A Novel Approach to Investigating Stress-Pain Hypersensitivity

A Thesis Submitted to the Committee on Graduate Studies in Partial Full of the Requirements for the Degree of Master of Science in the Faculty of Arts and Science

TRENT UNIVERSITY

Peterborough, Ontario, Canada

Psychology M.Sc. Graduate Program

January 2025

ABSTRACT

A Novel Approach to Investigating Stress-Pain Hypersensitivity

Kirkland Johnston

It is well known that pain can heighten sensitivity to stimuli that signal threat in most species. In rodents, exposure to predator odor, such as 2,5-dihydro-2,4,5-trimethylthiazoline (TMT), induces anxiety and alters pain sensitivity. This study explored the effect of predator odor stress on mechanical pain sensitivity in a rat model of acute inflammatory pain induced by suboptimal doses of Complete Freund's Adjuvant (CFA). Male Sprague-Dawley rats were injected intraplantarly with 50% or 25% (v/v) of CFA in the hindpaw and then exposed the next day to 5 minutes of either 10% TMT (synthetic fox urine) or a neutral odor. Both groups showed reduced paw withdrawal thresholds in the von Frey test. However, TMT-exposed rats displayed persistent mechanical hypersensitivity, which never returned to baseline (pre-CFA) levels when compared to CFA-rats exposed to the neutral odor or control rats exposed to TMT. In addition, TMT exposure after CFA induced greater anxiety-like behavior in the elevated plus maze without affecting locomotor activity in the open field or altering learned responses in a backward paired shock-tone conditioning task. Finally, systemic administration of a CCK_2 antagonist before exposure to TMT partially rescued the mechanical hypersensitivity in these animals but had little effect on CFA-treated rats exposed to the neutral odor. These results suggest that naturalistic stress can lead to a long-lasting nociceptive sensitization that extends beyond the duration of the initial inflammatory injury. Our findings also highlight the importance of CCK_2 signaling as a potential mediator of and therapeutic target for stress-induced pain hypersensitivity.

Keywords: TMT, stress, pain, CFA, CCK, mechanical sensitivity, hyperalgesia, allodynia.

ACKNOWLEDGEMENTS

I would like to express my deepest gratitude to Dr. Neil Fournier for his mentorship, invaluable guidance, and incredible support throughout my academic journey and the supervision of my thesis. His expertise and insight were instrumental in shaping the direction and outcomes of this work. I am especially grateful for his unwavering encouragement and belief in my abilities, which pushed me to achieve more than I thought possible.

I want to give special thanks to my second reader, Dr. Brenda Smith-Chant, and external examiner, Dr. Stephanie Tobin, for their collective insights, technical skills, and overall support in completing this project.

I would also like to acknowledge the contributions of my wonderful lab mates, Brendan O'Brien and Karolina Wawrzyczek, for their assistance with experimental procedures. Their dedication, diligence, and support significantly contributed to the successful completion of this research and the quality of the findings presented.

Finally, I sincerely thank my fellow graduate students, Evana Xiao, Gillian Silver, and Lexi Thivierge, who assisted in various stages of the project. Your collective contributions have been invaluable.

TABLE OF CONTENTS

CHAPTER 1

INTRODUCTION

Pain is a universal experience with adaptative evolutionary value (Crook et al., 2014; Williams, 2023). The experience of acute pain produces immediate responses to actual or potentially harmful stimuli. In addition, acute pain increases sensitivity at and around the site of injury, which triggers protective behaviors that prevent further injury and promote recovery (Basbaum et al. 2009; Crook et al., 2014; Sandkühler, 2009). Acute pain can also serve as a powerful motivator that fosters survival by increasing vigilance towards cues that signal the possibility of threat or injury (Kleshchova et al., 2019). This heightened awareness, in turn, enhances aversive learning and generates organized defensive responses that promote escape from and future avoidance of cues and situations that may be linked to a threat (Pak & Hashmi, 2023; Petrini & Arendt-Nielsen, 2020; Quartana et al. 2009). Thus, the importance of acute pain is evident in its ability to improve survival and enhance recovery after injury.

By contrast, pain that persists long after the initial injury has healed is considered to have limited adaptative or protective value for the sufferer and instead represents a maladaptive pathological state (Corder et al., 2013; De Felice et al., 2011; DosSantos et al., 2017; Timmers et al., 2019; Vachon-Presseau et al., 2016). Decades of research have shown that chronic pain develops through a complex series of molecular and physiological changes beginning at specialized sensory neurons located around the body (Dubin & Patapoutian, 2010; Nikolenko et al., 2022). These sensory neurons—called nociceptors—can detect noxious or harmful stimuli such as intense pressure, cold, heat or chemical irritants (Barrot, 2012). It has been shown that some injuries can cause long-lasting changes in nociceptor function, which can lead to the development of "sensitization" (Latremoliere & Woolf, 2009; Nijs et al., 2021). Sensitization of the spinal cord and brain is critical in transforming the function of nociceptive circuitry into a

state of overexcitability (Dansereau et al., 2008; Pak et al., 2018). Researchers widely view this process as enhancing behavioral and physiological sensitivity to painful stimuli (hyperalgesia) and ordinarily non-painful stimuli (allodynia), key events in chronic pain development (Geva & Defrin, 2018; Loffler et al., 2023; Tao et al., 2019). However, the long-lasting sensitivity of the nociceptive system raises an important question: What is the evolutionary and adaptive value of chronic pain? The answer to this question—while interesting from the perspective of fundamental neurobiology—also has important clinical and societal implications. The Canadian Pain Task Force (2021) estimates that over 8 million Canadians (1 in 5) live with chronic pain. Given the prevalence of chronic pain conditions and the decline in the quality of life that it causes not only to the affected individuals but also their families, understanding how acute pain transitions into a state of chronic pain is of paramount importance.

Yet, despite the common view that chronic pain is always maladaptive, there is limited experimental evidence available that has directly examined this point. Interestingly, sensitization of nociceptors in response to injury can confer benefits in some situations. For example, Crook, Walters and colleagues (2014) showed that heightened pain sensitivity in squid after a sub-lethal injury enhanced survival from predators. These findings suggest that injury-induced nociceptor hyperexcitability may increase survival under high predation risk by promoting hypervigilance. In support of this idea, a recent study showed that mice experiencing neuropathic pain display increased avoidance during a food reward task, which required them to obtain a food reward from a route that exposed them to the smell of fox urine (Lister et al., 2020). This suggests that ongoing awareness of chronic injury altered their behavior to reduce predator risk (Lister et al., 2020).

1. Biology of Pain

Acute and chronic pain cause significant clinical, economic, and social burden. Pain is the most common reason for visits to primary care physicians. In Canada alone, there are approximately 16 million emergency department visits annually; an estimated 10-16% of these visits are related to pain-associated conditions (e.g., fibromyalgia) (Small et al., 2019). The total direct and indirect economic costs of persistent pain are placed at over $38 billion annually, which surpasses the costs of several major diseases, including cardiovascular ($21.2 billion), neoplasms ($26 billion), and gastrointestinal ($2 billion) disease. In addition, the comorbidities associated with pain impose an additional burden on patients and their families. These include opioid medication overuse, misuse, addiction, depression, anxiety, social isolation, and financial difficulties. With the rising incidence and prevalence of pain, particularly following the COVID-19 pandemic, identifying factors that affect the transition from acute pain to chronic pain states has become increasingly important. Exposure to stress, as will be discussed, is one well-known factor that impacts the progression of pain symptoms and hinders recovery (Apkarian et al., 2009; Ridder et al., 2021; Vasic & Schmidt, 2017; Yu et al., 2020). Thus, understanding how stress impacts neurobiological mechanisms associated with the development of pain is vital to providing better support for patients and improving pain management strategies.

1.1. Pain Characteristics

"Pain" describes a broad range of unpleasant sensations but comprises complex and multidimensional experiences that can be initiated by a diverse range of conditions. This point is critical as each pain condition can involve distinct pathophysiological mechanisms requiring different therapeutic approaches. The International Association for the Study of Pain (IASP) defines pain as "an unpleasant sensory and emotional experience associated with, or resembling that associated with actual or potential tissue damage" (Raja et al., 2020). It has long been speculated that the dysregulation of the affective component of pain is a crucial contributor to the

long-term continuation of pain symptoms and pain-related distress (DosSantos et al., 2017; Yang & Chang, 2019). Increasing evidence suggests that pain's affective/motivation and sensory components involve a distinct but interacting network of brain regions, as evidenced by human and animal studies showing that even when the transmission of peripheral pain signals is silenced, the experience of pain persists (Auvray et al., 2010). However, it is essential to acknowledge that some aspects of pain are truly physiological, for example, the capacity to detect potentially damaging external stimuli such as intense heat or cold, excessive mechanical force or pressure, and chemical irritants. This constitutes nociceptive pain, which, as I will discuss, is driven by the activity of high-threshold sensory neurons adapted to transduce such noxious stimuli into signals that are ultimately relayed to and processed by various components of the central nervous system (CNS), leading to the sensation and experience of pain.

Pain can also be a powerful motivator for learning about real or perceived environmental or predation-related dangers that can lead to tissue damage, injury, or even death. It is expected that mechanisms and behaviors necessary for nociception and pain would have been subject to intense evolutionary pressures, resulting in even simpler organisms early in phylogenesis demonstrating rudimentary features of the affective and sensory components of the pain experience. For example, hagfish and lampreys are among the earliest vertebrates to exhibit a complex CNS with structures homologous to those found in the mammalian extended limbic system, such as the amygdala, ventral striatum, and habenula (Loonen & Ianova, 2015). These structures have been implicated in affective, arousal, and motivational behaviors in many mammalian species, including humans.

1.2. Pain Pathways and Mechanisms

Acute pain is the physiological sensation of hurt that results from the immediate activation of nociceptive pathways by peripheral stimuli of sufficient intensity that lead to or

could lead to tissue damage. Nociception, on the other hand, can be viewed as the detection of noxious stimuli and is seen as a protective process that helps prevent injury by generating automated reflexive responses (i.e., withdrawal) from a noxious stimulus along with an evocation of an unpleasant sensation that triggers complex behaviors to avoid further contact with such stimuli.

In the following section, I will review mechanisms that result in both acute and chronic pain, as well as the process by which unpleasant noxious stimuli from the periphery are transmitted through the spinal cord to various regions of the brain, resulting in the physiological sensation of pain and associated negative emotional responses that ultimately contribute to the experience of pain.

1.2.1. Mechanisms of Nociception

Acute pain involves the transduction of noxious signals into an appropriate signal by specialized primary (unipolar) sensory neurons called nociceptors (Dubin & Patapoutian, 2010; Nikolenko et al., 2022). The cell bodies of the nociceptors are in the dorsal root ganglion, and they have a peripheral process that innervates the skin, muscles, joints, and viscera and a central process that connects with neurons within the dorsal horn of the spinal cord or the trigeminal ganglion (Tracey, 2017). Nociceptors can be divided into two main types: the unmyelinated C-fibers or the lightly myelinated A-δ fibers. The A-δ fibers tend to be responsible for the conduction of fast, sharp pricking pain that is well localized, while the C-fibers are associated with dull aching or burning pain that is poorly localized (Yam et al., 2018). These differences likely serve distinct functions. For example, research suggests A-δ fibers may have protective mechanisms, but C-fibers might play a role in signaling when there is tissue damage that is continuously experienced (Hsieh et al., 2015).

Information related to pain and temperature is relayed through A-δ and C fibers that enter the spinal cord via Lissauer's tract and terminate onto secondary projection neurons located in the various laminae of the dorsal horn of the spinal cord (mainly Rexed's laminae I, II and V) (Khalid & Tubbs, 2017). From here, the experience of pain is mediated by two distinct pathways. The first pathway (anterolateral pathway) involves the crossed fibers of the ascending lateral spinothalamic tract, which terminates mainly within the ventroposterior thalamus (Khalid & Tubbs, 2017). Subsequently, information is sent to the primary and secondary somatosensory cortices (S1 and S2), which are critical for detecting the sensory-discriminative features of a painful stimulus, such as its location, intensity, and quality (Khalid & Tubbs, 2017). The other pathway is an essential affective and visceral component of pain. This pathway projects rostrally and bilaterally from the spinal cord to diffusely innervate the posterior and intralaminar thalamic nuclei (Khalid & Tubbs, 2017). These thalamic structures send projections to the anterior cingulate cortex (ACC), brain stem nuclei, and limbic areas, thereby contributing to pain's emotional and aversive experience (Xue et al., 2022).

1.2.2. Chemistry of Pain

Pain is typically associated with inflammation, which is a complex biological response involving the somatosensory, neural, immune, autonomic and vascular/circulatory systems to tissue damage, pathogens and/or irritants. Acute inflammation is a natural biological response and ultimately serves as a protective function to remove harmful (toxic) stimuli and initiate healing processes. Tissue injury triggers the release of various chemical mediators, including bradykinin, prostaglandins, nerve growth factor, histamine, ATP, serotonin, proinflammatory cytokines (tumour necrosis-alpha, interleukin n1B), and others.

Complete Freund's Adjuvant (CFA) is a reliable technique for inducing inflammatory pain, which contains heat-killed and dried mycobacterium Tuberculosis emulsified in mineral oil.

Intraperitoneal (i.p.), subcutaneous (s.c.), or intraplantar (i.pl) CFA typically induce a robust immune response and inflammation in peripheral nerves and surrounding tissues (Liu et al., 2021). CFA is an ideal technique for investigating chronic pain in rodents due to its ability to induce inflammatory injury, which is associated with pain but can arise independently of inflammation (e.g., neuropathic pain). The current model can prolong the injury's healing for up to 10 or more days (Abaddie et al., 2009; Hilfiger et al., 2020). The hydrophobic nature of mineral oil makes it more difficult for the body to metabolize, producing a depot effect at the injection site and resulting in the gradual absorption of antigens (Fan et al., 2022). This extends the immune response's duration and prolongs the injury's overall recovery.

As these antigens are slowly released, they activate pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs) on resident immune cells and local tissues (Pasare & Medzhitov, 2004). This activation leads to transcription and release of pro-inflammatory cytokines (e.g., tumour necrosis factor-alpha, TNF-α; interleukin-1 beta, IL-1β; interleukin-6, IL-6) due to triggering downstream pathways nuclear factor-kappa B (NF-κB) (Abaddie et al., 2009; Coderre, 2008). Consequently, high levels of cytokines foster an inflammatory milieu that elicits immune protection from macrophages, neutrophils, and lymphocytes to defend the area of injection and body from further infection and damage (Fontes et al., 2017). This primary line of defence also reinforces the immune system by releasing additional cytokines, chemokines, and reactive oxygen species (ROS) (Manoharan et al., 2024). Cytokines and ROS work together to sensitize nociceptors by phosphorylating and upregulating ion channels such as TRPV1, voltage-gated sodium channel Nav1.7, and Nav1.8 (Breese et al., 2005; Dib-Hajj et al. 2007). Likewise, chronic activation of nociceptors stimulates the persistent release of neuropeptides (substance P and CGRP) that escalate inflammation (Lyengar et al., 2017). When substance P is released, it binds to neurokinin-1 receptors on immune and endothelial cells, promoting vasodilation and

increasing vascular permeability (Suvas, 2017). CGRP potently contributes to vasodilation and acts on CGRP receptors located on immune and neuronal cells; see Russell et al. (2014) for a general breakdown and graphical schematic. The increased vasodilation and weakening in vascular permeability contribute to the extravasation of immune cells into the affected tissue, thereby amplifying the local inflammatory response (Suvas, 2017).

1.2.3. Chronification and Persistence of Pain

As discussed earlier, acute pain is normal and has a protective function. Repeated subthreshold stimulation or particularly intense noxious stimulation can both lead to the phenomenon of sensitization of the nociceptive system, a process in which the threshold for activation of the system is substantially reduced, and responses to subsequent stimuli are amplified. Without ongoing tissue injury, this heightened sensitivity eventually returns to the original baseline, where above-threshold stimuli are again required to initiate nociceptive signaling. Ultimately, the adaptative function of peripheral sensitization is to promote pain awareness during heightened vulnerability after injury so that the detection of threats and the production of appropriate defensive reactions occur more rapidly. However, in some situations, when pain outlasts the initial inflammatory, tissue or nerve injury, it ceases to be adaptive. Stress can significantly contribute to pain modulation, with strong evidence supporting its ability to induce hyperalgesia (i.e., a painful stimulus is now perceived as more painful) and allodynia (i.e.,, a non-painful stimulus is now perceived as painful) or even spread beyond the initial injury site (i.e., secondary hyperalgesia) (Geva & Defrin, 2018; Loffler et al., 2023). Initiation of the fight-or-flight response via the release of glucocorticoids (GCs) and catecholamines (CAs) (e.g., cortisol or corticosterone, and epinephrine) ensures swift bodily reaction to danger (LeDoux, 2000; LeDoux, 2007). Acute and chronic stress can lead to an overreaction of this process, activating the hypothalamic-pituitary-adrenal (HPA) axis and the sympathetic nervous system

(SNS). This stress-induced activation may enhance fear-driven hypervigilance by shifting attentional resources toward pain-related stimuli (Han et al., 2015). The mechanisms underlying these phenomena seem analogous to chronic stress' sustained release of GCs and CAs (Timmers et al., 2019).

Researchers investigating the impacts of acute and chronic stress on pain in murine models of inflammatory pain have found elevated levels of GCs and CAs, along with neuronal atrophy and synaptic loss in the hippocampus compared to control groups (Mutso et al., 2012; Timmers et al., 2019). These observations suggest that stress potentiates pain by disrupting the negative feedback loop and immune system, promoting the sustained release of cortisol/corticosterone and pro-inflammatory cytokines (Chapman et al., 2008; de Kloet et al., 2005). This disruption contributes to the persistence of pain even in the absence of an ongoing injury and highlights the hippocampus's critical role in central sensitization (Apkarian et al., 2016; Ridder et al., 2021; Vasic & Schmidt, 2017; Yu et al., 2020). First, the loss of synapses and weakening of pathways important for silencing the release of stress hormones diminishes the hippocampus' ability to regulate an organism's stress (Mutso et al., 2012; Ridder et al., 2021; Timmers et al., 2019). Second, sensitization strengthens and increases synapses involved in enhancing threat detection and release of stress hormones (Ridder et al., 2021; Yu et al., 2020). Such maladaptive alterations to the negative feedback loop reduce the efficacy of the mechanism's ability to silence the release of stress hormones, and the strengthening of the CNS nociceptive neurons and pathways enhances pain perception and symptoms.

In such situations, mechanisms that can account for the temporal, spatial and threshold changes in pain sensibility during chronic pain states have been linked to neuroplastic changes across the CNS through a process referred to as central sensitization (Apkarian et al., 2009; Ridder et al., 2021; Vasic & Schmidt, 2017; Yu et al., 2020). Loeser and Treede (2008) define

central sensitization as the "increased responsiveness of nociceptive neurons in the central nervous system to their normal or subthreshold afferent input". In other words, central sensitization is a pathological process wherein the CNS undergoes a persistent state of high reactivity, which amplifies pain signals (Loeser & Treede, 2008; Ridder et al., 2021; Yu et al., 2020). This phenomenon is considered pathological as it is often a consequence of chronic pain conditions, such as fibromyalgia, migraine, and irritable bowel syndrome, where typical nociceptive and pain perception thresholds diminish (Latremoliere & Woolf, 2009; Nijs et al., 2021). Mechanistically, central sensitization involves changes at the level of the nociceptive pathways in the CNS, where repeated or intense noxious stimuli lead to long-term potentiation (LTP) of synaptic transmission in pain pathways (Latremoliere & Woolf, 2009; Bazzari & Bazzari, 2022).

