Internship Report

Functional Effects of the Relaxin-3/RXFP-3 Pathway on the Affective Dimension of Chronic Pain

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Abstract

Chronic pain, defined as pain lasting more than 3-6 months, affects approximately 20% of the global population and has a profound socioeconomic effect, inducing a severe drop in the quality of life of patients with few effective treatment and management options available. The Relaxin-3 neuropeptide has been identified as a potential pharmacotherapeutic agent, having been shown to attenuate hyperalgesia stemming from CFA-induced chronic pain in mice in the Von Frey and Plantar behavioral assays. However, it is unclear whether Relaxin-3 is able to induce analgesia against spontaneous pain and to modulate the affective-emotional dimension of nociception, two key components for treatment of chronic pain. Here, we developed a modified Conditioned Place Preference paradigm utilizing pain relief as a reward to assess Relaxin-3's effects on these facets of chronic pain. We found that Relaxin-3 is able to induce Conditioned Place Preference in CFA-treated mice, but not in NaCl-treated mice, indicating that it is reducing spontaneous pain and modulating the affective dimension of nociception in this chronic pain mouse model . We additionally utilized this modified CPP in conjunction with a pharmacogenetic technique employing DREADD/CNO to examine the Relaxin-3 circuitry, finding strong evidence that Relaxin-3 inhibits Somatostatin neurons in the BLA in its pain modulation pathway. Finally, we induced continuous activation of Relaxin-3 Receptor RXFP-3 in the ACC through the tonic production of Relaxin-3 agonist R3/I5 in order to assess its effects on the development of chronic pain. Utilizing the Von Frey and Plantar nociceptive assays, we found that tonic R3/I5 release attenuated CFA-induced hyperalgesia in a manner similar to intracerebral injections of Relaxin-3 agonist A5, the current golden standard for Relaxin-3 research, without apparent extraneous effects in regards to behavior or the development of chronic pain in mice with or without pain, and as such propose it as an alternative to A5 injections.

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Introduction

Pain is, despite its negative connotations, an essential tool. Our ability to feel pain following trauma aids both in the healing process, and in the avoidance of future trauma. Further, pain plays an important role in learning, social behaviors, and other essential mechanisms (Leknes 2014). However, when pain lasts far beyond the trauma which initially elicited it or arises and persists from no recognizable source of trauma, it can become a maladaptive experience. Chronic pain is broadly defined as pain lasting more than 3-6 months (Bouhassira 2008, Ianniti (Werner and Bischoff) 2014), and can greatly decrease the quality of life of those affected. Furthermore, chronic pain has a high prevalence, with approximately 20% of the global population estimated to suffer from chronic pain (Breivik 2006, Bouhassira 2008, Goldberg 2011). However, despite one in five people living with this condition, treatment and management options remain limited and largely inadequate: 40% of Europeans with chronic pain reported dissatisfaction with the efficacy of their pain treatment, and 56% reported that their prescription medication for chronic pain was somewhat effective to not effective (Breivik 2006). Indeed, common pain medications, such as opioids, have been reported to achieve merely a 30% mean pain relief in chronic pain patients (Hylands-White 2016). As such, there is a pertinent need to research novel compounds and develop new therapeutic methods to address chronic pain in patients. Research at the Institute of Neurodegenerative Diseases at the University of Bordeaux has recently implicated the Relaxin-3 neuropeptide in the modulation of pain and identified it as a possible candidate for chronic pain management. In this study, we expand on the work already done in this field, assessing the ability of Relaxin-3 to modulate the affective component of nociception, as well as laying the groundwork for future research on this neuropeptide.

Pain:

The Three Dimensions of Pain

According to the International Association for the Study of Pain IASP), pain is defined as an “unpleasant sensory emotional experience with actual or potential tissue damage, or described in terms of such damage”. It is characterized by three principal components: the sensory-discriminative dimension, the affective-emotional dimension, and the cognitive-evaluative dimension. The sensory-discriminative dimension principally involves the somatosensory cortex and allows for the identification of various physical qualities of pain, such as presence, character, intensity, and location of the pain. The affective-emotional dimension is mediated via the amygdala, the reticular formation, and the brainstem, and allows for the emotional response to pain and the associated conditioned avoidance behaviors. The cognitive-evaluative dimension, meanwhile, is primarily associated with the cerebral cortex, and mediates the learned behaviors concerning pain — including sociocultural aspects, attention, context, and previous experiences — and as such is primarily involved in pain modulation (Ellison 2017). The combined actions of these systems form the “pain matrix”, from which arises our pain experience.

Pain Processing: From Noxious Stimulus to Conscious Perception

The process through which painful stimuli are received and consciously perceived is composed of four major components: Transduction, Transmission, Perception, and Modulation.

Transduction is the process through which noxious stimuli (thermal, mechanical, chemical) are converted into action potentials, and thus integrated into the nervous system (Ellison 2017). This process occurs in the peripheral nervous system and is mediated by peripheral terminals of nociceptor sensory fibers, with different modalities of stimuli activating different specialized nociceptors. For example, while TRPV1 is activated by noxious heat, noxious cold activates TRPM8, mechanical stimuli activate TRPV4, and acid-based chemical stimuli activates ASIC3 (Basbaum 2009, Iannitti (Gregory and Sluka) 2014).

Once these stimuli have been converted into action potentials by the nociceptors, they are relayed from these peripheral neurons to the central nervous system in a process referred to as Transmission. This process occurs through medium-diameter myelinated Aδ fibers — fast mechanical/thermal nociceptive transmission responsible for rapid, sharp pain and the initial reflex response — and small-diameter unmyelinated C fibers — slow polymodal nociceptive transmission responsible for slow, burning pain — which conduct the signals from the periphery to the Dorsal Horn of the spinal cord (Basbaum 2009, Reddi 2013, Ellison 2017). From the DRG, the nociceptive information is transmitted to the brain through ascending pathways, principal of which are the spinothalamic (STT) and the spinoreticular tracts (SRT). The STT transmits information primarily to the thalamus, with further connections to the reticular formation, the limbic system, and the hypothalamus, and is associated with the sensory-discriminative component of pain. It is further subdivided into the neospinothalamic tract, which carries fast impulses for acute sharp pain, and the paleospinothalamic tract, which carries slow impulses for dull or chronic pain. (Reddi 2013, Ellison 2017). Meanwhile, the SRT transmits information primarily to the brainstem reticular formation and is implicated in the emotional-affective dimension of pain (Reddi 2013, Elison 2017).

Following transmission, Perception takes place, in which the information from the ascending pathways is integrated at the level of the brain, consolidating information throughout a network of regions which form up the “pain matrix”, and conscious awareness of pain is ultimately developed. From the STT, information reaches the somatosensory cortex for interpretation, as well as other regions such as the Periaqueductal Grey (PAG) for an integrated response, ultimately forming the sensory-discriminative dimension of nociception. From the SRT, projections are sent to the thalamus and the hypothalamus, eventually reaching the ACC and the amygdala and forming the affective-emotional dimension of nociception. Finally, projections to the cerebral cortex are principally involved in forming the cognitive-evaluative dimension of nociception (Gao 2004, Basbaum 2009, Ellison 2017).

The final process of the pain pathway is Modulation: the alteration of pain perception through supraspinal influences referred to as descending pathways. These pathways can have both inhibitory, suppressing painful stimuli, or facilitatory, enhancing nociception, and mediate the effects of many analgesic/proalgesic effects. These include modulatory effects stemming from emotion, expectation, stress, and the placebo/nocebo effects, as well as many analgesic compounds (both endogenous and exogenous) such as opiates, non-steroidal anti-inflammatory drugs (NSAIDs), and serotonin and norepinephrine reuptake blockers (Heinricher 2009, Ossipov 2010, Reddi 2013). The most prominent of these descending pathways is the PAG-RVM

(Periaqueductal Grey - Rostral Ventral Medulla) pathway. This pathway receives and integrates inputs from the frontal cortex, amygdala, insular cortex, hypothalamus thalamus, and parabrachial nucleus and sends projections to the spinal cord, utilizing serotonergic and noradrenergic projections to inhibit or augment nociception (Heinricher 2009, Ossipov 2010, 2014, Reddi 2013). Importantly, these descending pathways have also been linked to the development and maintenance of chronic pain, with both their innate facilitatory influences and the loss of their endogenous inhibitory control playing an important role in sensitization and in the chronification of pain (Porreca 2002, Heinricher 2009, Ossipov 2010).

Chronic Pain:

Primary Characteristics

Under normal circumstances, pain is a useful tool that aids in the survival of organisms by signalling and warning against noxious stimuli, warding against further injury and aiding in the healing process by discouraging exertion of damaged tissues, and encouraging future avoidance of the harmful behaviors which led to the injury in the first place. In some cases, however, pain may persist long after the offending injury, continuing for months, years, or in perpetuity; this is the chronification of pain. Chronic pain is a disease state of the nervous system in which pain becomes maladaptive and debilitating, no longer serving as a survival tool for the organism but instead hindering and harming it. It is defined by the International Association for the Study of Pain as pain lasting more than three months and can arise from a variety of factors: diseases such as cancer, HIV, and Multiple Sclerosis, persistent inflammation, and primary lesions or dysfunction of the nervous system all have been linked with the development of chronic pain (Basbaum 2009, Ellison 2017). Regardless of etiology, chronic pain is characterized by a few principal features: hyperalgesia (exacerbated pain response to painful stimuli), allodynia (pain response from stimuli which were not previously painful), and spontaneous pain (pain arising from no stimuli or activity). Chronic pain has also been linked with emotional and cognitive disturbances, such as depression and anxiety, both in the development of chronic pain and as a result thereof (Sufka 2000, Finnerup 2001, Ossipov 2014, Reddi 2017, Velly 2018).

Mechanisms of Induction and Maintenance

Chronic pain can be initiated and maintained through both peripheral and central mechanisms. A major player in peripheral sensitization is the chemical milieu of inflammation, which refers to the inflammation-induced changes in the chemical environment of nerve fibers resulting from tissue damage, which releases a collection of endogenous factors collectively known as “the inflammatory soup”. This “soup” is composed of various neurotransmitters and neuropeptides, eicosanoids and neurotrophins, cytokines and chemokines, and extracellular proteases and protons (Basbaum 2009). Notably, nociceptive neurons express cell surface receptors for these proinflammatory/proalgesic compounds, leading to the inflammatory soup being able to directly interact with nociceptive fibers to enhance their excitability and sensitivity (Basbaum 2009, Gregory 2013).

Central sensitization, meanwhile, occurs through three primary mechanisms: The first, Glutamate/NMDA Receptor-Mediated Sensitization, refers to recruitment of normally silent

NMDA glutamate receptors in the lamina I of the superficial dorsal horn of the spinal cord following intense/persistent stimulation of Aδ and C fibers. These receptors, now responsive to signalling from nociceptive fibers, leads to an increase of intracellular calcium and a subsequent cascade of calcium-dependent signalling pathways and second messengers leading to both increased excitability of the output nociceptive neuron, and a facilitation of the transmission of nociceptive signals to the brain (Sufka 2000, Basbaum 2009). The second is Disinhibition, in which normally inhibitory GABAergic and Glycinergic interneurons in the spinal cord cease to inhibit the lamina I output neurons following an injury event, thus inducing hyperalgesia. Not only that, disinhibition has been shown to lead to the recruitment of the normally non-nociceptive Aβ fibers towards transmission of pain signals, as they are no longer prevented from doing so by the inhibitory interneurons. This, in turn, leads to allodynia, as normally innocuous stimuli become painful (Sufka 2000, Basbaum 2009). Finally there is Microglial Activation. Following peripheral nerve injury, ATP and chemokines are released which stimulate microglial cells; these in turn enhance excitability and pain responses to both noxious and innocuous stimuli, as well as releasing cytokines and other factors which themselves contribute to central sensitization. Further, microglial activation at the level of the brainstem has been shown to counteract feedback inhibitory controls, thus resulting in further facilitatory influences on pain processing (Porreca 2002, Basbaum 2009)

Chronic Pain as a Global Health Crisis

With 20% of the global population estimated to suffer from chronic pain and with this number expected to increase in the following decades due to the aging population, it is clear that chronic pain is of significant societal concern (Breivik 2006, Bouhassira 2008, Goldberg 2011). Despite this, treatment and management of persistent and recurring pain is lacking, with 40% of Europeans suffering from chronic pain reporting dissatisfaction with their pain treatment's efficacy, and with 56% reporting their prescription medication to be between somewhat effective to not at all effective (Breivik 2006). The current WHO guideline regarding pharmacological pain management counsels physicians to utilize a "stepladder" approach, starting with non-opioids such as paracetamol, and increasing to weak, then strong, opioids based on the patient's response (Hylands-White 2016). However, even opioids have not been conclusively demonstrated to be efficacious in treating persistent pain; chronic opioid administration has been associated with hyperalgesia in patients, and opioids have been reported to only achieve a 30% mean pain relief in chronic pain patients (Hylands-White 2016, Tompkins 2016). In fact, there have been no well-controlled long-term studies indicating that opioids can effectively relieve pain or improve function in pain beyond twelve weeks (Tompkins 2016).

When combined with the potential risk for addiction, abuse, and harm stemming from chronic opioid use, as exemplified by the ongoing opioid crisis, it is clear that novel approaches to pain management are necessary. A multimodal approach incorporating both pharmacological and non-pharmacological therapies is currently considered one of the most promising approaches, as outlined in the 2021 National Institute for Health and Care Excellence (NICE) Guideline for Chronic Pain. Within the realm of novel pharmacological therapies, non-opioid analgesics, and specifically neuropeptides, has been encouraged as a promising field (Hoyer and Bartfai 2012, Tompkins 2016, NICE 2021). In light of this, the neuropeptide Relaxin-3, which has been

recently linked to pain relief in persistent inflammatory pain, is a promising avenue of research for a novel pharmacological tool for the treatment and management of chronic pain.

Relaxin-3 and RXFP-3:

Background

Relaxin-3, first discovered in 2001, is a neuropeptide belonging to the Relaxin family, a group of seven insulin-like peptides (Relaxin-1 through Relaxin-3, and INSL-3 through INSL-6) which are involved in a diverse array of physiologic functions ranging from male and female reproduction, involvement in the cardiovascular system and, most importantly for this report, acting as a neuropeptide in the central nervous system: Relaxin-3 is almost exclusively expressed in the brain (Wilkinson 2005, Bathgate 2013, Ma 2017). Though some Relaxin peptides have species-dependent variations in what functions and processes they are involved in, others are well conserved across all mammalian species, with Relaxin-3 being especially highly conserved and widely accepted as the ancestral peptide of the Relaxin Family Peptides (Wilkinson 2005, Bathgate 2013).

These peptides bind to and activate their effects through a family of four G protein-coupled receptors (GPCRs), which are referred to as the Relaxin Family Peptide Receptors (RXFP) 1 through 4 (Halls 2007, Bathgate 2013). These receptors are by and large not exclusively linked to a particular Relaxin peptide; for instance, RXFP-1 is able to be activated by both Relaxin-1 and Relaxin-3, and RXFP-4 is stimulated by both INSL5 and by Relaxin-3 (Bathgate 2013). Despite this, RXFP3, discovered in 2000 and formerly named the “Somatostatin and Angiotensin-like Receptor” (SALRP), was discovered in 2000, is considered the *de facto* Relaxin-3-associated receptor.This is due to RXFP-3 being solely stimulated by Relaxin 3, and because it is the only receptor activated by Relaxin-3 to have a significant overlap in expression areas with endogenous Relaxin-3 (Matsumoto 2000, Bathgate 2013).

RXFP-3 is coupled to a $G_{i/o}$ protein, with its activation leading to the inhibition of adenylyl cyclase and the induction of ERK1/2 phosphorylation. It can be found in small quantities in the testicles of mice and humans, and in the salivary glands and thymus of humans; however, is primarily expressed in the brains of numerous mammalian species (Halls 2007, Bathgate 2013). The distribution of RXFP-3 in the brain of rodents has been extensively studied, with significant overlap between expression of RXFP-3 and Relaxin-3 fibers found in the hypothalamus, septum, hippocampus, median raphe, PAG, and central and medial amygdala (Bathgate 2013). This distribution has contributed in inciting studies linking Relaxin-3 to various domains, such as appetite, stress, arousal, learning, memory, and the metabolism (Bathgate 2013). Previous research in this laboratory has also demonstrated the presence of both Relaxin-3 neurons and RXFP-3 expression in the Bilateral Amygdala (BLA) and the Anterior Cingulate Cortex (ACC). In both these regions, somatostatin neurons were shown to interact closely with the Relaxin-3 circuitry: there was significant contact between Relaxin-3 neurons and somatostatin neurons in the ACC and BLA, and the majority of neurons expressing RXFP-3 in these areas were somatostatin neurons (Figures 1 and 2) (Abboud 2021). Given the involvement of the BLA and the ACC in the pain matrix, Relaxin-3 was investigated for its role in nociceptive pathways, and was found to have a significant role therein.

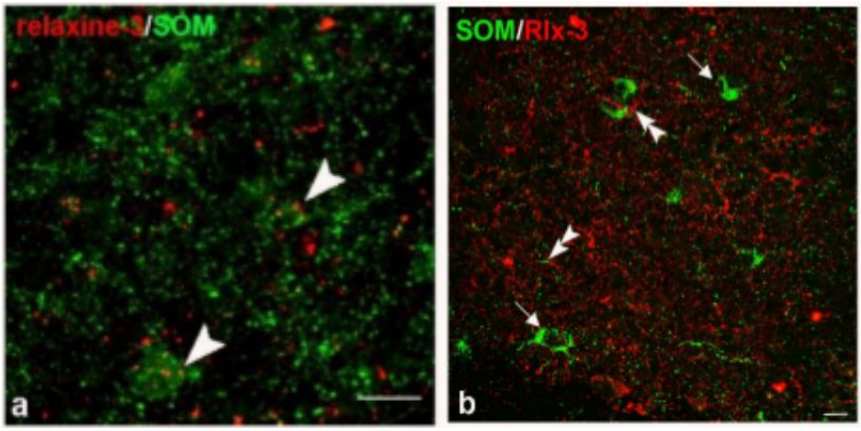


Figure 1: Immunohistochemistry of Relaxin-3 and Somatostatin neurons in the BLA and ACC (Abboud 2021) IMH images demonstrating contact between Relaxin-3 and Somatostatin neurons in the naive mouse BLA (A) and the ACC (B). Relaxin-3 neurons are shown in green (Alexa 488), Somatostatin neurons are shown in red (Alexa 594), points of Relaxin-3/Somatostatin neuron contact are indicated by the arrows. Bar is scaled at 10 μm

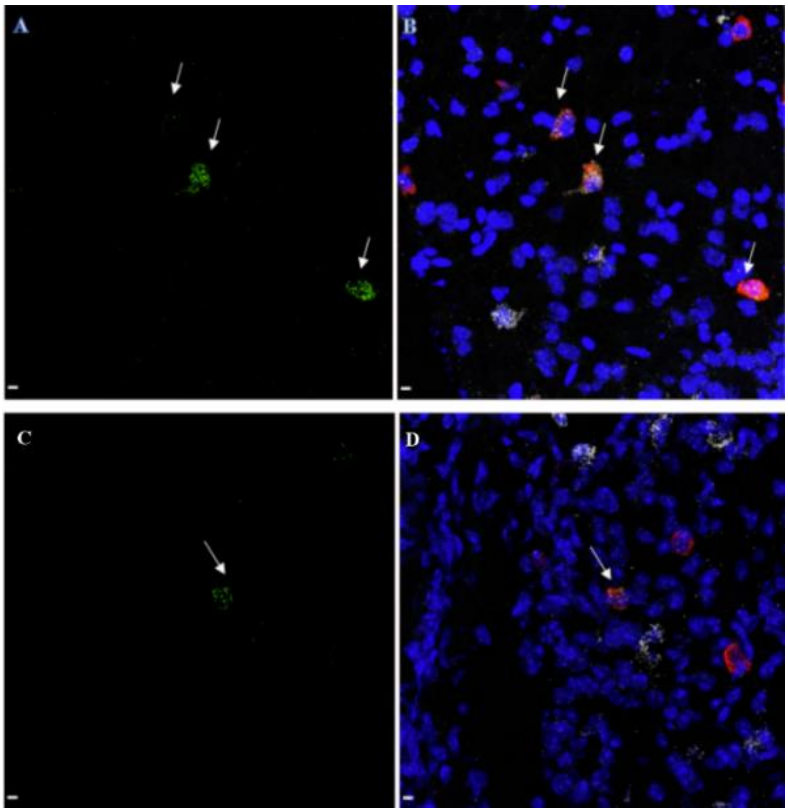


Figure 2: Immunohistochemistry of RXFP-3 and Som expressing neurons in the BLA and ACC (Abboud 2021) RNAscope in situ hybridization images showing RXFP-3 (A and C) and Som (B and D) RNA in the BLA (A and B) and ACC (C and D) of mice treated with saline intraplantar injection (control mice). The majority of neurons expressing RXFP-3 RNA are Somatostatin neurons. RXFP-3 RNA is shown in green, Som RNA is shown in red, neurons showing RXFP-3 and Som RNA overlap are pointed out by white arrows. Bars are 20μm.

Relaxin-3 and Nociception:

The research team of Professor Marc Landry, under the auspices of the Institute of Neurodegenerative Diseases at the University of Bordeaux and alongside partner teams in

Australia, Canada, and France, has been investigating the involvement of the Relaxin-3/RXFP-3 system in nociception. Utilizing a complete Freund's Adjuvant (CFA) inflammatory persistent pain model in the Von Frey and Plantar pain assays, they demonstrated that BLA injection of RXFP-3 agonist A5 produced a transient reduction of mechanical and thermal hyperalgesia, while ACC injections of A5 produced a transient reduction of mechanical, but not thermal, hyperalgesia. Importantly, these applications of A5 produced no changes in the control, pain-less animals, supporting the conclusion that Relaxin-3 is indeed producing these changes through the modulation of pain (Figures 3 and 4) (Abboud 2021).

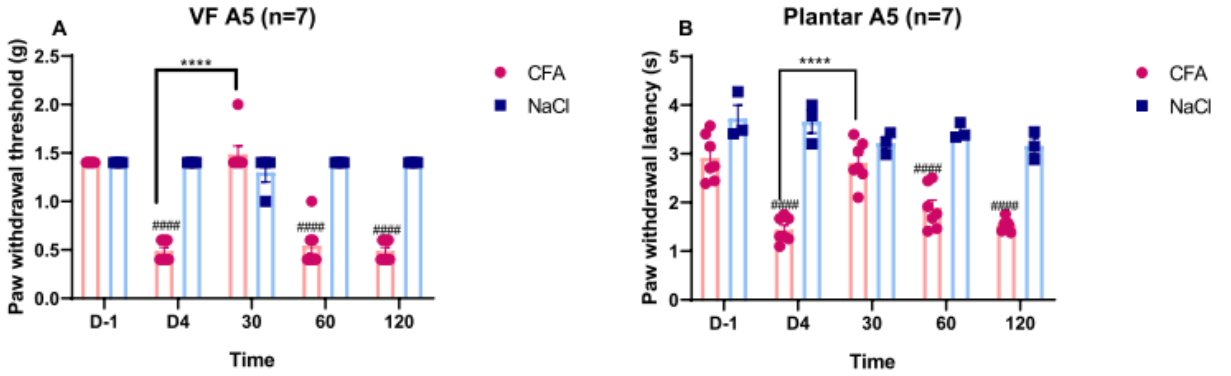


Figure 3: A5 BLA effects on mechanical and thermal hypersensitivity (Abboud 2021)
Baseline values were obtained 1 day prior to CFA/NaCl injection to the posterior paw, and A5 injection to the BLA occurred 4 days after CFA/NaCl injection. A5 injection led to the transient attenuation of CFA induced mechanical (A) and thermal (B) ipsilateral hypersensitivity. This attenuation was present 30 minutes after A5 injection, and had disappeared by the 60 minutes post-injection. CFA animals are indicated in red (n = 7); NaCl animals are indicated in blue (n not provided). **** indicates $p \leq 0.0001$ as compared to D4. #### indicates $p \leq 0.0001$ as compared to D1. Mean and SEM are indicated.

