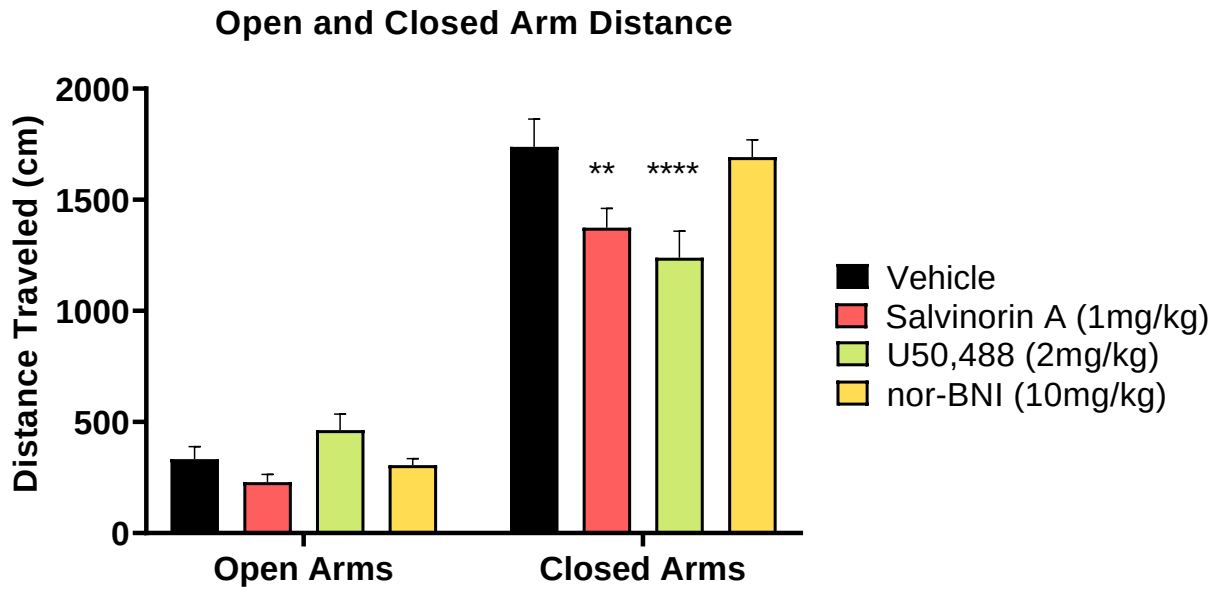


*Figure 16: Distance Traveled Open vs. Closed Arms: Closed Arms; Salvinorin A p<0.005, U50,488 p<0.0001 when compared to vehicle-treated animals*

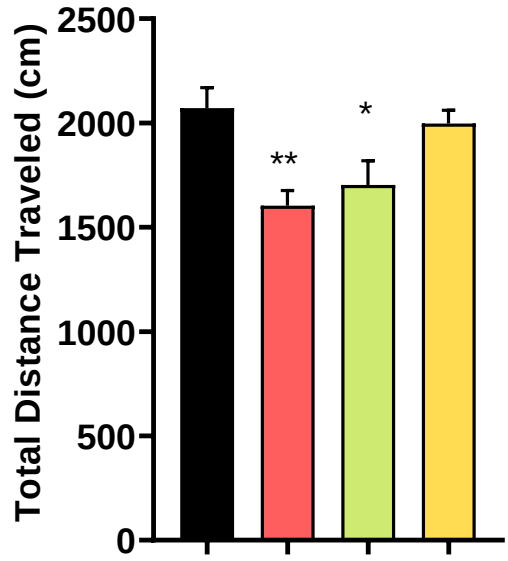


*Figure 17: Total Distance: Salvinorin A p<0.005, U50,488 p<0.05 when compared to vehicle-treated animals*

**Fear Conditioning**

For Fear Conditioning, percent freezing was measured during the contextual and cued sessions. Due to the binary nature of this data (freezing versus non-freezing), a logarithmic transformation to normalize the data was completed for the ANOVA. For the cued sessions, freezing was separated into bins denoted by pre-cue, cue, and post-cue. The contextual fear portion (Fig. 18) revealed that Salvinorin A and U50,488 exhibited significantly less freezing behavior ($p$=0.0437, $p$=0. 0.0119) when compared to the vehicle group. For the cued portion (Fig. 19), Salvinorin A and nor-BNI treatment groups showed significantly less freezing behavior ($p$=0.0107, $p$=0.0315 respectively) when compared to the vehicle group. The change in freezing was insignificant for all groups as determined by the ANOVA ($p$=0.1215) during the cue. During the post-cue bin, Salvinorin A and U50,488 groups showed significantly less freezing behavior ($p$=0.0046, $p$=0.0139 respectively) when compared to the vehicle group.