Molecular actors involved in central sensitization include ionotropic glutamate (Glu) receptors (Latremoliere & Woolf, 2009; Liu et al., 2022). One of the most well-researched receptors that alter synaptic plasticity and consequently amplify pain signaling is the N-methyl-D-aspartate (NMDA) receptor (Liu et al., 2022). Other well-documented receptors involved in these processes are α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and kainate receptors (Bleakman et al., 2006). The binding of Glu to these receptors, coupled with neuronal depolarization, results in elevated intracellular calcium levels (Bleakman et al., 2006; Latremoliere & Woolf, 2009; Liu et al., 2022). This calcium influx activates a range of intracellular signaling cascades in pathways such as the calcium/calmodulin-dependent protein kinase II (CaMKII) and mitogen-activated protein kinase (MAPK) (Crown et al., 2012; Wang et al., 2007). These cascades promote several downstream effects that enhance neuronal excitability by lowering the threshold for cellular depolarization. A critical effect is the phosphorylation of ion channels (e.g., TRPV1; transient receptor potential vanilloid), which increases their activity

and sensitivity (Liu et al., 2022; Maio et al., 2023). Additionally, activating CaMKII and MAPK pathways can facilitate changes in gene expression, such as the upregulation of immediate early genes like c-fos and genes involved in neuroinflammation and synaptic plasticity (Minatohara et al., 2016; Wang et al., 2007).

Furthermore, the release of neurotransmitters such as substance P and calcitonin gene-related peptide (CGRP) from primary afferent neurons contributes to the sensitization of dorsal horn neurons in the spinal cord (Dansereau et al., 2008; Pak et al., 2018). This process is compounded by the recruitment of glial cells, which release pro-inflammatory cytokines and chemokines that further exacerbate neuronal excitability (Abbadie et al., 2009; Charo & Ransohoff, 2006). Microglia and astrocytes, the primary glial cell types involved, become activated in response to injury or prolonged pain stimuli, perpetuating a state of neuroinflammation (Clark et al., 2007a; Fu et al., 1999). The combined effect of these molecular and cellular changes results in a neuroinflammatory response that not only sustains but also escalates the sensation of pain, leading to a nervous system conducive to allodynia and hyperalgesia. Clinically, central sensitization presents significant challenges in the management of chronic pain. Traditional analgesics, which primarily target peripheral mechanisms of pain, often prove ineffective. Therefore, treatment strategies are increasingly focusing on interventions targeting central mechanisms, including using NMDA receptor antagonists, gabapentinoids, and therapies aimed at modulating glial cell activity, with interesting research discussed later (see Neurochemistry of pain and threat) demonstrating that attenuation of stress may aid in preventing or treating chronic pain (Adamec et al., 1997; Rivat et al., 2010).

1.4 Models of Ecologically Relevant Predator Odor Stress

Predators release chemicals known as kairomones (metabolite) through their excreta—feces, urine, or bodily secretions (Brechbühl et al., 2013). These kairomones can be a type of

hormone, pheromone or allomone, but predator kairomones serve as crucial signals for prey species, alerting them to the presence of predators, triggering activation of the HPA axis, and aiding in the initiation of escape behaviors to avoid predation (Fortes-Marco et al., 2013). Research has sought to utilize predator odour through various methods to investigate their effects on stress by observing prey behaviors, including fleeing, freezing, and burrowing. Genuine predator excreta has shown significant and insightful implications for understanding how prey may respond in natural environments. This is exemplified by studies showing next to or no behavioral influences on prey species after exposure to genuine feces or urine of predator species that do not naturally prey on them (Cox et al. 2010). Cox et al. (2010) demonstrate that contextual learning can override natural predation patterns, as kangaroos exhibited threat responses to Tasmanian devil urine when the devils were fed kangaroo meat, despite Tasmanian devils not being natural predators of kangaroos. However, concerns have been raised about using genuine predator excreta in laboratory settings, particularly due to the challenges of standardizing natural excreta and maintaining odour consistency across different laboratories (Apfelbach et al., 2015). Research also shows that the aging of predator excreta may degrade its efficacy in eliciting anxiety and fear-related behaviors (Apfelbach et al., 2005; Apfelbach et al., 2015). Artificially synthesized predator odours offer a viable alternative, allowing for greater standardization across laboratories and more precise control over the potency of the odours used.

Among the most studied synthetic odours is 2,4,5-trimethylthiazoline (TMT), derived from red fox (*Vulpes vulpes*) excreta. Its first use by Vernet-Maury (1980) cascaded much subsequent research, culminating in a large body of work supporting TMT's ability to induce unconditioned (i.e.,, in naïve laboratory animals) anxiety and fear-related responses (Rosen et al., 2008; Taugher et al., 2015). Indeed, its robustness for eliciting freezing and fear-related behaviors in rodents seemingly precipitated researchers' concerns that synthetic TMT may be sufficiently

irritative to act on nociceptive pathways via the trigeminal nerve. Hacquemand et al. (2010) demonstrate that intranasal administration of zinc sulphate ($ZnSO_4$), which selectively impairs olfactory sensitivity while sparing trigeminal function, abolishes freezing responses to natural fox feces but not synthetic TMT. Research has since shown that olfaction is likely a critical component of TMT-induced freezing (Kobayakawa et al., 2007), as undiluted TMT triggered freezing in trigeminally ligated animals but not in those with surgically removed olfactory bulbs (Ayers et al., 2013).

Concurrently, trading natural fox excreta for synthetics may impede ecological validity due to the increased potency of synthetic TMT, particularly at elevated concentrations, which has been shown to induce greater freezing responses compared to natural fox excreta (Buron et al., 2007; Galliot et al., 2012). Thus, researchers have sought to investigate TMT concentrations more akin to genuine fox excreta. These studies show variability in what is considered optimal, with some research (Day et al., 2004; Falconer & Galea, 2003) implementing higher concentrations and/or large volumes, while others, like Hacquemand et al. (2013), propose that a 1% concentration of TMT most closely resembles the effects of genuine fox odour. Most evidence-based opinions tend to be somewhere in between, either implementing very minute volumes of pure TMT (Blanchard et al., 2003b; Endres & Fendt, 2007) or significantly diluting it. Buron and colleagues (2007) find that concentrations of 50-100% pure TMT seem to elicit more significant stress behaviors than genuine fox feces but only marginal differences when diluting TMT to 10%. TMT is a noxious chemical characterized by a potent repugnant odor. Research comparing TMT to other noxious chemicals with similar odors but lacking kairomone properties, such as butyric acid, demonstrates that TMT more effectively elicits freezing behaviors and more significant stress responses (e.g., elevated corticosterone levels) (Day et al., 2004; Endres &

Fendt, 2009). These findings support the notion that subjects respond not solely to the unpleasant smell of TMT but rather that it activates internal stress responses.

Furthermore, stress is a critical factor in pain modulation, influencing the intensity and the chronicity of pain experiences. In animal models, stress is often induced to study its effects on nociception, pain behaviors, and the transition from acute to chronic pain states. Much research examining stress-related effects on pain involves fear conditioning paradigms (e.g., electrical foot shock). Assessing subjects' ability to associate cues with fear can help elucidate pain's interconnectedness with learning and memory processes. These conditioned fear responses often involve pain stimuli, effectively integrating nociceptive pathways with fear-related ones. Stress paradigms that do not involve conditioning may thus assist investigators in better isolating the impact of stress on pain by mitigating the confounding influence of pain-related learning.

Like most other prey species, rodents suppress behavioral signs of pain or weakness when in the presence of a potential threat. Interestingly, predators prefer preying on injured animals, especially prey exhibiting expressive behaviors that signal pain or injury (e.g., limping) (Walters, 2019). Researchers suggest that stress-induced analgesia (SIA) evolved to temporarily dull pain, enhancing physical escape from predators and reducing visible signs of vulnerability to avoid being targeted. Predator odours such as TMT effectively evoke unconditioned stress, as demonstrated by Müller & Fendt (2006), who showed that TMT induces fear-related behaviors (e.g., freezing) without activating the central amygdala (CeA), a region crucial for conditioned fear and norepinephrine-mediated antinociception (Maire et al., 2016; Pagliusi & Gomes, 2023).

TMT's primary engagement of the medial amygdala (MeA) and bed nucleus of the stria terminals (BNST) is cited as potentially crucial in sustaining anxiety and fear (Deyama et al., 2008b; Walker et al., 2009) and modulating pain, especially due to these regions’ connections with the periaqueductal grey (PAG). Most recent research (Kim et al., 2018; Lubejko et al., 2024;

Maire et al., 2016) indicates the PAG modulates pain signally via its descending pathways with the RVM and LC (rostral ventromedial medulla and locus coeruleus, respectively). The PAG-LC pathway exerts antinociceptive effects through NE release (Biagioni et al., 2013; de Oliveira et al., 2017) and binding to dorsal horn receptors (Kim et al., 2018; Lubejko et al., 2024; Maire et al., 2016). Recent findings by Lubejko et al. (2024) reveal that these antinociceptive effects are mediated, in part, by opioid-dependent mechanisms within the PAG-LC pathway. The serotonergic PAG-RVM pathway is a critical endogenous modulator of pain and a key target for opioid analgesia (Kim et al., 2018; Lubejko et al., 2024). Research demonstrates this pathway's analgesic effects rely on Glu neurons, while pain is initiated via inhibitory mechanisms (Chen et al., 2024; Samineni et al., 2017; Taylor et al., 2019).

1.4.3. Neurochemistry of Pain and Threat

Cholecystokinin (CCK), mainly through its CCK_2 receptors, acts as a neuromodulator within the CNS, enhancing anxiety, nociception, and gastric acid secretion (Gallopin et al., 2005; Jennings, 2014; Kovelowski et al., 2000). These enhancements in nociceptive signaling may exacerbate pain perception, particularly in chronic pain conditions (Lovick, 2008). This effect is believed to result from CCK's interaction with endogenous opioid systems, where it may antagonize opioid-mediated analgesia and thus contribute to sustaining pain (Jennings, 2014; Lovick, 2008). CCK is also implicated in the regulation of anxiety, exerting anxiogenic effects primarily mediated via CCK_2 receptors in the amygdala (Jennings, 2014; Lovick, 2008). Mechanistically, CCK_2 receptors (or CCK subtype B receptors, CCK-B) couple with guanine nucleotide-binding proteins (G-proteins), specifically the Gq/11 subtype (Piiper et al., 1997). G-proteins are critical in transducing action potentials from transmembrane receptors to intracellular pathways (Tuteja, 2009). When a ligand binds to a G-protein-coupled receptor (GPCR) (i.e.,, CCK_2), the receptor undergoes a conformational change that activates the associated G-protein

(Tuteja, 2009). G-proteins are heterotrimeric (i.e.,, composed of three subunits: alpha (α), beta (β), and gamma (γ)). In its inactive state, the G-protein is bound to guanosine diphosphate (GDP) and is associated with the receptor (Tuteja, 2009).

Upon receptor activation, however, guanosine diphosphate (GDP) bound to the alpha subunit is replaced by guanosine triphosphate (GTP), leading to the dissociation of the G-protein into an active GTP-bound alpha subunit (Gα) and a beta-gamma dimer (Gβγ) (Tuteja, 2009). These activated subunits can subsequently interact with and regulate various downstream effectors (e.g., enzyme phospholipase C, PLC). CCK_2 receptor's coupling with Gq/11 can potentiate the activation of PLC, which then catalyzes the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP2) into two important second messengers: inositol trisphosphate (IP3) and diacylglycerol (DAG) (Piiper et al., 1997). IP3 facilitates the Ca_2 intracellular release, and DAG activates protein kinase C (PKC) (Li et al., 2004). These processes can consequently affect metabolism, gene expression, cell proliferation and growth.

Research demonstrates that administering (i.p.) CCK_2 receptor antagonists either 30 minutes before or after exposure to cat predator odour significantly reduces long-lasting anxiety-related behaviors in rats, with the most pronounced reductions observed in pretreated rats (Adamec et al., 1997; Adamec et al., 2004). Further research provides evidence for high levels of CCK expression in brain regions that process pain and emotions, including pathways such as PAG-RVM, PAG-LC, BNST and amygdala (Jennings, 2014; Lovick, 2008; Pagliusi & Gomes, 2023; Rivat et al., 2010). CCK has been shown to induce panic attacks in both humans and animals, and its pro-nociceptive effects are well-documented in various models of acute and chronic pain (Jennings, 2014; Lovick, 2008; Rivat et al., 2010). Specifically, the persistent activation of CCK_2 receptors has been linked to the enhancement and sustaining of pain, likely through the inhibition of the body's natural endogenous opioid system (Jennings, 2014; Lovick,

2008; Rivat et al., 2010). Research suggests that CCK's ability to reduce the efficacy of this system may increase the difficulty of treating chronic pain patients, as they may be less responsive to opiates (Kovelowski et al., 2000; Lovick, 2008).

Consequently, research has sought to investigate the efficacy of CCK_2 receptors as potential therapeutic targets for pain and anxiety (Adamec et al., 1997; Rivat et al., 2010). Selective CCK_2 receptor antagonists are potentially significant intervention strategies due to their ability to cross the blood-brain barrier (BBB), a crucial factor for its central pharmacological action (Loonam et al., 2003). CCK_2 receptors are predominantly expressed in the amygdala, hippocampus, and PAG—areas strongly involved in regulating fear, anxiety, and pain perception (Bernard et al., 2021). CCK_2 receptor antagonists exhibit a high affinity for CCK and gastrin peptides and effectively inhibits the binding of endogenous ligands, such as CCK-8 and gastrin, to the CCK_2 receptor (Bernard et al., 2021; Pagliusi & Gomes, 2023). This inhibition disrupts the downstream signaling pathways typically activated by these peptides. The antagonism of CCK_2 receptor activity seems to exert both anxiolytic effects and attenuation of nociceptive signaling. This dual mechanism is particularly relevant in chronic pain, where the interaction between anxiety and persistent pain may lead to a cycle of escalated severity.

1.5. Objectives and Hypotheses

Stress and pain are intimately linked, and while acute stress can induce analgesia, it remains unclear whether brief exposure to predator odours can increase pain hypersensitivity. The current investigation thus seeks to provide informative results on the interaction between brief stress responses and their potential to exacerbate acute pain conditions. In particular, predator odour TMT is a potent stressor that elicits defensive behaviors in rodents, including increased vigilance, risk assessment, avoidance, and freezing. These and other defensive and nocifensive behaviors will be examined to explore the influence of TMT-induced stress on central

sensitization mechanisms and their potential to amplify nociceptive and pain sensitivity. Most research has employed more intense CFA pain models, resulting in fewer investigations using mild doses of CFA. Thus, the current study uses a less invasive pain-inducing procedure to garner insights into the combined strength of TMT and CFA on persistent pain. The rationale behind this approach is to minimize confounding factors introduced by severe and prolonged tissue damage, potentially allowing for a more precise examination of how stress modulates central sensitization during the acute phase of pain.

Based on previous research (Baumbach et al., 2024; Buron et al., 2007), the current study introduces a low concentration of TMT (10%) during this acute pain phase, positing that TMT may interact with central sensitization mechanisms, thereby facilitating the transition from acute to chronic pain. It is hypothesized that animals exposed to both pain and stress will exhibit greater levels of anxious behaviors and longer-lasting reductions in paw withdrawal thresholds (PWTs) in their CFA-injected hindpaws compared to those subjected to control conditions, such as stress without pain. Ultimately, the findings from this study are expected to contribute to a deeper understanding of how stress interacts with pain pathways. Additionally, investigating the potential of a CCK_2 inhibitor to protect against the impact of acute stress on stress-induced persistent pain is hypothesized to mitigate these effects and reduce their long-term persistence.

CHAPTER 2

Establishing the Model

1. Introduction

In behavioral neuroscience, predator odors such as TMT, a component found in fox feces, are often used to elicit innate fear responses in rodents. TMT acts as a kairomone, a chemical signal released by one species that benefits another by triggering adaptive behaviors such as avoidance or defensive responses in prey organisms. Synthetic TMT, which is nearly 100% pure, is widely applied in laboratory settings to simulate natural predatory threats. Its high purity enhances its potency, likely making it more effective than natural excreta at provoking robust nocifensive (defensive) behaviors such as freezing, flight, or avoidance (Buron et al., 2007). This heightened response suggests that synthetic TMT evokes exaggerated fear and anxiety-like behaviors compared to the less concentrated natural stimuli observed in the wild (Hacquemand et al., 2010).

The potency of TMT in inducing stress makes it an invaluable tool for examining the physiological and behavioral consequences of predator-induced fear in laboratory animals. However, most research employs relatively high concentrations of TMT, which can lead to overwhelming fear responses. Such levels may overshadow the interactions between stress and other variables, such as inflammatory pain. For the current study, a moderate and more ecologically relevant concentration of TMT was selected to avoid confounding the effects of stress and nociception (Baumbach et al., 2024). This study aimed to explore how predator-induced stress might potentiate acute inflammatory pain, modeled by CFA, into a more persistent or chronic state in Sprague Dawley (SD) rats.

Chronic pain involves both physiological and psychological dimensions. One key hypothesis in pain research is that stress, particularly when superimposed on an existing injury,

can exacerbate pain and prolong its duration. Stress activates the HPA axis, leading to the release of GCs, which modulate inflammatory responses and can alter the progression of pain (McEwen, 2007). This stress response has been shown to influence both the sensory perception of pain and the emotional experience associated with it. Therefore, this study examines whether predator stress might prolong an acute inflammatory pain state, making it persist beyond the normal healing period of 7 to 10 days typically observed with CFA-induced inflammation (Baumbach et al., 2024).

CFA is a well-established model for inducing acute inflammatory pain in rodents due to its ability to elicit a localized immune response. Upon injection, the CFA's Mycobacterium tuberculosis antigens provoke an immune cascade, resulting in the release of pro-inflammatory cytokines and the recruitment of immune cells to the site of injection (Noh et al., 2021). This causes localized inflammation, erythema, edema, and hyperalgesia, modeling the human inflammatory pain response. While CFA-induced inflammation generally resolves within 7 to 10 days, the dose and concentration of CFA can impact the duration and intensity of the pain. Higher doses may extend the pain response beyond 20 days, modeling chronic conditions like rheumatoid arthritis (Abbadie et al., 2009).

The current study focused on establishing a reliable model of acute inflammatory pain that resolves within this standard timeframe. The experiments use suboptimal CFA doses to induce a mild, localized inflammatory response without extending into chronic pain unless modulated by stress. We aim to provide a baseline for evaluating how predator stress, elicited by TMT exposure, can interact with the inflammatory pain model and potentially extend its duration. The von Frey test, a widely accepted method for assessing mechanical pain thresholds, was employed to quantify changes in mechanical hypersensitivity across time (Chaplan et al., 1994).

This method allows precise measurement of the force required to elicit paw withdrawal, providing insight into the development and resolution of pain in response to both CFA and stress. In summary, this study builds on the understanding that stress can enhance pain sensitivity by activating central pathways involved in pain modulation. The use of predator odor stress alongside an inflammatory pain model allowed for a nuanced exploration of how stress influences the transition from acute to chronic pain. By using moderate doses of both stress and inflammatory stimuli, the study aimed to isolate the impact of stress on pain, avoiding the confounding effects of overwhelming nocifensive or inflammatory responses.

2. Methods

2.1. Animals

Male SD rats (Charles River, Montreal QB, Canada) weighing 150-200g at arrival were maintained in Trent University's Animal Care Facility. All rats were housed in groups of 2-3 in conventional rectangular polypropylene cages with standard corncob bedding in a temperature-controlled (21°C) room with a 12:12 hr light: dark cycle (lights on at 0700 h local time). Food and water were freely available (*ad libitum*) throughout the experiment, and experimental procedures were conducted exclusively during the light period. All procedures were performed in accordance with the guidelines of the Canadian Council on Animal Care and approved by Trent University's Animal Care Committee. Efforts were made to minimize the number of animals used.