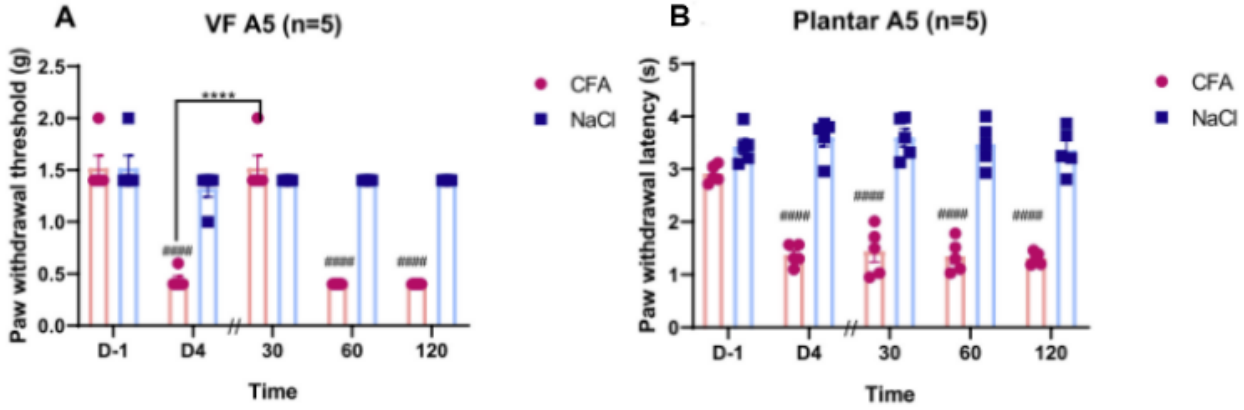


Figure 4: A5 ACC effects on mechanical and thermal hypersensitivity (Abboud 2021)
Baseline values were obtained 1 day prior to CFA/NaCl injection to the posterior paw, and A5 injection to the ACC occurred 4 days after CFA/NaCl injection. A5 injection led to the transient attenuation of CFA induced mechanical (A), but not thermal (B), ipsilateral hypersensitivity. This attenuation was present 30 minutes after A5 injection and had disappeared by the 60 minutes post-injection. CFA animals are indicated in red (n = 5); NaCl animals are indicated in blue (n not provided). **** indicates $p \leq 0.0001$ as compared to D4. #### indicates $p \leq 0.0001$ as compared to D1. Mean and SEM are indicated.

While these results demonstrate the analgesic action of the Relaxin-3/RXFP-3 circuit, the Von Frey and Plantar tests are limited in what they are able to test. While they are able to provide information regarding effects involving evoked stimuli and the sensory dimension of pain, they provide little to no information regarding the affective-emotional dimension of pain, nor do they show whether this system has any effects of spontaneous, non-evoked pain, a major hallmark in clinical cases of chronic pain. Indeed, the inability of these assays to reveal information on these factors has been repeatedly criticized and is theorized to be a cause of the failure of novel pharmaceutical compounds for chronic pain in human trials after having shown great potential in animal trials (Sufka 1994, Finnerup 2001, King 2009, Rutten 2011, Navratilova 2013). As such, it is important to assess whether the Relaxin-3/RXFP-3 system modulates nociception at an affective level and in response to non-evoked pain, in addition to its already established effects on evoked stimuli and on the sensory dimension of pain. However, behavioral assessment of the emotional-affective dimension and of non-evoked pain responses in animals is a challenging task, owing to its subtlety and the need of indirect measurements. For evoked pain, paw withdrawal latencies and vocalizations make for clearly defined observations. Meanwhile, non-evoked pain has no such explicitly linked measurements. To address this challenge, Sufka et al. developed a modified Conditioned Place Preference (CPP) in 1994.

Conditioned Place Preference for Nociception Studies:

The CPP assay is commonly utilized to investigate the reinforcing/aversive effects of drugs, typically in association with studies on addiction and reward. In the traditional CPP paradigm, a drug state is repeatedly paired to an environment (i.e. a chamber) with distinctive stimuli (e.g. visual cues), while a control “sober” state is repeatedly paired to an environment containing a different set of distinctive stimuli. Following several rounds of these pairings, preference is determined by comparing their interactions to the drug-associated environment and the sober-associated environment; more frequent/prolonged interactions with the drug-associated environment demonstrate the formation of a conditioned place preference.

The CPP test has also been shown to be useful in studies on pain and (putative) analgesic compounds. As Sufka et al. detailed in their study, by utilizing analgesic-induced pain relief as the rewarding component in the conditioning of animals with chronic pain, effects on the affective dimension of nociception and on spontaneous pain can be elucidated. They demonstrated this application of the CPP by analyzing the analgesic action of morphine, indomethacin, and MK-801 on CFA mice in both the traditional nociceptive assays (Von Frey, Plantar) and in the modified CPP assay. Since their work, this CPP paradigm has been successfully utilized in various other studies with different compounds and pain models (Tzschentke 2007, King 2009, Davoody 2011, Okun 2011, Rutten 2011, Wei 2013, Agarwall 2018). It is worth noting that treatments demonstrated to be clinically ineffective at addressing chronic pain in humans were shown to not elicit CPP in animal pain models; given this, CPP appears to be an important indicator for the potential applicability of novel treatments and compounds in a clinical setting (Navratilova 2013). As such, we have chosen to adapt this CPP to our relaxin-3/RXFP-3 studies, utilizing the CFA-induced persistent inflammatory pain mouse as our chronic pain model.

Chronic Pain Animal Model

Complete Freund's Adjuvant (CFA) injection is a frequently utilized model for persistent inflammatory pain and has been the model of choice for Relaxin-3-related experiments in this laboratory (Okun 2011, Fehrenbacher 2012, Abboud 2021). By subcutaneously injecting the suspension of heat-killed *Mycobacterium tuberculosis* into the hindpaw of mice, induction of inflammatory pain is achieved within 24 hours. This inflammation manifests as visible swelling and as pain hypersensitivity (hyperalgesia and allodynia), which lasts between 1 and 2 weeks; this persistency in pain modulation makes CFA a commonly utilized inflammatory chronic pain model (Fehrenbacher 2012, Abboud 2021) This hypersensitivity to pain can be experimentally observed in the Von Frey and Plantar nociceptive assays, presenting as a decrease in Paw Withdrawal Thresholds (PWT) in the ipsilateral paw which is not reflected in the contralateral paw's PWT, nor in mice injected with vehicle (NaCl 0.9%). In this laboratory, CFA has been determined to last 9 days before pain hypersensitivity begins to disappear significantly. As such, experiments related in this report were designed with this time limit taken into consideration.

Aim of the Project:

Prior research in this laboratory and associated institutions has linked Relaxin-3 and pain modulation, demonstrating its ability to attenuate mechanical and thermal pain hypersensitivity in a chronic inflammatory pain mouse model. However, given the limitations of the Von Frey and Plantar assays, Relaxin-3's ability to affect spontaneous pain and to modulate the affective-emotional dimension of nociception has not been conclusively demonstrated. This project seeks to develop a CPP protocol utilizing the pain-relief paradigm introduced by Sufka et al. in order to assess Relaxin-3's effects on these parameters, and in this manner complement the research previously completed in this laboratory regarding Relaxin-3's ability to modulate chronic pain. We additionally sought to characterize the Relaxin-3/Somatostatin neuron interactions in the BLA, utilizing a pharmacogenetic approach for in-vivo modulation of Somatostatin neurons in the CPP paradigm developed herein. Finally, we sought to determine the effects of chronic activation of the Relaxin-3 pathway in the ACC, virally inducing the tonic production of Relaxin-3 agonist R3/I5 and longitudinally assessing its effects via the Von Frey and Plantar nociceptive assays.

Materials and Methods

Animals:

Adult C57BL6/J mice were housed under standard conditions with *ad libitum* access to food and water. Somatostatin mice (SOM-IRES-Cre) were obtained from The Jackson Laboratory and kept under identical conditions to the C57BL6/J mice. Mice were housed socially, with 2-5 littermates in a cage when possible. Males and females were housed in separate cages and tested in separate sessions. Mice utilized in Von Frey, Plantar, and Open Field experiments were kept in a 12-hour standard light cycle, while mice utilized in CPP experiments were kept in a 12-hour reversed light cycle. Mice placed in a reversed light were allowed to habituate to the change for two weeks before any behavioral experimentation. All efforts were made to minimize the suffering and the number of animals used for the study presented here.

Starting three days prior to their first testing day, all animals were handled once a day to familiarize them with handling. All handling was done utilizing the palming method. Mice were allowed to habituate to the testing chamber for 15-20 minutes prior to the start of each testing session. To prevent interference from olfactory cues, all experimental apparatus were cleaned thoroughly at the start of each testing day and in between trials. Researchers were blinded to the experimental conditions of the mice for the duration of the experiments. Male and female mice were not tested in the same testing session.

Compounds

Complete Freund's Adjuvant (CFA) was obtained from Sigma Aldrich (F5881) and stored at 4°C. Buprenorphine was obtained from the PIV-EXPE of the University of Bordeaux at a concentration of 0.3 mg/ml diluted in NaCl 0.9% and kept at room temperature. Ibuprofen sodium salt (I1892) was obtained via Sigma-Aldrich and diluted in NaCl 0.9% to a concentration of 20 mg/ml. Ibuprofen sodium salt was stored at room temperature, while the ibuprofen solution was stored at 4°C. Clozapine N-Oxide (CNO) dihydrochloride (HB6149) was obtained from HelloBio and diluted in NaCl 0.9% to a concentration of 2 mg/ml. Both CNO powder and solution were stored at -20°C. A5 agonist was obtained from the Florey Institute, Australia, and diluted in 1x Artificial Cerebrospinal Fluid (ACSF) to a concentration of 5µg/µl. Both A5 powder and A5 solution were stored at -20°C. ACSF is a solution at pH 7.4 consisting of: NaCl (130.5mM), KCl (2.4mM), $CaCl_2$ (2.4mM), $NaHCO_3$ (19.5mM), $MgSO_4$ (1.3mM), KH_2PO_4 (1.2mM), HEPES (1.25mM), Glucose (10mM). The construct utilized in the DREADD CPP was obtained from Bryan Roth through AddGene (50474-AAV5), and consisted of a Gq-coupled hM3D DREADD fused with mCherry under the control of human synapsin promoter (*p-AAV5-hSyn-hM3D(Gq)-mCherry*), serving as an excitatory receptor in conjunction with CNO application. The construct utilized for the tonic expression of RXFP-3 agonist R3/I5 was rAAV1/2-FIB-R3/I5 and was obtained from a private laboratory; construct information is detailed in Ganella 2013.

Stereotaxic Surgeries — Cannula Implantation and Viral Injections (DREADD, I5):

Anesthesia is induced in an induction chamber with 5% isoflurane, and then maintained with 2% isoflurane via the mask of the stereotaxic frame with 0.2 bar LMP air. After being mounted on the stereotaxic frame and secured in place utilizing the ear bars, a heating mat set to 37°C is placed beneath the mouse, ocular gel is applied to their eyes, and the head of the animal is shaved utilizing an electric razor. The mouse is then subcutaneously injected with 100 ul buprenorphine (0.1 mg/kg) in its back, and subcutaneously injected with 100 ul 1% lidocaine under the scalp. The surgical area is disinfected utilizing Betadine Soap, then yellow betadine, before being allowed to dry.

The skin of the surgical area is incised to expose the skull, which is cleaned with NaCl 0.9%. One (1) drop of Green Activator (*Super-Bond C&B*) is applied to the skull and removed after 30 seconds. Then, one (1) drop of Red Activator (*Super-Bond C&B*) is applied to the skull and removed after 15 seconds, after which the area is cleaned with NaCl 0.9% again. The Bregma is located and utilized to confirm the horizontality of the skull; if it is not, it is adjusted in the

stereotaxic frame until it is planar. Then, the target coordinates are located relative to the Bregma. The target coordinates for the BLA are: AP = -1.6 mm; ML = +/- 3.3 mm; DV = -4.8 mm, and are utilized for the BLA cannula insertions and the DREADD injections. The target coordinates for the ACC are: AP = +0.9 mm; ML = +/- 0.4 mm; DV = -1.4 mm, and are utilized for the I5 injections. A hole is manually drilled into the skull at the target coordinates utilizing the tip of the drill.

Cannula Implantation:

The guide cannula (for cannula specifications, refer to Table 1) is placed on the cannula holder with a dummy already inserted into the guide cannula, which is then mounted onto the frame. The Bregma is located and utilized to recalibrate the target coordinates to the cannula. The cannula is then moved to the target coordinates, and slowly inserted into the brain to the target depth. Dental cement (*Super Bond C&B*) is then prepared by mixing 0.75 teaspoons of polymer with 3 drops of monomer and one drop of Catalyst V; the mixture is applied around the cannula and the entirety of the surgical area, covering all of the exposed skull. After the cement has dried (5-10 minutes), the mouse is taken off the stereotaxic frame and moved into a clean recuperation cage, which is placed on a warming blanket set to 37°C. The mouse is allowed to recover until it has awakened and regained locomotion, at which point it is returned to its home cage. The mouse undergoes post-operational checkups for three days following the procedure to ensure proper recovery.

Viral Injection (hM3D(Gq), R3/I5)

A 10µl Hamilton syringe is placed on the syringe holder connected to the infusion pump, which is then mounted onto the stereotaxic frame. The Bregma is located and utilized to recalibrate the target coordinates to the syringe. The syringe is then moved to the target coordinates, and slowly inserted into the brain to the target depth. The injection of the substance is then initiated. pAAV-hSyn-hM3D(Gq)-mCherry is injected at a rate of 35 nl/min to a volume of 350 nl. rAAV1/2-FIB-R3/I5 is injected at a rate of 25 nl/min to a volume of 250 nl. Ten minutes after the end of the injection process, the needle is slowly retracted. Then, after a 5 minute waiting period, the incision is sutured utilizing 6.0 thread, and the area is disinfected with Betadine. The mouse is taken off the stereotaxic frame and moved into a clean recuperation cage, which is placed on a warming blanket set to 37°C. The mouse is allowed to recover until it has awakened and regained locomotion, at which point it is returned to its home cage. The mouse undergoes post-operational checkups for three days following the procedure to ensure proper recovery.

Induction of Inflammatory Pain Model:

Anesthesia is induced in an induction chamber with 5% isoflurane, and then maintained with 2% isoflurane via a surgical mask, with the mouse resting on a heating mat set to 37°C. The mouse then undergoes a 20 µl intraplantar injection with Freund's Complete Adjuvant (CFA) to the hindpaw contralateral to the implanted cannula/viral injection site. Mice with the Control Group designation are instead injected with 20 µl NaCl 0.9% in a similar manner. The mouse is then moved into a clean recuperation cage, which is placed on a warming blanket set to 37°C. The mouse is allowed to recover until it has awakened and regained locomotion, at which point it is returned to its home cage. Successful induction of the chronic inflammatory pain model via CFA

injection is visually assessed the day after the injection via visual inspection of the injection site, with visible swelling of the hindpaw indicating a successful procedure.

Conditioned Place Preference (CPP):

CPP Procedure

CPP testing was performed in a 2-chamber CPP constructed from opaque, smooth, dark grey PVC sheets 0.5 cm thick. Both chambers measured 20 cm x 21 cm x 30 cm, and each had their walls entirely covered by proximal visual cues on plasticized sheets. Visual cues for the ibuprofen CPP consisted of vertical black and white stripes in one chamber, and solid gray in the other. For all other CPP experiments, visual cues consisted of vertical black and white stripes in one chamber, and horizontal black and white stripes in the other. The chambers were connected by a narrow corridor measuring 10.5 cm x 16 cm x 30 cm, which did not have any visual cues. The floor of the arena was uniformly composed of the same material as the chamber/corridor walls. Figure 5 is a visual representation of the CPP Arena as seen in the A5 CPP recordings. A removable gate constructed from the same material and measuring 11.5 cm x 0.5 cm x 33 cm was utilized during conditioning sessions to close off access to chambers not being currently conditioned. Testing was performed under dim light conditions to reduce stressors; red light was not utilized as the mice needed to be able to discern the visual cues in each chamber. Testing took place over 10 days and was composed of three phases: pre-conditioning (days 1-3), conditioning (days 4-9), and testing (day 10).

In the preconditioning sessions, the mouse was placed in the corridor of the arena in an undrugged state and allowed to freely explore all chambers of the arena for 15 minutes before being returned to their cage. On the 3rd day of preconditioning, mouse behavior was recorded and analyzed to determine innate chamber biases. Innate chamber biases were considered at an individual level for the purpose of determining whether a biased or an unbiased conditioning strategy would be utilized. If the mouse presented a difference in chamber preference greater than 10%, a biased conditioning strategy was utilized, and the experimental substance was paired to the least-preferred chamber. If the mouse presented a difference in chamber preference less than 10%, an unbiased conditioning strategy was utilized, and the experimental substance was paired at random, utilizing a counterbalancing approach. At the end of the 3rd day of preconditioning, mice were injected with CFA (chronic pain group) or NaCl 0.9% (no pain group).

In the conditioning sessions, the mouse was injected with either the experimental substance (days 4, 6, and 8) or the vehicle substance (days 5, 7, 9), then after a waiting period isolated in a holding cage (5 minutes for Buprenorphine testing, 15 minutes for Ibuprofen and A5 testing), placed in the paired chamber associated with the compound injected for 30 minutes, with access to the remainder of the arena being blocked off by the gate, before being returned to their cage. In the DREADD CPP, to ensure simultaneous activation of DREADD and A5 activity, CNO injections occur 30 minutes before they are placed in the CPP arena (15 minutes prior to A5 injection). Finally, in the test session, the mouse was placed in the corridor of the arena in an undrugged state and allowed to freely explore all chambers of the arena for 15 minutes before being returned to their cage, with their behavior being recorded and analyzed to determine

Conditioned Place Preference. For further details concerning the CPP Procedure, including variations in the procedure for the CPPs performed in the development phase (Ibuprofen, Buprenorphine), refer to the CPP Protocols included in Supplement S1

Figure 5: Conditioned Place Preference Arena:
Conditioned Place Preference arena as seen in the recordings created. The leftmost chamber is covered with horizontal stripes, while the rightmost chamber is covered with vertical stripes; otherwise, the two chambers are functionally identical. The two chambers are connected by a narrow corridor, whose walls are bare and contain no visual clues. Sample taken during the Testing phase of this mouse; as such, all chambers are accessible by the mouse. During conditioning, a gate would be utilized to block access to both the corridor and the chamber not being paired during that session.

CPP Injections

A5 (5µg/µl) and ACSF were injected intracerebrally into the BLA via the guide cannula following anesthetization of the mouse via isoflurane; injection was performed utilizing a 25 µl Hamilton Syringe and an infusion pump at a rate of 100 nl/min to a volume of 200 nl. Ibuprofen and Buprenorphine were injected intraperitoneally utilizing a 1 ml syringe with a 29G needle in awake and restrained mice. Buprenorphine (0.3 mg/ml) was injected to a volume of 3.33 ml/kg. while Ibuprofen (20 mg/ml) was injected to a volume of 5 ml/kg. CNO was injected intraperitoneally utilizing a 1 ml syringe with a 29G needle following anesthetization of the mouse via isoflurane, prior to the injection of A5/ACSF. CNO (2 mg/ml) was injected to a volume of 2 ml/kg. Of note is that this volume was injected erroneously, with the correct target volume being of 1 ml/kg of 2 mg/ml CNO; as such, while this study reports the effects from the actually injected volume of 2 ml/kg, it recommends utilizing the correct volume of 1 ml/kg.

CPP Analysis

Experiment videos were recorded utilizing the camera Windows 10 application and an external webcam and scored utilizing Ethovision XT 13. Time spent in each chamber was utilized to assess chamber preference ($Preference\ (Chamber\ X) = \frac{Time\ spent\ in\ Chamber\ X}{15\ minutes} * 100\%$), with time spent in the corridor being recorded and calculated but not utilized in the statistical analyses. Changes in chamber preference, and as such assessment of the development of conditioned place preference, was assessed through the calculation of CPP Scores. CPP scores were determined individually for each mouse, with each mouse receiving a CPP score

for each chamber; CPP score averages were subsequently determined per experimental group and utilized to measure changes in chamber preference. CPP score was calculated utilizing the following formula: $CPP\ Score\ (Chamber\ X) = \frac{Time\ spent\ in\ Chamber\ X_{Postconditioning} - Time\ spent\ in\ Chamber\ X_{Preconditioning}}{15\ minutes} * 100\%$. Area transition was said to occur when the animal's centerpoint, as defined by Ethovision, crossed the threshold between the chambers and the corridor. Chance level for time spent in a chamber was set at 41.67%, calculated from the area of the arena ($Chance\ level\ = \frac{380\ cm^2}{912\ cm^2}\%$). Mice with a chamber preference greater than 80% and/or less than 20% during the preconditioning and/or the test phase were excluded from the test results, as this is a sign of non-active participation in the task (King 2009, Navratilova 2013, McKendrick 2020).

Von Frey:

Von Frey Procedure

The Von Frey apparatus was obtained from Ugo Basile, and consisted of 12 10x10x14 cm plastic compartments (opaque divisions between compartments, transparent wall facing researcher, no floor, removable ceiling) over a black mesh grid (1 mm thick, 5x5 mm square holes) coated in polymer resin. Von Frey filaments were also obtained from Ugo Basile, and consisted of monofilaments of various target forces; forces utilized in these experiments were as follows: 0.04 g; 0.16 g; 0.40 g; 0.60 g; 1.0 g; 1.4 g; 2.0 g; 4.0 g. Testing was performed under bright white light conditions. No more than 7 mice were tested at a time.

Von Frey testing was done utilizing an ascending/descending scoring method. The starting filament is applied perpendicularly to the mouse's hindpaw, and the response and lack thereof is recorded. This is repeated until stimulation of that paw with the starting filament resulted in three withdrawal responses, or three lack of responses. If three withdrawal responses were recorded, the next lower force filament is utilized, and the process is repeated, utilizing subsequently lower filaments until three lack of responses are recorded. If three lack of responses were recorded with the starting filament, the next higher force filament is utilized, repeating the process until three withdrawal responses are recorded. In both cases, the Paw Withdrawal Threshold (PWT) is the last filament which elicited three withdrawal responses. After a paw is stimulated, that same paw is not stimulated for at least 30 seconds to avoid sensibilization. To prevent paw withdrawal in response to touch instead of nociception, mice are periodically stimulated utilizing a 0.04 g filament. The starting filament is 1.0 g for baseline measurement. For post-CFA measurement, the starting filament is 1.0 g for the paw contralateral to the injection, and 0.6 g for the paw ipsilateral to the injection.

In the Ibuprofen and Buprenorphine Von Frey experiments, Baseline PWTs were obtained up to 1 week prior to CFA injection, with the mice in an undrugged state. Mice were tested for pain responses 24 hours after CFA injection in the manner described above. In the I5 Von Frey experiment, two baseline PWTs were obtained: the Pre-Expression Baseline was obtained up to 1 week prior to the injection of the R3/I5 viral construct, while the Post-Expression Baseline was obtained 4 weeks after R3/I5 construct injection. CFA was applied the day after the Post-

Expression Baseline was obtained, and mice were tested for pain responses on days 1, 3, 5, and 7 after CFA injection, in the manner described above.

Von Frey Injections:

Ibuprofen and Buprenorphine were injected intraperitoneally utilizing a 1 ml syringe with a 29G needle in awake and restrained mice. Buprenorphine (0.3 mg/ml) was injected to a volume of 3.33 ml/kg. while Ibuprofen (20 mg/ml) was injected to a volume of 5 ml/kg.

Plantar:

Plantar Procedure

The Plantar apparatus was obtained from Ugo Basile, and consisted of 12 10x10x14 cm plastic compartments (opaque divisions between compartments, transparent wall facing researcher, no floor, removable ceiling) over a transparent glass pane, underneath which a movable I.R. (infrared) generator with embedded paw withdrawal detector was placed.Testing was performed under bright white light conditions. No more than 7 mice were tested at a time. Radiant intensity was set to 50, and automatic cutoff was set at 7 seconds. Mice were tested for PWTs 5 times per session per hindpaw, or until 3 PWTs within 1 second of each other were obtained for that particular mouse and that particular paw. The average of these 3-5 PWTs was taken as a measurement of their thermal pain sensitivity. In calculating this average, PWTs measurements falling outside of 1.5 seconds from the next closest measurement were considered outliers and excluded. After a paw is stimulated, that same paw is not stimulated for at least 30 seconds to avoid sensibilization.

In the Ibuprofen and Buprenorphine Von Frey experiments, Baseline PWTs were obtained up to 1 week prior to CFA injection, with the mice in an undrugged state. Mice were tested for pain responses 24 hours after CFA injection in the manner described above. In the I5 Von Frey experiment, two baseline PWTs were obtained: the Pre-Expression Baseline was obtained up to 1 week prior to the injection of the R3/I5 viral construct, while the Post-Expression Baseline was obtained 4 weeks after R3/I5 construct injection. CFA was applied the day after the Post-Expression Baseline was obtained, and mice were tested for pain responses on days 1, 3, 5, and 7 after CFA injection, in the manner described above.

Plantar Injections:

Ibuprofen and Buprenorphine were injected intraperitoneally utilizing a 1 ml syringe with a 29G needle in awake and restrained mice. Buprenorphine (0.3 mg/ml) was injected to a volume of 3.33 ml/kg. while Ibuprofen (20 mg/ml) was injected to a volume of 5 ml/kg.

Open Field:

Open field testing was performed in a square chamber made out of gray PVC measuring 40 cm x 40 cm under dim white lighting conditions. The animal was placed in the center of the arena and was allowed to explore the area for 10 minutes, after which the animal was returned to its individual cage.