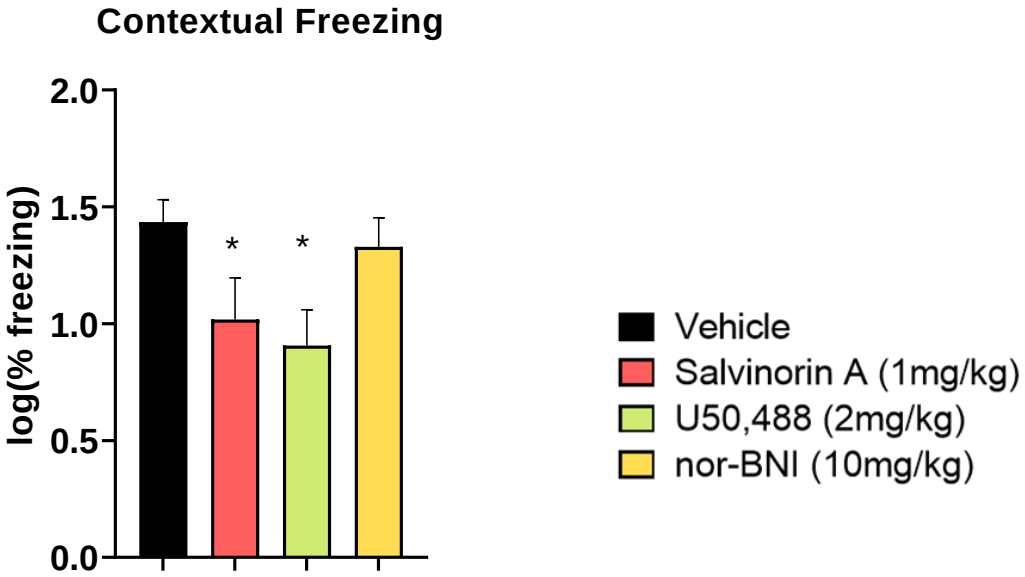


*Figure 18: Contextual Fear: Salvinorin A $p<0.05$, U50,488 $p<0.05$ when compared to vehicle-treated animals*

