MCGILL UNIVERSITY

Pain at Face Value:

Lateralization and Neuroanatomical Mediation of Pain-Induced Facial Grimacing

A THESIS

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Brief Abstract in English

Pain includes sensory and affective dimensions that influence behaviour and quality of life. Our current clinical and preclinical measures of pain are hindered by substantial variability and lack of translatability. Existing preclinical measures that rely on withdrawal thresholds (e.g., von Frey, Hargreaves, tail flick) or quantification of nocifensive behaviour (e.g., licking a paw that has had an algogen administered) are inadequate at modelling the type of spontaneous pain that is most clinically valuable. Chapter 2 of this thesis shows that in a large-scale normative data set, many of the classic default algesiometry show startling variability—further, models such as these only capture the sensory component of pain. Critically, the affect component of pain conveys the aversive aspects of pain, which engender the suffering that makes pain unpleasant. Facial expression of emotion is a behaviour that is exquisitely phylogenetically conserved across species and includes pain-induced facial grimacing. There is a dearth of studies that directly probe the relationship between pain-induced facial grimacing and the emotional component of pain. Thus, we do not have a conclusive answer to the question, *to what degree does pain face reflect the affective aspect of pain?* The studies presented in chapters 3 and 4 take a two-pronged approach to attempt to answer this question. Chapter 3 is a study that used designer receptors exclusively activated by designer drugs (DREADDs) to inhibit areas of the mouse brain primarily involved in pain sensation or pain emotion while mice are video recorded. Videos were used to score mice using the Mouse Grimace Scale (MGS) and for quantifying reflexive pain behaviour. We found that inhibition of affective ROIs significantly attenuated pain-induced facial grimacing while leaving reflexive pain behaviour intact, suggesting that the MGS is primarily a measure of pain affect. Chapter 4 focuses on the fact that other emotional states are broadcast on the face and (e.g., fear, anger) are lateralized such that the left side of the face

expresses more strongly than the right. Comparing pain-induced facial grimacing to facial expressions of emotion is one way to determine the degree to which the Mouse Grimace Scale captures pain affect. To our knowledge, the lateralization of pain-induced facial grimacing has never been examined. After comparing left-face and right-face images, we found that pain-induced facial grimacing is also lateralized but always more strongly on the right, opposite to all other facial emotions. Cumulatively, the three manuscripts presented here show that our current preclinical measures of pain are insufficient if we want to capture the pain experience as a whole and that the Mouse Grimace Scale is decidedly a measure of pain affect and should be used in combination with other classical pain assays for a more valid and thus more valuable understanding of pain.

Brief Abstract in French

La douleur comprend des dimensions sensorielles et affectives qui influencent le comportement et la qualité de vie. Nos mesures cliniques et précliniques actuelles de la douleur sont entravées par une variabilité importante et un manque de traductibilité. Les mesures précliniques existantes qui s'appuient sur des seuils de sevrage (par exemple, von Frey, Hargreaves, coup de queue, etc.) ou sur la quantification d'un comportement nocifensive (par exemple, lécher une patte ayant reçu un algogène) sont inadéquates pour modéliser le type de douleur spontanée qui est cliniquement la plus précieuse. Le chapitre 2 de cette thèse montre que dans un ensemble de données normatives à grande échelle, de nombreuses algésiométries classiques par défaut présentent une variabilité surprenante. De plus, de tels modèles ne capturent que la composante sensorielle de la douleur. De manière critique, la composante affective de la douleur transmet les aspects aversifs de la douleur, qui engendrent la souffrance qui la rend désagréable. L'expression faciale de l'émotion est un comportement qui est remarquablement conservé phylogénétiquement à travers les espèces et comprend les grimaces faciales induites par la douleur. Il existe peu d'études qui examinent directement la relation entre les grimaces faciales induites par la douleur et la composante émotionnelle de la douleur. Ainsi, nous n'avons pas de réponse concluante à la question : dans quelle mesure le visage douloureux reflète-t-il l'aspect affectif de la douleur ? Les études présentées dans les chapitres 3 et 4 adoptent une approche à deux volets pour tenter de répondre à cette question. Le chapitre 3 est une étude qui a utilisé des récepteurs de synthèse activés exclusivement par des drogues de synthèse (DREADD) pour inhiber les zones du cerveau de souris principalement impliquées dans la sensation de douleur ou l'émotion de la douleur pendant que les souris sont enregistrées en vidéo. Des vidéos ont été utilisées pour évaluer les souris à l'aide de l'échelle de grimace de la

souris (MGS) et pour quantifier le comportement réflexif de la douleur. Nous avons constaté que l'inhibition des ROI affectives atténuait de manière significative les grimaces faciales induites par la douleur tout en laissant intact le comportement réflexif de la douleur, ce qui suggère que le MGS est principalement une mesure de l'effet de la douleur. Le chapitre 4 se concentre sur le fait que d'autres états émotionnels sont diffusés sur le visage et (par exemple, la peur, la colère) sont latéralisés de telle sorte que le côté gauche du visage s'exprime plus fortement que le droit. Comparer les grimaces faciales induites par la douleur aux expressions faciales d'émotion est un moyen de déterminer dans quelle mesure l'échelle de grimace de la souris capture l'effet de la douleur. À notre connaissance, la latéralisation des grimaces faciales induites par la douleur n'a jamais été étudiée. Après avoir comparé les images du visage gauche et du visage droit, nous avons constaté que les grimaces faciales induites par la douleur sont également latéralisées mais toujours plus fortement à droite, à l'opposé de toutes les autres émotions faciales. Cumulativement, les trois manuscrits présentés ici montrent que nos mesures précliniques actuelles de la douleur sont insuffisantes si nous voulons capturer l'expérience de la douleur dans son ensemble et que l'échelle de grimace de la souris est résolument une mesure de l'effet de la douleur et doit être utilisée en combinaison avec d'autres méthodes passe-partout. tests de douleur pour une compréhension plus valide et donc plus précieuse de la douleur.

Preface

Chapter 1 is a general introduction and comprehensive review of literature necessary to contextualize data and discussion in subsequent chapters.

Chapter 2 is an empirical data chapter which analyzed a large-scale data set and provides normative values and reveals substantial variability in classic preclinical algesiometry and is published in *The Journal of Pain*: Zumbusch, A. S., McEachern, E. L., Morgan, O. B., Nickner, E., & Mogil, J. S. (2024). Normative Preclinical Algesiometry Data on the von Frey and Radiant Heat Paw-Withdrawal Tests: An Analysis of Data from More Than 8,000 Mice Over 20 Years. *The Journal of Pain*.

Chapter 3 is a manuscript examining the role of select sensory and affective regions of the pain matrix in pain-induced facial grimacing in mice to determine the degree to which grimace scale reflect pain sensation vs pain affect: Affective and Sensory Neuroanatomical Substrates of Pain-Induced Facial Grimacing. Alicia S. Zumbusch, Elodie Nickner, Sijie Xu, Dana Harell, Susana Sotocinal, Milan Valyear, Jonathan Britt, Jeffrey S. Mogil

Chapter 4 is a manuscript focused on lateralization of pain induced facial grimacing: Lateral Asymmetry of Pain-Induced Facial Grimacing in Mice. Alicia S. Zumbusch, Elodie Nickner, Sijie Xu, Dana Harell, Oakley B. Morgan, Heewon Jang, Sol Blanco, Melanie Di Maria, Laila Chaudhry, Louise Castillo, Susana Sotocinal, Jeffrey S. Mogil.

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My favourite thing about writing my acknowledgments when I wrote my MSc thesis years ago was that it invited to me to reflect on my experience as a graduate student in hindsight. This time with my PhD it feels far more natural to look forward. It would be so easy to have a list of lamentations; of "could haves" and "should haves" and ways that I wish it had been during my degree. Yet, the most tacit feeling this degree has given me is the assurance that tenacity is the lynchpin for all the other inevitable metaphorical and literal MacGyvering that one ends up doing as a grad student. The grit that got me to this point was the result of the encouragement and support of myriad individuals along this journey whom I hope I can show some modicum of gratitude to in this text.

The most salient person to thank is Doctor Jeffrey Mogil. I frequently espouse my admiration for Jeff not because he is *the* Jeff Mogil, but because of the way he thinks about and communicates science. When I first sought to work with Jeff, it was because I had been in a few seminar classes with him. I remember walking away from them thinking that the way Jeff thinks about science is the way *I* think about science. Prioritizing answering meaningful questions with simple and elegant experiments and not being waylaid trying to stuff every glossy technique you can into a study to try and wring the impact factor sponge. This and his intellectual enthusiasm for behaviour and translation was something that deeply resonated with me and is something I intend to always keep as a central tenet of my research. Beyond my intellectual admiration of Jeff, I also have immense gratitude for his, kindness, patience and understanding while I navigated some of the most challenging personal times in my life. I doubt many PIs would have given me such grace and there are very few words that can convey what it has meant to me that

he didn't give up on me. Thank you, Jeff, your integrity and forbearance are something I hope I can take and extend to others.

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Contributions to Original Scholarship & Knowledge

The exploration of pain processing through the lens of facial grimacing provides valuable insights into the neurobiological and affective dimensions of pain. The synthesis of findings from Chapters 2, 3, and 4 underscores the importance of considering both sensory and emotional components in pain assessment and management. By advancing our understanding of pain lateralization, affective neuroanatomy, and variability in preclinical measures, this dissertation contributes to the development of more nuanced and effective pain research methodologies. These insights hold promise for improving pain treatment and alleviating suffering, emphasizing the critical need for integrated approaches in pain research and clinical practice.

Contribution of authors

The following is a list of co-authors and their contributions to the manuscripts in this dissertation:

Jeffrey Mogil: Principal Investigator of all projects, experiment conceptualization, manuscript writing and editing

Eleri McEachern: Analysis of data in Chapter 2 in R

Oakley Morgan: Intellectual feedback and editing of chapter 2, cFos in chapter 4

Elodie Nickner: Video taking and processing for all MGS in chapter 3 and 4. Assisted in stereotaxic surgeries, as manuscript writing, editing and data entry for all chapters.

Sijie Xu: Video taking and processing for both chapter 3 and 4 projects. Assisted in brain slicing and cFos staining for chapter 4.

Dana Harell: Video taking and processing for both chapter 3 and 4 projects.

Susana Sotocinal: Video processing and all MGS scoring for both chapter 3 and 4 projects. All von Frey.

Milan Valyear: Project strategizing and intellectual contribution for chapter 3.

Jonathan Britt: Co-investigator provided initial virus for chapter 3.

Melanie Di Maria: Assisted in brain slicing, fluorescent microscopy, cFos staining and H&E staining for chapter 3 and 4.

Sol Blanco: Assisted in brain slicing, fluorescent microscopy, cFos staining and H&E staining for chapter 3 and 4.

Heewon Jang: Assisted in brain slicing, fluorescent microscopy, cFos staining and H&E staining for chapter 3 and 4.

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List of Symbols, Abbreviations, Nomenclature

ACC - Anterior Cingulate Cortex

AA - Acetic Acid

AM - Affective Motivational

ANOVA - Analysis of Variance

AU - Action Unit

CCI - Chronic Constriction Injury

CeA - Central Amygdala

CFA - Complete Freund's Adjuvant

CNS - Central Nervous System

CNO - Clozapine-N-oxide

DMSO - Dimethyl Sulfoxide

DREADD - Designer Receptors Exclusively Activated by Designer Drugs

DV - Dorsal Ventral

FACS - Facial Action Coding System

Fos - Immediate Early Gene Product (c-Fos)

HT - Hargreaves' Test

KO - Knockout

ML - Medial Lateral

MGS - Mouse Grimace Scale

PBN - Parabrachial Nucleus

PCR - Polymerase Chain Reaction

PET - Positron Emission Tomography

PDF - Postdoctoral Fellow

RA - Research Associate

rACC - Rostral Anterior Cingulate Cortex

rAI - Rostral Anterior Insular Cortex

RNA - Ribonucleic Acid

S1 - Primary Somatosensory Cortex 1

SD - Sensory Discriminative

SD/mean - Coefficient of Variation

SEM - Standard Error of the Mean

SNI - Spared Nerve Injury

SPSS - Statistical Package for the Social Sciences

UG - Undergraduate

vF - von Frey

ZYM - Zymosan

Chapter 1

Introduction

Pain has a crucial adaptive role in protecting an organism despite being a largely unpleasant sensation. The experience of pain is both sensory and emotional. Melzack and Casey formally identified this sensory and emotional dichotomy in the pain experience and labelled them as the sensory-discriminative (SD) and the affective-motivational (AM) components of pain (Melzack & Casey, 1968). Following this theoretical segregation of pain sensation and pain emotion many studies demonstrated how the painfulness or "algosity" of pain is distinct from the "suffering" aspect of it (Berthier et al., 1988; Craig, 2003; Fields, 1999). Indeed, even research predating this formal distinction noted that a frontal lobotomy changed the patient's "mental attitude" toward their chronic pain without eradicating the sensation of pain itself (Lyerly, 1951). The formal definition of pain provided by the International Association for the Study of Pain reflects this theoretical framework and states that pain is both a "sensory *and* emotional experience" (Raja et al., 2020). Despite the ubiquity of the SD versus AM understanding of pain, there is no agreement on the degree to which the sensory or emotional aspects of pain are dissociable, or whether other components of the pain experience, such as cognitive or social aspects, are similarly independent. Indeed, this question is of such profound importance that it is reflected in non-academic media, such as the article "Is Pain a Sensation or Emotion?" published in the Sunday edition of *The New York Times* (2019). Before reviewing evidence for the emotional and sensory aspects of pain, it is necessary to define several terms.

Defining the word "affect" for use in the research described here requires incorporating several related terms. First is "unpleasantness," which includes pain aversiveness and intensity.

A seminal text by Howard Fields separates unpleasantness into primary and secondary unpleasantness. Primary unpleasantness is that which is proportional or relative to the stimulus causing the pain (Fields, 1999). Secondary unpleasantness is that which is informed highly by context and memory and where the relationship to stimulus intensity is variable. Importantly, secondary unpleasantness is related to cognition and mood and is highly subjective (Fields, 1999). Another term introduced by Fields is "algosity," which he asserts is the characteristic of pain that makes it unique from other sensations, including unpleasant but not painful sensations such as itch and other various dysesthesias (Fields, 1999). In clinical psychology, "affect" refers to the outward expression of internal mood, feelings, and emotions (American Psychiatric Association & Association, 2013). This definition is distinct from Fields' definition, which asserts that affect *is* the subjective emotional state and is unverifiable except by report from the one experiencing the emotion (Fields, 1999). While parsing the term at this level may seem superfluous, the inclusion of both in the present definition of affect is essential when considering both the human and animal literature. Here, "affect" will refer to the actual (or presumed in the case of non-human research) internal emotional state and the outward presentation or expression of emotion by the subject.

Additionally, a meaningful conceptual separation of the SD from the AM necessitates an understanding of the current research on pain-related neuroanatomy and physiology. Thus, the following sections will review peripheral, spinal, supraspinal, and cortical anatomy and their role in our understanding of pain as a sensation and emotion.

Peripheral Nociceptive Circuitry and Spinal Pain Processes

Sensory aspects of pain have been acknowledged for longer than the emotional aspects, even so far as to be recognized as a fifth vital sign (Araujo & Romero, 2015; Bertagnolli,

2004; Lynch, 2001; Walid et al., 2008). When a noxious or painful stimulus is detected, it is called nociception. Nociception is a type of somatosensation specific to the transmission of noxious sensory stimuli. Importantly, not all nociception is sufficient to produce the conscious and subjective experience of pain, nor is it necessary to produce pain. However, the sensory component of the pain experience usually refers to nociception specifically. Before arriving in the brain, nociceptors detect noxious sensory stimuli in the periphery, which convey the signal to the central nervous system (CNS). These peripheral sensory receptors, also known as primary afferents or first-order neurons, are highly heterogeneous and include Aβ, Aδ and C-fibres.

These primary peripheral nociceptors are delineated according to the character of stimuli they respond to, their conductance, and the location of their downstream spinal and supraspinal targets. Highly myelinated and large in diameter, Aβ fibres are typically responsible for transmitting non-nociceptive mechanosensory information, though they can sometimes play a role in mechanical pain transmission (Zeng et al., 2011). A-delta (Aδ) fibres are also myelinated and relatively large in diameter, yielding high conduction velocity of ascending pain signals and are responsible for "fast pain" (Bishop & Landau, 1958; Garland, 2012; Loeser & Melzack, 1999). Fast pain is that which immediately follows the application of the noxious stimuli and is typically more localized, but less intense and unpleasant. Conversely, C-fibres are unmyelinated and are responsible for "slow pain." In addition to having slower conductance velocity due to the lack of a myelin sheath, slow pain conducted by C-fibres is more diffuse and persistent while generally being more unpleasant.

In addition to being functionally and anatomically distinct, these fibres comprise highly heterogeneous but precise connections at distinct laminae of the dorsal horn of the spinal cord. Briefly, C fibres preferentially synapse to second-order neurons in Rexed’s laminae I and II, with

the delineating factor being whether they are peptidergic (lamina I) or non-peptidergic (lamina II). A-delta fibres also terminate in lamina I along with the peptidergic c-fibres, however, they also project to lamina V (Basbaum et al., 2009)[1]. These fibres then synapse with second-order interneurons, which convey the signal to the ventral horn on the contralateral side.

To this point, there is little evidence for a meaningful physiological distinction between the sensory and emotional aspects of pain signalling. Though there are many identifiable segregations of spinal cell subpopulations according to what type of stimuli they respond to (i.e., labelled lines), a more pertinent distinction for the studies presented here is to distinguish spinal pathways according to their eventual targets in the brain and their apparent contributions to the lived pain experience. Thus, the following subsections will discuss how parallel but discrete ascending tracts convey incoming nociceptive information to their respective terminuses in the brain.

Pain as a Sensation

Once the sensory signal reaches the spinal cord, it ascends along the spinothalamic tract (STT) of the spinal cord and is trafficked through the thalamus to the primary somatosensory cortex (S1; (De Ridder et al., 2021). This pain pathway that terminates in S1 is responsible for the discriminative and sensory components of pain (De Ridder et al., 2021; Rainville, 2002; Renthal, 2020). The sensory aspects of pain include things such as the location of the pain, the intensity of the pain, and the nature of the pain (e.g., aching, throbbing, burning), but unpleasantness is part of the affective pain experience (Bushnell et al., 2013; Flor et al., 1995; Kulkarni et al., 2005).