2.2. CFA Model of Inflammatory Pain

Rats were placed under light anesthesia (<3 min) with 2-2.5% isoflurane (flow rate of 1 L/min in pure oxygen) to reduce handling and stress before the procedure. Inflammation was induced by unilateral intraplantar (i.pl.) injections of 50 ul or 25 ul of diluted (50% or 25%) Complete Freund's adjuvant (CFA; Sigma Aldrich, Oakville ON Canada) into either the left or right plantar hind paw using a 100 uL microsyringe with a 27-gauge needle (see Figure 1). The

choice of intraplantar injection rather than intraperitoneal administration was made to induce localized inflammation directly at the injection site in the hind paw, potentially allowing for a more accurate assessment of how mild stress may potentiate nociceptive sensitivity and allodynia. CFA containing 1 mg of heat-inactivated and dried *Mycobacterium butyricum* per milliliter of emulsion was diluted with saline (0.9%), resulting in a final concentration of 0.5 mg/ml (Experiment 1) or 0.25 mg/ml (Experiment 2, 3, and 4) of killed mycobacteria. In adult rodents, CFA injection rapidly produces localized cutaneous inflammation (erythema, edema and tenderness) in the injected paw along with mechanical hypersensitivity and thermal hyperalgesia that can continue for weeks post-injection which closely mimics aspects of inflammatory pain conditions seen in humans (Abaddie et al., 2009; Cao et al., 2024; Hilfiger et al., 2020; Noh et al., 2021). For comparison, control rats received light isoflurane anesthesia but did not receive a vehicle injection as past work found that intraplantar injection (i.e., needle prick) alone is still part of the pain experience and could still induce a modest change in mechanical sensitivity lasting for ~3 days (Smith et al. 2016).

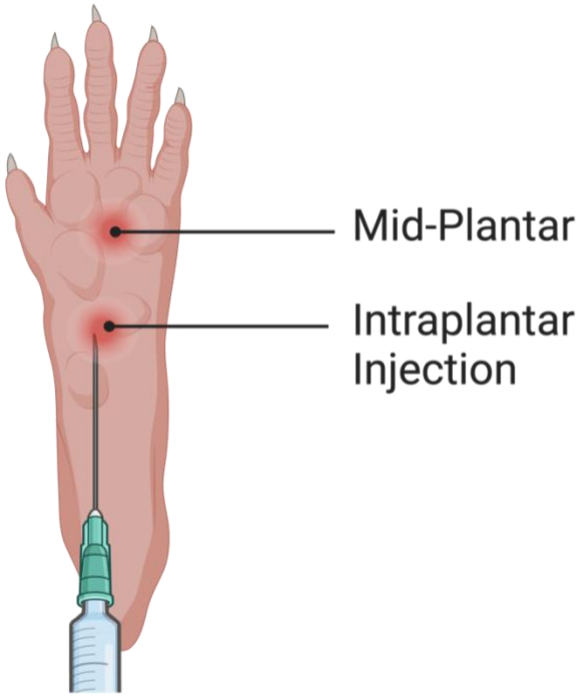

Figure 1. **CFA Injection Sites in Subjects' Hindpaws – Counterbalanced Administration Across Animals.**

2.3. Mechanical Threshold Testing

Rats were placed in individual chambers (19.5 x 14.2 x 9.5 cm) on an elevated metal mesh platform (see Figure 2). Rats were allowed to habituate to the chambers for at least 30 min before assessing for mechanical nociceptive thresholds. Mechanical paw withdrawal thresholds of the ipsilateral (CFA-injected) and contralateral (non-injected) paw were determined using a series of calibrated von Frey monofilaments (filament # 7-14, North Coast Mecial Inc. Gilroy California USA). In this procedure, the filament was applied perpendicularly to the lateral edge of the paw. The application of the filament and the delivery of force was noted by the bowing of the filament for at least 2 seconds. An “up-down” method (Chaplan et al., 1994) was used to identify each animal’s 50% PWT. A quick withdrawal or shaking of the stimulated paw, lifting, guarding, biting or licking of the paw was considered a positive withdrawal response, while the absence of these

responses was regarded as a negative withdrawal response. A positive withdrawal response (X) was followed by the application of the next lower force filament, whereas a negative (no) withdrawal response (O) resulted in the application of the next highest force filament in the series. Filaments (starting at filament # 10, 2.0 g) were applied until the first cross-over point (XO / or OX) was met, after which another four filament presentations were given according to up-down rules. Testing ended if a positive response to the lowest possible filament (#7, 0.6 g) or a negative response to the highest possible filament was observed (#13, 8.0 g). Subjects' right and left hind paws were tested twice in each von Frey session, with an average of 5 minutes between successive tests.

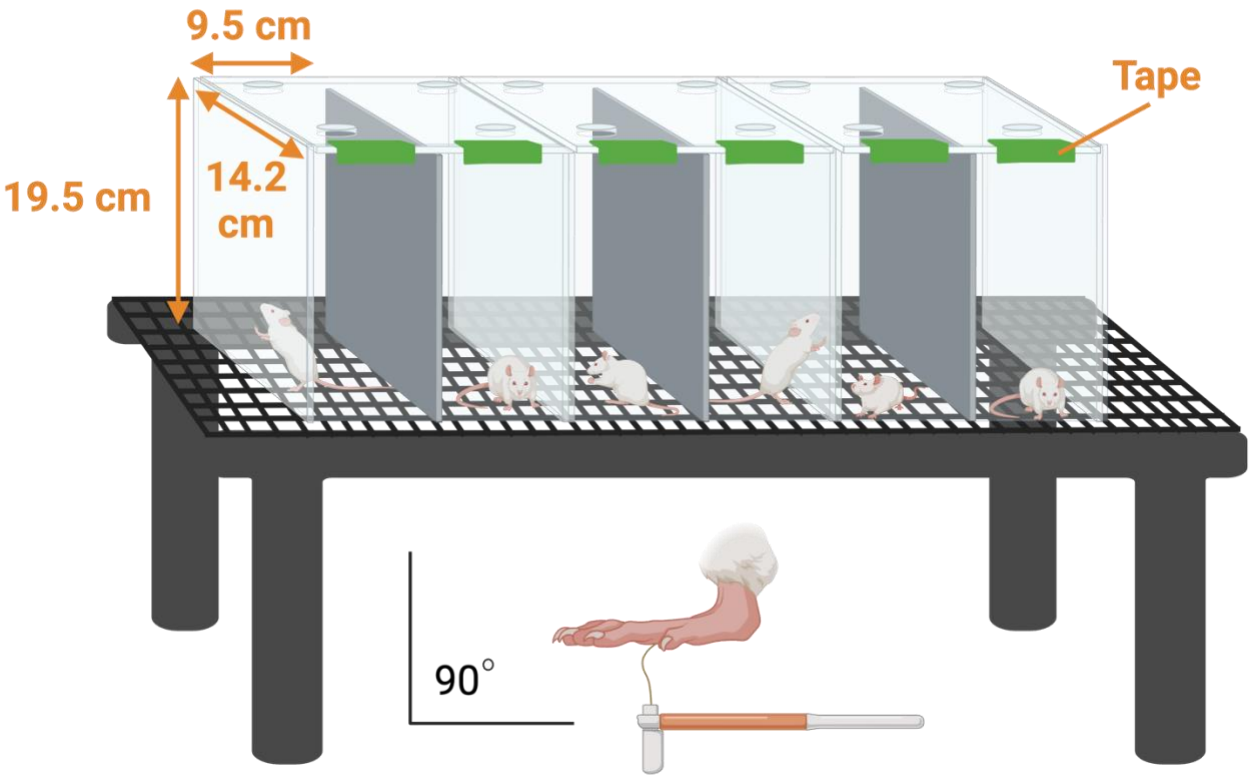

Figure 2. **von Frey Apparatus & Filament Position.**

2.4. Predator Odor Stress

The exposure chamber was made of glass (29.5 cm x 23.9 cm x 49 cm), contained a small layer of bedding, and was housed in a Biosafety Cabinet to reduce dispersion (BSC-Level 2). A small piece of filter paper (6 cm x 2 cm) containing 35 ul of deionized water (diH_2O; stimulus control) was taped to one side wall of the chamber, with the location of the filter paper counterbalanced across subjects. Each rat was allowed to freely explore the chamber for 5 or 10 minutes during a single habituation session, depending on the experiment (see Experimental Timeline 1 – 4). To minimize scent cues, the chambers were thoroughly cleaned with 70% (v/v) ethanol and Oxivir Five 16 Concentrate (Diversy, Canada), and bedding, filter paper and diH_2O were freshly replaced after each subject. The habituation session occurred the day before the CFA injection and two days before exposure to the odorant stimuli.

For TMT or neutral odor exposure, rats were placed into the chamber for 5 minutes and exposed to either 35 ul of 10% TMT (BioSRQ, Sarasota Florida, USA) or water applied to filter paper that was taped to the interior of one side wall of the apparatus. This concentration of TMT has been previously shown to produce robust and consistent freezing responses in rodents (Buron et al., 2007). The location of the filter paper was counterbalanced across subjects. The exposure sessions were recorded by an overhead camera, and activity behaviors were quantified using AnyMaze software. To minimize potential cross-contamination of TMT to the stimulus control conditions, rats in the control conditions were tested first. The chambers were thoroughly cleaned between subjects with 70% ethanol and Oxivir Five 16 Concentrate (Johnson Diversy, Canada), and bedding, filter paper and diH_2O were freshly replaced after each subject.

2.5. Behavioral Measures

2.5.1. Open Field Test

Activity and exploratory behavior were examined in an open-field test (OFT). The arena was a stainless steel box (60 cm x 60 cm x 60 cm) with a small amount of corn bedding covering

the floor. Upon placing the rat into one of the four corners, they were allowed to explore the open field arena for 10 minutes. The session was recorded with a camera situated above the open field and analyzed using AnyMaze software (Stoelting, Wood Dale, IL). The open field was digitally divided into a center zone, which was defined as a 400 cm^2 square in the middle of the open field, and a peripheral zone, which was considered as the space between the walls of the enclosure and the middle zone border (i.e., the 3200 cm^2 area surrounding the middle zone). During the exploration session, the total distance travelled, the mean movement velocity, the time spent and distance travelled in the center zone. After testing each animal, the arena was cleaned with Oxivir Five 16 concentrate (Diversey Inc. Canada).

2.5.2. Elevated Plus Maze

Anxiety-like behavior was assessed using an elevated plus maze (EPM) that was elevated 50 cm from the floor, consisting of two open arms (50 cm x 10 cm x 40cm) and two enclosed arms (50 cm x 10 cm x 40 cm). The plus maze was placed in the center of a homogeneously illuminated room. Each rat was placed in the intersection between the arms facing the open arm opposite to the investigator. Each session was video recorded for 5 min, and the rat's position was determined by automatic video tracking (AnyMaze, Stoelting, Co.). The percentage of open arm entries, time in open arms (in seconds, s), time in closed arms (s), and time in the center square (s) were recorded. After testing each animal, the elevated plus maze was cleaned with Oxivir Five 16 concentrate (Diversey Inc. Canada).

2.5.3. Relief Conditioning

Rats were placed in a Plexiglas chamber (25.4 x 25.4 x 36.5 cm, Ugo Basile) housed in a sound-attenuating cabinet. The chamber had a standard grid floor consisting of 21 stainless steel rods (4 mm diameter, 1 cm distance) connected to an adjustable shock generator (Ugo Basile, Varese, Italy) to deliver a scrambled foot shock. A ventilation fan produced a constant background

noise of 55 dB within the sound-attenuating cabinet. The chamber was illuminated by a 2.5 W white LED light. A tone CS was presented through a loudspeaker mounted on the ceiling of the cabinet. All conditioning testing sessions were video recorded using a webcam placed above the conditioning chamber and connected to a laptop computer. After running each subject, the chambers were cleaned with Oxivir Five 16 concentrate (Diversey Inc. Canada).

Relief conditioning was carried out over three days. The first day consisted of a 10-minute acclimation session to the conditioning chambers. The next day, animals underwent a relief conditioning protocol. This protocol consisted of a 90 s habituation period, which was then followed by 6 explicitly backward paired foot-shock (UCS, 1.5 s, 1.0 mA) and tone (CS, 20 s, 5000 Hz, 80 dB) pairings. The interval from the shock's end and the tone's onset was always 2 s, and the mean inter-shock interval was 90 s. The next day, animals were returned to the conditioning chamber, and after a 90 s acclimation period, 6 test tones (20 s, 80 dB) were delivered with an interval of 60 s between successive tones. Defensive freezing, which was defined as the absence of observable movement except those necessary for respiration, was measured using an automated freeze detection system (AnyMaze). The percentage of freezing displayed during each test tone was used as a measure of conditioned fear.

2.6. Euthanization and Tissue Collection

After the completion of each study, rats were deeply anesthetized with sodium pentobarbital (340 mg/ml, Euthansol, Merck Animal Health Canada) and then underwent transcardiac perfusion with room temperature 0.1 M phosphate-buffered saline (PBS, pH=7.4) followed by ice-cold 4% (w/v) formaldehyde fixative that was freshly prepared from depolymerized paraformaldehyde. The brains were extracted and post-fixed in the same fixative overnight before being placed in PBS with 0.01% (w/v) sodium azide for long-term storage and future immunohistochemical probing.

In some experiments, animals were briefly sedated with isoflurane and were sacrificed by rapid decapitation. The brain was removed, and specific brain regions (e.g., medial prefrontal cortex, amygdala, hippocampus) were rapidly microdissected on ice and then stored in RNAlater at 4 °C for future molecular analysis.

2.7. Experimental Design

2.7.1. Experiment One – Optimizing CFA Model

Experiment one explores whether stress could enhance nociceptive sensitivity and potentiate acute pain into a more persistent state. To investigate this interaction, a suboptimal dose (50 µl; 50%) of CFA followed by a brief (5-minute) exposure to naturalistic predator odor was employed. Thirty-two male SD rats were randomly assigned to three groups: No Stress and Pain, Stress and Pain, and Stress and No Pain. Mechanical sensitivity assessments were conducted at multiple intervals over 30 days to evaluate nociceptive thresholds. Developing a model of acute inflammatory pain that resolves within the typical healing period may thus provide insight into the impact of stress on pain progression.

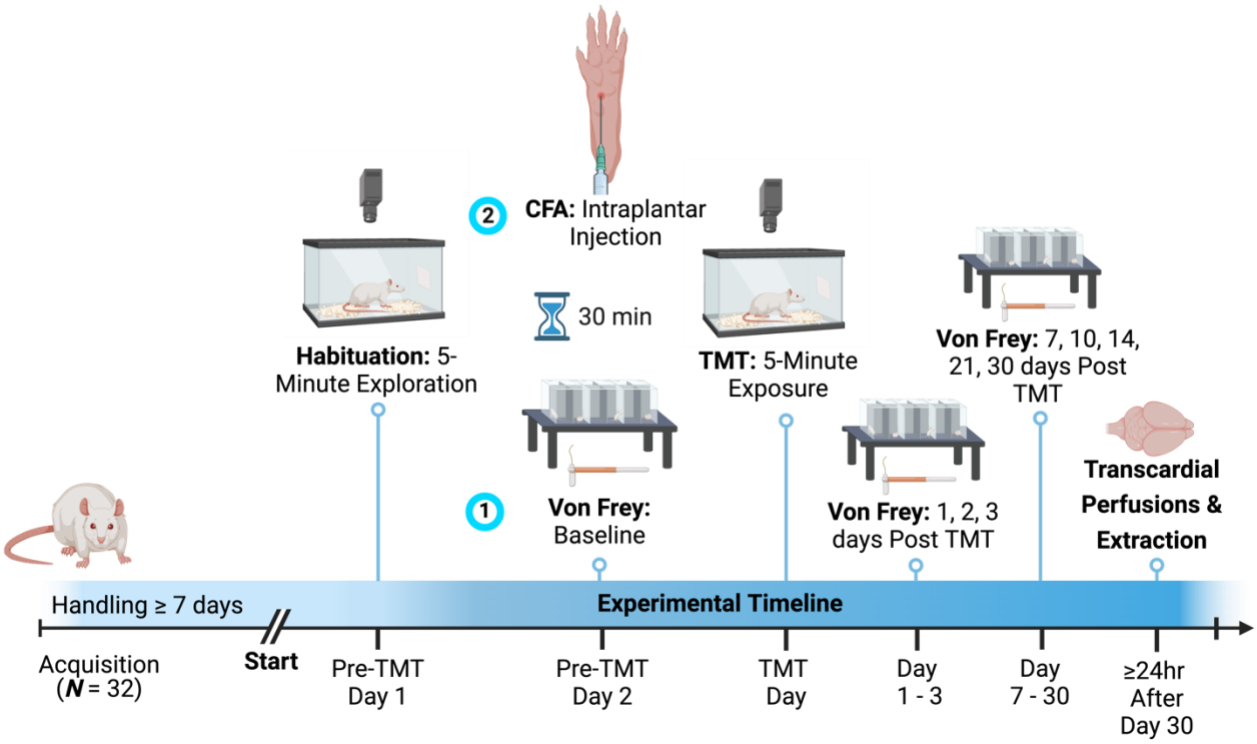

Figure 3. **Experiment One – Timeline.**

2.7.2. Experiment Two – Establishing the CFA Model

The second experiment similarly examines the interaction between CFA-induced inflammatory pain and predator odor stress but distinctly utilizes a reduced concentration of CFA (50 µl; 25%), with only two von Frey assessments (baseline and 7 days post-TMT) and rapid decapitations and microdissections of brain tissues occurring eight days post-TMT. To refine the model from experiment one, it was hypothesized that reducing the CFA dose could sufficiently trigger an immune response that induces pain within the optimal healing period. Experiment two consisted of nine male SD rats with two conditions: Stress and Pain (n=5) and No Stress and Pain (n=4). Four additional subjects were included as naïve controls for comparison in future molecular analyses to ensure accurate baseline measurements from decapitation without prior exposure to pain or stress. Therefore, experiment two aimed to optimize the CFA dose to ensure the induction

of acute inflammatory pain without progressing into a chronic phase due to CFA alone. This adjustment is crucial for attributing any prolonged pain specifically to predator stress rather than an exaggerated inflammatory response from the CFA injection.