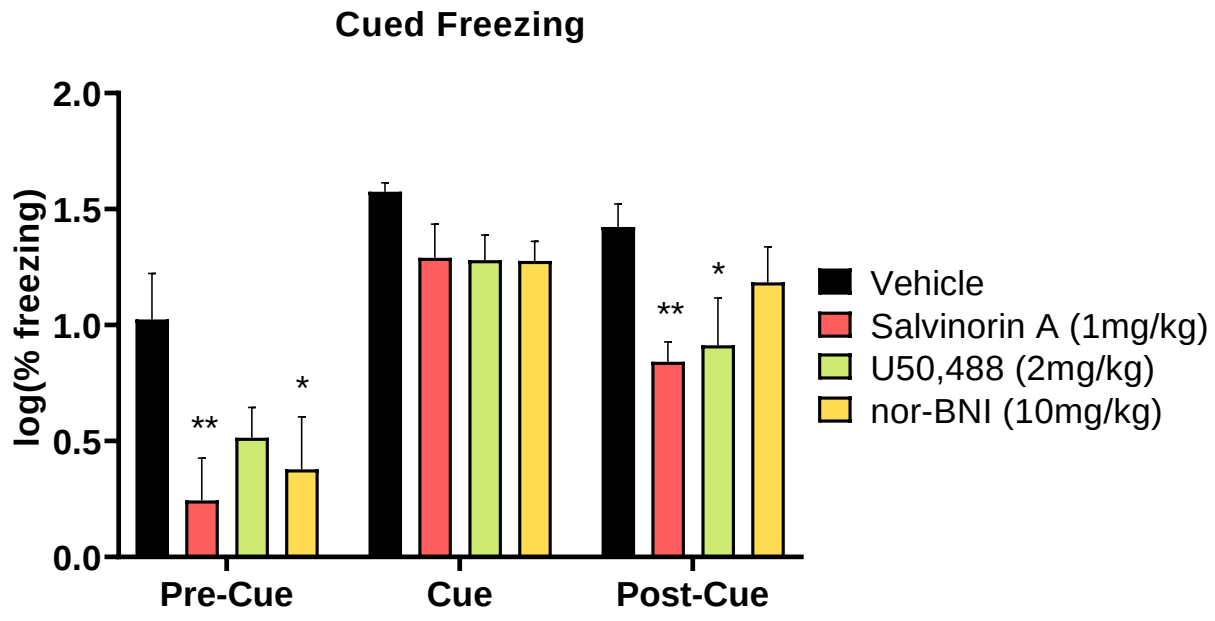


*Figure 19: Cued Fear: Pre-Cue; Salvinorin A p<0.005, nor-BNI p<0.05; Post-Cue; Salvinorin A p<0.005, U50,488 p<0.05 when compared to vehicle-treated animals*

## Conclusions

Sociability testing shows that all four groups independently retained normal social behavior as indicated by significant interest in the mouse over the object (Fig. 2A-D). The Salvinorin A and the U50,488 groups showed significant hypolocomotion during testing (Fig. 6). The Salvinorin A group showed no difference in chamber preference (Fig. 4); however, it did show a significant decrease in entries into all chambers (Fig. 5). The Salvinorin A group also showed a significant decrease in time spent sniffing the mouse when compared to the other treatment groups (Fig. 3), indicating Salvinorin A at 1mg/kg i.p. has a detrimental effect on sociability. U50,488 treated

groups showed a significant increase in preference for the mouse chamber and a significant decrease in preference for the object chamber (Fig. 4) however, since they showed no significant increase in time spent sniffing the mouse over the object (Fig. 3) these results are not enough to conclude that U50,488 induces an increase in sociability.

Social novelty testing shows that the vehicle group and the Salvinorin A group independently retained a normal preference for social novelty, while the U50,488 and nor-BNI groups did not (Fig. 7A-D). The Salvinorin A and the U50,488 groups showed significant hypolocomotion during testing (Fig. 11). When compared across groups, no group showed a significant difference in chamber preference (Fig. 9). Salvinorin A treated mice showed a significant decrease in entries into the center and novel mouse chamber (Fig. 10), as well as a significant decrease in time, spent sniffing the novel mouse (Fig. 8) when compared across groups. This indicates that although the Salvinorin A group retained a preference for social novelty when other groups did not, treatment had a significant detrimental effect on preference for social novelty. The U50,488 group showed a significant reduction in entries to all chambers (Fig. 10), however, failed to show a significant decrease in preference for social novelty, measured by time spent sniffing (Fig. 8), when compared across groups. Nor-BNI treated mice, while showing a significant decrease in entries into the known mouse chamber when compared across groups (Fig. 10) and a significant lack of preference for social novelty independently (Fig. 7D), it failed to show a significant change in time spent sniffing for the novel mouse (Fig. 8) when compared across groups. This data is conflicting and therefore a conclusion, either way, cannot be drawn.

In the Elevated Plus Maze, a decrease in % time spent in as well as % entries into the open arms are the strongest indicators of increased anxiety. There was no significant difference across groups in % time (Fig. 12) however U50,488 showed a significant increase in % entries (Fig. 13)

which suggests an anti-anxiety phenotype. Salvinorin A and U50,488 treated groups showed a significant reduction in exploration and locomotion as indicated by the total distance traveled (Fig. 17) as well as the total arm entries (Fig. 14). This decrease in distance traveled in the closed arms also suggests these groups were more inclined to rest in the closed arms than in the open arms, suggesting an anxious phenotype, however, this is not supported enough by the experiment to conclude. This is partly because of the conflicting data obtained from the analysis of % entries into the open arms. Further testing would be required to see if this is the result of sedation brought on by KOR-agonist treatment. Elevated Plus Maze testing indicates that Salvinorin A and U50,488 have a significant detrimental effect on locomotion (Fig. 17), which is also supported by data obtained during 3-Chamber Social Interaction testing (Fig. 6, 11).