Experiment videos were scored utilizing Ethovision XT 13, with the center area being defined as a square at the center of the arena comprising exactly 50% of the entire open field surface, and with the periphery comprising the remaining area. Time spent in the periphery vs. time spent in the center was utilized to assess anxiety, while distance travelled was utilized to assess locomotion.

Pain Transfer:

Pain Transfer experiments were performed as described in Smith 2021, with Von Frey testing following the protocol delineated in this report. Pairs were formed out of littermates housed in the same cage. Baseline PWTs were obtained one week prior to CFA injection. Pain-group mice were injected with CFA, while Bystander-group mice were injected with saline. CFA injection was performed on awake mice, as per the protocol set by Smith et al; other than this variation, CFA injections followed the protocol delineated in this report. Immediately following CFA injection, pairs were placed in new cages with no access to food, water, or enrichment, and left in the Von Frey testing room to socially interact undisturbed for 1 hour. At the end of the hour, mice were moved to the Von Frey apparatus and their PWTs were obtained. Mice were not returned to their original cages, instead being housed in the pairs formed for this experiment.

Immunohistochemistry:

Following behavioral experiments, mice were perfused and their brain tissues were harvested. Perfusion-fixed tissues were cut utilizing a cryostat, and then underwent the lab-standard RLN3/GFP immunohistochemistry to determine successful I5 AAV expression, utilizing its GFP tag. Tissues were washed three times with PBS 1X (0.1 M) for 10 minutes, then incubated in BB BSA 1% and Triton 0.3% diluted in PBS 1X for 30 minutes at room temperature under constant agitation. Tissues were then incubated overnight at 4°C under constant agitation in the primary antibodies (Mouse anti-RLN3 1/10 and Chicken anti-GFP 1/1000) diluted in PBS 1X and BSA 1%. The next day, tissues were washed three times with PBS 1X for 10 minutes, then incubated for 2 hours at room temperature under constant agitation with the secondary antibodies (Goat anti-mouse A568 1/500, Goat anti-chicken A488 1/500) diluted in PBS 1X. Afterwards, tissues were once again washed three times with PBS 1X for 10 minutes, then mounted on slides utilizing Fluoromount G for imaging.

Statistical Analysis:

For statistical analysis GraphPad Prism 9.1.0 (221) was used. First, outliers were removed utilizing ROUT (Q = 1%). For analysis of differences between three or more different groups, a 2-way ANOVA with Tukey's or Sidak's Multiple Comparison was used ($\alpha = 0.05$). In addition to these, Fisher's LSD Multiple Comparison was used to determine trends in the data ($\alpha = 0.05$). Performance of groups above or below chance level was determined through one sample T-Tests.

Results

Pain Transfer:

Von Frey: Bystander mice do not exhibit hyperalgesia following social interaction with CFA-treated mice

The Pain Transfer experiment was performed in order to determine whether the behavior of mice with no pain would be affected if they were housed in the same cage as mice with CFA-induced chronic inflammatory pain. This was done following the results found reported in Smith 2021, which reported that when CFA mice and Bystander mice socially interact, Bystander mice will behave as if they are in pain in a manner that is detectable through tests such as the Von Frey.

Figure 6 shows the paw withdrawal threshold (PWT) of CFA and Bystander Ipsilateral and Contralateral paws at baseline and after pain transferal. No significant difference in baseline values was observed between groups ($p \geq 0.896$). CFA animals showed a significant reduction in PWT following pain transferal in their ipsilateral paw ($p = 0.025$), but not in their contralateral paw ($p \geq 0.999$). Bystander animals showed no significant change following pain transferal in either paw ($p \geq 0.850$). Only the test-phase ipsilateral PWT of the CFA mice showed a significant deviation from the overall average baseline of 1.575 g ($p = 0.003$); the PWT of all other groups were not significantly different from this overall baseline ($p \geq 0.812$).This represents a significant deviation from the results presented in Smith 2021, in which Bystander animals demonstrated a significant decrease in PWT in both ipsilateral and contralateral paws following pain transfer, matching that of the CFA ipsilateral paw. Our results appear to contradict theirs, suggesting instead that there is no social transfer of pain from our CFA-treated mice to naive mice. Table 2 contains more detailed data on the Pain Transfer test

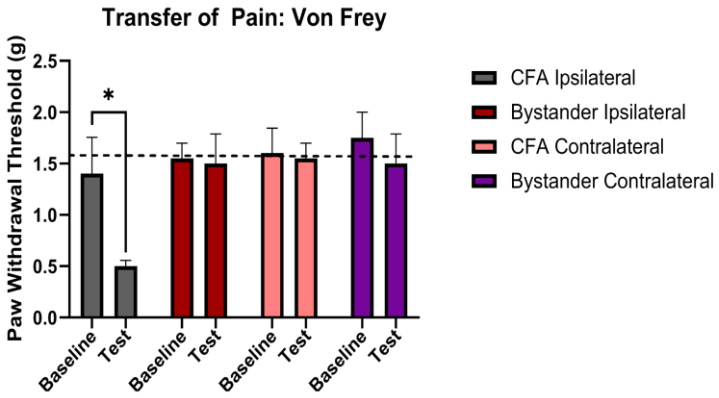


Figure 6: Pain Transfer Von Frey

Only the Ipsilateral paw of the CFA mice exhibited a significant decrease in PWT from baseline after the pain transfer procedure, with the CFA contralateral paw and both Bystander paws showing no change (CFA Ipsilateral: Baseline: 1.40 ± 0.71 g, Test: 0.50 ± 0.12 g; Bystander Ipsilateral: Baseline: 1.55 ± 0.30 g; Test: 1.50 ± 0.58 g; CFA Contralateral: Baseline: 1.60 ± 0.49 g; Test: 1.55 ± 0.30 g; Bystander Contralateral: Baseline: 1.75 ± 0.50 g; Test: 1.50 ± 0.58 g). Dotted line represents the average baseline value for all mice (1.575 g). Only the Ipsilateral PWT of the CFA mice differed significantly from this value. CFA Ipsilateral Paw is indicated in gray; Bystander Ipsilateral Paw is indicated in red; CFA Contralateral Paw is indicated in pink; Bystander Contralateral Paw is indicated in purple. For all groups, n = 4. All mice utilized in this experiment were Male. * indicates $p \leq 0.05$. Mean and SEM are indicated.

Ibuprofen:

Ibuprofen was selected, in conjunction with buprenorphine, as a pilot and a positive control for the Relaxin-3 CPP by providing a point of comparison from a more well-established antinociceptive drug and allowing us to determine points of improvement and potential problems with our CPP apparatus and protocol. Ibuprofen was specifically selected based on Lim 2014, in which the effects of ibuprofen on an CFA-induced arthritic chronic pain model was tested in the CPP paradigm. The Von Frey test was utilized to test for anti-nociception to mechanical stimuli on our CFA mice, while the Plantar test was utilized to test the same regarding thermal stimuli. Meanwhile, the CPP test was utilized to determine anti-nociception to non-evoked stimuli and effects on the affective facet of pain. At this stage, the CPP protocol utilized a one-session conditioning paradigm; ibuprofen and saline were only administered and paired to their respective chambers once, for a total of two conditioning sessions throughout the experiment. Furthermore, visual cues consisted of vertical stripes in one chamber, and solid gray walls in the other.

Von Frey: Ibuprofen does not significantly attenuate CFA-induced mechanical allodynia

Figure 7 compares the PWT of CFA mice treated with either i.p. ibuprofen or i.p. saline. No significant difference in baseline values were observed between all groups ($p \geq 0.993$). As such, all baselines were merged to produce an overall average baseline (1.53 g). Both ibuprofen- and saline-treated animals demonstrated a significant decrease from baseline in ipsilateral PWT during the test phase ($p \leq 0.036$), but not in contralateral PWT ($p \geq 0.980$). Additionally, ipsilateral PWT of both ibuprofen- and saline-treated animals were significantly different from the overall average baseline PWT ($p \leq 0.006$). Interestingly, no significant difference between ipsilateral PWT and contralateral PWT within groups was observed in the test phase ($p \geq 0.052$). However, Fisher's LSD revealed a significant trend towards the contralateral PWT during the test phase being higher than their ipsilateral counterparts ($p \leq 0.005$). Table 3 contains more detailed data on the ibuprofen Von Frey. Overall, these results appear to suggest that ibuprofen at the administered dosage does not have an effect on evoked mechanical nociception. However, as there may be a dissociation between modulation of nociception in the somatosensory dimension and in the affective dimension (van der Kam 2008), a failure to affect evoked mechanical allodynia does not predict a failure to affect non-evoked pain. As such, the CPP experiment was conducted as planned.

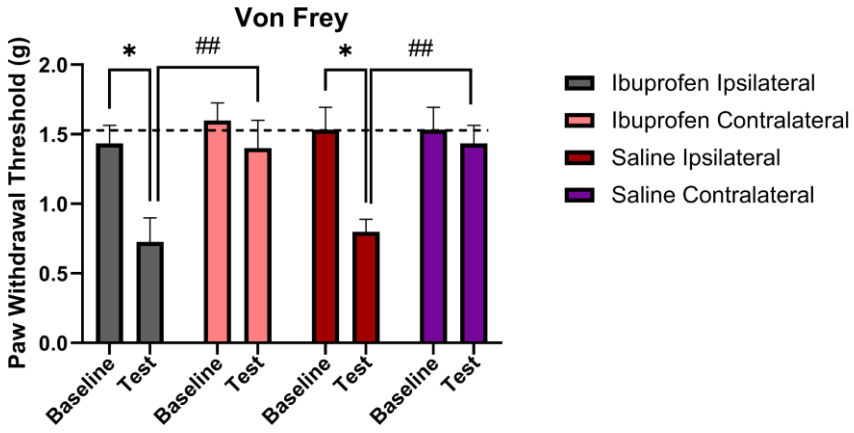


Figure 7: Ibuprofen Von Frey

Both mice treated with i.p. ibuprofen and i.p. saline exhibited a significant decrease in PWT in the ipsilateral paw following CFA injection; no significant change was observed in the contralateral paw of either group. The ipsilateral paw of the ibuprofen-treated mice is indicated in gray; their contralateral paw is indicated in pink. The ipsilateral paw of the saline-treated mice is indicated in red; their contralateral paw is indicated in purple. The dotted line indicates the average baseline for all the animals (1.53 g). For all groups, n = 6. All mice utilized in this experiment were Male. * indicates $p \leq 0.05$. ## indicates $p \leq 0.005$ per Fisher's LSD. Mean and SEM are indicated.

CPP: Ibuprofen does not induce significant conditioned place preference in either CFA- or NaCl-treated mice

Figure 8A compares the preferences for the ibuprofen-paired and saline-paired chambers before and after conditioning for the CFA- and NaCl treated mice. As can be observed, there is no significant difference in preference between the preconditioning and test phases for any group or chamber pairings ($p \geq 0.914$). However, as demonstrated in Figure S5A, mice showed a significant bias for the solid chamber over the striped chamber during the preconditioning phase ($p = 0.003$). Figures 8B-C show the CPP score for the ibuprofen-paired (B) and saline-paired (C) chambers between the preconditioning and test phases. Neither CFA nor NaCl mice showed a significant CPP score for either chamber ($p \geq 0.462$), nor was there a significant difference in the CPP score between groups in either chamber ($p \geq 0.495$). Overall, this data suggests that ibuprofen was unable to induce CPP in neither the CFA-treated nor the NaCl treated groups. In light of these results, the conditioning protocol was changed to a three-session conditioning paradigm in order to enhance the conditioning effect and ensure that it may take place. Furthermore, in order to diminish the inherent bias towards the solid chamber over the striped chamber, the visual cues were changed to consist of vertical stripes in one chamber, and horizontal stripes in the other.

Table 4 contains more detailed data on the ibuprofen CPP. The data presented here reflects a conditioning protocol which utilizes individual preconditioning chamber preferences as a basis for the substance-chamber pairings (for another pairing method, refer to Supplement S2).

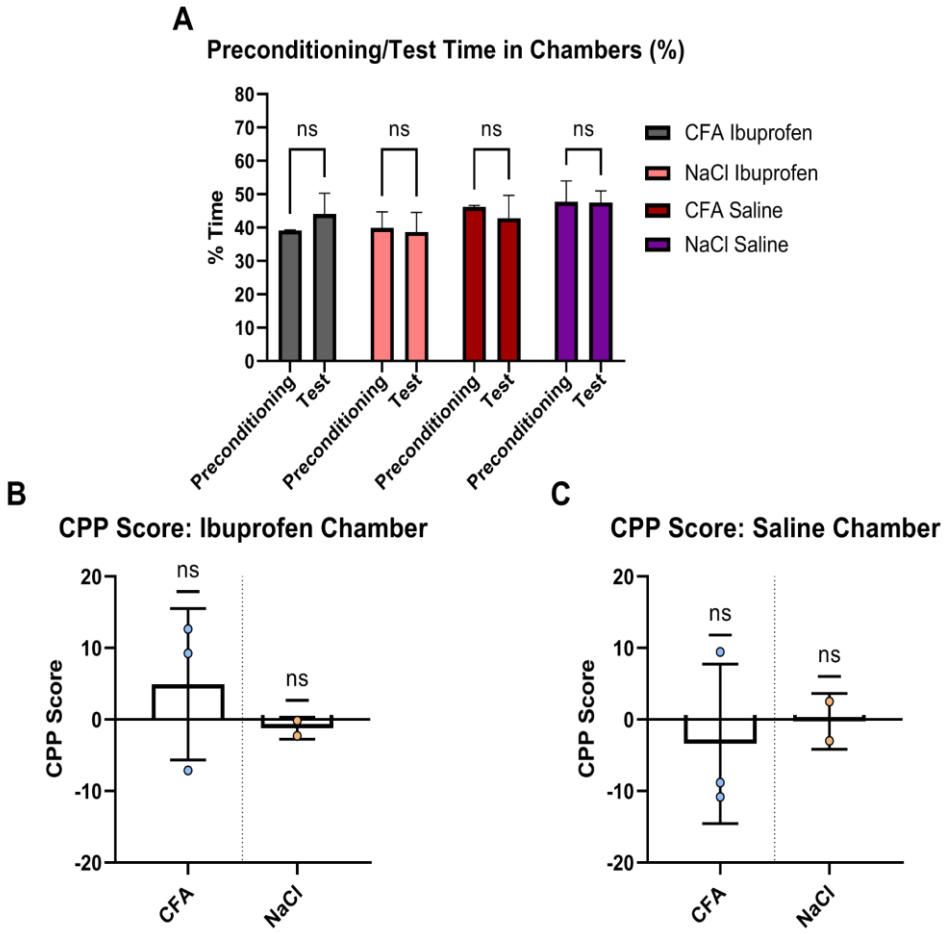


Figure 8: Ibuprofen CPP

A) Neither CFA nor NaCl mice exhibited a significant difference in chamber preference between preconditioning and test sessions for either the ibuprofen or the saline chamber (CFA Ibuprofen: Preconditioning: 39.08 ± 0.43%; Test: 44.00 ± 10.95%; NaCl Ibuprofen: Preconditioning: 39.91 ± 6.80%; Test: 38.70 ± 8.33%; CFA Saline: Preconditioning: 46.15 ± 0.81%; Test: 42.75 ± 11.95%; NaCl Saline: Preconditioning: 47.71 ± 8.83%; Test: 47.47 ± 4.93%). No value was significantly different from chance level (41.67%). B-C) Neither CFA nor NaCl mice exhibited a CPP score significantly different than 0 in either the ibuprofen or the saline chamber, nor did CPP scores differ between CFA and NaCl mice (B: CFA: 4.93 ± 10.58; NaCl: -1.22 ± 1.53; C: CFA: -3.40 ± 11.16; NaCl: -0.24 ± 3.90). A) CFA Ibuprofen mice are indicated in Grey; NaCl Ibuprofen mice are indicated in pink; CFA Saline mice are indicated in red; NaCl Saline mice are indicated in Purple. B-C) CFA animals are indicated in Blue; NaCl animals are indicated in Beige. CFA: n = 3; NaCl: n = 2. Mice were both Male (CFA: n = 1; NaCl: n = 1) and Female (CFA: n = 2; NaCl: n = 1). ns indicates $p \geq 0.05$. Mean and SEM are indicated.

Buprenorphine:

As with ibuprofen, buprenorphine was selected as a pilot and a positive control for the Relaxin-3 CPP to provide a point of comparison from a more well-established antinociceptive drug, in this case a partial μ-opioid receptor (MOR) agonist, and to allow us to determine points of improvement and potential problems with our CPP apparatus and protocol (Marquez 2007, Canestrelli 2014). Buprenorphine was selected based primarily on Tzschentke 2004, which outlined buprenorphine usage in CPP experiments, though not in a pain-relief paradigm. As with the ibuprofen experiments, Von Frey was utilized to assess buprenorphine's effects on evoked

mechanical nociceptive stimuli, while the CPP was utilized to determine effects on non-evoked nociception. At this stage, the CPP protocol utilized a three-session conditioning paradigm; ibuprofen and saline were administered and paired to their respective chambers three times, for a total of six conditioning sessions throughout the experiment. Furthermore, visual cues consisted of vertical stripes in one chamber, and horizontal stripes in the other.

Von Frey: Buprenorphine attenuates CFA-induced mechanical allodynia

Figure 9 compares the PWT of CFA mice treated with either i.p. buprenorphine or i.p. saline. No significant difference in baseline values were observed between all groups ($p \geq 0.959$). As such, all baselines were merged to produce an overall average baseline (1.43 g). Neither buprenorphine-treated nor saline-treated animals demonstrated a significant difference from baseline PWT in either their ipsilateral or contralateral paws ($p \geq 0.098$). However, Fisher's LSD analysis unveiled a trend in which the ipsilateral PWT of the saline-treated mice was decreased from its baseline value ($p = 0.006$). Furthermore, saline-treated mice had significantly lower ipsilateral PWT than contralateral PWT in their test phase ($p = 0.006$). This was not true for buprenorphine-treated mice ($p = 0.136$), however Fisher's LSD did show a trend for a lower ipsilateral PWT ($p = 0.009$). When comparing to the overall average baseline, only the ipsilateral PWT of saline-treated mice differed, being significantly lower ($p \leq 0.0001$). Overall, this suggests that buprenorphine is able to attenuate evoked pain from mechanical nociceptive stimuli. Table 5 contains more detailed data on the Buprenorphine Von Frey.

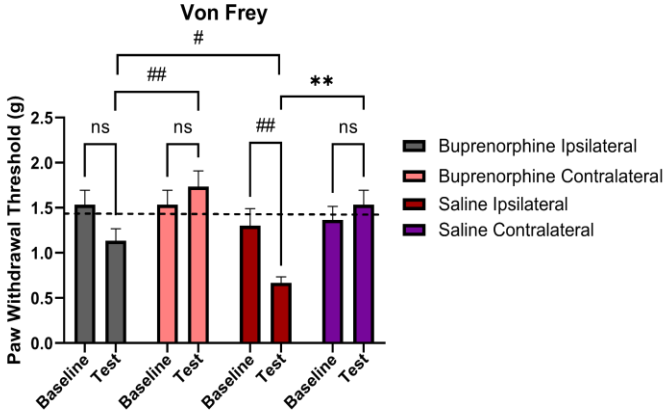


Figure 9: Buprenorphine Von Frey

No significant differences between the baseline PWT and the test phase PWT were observed in either the ipsilateral or contralateral paws of all groups (Buprenorphine Ipsilateral: Baseline: 1.53 ± 0.39 g; Test: 1.13 ± 0.33 g; Buprenorphine Contralateral: Baseline: 1.53 ± 0.39 g; Test: 1.73 ± 0.43 g; Saline Ipsilateral: Baseline: 1.30 ± 0.47 g; Test: 0.67 ± 0.16 g; Saline Contralateral: Baseline: 1.37 ± 0.37 g; Test: 1.53 ± 0.39 g). However, Fisher's LSD revealed a trend for the decrease in the ipsilateral PWT of saline-treated mice from baseline during the test phase. Test phase Ipsilateral PWT of buprenorphine-treated animals did not differ significantly from their contralateral buprenorphine-treated and ipsilateral saline-treated test phase counterparts, though these were marked significantly different by Fisher's LSD. The test phase ipsilateral and contralateral PWT of saline-treated mice differed significantly. The dotted line represents the mean baseline of all animals (1.43 g). The Ipsilateral PWT of the Saline-treated mice during the test phase was the only test phase PWT to significantly differ from this baseline. The ipsilateral paw of the buprenorphine-treated mice is indicated in gray; their contralateral paw is indicated in pink. The ipsilateral paw of the saline-treated mice is indicated in red; their contralateral paw is indicated in purple. Mice were both Male (n = 6) and Female (n = 6). ** indicates $p \leq 0.005$; ns indicates $p \geq 0.05$. Per Fisher's LSD # indicates $p \leq 0.05$; ## indicates $p \leq 0.005$. Mean and SEM are indicated.

CPP: Buprenorphine does not induce significant conditioned place preference in either CFA- or NaCl-treated mice

Figure 10A compares the preferences for the buprenorphine-paired and saline-paired chambers before and after conditioning of the CFA- and NaCl-treated mice. As can be observed, there is no significant difference in preference between the preconditioning and test phases for any group or chamber pairings ($p \geq 0.987$). In addition, as demonstrated in Figure S6A, mice do not demonstrate a significant preference for either chamber in the preconditioning phase when considered as a group ($p \geq 0.404$). Figures 10B-C show the CPP score for the buprenorphine-paired (B) and saline-paired (C) chambers between preconditioning and test phases. Neither CFA nor NaCl showed a significant CPP score for either chamber ($p \geq 0.639$), nor was there a significant difference in the CPP score between groups in either chamber ($p \geq 0.857$). Overall, this data suggests that buprenorphine was unable to induce CPP in neither the CFA-treated nor the NaCl-treated groups. This may be due to the necessity to include washout periods between conditioning sessions due to buprenorphine's long lasting effects. Given that A5 has been noted to function only for a small period of time, including washout periods was deemed unnecessary for this experiment. Additionally, as demonstrated by the buprenorphine CPP, utilizing vertical and horizontal stripes as visual cues results in less pronounced innate chamber bias differences. As such, this visual cue setup was utilized for all other CPP experiments. Table 6 contains more detailed data on the buprenorphine CPP. The data presented here reflects a conditioning protocol which utilizes individual preconditioning chamber preferences as a basis for the substance-chamber pairings (for another pairing method, refer to Supplement S3).

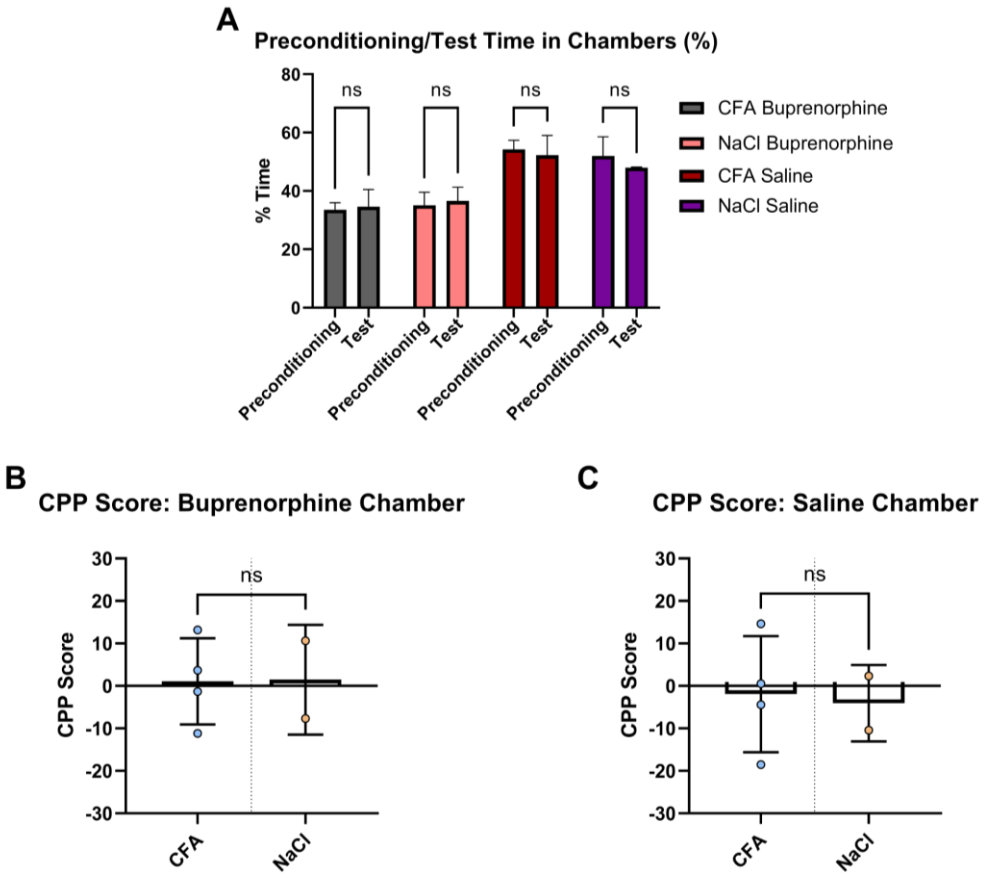


Figure 10: Buprenorphine CPP

A) Neither CFA nor NaCl mice exhibited a significant difference in chamber preference between preconditioning and test sessions for either the buprenorphine or the saline chamber. (CFA Buprenorphine: Preconditioning: 33.55 ± 4.79%; Test: 34.63 ± 11.74%; NaCl Buprenorphine: Preconditioning: 35.12 ± 6.31%; Test: 36.59 ± 6.63%; CFA Saline: Preconditioning: 54.21 ± 6.27%; Test: 52.26 ± 13.55%; NaCl Saline: Preconditioning: 52.02 ± 9.27%; Test: 47.96 ± 0.26%). CFA Preconditioning and Test values were significantly different from chance level (41.67%). B-C) Neither CFA nor NaCl mice exhibited a CPP score significantly different than 0 in either the buprenorphine or the saline chamber, nor did CPP scores differ between CFA and NaCl mice (B: CFA: 1.08 ± 10.15; NaCl: 1.47 ± 12.93; C: CFA: -1.95 ± 13.69; NaCl: -4.06 ± 9.00). A) CFA Buprenorphine mice are indicated in Grey; NaCl Buprenorphine mice are indicated in pink; CFA Saline mice are indicated in red; NaCl Saline mice are indicated in Purple. B-C) CFA animals are indicated in Blue; NaCl animals are indicated in Beige. CFA: n = 4; NaCl n = 2. Mice were Male (CFA: n = 2) and Female (CFA: n = 2; NaCl: n = 2). ns indicates $p \geq 0.05$. Mean and SEM are indicated.