[1] Research has allowed for further delineation of primary afferents within discrete laminae not discussed here

One study differentiated the cortical areas involved in the sensory aspect of pain from those involved in affect using positron emission tomography (PET). This study revealed that activity in affective regions of the brain, like the anterior cingulate cortex (ACC), positively correlated with pain unpleasantness, while activity in somatosensory areas was unchanged (Rainville et al., 1997). This evidence supports the separation of pain affect from the sensory aspects of pain, which are vital in treating pain. For instance, a very intense aching pain in the jaw would be treated differently than a sharp stabbing pain in the abdomen. Indeed, these are the characteristics used in life-saving emergency paramedical and field diagnoses and triaging (Beveridge, 1999; Cordell et al., 2002); (Janati et al., 2018). Despite the importance of pain as a sensation and the primary role of ascending sensory signals of that sensation, it does not encapsulate the entire pain experience.

Pain as an Emotion

Though somewhat nebulous, the affective component of pain is crucial to understand as it is the component that causes the suffering that accompanies pain sensation. Arguably, without the suffering that accompanies pain, it would be just another sensation that could easily ignored (e.g., the hum of electronics, the sensation of your back against a chair). In contrast to the sensory spinothalamic tract discussed above, the signal transmitting the affective component of pain, or the "suffering" component is separate. This ascending affective pain signal travels *via* the spinoparabrachial tract and synapses in the parabrachial nuclei (Pb) of the pons where it then projects to limbic structures such as the amygdala, nucleus accumbens (NAc) and ventral tegmental area (VTA) (Deng et al., 2020; Gauriau & Bernard, 2002; Renthal, 2020; Yan et al., 2022; Yang et al., 2021). There is also a more medial aspect of the STT that projects through the more medial aspects of the thalamus and lands in the cingulate cortex which contributes to the emotional aspect of pain (Dum et al., 2009). Further research has identified several cortical

regions that relate specifically to pain affect such as the anterior cingulate cortex (ACC) and the anterior insula (aI) (Rainville, 2002; Renthal, 2020).

Though people do learn to live with pain, learn to reduce pain, or heal and resolve associated injuries, the full or partial remediation of pain does not necessarily resolve the affective sequelae of that pain. For example, fear of aggravating an old injury or reluctance to activity for fear of causing a pain flare is common and can be more debilitating to quality of life than the pain itself (Crombez et al., 1999; Zale et al., 2013).

Outside of neurobiology, studies about the affective component of pain focus on either cognition or perception. Cognitive approaches utilize functional neuroimaging and existing learning models in humans and non-human animals (Wiech, 2016). Approaches prioritizing pain perception in the affective experience of pain tend towards the more abstract and ontological. For instance, De Ridder et al., (2021) define perception as the interpretation and organization of sensory stimuli to produce a meaningful experience of self and the world. They assert that "When a person says he or she is "in pain", what the person actually says is 'I have a certain amount of painfulness associated with a certain amount of suffering during a certain amount of time'" (De Ridder et al., 2021).

Measuring Affect

Unfortunately, existing preclinical models of emotion only measure behaviours that researchers *presume* to relate to emotion. Whether they are valid in terms of informing human pain treatment is hardly assured. For instance, fear and anxiety assays in rodents (e.g., open field, elevated plus maze, light-dark box) rely heavily on prey behaviour that human beings lack entirely. Additional models such as the forced swim and tail suspension tests also have limited

human generalizability and are now believed to likely relate more to stress coping than to affect *per se* (Commons et al., 2017*)*. The absence of verbal communication in rodents means we cannot confirm the meaning and relationship to affect inherent in these behaviours, let alone how they relate to pain behaviours.

Even when working with a species that is capable of verbal expression there are scarcely few studies that adequately bridge the translational gap. Human research examining emotion ranges from pen and paper inventories (e.g., Beck's Depression Inventory; BDI), to observing naturalistic behaviours (e.g., aggression), to physiological measures (e.g., blood pressure, skin conductance). We cannot give rodents surveys, and behavioural observation is subject to the same species-specific confounds described in rodent affective assays. Even physiological measures are subject to practical barriers (e.g., measuring blood pressure in a mouse) in addition to the confound that handling them produces stress that will impact most if not all the physiological outputs. Importantly, the definition of pain set by the International Association for the Study of Pain was recently updated to account for the obvious fact that the inability to describe one's pain does not mean that one cannot or does not experience pain (Raja et al., 2020). Thus, a non-verbally dependent measure of affect would have the most utility for humans with and without full verbal abilities (e.g., neonates) and be the most translationally relevant for use in non-humans without species-specific caveats.

Measuring Pain

A literature search of pain measurement or "algesiometry" in humans and experimental animals yields many widely used and validated measures of sensory aspects of pain in rodents (Deuis et al., 2017; Mogil, 2022; Turner et al., 2019). Of the various aspects of pain experience that

an algesiometric measure or assay could account for, four broad themes emerge (Mogil, 2022). First, is *pain duration*. Many existing algesiometry measures pain transiently on a scale of seconds or minutes. In rodents, these are measures like the tail-flick, hot-plate and Randall-Selitto measures which transiently apply the noxious stimulus to an extremity and measure latency to a withdrawal or other nocifensive response. In humans, measures such as the cold pressor test, or the application of heat or pressure, are measures of acute pain. However, the reality is that most clinically relevant pain occurs on a scale of hours to years (Mogil, 2022). The second characteristic of pain that algesiometry must consider is *location*. All the above measures and other popular rodent pain measures, such as the von Frey and Hargreaves' (radiant heat paw-withdrawal) tests, are assessed by applying the stimulus to the skin (usually of the plantar hind paw) and at a precise location. Many common clinical presentations of pain, such as headache, joint pain, and visceral pain are not superficial and may be far less localized. A third consideration is *pain modality*. That is, pain can be evoked through pressure, heat, cold, electricity, or chemicals applied externally or ingested. Such experimenter-delivered noxious stimuli may not be analogous to spontaneous pain, such as that due to inflammation or nerve damage (Mogil, 2022). The fourth and last consideration is the type of response being quantified (e.g., outcome measure or dependent variable). In humans, this ranges from quantitative measures such as pain thresholds and tolerance to those that extrapolate information about the pain via ratings or questionnaires (Mogil, 2022). In rodents, the measures we use are interpreted based on behaviours such as nocifensive responding (e.g., licking and biting) or withdrawal latencies. Of course, there are also proposed biomarkers, such as those generated in genetic, epigenetic, or functional neuroimaging studies, which are limited by being correlational in how they are tied to pain experience and pain behaviours.

Though there are myriad options for the quantitative measurement of pain in humans and animals, they are all subject to the aforementioned translational caveats. Additionally, established measures like the hot-plate, tail-flick, von Frey and Hargreaves' tests only capture the presumed pain in that epoch of time which the measure is taken. Replicate measures are required to verify any temporal dynamics in the pain experience. Though techniques like Hargreaves' test (HG) and the von Frey test (VF) are mainstays of algesiometry, they show considerable and potentially concerning variability especially when the aforementioned potentially requisite replicate measures are taken. *The second chapter of this dissertation is a study examining over 8000 over 20 years and highlights the significant limitations of VF and HG and highlight the need for an objective, translational measure of spontaneous pain.* The variability observed in this study combined with the sections above on the pitfalls of measuring pain and affect independently emphasizes the need for an easily observable, non-verbal output that pertains to both pain sensation and affect. Fortunately, there is such a real-time measure of spontaneous pain and pain affect that is also highly phylogenetically conserved across species: facial expressions.

Development of Grimace Scales

The utility of facial expressions has been cemented into evolutionary biology since initially being described by Darwin (Darwin, 1872). A literature search of even one of the 60+ recognizable emotions described by Darwin yields an immense volume of research dedicated to measuring and quantifying facial expression of emotions as well as understanding their neurobiological substates. Despite facial expressions being historically entrenched into the collective scientific mind, the recognition of the importance of pain-related facial expressions is comparatively recent. Clinicians have long acknowledged the importance of non-verbal

expression of pain, which is well evidenced by its inclusion in the behavioural "wave" of pain psychology that Fordyce and others pioneered (Fordyce, 1976). Acknowledging the importance of non-verbal communication of emotions led to the development of a Facial Action Coding System (FACS) designed to measure and categorize different movements of facial musculature into different emotions, including pain (Ekman & Friesen, 1978).

This FACS system developed by Ekman allowed for coding of facial features base on muscle movement. These muscle movements combine to produce the recognizable facial expression of pain which includes brow furrowing, squinting, nose scrunching, upper lip retraction and open oral posture (Kappesser, 2019; Grunau & Craig, 1987). Facial grimacing is used in the evaluation of pain and informs the social communication model of pain (Craig et al., 2001; K. D. Craig, 2009). Pain-induced facial grimacing, like the other major emotions, can be easily interpreted by others and distinguished from the other emotions (Kappesser & Williams, 2002; Prkachin & Solomon, 2008; Williams, 2002). Importantly, it is also highly consistent across the lifespan and between species (as discussed above) making it highly generalizable (Chambers & Mogil, 2015; Grunau & Craig, 1987; Prkachin, 2009).

While beneficial, the FACS system is limited to use in humans. The efforts of two pain researchers, Kenneth Craig and Jeffrey Mogil, and their colleagues bridged this gap between clinical and preclinical work and developed a scale for use in laboratory mice, the Mouse Grimace Scale (MGS; see (Mogil et al., 2020) for a detailed account of how the technique was developed). Briefly, the MGS measures alterations to several facial action units. In white-coated mice (i.e., CD-1 strain) the action units are cheek bulge, whisker position, nose bulge, ear position, orbital tightening. Action units are assigned a value based on how strongly visible they are to the coder (0: not present, 1: moderate, 2: severe). This coding can be evaluated at any time

interval determined by the experimenter and yields a composite score for all the action units (i.e., MGS Score).

The application of the MGS scale has extensive utility and has been adapted for use in more than 10 other mammalian species (Mogil et al., 2020). The MGS uniquely allows us to measure how a stimulus is being broadcast facially by a mouse (or other preclinical research species). The most important quality of the MGS that differentiates it from other pain measures is that it measures spontaneous pain. As described in the above section on pain measurement, one of the main drawbacks to most pain measures is that they are experimenter evoked and thus not necessarily translatable to clinical pain. Spontaneous pain is the most important type of pain to study as it is the most clinically relevant (Sadler et al., 2022; Schmelz, 2021; Tappe-Theodor & Kuner, 2014).

Studies of pain symptomatology reveal that spontaneous and non-evoked ongoing pain was reported in almost all patients (Backjona et al., 2004). In contrast, evoked pain similar to that which is used in laboratory evaluations of pain using pressure and heat were only reported in 64% and 38% of patients, respectively. Additionally, neuropathic pain tends to be evaluated in animals by inducing nerve damage and applying mechanical stimuli (e.g., von Frey fibres). This methadological mismatch is inadequate for clinical translation as studies show that patients with neuropathic conditions such as complex regional pain syndrome (CRPS) or peripheral nerve injury tend to experience dynamic allodynia (pain caused by non-painful stimuli), hyperalgesia and paradoxical thermal sensation (Maier et al., 2010). Importantly, grimace evaluations can be applied to all of the above non-evoked variations in clinical pain presentations and symptomatology.

Other pain measures, such as those where the dependent measure is paw-withdrawal threshold or latency (e.g., VF, HG) are useful for testing pain sensitivity but don't allow for a concurrent measure of other pain behaviours. Another benefit of the MGS is that it can yield a rich gradient of more subtle differences in pain expression across time. This allows for integrating more advanced and temporally specific techniques such as chemogenetics and optogenetics. What remains unknown and almost completely unexplored is what aspect(s) of the pain experience facial grimacing reflects. Thus, a primary question that my doctoral research centres around asking is: *is pain-induced facial grimacing a measure of sensation or emotion*?

Grimacing: Sensation or Emotion?

Despite knowledge of the social utility, ontogeny, and phylogeny of facial grimacing (see above section on development of grimace scales) it is still unclear to what degree facial grimacing is reflecting a sensation or an emotion. There are correlational human data to suggest that facial expression of pain relates to both the intensity of pain (i.e., sensory aspect) as well as the unpleasantness (i.e., emotional aspect) of pain (Kunz et al., 2004; Prkachin, 1992; Prkachin & Solomon, 2008). Other research has demonstrated that different facial musculature and thus different action units (AUs) are activated by pain affect and pain sensation (Kunz et al., 2012). This study showed that the sensory aspect of pain was displayed more by contraction of muscles around they eyes (e.g., squinting), whereas the affective component of pain was displayed more by raising of the lip, scrunching of the nose and brow contraction (Kunz et al., 2012). In conjunction with these behavioural data are functional neuroimaging studies showing that brain activity in areas linked with both pain sensation (e.g., S1, S2) and pain affect (e.g., ACC, insula) are enhanced (Budell et al., 2015). Interestingly, one study also showed a greater correlation between the self-reported pain ratings made by pain patients and their facial response to painful

stimuli than controls do (Lautenbacher et al., 2017). These data suggest that facial grimacing and its component subunits may reflect different aspects of the pain experience and that clinical presentations of affect alter the relationship between pain expression and the pain experience.

Lateralization of Facial Expression

Another aspect of facial expressions that relates to emotional expression is their symmetry. That is, other emotions are asymmetrical in how they are expressed facially, how an observer interprets them, and which areas of the brain are involved (Lindell, 2018; Lindell, 2013). Research shows that facial expressions relating to various experiences (e.g., fear, pleasure) in humans, non-human primates and other non-human animals are lateralized (Lindell, 2018). That is, emotional expression is asymmetrical both in the observable facial output and in the neural structures and circuitry that govern their expression. An examination of the emotions of happiness, sadness, anger, and fear showed that both posed, and naturally evoked expressions of these emotions were asymmetrical such that the left expressed the emotion more strongly (Indersmitten & Gur, 2003; Sackeim et al., 1978). This is in line with the pop-psychology heuristic that the right side of the brain is the "more emotional" side. However, as far as we know whether pain is similarly lateralized has never been assessed.

Despite the absence of any research examining the lateralization of pain-induced facial grimacing specifically, there is a substantial body of literature that demonstrates asymmetrical contributions of certain brain nuclei in pain and emotional processing; prime amongst them is the amygdala. The role of the amygdala in general pain and emotional processing, though well characterized, is a burgeoning area of research within the pain community and the broader scientific purview. A comprehensive review of the amygdala's role in emotion and pain

processing is beyond the scope of this thesis and has been covered by others extensively. Nonetheless, there are several studies that supplement the research discussed in later chapters.

Unilateral Contributions of the Amygdala to Pain and Emotion

Distinct Roles of the Left and Right Amygdala

The left and right amygdalae exhibit distinct yet complementary roles in pain and emotional processing. The right amygdala is primarily associated with the processing of negative emotions and initiating automatic responses to threatening stimuli. For instance, when participants were presented with fear-inducing images, greater fMRI activation was observed in the right amygdala (Morawetz, 2017). In contrast, the left amygdala is involved in processing both positive and negative emotions and plays a more complex role in the conscious evaluation and interpretation of emotional stimuli. This includes verbal and cognitive aspects of emotional processing, such as labeling emotions and integrating them with contextual information (Burklund et al., 2014).

Functional Asymmetry in Emotional Processing

The left amygdala is particularly active during tasks that require emotional labeling and regulation, underscoring its role in the cognitive appraisal of emotions (Burklund et al., 2014). A meta-analysis by Sergerie et al. (2008) highlighted that neuroimaging studies consistently show greater right amygdala activation in response to fearful faces compared to neutral faces, whereas the left amygdala is more engaged in tasks requiring emotional contextualization. Patients with unilateral damage to the amygdala exhibit asymmetrical deficits in emotional processing, further illustrating this functional asymmetry. Conversely, damage to the left amygdala affects the processing of complex emotional and social cues, impairing cognitive aspects of emotional regulation (Adolphs, 2001; Phelps & LeDoux, 2005); Calder et al., 2002).

Right Amygdala: Immediate and Unconscious Processing

The right amygdala is heavily involved in the immediate, automatic processing of emotional responses to pain. It is particularly responsive to negative and threatening stimuli, including pain. Damage to the right amygdala can impair the recognition of fearful facial expressions and other negative emotions, reflecting its role in processing such stimuli (Adolphs, 2001). Functional MRI (fMRI) studies have shown significant activation of the right amygdala in response to acute pain stimuli. For example, Ploner et al. (2010) applied painful heat stimuli to subjects and observed significant right amygdala activation, supporting its role in the immediate emotional reaction to nociceptive stimuli. Baliki et al. (2006) further demonstrated the right amygdala's involvement in the emotional modulation of pain perception. They reported increased right amygdala activity in response to pain anticipation and experience, highlighting its role in the emotional reaction to pain. Despite the apparent role of the right amygdala in the more immediate processing of pain, there is evidence for its role in chronic pain as well. For instance, chronic pain patients exhibit structural and functional changes in the amygdala, particularly the right amygdala (Apkarian et al., 2005).

Though there is vast evidence for the right amygdala's role in affective pain processing in and of itself, we also know that the interaction of the right amygdala and various cortical regions is crucial for regulating pain-related emotions. This connection appears to modulate the immediate emotional response to pain and influences pain-related behaviours. Bushnell et al. (2013) found that the right amygdala-prefrontal cortex pathway is involved in downregulating pain-related anxiety. Their study showed that individuals with higher connectivity between these regions reported lower pain-related anxiety.

Left Amygdala: Cognitive and Evaluative Processing

In contrast, the left amygdala is more involved in the cognitive-evaluative aspects of pain processing, including the contextual evaluation of pain and its integration with other cognitive processes. Simons et al. (2014) examined the differential activation of the left and right amygdala in response to pain-related fear and found that the left amygdala was more active during tasks requiring cognitive evaluation and labelling of the pain experience.Liberzon et al. (2000) reported that the left amygdala is more engaged during tasks involving the recall and contextualization of pain memories. Their PET imaging study found that the left amygdala was particularly active when subjects recalled painful experiences.

Like the right amygdala, the left amygdala's connection with cortical regions expands its role in pain processing. Connections between the left amygdala and the anterior cingulate cortex (ACC) is crucial for the cognitive appraisal of pain. This pathway facilitates the integration of pain with cognitive and emotional contexts, influencing how pain is perceived and remembered (Vogt et al., 2003) found that the left amygdala and ACC are co-activated during tasks requiring the cognitive evaluation of pain, suggesting a role for the left amygdala in integrating pain with higher cognitive processes.

Thus, *this branch of my doctoral research investigates lateralization of pain-induced facial grimacing with a focus on how the pain model and location impacts grimace lateralization, and the selective role of the left and right CeA in processing and conveying pain affect.*

Using Anatomy to Determine Sensation and Emotion in Grimacing

The above sections discuss literature on the vastly different cortical and subcortical brain regions areas implicated in pain sensation and pain affect. Using research from wider bodies of

non-pain-specific literature such as the vast reward literature can supplement the above discussion of pain circuitry.