During fear conditioning, pretreatment before the training phase resulted in Salvinorin A and U50,488 treated mice showed a decrease in freezing behaviors during the contextual fear test (Fig. 18), indicating a significant deficit in contextual fear memory. No groups showed a significant difference in startle response during the cue portion of the test (Fig. 19). Since the cued portion is the metric for the cued conditioning test, the conclusion is that all three treatments failed to produce a significant change in cued fear memory.

## Discussion

The purpose of this experiment is proof of concept – if modulation of the kappa opioid receptor can lead to behavior changes that mimic the ones seen in PTSD, then we would have taken the first step in creating a new paradigm for studying the disorder. We were able to provide evidence that treatment with Salvinorin A (1mg/kg) 15 minutes before testing can negatively affect sociability and preference for social novelty behavior in 14-week-old male mice regardless of

housing conditions. We also showed that treatment with Salvinorin A (1mg/kg) and U50,488 (2mg/kg) 15 minutes before testing was sufficient to produce hypolocomotor effects in this population as supported by Elevated Plus Maze and 3-Chamber Social Interaction testing. Lastly, we were able to show that pretreatment with Salvinorin A (1mg/kg) and U50,488 (2mg/kg) 15 minutes before testing dampened the contextual fear response significantly.

Studies involving U50,488 as related to 3-Chamber social interaction (Dogra et al. 2016) as well as Elevated Plus Maze were able to show that dosages of 5mg/kg were able to produce a significant increase in time spent with the object over the mouse, as well as negatively affect the time spent in the open arms of the EPM (Bruchas et al. 2009). Prior experiments (Liu et al. 2019) show that U50,488 produced a dose-dependent effect on locomotor activity in male mice, which supports our data from EPM and 3-Chamber tests. This study involving EPM (Bruchas et al. 2009) also discussed using a pretreatment time of 30 minutes to reduce the hypolocomotor effect of the KOR agonist. It is with this information that, if this experiment would be conducted again, the dosage for U50,488 would be increased to 5mg/kg and pretreatment time would be increased to 30 minutes. It is important to note that pretreating with Salvinorin A at 30 minutes would not be advantageous, since this compound is known to have a short half-life and rapid onset of action, with studies relating a pretreatment time of more than 20 minutes with the failure of behavioral effect production (Kivell et al. 2014). We have determined that to accurately assess anxiety and to diminish the effect of KOR-related hypolocomotion, the EPM test would be replaced with the open field test. This test would be able to quantify anxiety by measurement of time spent in the center versus the corner of the apparatus. Hypolocomotor activity would have less of an effect here since theoretically a hypolocomotive mouse presenting with anti-anxiety

behavior would remain in the center, and a phenotypically anxious mouse would remain on the sides or in the corner.

For the 3-Chamber test, this protocol involved chamber acclimation without the presence of the wire cups (Stanford Medicine [date unknown]). However, other protocols exist that include the wire cup during acclimation (Kaidanovich-Beilin et al. 2011). Since this test only provided significance for the Salvinorin A group, it may be beneficial to include the cups during acclimation in a follow-up experiment to exclude the factor of social novelty with respect to the cup.

While fear conditioning did not result in a significant change in cued fear memory, it did result in a significant decrease in post-cue freezing. While this does not indicate anything directly, it does suggest that these compounds could influence fear extinction. A prior study conducted by Bruchas et. al. showed that when nor-BNI was administered to groups following the first extinction trial it had a negative effect on fear extinction. (Bruchas et al. 2009). Therefore, it may be beneficial to follow up this study with a fear extinction study, since impaired fear extinction is considered to be a significant marker of PTSD (Zuj et al. 2016). Also, it has been shown that 0.1, 1, and 10mg/kg doses of U50,488 were able to acutely exacerbate the fear response in contextual and cued fear conditioning. However, this experiment utilized a protocol that involved dosing before the contextual test, which differs from this experiment where subjects were dosed before training (Vunck et al. 2011). Therefore, it may be beneficial to alter the fear conditioning protocol slightly to include pretreatment before the contextual test as opposed to before the training trial.