A5:

A5 is an RXFP3 agonist which has been demonstrated to elicit analgesic effects following injection to the BLA in the Von Frey and Plantar behavioral tests. However, it is unknown whether it is able to affect non-evoked pain, nor whether it can modulate the affective dimension of nociception. Having fine-tuned the CPP apparatus and protocol with the ibuprofen and buprenorphine pilots, we ran CFA- and NaCl-treated mice through the CPP experiment utilizing intracerebral injections of A5 and ACSF to the BLA as the drug and vehicle conditioning substances, respectively. These experiments utilized a 6-day conditioning scheme, and vertical and horizontal stripes as visual cues.

CPP: A5 induces significant conditioned place preference in mice treated with CFA, but not in those treated with NaCl

Figure 11A shows the preferences for the A5-paired and ACSF-paired chambers before and after conditioning of the CFA- and NaCl-treated mice. As can be observed, CFA mice demonstrate a both a significant trend for increased preference for the A5-paired chamber following conditioning, and a significant trend for decreased preference for the ACSF-paired chamber following conditioning, as demonstrated by Fisher's LSD ($p = 0.037$, $p = 0.021$). NaCl mice show no significant difference in preference after conditioning in either chamber ($p \geq 0.667$). Figures 11B-C show the CPP score for the A5-paired (B) and ACSF-Paired (C) chambers between preconditioning and test phases. CFA animals demonstrated a significant positive CPP score in the A5 chamber ($p = 0.030$), and a significant negative CPP score in the ACSF chamber ($p = 0.036$). NaCl animals did not demonstrate significant CPP scores in either chamber ($p \geq 0.232$). There additionally was no significant difference between CFA and NaCl CPP scores in either chamber ($p \geq 0.400$). Overall, these results indicate that A5, and as such Relaxin-3, induces conditioned place preference in CFA mice but not in NaCl mice. This in turn suggests that Relaxin-3 is able to effectively modulate nociception in the affective dimension, and that it can attenuate non-evoked pain. Table 7 contains more detailed data on the A5 CPP. The data presented here reflects a conditioning protocol which utilizes preconditioning chamber preferences as a basis for the substance-chamber pairings (for another pairing method, refer to Supplement S4).

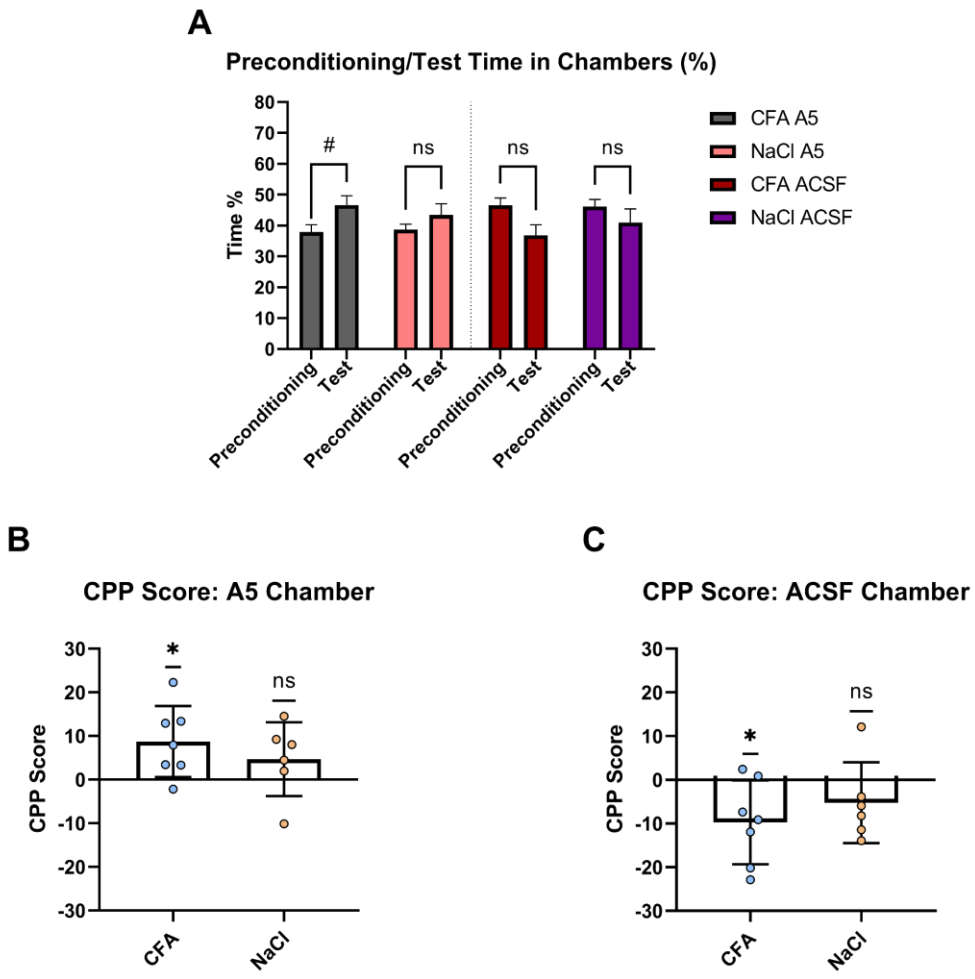


Figure 11: A5 CPP

A) NaCl animals did not present a significant difference in chamber preference between preconditioning and test sessions for either A5 or ACSF chambers. Fisher's LSD revealed a trend in the CFA animals for higher preference for the A5 chamber after conditioning, and a trend for lower preference for the A5 chamber after conditioning (CFA A5: Preconditioning: 37.93 ± 6.15%; Test: 46.66 ± 7.91%; NaCl A5: Preconditioning: 38.73 ± 4.23%; Test: 43.41 ± 8.80%; CFA ACSF: Preconditioning: 46.59 ± 6.19%; Test: 36.85 ± 9.00%; NaCl ACSF: Preconditioning: 46.18 ± 5.51%; Test: 40.97 ± 10.81%). B-C) CFA animals showed a significantly higher CPP score in the A5 chamber, and a significantly lower CPP score in the ACSF chamber. NaCl animals CPP scores were not significant for either chamber (B: CFA: 8.73 ± 8.16; NaCl: 4.69 ± 8.44; C: CFA: -9.74 ± 9.60; NaCl: -5.21 ± 9.24). A) CFA A5 mice are indicated in Grey; NaCl A5 mice are indicated in pink; CFA ACSF mice are indicated in red; NaCl ACSF mice are indicated in Purple. B-C) CFA animals are indicated in Blue; NaCl animals are indicated in Beige. CFA: n = 7; NaCl n = 6. Mice were Male (CFA: n = 4, NaCl: n = 4) and Female (CFA: n = 3; NaCl: n = 2). * indicates $p \leq 0.05$; ns indicates $p \geq 0.05$. # indicates $p \leq 0.05$ according to Fisher's LSD. Mean and SEM are indicated.

DREADD modulation of BLA Somatostatin Neurons in the A5 CPP

While the exact circuitry of the Relaxin-3 pathway is still to be determined, this lab has previously demonstrated that Somatostatin neurons in the BLA, which contain RXFP3, appear to be involved in the modulation of pain by Relaxin-3. Following the hypothesis that Relaxin-3 inhibits these Somatostatin neurons as part of this pathway and having demonstrated the conditioning effect of A5 in the CPP paradigm, we wanted to determine whether excitation of these neurons could effectively block A5's induced effects. To do so, we expressed Gq(excitatory)-DREADD in Somatostatin neurons in the BLA and coincided their activation with the conditioning sessions in the A5 CPP paradigm utilized in the previous experiment. Unfortunately, due to the unavailability of Somatostatin-Cre mice this experiment was performed with a small sample size (n = 4), and as such should be considered preliminary data with further testing being required.

CPP: Excitation of BLA Somatostatin Neurons via DREADD/CNO prevents A5 from inducing conditioned place preference in CFA mice

Figure 12A shows the preferences for the A5-paired and ACSF-paired chambers before and after conditioning of the DREADD-expressing CFA-treated mice. It additionally shows the same values for the non-DREADD CFA- and NaCl-treated mice, which were taken from the A5 CPP (Figure 11) as a basis of comparison. As can be observed, DREADD CFA mice show no significant difference in chamber preference for either chamber after conditioning ($p = 0.970$). Meanwhile, non-DREADD CFA mice exhibit an increased preference for the A5-paired chamber and a significantly decreased preference for the ACSF chamber after conditioning, while non-DREADD NaCl mice showed no significant differences. Figures 12B-C show the CPP score for the A5-paired (B) and ACSF-paired (C) chambers between preconditioning and test phases. DREADD CFA mice did not have significant CPP scores in either the A5 chamber nor the ACSF chamber ($p \geq 0.861$). Meanwhile, as shown in Figure 11, non-DREADD CFA animals demonstrated significant CPP scores in both chambers. Overall, these results appear to indicate that excitation of the Somatostatin neurons effectively blocks the effects of A5, as the post-conditioning chamber preferences exhibited by the DREADD CFA mice more closely resembles that of the non-DREADD NaCl mice than that of the non-DREADD CFA mice. However, as previously highlighted, further experimentation is necessary to complement these preliminary results. Table 9 contains more detailed data on the DREADD/A5 CPP.

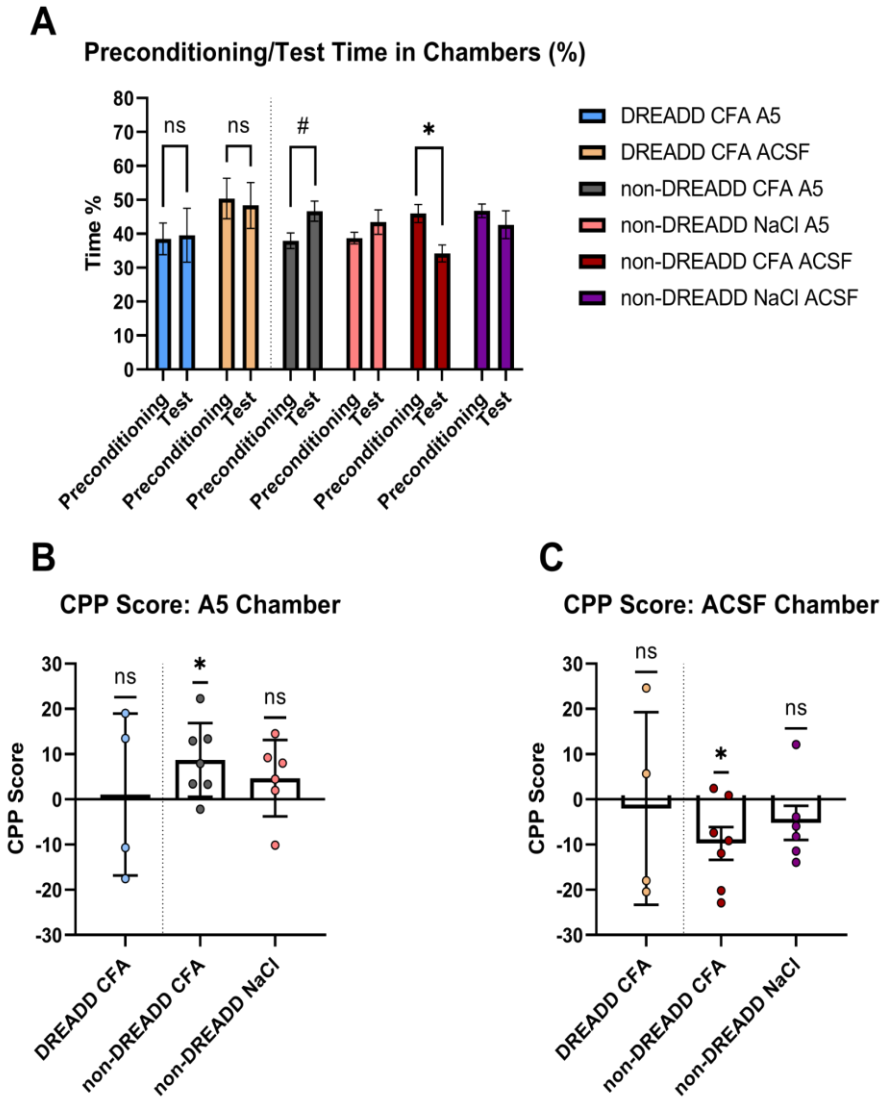


Figure 12: A5 CPP in DREADD Mice

A) DREADD CFA animals did not exhibit a significantly different chamber preference after conditioning in either A5 or ACSF chambers. non-DREADD CFA and NaCl animals from the A5 experiment are included for comparison, demonstrating either a significant change or a trend for significant change in the CFA animals but not in the NaCl animals (DREADD CFA: A5: Preconditioning: 38.49 ± 9.32%; Test: 39.55 ± 15.86%; ACSF: Preconditioning: 50.38 ± 11.91%; Test: 48.36 ± 13.52%). B-C) DREADD- CFA animals did not exhibit a significant CPP score in either the A5-paired (1.07 ± 17.89%) or the ACSF-paired chamber (-2.03 ± 21.31%). DREADD CFA scores did not significantly differ from non-DREADD CFA and NaCl scores in either chamber. For non-DREADD information, refer to Figure 11. DREADD CFA A5 mice are indicated in blue; DREADD CFA ACSF mice are indicated in beige; non-DREADD CFA A5 mice are indicated in grey; non-DREADD NaCl A5 mice are indicated in pink;non-DREADD CFA ACSF mice are indicated in red; non-DREADD NaCl ACSF mice are indicated in purple. DREADD CFA: n = 4. Mice were Male (n = 3) and Female (n = 1). * indicates $p \leq 0.05$; ns indicates $p \geq 0.05$. # indicates $p \leq 0.05$ according to Fisher's LSD. Mean and SEM are indicated.

Chronic excitation of RXFP3 via R3/I5 expression:

The application of the rAAV1/2-FIB-R3/I5 vector leads to chronic expression of the Relaxin-3 agonist R3/I5 in the targeted area, resulting in constant activation of RXFP3 and the Relaxin-3 circuit. We sought to determine the functional effects of this chronic activation in the ACC on stress and locomotion, as well as on mechanical and thermal nociception. For this purpose, we tested mice expressing R3/I5 on the Open Field, Von Frey, and Plantar tests. Successful injection and expression was determined indirectly through imaging analysis of GFP, which is co-expressed with I5, as currently no method exists to directly confirm I5 expression (Figure 13).

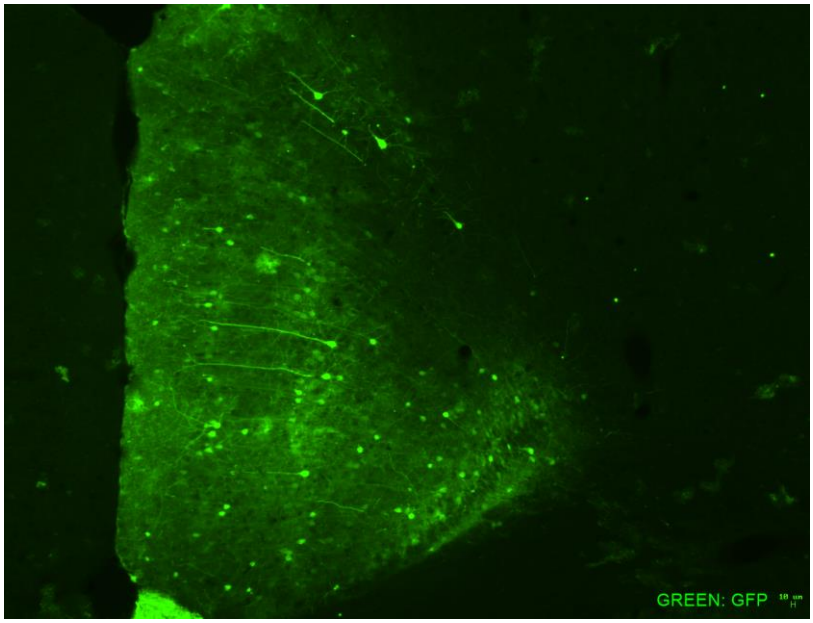


Figure 13: rAAV1/2-FIB-R3/I5 expression in ACC neurons confirmed via GFP imaging
Microscopy image of the ACC at 5x magnification demonstrating localization of GFP in green. Neurons tagged with GFP are presumed to also express R3/I5, as the viral construct is designed so that the neuron co-expresses these. Image provided courtesy of Louison Brochoire.

Open Field: R3/I5 expression in the ACC results in no alteration in locomotion and in stress behavior:

Figure 14A shows the percentage of time spent by R3/I5 and WT mice in the center area of the open field arena. No significant difference was found between groups ($p = 0.543$). Figure 14B shows the total distance travelled by R3/I5 and WT mice during the experiment. As before, no significant difference is present between groups ($p = 0.897$). These results suggest that there is no significant alteration in stress or locomotion resulting from chronic expression of R3/I5 in the ACC. As such, behavioral alterations revealed in the nociceptive assays can be reasonably assumed to not stem from differences in these factors. For more detailed results on the Open Field data, refer to Table 10.

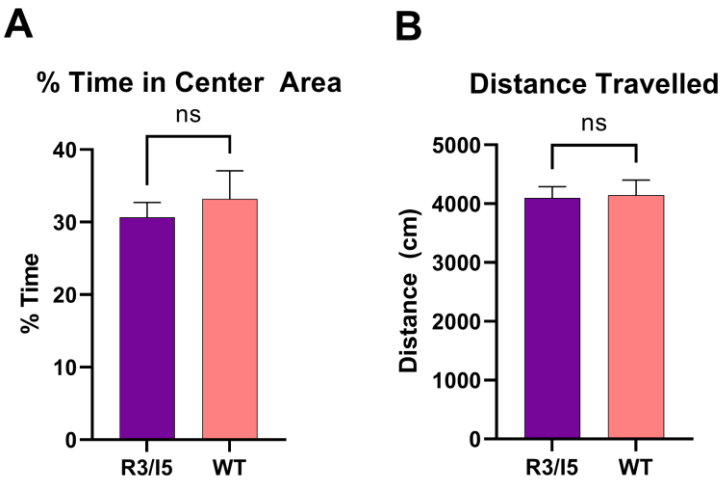


Figure 14: R3/I5 Open Field

A) R3/I5 mice and WT mice spent the same time in the center of the Open Field Arena (R3/I5: 30.65 ± 7.45%; WT: 33.20 ± 8.71%). B) R3/I5 mice and WT mice travelled the same distance during the course of the experiment (R3/I5: 4097 ± 700 cm ; WT: 4143 ± 575 cm). R3/I5 mice are indicated in purple; WT mice are indicated in pink. R3/I5: n = 13 (Male: n = 6); WT: n = 5 (all Male). ns indicates $p \geq 0.05$. Mean and SEM are indicated.

Von Frey: R3/I5 expression in the ACC alleviates CFA-induced mechanical allodynia

Figures 15A-B show the Von Frey PWT for the ipsilateral and contralateral paws of CFA and NaCl animals before R3/I5 injection, after R3/I5 injection, and for 7 days after the start of R3/I5 chronic expression. There was no significant difference in ipsilateral PWT (Figure 15A) as a result of pain condition ($p = 0.139$), time ($p = 0.121$), or interaction thereof ($p = 0.492$). Additionally, no subject-based variation was reported ($p = 0.520$). There was no significant difference in contralateral PWT (Figure 15B) as a result of pain condition ($p = 0.126$) or the interaction between time and pain condition ($p = 0.504$), nor was intra-subject variation reported ($p = 0.210$). However, a significant variation due to time was present ($p = 0.017$); Tukey's multiple comparisons test revealed that CFA animals differed significantly in contralateral PWT on day 5 post-CFA injection as compared to the pre-I5 baseline and Days 1 and 7 post-CFA injection ($p \leq 0.007$). However, no significant difference is present between CFA contralateral PWT and NaCl contralateral PWT on day 5 post-CFA ($p = 0.104$). This, along with the fact that this variation in PWT is not maintained or found in any other day, indicates that this change may be due to random variation or experimental error. Overall, these results suggest that the chronic expression of R3/I5 in the ACC has an analgesic effect on evoked mechanical pain sensitivity resulting from CFA-induced chronic inflammatory pain. For more detailed results on the R3/I5 Von Frey data, refer to Table 10.

Plantar: R3/I5 expression in the ACC has no effect on CFA-induced thermal allodynia

Figures 15C-D show the Plantar PWT for the ipsilateral and contralateral paws of CFA and NaCl animals before R3/I5 injection, after R3/I5 injection, and for 7 days after the start of R3/I5 chronic expression. There was a significant difference in ipsilateral PWT (Figure 15C) as a result of pain condition ($p = 0.0001$), subject ($p = 0.0009$), and interaction between time and pain condition ($p \leq 0.0001$). There was, however, no significant effect due to time ($p = 0.125$). Sidak's multiple comparison revealed that NaCl ipsilateral PWT did not significantly change throughout the experiment ($p \geq 0.066$). However, CFA ipsilateral PWT differed significantly from baseline values and their NaCl counterparts at various points in time. At day 1 post-CFA, CFA ipsilateral PWT was significantly lower than pre-I5 baseline ($p = 0.021$), the post-I5 baseline ($p = 0.002$), and day 7 post-CFA ($p = 0.018$) PWTs, as well as the PWT of the NaCl mice in the same day ($p = 0.0004$). At day 3 post-CFA, CFA ipsilateral PWT was significantly lower than post-I5 baseline ($p = 0.041$), as well as their same-day NaCl counterpart's PWT ($p = 0.0006$). Finally, at day 5 post-CFA, CFA ipsilateral PWT was significantly lower than post-I5 baseline ($p = 0.005$). Overall, these results suggest that chronic expression of R3/I5 in the ACC has no functional effect on evoked thermal pain sensitivity resulting from CFA-induced chronic inflammatory pain. For more detailed results on the R3/I5 Plantar data, refer to Table 10.

Notably, the results obtained in both the Von Frey and Plantar assays very closely match those previously obtained in the lab, in which A5 was injected into the ACC of CFA-treated mice (Figure 4). This suggests that R3/I5 expression operates effectively identically to A5 injections, opening the possibility of replacing the time-consuming, labor-intensive process of repeated A5 injections with the singular viral injection.