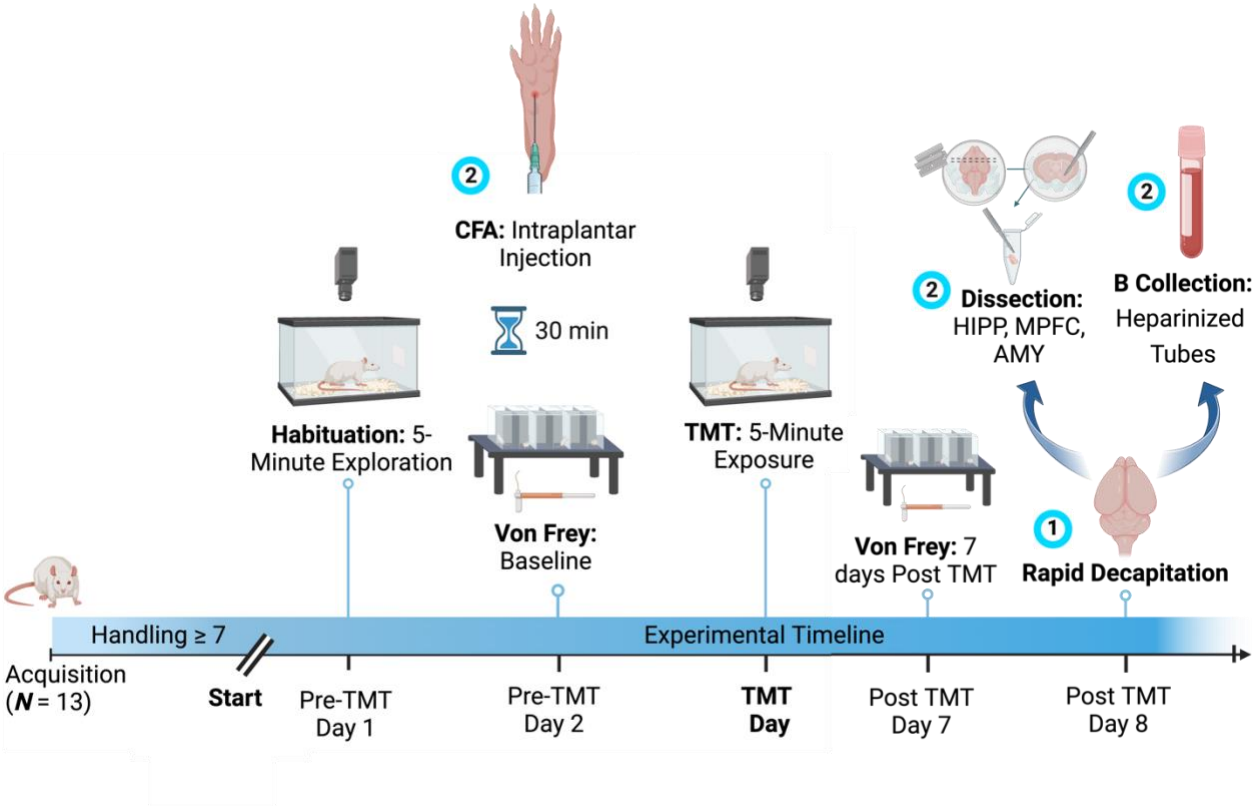

Figure 4. **Experiment Two – Timeline.**

2.7.3. Experiment Three – Replicating the CFA Model and Assessing Anxiety-Related Behavior

The third experiment aims to replicate the findings from experiment two, demonstrating that lowering the dose of CFA to 50 µl of 25% concentration may produce an immune response sufficient to elicit pain within the optimal period without the risk of the pain model inadvertently extending beyond this window. Additionally, we employed a combination of behavioral assays to test this hypothesis, including the von Frey test for assessing mechanical nociceptive sensitivity over fourteen days, the OFT, and the EPM to evaluate anxiety-related behaviors. The addition of

the OFT and EPM tests may provide additional insight into the emotional aspects of pain or affective behaviors not completely captured in the previous experiments. We also incorporated a relief learning paradigm to explore the cognitive aspects of pain modulation. This paradigm allows us to investigate how prior experiences with pain and stress may influence subsequent pain responses. Perfusions were conducted 60 minutes after completing the relief learning test for future quantification of protein markers. There were two conditions within these two studies: Stress and Pain (n=5), No Stress and Pain (n=5), and five additional naïve controls. The five naïve controls exclusively underwent relief learning to provide baseline data for future immunohistochemistry.

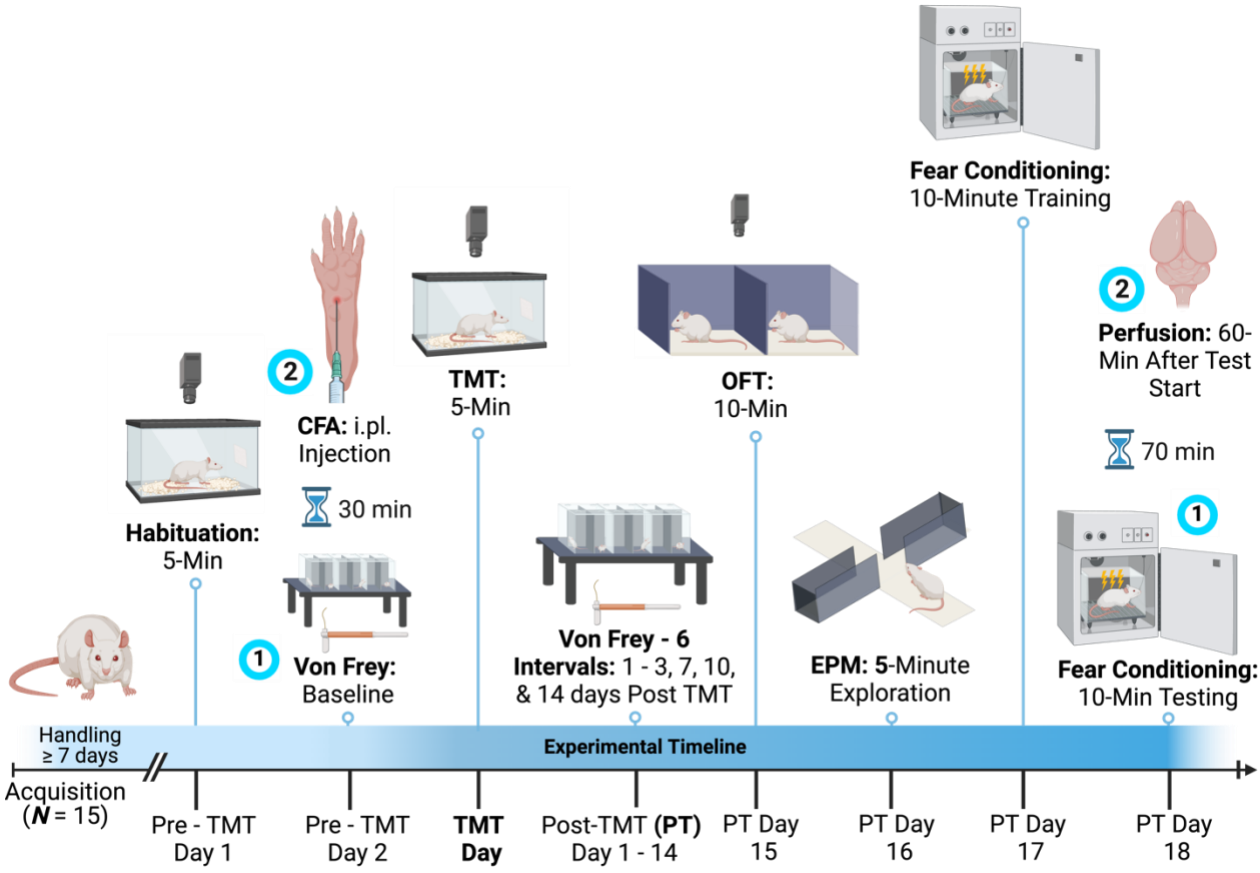

Figure 5. **Experiment Three – Timeline.**

2.8. Statistical Analyses

All statistical analyses were performed using R-studio version 4.4.1. Comparisons between groups were examined using repeated measures ANOVAs, and post-hoc analyses using Bonferroni correction on pairwise t-tests were implemented. All diagrams of experimental procedures were generated using Biorender, and aligned with data figures generated in R.

3. Results

3.1. Experiment One – Effect of predator stress on mechanical hypersensitivity in the CFA model

In the present study, rats were assigned to receive a single unilateral injection of 50% CFA into the left or right hind paw (CFA rats). The following day, CFA rats were exposed to either TMT as the predator odor (n=11) in an attempt to induce persistent mechanical allodynia or were exposed to water which served as a control odor (n=8). An additional group of rats (n=12) not injected with CFA but exposed to predator odor served as a reference. Mechanical PWTs were assessed using the von Frey test at 24 hrs, 48 hrs, 72 hrs, 7 days, 12 days, 14 days, 21 days and 31 days following exposure to either TMT or the control odor. A schematic of the experimental design is shown in Figure 3. We intentionally chose not to include a group of control rats assigned to the water odor condition because previous studies have demonstrated that mechanical withdrawal thresholds remain consistent across repeated testing in non-injured rats (Noh et al., 2021). Moreover, exposure to TMT does not appear to alter mechanical thresholds in non-injured rodents but instead acutely induces stress-induced analgesia and anti-nociceptive effects that dissipate within 24 hrs (Butler et al., 2009; Hotsenpiller & Williams, 1997). Therefore, we considered the inclusion of control rats exposed to TMT as a suitable reference for contrasting withdrawal thresholds in CFA-injected rats.

To address changes in mechanical sensitivity after inflammatory injury induced by CFA, a two-way repeated measures ANOVA was used to examine paw withdrawal responses in the ipsilateral and contralateral hindpaws separately. For the ipsilateral paw results, a significant

interaction between group and time [$F(12, 174) = 1.893$, $p = .038$], indicating that exposure to predator stress differentially impacted the time course of recovery after CFA injection. Additionally, significant main effects were also observed for group [$F(2, 29) = 32.929$, $p < .001$] and time [$F(6, 174) = 11.24$, $p < .001$]. As shown in Figure 6. A and B, animals injected with 50% CFA but not exposed to predator odor resulted in marked mechanical sensitivity in the injected paw at 48 hours [all Ps < .023], 72 hours [all Ps < .001], 7 days [all Ps < .001], 10 days [all Ps < .001], and 14 days [all Ps < .028] compared to the control group. Importantly, the withdrawal thresholds for these animals reached values comparable to those of controls by 21 days ($p = .098$) and 31 days ($p = .538$), suggesting that mechanical sensitivity was resolved primarily by the third week for the CFA-injected paw. In contrast, animals exposed to TMT 24 hours after 50% CFA injection produced pronounced and ongoing sensitization at all 8 time points examined (all Ps < .01).

Examination of mechanical sensitivity for the contralateral (non-injected) paw revealed similar effects (see Figure 6. C and D). The analysis revealed significant main effects of group [$F(2, 29) = 19.03$, $p < .001$] and time [$F(6, 174) = 4.93$, $p = .013$], but no significant interaction between group and time [$F(12, 174) = 0.42$, $p = .865$]. Animals injected with 50% CFA but not exposed to predator odor exhibited significant mechanical hypersensitivity at 72 hours [$p < .001$] and 7 days [$p < .001$] compared to control rats. However, their withdrawal thresholds gradually returned to baseline by 14 days, with no significant differences observed at 14 days [$p = .076$] and beyond (all Ps > .1). Rats injected with CFA and exposed to predator odor demonstrate reductions in PWTs as early as 48 hours [$p < .05$], continuing to day 10 [$p < .01$] and trending towards recovery from then on.

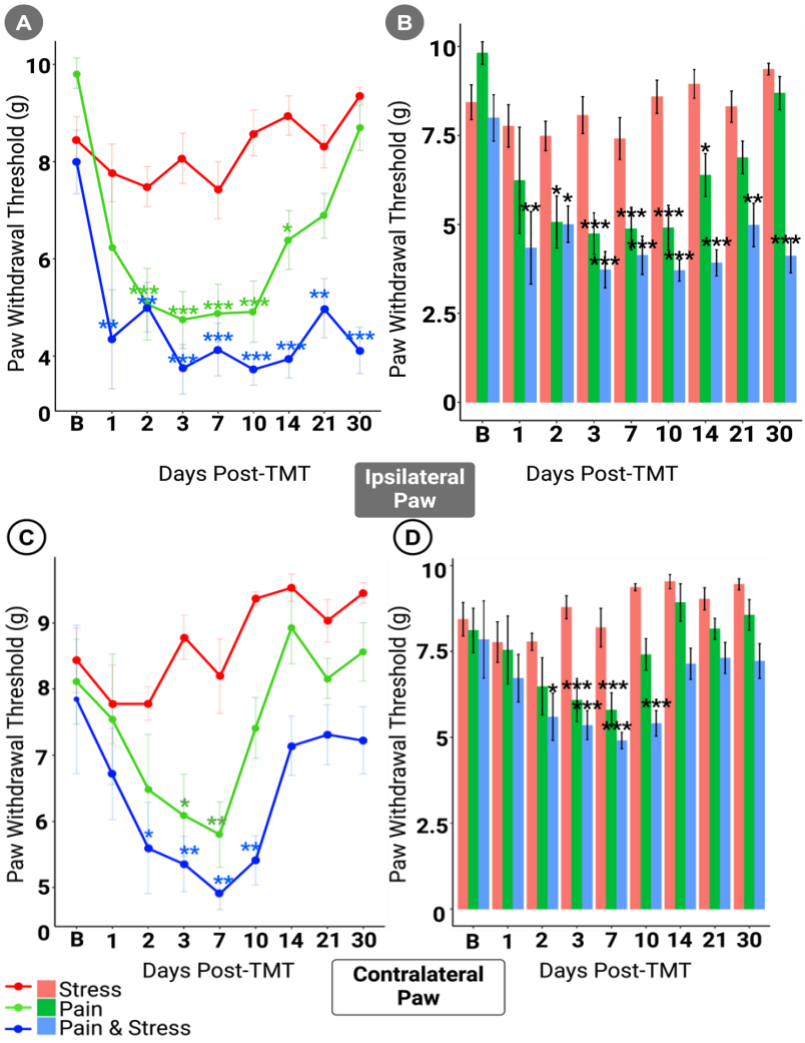

Figure 6. **CFA-injured subjects exposed to predator odor show reduced PWTs.** (*A*) Within-group differences show that injured animals exposed to predator stress exhibited a marked reduction in PWT earlier and for a greater total duration than other groups. (*B*) Between-group analyses revealed injured animals that underwent stress exposure show consistently lower PWTs across time than the other groups (*C*) Animals in the pain and stress group exhibit a significant decline in PWT compared to other groups but recovered by day 21. (*D*) Animals in the no pain and stress group display consistently higher PWTs than the other two groups. *Note*. "B" on x-axes indicates baseline measurement, and data are presented as mean ± *SEM*, with asterisks denoting the degree of statistical significance (*$p < .05$, **$p < .01$, ***$p < .001$).

3.2. Experiment Two – Predator stress induces long-lasting mechanical allodynia even with milder doses of CFA

The findings above suggest that exposure to predator odour after inflammatory injury enhances sensitivity to otherwise innocuous sensory stimuli. However, the prolonged recovery period observed with this higher concentration of CFA used in this first study made it unclear whether similar hypersensitization could be induced with a milder inflammatory insult—a situation that might better mimic naturalistic situations of subthreshold or subacute inflammation. To address this, we attempted to extend our previous finding by reducing the dose of CFA administered to produce a less severe inflammatory response and then evaluated the impact of this manipulation on the development of CFA-inflammatory pain after predator odor stress.

A two-way repeated measures ANOVA with time as the within-subject factor and group as the between-subject factor was used to examine ipsilateral paw withdrawal thresholds. The interaction between group and time [$F(6,48)=4.40$, $p < .001$] was found to be significant, as well as the main effects for group [$F(6,48)=7.302$, $p < .001$] and time [$F(1,8)=25.20$, $p < .001$]. Unilateral intraplantar injection of 25% CFA significantly decreased withdrawal thresholds within the first 72 hours for all animals, as shown in Figure 7. By day 7, CFA rats previously exposed to the control odor no longer displayed a significant difference in withdrawal threshold compared to their baseline measurements (paired t-test, day 7 vs. baseline, $p = .851$), indicating that CFA produced robust mechanical sensitivity that persisted for at least three days but resolved within

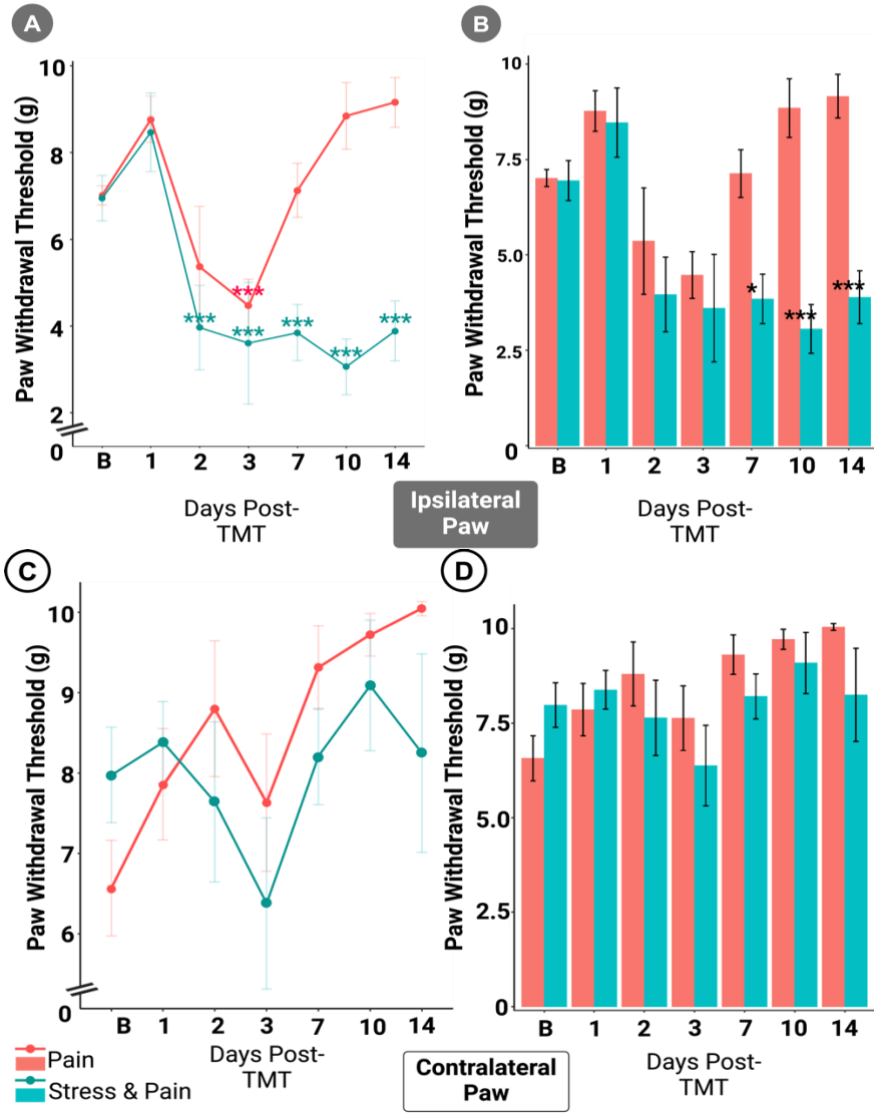

Figure 7. **A mild dose of CFA induces similar reductions in PWTs in CFA-injured rats exposed to predator odor.** (*A*) Injured animals exposed to stress show sustained reductions in PWTs compared to baseline from 7 days onward. (*B*) Between-group differences are first observed in the Pain X Stress condition at 48 hours and sustained to the last Von Frey measurement. (*C*) Downward trends in contralateral PWTs are more pronounced in the Pain X Stress group, but there is no statistically significant difference compared to the baseline in either group. (*D*) No between-group differences were detected. *Note*. "B" on X-axes indicates baseline measurement, and data are presented as mean ± SEM, with asterisks denoting the degree of statistical significance (*$p < .05$, **$p < .01$, ***$p < .001$).

one-week post-administration. CFA rats exposed to TMT continued to exhibit reduced mechanical thresholds on day 7 ($p < .043$), day 10 ($p < .001$) and day 14 ($p < .001$) compared CFA rats given the control odor (All ps <.043), and when contrasted with their own baseline measurement (All p

<.001). No significant differences were observed in the subjects' contralateral paws; however, animals that underwent CFA injection and TMT exposure displayed a downward trend in PWTs between 48 and 72 hours.

We also replicated these ipsilateral paw findings in a separate cohort of rats (CFA/control odor, n = 4; CFA/TMT, n = 5), confirming that CFA-induced mechanical sensitivity largely recovered by day 7 following exposure to the control odor (paired t-test, day 7 vs. baseline, p = .999) (see Figure 8). However, consistent with our previous results, mechanical hypersensitivity still remained on day 7 in CFA-injected rats exposed to TMT (paired t-test, day 7 vs. baseline, p < .001). Interestingly, both ipsilateral and contralateral hindpaws show this marked difference, which was not seen in the previous experiment. Taken together these results demonstrate that a mild inflammatory injury induced by a reduced dose of CFA produces transient pain, characterized by a decrease in mechanical pain thresholds, which typically resolves within one week. In contrast, brief exposure to predator odor stress in animals already sensitized or primed by a small dose of CFA appears to induce a state of prolonged hypersensitivity and allodynia that extends beyond the site of injury.