One such study that is highly relevant is a recent study demonstrating that optogenetic control of the central amygdala (CeA) can alter motivational states such that animals will self-inflict pain (Warlow et al., 2020). Crucially, while the mice will approach the noxious stimulus and inflict pain on themselves, the pain stimulus still retains its aversive qualities, as evidenced by the retained defensive treading and conditioned fear response. The quantification of Fos protein activation in the same study suggests likely differences in circuit recruitment that determine whether the noxious stimuli are approached or avoided. In control mice that demonstrated avoidance of the pain stimulus, Fos activation was enhanced in the ventral lateral periaqueductal grey (vlPAG), bed nucleus of the stria terminalus (BNST) and basolateral amygdala (blA). Conversely, in those mice that had their CeA optogenetically activated and demonstrated attraction to the noxious shock rod, there was enhanced activation in the ventral tegmental area (VTA), substantia nigra pars compacta (SNc), NAc shell (NAcS), dorsolateral neostriatum, lateral hypothalamus, medial orbitofrontal cortex (mOFC) and posterior insula. Utilizing existing knowledge of the functional role of brain regions to inform our understanding of behaviour in this manner can be tuned to examine pain affect using the MGS. That is, we can inactivate or activate regions other data have shown to related to aspects of pain affect versus pain sensation and evaluate their impact on pain-induced facial grimacing.

Studies examining the affective components of pain-induced grimacing and how it relates to pain more broadly are almost non-existent. Indeed the 2010 study performed by Langford et al. (2010) appears to be the only one directly examining this question, but that study used non-reversible lesion methods for inactivation and had an extremely small sample size. Fortunately,

the prevalence of viral methods for temporarily inhibiting or exciting specific regions of interest (ROIs) such as Designer Receptors Exclusively Activated by Designer Drugs (DREADDs) and optogenetics allows for a more temporally precise and fine-grained evaluation of facial grimacing than the lesion methods used in Langford et al. (2010). Importantly, optogenetics, though in many ways more tuneable and temporally precise, also includes possible confounds. First, cementing of permanent optical fibres onto the dorsal side of the skull may inhibit the ability of the mouse to express normal grimacing, particularly for more anterior regions of the brain that require implantation anterior to bregma (e.g., rAI, ACC). Particularly, the ear movement and eye squint may be impeded and although controls can account for between-group differences, there may be a floor effect such that both animals in pain and controls can only express minimal grimacing. Additionally, simple considerations such as fibre placement and maintenance of general mouse health are eliminated or much more easily managed via other methods. Thus, DREADDs are the ideal tool for the studies described below.

Regions of Interest

In the above sections on pain as a sensation and pain as an emotion, I briefly review the ascending pain pathways and some of the major supraspinal brain structures where pain signalling is processed and whether these regions have been identified as primarily affective or sensory. In choosing which areas specifically to chemogenetically manipulate, my focus was to prioritize areas of the "pain matrix" that are well known to be preferentially contributing to the SD or the AM components of pain. The SD areas I focused on were the thalamus and primary somatosensory cortex, and the AM areas I chose were the insula, cingulate and amygdala. Within each other these broad areas, I focused more precisely on subnuclei that have substantial literature demonstrating their role in their respective pain processes. Although the pain signal is

at some point processed in a multitude of brain areas (i.e., the pain matrix), there are several candidate areas that are ideal for investigation of how pain-induced facial grimacing reflects sensation versus affect.

Sensory-Discriminative ROIs

Thalamus (Ventral Posterior Nucleus)

The thalamus has a role in many bodily processes, especially perceptions like pain that are closely tied to survival, and is one of the first supraspinal structures that relays pain information to subsequent areas. The thalamus comprises several nuclei and subnuclei, and is organized into distinct functional domains, each with specialized roles in sensory and thus pain processing. The ventral posterior nucleus (VPN), comprising the ventral posterolateral (VPL) and ventral posteromedial (VPM) subnuclei, serves as the primary relay for somatosensory information, including nociceptive input from the spinal cord and trigeminal system (Jones, 2007; (Giesler et al., 1994). Additionally, the medial and lateral thalamic nuclei, along with their respective subnuclei, participate in the integration and transmission of nociceptive signals to cortical regions associated with pain perception (Hirsch & Burkhalter, 2016).

When it comes to distinct contributions to the SD and AM aspects of pain, the medial versus lateral distinction remains the primary demarcation. The more medial aspect of the thalamus receives input from the deeper lamina in the spinal cord and project to places like the anterior cingulate cortex (ACC) as well as the rostral anterior insular cortex (rAI). These two regions are regarded as primarily contributing to the AM aspects of pain and thus are discussed directly in their own right in subsequent sections discussing my chosen ROIs. The subnuclei in the more lateral aspect of the thalamus are collectively referred to as the ventrobasal complex and project to the primary somatosensory cortex (S1). This is also known as the lateral pain

pathway and is largely acknowledged to convey SD information regarding pain (Andersson et al., 1997; Kenshalo & Isensee, 1983). Neurons within these nuclei exhibit precise somatotopic organization, ensuring accurate localization of nociceptive stimuli (Apkarian et al., 2005). Moreover, recent studies have highlighted the role of VPL/VPM neurons in encoding the intensity and duration of noxious stimuli, contributing to the discriminative aspects of pain perception (Alvarez et al., 2019).

Primary Somatosensory Cortex (S1 Trunk Region)

The primary somatosensory cortex (S1) plays a fundamental role in encoding and discriminating the sensory qualities of noxious stimuli. Located in the postcentral gyrus of the parietal lobe, S1 receives direct input from thalamic nuclei, particularly the ventral posterior nucleus (VPN), conveying nociceptive signals from the periphery (Jones, 2007). S1 is organized into distinct cytoarchitectonic areas, including Brodmann areas 3a, 3b, 1, and 2, each exhibiting specialized functions in somatosensory processing (Kaas, 2012). Area 3b is traditionally associated with the processing of nociceptive and tactile stimuli, whereas areas 1 and 2 integrate additional sensory modalities, contributing to the multimodal representation of pain perception within S1 (Chudler & Dong, 1995).

Nociceptive stimuli elicit robust neuronal responses in S1, with neurons exhibiting receptive fields corresponding to the location and modality of the noxious input (Iwamura et al., 1993). Moreover, S1 neurons demonstrate remarkable specificity in discriminating between different sensory qualities of noxious stimuli, such as intensity, duration, and thermal properties (Mouraux et al., 2011). S1 neurons exhibit precise somatotopic organization, with distinct populations of neurons responding to specific body regions (Mountcastle, 1957). Because of this precise somatotopy I aimed specifically to inhibit only the relevant sub area of the S1 for the

pain model I chose (i.e., acetic acid writhing). The goal of this was that if we globally inhibited the entirety of the S1 alter the ability of the mouse to exhibit grimacing at all because the mouse would be getting no proprioceptive sensory feedback.

Affective-Motivational ROIs

Insular Cortex (Rostral Anterior Insula)

The insular cortex is a key node in the affective processing of pain and is anatomically and functionally divided into anterior and posterior regions. The anterior insula, encompassing the agranular and dysgranular cortices, is further subdivided into rostral anterior and dorsal anterior regions (Nieuwenhuys, 2012). Neuroimaging studies show that increased activation of the insular cortex during the experience of acute and chronic pain, with greater involvement of the anterior insula in processing the affective (Wiech & Tracey, 2009). Additionally, lesions or disruptions of insular activity have been linked to alterations in affective responses to pain, highlighting its significance in emotional processing (Craig, 2009; Langford et al., 2010). The insular cortex also serves as a convergence zone for interoceptive signals arising from the body, including nociceptive inputs from the periphery. Because the insula is extensively connected to the thalamus, somatosensory cortex, and limbic structures it appears to serve as an integrative hub for interoceptive sensory and emotional information that converge upon it (Gasquoine, 2014).

The rostral anterior insula (rAI) particularly is implicated in the integration of emotional and interoceptive information, making it a critical substrate for affective processing (Chang et al., 2013). This particular region within the insular cortex receives direct projections from the ventral posterolateral nucleus of the thalamus as well as further connection to limbic structures, including the amygdala and the anterior cingulate cortex (ACC; Augustine, 1996). Activation of the insula during pain processing elicits autonomic, and endocrine responses known for their role

in the regulation of emotional states (Phan et al., 2002). Moreover, the insula has reciprocal connection to prefrontal cortical areas involved in the cognitive appraisal and regulation of affect that shape one's emotional experience of pain (Wiech & Tracey, 2009)

Amygdala (Central Nucleus of the Amygdala)

The amygdala and its component subnuclei including the central amygdala (CeA) receive considerable research attention outside the realm of pain. Particularly, research relating the central amygdala to motivation and emotion is plentiful. While the CeA is known for its involvement in emotional processing in the context of pain, it plays a significant role in the modulation and integration of pain (Neugebauer, 2015). Even within the CeA there are further anatomical and functional distinctions, namely the lateral (CeL) and medial (CeM) aspects of the CeA. The CeL primarily serves as a site of integration for nociceptive information and is linked to emotional responses to pain and pain-related anxiety (Neugebauer, 2015). The CeM relates the behavioral aversion and the regulation of autonomic responses (Johansen, 2010). Both the CeL and the CeM receive inputs from multiple brain regions involved in pain processing, including the thalamus, prefrontal cortex (PFC), insular cortex, and periaqueductal gray (PAG) (Johansen, 2010).

Electrophysiological studies have demonstrated that CeA neurons exhibit increased firing rates in response to aversive stimuli, including nociceptive inputs (Neugebauer et al., 2004). Moreover, optogenetic manipulations targeting CeA neurons have been shown to modulate pain affect, further highlighting its role in affective processing (Johansen, 2010). Additionally, behavioural studies examining pain behaviours and measure of anxiety in rodents have shown that optogenetic activation of CeA enhances anxiety and pain behaviour, while inhibition decreases them (Carrasquillo and Gereau (2007). Human literature echoes these

findings as well. For example, Loggia et al. (2015) demonstrated that increased fMRI activity in the CeA correlates with heightened pain-related fear and anxiety in chronic pain patients. Activation of CeA projections to the PAG elicits defensive behaviors and autonomic responses, facilitating the avoidance of aversive stimuli (Han et al., 2015). Conversely, inhibition of CeA activity attenuates pain-induced fear and enhances pain tolerance, underscoring its role in pain modulation (Neugebauer et al., 2004). For instance, pharmacological inhibition of CRF receptors in the CeA reduced pain and anxiety in animal models (Ji & Neugebauer, 2007). Additionally, neuromodulation techniques such as deep brain stimulation (DBS) of the amygdala are being explored for their potential to alleviate chronic pain and its associated affective components (Zhang et al., 2021).

Anterior Cingulate Cortex (Rostral ACC)

The anterior cingulate cortex (ACC), particularly its rostral region is a crucial region for the affective dimension of pain processing. Neuroimaging studies have consistently demonstrated increased activation in the rACC during pain. For instance, Rainville et al. (1997) used positron emission tomography (PET) and found that the intensity of pain-related unpleasantness correlated with rACC activity. The rACC also plays a role in pain modulation as demonstrated by studies using functional magnetic resonance imaging (fMRI) showing that placebo-induced pain relief is associated with increased activity in the rACC (Wager et al., 2004). The rACC is connected to various brain regions involved in pain processing, including the periaqueductal gray (PAG), thalamus, amygdala and prefrontal cortex. Lastly, Kong et al. (2010) used resting-state fMRI to show that the rACC has altered connectivity with the default mode network (DMN) in chronic pain patients. This altered connectivity may underlie the persistent affective and motivational disturbances seen in pain conditions and hint at the role of the ACC

not as a region that assigns valence or algosity to a stimulus, but rather as and integration site for the anticipatory processing of various types of stimuli, including pain.

The role of the ACC as a processor for the anticipatory aspect of pain emotionality is perhaps the strongest support for the dichotomy of pain affect from pain sensation. Disentangling the sensory component of pain from the affective component during ongoing pain is murky given the utter lack of translatable measure of pain affect (the very issue this thesis seeks to resolve) to compare to any measure of pain sensation. Anticipation of pain, however, can occur in the total absence of pain. Thus, any alterations in brain or behaviour during pain anticipation can more safely be assumed to be a sequalae of altered pain affect, or at the very least not a consequence of pain sensation.

Significant literature exists to support this more nuanced role for the ACC in pain processing. Importantly, however, the differences in nomenclature for various model organisms compared with that of humans means that functionally and anatomically analogous regions are referred to differently. To uphold the readability and clarity of this section, note that the pregenual (pACC) and subgenual ACC (sACC) are analogous to the rACC in rodents (see van Heukleum 2020 for comprehensive review or this paper). Preclinical literature in non-human primates also appears to support the role of the rACC in emotional aspects of pain as set of studies done by Kayoma et al. (1998, 2000, 2001) show selective activation of single neurons to pain avoidance behaviours but not reward behaviours. Lesion studies suggest that the rACC is involved in the motivational drive to avoid pain as ablation of the rACC impairs the ability to learn and perform tasks that require avoidance of painful stimuli (Johansen et al., 2001). Studies have shown that activation of the rACC can reduce amygdala activity, thereby modulating fear and anxiety associated with pain (Etkin A, 2011).

Primary Research Questions, Experimental Objectives & Hypotheses

Is pain-induced facial grimacing a measure of sensation or emotion?

A prudent investigation of how pain induced facial grimacing relates to pain affect versus pain sensation begins with the inactivation of regions believed to be involved in those aspects of pain and examines the effect of that inactivation on grimacing behaviour. To dissociate general inhibition of the pain experience as a whole (i.e., sensation *and* emotion), a simultaneous evaluation of reflexive (i.e., sensory) pain behaviour is necessary. If inhibition of the areas involved in pain affect decreases grimacing more than reflexive pain behaviour, this would suggest that facial grimacing is more reflective of the affective aspect of pain than the sensory. Reciprocally, inhibition of areas devoted to the sensory aspects of pain may attenuate the reflexive pain behaviour and leave the grimacing response intact. Thus, *the first objective of my doctoral work is to examine the effect of chemogenetic inhibition of affective and sensory pain neuroanatomy on pain-induced facial grimacing as measured by the MGS.*

To achieve this aim, I expressed an inhibitory DREADD into the rostral anterior insula, central amygdala and rostral anterior cingulate cortex of adult CD-1 mice. After adequate transfection time mice were subject to recording of baseline and post pain induction videos. Between baseline and post, all mice were given either an intraperitoneal (i.p.) injection of the DREADD ligand CNO or vehicle followed immediately by an injection of acetic acid. Still frames of mouse faces during baseline and post videos were taken every 3 min and subsequently scored using the MGS. Inhibition of sensory neuroanatomical pain nodes were executed in the exact above fashion in the thalamus and primary somatosensory cortex. *I hypothesized that inhibition of the affective regions (i.e., rAI, CeA and rACC) would attenuate pain-induced facial*

grimacing and not effect reflexive pain behaviours. I also hypothesized that inhibition of the sensory regions would inhibit reflexive pain behaviours and leave pain-induced facial grimacing intact.

Is pain-induced facial grimacing lateralized?

Examining the degree to which pain-induced facial grimacing is lateralized like other emotions will reveal how pain is similar and different to the other emotions that are expressed asymmetrically. If the "pain face" is like other emotions and is lateralized, this would suggest that grimace scales are indeed reflective of the affective experience of pain and thus a valid and useful tool in the arsenal of researchers and clinicians alike. Additionally, I was interested in an initial exploration of the neuroanatomy that may contribute to lateralization of pain-induced facial grimacing. I also wanted to evaluate the asymmetry of pain signalling in the ascending and descending tracts as well as pain–relevant brain areas we know to be lateralized (i.e., CeA). Thus, *the second set of objectives that my doctoral research focused on are a) to evaluate the degree to which pain face is lateralized using the MGS, b) examine lateralization of neuronal activation at various levels of the neuraxis using cFos, c) to gain chemogenetic control of pain face lateralization through unilateral inhibition of the central amygdala, and d) to begin evaluating if pain face lateralization is observable in human subjects.*

For objective 2a, I examined lateralization of pain-induced facial grimacing through several aspects of pain (e.g., modality, duration, location). I induced acute, generalized and non-localized visceral pain (i.e., acetic acid), localized, unilateral chronic neuropathic pain (i.e., SNI) and inflammatory pain (i.e., CFA, carrageenan and zymosan) and evaluated asymmetry of facial grimacing for all five main assays using the MGS. The unique research question required

additional images beyond the standard MGS to include exclusively unilateral views of the mouse face from various angles (described in detail in the methods section). The images were scored using the MGS and them left and right images were compared. *I hypothesized that the left side of the face would show stronger pain-induced facial grimacing than the right in alignment with the established literature of other facially expressed emotions.*

For objective 2b, I quantified the expression of cFos as a proxy for the neural activity at various points in the pain signalling pathway. These were, the spinal cord, PBN, Thalamus, CeA, S1 and rAI. In this experiment, I quantified cFos expression at various levels of the CNS on the ipsilateral and contralateral sides of the brain to the pain location. The algogen I chose was that which yielded the strongest lateralization of pain-induced facial grimacing and the most straightforward for precisely timed tissue collection (i.e., carrageenan). *I hypothesized that the pro-nociceptive left CeA would show greater cFos expression. I had no* a priori *hypotheses regarding other supraspinal regions.*

For objective 2c, I used an inhibitory DREADD to unilaterally silence the CeA and evaluated pain face lateralization before and after activation of the DREADD with CNO. This was performed on the ipsilateral and contralateral sides of the brain to the pain location and pain was induced using CFA. *I hypothesized that inhibition of the CeA would abolish any observable pain face lateralization.*

Finally, for objective 2d, I performed a post-hoc reanalysis of videos of human subjects experiencing cold pain for a separate experiment. Here, I evaluated differences in facial expression using the FACS on the left and right while participants were subjected to the cold pressor test on both hands sequentially. *I hypothesized that the right side of the face would show*

greater activation regardless of pain side in alignment with the mouse data we had collected at that point.

The overall goal of the studies described here is to greatly enhance our understanding of how grimacing is a powerful measure of both pain *and* affect. This increased nuance in our understanding of the affective component of pain and the optimal measure of it can be harnessed to better study pain preclinically and most importantly, treat pain suffering more effectively.