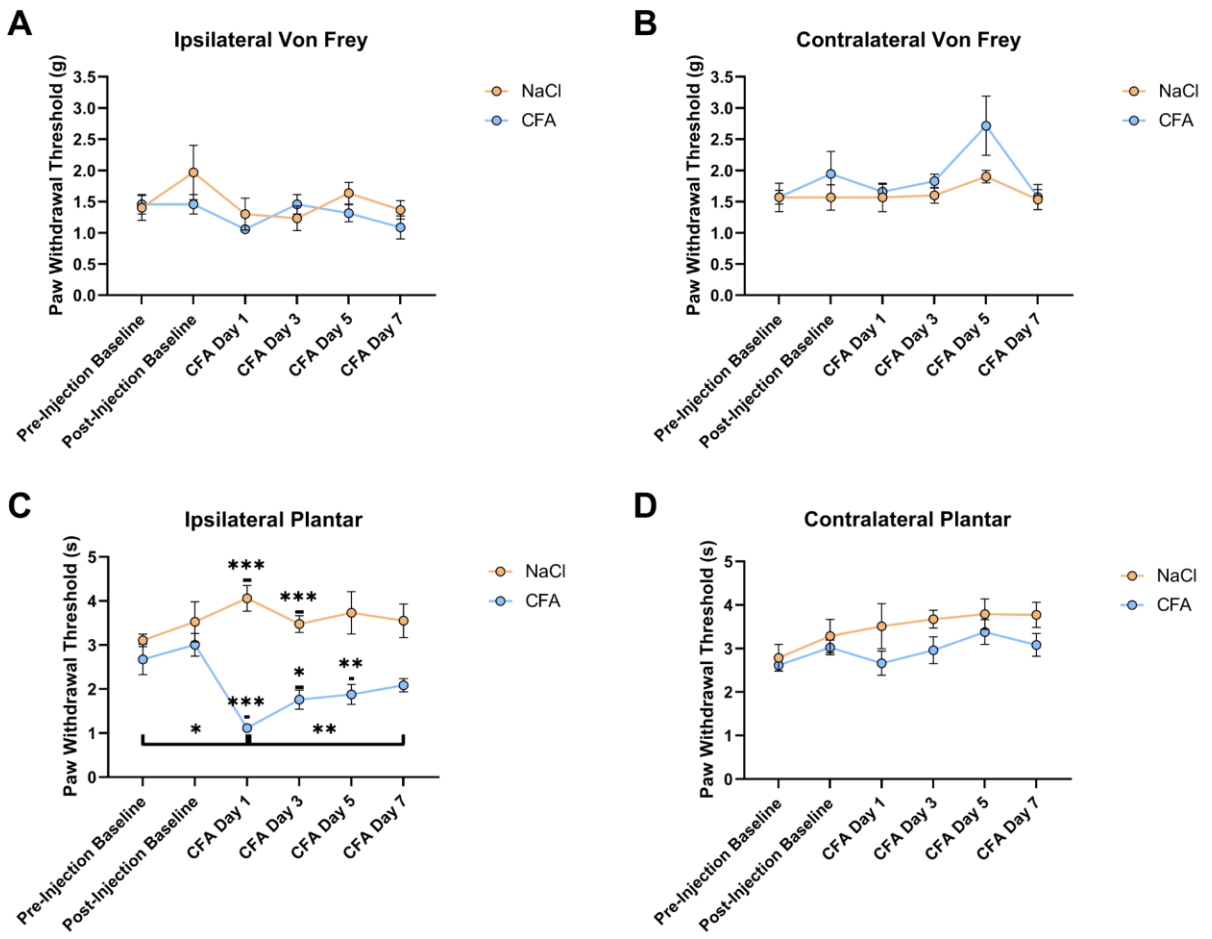


Figure 15: R3/I5 Von Frey and Plantar

A) CFA and NaCl animals did not have significantly different ipsilateral PWTs, nor did their PWTs differ significantly during the course of the experiment (CFA: Pre-injection: 1.46 ± 0.41 g; Post-injection: 1.46 ± 1.42 g; Day 1: 1.06 ± 0.15 g; Day 3: 1.46 ± 0.41 g; Day 5: 1.31 ± 0.36 g; Day 7: 1.09 ± 0.49 g; NaCl: Pre-injection: 1.40 ± 0.49 g; Post-injection: 1.97 ± 1.07 g; Day 1: 1.30 ± 0.63 g; Day 3: 1.23 ± 0.48 g; Day 5: 1.63 ± 0.43 g; Day 7: 1.37 ± 0.37 g). B) CFA and NaCl animals did not have significantly different contralateral PWTs, nor did their PWTs differ significantly during the course of the experiment. The exception to this is CFA contralateral PWT on day 5, which was significantly higher than the pre-I5 baseline and the days 3 and 7 PWTs. (CFA: Pre-injection: 1.57 ± 0.29 g; Post-injection: 1.94 ± 0.95 g; Day 1: 1.66 ± 0.32 g; Day 3: 1.83 ± 0.29 g; Day 5: 2.71 ± 1.25 g; Day 7: 1.57 ± 0.54 g; NaCl: Pre-injection: 1.57 ± 0.56 g; Post-injection: 1.57 ± 0.50 g; Day 1: 1.57 ± 0.56 g; Day 3: 1.60 ± 0.31 g; Day 5: 1.90 ± 0.25 g; Day 7: 1.53 ± 0.39 g). C) NaCl ipsilateral PWT did not significantly change throughout the experiment. However, CFA ipsilateral PWT significantly decreased from baseline and from their NaCl counterparts on days 1, 3, and 5 post-CFA injection. (CFA: Pre-injection: 2.67 ± 0.91 s; Post-injection: 3.01 ± 0.68 s; Day 1: 1.11 ± 0.24 s; Day 3: 1.76 ± 0.57 s; Day 5: 1.88 ± 0.60 s; Day 7: 2.09 ± 0.40 s; NaCl: Pre-injection: 3.11 ± 0.35 s; Post-injection: 3.53 ± 1.12 s; Day 1: 4.06 ± 0.71 s; Day 3: 3.48 ± 0.46 s; Day 5: 3.73 ± 1.17 s; Day 7: 3.55 ± 0.94 s). D) CFA and NaCl animals did not have significantly different contralateral PWTs, nor did their PWTs differ significantly during the course of the experiment. (CFA: Pre-injection: 2.62 ± 0.34 s; Post-injection: 3.02 ± 0.44 s; Day 1: 2.66 ± 0.73 s; Day 3: 2.96 ± 0.82 s; Day 5: 3.38 ± 0.75 s; Day 7: 3.08 ± 0.69 s; NaCl: Pre-injection: 2.79 ± 0.75 s; Post-injection: 3.29 ± 0.94 s; Day 1: 3.51 ± 1.27 s; Day 3: 3.67 ± 0.50 s; Day 5: 3.79 ± 0.86 s; Day 7: 3.77 ± 0.71 s). CFA mice are indicated in Blue; NaCl mice are indicated in Beige. CFA: n = 7 (Male: n = 3); NaCl: n = 6 (Male: n = 3) . * indicates $p \leq 0.05$; ** indicates $p \leq 0.005$; *** indicates $p \leq 0.0005$. Mean and SEM are indicated.

Discussion

Chronic pain is one of the most insidious problems facing the healthcare community, due to its widespread prevalence, the difficulty in addressing and treating this condition, and its impact on individual quality of life and on society, especially in its role in the opioid epidemic. As such, it remains imperative to pursue avenues of research through which we may further understand the mechanisms which govern chronic pain, and through which we may uncover and develop treatment and management options. Relaxin-3, a neuropeptide implicated in pain modulation, is one such avenue of research. Here, we adapt the Conditioned Place Preference paradigm in order to assess the functional effect of Relaxin-3 on non-evoked pain and the affective facet of nociception utilizing a chronic inflammatory pain mouse model. We additionally perform preliminary pharmacogenetic experiments in the CPP paradigm utilizing excitatory DREADD in BLA Somatostatin neurons containing RXFP3 in order to further characterize the Relaxin-3 circuitry. Finally, we evaluate the effects of chronic ACC RXFP3 activation on the development of chronic inflammatory pain and the resulting changes in nociception utilizing the rAAV1/2-FIB-R3/I5 viral construct and determine its potential as a tool for further experimentation in this avenue of research.

Social Transfer of Pain

In our laboratory, CFA and control groups are traditionally housed together, to allow for ease of blinding during experimentation and to mitigate the effect of cage-based differences. While this had presented no problems previously, the data presented in Smith 2021 raised a significant issue with this arrangement: as reported in their article, mice which socially interact with cagemates experiencing CFA-induced pain exhibit mechanical analgesia for hours after they have been separated, notably represented by a reduced PWT in the Von Frey assay. In light of this publication, our housing scheme was put into question; it is possible that control groups were being affected by the CFA mice with which they were housed, thus skewing our results due to the social transfer of pain. In order to address this concern, we replicated the experiment detailed in Smith 2021.

Our testing yielded no indication that our mice experienced a significant social transfer of pain, with Bystander PWT remaining at baseline levels while CFA ipsilateral PWT significantly decreased. This conclusion is further supported by previous results obtained by other researchers in this laboratory, as none report significant hyperalgesia in control mice which were housed alongside CFA mice. It is unclear at the time what the source of this difference between our and Smith et al.'s results may be. Stress may play a prominent role, as mice with greater stress levels may be more susceptible to exhibit hyperalgesia following social interaction with a mouse in pain. However, this is difficult to confirm, as there may be a number of sources of variation; for example, while we habituated the mice for three days prior to experimentation utilizing the palming method, Smith et al. did not describe their procedure for habituation to handling, thus potentially presenting a point of divergence in our protocols. As such, further investigation into this would be necessary to determine the source of this phenomena. However, within the scope of the experiments detailed herein, our results suggest that no effect due to social transfer of pain owing to housing arrangements is present.

Ibuprofen and Buprenorphine Experiments

The ibuprofen and buprenorphine experiments served as pilots for the CPP test we developed, allowing us to ensure the proper functioning of the apparatus and protocol and to fix any issues in them prior to the A5 CPP experiments. As these substances are also known analgesics of widespread use, and both have been previously utilized in CPP tests (Lim 2014, Tzschentke 2004), they also were to fulfill the role of positive controls for the later CPP experiments. However, as was previously detailed, neither substance yielded the results which were expected.

In Lim 2014, a CFA-induced arthritic chronic pain rat model was utilized to determine the ability of ibuprofen to alleviate pain in both the Von Frey test and in the same CPP paradigm as used in this project, with pain relief utilized as a reward. There, ibuprofen was demonstrated to attenuate mechanical allodynia in pain condition mice, and to produce significant CPP in pain condition mice, but not sham mice. These results thereby suggest that ibuprofen is effective at alleviating pain both at a somatosensory and at an affective level, and that it provides no innate reward in the CPP paradigm beyond pain relief. Remarkably, our results differed from those published by Lim et al. in every aspect. As demonstrated in our experiment, ibuprofen did not have a significant effect in alleviating mechanical allodynia in our CFA mice; mice treated with either ibuprofen or saline had identical PWTs. Further, our CPP results suggest that ibuprofen has an overall rewarding effect independent of pain relief. Neither CFA- nor NaCl-treated mice demonstrated a significant preference for either chamber after conditioning, with no significant CPP scores in either group or chamber. Thus, our results suggest that ibuprofen was neither innately rewarding, nor able to provide pain relief in our mice.

One possible explanation for this significant deviation from previous literature is the difference in pain animal models. Whereas Lim et al. utilized a CFA-induced arthritic chronic pain rat model, we utilized CFA-induced plantar inflammatory chronic pain mouse model. It is possible that ibuprofen has little to no effect on plantar inflammatory pain, as suggested by our results, while still being effective at alleviating arthritic pain, as suggested by Lim et al. Similarly, while similar doses of ibuprofen were utilized in both experiments, utilizing mice instead of rats may have necessitated a different dose to account for species-based differences. Different animal models may also account for the apparent innately rewarding effect of ibuprofen that was not observed in Lim 2014, though further inquiries would have to be made to determine the source and validity of these deviations. It is worth noting that a separate study (Rutten 2011) tested ibuprofen's effects on carrageenan-induced plantar inflammatory pain, resulting in an alleviation of mechanical allodynia as measured by the Randal Selitto test, but not inducing CPP in either pain or pain-free conditions. Ibuprofen did, however, reduce carrageenan-induced Conditioned Place Aversion (CPA). As such, it is apparent that there is some variation in findings regarding ibuprofen's analgesic effects.

Buprenorphine has not, to the best of our knowledge, been utilized previously in a CPP paradigm utilizing pain relief as the incentivizing reward. However, due to its widespread use as a powerful analgesic (Lewis 1985), it is a promising positive control for the CPP experiments

described in this report. This is somewhat complicated by its innately rewarding properties; buprenorphine has been found to produce significant CPP in pain-free rats, which would mean that pain relief-based rewards may be cloaked or overshadowed. However, a dissociation between the innately rewarding effects and the pain relief-based rewarding effects of morphine has been previously obtained in the CPP test; this was done by utilizing doses which were sub-threshold for innate reward, but still able to produce an effect on the affective component of pain (Armendariz 2018). In the same manner, buprenorphine's effects may be similarly dissociated, allowing it to serve as a positive control. It is also possible that buprenorphine's rewarding properties, both innate and via pain relief, may have an additive effect in the resulting CPP, resulting in higher CPP scores in mice with pain condition than those without. As such, buprenorphine remained as a candidate for the pilot CPP tests, with the starting dose of 1.0 mg/kg, which has been demonstrated sufficient to induce CPP in control mice (Tzschentke 2004).

As expected, our Von Frey results revealed that buprenorphine alleviated CFA-induced mechanical allodynia. However, buprenorphine did not induce CPP in either CFA or control mice. While this would seem to indicate that buprenorphine has no innately rewarding properties, nor is it capable of modulating the affective facet of pain, this failure in eliciting CPP may be due to buprenorphine's pharmacokinetics. Buprenorphine's long duration of action and slow receptor dissociation rates result in a single dosage potentially having an effect for several days post-administration; as such, carry-over effects from the buprenorphine conditioning sessions may impact vehicle conditioning sessions, thus preventing CPP from forming (Tzschentke 2004). Ideally, 24-48 hours of washout between conditioning sessions would be set in place in order to prevent these crossover effects (Tzschentke 2004). However, due to the limitations of our animal model, with CFA effects only lasting approximately 9 days), the 6-session conditioning protocol could not accommodate these washout periods. It was our hope that a low buprenorphine dose would minimize the impact of this cross-over, but it appears that this was not the case. Buprenorphine may still be successfully utilized as a positive control for our CPP paradigm by A) further reducing the dose of buprenorphine, B) decreasing the number of conditioning sessions, or C) a combination thereof; however, such follow up experiments were not feasible within the scope and duration of this project.

A5 CPP

As detailed previously, prior research on Relaxin-3/RXFP-3 in this laboratory has revealed that it possesses modulatory properties in pain signaling, inducing analgesia in CFA-mediated inflammatory chronic pain in a mouse model. Specifically, administration of A5 to the BLA resulted in significant transient mechanical (Von Frey) and thermal (Plantar) analgesia (Abboud 2021). However, given the limitations of these pain assays, these findings concern primarily the sensory aspect of nociception and the reflexive response to induced pain, without providing significant measures for assessing Relaxin-3's effects on the affective aspect of nociception and on spontaneous pain. Given the relevance of these factors in chronic pain at a clinical level, analyzing Relaxin-3's ability to modulate nociception at these levels is essential (Sufka 1994, Finnerup 2001, King 2009, Rutten 2011, Navratilova 2013). Here, we adapted the CPP protocol to utilize pain relief as a conditioning reward following the paradigm first described in Sufka

1994. This allows us to determine Relaxin-3's effect on tonic pain, measuring the response to non-evoked painful stimuli in the affective dimension (Sufka 1994, King 2009, Okun 2011, Davoody 2011, Wei 2013).

An important consideration worth noting regarding the CPP protocol is the method through which innate chamber bias is utilized to determine the conditioning method of the experiment. Two primary pairing techniques exist for conditioning in the CPP test: biased and unbiased. In biased pairing, the experimental drug (in this case, A5) is paired with the least preferred chamber, while the vehicle (in this case, ACSF) is paired with the chamber the mice showed the greatest innate preference for in the preconditioning session. This technique is easier to implement, as it allows for mice to have significant preferences towards one chamber over the other; however, results may be obscured by these innate preferences, thus resulting in a potentially less sensitive CPP. In the unbiased pairing model, chamber/drug pairings are randomly assigned, and differ from mouse to mouse in a counterbalanced manner. For this to function, it is required that there be no significant preference for one chamber over another, resulting in a harder setup; however, clearer results may be obtained (Mueller and de Wit 2010, Tappe-Theodor 2019). Following these guidelines, we originally sought to utilize the unbiased pairing method. Testing of the second iteration of the CPP apparatus (horizontal and vertical stripes) resulted in no significant innate bias for one chamber over the other (Supplement S3A), and the experiment thus proceeded. However, as we later discovered, this lack of innate bias was only present when one considered average values; when looking at the level of individual mice, clear preferences appeared in many. Regrettably, this revelation only occurred after the completion of all CPP experiments, as no sources indicated that individual chamber preferences were to be utilized when considering pairing techniques; only Calcagnetti 1994 indicated this potential concern, and this source was unfortunately found late in the study. The resulting fallout led to the exclusion of several mice from the study, as many of them were improperly conditioned, with A5 being paired to the most-preferred chamber rather than the least. The results presented in this report represent the data from the mice which had been properly paired according to the biased conditioning method.

Our results revealed that the intracerebral injection of A5 to the BLA elicits significant CPP in CFA-treated mice, but not in saline-treated mice. This was demonstrated both when considering overall group results, as done when comparing the percentage of time spent in a chamber before and after conditioning (Fig 11A), as well as when considering individual changes in preference, as done when calculating CPP scores (Fig 11B/C). As such, in addition to its analgesic effects on evoked pain and the sensory dimension of nociception (Abboud 2021), these results suggest that Relaxin-3 is also able to attenuate spontaneous pain and modulate nociception in the affective dimension. While the relatively small sample size creates the need for further testing to supplement these findings and to increase their robustness, these results, in combination with what has been previously found regarding Relaxin-3's role in nociception, are promising. Relaxin-3's apparent ability to affect nociception at this level further reinforces its potential to become clinically relevant and underscores the importance of continuing in this avenue of research. Furthermore, the apparent success in this CPP protocol in detecting the conditioned place preference elicited by Relaxin-3-mediated pain relief in CFA mice presents

the possibility of utilizing this protocol in further research of the Relaxin-3/RXFP-3 system, as the experiment described in this report presents both a baseline of comparison for further investigations, and a base protocol which can be adapted for future inquiries into this system. The pharmacogenetic manipulation of BLA Somatostatin neurons for the investigation of their involvement in the Relaxin-3/RXFP-3 circuitry, as presented in this report, comprises one such possible use of this CPP protocol.

DREADD modulation of BLA Somatostatin Neurons in the A5 CPP

In conjunction with the functional effects of Relaxin-3 on nociception and its role in pain modulation, the circuitry underlying the Relaxin-3/RXFP-3 neural pathways is of particular interest. Investigations into this topic are already taking place in our laboratory, as well as those of our partners. Imaging analysis of immunohistochemical stainings of the ACC and BLA have already highlighted the interplay of Somatostatin and Relaxin-3 neurons (Figures 1-2) which, given that Relaxin-3 action in the BLA is primarily inhibitory, is hypothesized to involve Relaxin-3 inhibiting Somatostatin neurons in effectuating the analgesic modulation we observe. In order to test this hypothesis, and to determine the functional role of BLA Somatostatin neurons in the Relaxin-3/RXFP-3 pathway, we utilized Gq-coupled DREADDs activated by CNO to induce excitation of the BLA Somatostatin neurons in Som-Cre mice concurrently with the activity of A5 in the Relaxin-3 CPP paradigm previously described. In this manner, if BLA Relaxin-3 is indeed inhibiting BLA Somatostatin neurons as a central point in its analgesic pathway, we would expect to observe an extinction of the conditioned place preference induced by A5 in CFA mice in animals treated with DREADD/CNO, as the excitation counteracts the effects of Relaxin-3.

Utilizing this pharmacogenetic approach in the CPP test necessarily required certain adjustments to the CPP protocol we had previously developed. As highlighted above, it is important that DREADD activation occurs simultaneously with the start of A5 activity, and that both their effects last for the duration of the conditioning session. Following current literature guidelines regarding the pharmacokinetic properties of DREADD-activator CNO, mice utilized in this experiment received two injections prior to conditioning sessions: an i.p. CNO injection 30 minutes prior to conditioning, and an intracerebral A5 injection 15 minutes after, with the subsequent conditioning session lasting 30 minutes (Smith 2016, Roth 2016, Jendryka 2019). Another consideration is the use of CNO. Though a traditional drug of choice for DREADD activation, recent studies have found that a proportion of CNO is reverse-metabolized into Clozapine in what is thought to be a major factor in CNO activation of DREADD (Gomez 2017, Manvich 2018, Jendryka 2019). Clozapine itself is an antipsychotic drug whose accumulation in high concentrations due to CNO injections may result in effects beyond those mediated by DREADD activity, thus posing a potential complication in behavioral experiments utilizing CNO (Manvich 2018, Mahler 2018, Jendryka 2019). While Clozapine reaches maximal CSF concentration 2 to 3 hours after CNO injection, thus eliciting its greatest effects outside of the context of the conditioning session, injecting CNO on consecutive days may result in an accumulation of Clozapine, exacerbating secondary effects (Gomez 2017, Mahler 2018). In order to address this concern, we injected CNO at a dosage of 4 mg/kg. While significantly lower than the typical dose of 10 mg/kg, CNO at 4 mg/kg is still able to adequately activate

DREADD, and CNO injections at these lower doses have been suggested as a potential mitigator for the side-effects of reverse-metabolized Clozapine (Jendryka 2019).

Importantly, due to a shortage in the quantity of Som-Cre mice available during this project, we were unable to obtain a sufficient number of mice to determine effects with a sufficient level of significance. Given this limitation, rather than attempt to provide a definitive answer to our hypothesis, we sought to create a pilot experiment as a proof of concept for the use of pharmacogenetic approaches in this adapted CPP protocol, and in order to obtain preliminary data and an indication of trends with which to guide future inquiries into this research. In order to maximize the relevance of these results, we opted to forego the DREADD no-pain control group, and to utilize the non-DREADD CFA and NaCl groups from the A5 CPP as a basis of comparison for this experiment's results.

Despite these concerns regarding the experiment, the results from CPP testing suggest that excitation of BLA Somatostatin neurons does counteract the pain relief induced by A5, as DREADD CFA mice did not appear to develop the conditioned place preference that their non-DREADD CFA counterparts exhibited. However, despite these seemingly positive results, it is important to highlight the limitations of this experiment. The small sample size undermines the robustness of these results, especially when the particularly large standard deviation in the DREADD CFA group. Indeed, visual analysis of the CPP scores of this group (Fig 12A/B) reveals two apparent groups: two mice whose CPP Scores are significantly positive, and two whose CPP Scores are significantly negative. Though they average out to a CPP score of almost 0, these results may be more indicative of distinct subgroups responding differently to CNO/DREADD activity paired with A5. This uncertainty is compounded by the lack of control groups. Overall, and once again, these results present preliminary data from a pilot experiment; they may point towards A5 inhibiting Somatostatin in its analgesic pathway and appear to indicate that the adapted CPP protocol may be utilized for these purposes. However, continued testing is essential for significant results to be obtained. Key components for further experimentation are a significant increase of sample size, the addition of a DREADD NaCl control group, and the inclusion of a non-DREADD CFA and NaCl group receiving CNO injection, in order to reveal any side effects elicited from CNO and reverse-metabolized Clozapine.

R3/I5

The effects of chronic activation of RXFP-3 have been previously studied in feeding and arousal mechanisms, but never in relation to pain mechanisms (Ganella 2013, Smith 2013) . By utilizing an adeno-associated viral vector (rAAV1/2-FIB-R3/I5), we are able to constitutively secrete RXPF-3-agonist R3/I5 from locally affected neurons; by targeting the ACC in this manner, we are therefore able to elicit tonic activation of the Relaxin-3/RXFP-3 analgesic pathway. By subsequently running these animals through nociceptive assays, we sought to elucidate the functional effects of this continuous expression of R3/I5: how it may affect the development and progression of CFA-induced chronic pain, the effects of continuous activation on the analgesic potential of the Relaxin-3/RXFP-3 pathway, and overall effects on the behavior of the mice.

Given that Relaxin-3 has been previously implicated as having a role in stress, and that alterations to stress and to locomotion may impact results in the Von Frey and Plantar nociceptive assays, we performed an Open Field test on our R3/I5 mice in order to determine the presence of changes to locomotion and anxiety behavior (Watanabe 2011, Bathgate 2013). Open Field results showed no apparent changes to either locomotion or stress, indicating that R3/I5 tonic secretion in the ACC has no discernible effects in these areas in the period of time tested, and that results obtained from the nociceptive assays had no interference from changes in these fields.

Given the chronic nature of the R3/I5 secretion, we opted to perform a longitudinal study of nociception. In order to determine whether the tonic secretion of R3/I5 itself resulted in alterations to pain sensitivity in the absence of any further interference, we obtained two Baseline PWT measurements: one before the injection of the viral vector, and one 4 weeks after its injection, once R3/I5 had already begun to be produced in the ACC. No significant differences in these Baseline measurements were observed, indicating that in the absence of CFA-induced chronic inflammatory pain, chronic activation of the Relaxin-3/RXFP-3 has no apparent effect on nociception. This discovery is in line with previous experiments, in which A5 injection had no effect on NaCl mice in the Von Frey and Plantar tests (Abboud 2021).