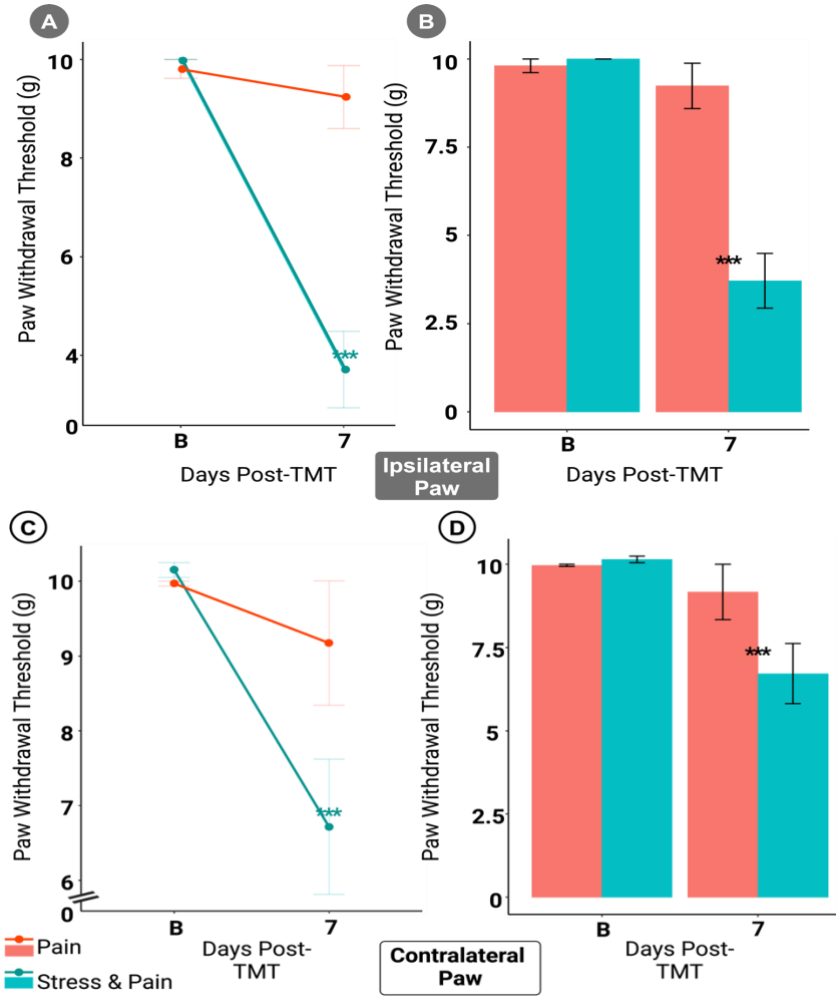

Figure 8. **A mild dose of CFA and predator odor replicates significant reductions in PWTs in CFA-injured subjects exposed to predator odor, further supporting the efficacy of this model.** (*A*) Compared to baseline, the mean ipsilateral PWTs for the pain and stress group show a significant reduction in ipsilateral PWTs at 7 days. (*B*) Between-group differences were observed in the pain and stress condition at 7 days post-TMT exposure. (*C*) A within-group difference of significantly lowered PWT is exhibited in the stress and pain condition. (*D*) The pain and stress condition was also significantly lower than the other group at 7 days. *Note*. "B" on x-axes indicates baseline measurement, and data are presented as mean ± *SEM*, with asterisks denoting the degree of statistical significance (***$p < .001$).

3.3. Experiment Three – Exposure to predator odor stress after inflammatory injury enhances anxiety and impairs relief learning following inflammatory injury

Hypersensitivity to touch is one hallmark of stress-related psychopathologies. Thus, if exposure to predator odor following an inflammatory injury results in persistent pain awareness,

we asked whether this hypersensitization might also affect stress-induced affective behaviors and threat-coping responses. To address this, rats were exposed to either TMT (n=5) or a control (water) odor (n=5) twenty-four hours after the induction of a mild inflammatory injury induced by CFA. The rats then underwent a series of behavioral testing starting 15 days later when CFA-evoked mechanical pain generally resolved in animals not exposed to predator odor (see Figure 7). The behavioral tests examined included (in order) the open field test, elevated plus maze and conditioned relief learning (please see Figure 5 for an experimental timeline).

The open field test was used to examine patterns of exploratory activity in CFA rats exposed to predator odor (TMT) or a control odor. Please also see Figure 9 for a breakdown of the experiment's timeline. Analysis of overall open field exploration revealed a trend towards reduced activity, as indicated by total distance traveled [$t(8) = 1.97$, $p = .084$] and movement speed [$t(8) = 1.98$, $p = .083$] in CFA-injected rats exposed to TMT; however, these differences did not reach statistical significance. There were no significant differences in either the total distance traveled [$p = .463$] or the time spent in the center [$p = .215$] or peripheral zones [All $ps > .098$].

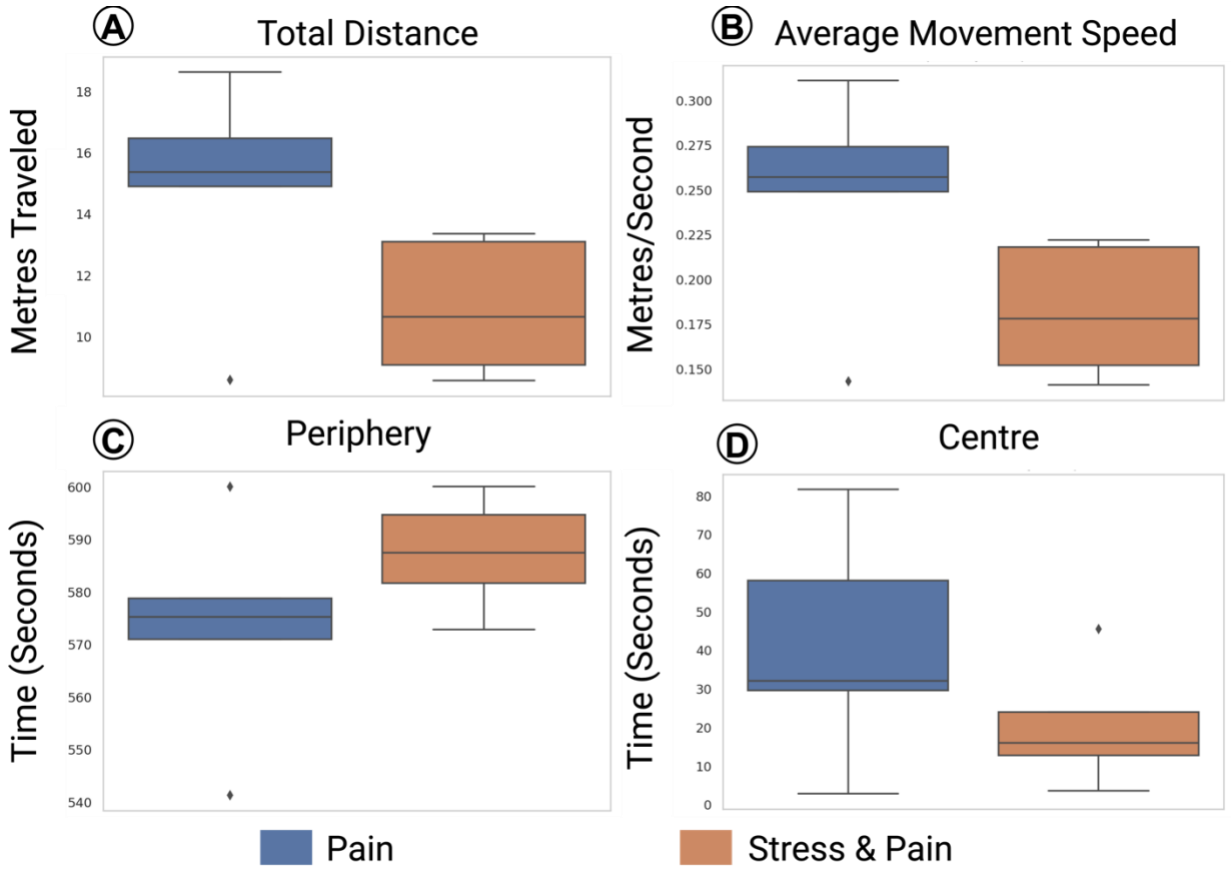

Figure 9. **Elevated anxiety-related behaviors and reduced exploration in CFA-injured animals under stress.** (*A*) Total distance traveled was slightly reduced in injured animals exposed to stress compared to the pain group, indicating trends of decreased exploratory behavior. (*B*) The average movement speed was also significantly lower in injured animals exposed to stress stress compared to pain controls. (*C*) Time spent in the periphery was higher in the stress exposed animals, suggesting an increase in anxiety-like behavior. (*D*) Conversely, the pain group spent more time in the center of the arena compared to animals exposed to stress, further supporting the anxiogenic effect of stress in combination with pain. Data are presented as mean ± *SEM*.

In the elevated plus maze, CFA rats exposed to TMT spent significantly less time in the open arms [$t(8) = 3.01$, $p < .017$] compared to those exposed to the control odor, indicating an anxiogenic response (see Figure 10 and Appendix Figure 14 for AnyMaze trackpad analysis output). This heightened anxiety was further reflected by this group engaging in significantly more episodes of freezing. Although there were no significant group differences in the number of entries into the open arms [$t(8) = 1.35$, $p = .212$], CFA rats exposed to TMT tended to make more

entries into the closed arms compared to those exposed to the control odor; however, this difference approached but did not reach statistical significance [$t(8) = 2.30$, $p = .051$]. Thus, further testing with greater sample sizes may be required in order to achieve statistical significance.

Persistent pain is also known to enhance the consolidation of fear memories, leading to heightened fear responses and an increased risk of developing anxiety disorders (Cardenas et al., 2019). This is believed to result from the strong association between pain and aversive stimuli generated during learning, which results in enhanced hypervigilance and exaggerated threat perception. However, less is known about how pain might influence the processing of signals that predict relief or safety from harm or threat. It has been shown that stimuli experienced upon the cessation of an aversive event can signal a moment of relief, leading to the formation of appetitive memories, as seen in both humans and rats (Mohammadi et al., 2019). Thus, we were interested in determining if TMT sensitization of CFA-evoked inflammatory pain might adversely impair learning of safety signals that predict relief from threat. A group of non-stressed pain naïve rats (n=5) served as a reference.

Our results indicate that freezing to the test tones was significantly lower than freezing demonstrated during the pre-tone period [RM ANOVA, $F(1,12)=18.78$, $p < .001$] (see Figure 11). Although there were no significant differences in mean tone freezing across groups during the recall test, individual analyses revealed distinct patterns between the groups. Specifically, the control group and the CFA rats exposed to the control odor exhibited significantly less freezing to the tones than during the pre-tone interval [naïve controls: paired t-tests, $t(4)=2.30$, $p < .05$; CFA only: paired t-tests, $t(4)=3.73$, $p < .020$]. In contrast, CFA rats exposed to TMT did not show a significant difference in freezing between the test tones and the pre-tone period [paired t-test,

t(4)=1.88, p=.133], suggesting that CFA-induced inflammatory pain, when in conjunction with predator odor stress, disrupts the ability to recognize safety signals associated with relief.

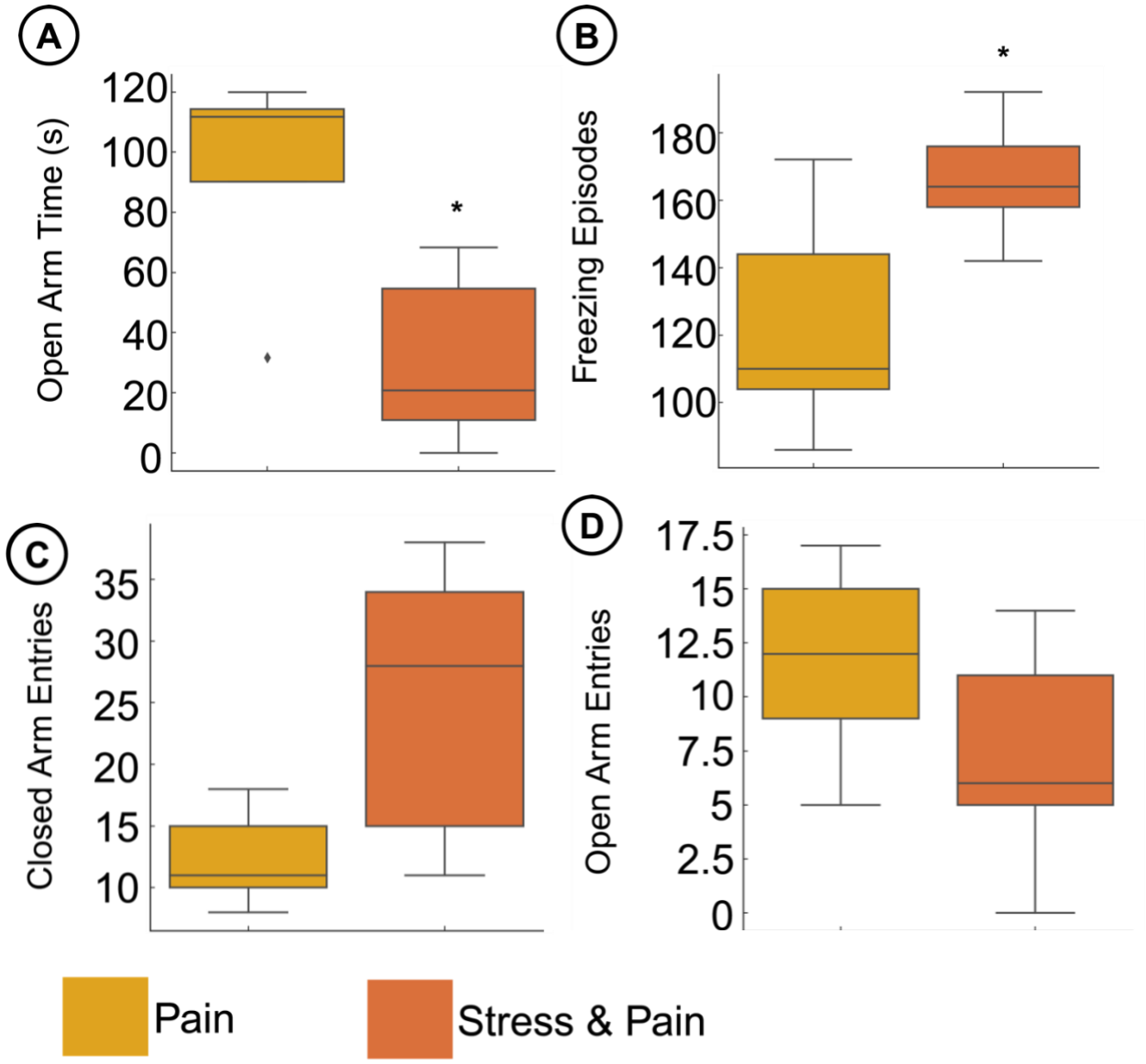

Figure 10. **CFA-injured rats exposed to predator odor spent significantly less time in the open arms, and exhibited greater freezing episodes overall.** (*A*) CFA-injured rats exposed to predator odor spent significantly less time in the open arms compared to those in the pain group. (*B*) The stress and pain group exhibited a significantly higher number of freezing episodes than animals experiencing CFA-injury without stress exposure, indicating an increased anxiety-like response. (*C*) Closed arm entries were greater in CFA-injured animals exposed to stress, suggesting enhanced avoidance behavior. (*D*) The pain group made more open arm entries relative to animals exposed to stress and injured via CFA. *Note*. "B" on x-axes indicates baseline measurement, and data are presented as mean ± *SEM*, with asterisks denoting the degree of statistical significance (*$p < .05$).

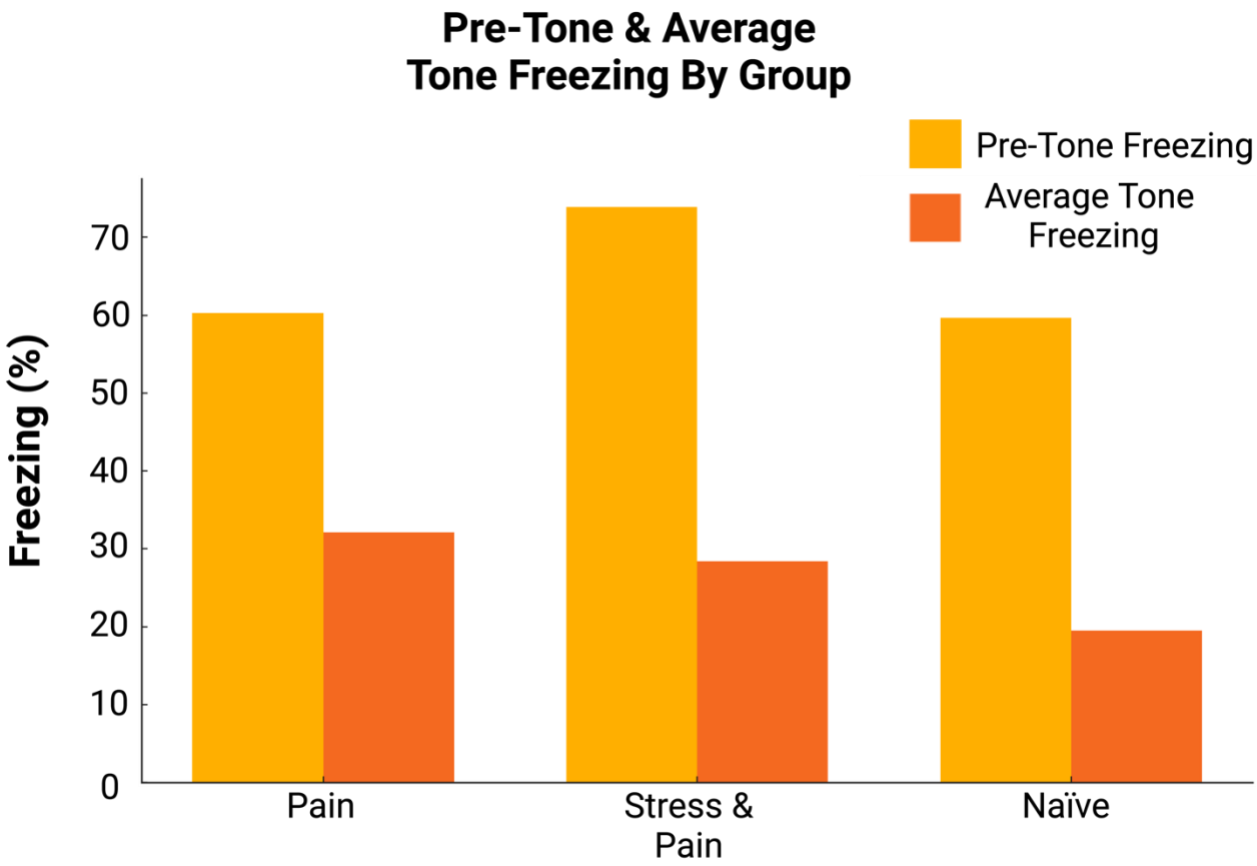

Figure 11**. All groups showed significantly less freezing during the tone than in the pre-tone period, except for CFA rats exposed to predator odor, indicating a disruption in safety signal recognition.** Pre-tone freezing was slightly higher for injured animals exposed to stress compared to the pain and naïve groups, with tone exposure freezing levels was minorily elevated in injured animals not exposed to stress. *Note*. Data are presented as mean ± *SEM*.

4. Discussion

The first experiment effectively demonstrated that the CFA injection produced significant hyperalgesia in both pain groups, as evidenced by the reduced PWTs. Notably, the combination of stress exposure with CFA injections led to prolonged reductions in PWTs compared to those that only received the CFA injection. However, the animals that only received CFA injection also exhibited sustained lower PWTs beyond the expected acute phase, complicating the interpretation of the acute pain model. Ideally, the CFA-induced hyperalgesia should resolve within 7 to 10 days to reflect a confined inflammatory response. The extended reduction in PWTs seen in this

group suggests that the inflammatory response was more robust than anticipated, potentially pushing the model beyond acute pain into a subacute phase.