Chapter 2

Normative preclinical algesiometry data on the von Frey and radiant heat paw withdrawal tests: an analysis of data from over 8,000 mice over 20 years

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Abstract

The measurement of withdrawal to experimenter-delivered mechanical stimuli (von Frey test) and to heat stimuli (radiant heat paw-withdrawal or Hargreaves' test) applied to the hind paws is ubiquitous in preclinical pain research, but no normative values for the most common applications of these tests have ever been published. We analyzed a retrospective data set of withdrawal thresholds or latencies in 8,150 mice in which these measures were taken using replicate determinations, before and after injection of inflammatory substances or experimental nerve damage producing pain hypersensitivity (a total of 97,332 measurements). All mice were tested in the same physical laboratory over a 20-year period using similar equipment and procedures. We find evidence of large interindividual variability, affected by tester, genotype, mouse sex, tester sex, replicate order, and injury. These factors are discussed, and we believe that these normative data will serve as a useful reference for expected values in preclinical pain testing.

1. Introduction

Despite advances in human imaging studies, RNA sequencing of human tissue, and *in vitro* approaches to pain research (e.g., using induced pluripotent stem cells and "organs-on-chips"), discovery research and preclinical testing of new treatments requires whole-animal behavioral studies and likely will for quite a long time to come (Mogil et al., 2010). A recent review co-authored by one of us provided insight into the status quo of preclinical pain testing (or algesiometry) as of 2020, as assessed by an analysis of papers published in the journal, *Pain* (Sadler et al., 2022*)*. As of that year, the mouse (*Mus musculus*) surpassed the rat (*Rattus norvegicus*) as the primary research subject in preclinical pain research. Within this species, the inbred C57BL/6 mouse strain was used in 60% of published papers, with outbred CD-1 mice in second place with almost 8% (Sadler et al., 2022). Since 1980, the study of acute pain in animals has declined from almost 80% of the total to just over 10%; the study of longer-lasting inflammatory and neuropathic pain now represents nearly 60% of total studies (Sadler et al., 2022). Of the inflammatory assays, complete Freund's adjuvant (CFA) and carrageenan (CARR) together represent 67% of the total, and four experimental nerve injuries together account for 77% of the total: spared nerve ligation, chemotherapeutic-induced neuropathy, spared nerve injury (SNI), and chronic constriction injury (CCI) (Sadler et al., 2022). Finally, in terms of dependent measures—although their use has been heavily and rightly criticized (Blackburn-Munro, 2004; Mao, 2002; Mogil & Crager, 2004; Yezierski & Hansson, 2018)—the measurement of experimenter-evoked mechanical sensitivity (overwhelmingly performed using the up-down psychophysical method of Dixon; (Chaplan et al., 1994)) and thermal sensitivity (overwhelmingly performed using the radiant heat paw-withdrawal or Hargreaves' test (Hargreaves et al., 1988)) occurred in almost three-quarters of published papers in 2020 (Sadler et al., 2022).

One problematic feature of algesiometry is the large interindividual and intraindividual variability encountered. For example, average baseline von Frey withdrawal thresholds (using manual fiber application and the up-down method) in mice reported by 55 papers published in *Pain* between 2000–2020 ranged from 0.3 g to 8.0 g (Sadler et al., 2022, see Supplementary Data 1). However, it is difficult to know how to interpret such data since these experiments all occurred in different laboratories using different equipment and procedures. A more useful exercise, perhaps, would be to consider variability *within* a laboratory, where equipment and procedures are more likely to be standardized. We performed such an analysis of over 8,000 baseline measurements of mouse tail-withdrawal latencies from 49 °C water taken in the senior author's laboratories from 1993–2001. We found that the two largest contributors to the considerable variability observed (withdrawal latencies ranging from <1 s to >7 s) were the individual tester and the mouse genotype, in that order (Chesler et al., 2002a, 2002b).

The senior author's laboratory has conducted a large number of mouse experiments since that time, all occurring in three small testing rooms in a single laboratory. Most of these studies have used extremely common assays of inflammatory and neuropathic pain, as described above, and replicate measurements of mechanical and thermal sensitivity before and at multiple time points after these injuries. We are unaware of any prior publication of normative values (measures of central tendency plus error) of these common algesiometric procedures. Here, we present an analysis of such data collected in over 8,000 mice over a 20-year period in a relatively constant testing environment.

2. Materials and Methods

2.1. Data identification and preparation

Data archives were inspected to identify data from individual experiments performed in the senior author's laboratory, all taking place in one of three contiguous testing rooms on the 7th floor of the Stewart Biology Building (1205 Dr. Penfield St.) on the downtown campus of McGill University in Montreal over a 20-year period from 2002–2022, with the following characteristics:

1) young adult mice (6–20 weeks at commencement of testing) as subjects.

2) algesiometry performed using the "manual" von Frey test of mechanical sensitivity (Stoelting Touch Test Sensory Probes [previously known as the Semmes-Weinstein monofilament kit]; Item 58011); using the up-down psychophysical method of Dixon (Chaplan et al., 1994), or the radiant heat paw-withdrawal (Hargreaves') test of thermal sensitivity (IITC Inc. Life Science Plantar Analgesia Meter; Model 390; set at 20% of maximum intensity);

3) testing of both hind paws.

4) at least two replicate determinations of sensitivity (for von Frey, withdrawal threshold in grams; for Hargreaves' test, withdrawal latency in seconds) per hind paw at baseline.

5) (where possible) experiments featuring an injury (nerve damage or inflammation) or non-drug manipulation producing mechanical or thermal hypersensitivity on a time course of hours to-days; and,

6) available data from at least 10 mice.

After identifying relevant data sets, all data were entered into four master Excel spreadsheets, two for von Frey and two for Hargreaves' test (provided as Supplementary Data 1–

4). Along with the withdrawal threshold or latency measurements themselves, the following information was entered: 1) experiment name, 2) tester, 3) tester gender (self-identified, from which sex was inferred), 4) tester level (e.g., graduate student), 5) year, 6) assay (either baseline only or particular nerve injury or inflammatory agent), 7) mouse genotype, 8) mouse sex, 9) experimental condition (i.e., nerve injury versus sham surgery; inflammatory agent versus vehicle), 10) post-injury/inflammation time point, and 11) side of nerve injury/inflammation. Note that data from multiple time points were often available; all such data recorded were entered. In many of these experiments, drugs were injected, or other experimental manipulations occurred following baseline and post-injury measures; all data analyzed here were measurements occurring *prior* to such manipulations.

Once all 97,332 individual data points and other information were entered, averages were calculated at every time point. To facilitate comparisons, mechanical and thermal hypersensitivity were quantified by calculating the percentage of the maximum possible hypersensitivity with reference to each subject's average baseline on the relevant hind paw. In the vast majority of cases, nerve injury or inflammatory injection was delivered to the left hind paw; care was taken to track the true ipsilateral and contralateral sides when the right hind paw was used instead. Von Frey test thresholds above 2.0 g (n=14) were removed from the data set, as under normal circumstances such fibers simply lift the hind paw of a mouse directly off the ground without bending. Similarly, Hargreaves' test latencies above 40 s (n=8) were removed from the data set since this latency represents our chosen cut-off to prevent tissue damage, and thus latencies >40 s were improperly obtained.

2.2. Statistical analyses

Data are presented in all cases as individual data points overlaid with median ± 25%/75% quartile box or violin plots. Data were analyzed using SPSS v. 24 or GraphPad Prism v. 10, and group comparisons used a criterion $\alpha = 0.05$.

Regression trees, a form of decision tree classification model built via machine learning, were constructed for baseline von Frey and Hargreaves' test data in R (using dplyr, tidyr, rpart, and rpart.plot packages). The technique involves recursive binary splitting to grow a tree, followed by cost complexity pruning to maximize goodness-of-fit. Only variable levels with high sample sizes were included. We calculated the ranking of each variable (genotype, tester, tester sex, mouse sex) in explaining variance in the tree using the var_importance function.

3. Results

3.1. Properties of the data sets

Identified and analyzed von Frey and Hargreaves' baseline data are provided in Excel format as Supplementary Data 1 and 2, respectively. The subset of data in which post-injury (nerve damage or inflammation) measurements were taken are provided as Supplementary Data 3 and 4.

Our search identified 115 distinct von Frey test experiments with sample sizes ranging from *n*=10–277 mice, and 101 distinct Hargreaves' test experiments with sample sizes ranging from *n*=10–792 mice. Data from a grand total of 3,642 mice tested for baseline von Frey thresholds were collected, as were data from a grand total of 4,508 mice tested for baseline Hargreaves' thresholds. Note that in some cases the very same mice were tested on both measures at different times; precise details about the timing were mostly irretrievable from the surviving digital records. Of the 8,150 mice tested for baseline mechanical or thermal sensitivity, 3,623 also provided post-injury data (2,717 mechanical; 906 thermal). Given the lab's conviction that using

only male subjects is unethical and poor research practice (Mogil, 2016) there was an almost equal representation of male (3,697) and female (3,996) mice. The remainder (457) were either of unknown sex or not gonadally intact (i.e., ovariectomized or castrated). The data analyzed herein represent averages of replicate determinations of withdrawal thresholds or latencies at all time points and are comprised of 20,004 individual measurements of baseline von Frey thresholds, 59,108 measurements of baseline Hargreaves' test latencies, 13,985 measurements of post-injury von Frey thresholds, and 4,235 measurements of post-injury Hargreaves' latencies.

A total of 25 testers were involved in data collection (10 male; 15 female); all were either undergraduate research assistants (n=9), graduate students (n=7), postdoctoral fellows (n=7), or career research associates (n=2). A total of 265 measurements (3.2% of the total) could not be attributed to any individual tester.

For many years the focus of the laboratory was on genetics (Mogil, 2012a), and thus data from 44 different mouse genotypes are represented. Note that substrains were not individually categorized, and different hybrid and congenic strains on the same genetic background were collapsed together. Mutant mice of any sort—whether conventional transgenic knockouts (the majority of mutants tested), heterozygous mice with one mutant allele, or other types of genetically altered mice—were analyzed for simplicity as "knockouts" (KOs), and categorized only by their background strain, which in almost all cases was C57BL/6. A total of 138 measurements (1.7% of the total) could not be attributed to any specific genotype.

Finally, 10 different assays producing hypersensitivity were employed in the data sets analyzed. Two of them were unilateral peripheral nerve injuries producing mononeuropathy: the spared nerve injury (SNI) and the chronic constriction injury (CCI). Four of them were inflammogens: carrageenan (CARR), complete Freund's adjuvant (CFA), lipopolysaccharide

(LPS), and zymosan (ZYM). CARR, CFA, and ZYM were all delivered into one hind paw; LPS was delivered by multiple injection routes. In almost every case, the SNI and CCI surgeries were performed by the same individual, a career research associate. Similarly, the same concentration of carrageenan (2%), CFA (50%), and zymosan (3 mg/ml) were used in all experiments, with the same injection volume, vehicle, and route for all (20 µl in saline, intraplantar). The remaining assays included: classically conditioned pain hypersensitivity (Martin et al., 2019), a cancer pain model in which fibrosarcoma cells were injected into the mouse calcaneus bone (Wacnik et al., 2001), the medial meniscus destabilization model of osteoarthritis (Fernihough et al., 2004), and swim stress induced- hyperalgesia (Quintero et al., 2000). Note that the data presented are only from mice expected to become hypersensitive; data from mice receiving sham surgery or vehicle injection were omitted from analysis.

3.2. Normative baselines and variability

The grand average baseline mechanical threshold of all mice tested was 0.77 g (median: 0.74 g), with an SD of 0.34 g and an SEM of 0.006 g (see Fig. 1A). Thresholds ranged from 0.04–2.0 g, and the interquartile range was 0.47 g. It is difficult to know whether very low baseline thresholds reflect tester error, the extreme sensitivity of certain mice (e.g., of sensitive genotypes), or data entry errors. Data variability might also derive from different latencies after von Frey fiber calibration (Bove, 2006). Skewness was 0.52 ± 0.04 g and kurtosis were 0.12 ± 0.08 g; thus, the overall distribution approximates normality.

The grand average baseline thermal latency of all mice tested was 11.8 s, with an SD of 5.8 s and an SEM of 0.09 s (see Fig. 1B). Latencies ranged from 2.2–40 s, and the interquartile range was 8.1 s. Very low or very high latencies might reflect tester error, genotype differences, or data entry errors. Although the same model radiant heat device was used over the entire 20-year

period, the energy output of the two individual devices used might differ slightly and/or drift over time. It is also possible that in some experiments the device was not actually set to 20% of maximum intensity, even though this is the default setting in the laboratory. Skewness was 0.86 ± 0.04 s and kurtosis was 0.55 ± 0.07 s, and thus the overall distribution does not deviate significantly from normality.

3.3. Laterality

The grand average baseline mechanical thresholds and thermal latencies of the left and right hind paws (before any insult had occurred to either) were compared by *t*-test. In both cases, no evidence of laterality was found, such that the mechanical ($t_{7262} = 0.5$, $p=0.59$) and thermal ($t_{9014} = 1.3$, $p=0.20$) sensitivity of the left and right hind paw were equal (Fig. 1C, D).

3.4. Tester and Strain

A prior analysis of over 8,000 49 °C tail-withdrawal test measurements revealed that the two largest sources of variance were tester and genotype (Chesler et al., 2002a, 2002b), and large differences were seen among testers and genotypes in the current data as well.

Figure 2 shows the data from the eight most-active testers on each assay (those with >100 individual mice tested), and data (including coefficients of variation; CV = SD/mean) from all testers grouped by their level as a proxy for experience. Notable is the relative uniformity of baseline mechanical thresholds among testers (one-way ANOVA on genotype: $F_{19,3622} = 23.0$) compared to thermal latencies (one-way ANOVA on genotype: $F_{22,4485} = 117.9$), although they are both highly statistically significant.

Figure 3 shows the data from the six most commonly represented mouse genotypes on each assay (those with >100 individual mice tested), along with CVs for the two most popular strains in both data sets (and in pain research more generally (Sadler et al., 2022)), CD-1 (Crl:CD1[ICR]) and C57BL/6 (a combination of C57BL/6J and C57BL/6NCrl substrains). Note that tester and strain differences are thoroughly conflated, as certain testers' experiments were focused on particular genotypes. Again, however, the relative uniformity of baseline mechanical thresholds among strains (one-way ANOVA on genotype: $F_{36,3605} = 16.0$) compared to thermal latencies (one-way ANOVA on genotype: $F_{21,4486} = 118.2$) is apparent.

3.5. Subject and Experimenter Sex

Overall, male and female mice displayed statistically equivalent baseline mechanical thresholds ($t_{3391} = 0.7$, p=0.48) (Fig. 4A), but female mice were significantly more sensitive (female: 11.1 s; male: 12.4 s) to thermal pain at baseline ($t_{4296} = 7.4$, p<0.0001) (Fig. 4B).

As we have previously reported (Sorge et al., 2014), male experimenters produce stress-induced analgesia in mice, and this manifests as higher apparent baseline mechanical thresholds ($t_{3567} = 5.0$, p<0.0001) and thermal latencies ($t_{4314} = 5.2$, p<0.0001) in mice tested by male versus female testers (Fig. 4C, D). The size of the difference is notable: male experimenters yield a 7.7% increase in baseline mechanical thresholds and a 9.4% increase in baseline thermal latencies compared to female experimenters. There was a significant interaction between tester sex and mouse sex for mechanical thresholds ($F_{1,3315} = 13.5$, p<0.0001) driven largely by a difference within female testers (male mice: 0.70 g; female mice: 0.77 g), but no such interaction for thermal latencies ($F_{1,4270} = 0.06$, p=0.81).

3.6. Regression Tree Analysis

To obtain a ranking of the variables affecting average baseline sensitivity, we performed regression tree analysis in R. The best-fit trees for von Frey and Hargreaves' test baseline data are shown as Supplementary Fig. 1. As shown in Table 1, variable importance scores derived from these trees reveal that for von Frey baseline thresholds, genotype explained more variance than tester, whereas for Hargreaves' test baseline latencies, tester and genotype explained equal (and higher) amounts of variance. For both measures, more variance was explained by the sex of the experimenter compared to the sex of the mouse.

3.7. Order of Measurements

Replicate measurements of baseline (average of left and right hind paws) mechanical thresholds and thermal latencies are shown in Figure 5. A repeated measures ANOVA on data from mice receiving all 6 (for von Frey) and all 8 (for Hargreaves') consecutive measurements revealed no significant effects of measurement at baseline for mechanical testing ($F_{5,1350} = 1.4$, $p=0.22$), but a significant repeated measures effect for thermal testing ($F_{7,19859} = 2.6$, $p=0.01$), such that withdrawal latencies decreased with repeated testing.

To assess variability independent of mean or sample size, we calculated the CV for each replicate measurement. For von Frey baseline replicates, CVs increased over the first four measurements, and then decreased (with much smaller sample sizes) thereafter. In contrast, the CVs of Hargreaves' test baseline replicates were maintained in a tight range over all eight measurements.

3.8. Hypersensitivity

Figures 6 and 7 show data from an analysis of mechanical and thermal hypersensitivity measurements, respectively (see Supplementary Data 3 and 4).

A comparison of peak mechanical hypersensitivity (at any post-injury time point) reveals higher modal levels in SNI versus CCI and ZYM versus CFA (Fig. 6A). Average mechanical hypersensitivity on the side contralateral to the injury (i.e., "mirror pain") was weak but statistically significant after SNI (one-sample *t*-test: $t_{821} = 5.8$, $p<0.001$) and CCI (($t_{172} = 2.7$, $p=0.008$) and statistically absent after CFA ($t_{857} = 51.2$, $p=0.24$) and ZYM ($t_{362} = 1.4$, $p=0.17$) (Fig. 6B). Clear evidence of the time course of these four pain-inducing insults was obtained, with SNI reaching peak levels of hypersensitivity by day 4 post-surgery and remaining stable thereafter (Fig. 6C), CCI reaching peak levels by day 7–10 post-surgery and then declining thereafter (Fig. 6D), CFA reaching peak levels by day 3 post-injection and declining thereafter (Fig. 6E), and ZYM hypersensitivity reaching peak levels by 3–4 hours post-injection and then declining at 6 hours.

A comparison of peak thermal hypersensitivity reveals high modal levels after CFA and CARR, and very weak levels (with considerable variability) after CCI (Fig. 7A). On the side contralateral to the injury (Fig. 7B), mice subjected to both CCI and CFA displayed statistically significant hypoalgesia ($t_{235} = 2.8$, $p=0.006$ and ($t_{165} = 2.6$, $p=0.01$, respectively), whereas mice subjected to CARR displayed hypersensitivity ($t_{75} = 2.8$, $p=0.006$). ZYM-injected mice showed no statistically significant changes on the contralateral hind paw ($t_{48} = 1.2$, $p=0.24$). In addition to displaying very weak peak thermal hypersensitivity, CCI-operated (Fig. 7C) and ZYM-injection mice displayed a flat time course (Fig. 7F). CFA produced thermal hypersensitivity that peaked on day 1 post-injection and decreased thereafter (Fig. 7D). CARR produced thermal hypersensitivity peaking at 3 hours post-injection, but no later time points were available (Fig. 7E).