Concerning nor-BNI administration, a pretreatment time of 60 minutes was chosen after discovering that a pharmacokinetic analysis had determined the Tmax to be 30 minutes (Munro

et al. 2012), and another study showed that a 60 minute pretreat was sufficient to significantly remove stress-induced odorant aversion (Land et al. 2008). Further research on the nor-BNI mechanism of action revealed that it is a non-competitive antagonist and works by desensitizing KORs – an effect that lasts days after administration. This, coupled with research of nor-BNI with a pretreatment time of 48 hours producing a significant increase in time spent in the open arms in EPM (Huang et al. 2016) and that nor-BNI increases time spent in the open arms in a dose dependent manner in rats (Knoll et al. 2007) leads us to believe that the choice of a 60-minute pretreatment time was too short for efficacy. Also, the pharmacological assay indicates that peak antagonism remains for at least 48 hours leads us to determine that if this experiment were conducted again the nor-BNI pretreatment time would be extended to 48 hours.

Both KOR agonists Salvinorin A and U50,488 induced hypolocomotion in this experiment. To ensure that results are not due to a sedative effect, further testing needs to be conducted. The best way to mitigate these effects would be to measure anxiety with an Open Field assay, where anxiety is quantified by time spent in the center and time spent on the edges of the apparatus, with an increase in the former being indicative of an anti-anxiety phenotype. This assay would need to be paired with a Hole-Board assay (Labots et al. 2015), where the mouse is able to explore the holes in the field without the need to ambulate. Rearing, another measure of exploration, is also measured in this assay. If the ambulatory distance is significantly decreased and exploratory behavior remains, then this would tell us that the effects are not due to sedation (Seibenhener and Wooten 2015). Lastly, studies have shown that a pretreatment time of 0.5 hours is sufficient to reduce the hypolocomotor effects of KOR agonists, therefore adoption of this practice may be beneficial (Bruchas et al. 2009).

To increase the strength of a KOR-induced PTSD model, the inclusion of additional groups would be beneficial. The addition of opioid receptor gene kappa 1 (OPRK1) knockout mice should be included and would be useful in seeing exactly how much of an effect the KOR is contributing to these behaviors (Maldonado et al. 2018). By the same notion, a prodynorphin (PDYN) knockout group would also be useful as it would offer a more granular idea of what mechanisms are at play in relation to KORs and these behaviors. Lastly, a co-treated U50,488/nor-BNI group would be a welcome addition. For one, it would act as a reference group since co-treatment with these compounds regularly shows rescuing of the phenotype and blockage of the agonistic effect of U50,488 (Bruchas et al. 2009: 1; Hung et al. 2015; Dogra et al. 2016).

This study used fear conditioning to examine Intrusion, EPM for avoidance, and 3-Chamber testing for negative alterations in cognition and mood. It is important to note that this is not the end of testing for these behaviors. The DSM-V offers a wide array of tests that it has deemed viable for studying these behaviors as related to PTSD (Verbitsky et al. 2020). Furthermore, the addition of a fourth test for alterations in arousal and reactivity would be necessary to round out the model, as these tests are good for examining behavior analogous to the hyper-vigilance that is often seen in PTSD cases (Verbitsky et al. 2020).

Lastly, this study utilized a design that included mice that were group-housed and single housed balanced across groups. This was to mitigate the effect of different types of stressors that can arise from several types of housing environments. One study was able to show that single housing mice resulted in less stressed subjects when compared to group-housed mice (Kamakura et al. 2016). Another study showed that mice that were socially isolated during adolescence had higher levels of anxiety and depressive-like behaviors, as well as impaired social behaviors

(Medendorp et al. 2018). These two studies represent the idea that mice housed in a research setting are subject to certain stressors no matter what their housing conditions are. However, in this study, subject subgroups related to housing were small, n=4 group-housed, n=6 single housed; therefore, it may be advantageous to increase population sizes for these groups to n=10 to get a better representation of the mixed population.

This study was able to show that Salvinorin A (1mg/kg) pretreatment at 15 minutes has a detrimental effect on sociability and locomotion, U50,488 (2mg/kg) pretreatment at 15 minutes has a detrimental effect on contextual fear memory and locomotion, and that nor-BNI (10mg/kg) pretreated at 60 minutes does not affect sociability, social novelty, anxiety, or fear memory. However, previous studies were able to show effects related to these behaviors with different treatment concentrations and pretreatment times. Further research into this paradigm is required to create a new acute model of PTSD. With the completion of that paradigm and the confirmation that activation or inhibition of a target receptor can induce these symptoms as opposed to a battery of stress tests, it has the potential to improve the efficiency of efforts to study PTSD. Furthermore, this discovery will lead to the ability to evaluate novel compounds that combat these symptoms with the hope of discovering a practical pharmaceutical target for the treatment of PTSD.

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