To address this, subsequent experiments (two and three) focused on refining the dose of CFA to create a more reliable acute inflammatory pain model. By reducing the CFA dose concentration, we aimed to generate a localized immune response that would elicit hyperalgesia within the desired time frame without inadvertently promoting prolonged or chronic pain in the absence of stress. This approach is critical for isolating the effects of predator odor stress on the extension of pain, ensuring that any increase in pain duration can be attributed to the stressor rather than an excessive initial inflammatory reaction. The results from these experiments support the hypothesis that lowering the CFA dose induces acute inflammation that resolves within the optimal 7 to 10-day period, creating a more controlled baseline for further manipulation by stress.

Importantly, behavioral data from the EPM and fear conditioning paradigms provide additional insights into the interaction between stress, pain, and anxiety-like behaviors. The stress-exposed groups demonstrated heightened anxiety-like behaviors, consistent with the notion that predator odor stress engages fear-related neurocircuitry. This finding complements the primary hypothesis that stress may exacerbate pain by activating overlapping central pathways involved in both fear and pain processing. The current study's third experiment further refined the CFA model, reducing the concentration to 50 µl of a 25% CFA solution. This adjustment aimed to avoid excessive immune activation while still eliciting a sufficient inflammatory response to model acute pain. Behavioral assays, including the von Frey test, OFT, and EPM, were employed to assess nociceptive sensitivity and anxiety-related behaviors across the experimental timeline. The inclusion of both OFT and EPM allowed for a more comprehensive evaluation of the emotional components of pain, revealing that stress can influence not only the sensory dimensions of pain but also its affective dimensions.

Moreover, the incorporation of a relief learning paradigm allowed us to explore the cognitive aspects of pain modulation, particularly how prior experiences with stress and pain shape subsequent responses. The data suggest that exposure to predator odor stress, in conjunction with the CFA-induced pain, may enhance fear conditioning and modulate pain processing through learned associations. This finding is critical for understanding the cognitive underpinnings of chronic pain, particularly in how stress-induced memory and learning processes might perpetuate pain long after the initial injury has healed.

CHAPTER 3

Attenuation of Stress and its Impact on Pain Prolongation

1. Introduction

Chronic pain remains a significant clinical challenge, and researchers suggest that the comorbidity between chronic pain and mental health disorders may worsen pain conditions (Antioch et al., 2020). Research has largely focused on investigating the impact that brief or chronic stressors can have on chronic pain models. However, limited research examines how brief stressors could potentiate acute pain in rodent models. Experiment three in our research highlighted how stress induced by predator odour exposure may prolong hyperalgesia in a rodent model of inflammatory pain. These findings suggest that stress plays a role in potentiating pain, raising the need for interventions that can disrupt this process and prevent the chronification of pain.

In light of these findings, study four aims to explore the potential of targeting the CCK_2 receptor as a strategy to mitigate the stress-influenced transition from acute to chronic pain in rats. Selective CCK_2 receptor antagonists (e.g., LY225910) are of particular interest due to their ability to cross the BBB and inhibit CCK_2 receptor activity centrally (Loonam et al., 2003). Previous studies have shown that CCK_2 receptor antagonists can enhance the efficacy of opioid analgesics and reduce anxiety-related behaviors (Kovelowski et al., 2000; Lovick, 2008). Specifically, it is hypothesized that the administration of LY225910 will reduce the amplification of nociceptive signals and lessen anxiety and emotional stress that contribute to the persistence of pain (Kovelowski et al., 2000; Lovick, 2008).

To test this hypothesis, experiment four utilized the CFA-TMT model of inflammatory pain with stress, building on the framework established in experiments two and three. The focus was on assessing nociceptive sensitivity through the von Frey test, which allows for continuous

monitoring of LY225910's impact on subjects' nociceptive thresholds and the duration and intensity of hyperalgesia following pain and stress exposure. Overall, study four aims to investigate the potential of LY225910 as a pharmacological intervention to mitigate stress' contributions to the chronification of pain by targeting CCK_2 receptors. It is further hypothesized that CCK_2 receptor antagonists will attenuate PWT reductions after CFA administration and an additional injury via formalin 12 days after TMT exposure. Overall, study four aims to investigate the potential of LY225910 as a pharmacological intervention to mitigate stress' contributions to pain chronification by targeting CCK_2 receptors.

2. Methods

2.1. Animals

34 male SD rats (weight; *M*= 200g) were purchased from Charles River Laboratories in QB, Canada, and maintained in our colony at Trent University's Animal Care Facility. Rats underwent the same housing conditions as the previous studies. The methods employed in Chapter 3 generally follow those described in Chapter 2 (refer to Section 2) regarding animal housing and experimental procedures (i.e.,, CFA, von Frey, TMT, euthanization and tissue collection). The following sections describe the differences and modifications specific to the current study. All procedures were approved by Trent University's Animal Care Committee and complied with institutional guidelines and the Canadian CCAC.

2.2. Habituation, von Frey & CFA Administrations

Experiment four's subjects were allocated to five different groups (see Figure 13). Please also see Figure 12 for a breakdown of experiment four's experimental timeline. On the pre-test day, rats underwent a 5-minute habituation to a glass enclosure in which TMT exposures would occur two days later. The remaining details of the habituation, von Frey, and TMT-exposure remained the same as in Chapter 2. The only difference in von Frey testing was that the last

assessment occurred 12 days post-TMT, and subjects were injected with formalin to simulate a repeated injury to subjects' previously injected hindpaws (i.e.,, ipsilateral).

2.3. CCK_2 Receptor Inhibitor & Predator Odour Exposure

Per biotechne's product specifications, LY225910 is a potent CCK_2 receptor antagonist with a molecular weight of 502.41 g/mol and an IC50 (half-maximal inhibitory concentration) of 9.3 nM in inhibiting [125I]-labelled CCK-8 sulphate binding in mouse brain membranes. Upon the drug's arrival, the compound was stored at -20°C with desiccants to maintain its stability per the manufacturer's recommendations. On the day of the experiment, LY225910 was prepared by dissolving 10 mg of the compound in 1 ml of 100% dimethyl sulfoxide (DMSO), creating a 10 mg/ml stock solution. This solution was thoroughly mixed until fully dissolved and then aliquoted into 100 µl volumes, resulting in 10 vials. These vials were labelled and stored at -20°C with desiccants to prevent degradation from repeated freeze-thaw cycles.

For administration, 50 µl of the stock solution was diluted with 950 µl of 5% DMSO in .9% NaCl, yielding a .5 mg/ml working solution. This dilution was performed immediately before injection to ensure the drug's stability. A vehicle control solution, consisting of 5% DMSO in .9% saline, was prepared in parallel to maintain consistency across experimental conditions. Each animal was thus injected with LY225910 (.5 mg/kg in 5% DMSO/.9% saline) or vehicle (5% DMSO/.9% saline) 30 minutes before a 5-minute exposure to either TMT or diH_2O. Animals underwent a 5-minute re-exposure to the glass chamber 11 days after TMT exposure, adhering to the same experimental conditions as habituation.

2.4. Formalin Administrations, von Frey, & Tissue Collection

On the day of tissue collection, animals received an (i.pl.) injection of 50 µl of 1.5% formalin in .9% saline into the CFA-treated hindpaw. Injections were staggered to ensure the timely measurement of PWTs using the Von Frey test. Specifically, two subjects were injected

and tested 70 minutes later. During this interval, additional subjects were injected at 35-minute intervals, facilitating a workflow where one experimenter administered injections, another conducted the Von Frey tests, and one or two additional experimenters performed transcardial perfusions 90 minutes after formalin administration. Immediately following Von Frey testing, subjects were deeply anesthetized via an injection (i.p.) of .3 mL sodium pentobarbital (Euthansol, Merck Animal Health Canada) and then transcardially perfused. Details on the remaining tissue collection procedures can be referenced in Chapter 2.

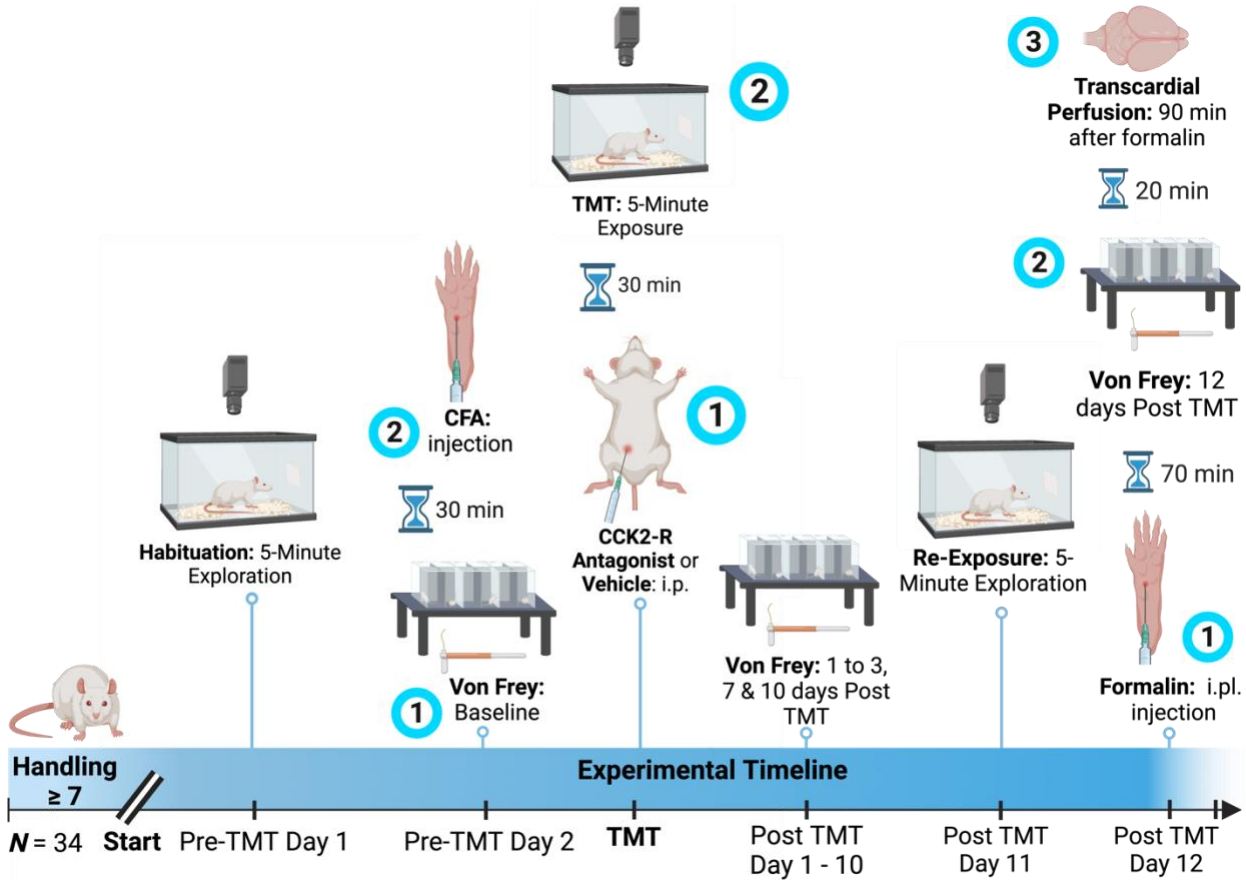

Figure 12. **Experiment Four – Timeline.**

3. Results

3.1. CCK_2 receptor antagonist attenuates acute inflammatory pain in odor control and predator odor exposed subjects

To examine the effects of TMT stress on CFA-induced mechanical hypersensitivity, PWTs were measured using the von Frey test over a 12-day period (see Figure 13). A two-way repeated measures ANOVA, with time as the within-subject factor and group as the between-subject factor, was performed to evaluate withdrawal thresholds in the ipsilateral and contralateral paws, respectively.

The analysis revealed significant main effects of group [$F(4, 203) = 93.08$, $p < .001$] and period [$F(6, 203) = 25.72$, $p < .001$], indicating that both TMT exposure and CFA-induced inflammation significantly influenced PWTs over time. Additionally, there was a significant group-by-period interaction [$F(24, 203) = 6.70$, $p < .001$], suggesting that the groups responded differently across the time points. Subjects exposed to TMT following CFA injection exhibited significantly reduced PWTs compared to controls 24 hours ($p < .05$) after exposure and continuing through day 12 ($p < .001$). In contrast, animals in the CFA + vehicle group partially recovered by day 12, with PWTs returning closer to baseline levels.

Similarly, significant group effects [$F(4, 203) = 76.04$, $p < .001$] and period effects [$F(6, 203) = 11.01$, $p < .001$] were observed in the contralateral paw, along with a significant group by period interaction [$F(24, 203) = 5.34$, $p < .001$]. Rats exposed to TMT showed prolonged reductions in contralateral PWTs compared to controls, with significant differences detected starting on day 3 ($p < .05$) and persisting through day 12 ($p < .001$). However, animals that received both CFA and vehicle injections showed more rapid recovery of contralateral PWTs, with thresholds nearing baseline by day 12. These findings demonstrate that exposure to predator stress exacerbates mechanical allodynia in both the ipsilateral and contralateral paws, significantly prolonging pain sensitivity compared to vehicle-treated animals.

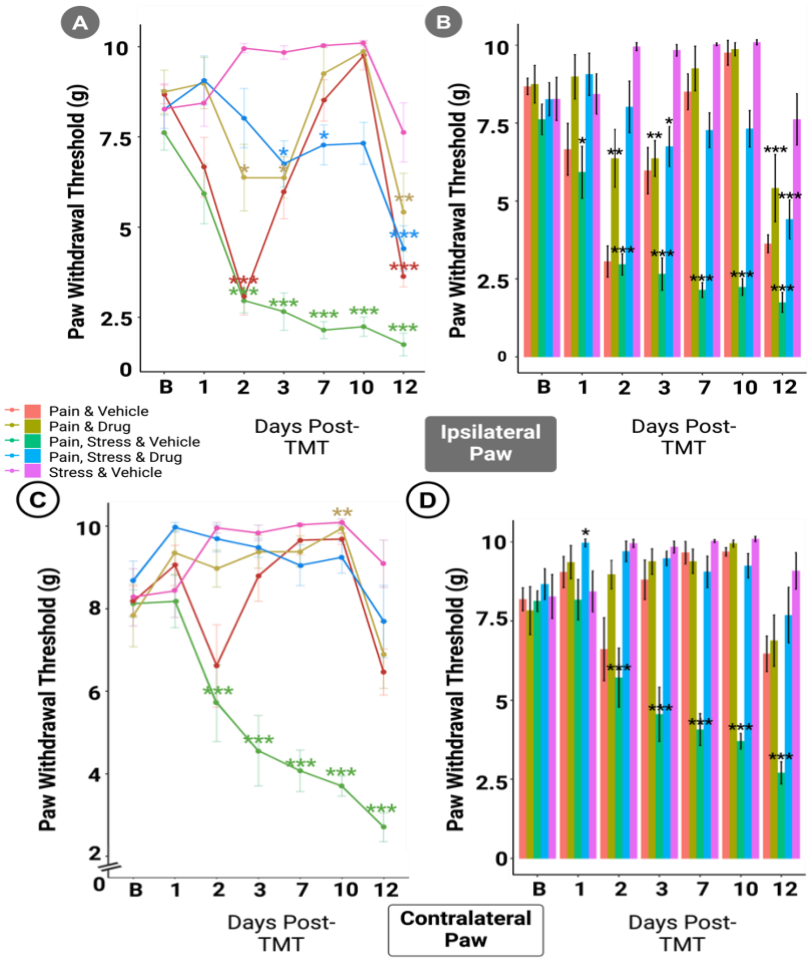

Figure 13. **CCK$_2$ receptor antagonist protects against PWT reductions in CFA-injured subjects exposed to control odor or predator odor.** (*A*) Compared to baseline, the mean ipsilateral PWTs for the pain and stress group show a significant reduction starting at 3 days post-TMT exposure and persisting through day 12. Drug administration in the pain, stress, and drug group attenuated these reductions, showing significant recovery at days 7 and 10. (*B*) Between-group differences are evident at all post-exposure time points, indicating that the pain and stress condition had significantly lower PWTs compared to all other groups. (*C*) A within-group comparison of contralateral paw PWTs shows significant reductions in the pain and stress group from day 3 through day 12. (*D*) Between-group comparisons for the contralateral paw also reflect significant reductions in PWTs in the pain and stress group at days 3, 7, and 10 compared to baseline. *Note*. "B" on the x-axes represents baseline measurement. Data are presented as mean ± *SEM*, with asterisks denoting the degree of statistical significance (*$p < .05$, **$p < .01$, ***$p < .001$).

4. Discussion

In the final von Frey test, all groups demonstrated a decline in PWTs following an injection of 50 µl of 1.5% formalin, administered 60 minutes before testing. As depicted in Figure 13, the

highest mean PWTs were observed in animals exposed to predator odor and treated with vehicle, while the lowest were in CFA-injured animals also exposed to predator odor and receiving vehicle injections. CFA-injured animals that received vehicle exhibited the most substantial reduction in PWTs. Notably, animals treated with the CCK_2 receptor antagonist showed a reduction in PWTs during the final von Frey test, but their means were higher than those of CFA-injured, vehicle-treated animals, positioning them between the CFA-naïve (stress + vehicle) group and CFA-injured animals. These findings suggest that the CCK_2 antagonist may offer protection from prolonged pain symptoms and repeated injury. Interestingly, CFA-injured animals exposed to predator odor and treated with vehicle also exhibited a slight decrease in PWTs compared to prior time points, though this reduction was less pronounced than in other groups, potentially indicating a floor effect that potentially warrants further investigation.

CHAPTER 4

DISCUSSION

We demonstrate that (1) suboptimal administration (25%, 50 µl) of CFA alone does not prolong mechanical hypersensitivity beyond the typical acute pain window, (2) combining TMT (10%) exposure and CFA injury potentiates mechanical hypersensitivity, (3) TMT exposure and CFA injury increases anxiety and fear-related behaviors in the EPM 15 days after TMT exposure, and (4) CCK_2 receptor antagonist ameliorates stress-potentiated pain. These results suggest that naturalistic predator odour enhances pain sensitivity in CFA-induced acute inflammatory pain and that inhibiting molecular stress modulators like CCK could mitigate the transition from acute to chronic pain.

4. TMT-induced stress potentiates the chronification of acute pain

The present findings indicate that a suboptimal injection (i.pl.) of CFA and brief exposure to predator odour TMT sustains significant reductions in PWTs relative to control animals. CFA injury fosters a slow release of antigens that elicit continuous immune system activation, resulting in inflammation, swelling and edema. Acute inflammatory pain models consist of administering a low dose of CFA to a rodent's hind paw, with typical recovery observed from 7-10 days post-injection. Thus, we sought to establish an acute inflammatory pain model that could reflect this timeline. The first attempt to do so (see Study Two), consisting of 50 µl of 50% CFA, was successful, with 10% TMT seemingly prolonging pain experienced in the Pain X Stress condition. However, the Pain X No Stress condition exhibited reduced PWTs past the typical recovery period, prompting the 25% decrease in CFA concentration. Results from the second attempt at half concentration but equal volume were consistent with the usual recovery period (see Study Three), transcending the results of study two but strongly emulating their meaningfulness.

Stress likely interferes with typical recovery due to its potential to perturb neuroendocrine function and enhance inflammation, contributing to the sensitization of the CNS. Central sensitization is a pathological process characterized by reduced thresholds required in nociceptive neuronal firing within the CNS, resulting in amplified pain signals. In our study, stress-induced sensitization seems to enhance responses to a suboptimal dose of CFA, preventing the normal resolution of pain and driving it into a chronic state. This is consistent with previous research showing that stress can lower pain thresholds and prolong the duration of pain by promoting neuroplastic changes in pain pathways, particularly in pain-associated pathways (e.g., PAG-RVM and PAG-LC) (see Models of ecologically relevant predator odor stress).