4. Discussion

The data analyzed here are, to our knowledge, the largest set of retrospective preclinical pain data ever amassed, and all data points were collected in one of three adjacent rooms in a single building. All testers were trained and vetted by collecting pilot data judged to be sufficiently accurate and stable (i.e., having acceptably low intraindividual variability) prior to testing the mice comprising the data set analyzed here. Factors leading towards uniformity include the use of similar equipment and reagents (i.e., the same brand of von Frey filaments, the same method of von Frey threshold determination, the same model Hargreaves' test radiant heat device, the same doses of inflammatory agents, the same individual performing SNI and CCI surgeries), similar procedures (e.g., habituation time, criterion for non-response to von Frey fiber application, von Frey floor design, testing cubicle dimensions), and the fact that baseline and most post-injury measures reported here represent the grand average of multiple determinations on each hind paw. Factors leading towards variability include: the testing of different genotypes, cohorts, and sexes; the contribution of different testers of different sexes and experience level; and drift of actual stimulus intensity in aging equipment.

4.1. Normative baseline values

The median baseline mechanical withdrawal threshold of 3,642 mice was 0.74 g and the median baseline thermal withdrawal latency of 4,508 mice was 10.7 s. These absolute values obviously apply only to the use of Stoelting von Frey filaments with the up-down method, and an IITC radiant heat device set at 20% maximum intensity (≈35 W/mm^2) through a 0.5-cm-thick glass

floor, respectively. These are, however, commonly employed instantiations of these tests. More important, perhaps, is the variability observed, which is still quite large, even though all data were collected in essentially the same manner.

4.2. Tester effects

Tester effects were rather obvious in the von Frey and especially the Hargreaves' test data set. We do not believe this is largely due to differences in the criteria used to decide whether a mouse "responded" by withdrawing from the stimuli (although such determinations are wholly subjective), because all testers were trained and/or vetted by SS, who was trained by JR. Thus, JR's criteria were, in theory, applied to every mouse tested. Some of the tester differences are likely strain differences, of course. The large role of the tester in contributing to variability in preclinical data has been demonstrated previously for pain (Chesler et al., 2002a, 2002b) and more generally (Crabbe et al., 1999).

Using level (undergraduate, graduate, postdoctoral, career research associate) as a proxy for experience testing mice on the von Frey and Hargreaves' assays, we found some evidence that von Frey data collected by undergraduates was more variable (CV=0.51) than those collected by testers at other levels (CV=0.42–0.44). It is true that undergraduates on average had less experience when collecting these data. However, no such difference was seen on the Hargreaves' test, which is likely easier to learn to perform. Differences among the other groups are complicated by the fact that level is not necessarily an accurate reflection of experience; for example, certain postdoctoral fellows were new to these assays when they joined the lab.

4.3. Strain differences

Strain differences, especially among inbred mouse strains, have long been demonstrated in the pain field (Mogil et al., 1999). In fact, a surprising result from the current analysis is the relative uniformity of baseline mechanical thresholds among strains, especially compared to baseline thermal latencies. A perusal of Figure 3 suggests that in the von Frey test, within-strain variability predominates whereas in Hargreaves' test between-strain variability predominates.

Comparing within-strain variability (i.e., CV) between outbred CD-1 and inbred C57BL/6 mice revealed no evidence for tighter, or less variable, data using inbred mice. In fact, on Hargreaves' test it was the outbred strain showing a substantially lower CV. These findings are in accord with biomedical literature at large, in which there is no empirical evidence that the lack of genetic variance leads to lower phenotypic variance in inbred mice (Tuttle et al., 2018).

4.4. Mouse and tester sex

Sex differences in pain are receiving increased attention in recent years because of new funding agency mandates regarding sex as a biological variable (e.g., Clayton & Collins, 2014), and it has long been known that women are more sensitive to pain than men (see Mogil, 2012b). The situation in mice is more complicated, as sex differences are entirely strain-dependent (Mogil et al., 2000). We found no overall sex difference in baseline mechanical sensitivity but observed a robust sex difference in baseline thermal sensitivity, with females more sensitive than males. This pattern contrasts with the situation in humans, in which sex differences to experimental pressure pain are generally larger than those for heat pain (Riley III et al., 1998). Within this overall conclusion, however, sex x strain interactions can still be found. For example, in terms of mechanical sensitivity, C57BL/6 mice show no sex difference (males: 0.72 ± 0.02 g; females:

0.74 ± 0.02 g), but CD-1 male mice (0.73 ± 0.01 g) are significantly more sensitive than CD-1 female mice (0.80 ± 0.01 g). Note that regardless of sex differences in pain sensitivity *per se*, evidence continues to be amassed showing robust, qualitative sex differences in pain biology (see Mogil, 2020).

Surprisingly, tester sex appears to influence baseline pain sensitivity more than the sex of the mouse itself. For both mechanical and thermal pain, mice of both sexes tested by men are less sensitive (by ≈8%) than those tested by women. This is because male experimenters produce olfactorily mediated stress in mice and rats (Faraji et al., 2022; Georgiou et al., 2022; Sorge et al., 2014), leading to stress-induced analgesia (see Butler & Finn, 2009). That is, the *true* baseline sensitivity is that produced by female testers; male testers' baselines are inflated the stress-induced analgesia unless extensive habituation occurs.

4.5. Replicate determinations of pain sensitivity

In theory, taking more than one measurement of withdrawal threshold or latency is a good idea for increasing accuracy; for example because of the influence of the mouse's current behavioral state at the precise moment of stimulus impact on pain sensitivity (Callahan et al., 2008). On the other hand, replicate determinations might be associated with systematic changes due to the passage of time, habituation, and/or changing stress levels in the tested mice. Here we found no evidence of significant changes in withdrawal thresholds upon replicate von Frey application, but a consistent, albeit small, decrease in latencies with repeated Hargreaves' testing. In addition, repeated von Frey testing led to higher variability of later measurements, a shift not seen with Hargreaves' testing. Thus, it seems that replicate determinations may not be as advantageous for mechanical testing as they are for thermal testing, although why this might be remains unclear.

4.6. Pain hypersensitivity

The amount of hypersensitivity following hind paw inflammation or experimental nerve damage to nerves innervating the hind paw was assessed, and regardless of injury mechanical hypersensitivity (usually referred to as mechanical allodynia) appeared to be more robust overall than thermal hypersensitivity (usually referred to as thermal hyperalgesia). Not only were peak hypersensitivity scores higher for mechanical hypersensitivity (74–94% of maximum versus 24–67%), but CVs were lower (13–70% versus 49–229%). That SNI produces more mechanical allodynia than CCI is well known and a major reason for its popularity, and that SNI inconsistently results in thermal hyperalgesia was reported in the original paper describing the surgery (Decosterd & Woolf, 2000). Although CCI was originally characterized as featuring thermal hypersensitivity (in rats) (Bennett & Xie, 1988), the average maximal decrease reported in that paper was a ≈3-second decrease from a ≈10-second baseline, which is only slighter more robust than the ≈20% of maximal hypersensitivity seen here. We found that CFA produces less robust mechanical allodynia than ZYM, and less robust thermal hypersensitivity than CARR.

The current analysis makes plain the expected time course of hypersensitivity in these assays. The persistent nature of SNI mechanical hypersensitivity versus the resolution of other neuropathic pain states (including CCI) is well known and without adequate explanation, although ongoing experiments in our laboratory suggest the answer might involve differential levels of inflammation, which appears to be required to program pain resolution (Parisien et al., 2022). There is no similarly long-lasting inflammatory injury in common use, with even CFA hypersensitivity abating a week after injection. We have argued previously that important mechanisms contributing to chronic pain occur with a surprisingly long latency after injury

(Millecamps et al., 2023; Muralidharan et al., 2022), suggesting that preclinical pain research studies should probably last much longer than they currently do (modal duration after injury: 14-28 days (Sadler et al., 2022)).

4.7. Laterality and mirror pain

We found absolutely no evidence for lateralization of pain sensitivity, with equivalent mechanical thresholds and thermal latencies at baseline on the left versus right hind paw. In humans, there is some evidence for a left-sided predominance of pain in right-handed individuals (Brennum et al., 1989; Merskey & Watson, 1979). The 'pawedness' of mice is practically difficult to determine. We were unable to arrive at a robust conclusion regarding whether pain hypersensitivity is more robust when the injury is delivered to one paw or another because of our lab's almost complete (and arbitrary) preference for delivering that injury to the left side; an experiment is currently underway to address this question.

We were struck by the extreme variability of both mechanical and thermal hypersensitivity on the contralateral paw. Mirror pain is a long-studied phenomenon (see ref. Koltzenburg et al., 1999), the mechanism of which is still a source of considerable debate (e.g., Aloisi et al., 1993; Cheng et al., 2014; Coderre & Melzack, 1985). We previously observed that contralateral mechanical hypersensitivity is strain-dependent; in a survey of 31 inbred mouse strains, 13 displayed hypoalgesia on the contralateral side (as might be expected, since the ipsilateral side hurts when weight is borne on it) and 18 displayed contralateral allodynia, some quite robustly (Sorge et al., 2012).

4.8. Conclusions

We have argued previously that the reliance on mechanical and thermal evoked withdrawals as a measure of "pain" in preclinical experiments is far from optimal, given that these symptoms are rarer and less bothersome in chronic pain patients than spontaneous pain (Mogil, 2009, 2019; Mogil & Crager, 2004). Nonetheless, their use persists and will likely do so for some time. We hope that the analyses presented herein will lead to more thoughtful and effective use of these ubiquitous preclinical measures.

Figure Legends

Fig. 1. Grand average mechanical and thermal sensitivity of mice from repeated baseline measurements on the von Frey (VF) and Hargreaves' test (HT). A) Violin plot of VF baseline withdrawal thresholds (g) from 3,642 mice (see Supplementary Data 1), showing 25th, 50th (median), and 75th percentile values. B) Violin plot of HT baseline withdrawal latencies (s) from 4,508 mice (see Supplementary Data 2), showing 25th, 50th (median), and 75th percentile values. C) Baseline VF thresholds on the left versus right hind paw. D) Baseline HT latencies on the left versus right paw. Box plots in graphs C,D represent median and interquartile range. ns, not significant.

Fig. 2. Influence of tester (experimenter) on mechanical and thermal sensitivity. A,B) Baseline von Frey (VF; A) withdrawal thresholds (g) and Hargreaves' test (HT; B) withdrawal latencies (s) of mice tested by individual testers indicated by their initials; arranged in order of total sample size per tester. C,D) Baseline VF withdrawal thresholds (C) and HT withdrawal latencies (D) of all testers grouped by their level (UG, undergraduate; Grad, graduate student; PDF, postdoctoral fellow; RA, career Research Associate) as a proxy for experience. Coefficients of variation (CV=SD/mean) are provided for comparison purposes. Box plots represent median and interquartile range.

Fig. 3. Influence of genotype on mechanical and thermal sensitivity. A) VF withdrawal thresholds (g) of mice of the six most-common genotypes found in Supplementary Data 1. B) HT

withdrawal latencies (s) of mice of the six most-common genotypes found in Supplementary Data 2. Box plots represent median and interquartile range and are arranged in order of total sample size per genotype. Coefficients of variation (CV=SD/mean) are provided for comparison purposes.

Fig. 4. Influence of mouse sex and tester sex on mechanical and thermal sensitivity. A,B) Baseline von Frey (VF; A) withdrawal thresholds (g) and Hargreaves' test (HT; B) withdrawal latencies (s) of male and female mice. C,D) Baseline VF withdrawal thresholds (C) and HT withdrawal latencies (D) of mice of both sexes tested by male and female experimenters. Box plots represent median and interquartile range. ns, not significant. ****$p<0.0001$ as indicated.

Fig. 5. Influence of the order of repeated measurements of mechanical and thermal sensitivity. Baseline von Frey (VF; A) withdrawal thresholds (g) and Hargreaves' test (HT; B) withdrawal latencies (s) ordered by repeated measure instead of being averaged. Thus, BL1 represents the first of a series of repeated measures in the same testing session, BL2 represents the second, etc. Box plots represent median and interquartile range. Coefficients of variation (CV=SD/mean) are provided for comparison purposes.

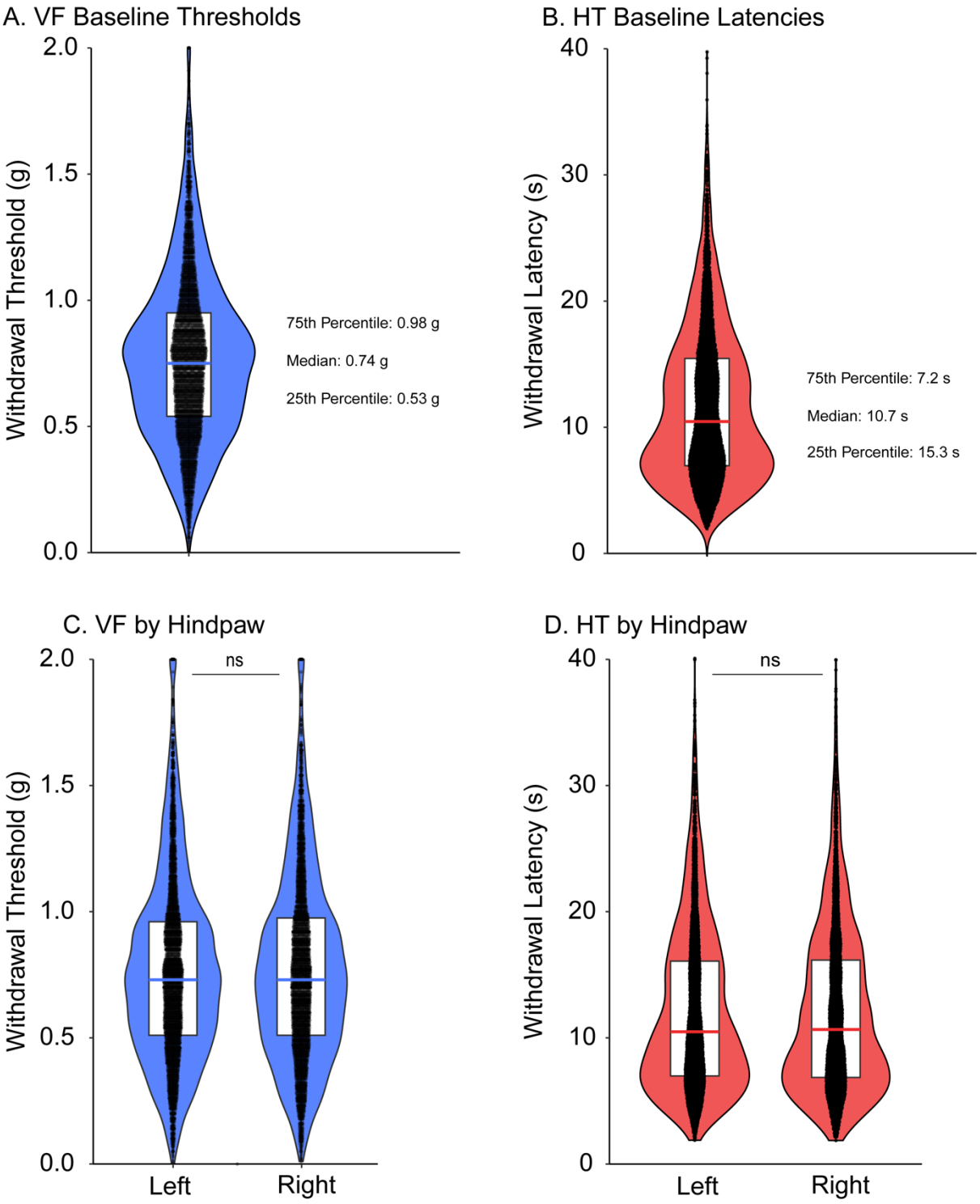
Fig. 6. Mechanical hypersensitivity after neuropathic and inflammatory injury in 2,717 mice (see Supplementary Data 3). A) Peak mechanical hypersensitivity (i.e., maximum hypersensitivity at any post-injury time point) of the hind paw ipsilateral to the injury in mice given spared nerve injury (SNI) or chronic constriction injury (CCI) surgeries, or complete Freund's adjuvant (CFA) or zymosan (ZYM) injections. B) Average mechanical hypersensitivity (of all post-injury time

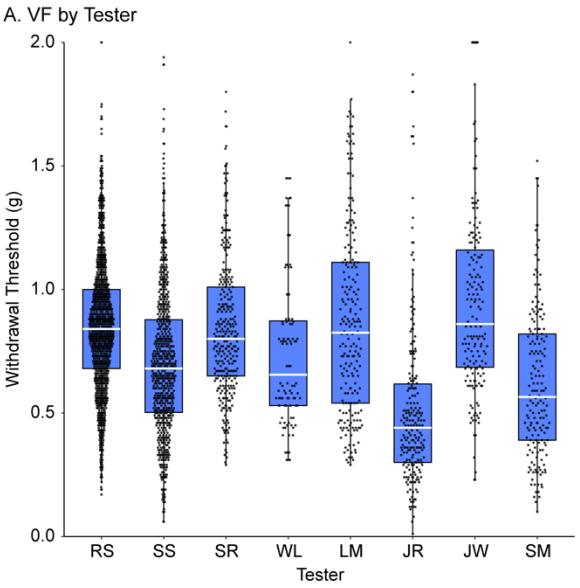
points) of the hind paw contralateral to the injury. Note that negative hypersensitivity represents hypoalgesia of the hind paw. C–F) Time course of SNI (C), CCI (D), CFA (E), and ZYM (F) hypersensitivity. Box plots represent median and interquartile range.