Prior testing in this laboratory has revealed that Relaxin-3 activity in the ACC transiently reduces CFA-induced mechanical hyperalgesia but has no effect on CFA-induced thermal hyperalgesia (Abboud 2021). In order to determine whether constitutive R3/I5 secretion affected the induction and progression of CFA-induced chronic inflammatory pain, and whether chronic activation of ACC RXFP-3 resulted in alterations to its analgesic pathway, we performed four Von Frey and Plantar tests at two-day intervals following CFA injection (Days 1, 3, 5, and 7). In the Plantar test, CFA injection induced significant ipsilateral thermal hyperalgesia which was not alleviated by R3/I5. This hyperalgesia persisted throughout the 7 days of testing, though a gradual increase of ipsilateral PWT in the CFA animals was observed, with the measured PWT at day 7 not differing significantly from its baseline; this is reflective of the gradual diminishing of pain sensitivity expected of CFA pain models. These results are suggestive of three things: 1) chronic R3/I5 expression does not appear interfere with the induction of CFA-induced chronic pain, 2) it does not seem to affect the progression of this pain, and 3) it has no significant analgesic effect on thermal hyperalgesia. Meanwhile, Von Frey testing notably resulted in no significant difference between NaCl and CFA animals at any testing day. While this could be interpreted as a failure to elicit chronic inflammatory pain, Plantar testing revealed that CFA-induced hyperalgesia is, indeed, very present despite R3/I5. As such, these results suggest that R3/I5 is inducing continuous analgesia in CFA-treated mice, with no apparent extraneous effects resulting from chronic activation of RXFP-3.

Strikingly, results from both assays resembled those previously produced by WT animals in Abboud 2021, with Von Frey and Plantar PWT values in the R3/I5 mice nearly matching those produced by their WT mice. This reproduction of results further solidifies the findings in this laboratory, reinforcing the apparent role of the Relaxin-3/RXFP-3 ACC pathway in reducing mechanical, but not thermal, hyperalgesia. These results additionally present the potential

opportunity to implement R3/I5 in further nociception-centered Relaxin-3 research, replacing the thus far typical A5 intracerebral injections. While A5 injections have been demonstrated to reliably activate RXFP-3, the process involved in doing so is time consuming, labor-intensive, and introduces additional stress to the experiment. Mice must undergo cannula implantation surgery, and thereafter have a guide cannula -- a large, bulky apparatus -- affixed to their skulls. Additionally, as A5 induces transient effects lasting no more than 60 minutes, repeated intracerebral injections are necessary, a process which involves the isoflurane-induced anesthetization of the mice and the insertion of a cannula into their skulls via the guide cannula (Abboud 2021). While the injection for the R3/I5 viral vector also necessitates stereotaxic surgery, it leaves the mice with no external attached apparatus, and given that it provides continuous activation of RXFP-3, it theoretically would not necessitate any further interventions or procedures for induction of Relaxin-3 mediated analgesia.

Of course, in order for this to be a feasible option, further testing would be necessary. While no apparent side effects of constitutive R3/I5 secretion have been observed in this experiment, more in depth inquiries regarding changes to behavior, physiology, and neural mechanisms would be advisable. Especially relevant would be an examination of longer-term changes, especially in regards to how it may affect Relaxin-3/RXFP-3 signaling. It is possible that, while no evident changes were observed after one to two weeks of R3/I5 expression, these may appear after a longer period of time as the body adapts to the chronic activation of this pathway. However, as it stands, the chronic release of R3/I5 presents itself as an attractive potential replacement, and a useful tool for future research in this field.

Future Directions

Ultimately, this overarching project aims to investigate the role of the Relaxin-3/RXFP-3 pathway in the modulation of nociception, and to determine whether targeting of this pathway may be of clinical relevance in treating chronic pain in patients. Here, we have developed a CPP protocol through which we demonstrated that BLA Relaxin-3/RXFP-3 activity provides pain relief in a chronic inflammatory pain mouse model, highlighting its ability to affect the affective-emotional dimension of nociception and to attenuate spontaneous, non-evoked pain. We additionally utilized this CPP protocol in conjunction with pharmacogenetic modulation of BLA Somatostatin neurons to functionally analyze the interaction between Relaxin-3/Somatostatin neurons, obtaining results which suggest that Relaxin-3 neurons inhibit Somatostatin neurons in their analgesic pathway. Finally, we virally induced constitutive secretion of Relaxin-3 agonist R3/I5 in the ACC in order to determine the effects of chronic RXFP-3 activation. From this, we observed that R3/I5 activity in the ACC relieves CFA-induced mechanical, but not thermal, hyperalgesia in a similar manner to A5 activity in the ACC. Additionally, no significant effects on the development or progression of chronic pain, nor any further apparent side effects, were observed from tonic ACC R3/I5, leading to the conclusion that R3/I5 induction may be a useful replacement for the currently-utilized intracerebral A5 injections.

In order to supplement the results obtained in this report, both to reinforce their robustness and to expand upon the information gathered, there are a number of additional experiments which may be worthwhile to pursue. Within the context of the A5 CPPs, testing of other Relaxin-

3/RXFP-3-associated brain regions would provide valuable information regarding the generalizability of the findings regarding BLA A5 application. This is especially relevant when considering the ACC, as distinct differences between the ACC and BLA Relaxin-3-mediated pain modulation have already been observed (Abboud 2021). Furthermore, given the diversity in chronic pain etiology and mechanisms, it would be interesting to utilize different chronic pain models in the context of these experiments. Finally, testing whether A5-antagonist R3 is able to suppress A5-induced conditioned place preference would add to the validity of the findings presented in this report.

While the DREADD CPP produced preliminary data suggesting that BLA Somatostatin neurons are inhibited in the Relaxin-3/RXFP-3 pain relief pathway, further testing with more animals is essential to obtain conclusive findings. Further experiments should include a pain-free control group (DREADD NaCl) to provide a basis of comparison from which we can more accurately analyze the results of the chronic pain group. Additionally, in order to determine whether CNO and reverse-metabolized Clozapine are producing effects which would interfere with or account for the results obtained in this report, non-DREADD CFA and NaCl control groups would be a valuable addition to this experiment. Finally, pharmacogenetic inhibition of BLA Somatostatin neurons in the absence of A5 within the context of the CPP test could provide relevant information regarding the functional role of Somatostatin neurons in the Relaxin-3/RXFP-3 analgesic pathway.

R3/I5 is a promising replacement for cannula-dependent intracerebral A5 injections. In foregoing the need for an external cannula attachment in the mice, and by reducing the overall number of procedures in which the mice are anesthetized, it provides a method of experimentally stimulating the Relaxin-3/RXP3 pathway that is less time consuming, requires less effort on the part of the researcher, is potentially more cost-effective, and is less stressful to the mice. However, while the results presented in this report are favorable, R3/I5 remains experimentally unproven relative to A5 injections; importantly, it remains unknown whether there exist side effects owing to R3/I5 expression, and whether there are long-term consequences of chronic stimulation of the Relaxin-3 pathway that may be undesirable in chronic pain studies. As such, further testing would be required before R3/I5 may be implemented in full force. A longitudinal study of the behavioral effects of tonic R3/I5 expression would be pertinent; similarly, a study determining the long term effects of chronic Relaxin-3/RXFP3 activation regarding the development of chronic pain and Relaxin-3's ability to modulate it would provide valuable information regarding the utility of R3/I5. Testing with R3/I5 expression within the BLA, as well as within the CPP paradigm, would also provide valuable insight regarding the potential of R3/I5 as a replacement of A5 injections.

Overall, this project has assessed Relaxin-3's ability to modulate spontaneous pain in the affective dimension of nociception utilizing an adapted Conditioned Place Preference testing paradigm, supplementing previous findings regarding its analgesic properties in long lasting pain and highlighting its potential as a therapeutic target in chronic pain. It has additionally laid the groundwork for further research into this area, piloting a pharmacogenetic approach in studying the neural circuitry of the Relaxin-3/RXFP-3 pathway and finding strong evidence

towards the inhibition of BLA Somatostatin neurons by Relaxin-3, and introducing a potential replacement for intracerebral injections of A5 via the use of R3/I5.Through the research presented in this report, it is our hope to gain greater insight into the mechanisms involved in chronic pain, and to aid in the development of a treatment for this condition.

Tables

Table 1: Cannula Specifications
Specifications of BLA cannulae utilized in these experiments

	Guide Cannulae	***Internal Cannulae***	***Dummy Cannulae***
BLA	C315GS-5/SPC/1.5 mm	C315IS-5/SPC/ to fit 1.5 mm C235I with 0.5 mm projection	C315DCS-5/SPC to fit 1.5 mm with 0.5 mm projection

Table 2: Pain Transfer
Results for CFA and Bystander mice in the Pain Transfer experiment. Values are mean ± SD.

	CFA Ipsilateral	***Bystander Ipsilateral***	***CFA Contralateral***	***Bystander Contralateral***
Baseline PWT (g)	1.40 ± 0.71 N = 4	1.55 ± 0.30 N = 4	1.60 ± 0.49 N = 4	1.75 ± 0.50 N = 4
Pain Transfer PWT (g)	0.50 ± 0.12 N = 4	1.50 ± 0.58 N = 4	1.55 ± 0.30 N = 4	1.50 ± 0.58 N = 4

Table 3: Ibuprofen Von Frey
Results for ibuprofen and saline CFA mice in the Ibuprofen Von Frey experiment. Values are mean ± SD.

	Ibuprofen Ipsilateral	***Saline Ipsilateral***	***Ibuprofen Contralateral***	***Saline Contralateral***
Baseline PWT (g)	1.43 ± 0.32 N = 6	1.53 ± 0.39 N = 6	1.60 ± 0.31 N = 6	1.53 ± 0.39 N = 6
Test Phase PWT (g)	0.73 ± 0.42 N = 6	0.80 ± 0.22 N = 6	1.40 ± 0.49 N = 6	1.43 ± 0.32 N = 6

Table 4: Ibuprofen CPP
Results for CFA and Saline mice in the Ibuprofen CPP experiment. Values are mean ± SD.

	Striped Chamber		***Solid Chamber***	
Time spent in chamber (%): Pre-conditioning	36.93 ± 6.43 N = 10		50.43 ±7.27 N = 10	
	Ibuprofen	***Ibuprofen***	***NaCl Chamber:***	***NaCl Chamber:***

	Chamber: CFA	***Chamber: Saline***	***CFA***	***Saline***
Time Spent in chamber (%): Pre-conditioning	39.08 ± 0.43 N = 3	39.91 ± 6.80 N = 2	46.15 ± 0.81 N = 3	47.71 ± 8.83 N = 2
	Ibuprofen Chamber: CFA	***Ibuprofen Chamber: Saline***	***NaCl Chamber: CFA***	***NaCl Chamber: Saline***
Time Spent in chamber (%): Post-conditioning	44.00 ± 10.95 N = 3	38.70 ± 8.33 N = 2	42.75 ± 11.95 N = 3	47.47 ± 4.93 N = 2
	Ibuprofen Chamber: CFA	***Ibuprofen Chamber: Saline***	***NaCl Chamber: CFA***	***NaCl Chamber: Saline***
CPP Score	4.93 ± 10.58 N = 3	-1.22 ± 1.53 N = 2	-3.40 ± 11.16 N = 3	-0.24 ± 3.90 N = 2

Table 5: Buprenorphine Von Frey

Results for Buprenorphine and Saline CFA mice in the Buprenorphine Von Frey experiment. Values are mean ± SD.

	Buprenorphine Ipsilateral	***Saline Ipsilateral***	***Buprenorphine Contralateral***	***Saline Contralateral***
Baseline PWT (g)	1.53 ± 0.39 N = 6	1.30 ± 0.47 N = 6	1.53 ± 0.39 N = 6	1.37 ± 0.37 N = 6
Test Phase PWT (g)	1.13 ± 0.33 N = 6	0.67 ± 0.16 N = 6	1.73 ± 0.43 N = 6	1.53 ± 0.39 N = 6

Table 6: Buprenorphine CPP

Results for CFA and Saline mice in the Buprenorphine CPP experiment. Values are mean ± SD.

	Vertical Striped Chamber		***Horizontal Striped Chamber***	
Time spent in chamber (%): Pre-conditioning	41.90 ± 8.64 N = 9		46.03 ± 11.57 N = 9	
	Buprenorphine Chamber: CFA	***Buprenorphine Chamber: Saline***	***NaCl Chamber: CFA***	***NaCl Chamber: Saline***
Time Spent in chamber (%): Pre-conditioning	33.55 ± 4.79 N = 4	35.12 ± 6.31 N = 2	54.21 ± 6.27 N = 4	52.02 ± 9.27 N = 2
	Buprenorphine Chamber: CFA	***Buprenorphine Chamber: Saline***	***NaCl Chamber: CFA***	***NaCl Chamber: Saline***
Time Spent in chamber (%): Post-conditioning	34.63 ± 11.74 N = 4	36.59 ± 6.63 N = 2	52.26 ± 13.55 N = 4	47.96 ± 0.26 N = 2
	Buprenorphine	***Buprenorphine***	***NaCl***	***NaCl Chamber:***

	Chamber: CFA	*Chamber: Saline*	*Chamber: CFA*	*Saline*
CPP Score	1.08 ± 10.15 N = 4	1.47 ± 12.93 N = 2	-1.95 ± 13.69 N = 4	-4.06 ± 9.00 N = 2

Table 7: A5 CPP

Results for CFA and Saline mice in the A5 CPP experiment. Values are mean ± SD.

	Vertical Striped Chamber		***Horizontal Striped Chamber***	
Time spent in chamber (%): Pre-conditioning	42.67 ± 9.47 N = 19		43.24 ± 10.03 N = 19	
	A5 Chamber: CFA	***A5 Chamber: Saline***	***ACSF Chamber: CFA***	***ACSF Chamber: Saline***
Time Spent in chamber (%): Pre-conditioning	37.93 ± 6.15 N = 7	38.73 ± 4.23 N = 6	46.59 ± 6.19 N = 7	46.18 ± 5.51 N = 6
	A5 Chamber: CFA	***A5 Chamber: Saline***	***ACSF Chamber: CFA***	***ACSF Chamber: Saline***
Time Spent in chamber (%): Post-conditioning	46.66 ± 7.91 N = 7	43.41 ± 8.80 N = 6	36.85 ± 9.00 N = 7	40.97 ± 10.81 N = 6
	A5 Chamber: CFA	***A5 Chamber: Saline***	***ACSF Chamber: CFA***	***ACSF Chamber: Saline***
CPP Score	8.73 ± 8.16 N = 7	-4.68 ± 8.44 N = 6	-9.74 ± 9.60 N = 7	-5.21 ± 9.24 N = 6

Table 8: DREADD/A5 CPP

Results for CFA and Saline mice in the DREADD/A5 CPP experiment. Values are mean ± SD.

	Vertical Striped Chamber		***Horizontal Striped Chamber***	
Time spent in chamber (%): Pre-conditioning	38.47 ± 8.04 N = 5		50.31 ± 9.94 N = 5	
	A5 Chamber: CFA	***A5 Chamber: Saline***	***ACSF Chamber: CFA***	***ACSF Chamber: Saline***
Time Spent in chamber (%): Pre-conditioning	38.48 ± 9.32 N = 4	73.94 N = 1	50.38 ± 11.91 N = 4	20.69 N = 1

	A5 Chamber: CFA	***A5 Chamber: Saline***	***ACSF Chamber: CFA***	***ACSF Chamber: Saline***
Time Spent in chamber (%): Post-conditioning	39.55 ± 15.86 N = 4	73.94 N = 1	48.36 ± 13.52 N = 4	20.69 N = 1
	A5 Chamber: CFA	***A5 Chamber: Saline***	***ACSF Chamber: CFA***	***ACSF Chamber: Saline***
CPP Score	1.07 ± 17.89 N = 4	24.65 N = 1	-2.03 ± 21.31 N = 4	-19.39 N = 1

Table 9: R3/I5 Open Field, Von Frey, Plantar
Results for R3/I5 and WT mice in the R3/I5 Open Field, and of R3/I5 CFA and Saline mice in the R3/I5 Von Frey and Plantar experiments. Values are mean ± SD.

	R3/I5		***WT***	
Open Field: Time in Center Area (%)	30.65 ± 7.45 N = 13		33.20 ± 8.71 N = 5	
Open Field: Distance Travelled (cm)	4097 ± 700 N = 13		4143 ± 575 N = 5	
	CFA Ipsilateral	***Saline Ipsilateral***	***CFA Contralateral***	***Saline Contralateral***
Von Frey Pre-I5 injection baseline PWT (g)	1.46 ± 0.41 N = 7	1.40 ±0.49 N = 6	1.57 ± 0.29 N = 7	1.57 ± 0.56 N = 6
Von Frey Post-I5 injection baseline PWT (g)	1.46 ± 0.41 N = 7	1.97 ± 1.07 N = 6	1.94 ± 0.95 N = 7	1.57 ± 0.50 N = 6
Von Frey Day 1 Post-CFA PWT (g)	1.06 ± 0.15 N = 7	1.30 ± 0.63 N = 6	1.66 ± 0.32 N = 7	1.57 ± 0.56 N = 6
Von Frey Day 3 Post-CFA PWT (g)	1.46 ± 0.41 N = 7	1.23 ± 0.48 N = 6	1.83 ± 0.29 N = 7	1.60 ± 0.31 N = 6
Von Frey Day 5 Post-CFA PWT (g)	1.31 ± 0.36 N = 7	1.63 ± 0.43 N = 6	2.71 ± 1.25 N = 7	1.90 ± 0.25 N = 6
Von Frey Day 7 Post-CFA PWT (g)	1.09 ± 0.49 N = 7	1.37 ± 0.37 N = 6	1.567 ± 0.54 N = 7	1.53 ± 0.39 N = 6
	CFA Ipsilateral	***Saline***	***CFA***	***Saline***

		Ipsilateral	***Contralateral***	***Contralateral***
Plantar Pre-I5 injection baseline PWT (s)	2.67 ± 0.91 N = 7	3.11 ± 0.35 N = 6	2.62 ± 0.34 N = 7	2.79 ± 0.75 N = 6
Plantar Post-I5 injection baseline PWT (s)	3.01 ± 0.68 N = 7	3.53 ± 1.12 N = 6	3.02 ± 0.44 N = 7	3.29 ± 0.94 N = 6
Plantar Day 1 Post-CFA PWT (s)	1.11 ± 0.24 N = 7	4.06 ± 0.71 N = 6	2.66 ± 0.73 N = 7	3.51 ± 1.27 N = 6
Plantar Day 3 Post-CFA PWT (s)	1.76 ± 0.57 N = 7	3.48 ± 0.46 N = 6	2.96 ± 0.82 N = 7	3.67 ± 0.50 N = 6
Plantar Day 5 Post-CFA PWT (s)	1.88 ± 0.60 N = 7	3.73 ± 1.17 N = 6	3.38 ± 0.75 N = 7	3.79 ± 0.86 N = 6
Plantar Day 7 Post-CFA PWT (s)	2.09 ± 0.40 N = 7	3.55 ± 0.94 N = 6	3.08 ± 0.69 N = 7	3.77 ± 0.71 N = 6

References

Abboud, C. (2021). *CONTRÔLE PEPTIDERGIQUE DE LA DOULEUR : MODULATIONS DES VOIES DESCENDANTES PAR LES SYSTÈMES RELAXINE*.

Agarwal, N., Helmstädter, J., Rojas, D. R., Bali, K. K., Gangadharan, V., & Kuner, R. (2018). Evoked hypoalgesia is accompanied by tonic pain and immune cell infiltration in the dorsal root ganglia at late stages of diabetic neuropathy in mice. *Molecular Pain*, *14*. https://doi.org/10.1177/1744806918817975

Armendariz, A., & Nazarian, A. (2018). Morphine antinociception on thermal sensitivity and place conditioning in male and female rats treated with intraplantar complete freund's adjuvant. *Behavioural Brain Research*, *343*, 21–27. https://doi.org/10.1016/j.bbr.2018.01.031

Basbaum, A. I., Bautista, D. M., Scherrer, G., & Julius, D. (2009). Cellular and Molecular Mechanisms of Pain. *Cell*, *139*(2), 267. https://doi.org/10.1016/J.CELL.2009.09.028

Bathgate, R. A. D., Halls, M. L., van der Westhuizen, E. T., Callander, G. E., Kocan, M., & Summers, R. J. (2013). Relaxin family peptides and their receptors. *Physiological Reviews*, *93*(1), 405–480. https://doi.org/10.1152/physrev.00001.2012

Bouhassira, D., Lantéri-Minet, M., Attal, N., Laurent, B., & Touboul, C. (2008). Prevalence of chronic pain with neuropathic characteristics in the general population. *Pain*, *136*(3), 380–387. https://doi.org/10.1016/j.pain.2007.08.013

Breivik, H., Collett, B., Ventafridda, V., Cohen, R., & Gallacher, D. (2006). Survey of chronic pain in Europe: Prevalence, impact on daily life, and treatment. *European Journal of Pain*, *10*(4), 287. https://doi.org/10.1016/j.ejpain.2005.06.009

Calcagnetti, D. J., & Schechter, M. D. (1994). Nicotine place preference using the biased method of conditioning. *Progress in Neuropsychopharmacology and Biological Psychiatry*, *18*(5), 925–933. https://doi.org/10.1016/0278-5846(94)90108-2

Canestrelli, C., Marie, N., & Noble, F. (2014). *Rewarding or aversive effects of buprenorphine/ naloxone combination (Suboxone) depend on conditioning trial duration*. https://doi.org/10.1017/S146114571400025X

Davoody, L., Quiton, R. L., Lucas, J. M., Ji, Y., Keller, A., & Masri, R. (2011). Conditioned place preference reveals tonic pain in an animal model of central pain. *J Pain*, *12*(8), 868–874. https://doi.org/10.1016/j.jpain.2011.01.010

Ellison, D. L. (2017). Physiology of Pain. In *Critical Care Nursing Clinics of North America* (Vol. 29, Issue 4, pp. 397–406). https://doi.org/10.1016/j.cnc.2017.08.001

Fehrenbacher, J. C., Vasko, M. R., & Duarte, D. B. (2012). Models of inflammation: Carrageenan- or unit 5.4 complete freund's adjuvant (CFA)-induced edema and hypersensitivity in the rat. *Current Protocols in Pharmacology*, *0 5*(SUPPL.56), Unit5.4. https://doi.org/10.1002/0471141755.ph0504s56

Finnerup, NB., IL, J., SH, S., FW, B., & TS, J. (2001). Pain and dysesthesia in patients with spinal cord injury: A postal survey. *Spinal Cord*, *39*(5), 256–262. https://doi.org/10.1038/SJ.SC.3101161

Ganella, D. E., Callander, G. E., Ma, S., Bye, C. R., Gundlach, A. L., & Bathgate, R. A. D. (2013). Modulation of feeding by chronic rAAV expression of a relaxin-3 peptide agonist in rat hypothalamus. *Gene Therapy*, *20*(7), 703–716. https://doi.org/10.1038/gt.2012.83

Gao, Y. J., Ren, W. H., Zhang, Y. Q., & Zhao, Z. Q. (2004). Contributions of the anterior cingulate cortex and amygdala to pain- and fear-conditioned place avoidance in rats. *Pain*, *110*(1–2), 343–353. https://doi.org/10.1016/j.pain.2004.04.030

Goldberg, D. S., & McGee, S. J. (2011). Pain as a global public health priority. *BMC Public Health*, *11*, 770. https://doi.org/10.1186/1471-2458-11-770

Gomez, J. L., Bonaventura, J., Lesniak, W., Mathews, W. B., Sysa-Shah, P., Rodriguez, L. A., Ellis, R. J., Richie, C. T., Harvey, B. K., Dannals, R. F., Pomper, M. G., Bonci, A., & Michaelides, M. (2017). Chemogenetics revealed: DREADD occupancy and activation via converted clozapine. *Science*, *357*(6350), 503–507. https://doi.org/10.1126/science.aan2475

Gregory, N. S., Harris, A. L., Robinson, C. R., Dougherty, P. M., Fuchs, P. N., & Sluka, K. A. (2013). An overview of animal models of pain: disease models and outcome measures. *The Journal of Pain : Official Journal of the American Pain Society*, *14*(11), 1255–1269. https://doi.org/10.1016/J.JPAIN.2013.06.008

Halls, M. L., Westhuizen, E. T. van der, Bathgate, R. A. D., & Summers, R. J. (2007). Relaxin Family Peptide Receptors – former orphans reunite with their parent ligands to activate multiple signalling pathways. *British Journal of Pharmacology*, *150*(6), 677. https://doi.org/10.1038/SJ.BJP.0707140

Heinricher, M. M., Tavares, I., Leith, J. L., & Lumb, B. M. (2009). Descending control of nociception: specificity, recruitment and plasticity. *Brain Research Reviews*, *60*(1), 214. https://doi.org/10.1016/J.BRAINRESREV.2008.12.009