4.1. CCK_2 antagonist attenuates stress-induced persistent pain

After successfully establishing and replicating the acute inflammatory pain model (see Chapter 2), we aimed to ameliorate the effects of stress on acute pain by administering subjects with the CCK_2 receptor inhibitor, LY225910. Thirty minutes before subjects underwent TMT exposure, they were administered (i.p.) either LY225910 or a vehicle. CCK modulates nociceptive transmission by inhibiting GABAergic signaling in the dorsal root ganglia, thereby reducing inhibitory control and promoting the propagation of pain signals from the PAG to the RVM (Jennings, 2014; Lovick, 2008; Pagliusi & Gomes, 2023; Rivat et al., 2010).

After 10 days of Von Frey filament tests, subjects that received antagonists exhibited similar recovery time and PWTs compared to control groups. These results strongly support our hypothesis that stress exacerbates acute inflammatory pain, facilitating its transition to chronic pain and that mitigating the effects of stress could prevent this progression. Our findings align with existing literature, highlighting the potential of CCK_2 receptor antagonists to alleviate inflammatory pain. However, our findings also extend the existing literature by demonstrating

that LY22591 may protect against chronic pain symptoms over time and when an additional subsequent injury is experienced.

LIMITATIONS

4.3. Repeated von Frey assessments are reliable at assessing mechanical sensitivity over time

The potential for repeated filament application to induce central sensitization is a significant consideration when using the Von Frey test to assess mechanical sensitivity. The current study sought to combat this concern by implementing Chaplan et al.'s (1994) von Frey method due to its intentional design meant to minimize the number of applications needed to calculate an animal's 50% PWT robustly. These concerns may appear less critical in light of study two's findings, where non-injured animals that were exposed to predator odor did not show significant differences between baseline and subsequent measurements. It is still possible that animals experiencing pain and stress may either consciously or unconsciously develop strategies to reduce the discomfort associated with filament application, which could influence their responses and decrease the accuracy of our interpretations. Suppose animals perceive the filament alone as unpleasant and not necessarily the force it applies. In that case, they may learn to withdraw their paw in anticipation of the researcher removing the filament (Barrot et al., 2012). This learned response could result in less accurate measurements of the withdrawal threshold. Contrary to this concern, the results from experiment two show consistent findings with those observed in experiments one and three at the same time points. While further research is warranted to confirm these observations, the data from squad two mitigates concerns about filament-induced sensitization or learned paw withdrawal responses and reinforces the reliability of the von Frey test, supporting the hypothesis that repeated testing does not significantly alter PWTs.

4.4. Lack of a naïve control group in EPM and OFT may limit interpretation of anxiety-like behaviors

A notable limitation of the present study is the absence of a true naïve control group in both the EPM and OFT, which may have provided additional insight into the baseline levels of anxiety-like behaviors and overall locomotion. Without such a control, the interpretation of the results remains incomplete, as we cannot fully discern whether the observed behaviors in CFA-injected or TMT-exposed animals are due to the experimental manipulations or other underlying factors. A naïve control group would have served as a baseline comparison for uninjured, unstressed animals, helping to differentiate between the inherent anxiety and exploratory behaviors in normal rats versus those influenced by injury and stress. Future studies should incorporate such a control to strengthen the interpretation of results and provide a more comprehensive understanding of how experimental manipulations specifically alter behavior in anxiety and pain-related paradigms. By including a naïve control group, the ability to isolate the effects of CFA and predator odor on both emotional and pain-related responses would be significantly enhanced, leading to clearer conclusions about the interaction between pain, stress, and behavior.

4.5. Need for molecular and immunohistochemical analyses to further elucidate the mechanisms involved in stress-potentiated pain

The current study also lacks immunohistochemical and molecular analyses, which would have provided further insight into the cellular and molecular mechanisms underlying the observed behavioral effects. The inclusion of such analyses, particularly the investigation of protein markers like c-fos, could have identified specific brain regions activated by the combination of CFA and predator odor-induced stress. This may have allowed us to better understand how stress potentiates the transition from acute to chronic pain. Future studies should

examine tissues collected from key pain-related regions such as the PAG, RVM, LC, ACC, hippocampus, and PFC to quantify stress-induced changes in nociceptive processing pathways. Additionally, analyzing inflammatory markers or glial activation could reveal how stress modulates neuroinflammation and contributes to central sensitization. Molecular analysis could also help determine the role of CCK_2 signaling in stress-potentiated pain and further validate the mechanism by which antagonizing this receptor reduces pain progression.

CONCLUSION

In conclusion, the present study demonstrates that stress, in the form of predator odor exposure, potentiates the transition from acute to chronic pain in a CFA-induced inflammatory pain model. The observed prolongation of pain behaviors in stress-exposed animals suggests that acute stress can hinder pain resolution, possibly through mechanisms involving central sensitization. These findings are relevant for understanding how acute stress in humans, such as psychological trauma or anxiety, could exacerbate or extend pain states following injury. The potential engagement of anxiety-related circuits in this process, particularly within the amygdala and PAG, suggests these regions may be targets of interest for mitigating stress-induced pain potentiation. Further research should focus on characterizing the specific neural and molecular pathways involved in this interaction and exploring pharmacological agents, such as CCK2 receptor antagonists, that may prevent stress from amplifying pain sensitivity. Understanding these pathways could inform strategies aimed at reducing the risk of chronic pain development following stressful events or injury.

References

Abbadie, C., Bhangoo, S., De Koninck, Y., Malcangio, M., Melik-Parsadaniantz, S., & White, F. A. (2009). Chemokines and pain mechanisms. *Brain Research Reviews*, *60*(1), 125–134. https://doi.org/10.1016/j.brainresrev.2008.12.002

Adamec, R. E., Shallow, T., & Budgell, J. (1997). Blockade of CCKB but not CCKA receptors before and after the stress of predator exposure prevents lasting increases in anxiety-like behavior: Implications for anxiety associated with posttraumatic stress disorder. *Behavioral Neuroscience*, *111*(2), 435–449. https://doi.org/10.1037/0735-7044.111.2.435

Adamec, R., Walling, S., & Burton, P. (2004). Long-lasting, selective, anxiogenic effects of feline predator stress in mice. *Physiology & Behavior*, *83*(3), 401–410. https://doi.org/10.1016/j.physbeh.2004.08.029

Antioch, I., Ovidiu-Dumitru, I., Ciobica, A., Doroftei, B., & Fornaro, M. (2020). Preclinical Considerations about Affective Disorders and Pain: A Broadly Intertwined, yet Often Under-Explored, Relationship Having Major Clinical Implications. *Medicina*, *56*(10). https://doi.org/10.3390/medicina56100504

Apfelbach, R., Blanchard, C. D., Blanchard, R. J., Hayes, R. A., & McGregor, I. S. (2005). The effects of predator odors in mammalian prey species: A review of field and laboratory studies. *Neuroscience & Biobehavioral Reviews*, *29*(8), 1123–1144. https://doi.org/10.1016/j.neubiorev.2005.05.005

Apfelbach, R., Parsons, M. H., Soini, H. A., & Novotny, M. V. (2015). Are single odorous components of a predator sufficient to elicit defensive behaviors in prey species? *Frontiers in Neuroscience*, *9*. https://doi.org/10.3389/fnins.2015.00263

Apkarian, A. V., Baliki, M. N., & Geha, P. Y. (2009). Towards a theory of chronic pain. *Progress in Neurobiology*, *87*(2), 81–97. https://doi.org/10.1016/j.pneurobio.2008.09.018

Apkarian, A. V., Mutso, A. A., Centeno, M. V., Kan, L., Wu, M., Levinstein, M., Banisadr, G., Gobeske, K. T., Miller, R. J., Radulovic, J., Hen, R., & Kessler, J. A. (2016). Role of adult hippocampal neurogenesis in persistent pain. *Pain*, *157*(2), 418–428. https://doi.org/10.1097/j.pain.0000000000000332

Auvray, M., Myin, E., & Spence, C. (2010). The sensory-discriminative and affective-motivational aspects of pain. *Neuroscience & Biobehavioral Reviews*, *34*(2), 214–223. https://doi.org/10.1016/j.neubiorev.2008.07.008

Ayers, L. W., Asok, A., Heyward, F. D., & Rosen, J. B. (2013). Freezing to the predator odor 2,4,5 dihydro 2,5 trimethylthiazoline (TMT) is disrupted by olfactory bulb removal but not trigeminal deafferentation. *Behavioural Brain Research*, *253*, 54–59. https://doi.org/10.1016/j.bbr.2013.06.034

Barrot, M. (2012). Tests and models of nociception and pain in rodents. *Neuroscience*, *211*, 39–50. https://doi.org/10.1016/j.neuroscience.2011.12.041

Basbaum, A. I., Bautista, D. M., Scherrer, G., & Julius, D. (2009). Cellular and Molecular Mechanisms of Pain. *Cell*, *139*(2), 267–284. https://doi.org/10.1016/j.cell.2009.09.028

Baumbach, J. L., Mui, C. Y. Y., Tuz Zahra, F., & Martin, L. J. (2024). A single exposure to the predator odor 2,4,5-trimethylthiazoline causes long-lasting affective behavioral changes in female mice: Modulation by kappa opioid receptor signaling. *Pharmacology Biochemistry and Behavior*, *242*, 173822. https://doi.org/10.1016/j.pbb.2024.173822

Bazzari, A. H., & Bazzari, F. H. (2022). Advances in targeting central sensitization and brain plasticity in chronic pain. *The Egyptian Journal of Neurology, Psychiatry and Neurosurgery*, *58*(1), 38. https://doi.org/10.1186/s41983-022-00472-y

Bernard, A., Danigo, A., Bourthoumieu, S., Mroué, M., Desmoulière, A., Sturtz, F., Rovini, A., & Demiot, C. (2021). The Cholecystokinin Type 2 Receptor, a Pharmacological Target for Pain Management. *Pharmaceuticals*, *14*(11), 1185. https://doi.org/10.3390/ph14111185

Biagioni, A. F., De Freitas, R. L., Da Silva, J. A., De Oliveira, R. C., De Oliveira, R., Alves, V. M., & Coimbra, N. C. (2013). Serotonergic neural links from the dorsal raphe nucleus modulate defensive behaviours organised by the dorsomedial hypothalamus and the elaboration of fear-induced antinociception via locus coeruleus pathways. *Neuropharmacology*, *67*, 379–394. https://doi.org/10.1016/j.neuropharm.2012.10.024

Bleakman, D., Alt, A., & Nisenbaum, E. S. (2006). Glutamate receptors and pain. *Seminars in Cell & Developmental Biology*, *17*(5), 592–604. https://doi.org/10.1016/j.semcdb.2006.10.008

Brechbühl, J., Moine, F., Klaey, M., Nenniger-Tosato, M., Hurni, N., Sporkert, F., Giroud, C., & Broillet, M.-C. (2013). Mouse alarm pheromone shares structural similarity with predator scents. *Proceedings of the National Academy of Sciences*, *110*(12), 4762–4767. https://doi.org/10.1073/pnas.1214249110

Breese, N. M., George, A. C., Pauers, L. E., & Stucky, C. L. (2005). Peripheral inflammation selectively increases TRPV1 function in IB4-positive sensory neurons from adult mouse. *Pain*, *115*(1), 37–49. https://doi.org/10.1016/j.pain.2005.02.010

Buron, G., Hacquemand, R., Pourie, G., Lucarz, A., Jacquot, L., & Brand, G. (2007). Comparative behavioral effects between synthetic 2,4,5-trimethylthiazoline (TMT) and the odor of natural fox (Vulpes vulpes) feces in mice. *Behavioral Neuroscience*, *121*(5), 1063–1072. https://doi.org/10.1037/0735-7044.121.5.1063

Butler, R. K., & Finn, D. P. (2009). Stress-induced analgesia. *Progress in Neurobiology*, *88*(3), 184–202. https://doi.org/10.1016/j.pneurobio.2009.04.003

Cao, B., Xu, Q., Shi, Y., Zhao, R., Li, H., Zheng, J., Liu, F., Wan, Y., & Wei, B. (2024). Pathology of pain and its implications for therapeutic interventions. *Signal Transduction and Targeted Therapy*, *9*(1), 155. https://doi.org/10.1038/s41392-024-01845-w

Cardenas, A., Blanca, M., & Dimitrov, E. (2019). Persistent pain intensifies recall of consolidated fear memories. *Neurobiology of Stress*, *10*, 100163. https://doi.org/10.1016/j.ynstr.2019.100163

Chaplan, S. R., Bach, F. W., Pogrel, J. W., Chung, J. M., & Yaksh, T. L. (1994). Quantitative assessment of tactile allodynia in the rat paw. *Journal of Neuroscience Methods*, *53*(1), 55–63. https://doi.org/10.1016/0165-0270(94)90144-9

Chapman, C. R., Tuckett, R. P., & Song, C. W. (2008). Pain and Stress in a Systems Perspective: Reciprocal Neural, Endocrine, and Immune Interactions. *The Journal of Pain*, *9*(2), 122–145. https://doi.org/10.1016/j.jpain.2007.09.006

Chen, Q., Zhao, M., Dong, J., & Yang, K. (2024). Chronic restraint stress-induced hyperalgesia is modulated by the periaqueductal gray neurons projecting to the rostral ventromedial medulla in mice. *Biochemical and Biophysical Research Communications*, *710*, 149875. https://doi.org/10.1016/j.bbrc.2024.149875

Clark, A. K., Gentry, C., Bradbury, E. J., McMahon, S. B., & Malcangio, M. (2007). Role of spinal microglia in rat models of peripheral nerve injury and inflammation. *European Journal of Pain*, *11*(2), 223–230. https://doi.org/10.1016/j.ejpain.2006.02.003

Coderre, T. J. (2008). *5.26 Spinal Cord Mechanisms of Hyperalgesia and Allodynia*.

Corder, G., Doolen, S., Donahue, R. R., Winter, M. K., Jutras, B. L., He, Y., Hu, X., Wieskopf, J. S., Mogil, J. S., Storm, D. R., Wang, Z. J., McCarson, K. E., & Taylor, B. K. (2013). Constitutive μ-Opioid Receptor Activity Leads to Long-Term Endogenous Analgesia and Dependence. *Science*, *341*(6152), 1394–1399. https://doi.org/10.1126/science.1239403

Crook, R. J., Dickson, K., Hanlon, R. T., & Walters, E. T. (2014). Nociceptive Sensitization Reduces Predation Risk. *Current Biology*, *24*(10), 1121–1125. https://doi.org/10.1016/j.cub.2014.03.043

Crown, E. D., Gwak, Y. S., Ye, Z., Yu Tan, H., Johnson, K. M., Xu, G.-Y., McAdoo, D. J., & Hulsebosch, C. E. (2012). Calcium/calmodulin dependent kinase II contributes to persistent central neuropathic pain following spinal cord injury. *Pain*, *153*(3), 710–721. https://doi.org/10.1016/j.pain.2011.12.013

Day, H. E. W., Masini, C. V., & Campeau, S. (2004). The pattern of brain c-fos mRNA induced by a component of fox odor, 2,5-dihydro-2,4,5-Trimethylthiazoline (TMT), in rats, suggests both systemic and processive stress characteristics. *Brain Research*, *1025*(1–2), 139–151. https://doi.org/10.1016/j.brainres.2004.07.079

De Felice, M., Sanoja, R., Wang, R., Vera-Portocarrero, L., Oyarzo, J., King, T., Ossipov, M. H., Vanderah, T. W., Lai, J., Dussor, G. O., Fields, H. L., Price, T. J., & Porreca, F. (2011). Engagement of descending inhibition from the rostral ventromedial medulla protects against chronic neuropathic pain. *Pain*, *152*(12), 2701–2709. https://doi.org/10.1016/j.pain.2011.06.008

Deyama, S., Katayama, T., Ohno, A., Nakagawa, T., Kaneko, S., Yamaguchi, T., Yoshioka, M., & Minami, M. (2008). Activation of the β-Adrenoceptor–Protein Kinase A Signaling Pathway within the Ventral Bed Nucleus of the Stria Terminalis Mediates the Negative Affective Component of Pain in Rats. *The Journal of Neuroscience*, *28*(31), 7728–7736. https://doi.org/10.1523/JNEUROSCI.1480-08.2008

Dib-Hajj, S. D., Cummins, T. R., Black, J. A., & Waxman, S. G. (2007). From genes to pain: Nav1.7 and human pain disorders. *Trends in Neurosciences*, *30*(11), 555–563. https://doi.org/10.1016/j.tins.2007.08.004

DosSantos, M. F., Moura, B. D. S., & DaSilva, A. F. (2017). Reward Circuitry Plasticity in Pain Perception and Modulation. *Frontiers in Pharmacology*, *8*, 790. https://doi.org/10.3389/fphar.2017.00790

Dubin, A. E., & Patapoutian, A. (2010). Nociceptors: The sensors of the pain pathway. *Journal of Clinical Investigation*, *120*(11), 3760–3772. https://doi.org/10.1172/JCI42843

Endres, T., & Fendt, M. (2007). Conditioned behavioral responses to a context paired with the predator odor trimethylthiazoline. *Behavioral Neuroscience*, *121*(3), 594–601. https://doi.org/10.1037/0735-7044.121.3.594

Endres, T., & Fendt, M. (2009). Aversion- *vs* Fear-Inducing Properties of 2,4,5-Trimethyl-3-Thiazoline, a Component of Fox Odor, in Comparison with Those of Butyric Acid. *Journal of Experimental Biology*, *212*(15), 2324–27. https://doi.org/10.1242/jeb.028498

Falconer, E. M., & Galea, L. A. M. (2003). Sex differences in cell proliferation, cell death and defensive behavior following acute predator odor stress in adult rats. *Brain Research*, *975*(1–2), 22–36. https://doi.org/10.1016/S0006-8993(03)02542-3

Fan, J., Jin, S., Gilmartin, L., Toth, I., Hussein, W., & Stephenson, R. (2022). Advances in Infectious Disease Vaccine Adjuvants. *Vaccines*, *10*(7), 1120. https://doi.org/10.3390/vaccines10071120

Fontes, J. A., Barin, J. G., Talor, M. V., Stickel, N., Schaub, J., Rose, N. R., & Čiháková, D. (2017). Complete Freund's adjuvant induces experimental autoimmune myocarditis by enhancing IL-6 production during initiation of the immune response: IL-6 in the initiation of EAM. *Immunity, Inflammation and Disease*, *5*(2), 163–176. https://doi.org/10.1002/iid3.155

Fortes-Marco, Lluis, Enrique Lanuza, and Fernando Martinez-Garcia (2013). Of Pheromones and Kairomones: What Receptors Mediate Innate Emotional Responses?. *The Anatomical Record*, 9. https://doi.org/10.1002/ar.22745.