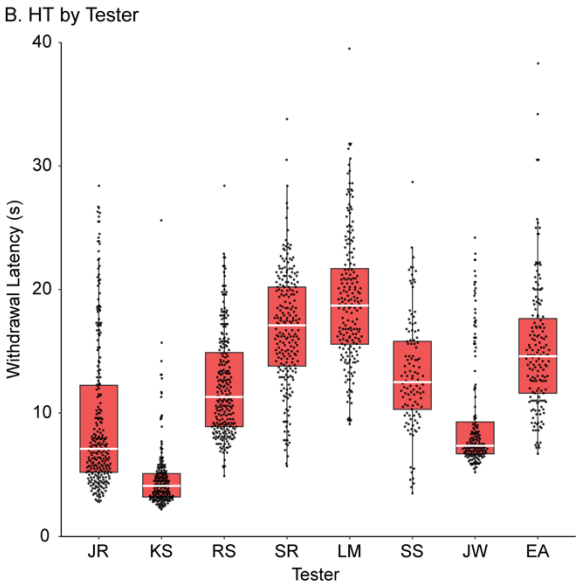
Fig. 7. Thermal hypersensitivity after neuropathic and inflammatory injury in 906 mice (see Supplementary Data 4). A) Peak thermal hypersensitivity (i.e., maximum hypersensitivity at any post-injury time point) of the hind paw ipsilateral to the injury in mice given chronic constriction injury (CCI) surgeries, or complete Freund's adjuvant (CFA), carrageenan (CARR), or zymosan (ZYM) injections. B) Average thermal hypersensitivity (of all post-injury time points) of the hind paw contralateral to the injury. Note that negative hypersensitivity represents hypoalgesia of the hind paw. C–F) Time course of CCI (C), CFA (D), CARR (E), and ZYM (F) hypersensitivity. Box plots represent median and interquartile range.

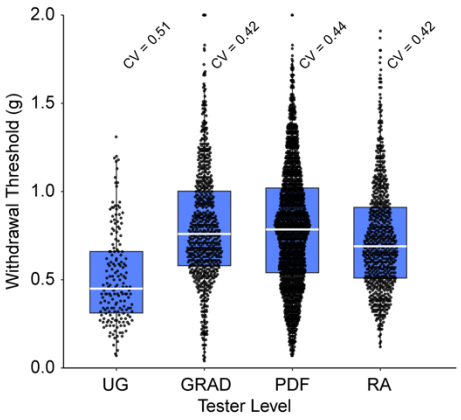
Table 1. Variable importance scores based on regression tree analysis.

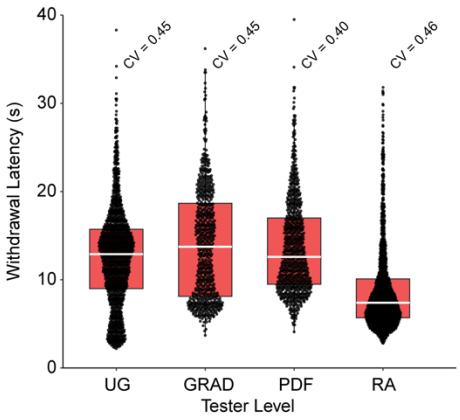
Measure	Variable	Variable Importance Score
von Frey	Genotype	23.3
	Tester	16.8
	Tester Sex	4.6
	Mouse Sex	1.3
Hargreaves'	Tester	36.5
	Genotype	36.4
	Tester Sex	7.2
	Mouse Sex	0.5

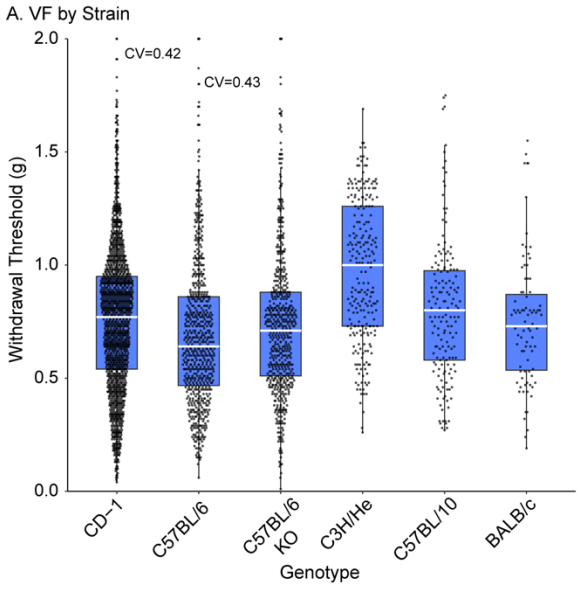

A. VF Baseline Thresholds
Withdrawal Threshold (g)
2.0
1.5
1.0
0.5
0.0
75th Percentile: 0.98 g
Median: 0.74 g
25th Percentile: 0.53 g
B. HT Baseline Latencies
Withdrawal Latency (s)
40
30
20
10
0
75th Percentile: 7.2 s
Median: 10.7 s
25th Percentile: 15.3 s
C. VF by Hindpaw
Withdrawal Threshold (g)
2.0
1.5
1.0
0.5
0.0
ns
Left
Right
D. HT by Hindpaw
Withdrawal Latency (s)
40
30
20
10
0
ns
Left
Right

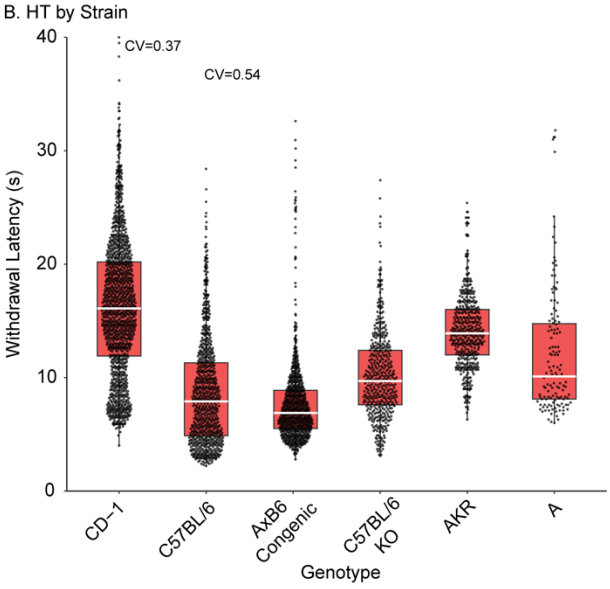

A. VF by Tester
Withdrawal Threshold (g)
2.0
1.5
1.0
0.5
0.0
RS
SS
SR
WL
LM
JR
JW
SM
Tester

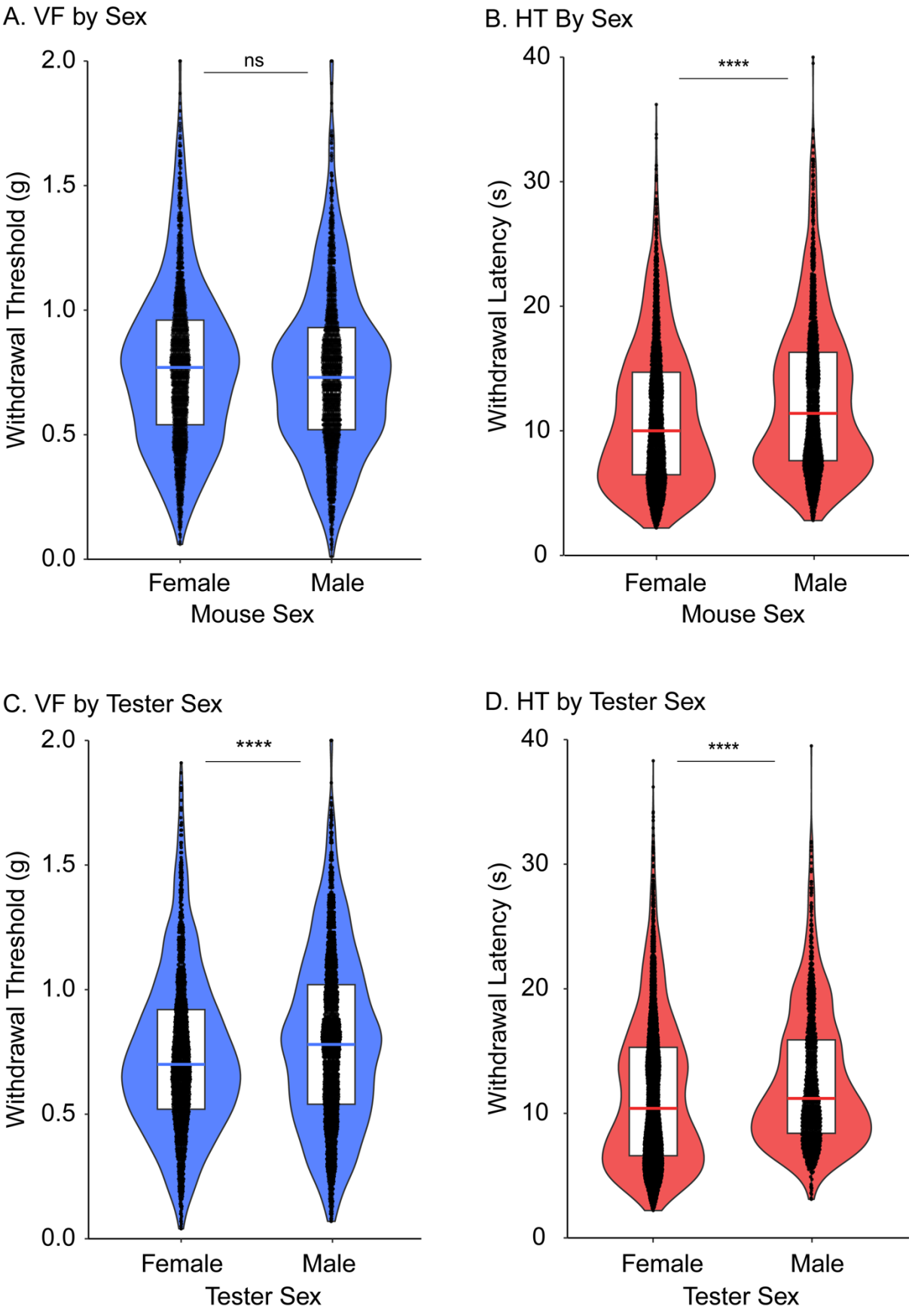

B. HT by Tester
Withdrawal Latency (s)
40
30
20
10
0
JR
KS
RS
SR
LM
SS
JW
EA
Tester

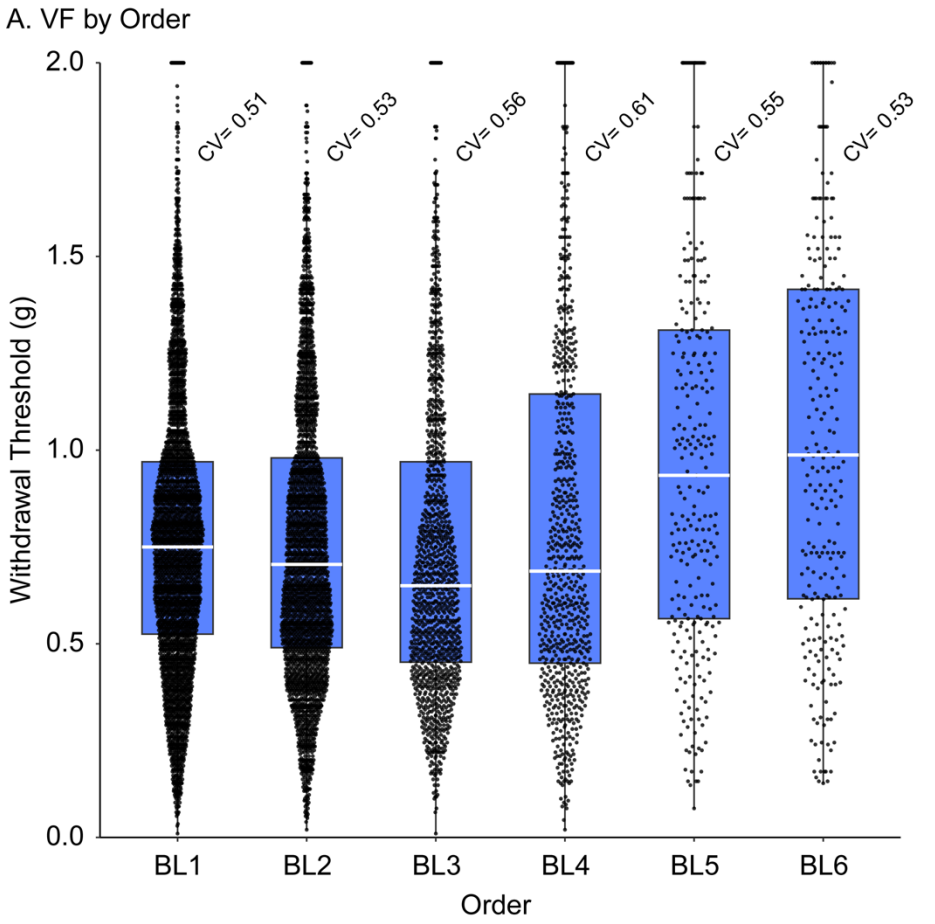

C. VF by Tester Level
Withdrawal Threshold (g)
2.0
1.5
1.0
0.5
0.0
CV = 0.51
CV = 0.42
CV = 0.44
CV = 0.42
UG
GRAD
PDF
RA
Tester Level

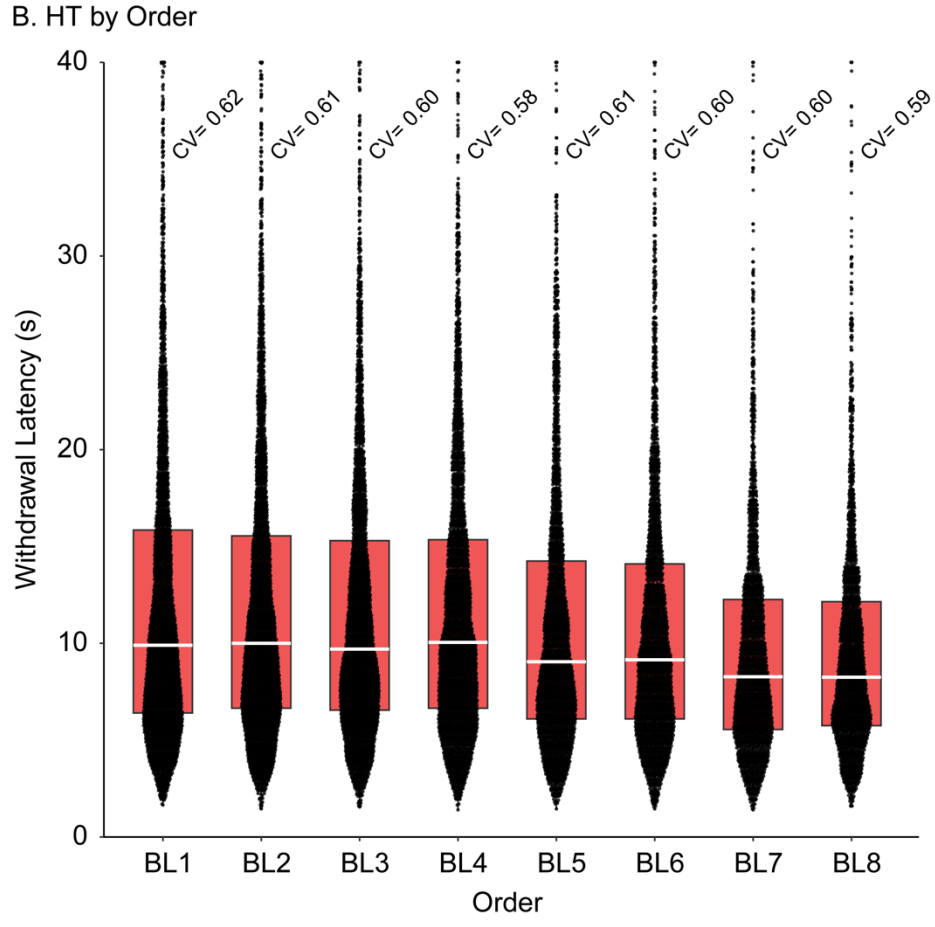

D. HT by Tester Level
Withdrawal Latency (s)
40
30
20
10
0
CV = 0.45
CV = 0.45
CV = 0.40
CV = 0.46
UG
GRAD
PDF
RA
Tester Level


A. VF by Strain
Withdrawal Threshold (g)
2.0
1.5
1.0
0.5
0.0
CV=0.42
CV=0.43
CD−1
C57BL/6
C57BL/6 KO
C3H/HeJ
C57BL/10
BALB/c
Genotype

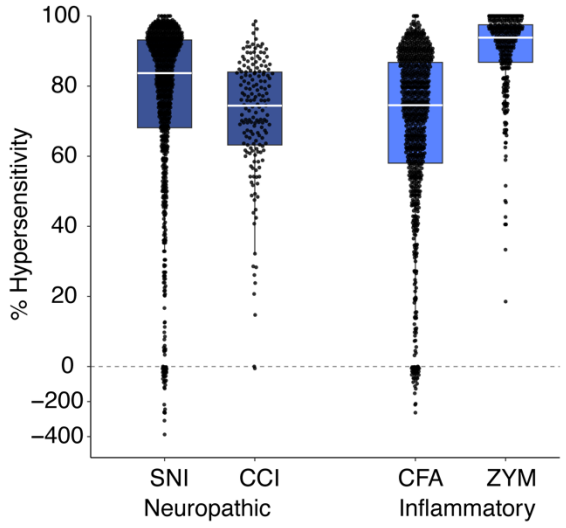

B. HT by Strain
Withdrawal Latency (s)
40
30
20
10
0
CV=0.37
CV=0.54
CD−1
C57BL/6
AxB6 Congenic
C57BL/6 KO
AKR
A
Genotype


A. VF by Sex
ns
Withdrawal Threshold (g)
2.0
1.5
1.0
0.5
0.0
Female
Male
Mouse Sex
B. HT By Sex
****
Withdrawal Latency (s)
40
30
20
10
0
Female
Male
Mouse Sex
C. VF by Tester Sex
****
Withdrawal Threshold (g)
2.0
1.5
1.0
0.5
0.0
Female
Male
Tester Sex
D. HT by Tester Sex
****
Withdrawal Latency (s)
40
30
20
10
0
Female
Male
Tester Sex

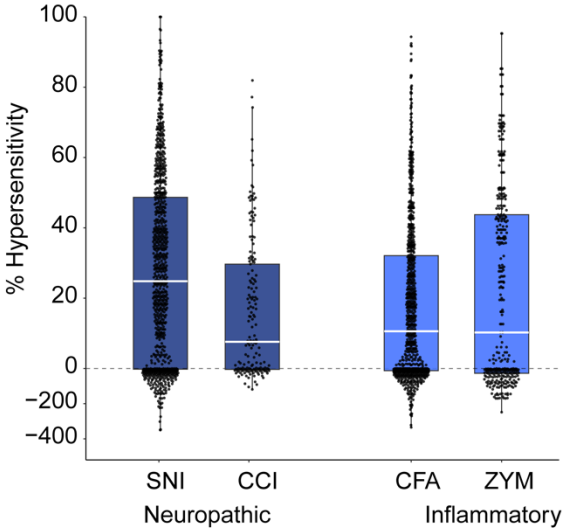

A. VF by Order
2.0
1.5
1.0
0.5
0.0
Withdrawal Threshold (g)
CV= 0.51
CV= 0.53
CV= 0.56
CV= 0.61
CV= 0.55
CV= 0.53
BL1
BL2
BL3
BL4
BL5
BL6
Order