Hoyer, D., & Bartfai, T. (2012). Neuropeptides and Neuropeptide Receptors: Drug Targets, and Peptide and Non-Peptide Ligands: a Tribute to Prof. Dieter Seebach. *Chemistry & Biodiversity*, *9*(11), 2367–2387. https://doi.org/10.1002/CBDV.201200288

Hylands-White, N., Duarte, R. V., & Raphael, J. H. (2017). An overview of treatment approaches for chronic pain management. In *Rheumatology International* (Vol. 37, Issue 1, pp. 29–42). Springer Verlag. https://doi.org/10.1007/s00296-016-3481-8

Iannitti, T., Kerr, B. J., & Taylor, B. K. (2014). Behavioral Neurobiology of Chronic Pain. In *Current topics in behavioral neurosciences* (Vol. 20). http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=4464806&tool=pmcentrez&rendertype=abstract

Jendryka, M., Palchaudhuri, M., Ursu, D., Veen, B. van der, Liss, B., Kätzel, D., Nissen, W., & Pekcec, A. (2019). Pharmacokinetic and pharmacodynamic actions of clozapine-N-oxide, clozapine, and compound 21 in DREADD-based chemogenetics in mice. *Scientific Reports*, *9*(1). https://doi.org/10.1038/S41598-019-41088-2

King, T., Vera-Portocarrero, L., Gutierrez, T., Vanderah, T. W., Dussor, G., Lai, J., Fields, H. L., & Porreca, F. (2009). Unmasking the tonic-aversive state in neuropathic pain. *Nature Neuroscience*, *12*(11), 1364–1366. https://doi.org/10.1038/nn.2407

Leknes, S., & Bastian, B. (2014). The Benefits of Pain. *Review of Philosophy and Psychology*, *5*(1), 57–70. https://doi.org/10.1007/s13164-014-0178-3

Lewis, JW. (1985). Buprenorphine. *Drug and Alcohol Dependence*, *14*(3–4), 363–372. https://doi.org/10.1016/0376-8716(85)90067-5

Lim, G., Kim, H., Mccabe, M. F., Chou, C.-W., Wang, S., Chen, L. L., Marota, J. J. A., Blood, A., Breiter, H. C., & Mao, J. (2014). *Neurobiology of Disease A Leptin-Mediated Central Mechanism in Analgesia-Enhanced Opioid Reward in Rats*. https://doi.org/10.1523/JNEUROSCI.0386-14.2014

Ma, S., Smith, C. M., Blasiak, A., & Gundlach, A. L. (2017). Distribution, physiology and pharmacology of relaxin-3/RXFP3 systems in brain. *British Journal of Pharmacology*, *174*(10), 1034–1048. https://doi.org/10.1111/bph.13659

Mahler, S. V., & Aston-Jones, G. (2018). CNO Evil? Considerations for the Use of DREADDs in Behavioral Neuroscience. In *Neuropsychopharmacology* (Vol. 43, Issue 5, pp. 934–936). Nature Publishing Group. https://doi.org/10.1038/npp.2017.299

Manvich, D. F., Webster, K. A., Foster, S. L., Farrell, M. S., Ritchie, J. C., Porter, J. H., & Weinshenker, D. (2018). The DREADD agonist clozapine N-oxide (CNO) is reverse-metabolized to clozapine and produces clozapine-like interoceptive stimulus effects in rats and mice. *Scientific Reports*, *8*(1), 3840. https://doi.org/10.1038/s41598-018-22116-z

Marquez, P., Baliram, R., Kieffer, B. L., & Lutfy, K. (2007). The mu opioid receptor is involved in buprenorphine-induced locomotor stimulation and conditioned place preference. *Neuropharmacology*, *52*(6), 1336–1341. https://doi.org/10.1016/j.neuropharm.2007.01.005

Matsumoto, M., Kamohara, M., Sugimoto, T., Hidaka, K., Takasaki, J., Saito, T., Okada, M., Yamaguchi, T., & Furuichi, K. (2000). The novel G-protein coupled receptor SALPR shares sequence similarity with somatostatin and angiotensin receptors. *Gene*, *248*, 183–189. www.elsevier.com/locate/gene

Mueller, D., & de Wit, H. (2011). Conditioned Place Preference in Rodents and Humans. *Animal Models of Behavioral Analysis*, *50*(June 2014), 267–305. https://doi.org/10.1007/978-1-60761-883-6

National Insititute for Health and Care Excellence. (2021). *Chronic pain (primary and secondary) in over 16s: assessment of all chronic pain and management of chronic primary pain NICE guideline*. www.nice.org.uk/guidance/ng193

Navratilova, E., Xie, J. Y., King, T., & Porreca, F. (2013). *Evaluation of reward from pain relief*. https://doi.org/10.1111/nyas.12095

Okun, A., DeFelice, M., Eyde, N., Ren, J., Mercado, R., King, T., & Porreca, F. (2011). Transient inflammation-induced ongoing pain is driven by TRPV1 sensitive afferents. *Molecular Pain*, *7*. https://doi.org/10.1186/1744-8069-7-4

Ossipov, M. H., Morimura, K., & Porreca, F. (2014). Descending pain modulation and chronification of pain. *Current Opinion in Supportive and Palliative Care*, *8*(2), 143. https://doi.org/10.1097/SPC.0000000000000055

Ossipov, M. H., Dussor, G. O., & Porreca, F. (2010). Central modulation of pain. *The Journal of Clinical Investigation*, *120*. https://doi.org/10.1172/JCI43766

Porreca, F., Ossipov, M. H., & Gebhart, G. F. (2002). Chronic pain and medullary descending facilitation. *Trends in Neurosciences*, *25*(6), 319–325. https://doi.org/10.1016/S0166-2236(02)02157-4

Reddi, D., & Curran, N. (n.d.). *An introduction to pain pathways and mechanisms*.

Roth, B. L. (2016). DREADDs for Neuroscientists. In *Neuron* (Vol. 89, Issue 4, pp. 683–694). https://doi.org/10.1016/j.neuron.2016.01.040

Rutten, K., Vry, J. De, Robens, A., Tzschentke, T. M., & Van Der Kam, E. L. (2011). Dissociation of rewarding, anti-aversive and anti-nociceptive effects of different classes of anti-nociceptives in the rat. *European Journal of Pain*, *15*(3), 299–305. https://doi.org/10.1016/j.ejpain.2010.07.011

Smith, K. S., Bucci, D. J., Luikart, B. W., & Mahler, S. V. (2016). DREADDs: Use and Application in Behavioral Neuroscience HHS Public Access. *Behav Neurosci*, *130*(2), 137–155. https://doi.org/10.1037/bne0000135

Smith, M. L., Asada, N., & Malenka, R. C. (2021). Anterior cingulate inputs to nucleus accumbens control the social transfer of pain and analgesia. *Science*, *371*(6525), 153–159. https://doi.org/10.1126/science.abe3040

Sufka, K. (2000). Chronic pain explained. *Brain and Mind*, *1*(2), 155–179. https://doi.org/10.1023/A:1010032508495

Sufka, K. J. (1994). Conditioned place preference paradigm: a novel approach for analgesic drug assessment against chronic pain. *Pain*, *58*(3), 355–366. https://doi.org/10.1016/0304-3959(94)90130-9

Tappe-Theodor, A., King, T., & Morgan, M. M. (2019). Pros and Cons of Clinically Relevant Methods to Assess Pain in Rodents. In *Neuroscience and Biobehavioral Reviews* (Vol. 100, pp. 335–343). Elsevier Ltd. https://doi.org/10.1016/j.neubiorev.2019.03.009

Tompkins, D. A., Hobelmann, J. G., & Compton, P. (2017). Providing chronic pain management in the "Fifth Vital Sign" Era: Historical and treatment perspectives on a modern-day medical dilemma. *Drug and Alcohol Dependence*, *173*(Suppl 1), S11. https://doi.org/10.1016/J.DRUGALCDEP.2016.12.002

Tzschentke, T. M. (2007). REVIEW ON CPP: Measuring reward with the conditioned place preference (CPP) paradigm: update of the last decade. *Addiction Biology*, *12*(3–4), 227–462. https://doi.org/10.1111/J.1369-1600.2007.00070.X

Tzschentke, T. M. (2004). Reassessment of buprenorphine in conditioned place preference: Temporal and pharmacological considerations. *Psychopharmacology*, *172*(1), 58–67. https://doi.org/10.1007/s00213-003-1626-4

van der Kam, E. L., Vry, J. De, Schiene, K., & Tzschentke, T. M. (2008). Differential effects of morphine on the affective and the sensory component of carrageenan-induced nociception in the rat. *Pain*, *136*(3), 373–379. https://doi.org/10.1016/j.pain.2007.07.027

Velly, A. M., & Mohit, S. (2018). Epidemiology of pain and relation to psychiatric disorders. *Progress in Neuro-Psychopharmacology and Biological Psychiatry*, *87*, 159–167. https://doi.org/10.1016/j.pnpbp.2017.05.012

Watanabe, Y., Tsujimura, A., Takao, K., Nishi, K., Ito, Y., Yasuhara, Y., Nakatomi, Y., Yokoyama, C., Fukui, K., Miyakawa, T., & Tanaka, M. (2011). Relaxin-3-Deficient Mice Showed Slight Alteration in Anxiety-Related Behavior. *Frontiers in Behavioral Neuroscience*, *5*(AUGUST). https://doi.org/10.3389/FNBEH.2011.00050

Wei, H., Viisanen, H., Amorim, D., Koivisto, A., & Pertovaara, A. (2013). Dissociated modulation of conditioned place-preference and mechanical hypersensitivity by a TRPA1 channel antagonist in peripheral neuropathy. *Pharmacology Biochemistry and Behavior*, *104*(1), 90–96. https://doi.org/10.1016/j.pbb.2012.12.014

Wilkinson, T. N., Speed, T. P., Tregear, G. W., & Bathgate, R. A. (2005). *Evolution of the relaxin-like peptide family*. https://doi.org/10.1186/1471-2148-5-1

Supplements

Supplement S1: CPP Protocols

Conditioned Place Preference Protocols

CPP Arena:

- Constructed from opaque,smooth, dark grey PVC sheets 0.5 cm thick
- Two chambers connected by a corridor, with a floor made from the same material on the entire arena.
    - Chamber outer dimensions
        - 20 cm width, 21 cm length, 30 cm height
    - Corridor dimensions
        - 10.5 cm width, 16 cm length, 30 cm height
- A removable gate made from the same material and measuring 11.5 cm length, 0.5 cm width, and 33 cm height was utilized during conditioning sessions to limit the mice to a single chamber
- Chamber walls were covered in a pattern printed on plastified paper. One chamber was completely covered in vertical black and white stripes, and the other was completely covered in horizontal black and white stripes. Black and white stripes were of the same width, as were vertical and horizontal stripes.

N.B.:

- Mice should be kept in a reverse light cycle so that testing occurs during their wakeful phase. Mice should be habituated to the reverse light cycle for at least 2 weeks prior to any behavioral experimentation.
- Mice should be habituated to handling utilizing the palming method for at least three consecutive days prior to behavioral experimentation.
- Testing room for the CPP should be kept under dim light illumination, as to reduce stress to the mice. All chambers of the CPP should be subject to the same level of illumination to reduce innate bias. Red light illumination should **not** be utilized (or must be utilized in conjunction with another form of illumination), as the mice must be able to see the intramaze visual cues to differentiate between the chambers.
- Testing of males and females should be done in separate sessions, as to prevent olfactory cues from interfering with behavior.
- The CPP arena should not be moved for the duration of the experiment (from preconditioning to final test) as to avoid changing the circumstances of the chambers and thus affecting the relative preference.
- Regarding Cannula Implantation Surgery: animals should be awarded a recovery period of no less than 1 week between the cannula implantation procedure and any behavioral experiments.
- Regarding i.p. Injections: animals should be injected in a room separate from the CPP arena and from the other mice being utilized in the CPP protocol, as to 1) prevent association of the room containing the CPP arena with fear/discomfort and as to 2) prevent anxiety/stress to the other animals.

Overview of Generalized CPP Protocol

Preconditioning Phase (Days 1 - 3)

The mice are placed in the corridor of the CPP arena in a substance-free state with full access to all chambers for 15 minutes on each day. On the 3rd day of preconditioning, behavior is recorded and analyzed utilizing Ethovision to determine the existence of innate preferences to the chambers, measuring time spent in each chamber. If a significant difference in time spent in each chamber is found to exist, an unbalanced conditioning procedure should be utilized. If no significant difference is present, a balanced conditioning procedure should be utilized. A balanced conditioning procedure is preferable, if achievable, in order to minimize noise in the results.

Animals spending more than an 80% (time spent > 720 sec) or less than 20% (time spent < 120 sec) of the total time in a chamber are considered outliers and excluded from further testing.

CFA Injection (Day 3)

Following the third preconditioning session, mice receive a 20 µl CFA or saline (NaCl 0.9%) subcutaneous injection to the left hindpaw. The mouse is anesthetized utilizing gaseous isoflurane (1 minute induction with 5% isoflurane), then kept unconscious through an anesthetic mask with 1-2% isoflurane throughout the procedure. Success in application is determined through visual assessment of inflammation the next day, preceding the start of the conditioning phase

Conditioning Phase (Days 4-9)

In the conditioning phase, the mouse is injected with a substance (the experimental or sham compound), then confined to the chamber associated with the substance for the duration of the conditioning session, with access to the corridor and other chamber being blocked by the gate. Conditioning to the experimental compound (the drug: A5, ibuprofen, buprenorphine) occurs on days 4, 6, and 8. Conditioning to the sham compound (the vehicle: ACSF, saline) occurs on days 5, 7, 9. Time of compound application and length of conditioning should be determined based on the compound's pharmacology. Examples for ibuprofen, buprenorphine, A5, and A5 paired with CNO are presented below.

For a balanced conditioning procedure, chamber/compound pairings should be counterbalanced between animals/groups. For an unbalanced conditioning procedure, the preferred chamber should be paired with the sham compound, while the avoided chamber should be paired with the experimental compound.

Note that this is the case for Conditioned Place Preference, in which the compound is expected/hypothesized to cause a preference. In Conditioned Place Aversion unbalanced conditioning procedures, the pairings should be reversed.

Test Phase (Day 10)

The animals are placed in the corridor of the CPP arena in a substance-free state with free access to all chambers and allowed to explore for 15 minutes, during which their behavior is recorded and later analyzed utilizing Ethovision for chamber preference.

A5 (Relaxin-3 Agonist) intracerebral injection to the BLA

Preliminary Preparation:

- Mice should have received BLA cannula implantation surgery at least one week prior, been placed in a reverse light cycle at least two weeks prior, and habituated to handling for at least 3 consecutive days.

    - Guide Cannula Specifications: C315GS-5/SPC/4mm
    - Internal Cannula Specifications: C3I5IS-5/SCP/ to fit 4 mm; C235I with 0.8 mm projection
    - BLA Stereotaxic Coordinates: X:+/-3.3; Y:-1.6; Z: -4.8
- Necessary Compounds
    - A5 should be diluted in ACSF to a concentration of 5 µg/µl
    - 1x ACSF should be prepared for sham injections
- Intracerebral Injection Apparatus
    - Internal cannula is connected by a polyethylene tube to a Hamilton Syringe (25 µl), which is mounted on an infusion pump.
    - Extra space in Hamilton Syringe and polyethylene tube is filled with silicone oil to remove air from these

Day 1-2: Preconditioning

1. Allow the mice to habituate to the testing room in their home cage for 30 minutes prior to experimentation
2. Thoroughly clean the CPP arena, then thoroughly dry the arena
3. Place the mouse in the corridor of the arena with free access to all chambers, then leave the room.
4. Allow the mouse to explore for 15 minutes, then remove them from the arena and place them into a clean holding cage.
5. Repeat steps 2-4 for all mice to be tested. When all mice from a home cage have been tested, return them from the holding cage to their home cage, and return the home cage to their housing room.

Day 3: Preconditioning Analysis and CFA Injection

1. Set up the video acquisition system, and ensure that all of the arena is clearly visible in video recordings.
2. Allow the mice to habituate to the testing room in their home cage for 30 minutes prior to experimentation
3. Thoroughly clean the CPP arena, then thoroughly dry the arena
4. Begin the recording session. Place the mouse in the corridor of the arena with free access to all chambers, then leave the room.
5. Allow the mouse to explore for 15 minutes, then remove them from the arena and place them into a clean holding cage. Stop the recording session, and save the video utilizing a standardized filing name (e.g.: "*date(ddmmyy)_CPP_Precon_BatchNumber_MouseSexNumber*")
6. Repeat steps 2-4 for all mice to be tested. When all mice from a home cage have been tested, return them from the holding cage to their home cage, and return the home cage to their housing room.
7. Analyze videos utilizing Ethovision to determine chamber preference (Vertical Stripes vs Horizontal Stripes). Exclude any mice with a preference score greater than 80% or less than 20% from further testing. Exclude mice spending less than 120 seconds in either chamber from further testing. Refer to the Appendix for Ethovision settings to be utilized

1 hour after testing, conduct CFA/saline injections

8. Anesthetize the mouse utilizing gaseous isoflurane (5% isoflurane for 1-2 minutes)
9. Place the mouse on a heating pad equipped with an anesthetic mask at 2% isoflurane

10. Inject 20 µl CFA or saline to the left hindpaw, depending on their designated pain condition, then place them in a clean holding cage on a heating pad until they are fully awakened. Then, return them to their home cage
11. Repeat steps 8 - 10 for all mice, then return the cages to their housing room.

Days 4-9: Conditioning

1. Thoroughly clean the CPP arena, then thoroughly dry the arena.
2. Place the gate so that chamber access is restricted to the relevant chamber for the particular conditioning day and mouse combination
    1. A5 conditioning occurs on days 4/6/8; ACSF conditioning occurs on days 5/7/9.
3. Anesthetize the mouse utilizing gaseous isoflurane (5% isoflurane for 1-2 minutes)
4. Place the mouse on a heating pad equipped with an anesthetic mask at 2% isoflurane
5. Utilizing a Hamilton Syringe, a polyethylene tube, a BLA internal cannula, and an infusion pump, inject A5 or ACSF into the BLA of the mouse at a rate of 100 nl/minute, up to a volume of 200 nl.
    1. A5 injections occur on days 4/6/8; ACSF injections occur on days 5/7/9.
6. After completion of injection, wait 60 seconds, then remove the internal cannula and place the unconscious mouse in a clean holding cage with no other mice. Place the cage in the testing room, and wait 15 minutes.
7. Place the mouse in the relevant chamber (as per step 2), then leave the room.
8. Allow the mouse to explore the chamber for 30 minutes, then remove them from the chamber and place them in a clean holding cage.
    1. Recommended: at the 15 minute mark of the mouse's exploration, start with the injection of the following mouse (steps 3-6) so that the next mouse's waiting period ends just after the current mouse's conditioning session ends
9. Repeat steps 1-8 for all mice, then return the cages to their housing room.

Day 10: Test

1. Set up the video acquisition system, and ensure that all of the arena is clearly visible in video recordings.
2. Allow the mice to habituate to the testing room in their home cage for 30 minutes prior to experimentation
3. Thoroughly clean the CPP arena, then thoroughly dry the arena
4. Begin the recording session. Place the mouse in the corridor of the arena with free access to all chambers, then leave the room.
5. Allow the mouse to explore for 15 minutes, then remove them from the arena and place them into a clean holding cage. Stop the recording session, and save the video utilizing a standardized filing name (e.g.: "*date(ddmmyy)_CPP_Test_BatchNumber_MouseSexNumber*")
6. Repeat steps 2-4 for all mice to be tested. When all mice from a home cage have been tested, return them from the holding cage to their home cage, and return the home cage to their housing room.
7. Analyze videos utilizing Ethovision to determine chamber preference (Vertical Stripes vs Horizontal Stripes). Refer to the Appendix for Ethovision settings to be utilized

A5 (Relaxin-3 Agonist) intracerebral injection to the BLA and CNO intraperitoneal injection for DREADD mice

Preliminary Preparation:

- Mice should have received DREADD injection to the BLA at least 2 weeks prior, BLA cannula implantation surgery at least one week prior, been placed in a reverse light cycle at least two weeks prior, and habituated to handling for at least 3 consecutive days.
    - Guide Cannula Specifications: C315GS-5/SPC/4mm
    - Internal Cannula Specifications: C3I5IS-5/SCP/ to fit 4 mm; C235I with 0.8 mm projection
    - BLA Stereotaxic Coordinates: X:+/-3.3; Y:-1.6; Z: -4.8
- Necessary Compounds
    - A5 should be diluted in ACSF to a concentration of 5 µg/µl
    - 1x ACSF should be prepared for sham injections
    - CNO should be diluted in saline to a concentration of 2 mg/ml and injected at a volume of 1 ml/kg
- Intracerebral Injection Apparatus
    - Internal cannula is connected by a polyethylene tube to a Hamilton Syringe (25 µl), which is mounted on an infusion pump.
    - Extra space in Hamilton Syringe and polyethylene tube is filled with silicone oil to remove air from these

Day 1-2: Preconditioning

1. Allow the mice to habituate to the testing room in their home cage for 30 minutes prior to experimentation
2. Thoroughly clean the CPP arena, then thoroughly dry the arena
3. Place the mouse in the corridor of the arena with free access to all chambers, then leave the room.
4. Allow the mouse to explore for 15 minutes, then remove them from the arena and place them into a clean holding cage.
5. Repeat steps 2-4 for all mice to be tested. When all mice from a home cage have been tested, return them from the holding cage to their home cage, and return the home cage to their housing room.

Day 3: Preconditioning Analysis and CFA Injection

1. Set up the video acquisition system, and ensure that all of the arena is clearly visible in video recordings.
2. Allow the mice to habituate to the testing room in their home cage for 30 minutes prior to experimentation
3. Thoroughly clean the CPP arena, then thoroughly dry the arena
4. Begin the recording session. Place the mouse in the corridor of the arena with free access to all chambers, then leave the room.
5. Allow the mouse to explore for 15 minutes, then remove them from the arena and place them into a clean holding cage. Stop the recording session, and save the video utilizing a standardized filing name (e.g.: "*date(ddmmyy)_CPP_Precon_BatchNumber_MouseSexNumber*")

6. Repeat steps 2-4 for all mice to be tested. When all mice from a home cage have been tested, return them from the holding cage to their home cage, and return the home cage to their housing room.
7. Analyze videos utilizing Ethovision to determine chamber preference (Vertical Stripes vs Horizontal Stripes). Exclude any mice with a preference score greater than 80% or less than 20% from further testing. Exclude mice spending less than 120 seconds in either chamber from further testing. Refer to the Appendix for Ethovision settings to be utilized

1 hour after testing, conduct CFA/saline injections

8. Anesthetize the mouse utilizing gaseous isoflurane (5% isoflurane for 1-2 minutes)
9. Place the mouse on a heating pad equipped with an anesthetic mask at 2% isoflurane
10. Inject 20 µl CFA or saline to the left hindpaw, depending on their designated pain condition, then place them in a clean holding cage on a heating pad until they are fully awakened. Then, return them to their home cage
11. Repeat steps 8 - 10 for all mice, then return the cages to their housing room.

Days 4-9: Conditioning

1. Thoroughly clean the CPP arena with, then thoroughly dry the arena.
2. Place the gate so that chamber access is restricted to the relevant chamber for the particular conditioning day and mouse combination
    1. A5 conditioning occurs on days 4/6/8; ACSF conditioning occurs on days 5/7/9.
3. Perform an i.p. injection of 2 mg/ml CNO to a quantity of 2 mg/kg (thus, inject 1 ml/kg), then place the mouse in a clean holding cage with no other mice. Wait approximately 10 minutes.
    1. Mice should be placed into the CPP arena 30 minutes after CNO injection, and 15 minutes after A5/ACSF injection. Timing-wise, A5/ACSF injection process should start approximately 10 minutes after CNO injection.
4. Anesthetize the mouse utilizing gaseous isoflurane (5% isoflurane for 1-2 minutes)
5. Place the mouse on a heating pad equipped with an anesthetic mask at 2% isoflurane
6. Utilizing a Hamilton Syringe, a polyethylene tube, a BLA internal cannula, and an infusion pump, inject A5 or ACSF into the BLA of the mouse at a rate of 100 nl/minute, up to a volume of 200 nl.
    1. A5 injections occur on days 4/6/8; ACSF injections occur on days 5/7/9.
7. After completion of injection, wait 60 seconds, then remove the internal cannula and place the unconscious mouse in a clean holding cage with no other mice. Place the cage in the testing room, and wait 15 minutes.
8. Place the mouse in the relevant chamber (as per step 2), then leave the room.
9. Allow the mouse to explore the chamber for 30 minutes, then remove them from the chamber and place them in a clean holding cage.
    1. Recommended: when a mouse is placed in their CPP Chamber, start with the CNO and A5/ACSF injection process of the following mouse (steps 3-8) so that the next mouse's waiting period ends just after the current mouse's conditioning session ends
10. Repeat steps 1-9 for all mice, then return the cages to their housing room.