Fu, K.-Y., Light, A. R., Matsushima, G. K., & Maixner, W. (1999). Microglial reactions after subcutaneous formalin injection into the rat hind paw. *Brain Research*, *825*(1–2), 59–67. https://doi.org/10.1016/S0006-8993(99)01186-5

Gallopin, T., Geoffroy, H., Rossier, J., & Lambolez, B. (2006). Cortical Sources of CRF, NKB, and CCK and Their Effects on Pyramidal Cells in the Neocortex. *Cerebral Cortex*, *16*(10), 1440–1452. https://doi.org/10.1093/cercor/bhj081

Geva, N., & Defrin, R. (2018). Opposite Effects of Stress on Pain Modulation Depend on the Magnitude of Individual Stress Response. *The Journal of Pain*, *19*(4), 360–371. https://doi.org/10.1016/j.jpain.2017.11.011

Hacquemand, R., Choffat, N., Jacquot, L., & Brand, G. (2013). Comparison between low doses of TMT and cat odor exposure in anxiety- and fear-related behaviors in mice. *Behavioural Brain Research*, *238*, 227–231. https://doi.org/10.1016/j.bbr.2012.10.014

Hacquemand, R., Jacquot, L., & Brand, G. (2010). Comparative Fear-Related Behaviors to Predator Odors (TMT and Natural Fox Feces) before and after Intranasal ZnSO4 Treatment in Mice. *Frontiers in Behavioral Neuroscience*, *4*. https://doi.org/10.3389/fnbeh.2010.00188

Han, S., Soleiman, M. T., Soden, M. E., Zweifel, L. S., & Palmiter, R. D. (2015). Elucidating an Affective Pain Circuit that Creates a Threat Memory. *Cell*, *162*(2), 363–374. https://doi.org/10.1016/j.cell.2015.05.057

Hotsenpiller, G., & Williams, J. L. (1997). A synthetic predator odor (TMT) enhances conditioned analgesia and fear when paired with a benzodiazepine receptor inverse agonist (FG-7142). *Psychobiology*, *25*(1), 83–88. https://doi.org/10.3758/BF03327031

Hsieh, M.-T., Donaldson, L. F., & Lumb, B. M. (2015). Differential contributions of A- and C-nociceptors to primary and secondary inflammatory hypersensitivity in the rat. *Pain*, *156*(6), 1074–1083. https://doi.org/10.1097/j.pain.0000000000000151

Jennings, E. M., Okine, B. N., Roche, M., & Finn, D. P. (2014). Stress-induced hyperalgesia. *Progress in Neurobiology*, *121*, 1–18. https://doi.org/10.1016/j.pneurobio.2014.06.003

Khalid, S., & Tubbs, R. S. (2017). Neuroanatomy and Neuropsychology of Pain. *Cureus*. https://doi.org/10.7759/cureus.1754

Kim, J.-H., Gangadharan, G., Byun, J., Choi, E.-J., Lee, C. J., & Shin, H.-S. (2018). Yin-and-yang bifurcation of opioidergic circuits for descending analgesia at the midbrain of the mouse. *Proceedings of the National Academy of Sciences*, *115*(43), 11078–11083. https://doi.org/10.1073/pnas.1806082115

Kleshchova, O., Rieder, J. K., Grinband, J., & Weierich, M. R. (2019). Resting amygdala connectivity and basal sympathetic tone as markers of chronic hypervigilance. *Psychoneuroendocrinology*, *102*, 68–78. https://doi.org/10.1016/j.psyneuen.2018.11.036

Kobayakawa, K., Kobayakawa, R., Matsumoto, H., Oka, Y., Imai, T., Ikawa, M., Okabe, M., Ikeda, T., Itohara, S., Kikusui, T., Mori, K., & Sakano, H. (2007). Innate versus learned odour processing in the mouse olfactory bulb. *Nature*, *450*(7169), 503–508. https://doi.org/10.1038/nature06281

Kovelowski, C. J., Ossipov, M. H., Sun, H., Lai, J., Malan, T. P., & Porreca, F. (2000). Supraspinal cholecystokinin may drive tonic descending facilitation mechanisms to maintain

neuropathic pain in the rat. *Pain*, *87*(3), 265–273. https://doi.org/10.1016/S0304-3959(00)00290-6

Latremoliere, A., & Woolf, C. J. (2009). Central Sensitization: A Generator of Pain Hypersensitivity by Central Neural Plasticity. *The Journal of Pain*, *10*(9), 895–926. https://doi.org/10.1016/j.jpain.2009.06.012

LeDoux, J. E. (2009). Emotion circuits in the brain. *Focus*, *7*(2), 274–274. https://doi.org/10.1176/foc.7.2.foc274

Li, C., Chen, X., & Williams, J. A. (2004). Regulation of CCK-induced amylase release by PKC-δ in rat pancreatic acinar cells. *American Journal of Physiology-Gastrointestinal and Liver Physiology*, *287*(4), G764–G771. https://doi.org/10.1152/ajpgi.00111.2004

Liu, J. A., Yu, J., & Cheung, C. W. (2021). Immune Actions on the Peripheral Nervous System in Pain. *Int. J. Mol. Sci.*

Liu, Y.-J., Li, Y.-L., Fang, Z.-H., Liao, H.-L., Zhang, Y.-Y., Lin, J., Liu, F., & Shen, J.-F. (2022). NMDARs mediate peripheral and central sensitization contributing to chronic orofacial pain. *Frontiers in Cellular Neuroscience*, *16*, 999509. https://doi.org/10.3389/fncel.2022.999509

Loeser, J. D., & Treede, R.-D. (2008). The Kyoto protocol of IASP Basic Pain Terminology ☆. *Pain*, *137*(3), 473–477. https://doi.org/10.1016/j.pain.2008.04.025

Loonam, T. M., Noailles, P. A. H., Yu, J., Zhu, J. P. Q., & Angulo, J. A. (2003). Substance P and cholecystokinin regulate neurochemical responses to cocaine and methamphetamine in the striatum. *Life Sciences*, *73*(6), 727–739. https://doi.org/10.1016/S0024-3205(03)00393-X

Lovick, T. A. (2008). Pro-nociceptive action of cholecystokinin in the periaqueductal grey: A role in neuropathic and anxiety-induced hyperalgesic states. *Neuroscience & Biobehavioral Reviews*, *32*(4), 852–862. https://doi.org/10.1016/j.neubiorev.2008.01.003

Lubejko, S. T., Livrizzi, G., Buczynski, S. A., Patel, J., Yung, J. C., Yaksh, T. L., & Banghart, M. R. (2024). Inputs to the locus coeruleus from the periaqueductal gray and rostroventral medulla shape opioid--mediated descending pain modulation. *S c i e n c e A d v a n c e s*.

Maire, J. J., Close, L. N., Heinricher, M. M., & Selden, N. R. (2016). Distinct pathways for norepinephrine- and opioid-triggered antinociception from the amygdala. *European Journal of Pain*, *20*(2), 206–214. https://doi.org/10.1002/ejp.708

Manoharan, R. R., Prasad, A., Pospíšil, P., & Kzhyshkowska, J. (2024). ROS signaling in innate immunity via oxidative protein modifications. *Frontiers in Immunology*, *15*, 1359600. https://doi.org/10.3389/fimmu.2024.1359600

Minatohara, K., Akiyoshi, M., & Okuno, H. (2016). Role of Immediate-Early Genes in Synaptic Plasticity and Neuronal Ensembles Underlying the Memory Trace. *Frontiers in Molecular Neuroscience*, *8*. https://doi.org/10.3389/fnmol.2015.00078

Moayedi, M., & Davis, K. D. (2013). Theories of pain: From specificity to gate control. *Journal of Neurophysiology*, *109*(1), 5–12. https://doi.org/10.1152/jn.00457.2012

Mohammadi, M., Bergado-Acosta, J. R., & Fendt, M. (2014). Relief learning is distinguished from safety learning by the requirement of the nucleus accumbens. *Behavioural Brain Research*, *272*, 40–45. https://doi.org/10.1016/j.bbr.2014.06.053

Müller, M., & Fendt, M. (2006). Temporary inactivation of the medial and basolateral amygdala differentially affects TMT-induced fear behavior in rats. *Behavioural Brain Research*, *167*(1), 57–62. https://doi.org/10.1016/j.bbr.2005.08.016

Mutso, A. A., Radzicki, D., Baliki, M. N., Huang, L., Banisadr, G., Centeno, M. V., Radulovic, J., Martina, M., Miller, R. J., & Apkarian, A. V. (2012). Abnormalities in

Hippocampal Functioning with Persistent Pain. *The Journal of Neuroscience*, *32*(17), 5747–5756. https://doi.org/10.1523/JNEUROSCI.0587-12.2012

Nijs, J., George, S. Z., Clauw, D. J., Fernández-de-las-Peñas, C., Kosek, E., Ickmans, K., Fernández-Carnero, J., Polli, A., Kapreli, E., Huysmans, E., Cuesta-Vargas, A. I., Mani, R., Lundberg, M., Leysen, L., Rice, D., Sterling, M., & Curatolo, M. (2021). Central sensitisation in chronic pain conditions: Latest discoveries and their potential for precision medicine. *The Lancet Rheumatology*, *3*(5), e383–e392. https://doi.org/10.1016/S2665-9913(21)00032-1

Nikolenko, V. N., Shelomentseva, E. M., Tsvetkova, M. M., Abdeeva, E. I., Giller, D. B., Babayeva, J. V., Achkasov, E. E., Gavryushova, L. V., & Sinelnikov, M. Y. (2022). Nociceptors: Their Role in Body's Defenses, Tissue Specific Variations and Anatomical Update. *Journal of Pain Research*, *Volume 15*, 867–877. https://doi.org/10.2147/JPR.S348324

Noh, A. S. M., Chuan, T. D., Khir, N. A. M., Zin, A. A. M., Ghazali, A. K., Long, I., Ab Aziz, C. B., & Ismail, C. A. N. (2021). Effects of different doses of complete Freund's adjuvant on nociceptive behaviour and inflammatory parameters in polyarthritic rat model mimicking rheumatoid arthritis. *PLOS ONE*, *16*(12), e0260423. https://doi.org/10.1371/journal.pone.0260423

Pagliusi, M., & Gomes, F. V. (2023). The Role of The Rostral Ventromedial Medulla in Stress Responses. *Brain Sciences*, *13*(5), 776. https://doi.org/10.3390/brainsci13050776

Pak, D. J., Yong, R. J., Kaye, A. D., & Urman, R. D. (2018). Chronification of Pain: Mechanisms, Current Understanding, and Clinical Implications. *Current Pain and Headache Reports*, *22*(2), 9. https://doi.org/10.1007/s11916-018-0666-8

Pak, V., & Hashmi, J. A. (2023). Top-down threat bias in pain perception is predicted by higher segregation between resting-state networks. *Network Neuroscience*, *7*(4), 1248–1265. https://doi.org/10.1162/netn_a_00328

Pasare, C., & Medzhitov, R. (2004). Toll-like receptors: Linking innate and adaptive immunity. *Microbes and Infection*, *6*(15), 1382–1387. https://doi.org/10.1016/j.micinf.2004.08.018

Petrini, L., & Arendt-Nielsen, L. (2020). Understanding Pain Catastrophizing: Putting Pieces Together. *Frontiers in Psychology*, *11*, 603420. https://doi.org/10.3389/fpsyg.2020.603420

Piiper, A., Stryjek-Kaminska, D., Klengel, R., & Zeuzem, S. (1997). CCK, carbachol, and bombesin activate distinct PLC-beta isoenzymes via Gq/11 in rat pancreatic acinar membranes. *American Journal of Physiology-Gastrointestinal and Liver Physiology*, *272*(1), G135–G140.

Quartana, P. J., Campbell, C. M., & Edwards, R. R. (2009). Pain catastrophizing: A critical review. *Expert Review of Neurotherapeutics*, *9*(5), 745–758. https://doi.org/10.1586/ern.09.34

Rivat, C., Becker, C., Blugeot, A., Zeau, B., Mauborgne, A., Pohl, M., & Benoliel, J.-J. (2010). Chronic stress induces transient spinal neuroinflammation, triggering sensory hypersensitivity and long-lasting anxiety-induced hyperalgesia. *Pain*, *150*(2), 358–368. https://doi.org/10.1016/j.pain.2010.05.031

Rosen, J. B., Asok, A., & Chakraborty, T. (2015). The smell of fear: Innate threat of 2,5-dihydro-2,4,5-trimethylthiazoline, a single molecule component of a predator odor. *Frontiers in Neuroscience*, *9*. https://doi.org/10.3389/fnins.2015.00292

Russell, F. A., King, R., Smillie S. -J., Kodji, X., & Brain, S. D. (2014). Calcitonin Gene-Related Peptide: Physiology and Pathophysiology. *Physiological Reviews*, *4*. https://doi.org/10.1152/physrev.00034.2013

Samineni, V. K., Grajales-Reyes, J. G., Copits, B. A., O'Brien, D. E., Trigg, S. L., Gomez, A. M., Bruchas, M. R., & Gereau, R. W. (2017). Divergent Modulation of Nociception by

Glutamatergic and GABAergic Neuronal Subpopulations in the Periaqueductal Gray. *Eneuro*, *4*(2), ENEURO.0129-16.2017. https://doi.org/10.1523/ENEURO.0129-16.2017

Sandkühler, J. (2009). Models and Mechanisms of Hyperalgesia and Allodynia. *Physiological Reviews*, *89*(2), 707–758. https://doi.org/10.1152/physrev.00025.2008

Smith, M. L., Hostetler, C. M., Heinricher, M. M., & Ryabinin, A. E. (2016). Social transfer of pain in mice. *SCIENCE ADVANCES*.

Suvas, S. (2017). Role of Substance P Neuropeptide in Inflammation, Wound Healing, and Tissue Homeostasis. *The Journal of Immunology*, *199*(5), 1543–1552. https://doi.org/10.4049/jimmunol.1601751

Tao, Z.-Y., Wang, P.-X., Wei, S.-Q., Traub, R. J., Li, J.-F., & Cao, D.-Y. (2019). The Role of Descending Pain Modulation in Chronic Primary Pain: Potential Application of Drugs Targeting Serotonergic System. *Neural Plasticity*, *2019*, 1–16. https://doi.org/10.1155/2019/1389296

Taugher, R. J., Ghobbeh, A., Sowers, L. P., Fan, R., & Wemmie, J. A. (2015). ASIC1A in the bed nucleus of the stria terminalis mediates TMT-evoked freezing. *Frontiers in Neuroscience*, *9*. https://doi.org/10.3389/fnins.2015.00239

Taylor, N. E., Pei, J., Zhang, J., Vlasov, K. Y., Davis, T., Taylor, E., Weng, F.-J., Van Dort, C. J., Solt, K., & Brown, E. N. (2019). The Role of Glutamatergic and Dopaminergic Neurons in the Periaqueductal Gray/Dorsal Raphe: Separating Analgesia and Anxiety. *Eneuro*, *6*(1), ENEURO.0018-18.2019. https://doi.org/10.1523/ENEURO.0018-18.2019

Timmers, I., Quaedflieg, C. W. E. M., Hsu, C., Heathcote, L. C., Rovnaghi, C. R., & Simons, L. E. (2019). The interaction between stress and chronic pain through the lens of threat learning. *Neuroscience & Biobehavioral Reviews*, *107*, 641–655. https://doi.org/10.1016/j.neubiorev.2019.10.007

Tracey, W. D. (2017). Nociception. *Current Biology*, *27*(4), R129–R133. https://doi.org/10.1016/j.cub.2017.01.037

Tuteja, N. (2009). Signaling through G protein coupled receptors. *Plant Signaling & Behavior*, *4*(10), 942–947. https://doi.org/10.4161/psb.4.10.9530

Vasic, V., & Schmidt, M. (2017). Resilience and Vulnerability to Pain and Inflammation in the Hippocampus. *International Journal of Molecular Sciences*, *18*(4), 739. https://doi.org/10.3390/ijms18040739

Walker, D. L., Miles, L. A., & Davis, M. (2009). Selective participation of the bed nucleus of the stria terminalis and CRF in sustained anxiety-like versus phasic fear-like responses. *Progress in Neuro-Psychopharmacology and Biological Psychiatry*, *33*(8), 1291–1308. https://doi.org/10.1016/j.pnpbp.2009.06.022

Walters, E. T. (2019). Adaptive mechanisms driving maladaptive pain: How chronic ongoing activity in primary nociceptors can enhance evolutionary fitness after severe injury. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *374*(1785), 20190277. https://doi.org/10.1098/rstb.2019.0277

Wang, J. Q., Fibuch, E. E., & Mao, L. (2007). Regulation of mitogen-activated protein kinases by glutamate receptors. *Journal of Neurochemistry*, *100*(1), 1–11. https://doi.org/10.1111/j.1471-4159.2006.04208.x

Xue, M., Shi, W.-T., Zhou, S.-B., Li, Y.-N., Wu, F.-Y., Chen, Q.-Y., Liu, R.-H., Zhou, Z.-X., Zhang, Y.-X., Chen, Y.-X., Xu, F., Bi, G.-Q., Li, X.-H., Lu, J.-S., & Zhuo, M. (2022). Mapping thalamic-anterior cingulate monosynaptic inputs in adult mice. *Molecular Pain*, *18*, 174480692210870. https://doi.org/10.1177/17448069221087034

Yam, M. F., Loh, Y. C., Tan, C. S., Khadijah Adam, S., Abdul Manan, N., & Basir, R. (2018). General Pathways of Pain Sensation and the Major Neurotransmitters Involved in Pain

Regulation. *International Journal of Molecular Sciences*, *19*(8), 2164. https://doi.org/10.3390/ijms19082164

Yang, S., & Chang, M. C. (2019). Chronic Pain: Structural and Functional Changes in Brain Structures and Associated Negative Affective States. *International Journal of Molecular Sciences*, *20*(13), 3130. https://doi.org/10.3390/ijms20133130

Yu, S., Li, W., Shen, W., Edwards, R. R., Gollub, R. L., Wilson, G., Park, J., Ortiz, A., Cao, J., Gerber, J., Mawla, I., Chan, S.-T., Lee, J., Wasan, A. D., Napadow, V., Kaptchuk, T. J., Rosen, B., & Kong, J. (2020). Impaired mesocorticolimbic connectivity underlies increased pain sensitivity in chronic low back pain. *NeuroImage*, *218*, 116969. https://doi.org/10.1016/j.neuroimage.2020.116969

APPENDIX

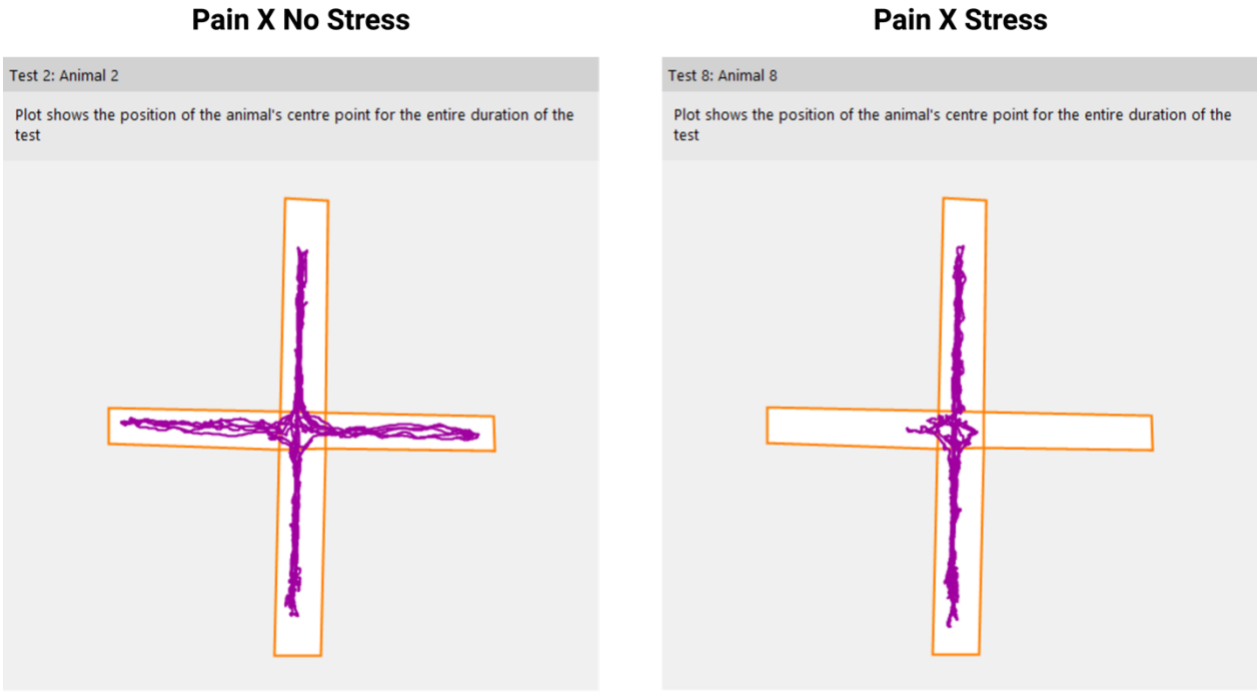

Figure 14. **AnyMaze trackpad analysis shows clear anxiety-like behaviors in CFA-injured rats exposed to predator odor compared to no stress controls.**