B. HT by Order
40
30
20
10
0
Withdrawal Latency (s)
CV= 0.62
CV= 0.61
CV= 0.60
CV= 0.58
CV= 0.61
CV= 0.60
CV= 0.60
CV= 0.59
BL1
BL2
BL3
BL4
BL5
BL6
BL7
BL8
Order

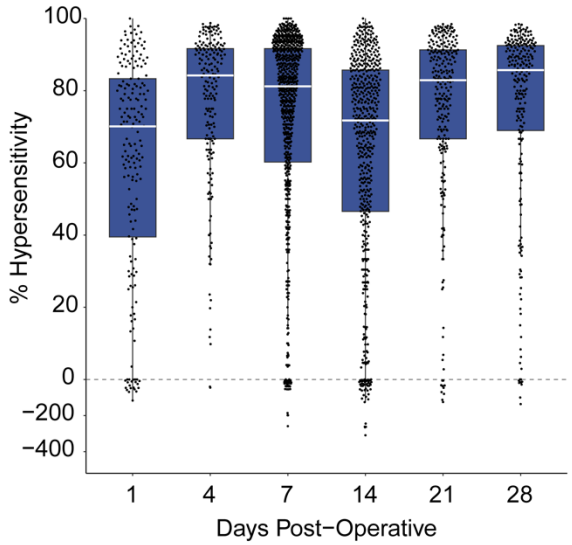
A. Peak Mechanical Hypersensitivity



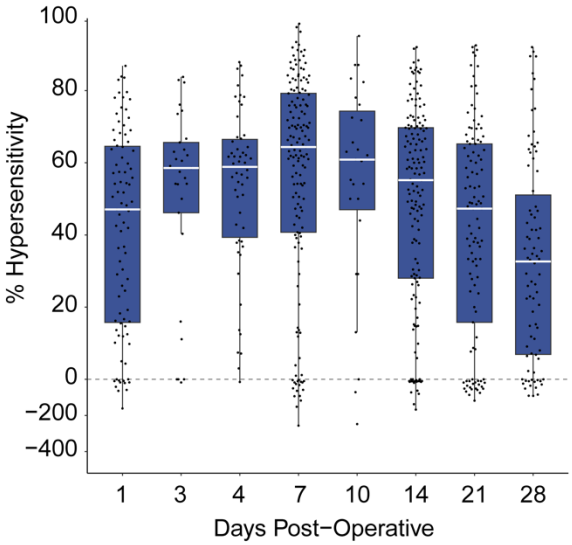
B. Contralateral Mechanical Hypersensitivity



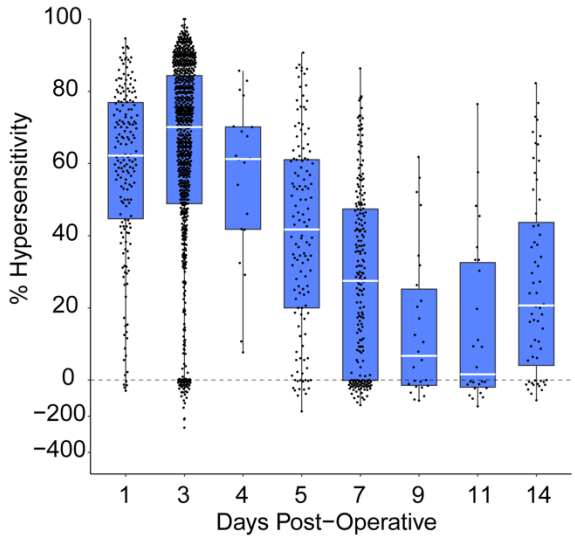
C. SNI Mechanical Hypersensitivity



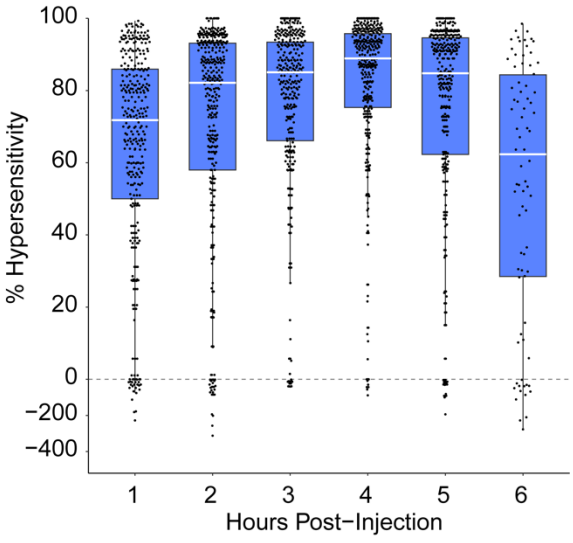
D. CCI Mechanical Hypersensitivity



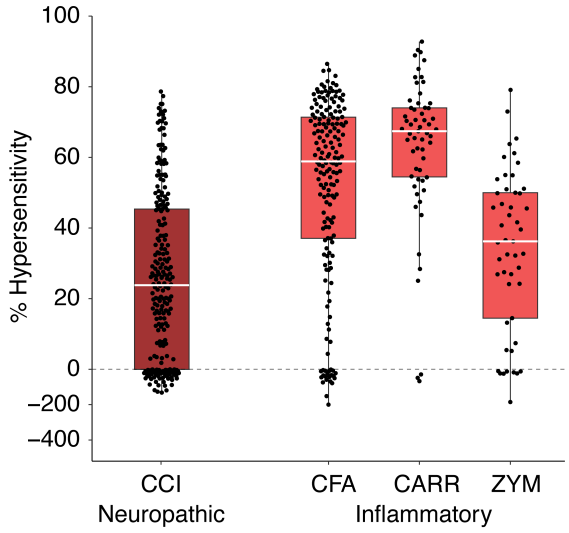
E. CFA Mechanical Hypersensitivity



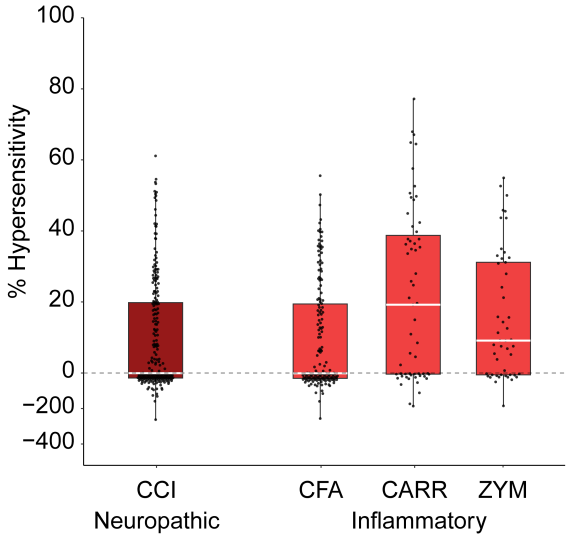
F. ZYM Mechanical Hypersensitivity



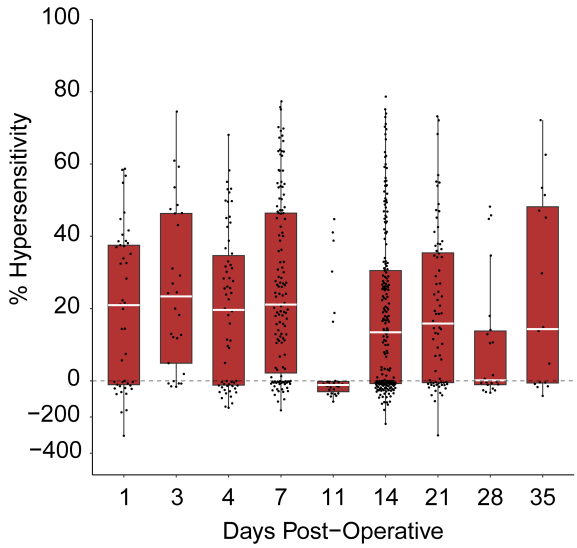
A. Peak Thermal Hypersensitivity



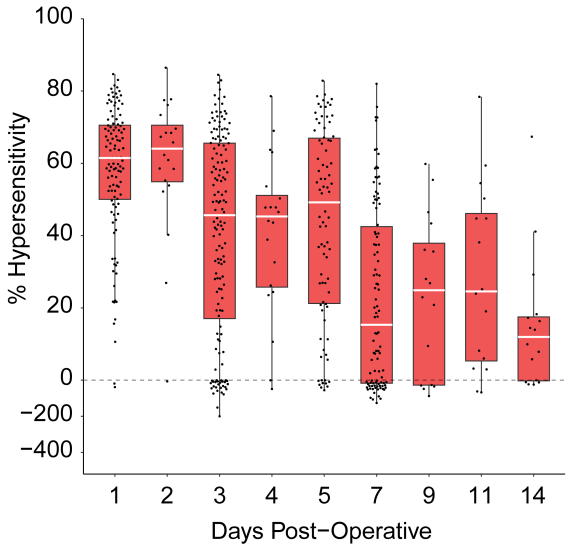
B. Contralateral Thermal Hypersensitivity



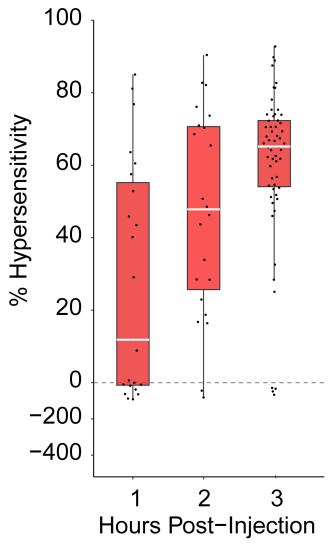
C. CCI Thermal Hypersensitivity



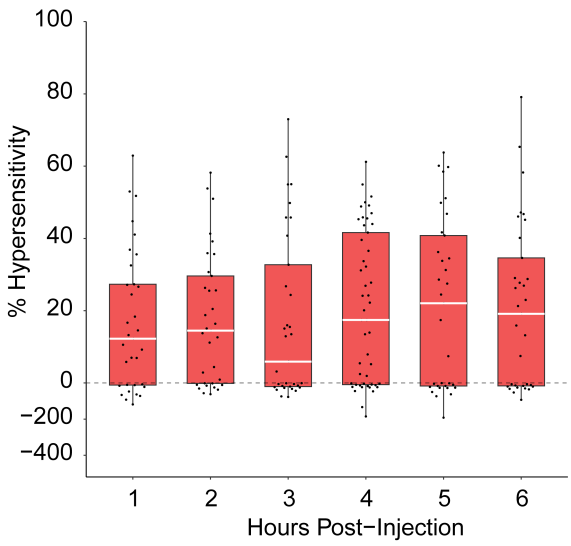
D. CFA Thermal Hypersensitivity



E. CARR Thermal Hypersensitivity



F. ZYM Thermal Hypersensitivity



Bridging Text between Chapter 2 and Chapter 3

Chapter 2 highlights the extensive variability found in classic preclinical algesiometry methods such as the von Frey and radiant heat paw-withdrawal tests. This variability underscores the necessity for more reliable and comprehensive methods to assess pain, particularly those that can effectively capture both sensory and emotional dimensions. Chapter 3 examines the neuroanatomical substrates of pain-induced facial grimacing in mice. This chapter aims to dissect the sensory and affective components of pain by focusing on the Mouse Grimace Scale (MGS) as a measure of pain affect. The use of Designer Receptors Exclusively Activated by Designer Drugs (DREADDs) allows for precise inhibition of targeted brain areas, offering a sophisticated approach to understanding the contribution of these regions to pain related facial expression. By targeting the thalamus and primary somatosensory cortex for sensory-discriminative aspects and the insular cortex, central amygdala, and anterior cingulate cortex for affective-motivational aspects, this study aims to delineate the distinct contributions of these regions to the pain affect as measured by the MGS and reflexive pain behaviors. The findings from this chapter provide critical insights into the neuroanatomical underpinnings of pain-induced facial expressions, ultimately contributing to a more nuanced understanding of pain as both a sensory and emotional experience, thus taking a step toward solving the issues brought up in chapter 2.

Chapter 3

Affective and Sensory Neuroanatomical Substrates of Pain-Induced Facial Grimacing

Alicia S. Zumbusch, Elodie Nickner, Sijie Xu, Dana Harrell, Susana Sotocinal, Milan Valyear, Jonathan Britt, Jeffrey S. Mogil

Abstract

Pain is a multidimensional experience involving sensory-discriminative (SD) and affective-motivational (AM) components. While the SD component is linked to the sensory properties of pain, the AM component is related to the emotional suffering associated with pain. This study aims to explore the neuroanatomical underpinnings of pain-induced facial grimacing in mice using chemogenetic inhibition. By targeting specific regions associated with SD (thalamus, primary somatosensory cortex) and AM (anterior insula, central amygdala, rostral anterior cingulate cortex) components, we assess their effects on facial grimacing and reflexive pain behaviors. Our findings suggest that facial grimacing is predominantly related to the affective dimension of pain, offering a translationally relevant, non-verbal measure of spontaneous pain.

Introduction

Pain serves an adaptive role by protecting an organism from harm, despite its unpleasant nature. The dual sensory and emotional experience of pain was formally identified (Melzack & Casey, 1968) as the sensory-discriminative (SD) and affective-motivational (AM) aspects of pain. This theoretical framework is reflected in the International Association for the Study of Pain's definition of pain as a "sensory and emotional experience" (Raja et al., 2020). Despite extensive research, there is still no consensus on the extent to which the sensory and emotional aspects of pain are dissociable or how other components, such as cognitive and social aspects, contribute to the pain experience.

The sensory signal of pain ascends via the spinothalamic tract (STT) to the thalamus and primary somatosensory cortex (S1), which are responsible for the sensory-discriminative aspects of pain, such as location, intensity, and nature (De Ridder et al., 2021; Rainville, 2002; Renthal, 2020). Positron emission tomography (PET) studies have shown that while sensory areas remain unchanged, affective regions like the anterior cingulate cortex (ACC) correlate with pain unpleasantness (Rainville et al., 1997). This separation is crucial for pain treatment and emergency medical diagnoses (Beveridge, 1999; Cordell et al., 2002; Janati et al., 2018). The affective component of pain, responsible for the suffering associated with pain sensation, follows a separate pathway via the spinoparabrachial tract to the parabrachial nuclei and subsequently to limbic structures such as the amygdala and nucleus accumbens (Deng et al., 2020) (Gauriau & Bernard, 2002) (Renthal, 2020); (Yan et al., 2022) (Yang et al., 2021)). Studies have shown that pain-related affect is mediated by regions like the anterior insula and ACC (Rainville, 2002) (Renthal, 2020). Fear of pain and activity avoidance can significantly impact quality of life (Crombez et al., 1999) (Zale et al., 2013).

Measuring Affect and Pain

Preclinical models of emotion rely on behaviors presumed to relate to emotion, but their validity in human pain treatment is uncertain (Commons et al., 2017). In humans, emotion research ranges from inventories (e.g., Beck's Depression Inventory) to physiological measures (e.g., blood pressure, skin conductance). Non-verbally dependent measures of affect are needed for translational relevance in both humans and animals.

Widely used pain measurement techniques, such as the tail-flick and hot-plate tests in rodents, primarily capture transient sensory aspects of pain (Mogil, 2022). These methods are inadequate for evaluating ongoing clinical pain. The development of the Mouse Grimace Scale (MGS) offers a non-verbal, phylogenetically conserved measure of spontaneous pain across species (Mogil et al., 2020). The MGS assesses facial action units, providing a rich gradient of pain expression over time, which is essential for integrating advanced techniques like chemogenetics and optogenetics.

Regions of Interest

The above section briefly covers the ascending pain pathways and key supraspinal brain structures involved in pain processing, identifying regions contributing to sensory-discriminative (SD) and affective-motivational (AM) aspects. Because our aim is to determine the degree to which facial grimacing does indeed predominantly reflect the AM as opposed to the SD component of pain, this study focuses on specific areas within the pain matrix that predominantly contribute to either the sensory-discriminative or affective-motivational components of pain. The SD regions targeted include the thalamus and primary somatosensory cortex, while the AM regions include the insula, cingulate, and amygdala. Given the functional heterogeneity of most brain regions including those listed above, we further focus on specific subnuclei within these

regions, which literature has shown to be critical in their respective pain processes. This approach helps elucidate how pain-induced facial grimacing reflects sensation versus affect.

Sensory-Discriminative Regions

Thalamus (Ventral Posterior Nucleus)

The thalamus, crucial for sensory and pain processing, contains several nuclei with distinct functions. The ventral posterior nucleus (VPN), including the ventral posterolateral (VPL) and ventral posteromedial (VPM) subnuclei, is the primary relay for somatosensory information, transmitting nociceptive input from the spinal cord and trigeminal system (Jones, 2007; (Giesler et al., 1994). The medial and lateral thalamic nuclei further integrate and transmit nociceptive signals to cortical regions associated with pain perception (Hirsch & Burkhalter, 2016).

The medial thalamus projects to the anterior cingulate cortex (ACC) and the rostral anterior insular cortex (rAI), which are involved in the affective-motivational aspects of pain. The lateral thalamus, particularly the ventrobasal complex, projects to the primary somatosensory cortex (S1), conveying sensory-discriminative pain information (Andersson et al., 1997; Kenshalo & Isensee, 1983)Neurons in these nuclei show somatotopic organization, ensuring accurate localization of nociceptive stimuli (Apkarian et al., 2005). Recent studies highlight the role of VPL/VPM neurons in encoding the intensity and duration of noxious stimuli, critical for discriminative pain perception (Apkarian et al., 2005); Alvarez et al., 2019).

Primary Somatosensory Cortex (S1 Trunk Region)

The primary somatosensory cortex (S1) is essential for encoding and discriminating the sensory qualities of noxious stimuli. Located in the postcentral gyrus of the parietal lobe, S1 receives

direct input from thalamic nuclei, particularly the VPN, conveying nociceptive signals from the periphery (Jones, 2007). S1 comprises distinct cytoarchitectonic areas, including Brodmann areas 3a, 3b, 1, and 2, each with specialized functions in somatosensory processing (Kaas, 2012). Area 3b is associated with processing nociceptive and tactile stimuli, while areas 1 and 2 integrate additional sensory modalities, contributing to the multimodal representation of pain perception within S1 (Chudler & Dong, 1995)

Nociceptive stimuli elicit robust responses in S1, with neurons displaying receptive fields corresponding to the location and modality of noxious input (Iwamura et al., 1993). S1 neurons also exhibit specificity in discriminating sensory qualities such as intensity, duration, and thermal properties (Mouraux et al., 2011). The precise somatotopic organization of S1 ensures accurate localization of nociceptive stimuli, a crucial factor in the sensory-discriminative aspect of pain (Mountcastle, 1957). To avoid potentially altering the mouse's ability to exhibit grimacing by inadvertently inhibiting proprioceptive feedback of the face, we specifically inhibited the relevant sub-area of S1 for the pain model used (i.e., trunk area due to using the visceral pain model of intraperitoneal acetic acid).