Day 10: Test

1. Set up the video acquisition system, and ensure that all of the arena is clearly visible in video recordings.
2. Allow the mice to habituate to the testing room in their home cage for 30 minutes prior to experimentation
3. Thoroughly clean the CPP arena, then thoroughly dry the arena
4. Begin the recording session. Place the mouse in the corridor of the arena with free access to all chambers, then leave the room.
5. Allow the mouse to explore for 15 minutes, then remove them from the arena and place them into a clean holding cage. Stop the recording session, and save the video utilizing a standardized filing name (e.g.: "*date(ddmmyy)_CPP_Test_BatchNumber_MouseSexNumber*")
6. Repeat steps 2-4 for all mice to be tested. When all mice from a home cage have been tested, return them from the holding cage to their home cage, and return the home cage to their housing room.
7. Analyze videos utilizing Ethovision to determine chamber preference (Vertical Stripes vs Horizontal Stripes). Refer to the Appendix for Ethovision settings to be utilized

Buprenorphine intraperitoneal injection

Preliminary Preparation:

- Mice should have been placed in a reverse light cycle at least two weeks prior, and habituated to handling for at least 3 consecutive days.
- Necessary Compounds
    - Buprenorphine should be obtained at a concentration of 0.3 mg/ml, for injection at 1 mg/kg (3.33 ml/kg)

Day 1-2: Preconditioning

1. Allow the mice to habituate to the testing room in their home cage for 30 minutes prior to experimentation
2. Thoroughly clean the CPP arena, then thoroughly dry the arena
3. Place the mouse in the corridor of the arena with free access to all chambers, then leave the room.
4. Allow the mouse to explore for 15 minutes, then remove them from the arena and place them into a clean holding cage.
5. Repeat steps 2-4 for all mice to be tested. When all mice from a home cage have been tested, return them from the holding cage to their home cage, and return the home cage to their housing room.

Day 3: Preconditioning Analysis and CFA Injection

1. Set up the video acquisition system, and ensure that all of the arena is clearly visible in video recordings.
2. Allow the mice to habituate to the testing room in their home cage for 30 minutes prior to experimentation
3. Thoroughly clean the CPP arena, then thoroughly dry the arena
4. Begin the recording session. Place the mouse in the corridor of the arena with free access to all chambers, then leave the room.

5. Allow the mouse to explore for 15 minutes, then remove them from the arena and place them into a clean holding cage. Stop the recording session, and save the video utilizing a standardized filing name (e.g.: "*date(ddmmyy)_CPP_Precon_BatchNumber_MouseSexNumber*")
6. Repeat steps 2-4 for all mice to be tested. When all mice from a home cage have been tested, return them from the holding cage to their home cage, and return the home cage to their housing room.
7. Analyze videos utilizing Ethovision to determine chamber preference (Vertical Stripes vs Horizontal Stripes). Exclude any mice with a preference score greater than 80% or less than 20% from further testing. Exclude mice spending less than 120 seconds in either chamber from further testing. Refer to the Appendix for Ethovision settings to be utilized

1 hour after testing, conduct CFA/saline injections

8. Anesthetize the mouse utilizing gaseous isoflurane (5% isoflurane for 1-2 minutes)
9. Place the mouse on a heating pad equipped with an anesthetic mask at 2% isoflurane
10. Inject 20 µl CFA or saline to the left hindpaw, depending on their designated pain condition, then place them in a clean holding cage on a heating pad until they are fully awakened. Then, return them to their home cage
11. Repeat steps 8 - 10 for all mice, then return the cages to their housing room.

Days 4-9: Conditioning

1. Thoroughly clean the CPP arena, then thoroughly dry the arena.
2. Place the gate so that chamber access is restricted to the relevant chamber for the particular conditioning day and mouse combination
    1. Buprenorphine conditioning occurs on days 4/6/8; saline conditioning occurs on days 5/7/9.
3. Perform an i.p. injection of Buprenorphine (0.3 mg/ml to a quantity of 1 mg/kg (thus, inject 3.33 ml/kg)) or Saline (volume of 3.33 ml/kg), as determined by the conditioning day, then place the mouse in a clean holding cage with no other mice.
4. After 5 minutes, place the mouse in the relevant chamber (as per step 2), then leave the room.
5. Allow the mouse to explore the chamber for 30 minutes, then remove them from the chamber and place them in a clean holding cage.
    1. Recommended: at the 25 minute mark of the mouse's exploration, start with the injection of the following mouse (steps 3-4) so that the next mouse's waiting period ends just after the current mouse's conditioning session ends
6. Repeat steps 1-5 for all mice, then return the cages to their housing room.

Day 10: Test

1. Set up the video acquisition system, and ensure that all of the arena is clearly visible in video recordings.
2. Allow the mice to habituate to the testing room in their home cage for 30 minutes prior to experimentation
3. Thoroughly clean the CPP arena, then thoroughly dry the arena
4. Begin the recording session. Place the mouse in the corridor of the arena with free access to all chambers, then leave the room.

5. Allow the mouse to explore for 15 minutes, then remove them from the arena and place them into a clean holding cage. Stop the recording session, and save the video utilizing a standardized filing name (e.g.: "*date(ddmmyy)_CPP_Test_BatchNumber_MouseSexNumber*")
6. Repeat steps 2-4 for all mice to be tested. When all mice from a home cage have been tested, return them from the holding cage to their home cage, and return the home cage to their housing room.
7. Analyze videos utilizing Ethovision to determine chamber preference (Vertical Stripes vs Horizontal Stripes). Refer to the Appendix for Ethovision settings to be utilized

Ibuprofen intraperitoneal injection

Preliminary Preparation:

- Mice should have been placed in a reverse light cycle at least two weeks prior, and habituated to handling for at least 3 consecutive days.
- Necessary Compounds
    - Ibuprofen should be diluted in saline to a concentration of 20 mg/ml, for injection at 100 mg/kg (5 ml/kg)

Day 1-2: Preconditioning

1. Allow the mice to habituate to the testing room in their home cage for 30 minutes prior to experimentation
2. Thoroughly clean the CPP arena, then thoroughly dry the arena
3. Place the mouse in the corridor of the arena with free access to all chambers, then leave the room.
4. Allow the mouse to explore for 15 minutes, then remove them from the arena and place them into a clean holding cage.
5. Repeat steps 2-4 for all mice to be tested. When all mice from a home cage have been tested, return them from the holding cage to their home cage, and return the home cage to their housing room.

Day 3: Preconditioning Analysis and CFA Injection

1. Set up the video acquisition system, and ensure that all of the arena is clearly visible in video recordings.
2. Allow the mice to habituate to the testing room in their home cage for 30 minutes prior to experimentation
3. Thoroughly clean the CPP arena, then thoroughly dry the arena
4. Begin the recording session. Place the mouse in the corridor of the arena with free access to all chambers, then leave the room.
5. Allow the mouse to explore for 15 minutes, then remove them from the arena and place them into a clean holding cage. Stop the recording session, and save the video utilizing a standardized filing name (e.g.: "*date(ddmmyy)_CPP_Precon_BatchNumber_MouseSexNumber*")
6. Repeat steps 2-4 for all mice to be tested. When all mice from a home cage have been tested, return them from the holding cage to their home cage, and return the home cage to their housing room.
7. Analyze videos utilizing Ethovision to determine chamber preference (Vertical Stripes vs Horizontal Stripes). Exclude any mice with a preference score greater than 80% or less

than 20% from further testing. Exclude mice spending less than 120 seconds in either chamber from further testing. Refer to the Appendix for Ethovision settings to be utilized

1 hour after testing, conduct CFA/saline injections

8. Anesthetize the mouse utilizing gaseous isoflurane (5% isoflurane for 1-2 minutes)
9. Place the mouse on a heating pad equipped with an anesthetic mask at 2% isoflurane
10. Inject 20 µl CFA or saline to the left hindpaw, depending on their designated pain condition, then place them in a clean holding cage on a heating pad until they are fully awakened. Then, return them to their home cage
11. Repeat steps 8 - 10 for all mice, then return the cages to their housing room.

Days 4-9: Conditioning

1. Thoroughly clean the CPP arena, then thoroughly dry the arena.
2. Place the gate so that chamber access is restricted to the relevant chamber for the particular conditioning day and mouse combination
    1. Ibuprofen conditioning occurs on days 4/6/8; saline conditioning occurs on days 5/7/9.
3. Perform an i.p. injection of Ibuprofen (20 mg/ml to a quantity of 100 mg/kg (thus, inject 5 ml/kg)) or Saline (volume of 5 ml/kg), as determined by the conditioning day, then place the mouse in a clean holding cage with no other mice.
4. After 15 minutes, place the mouse in the relevant chamber (as per step 2), then leave the room.
5. Allow the mouse to explore the chamber for 30 minutes, then remove them from the chamber and place them in a clean holding cage.
    1. Recommended: at the 15 minute mark of the mouse's exploration, start with the injection of the following mouse (steps 3-4) so that the next mouse's waiting period ends just after the current mouse's conditioning session ends
6. Repeat steps 1-5 for all mice, then return the cages to their housing room.

Day 10: Test

1. Set up the video acquisition system, and ensure that all of the arena is clearly visible in video recordings.
2. Allow the mice to habituate to the testing room in their home cage for 30 minutes prior to experimentation
3. Thoroughly clean the CPP arena, then thoroughly dry the arena
4. Begin the recording session. Place the mouse in the corridor of the arena with free access to all chambers, then leave the room.
5. Allow the mouse to explore for 15 minutes, then remove them from the arena and place them into a clean holding cage. Stop the recording session, and save the video utilizing a standardized filing name (e.g.: "*date(ddmmyy)_CPP_Test_BatchNumber_MouseSexNumber*")
6. Repeat steps 2-4 for all mice to be tested. When all mice from a home cage have been tested, return them from the holding cage to their home cage, and return the home cage to their housing room.
7. Analyze videos utilizing Ethovision to determine chamber preference (Vertical Stripes vs Horizontal Stripes). Refer to the Appendix for Ethovision settings to be utilized

Appendix

Ethovision 13 detection settings:

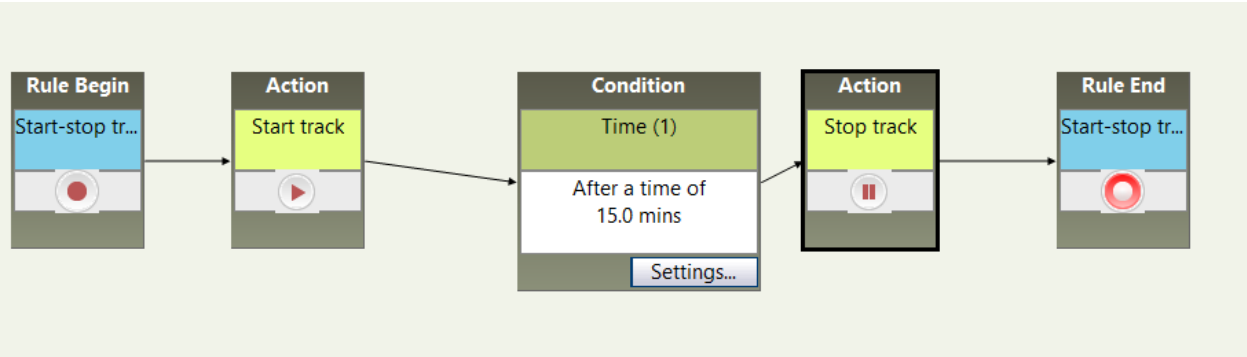
- New detection setting for each animal, to account for shifting camera/arena
- Static subtraction with image of the arena before the mouse is introduced being utilized as the background image
    - Background image is specific to trial; change for every video to account for shifting of the camera and/or arena
- Subject color comparison: Darker
    - 15 to 255
    - Contour erosion: 1
    - Contour dilation: 1
- These are generalized values; should be calibrated to special circumstances as needed

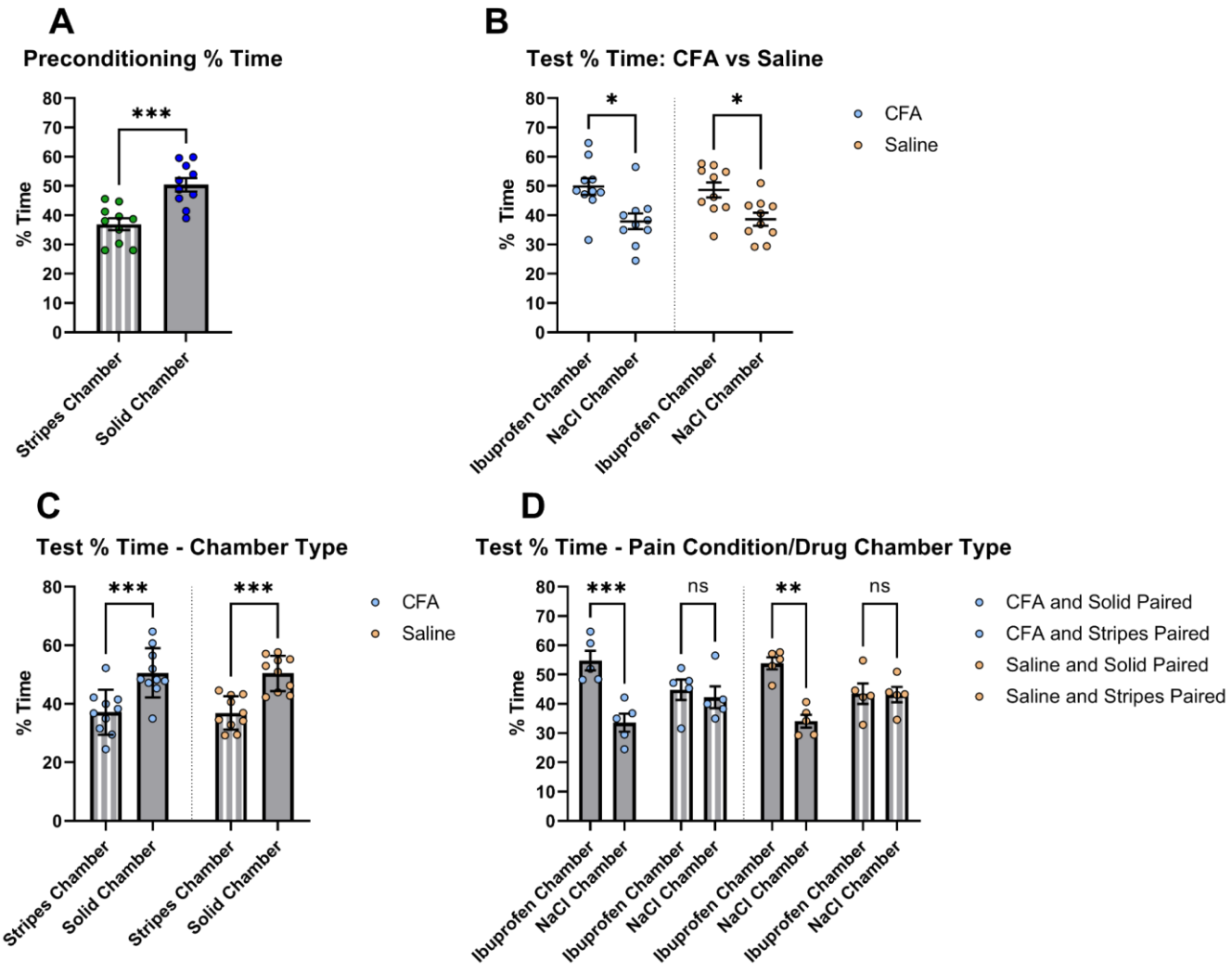
Measurements of interest

- In zone (Vertical, Corridor, and Horizontal Arenas)
    - Frequency, Cumulative Duration, Cumulative Duration (%)

Ethovision Arena for CPP

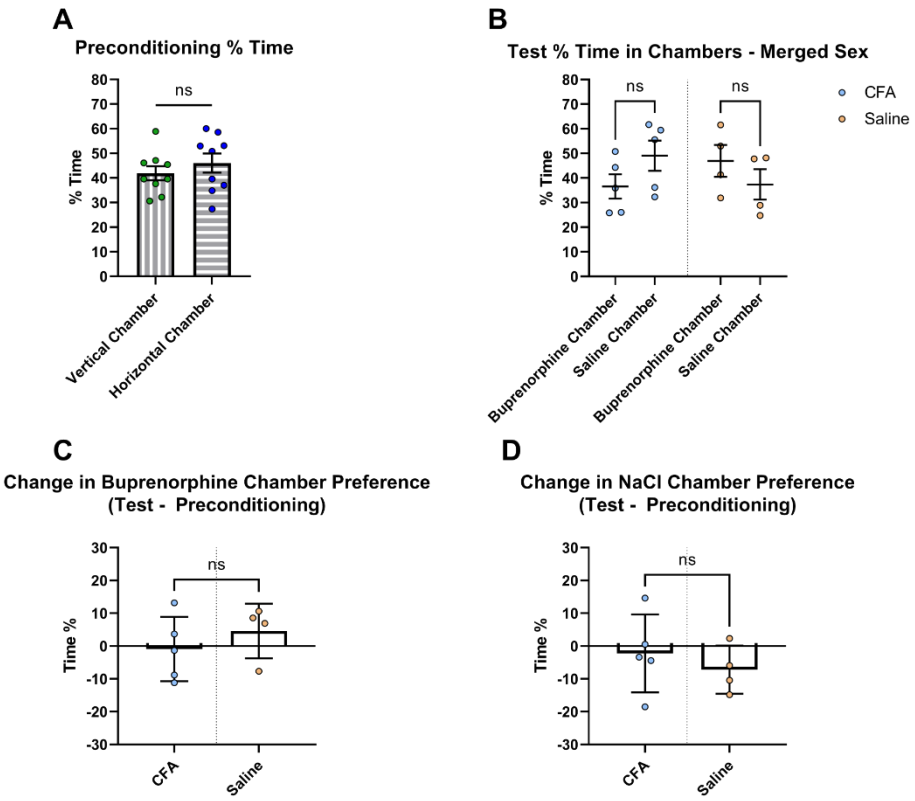
Trial Control





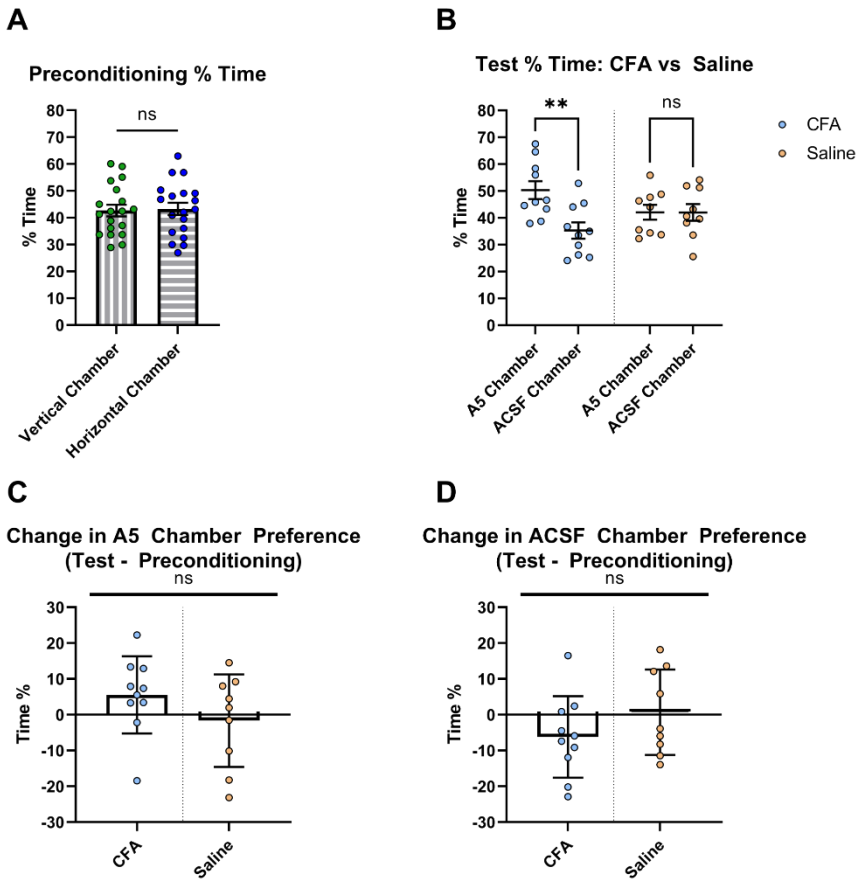
Supplement 2: Ibuprofen CPP Based on Group Preconditioning Values

A) Mice show a significant preference for the solid chamber over the striped chamber in the preconditioning phase (Stripes: 36.93 ± 6.43%; Solid: 50.43 ± 7.27%). B) Both CFA and saline groups show a significant preference for the ibuprofen-paired chamber over the NaCl-paired chamber post-conditioning (CFA: Ibuprofen: 49.81 ± 8.93%; NaCl: 37.91 ± 8.51%; Saline: Ibuprofen: 48.65 ± 8.17%; NaCl: 38.62 ± 6.97%). Time spent in the ibuprofen chamber was significantly above chance level (41.67%) for all groups. C) Both CFA and saline groups show a preference for the solid chamber over the striped chamber post-conditioning (CFA: Stripes: 37.14 ± 7.71%; Solid: 50.58 ± 8.42%; Saline: Stripes: 36.84 ± 5.73%; Solid: 50.41 ± 6.06%). D) Both CFA and Saline groups show a preference for the ibuprofen-paired chamber only when ibuprofen is paired to the solid chamber. When ibuprofen is paired to the striped chamber, there is no significant difference in preference in either group (CFA-Solid: Ibuprofen: 54.80 ± 7.48%; NaCl: 33.58 ± 6.81%; CFA-Stripes: Ibuprofen: 44.82 ± 7.82%; NaCl: 42.25 ± 8.34%; Saline-Solid: Ibuprofen: 53.84 ± 4.64%; NaCl: 34.07 ± 4.89%; Saline-Stripes: 43.47 ± 7.85%; NaCl: 43.17 ± 5.81%). CFA mice are indicated in light blue; Saline animals are indicated in beige; Striped chamber is indicated by vertical striped patterns, and in green in A; Solid chamber is indicated by a solid gray pattern, and in dark blue in A. A) n = 10. B-C) n = 10 for each group D) n = 5 for each group. Mice were both Male (n = 10) and Female (n = 10). * indicates $p \leq 0.05$; ** indicates $p \leq 0.005$; *** indicates $p \leq 0.0005$; ns indicates $p \geq 0.05$. Mean and SEM are indicated.



Supplement 3: Buprenorphine CPP Based on Group Preconditioning Values

A) Mice show no significant preference for either chamber in the preconditioning phase (Vertical: 41.90 ± 8.64%; Horizontal: 46.03 ± 11.57%). B) Neither CFA nor saline groups show a significant preference for the buprenorphine-paired chamber over the NaCl-paired chamber post-conditioning (CFA: Buprenorphine: 36.56 ± 11.05%; NaCl: 49.04 ± 13.77%; Saline: Buprenorphine: 46.92 ± 13.01%; NaCl: 37.39 ± 12.32%). Time spent in each chamber was not significantly different from chance level for all groups. C-D) Neither CFA nor saline mice exhibited a significant change in chamber preference between the preconditioning and the post-conditioning sessions in either the buprenorphine-paired chamber or the NaCl-paired chamber (C: CFA: -0.90 ± 9.84%; Saline: 4.61 ± 8.33%; D) CFA: -2.24 ± 11.87%; Saline: -7.22 ± 7.32%). A) n = 9. B-D) CFA: n = 5; Saline: n = 4. Mice were both Male (n = 4) and Female (n = 5). Male 5325 met exclusion criteria in the preconditioning phase and was excluded. ns indicates $p \geq 0.05$. Mean and SEM are indicated.



Supplement 4: A5 CPP Based on Group Preconditioning Values

A) Mice show no significant preference for either chamber in the preconditioning phase (Vertical: 42.67 ± 9.47%; Horizontal: 43.24 ± 10.03%). B) CFA mice show a significant preference for the A5-paired chamber over the ACSF-paired chamber (A5: 50.32 ± 10.54%; ACSF: 35.27 ± 9.61%). Saline animals show no preference for either chamber (A5: 42.05 ± 8.34%; ACSF: 41.99 ± 9.33%). Time spent in the A5 chamber by CFA mice was significantly above chance level. C-D) Neither CFA nor saline mice exhibited a significant change in chamber preference between the preconditioning and the post-conditioning sessions in either the A5-paired chamber or the ACSF-paired chamber (C: CFA: 5.55 ± 10.80%; Saline: -1.65 ± 12.91%; D) CFA: -6.21 ± 11.36%; Saline: -0.71 ± 11.92%). A) n = 19. B-D) CFA: n = 10; Saline: n = 9. Mice were both Male (n = 10) and Female (n = 9). Female 5406 met exclusion criteria during the preconditioning phase and was excluded. ** indicates $p \leq 0.005$; ns indicates $p \geq 0.05$. Mean and SEM are indicated.