Affective-Motivational Regions

Insular Cortex (Rostral Anterior Insula)

The insular cortex is a key region for affective pain processing, divided into anterior and posterior regions. The anterior insula, encompassing the agranular and dysgranular cortices, is further subdivided into rostral anterior and dorsal anterior regions (Nieuwenhuys, 2012). Neuroimaging studies show increased insular activation during acute and chronic pain, with the anterior insula involved in processing the affective dimension (Wiech & Tracey, 2009). Lesions or

disruptions in insular activity alter affective pain responses, highlighting its significance in emotional processing (Craig, 2009) (Langford et al., 2010)

The rostral anterior insula (rAI) integrates emotional and interoceptive information, making it critical for affective processing (Chang et al., 2013). This region receives projections from the ventral posterolateral nucleus of the thalamus and connects to limbic structures like the amygdala and ACC (Augustine, 1996). Activation of the insula during pain elicits autonomic and endocrine responses, regulating emotional states (Phan et al., 2002). The insula also has reciprocal connections to prefrontal cortical areas involved in the cognitive appraisal and regulation of affect (Wiech & Tracey, 2009).

Amygdala (Central Nucleus of the Amygdala)

The amygdala, particularly the central nucleus (CeA), is extensively researched in emotional processing and plays a significant role in pain modulation (Neugebauer, 2015). The CeA is divided into lateral (CeL) and medial (CeM) regions, with the CeL integrating nociceptive information and linking to emotional pain responses, while the CeM is involved in behavioral aversion and autonomic regulation (Johansen, 2010). Both regions receive inputs from multiple pain-processing brain areas, including the thalamus, prefrontal cortex, insular cortex, and periaqueductal gray (PAG).

Electrophysiological studies show that CeA neurons increase firing rates in response to aversive stimuli, including nociceptive inputs (Neugebauer et al., 2004). Optogenetic manipulations of CeA neurons modulate pain affect, further highlighting its role in affective processing (Johansen, 2010). Behavioral studies indicate that optogenetic activation of the CeA

enhances anxiety and pain behaviors, whereas inhibition reduces them (Carrasquillo & Gereau, 2007). Human studies support these findings, with increased fMRI activity in the CeA correlating with heightened pain-related fear and anxiety in chronic pain patients (Loggia et al., 2015). Activation of CeA projections to the PAG elicits defensive behaviors and autonomic responses, facilitating avoidance of aversive stimuli (Han et al., 2015). Conversely, inhibition of CeA activity reduces pain-induced fear and enhances pain tolerance ("The amygdala and persistent pain - PubMed," 2004 Jun). Pharmacological inhibition of CRF receptors in the CeA decreases pain and anxiety in animal models (Ji & Neugebauer, 2007). Neuromodulation techniques like deep brain stimulation (DBS) of the amygdala are being explored to alleviate chronic pain and its affective components (Zhang et al., 2021).

Anterior Cingulate Cortex (Rostral ACC)

The anterior cingulate cortex (ACC), especially its rostral region, is crucial for affective pain processing. Neuroimaging studies consistently show increased rACC activation during pain. For example, Rainville et al. (1997) found that pain-related unpleasantness correlated with rACC activity using PET. The rACC is also involved in pain modulation, with studies showing that placebo-induced pain relief is associated with increased rACC activity (Wager et al., 2004). The rACC connects to various pain-processing brain regions, including the PAG, thalamus, amygdala, and prefrontal cortex. Resting-state fMRI studies show altered connectivity between the rACC and the default mode network (DMN) in chronic pain patients, which may underlie persistent affective and motivational disturbances (Kong et al., 2010).

The rACC's role in processing the anticipatory aspect of pain emotionality supports the dichotomy between pain affect and pain sensation. Anticipation of pain can occur without actual

pain, allowing for safer assumptions about alterations in brain or behavior being related to pain affect rather than pain sensation. Preclinical studies in non-human primates show selective activation of rACC neurons in pain avoidance behaviors but not reward behaviors (Kayoma et al., 1998, 2000, 2001). Lesion studies suggest that the rACC is involved in the motivational drive to avoid pain, as ablation impairs learning and performing tasks requiring pain avoidance (Johansen et al., 2001). Activation of the rACC can reduce amygdala activity, modulating fear and anxiety associated with pain (Etkin, 2011).

Materials and Methods

Pain Expression

Stereotaxic Surgery

Adult male and female CD-1 mice were anesthetized with 2% isoflurane in oxygen (0.8 L/min flow rate). Adequate anaesthetic plane was met if there was a complete absence of plantar reflex in both hind paws, lack of blink reflex in response to gentle touch with a cotton swab and application of ocular lubricant, absence of whisking or withdrawal response to tail pinch, and appropriate rate of respiration as outlined by the McGill University Comparative Medicine and Animal Resources Centre (CMARC). We then head-fixed mice into the stereotaxic apparatus. Once adequately anesthetized, mice were secured in the stereotaxic apparatus (Stoelting, Woodlane, IL) such that the skull was horizontally level on the medial-lateral and rostral-caudal planes. Next, hair was removed from the scalp with forceps and ethanol (90%) and iodine will be used to disinfect the scalp before a ~1-cm sagittal incision was made in the scalp along the midline and the incision will be held open by bulldog clamps (Fine Science Tools). We verified the horizontality of the skull on the rostral-caudal axis by ensuring that the DV coordinate ~2

mm anterior to bregma was within at ± 0.2 μm range of the DV coordinate ~2 mm posterior to lambda. The DV of two coordinates at approximately ± 4 mm from the mid-sagittal cranial suture will be compared to verify the horizontality of the skull along the medial-lateral axis. Using a dental drill 0.2-mm burr holes will be drilled above the rostral anterior insula (rAI) or the central amygdala (CeA). A Hamilton microsyringe (33 G) was secured in the left and right arms of the stereotaxic apparatus and loaded with .4 μl of the inhibitory DREADD AAV-hSyn-hM4D(Gi)-mCherry (Addgene, Watertown, MA). The needle was then lowered into the rAI, CeA, rACC, S1 or thalamus. The virus was delivered bilaterally by gradually injecting .1μl at the initial DV coordinates and then subsequently retracting the needle by -.1 to 0.2 μm and a further 0.1 μl will be injected at each stop until all the virus had been delivered (Table 1). At that point, the needle was left in place for 10 min to allow for the virus to diffuse. After diffusion, the needle was slowly removed over the course of another 10 min. The scalp was then sutured with a 1-0 cutting needle pre-threaded with dissolvable suture material (Ethicon) using a simple-interrupted pattern of three single-throw surgeon's knots for each stitch. The mice were monitored on a heating source until recovery, at which point the mouse was returned to its home cage.

Table 1

Coordinates for Designer Receptors Exclusively Activated by Designer Drugs (DREADDs) Surgeries

	Angle°	AP	ML	DV
rAi	10	2.0	+/-3.07	-3.3 to -2.5
CeA	5	-1.1	+/-2.7	-4.6 to -3.8
ACC	10	1.7	+/-0.5	-2.0 to -1.7
S1	5	-1.5	+/-1.5	-1.5 to -1.2
Thalamus	10	-1.7	+/-2.5	-3.75 to -3.45

Note. Anterior and posterior (AP), medial and lateral (ML), dorsal and ventral (DV). Rostral anterior insula (rAi), central amygdala (CeA), anterior cingulate cortex (ACC), primary somatosensory cortex (S1). All coordinates above begin at the point of origin, bregma.

Video Capture and Pain Model Induction

Overview

After 3–6 weeks to allow for viral transfection of the DREADD (depending on the size of the area more or less diffusion was required) mice were randomly assigned to either the control group or the experimental group. Mice in both groups were subject to an initial baseline recording (BL; 1 h) after which all mice received a 0.2 mL i.p. injection of acetic acid to produce the characteristic reflexive “writhing” behaviours that accompany this type of visceral abdominal pain. Mice in the experimental group received also immediately received a follow-up injection of 3 mg/kg of the DREADD ligand clozapine-N-oxide (CNO) in a 5% solution of DMSO in saline. Control mice were injected with the same volume of the DMSO solution only. Directly after

receiving the two injections described mice the underwent post pain video recording (1 h; POST).

Video Capture

Mice were placed mice singly in custom stainless steel and Plexiglas observation cubicles (9 × 5 × 5 cm high) with HD digital video cameras positioned perpendicular to the cubicle facing the mouse sufficiently far to capture the entire cubicle and a 1cm perimeter outside. Facial grimacing and reflexive writhing behaviour were recorded and stored on SD cards before being stored and processed as described below.

Image Generation, Standard MGS Scoring and Reflexive Pain Behaviour

We grabbed frames from video taken before and after model induction at ~2-min intervals. Image captures were then cropped so the resultant JPEG file did not reveal body position. Blind scoring of the pictures was done as previously described (Langford et al., 2010) to get an average baseline and post-induction MGS score for each of the five action units (cheek, whiskers, nose, ears, eyes) as well as an overall score. A separate observer also analyzed the videos to quantify abdominal constrictions (writhes). Writhing is a specific behaviour characterized by the lengthwise stretching of the torso and concave dorsiflexion of the back similar to lordosis posture observed in sexually receptive female mice (Langford et al., 2010). Reflexive writhing behaviour was quantified by sampling post-pain induction videos every 60s and simply recording if the mouse was writhing or not. The total samples with writhes were then added up and recorded.

Results

Data were analyzed and figures were created in GraphPad Prism (version 10). We analyzed the raw scores on the MGS by comparing the averages for each action unit as well as the average MGS score and also reflexive writhing behaviour. MGS Score is the mean of the action units across all of the sampled sections of video. We then subtracted the pre-pain baseline period from the post-pain period of the acetic acid writhing test to yield a change score (**Δ** MGS).

Affective ROIs

Rostral Anterior Insula (rAI)

A one-tailed t-test comparing pre-pain baselines to post-pain induction revealed a main effect of pain in all AU's and overall MGS scores for the control group *(*$t_{V\text{-}ORB\text{-}BL}$ (10) = 7.21, p<.0001; $t_{V\text{-}NOSE\text{-}BL}$ (9) =4.22, p<.01; $t_{V\text{-}WHIS\text{-}BL}$(7) =9.29, p<.0001; $t_{V\text{-}EARS\text{-}BL}$(10) =2.80, p<.01; $t_{V\text{-}CHKS\text{-}BL}$ (8) =2.93, p<.01; $t_{V\text{-}AVG\text{-}BL}$ (10) =9.65, p<.0001) and most AU's of the experimental group ($t_{C\text{-}ORB\text{-}BL}$ (11) = 3.92, p<.01; $t_{C\text{-}NOSE\text{-}BL}$ (11) =2.16, p<.05; $t_{C\text{-}WHIS\text{-}BL}$ (9) =10.0, p<.0001; $t_{C\text{-}EARS\text{-}BL}$ (11) =.610, p=ns; $t_{C\text{-}CHKS\text{-}BL}$ (9) =3.30, p<.01; $t_{V\text{-}AVG\text{-}BL}$ (11) =6.61, p<.0001) demonstrating that the acetic acid produced an effect on each of the action units of the rAI mice in both groups, excluding the ears AU in the experimental group.

There was a significant interaction between group and pain *(*t(21) =1.950, p<.05; Fig 1. B) such that the control group (M_{VEH}= .613, SD_{VEH}= .211) showed a significantly higher overall **Δ** MGS score than the experimental group (M_{CNO}= .422, SD_{CNO}=.255) after pain induction. There was no significant interaction between group and pain on reflexive writhing behaviour (t (31) =1.289, p=ns; M_{VEH}= 13.93, SD_{VEH}= 8.689; M_{CNO}=9.944, SD_{CNO}=8.987; Fig 1. C).

For the **Δ** nose (t (19)=1.94, p<.05; M_{VEH}= .435, SD_{VEH}= .275; M_{CNO}= .187, SD_{CNO}= .300; Fig 1. E) and **Δ** ears (t (21)=2.281, p<.05; M_{VEH}= .2586, SD_{VEH}= .3061; M_{CNO}= .02858, SD_{CNO}=

.1620; Fig 1. H) AU's there was a significant interaction between group and pain such that the control group showed a significantly higher overall **Δ** MGS score than the experimental group after pain induction. For the **Δ** orbital AU there was a trend towards an interaction between group and pain *(t* (21)=1.20, *p*=ns; Fig 1. D) such that the control group (M_{VEH}= 1.04, SD_{VEH}= .480) showed a tendency towards a higher overall **Δ** orbital AU score than the experimental group (M_{CNO}= .751, SD_{CNO}= .664). There was not a significant interaction between group and pain for the **Δ** whiskers score *(t (*16)= .1443, *p*=ns; M_{VEH}= .7819, SD_{VEH}=.2383; M_{CNO}= .7987, SD_{CNO}= .2515; Fig 1. F) or for the **Δ** cheeks score (*t* (19)=.01650, *p*=ns, M_{VEH}= .4883, SD_{VEH}=.4831; M_{CNO}= .4916, SD_{CNO}= .4437; Fig 1. G).

Central Amygdala (CeA)

A one-tailed t-test comparing pre-pain baselines to post-pain induction revealed a main effect of pain in the following action units and overall MGS score for both the control group ($t_{V\text{-}ORB\text{-}BL}$ (9) = 6.92, *p*<.001; $t_{V\text{-}NOSE\text{-}BL}$ (7) =5.14, *p*<.001; $t_{V\text{-}WHIS\text{-}BL}$(7) =6.64, *p*<.0001; $t_{V\text{-}EARS\text{-}BL}$(9) = 2.65, *p*<.05; $t_{V\text{-}CHKS\text{-}BL}$ (7) =3.06, *p*<.01; $t_{V\text{-}AVG\text{-}BL}$ (9) =10.0, *p*<.0001) and the experimental group ($t_{C\text{-}ORB\text{-}BL}$(7) = 5.80, *p*<.001; $t_{C\text{-}NOSE\text{-}BL}$(7) =3.13, *p*<.01; $t_{C\text{-}WHIS\text{-}BL}$(6)=2.75, *p*<.05; $t_{V\text{-}AVG\text{-}BL}$ (7) =5.83, *p*<.001). The ears AU in the experimental group was trending towards significance ($t_{C\text{-}EARS\text{-}BL}$ (7) =1.19, *p*=ns) however the cheeks AU appeared not significant ($t_{C\text{-}CHEEKS\text{-}BL}$(6) =.537, *p*=ns).

There was a significant interaction between group and pain *(t (*15) = 2.265, *p<.05*, Fig 2. B) such that the control group (M_{VEH}= .6431, SD_{VEH}= .2030) showed significantly higher overall **Δ** MGS scores than the experimental group (M_{CNO}= .4103, SD_{CNO}= .2167) after pain induction. For writhing behaviour, there was no significant interaction between group and pain *(t (*18) = .552, *p*=ns; M_{VEH}= 11.2, SD_{VEH}= 9.39; M_{CNO}= 13.4, SD_{CNO}=8.78, Fig 2. C).

For the **Δ** cheeks AU there was a significant interaction between group and pain (t (12)=1.90, p<.05; Fig 2. G) such that the control group (M_{VEH}= .360, SD_{VEH}= .333) showed significantly higher overall **Δ** cheeks AU scores than the experimental group (M_{CNO}= .0262, SD_{CNO}= .316) only after pain induction. For the **Δ** orbital (t (15)=.375, p=ns; M_{VEH}= .985, SD_{VEH}= .450; M_{CNO}= .900, SD_{CNO}= .478, Fig 2. D), **Δ nose** (t(13)=1.11, p=ns; M_{VEH}= .453, SD_{VEH}= .249; M_{CNO}= .299, SD_{CNO}= .287, Fig 2. E), **Δ** ears (t (15)=1.17, p=ns; M_{VEH}= .414, SD_{VEH}= .494; M_{CNO}= .159, SD_{CNO}= .354, Fig 2. H), and **Δ** whiskers (t(3)=1.02, p=ns; M_{VEH}= .818, SD_{VEH}= .254; M_{CNO} .508, SD_{CNO}= .475, Fig 2. F) AU's there was no significant interaction between group and pain.

Rostral Anterior Cingulate Cortex (rACC)

A one-tailed t-test comparing pre-pain baselines to post-pain induction revealed a main effect of pain model in the following AU's and overall MGS score for both the control group ($t_{V\text{-}ORB\text{-}BL}$ (3) = 8.18, p<.01; $t_{V\text{-}NOSE\text{-}BL}$ (3) =5.44, p<.01; $t_{V\text{-}WHIS\text{-}BL}$(2) =18.8, p=<.01; $t_{V\text{-}CHKS\text{-}BL}$ (3) =4.72, p<.01; $t_{V\text{-}AVG\text{-}BL}$(3)=11, p<.001); and the experimental group ($t_{C\text{-}ORB\text{-}BL}$(3) = 18.1, p<.001; $t_{C\text{-}NOSE\text{-}BL}$(3) =3.59, p<.05; $t_{C\text{-}WHIS\text{-}BL}$(3)=11.8, p<.001; $t_{C\text{-}CHKS\text{-}BL}$(3) =5.02, p<.01; $t_{V\text{-}AVG\text{-}BL}$ (3) =20.0, p<.0001; demonstrating that the acetic acid did in fact produce an effect on these action units of the rACC mice. There was no main effect of pain model on the ears AU and overall MGS score in either the control ($t_{V\text{-}EARS\text{-}BL}$ (3) = 1.20, p=ns); or the experimental group ($t_{C\text{-}EARS\text{-}BL}$(3) =.1053, p=ns). There was no interaction between group and pain *(t (13*) = .2336, *p=ns*, Fig 3. B) for **Δ** MGS scores between the control group (M_{VEH}= .703, SD_{VEH}= .0834) and experimental group (M_{CNO}= .650, SD_{CNO}= .172). There was no significant interaction between group and pain *(t (13*) =.3059, p=ns, Fig 3. C) such that the control group (M_{VEH}= 16.3, SD_{VEH}=8.06) showed comparable writhing behaviour to the experimental group (M_{CNO}= 14.1, SD_{CNO}= 8.01).

For the **Δ** orbital AU there was a significant interaction between group and pain *(t (13*) = .4269, *p=ns*, Fig 3. D) such that the control group (M_{VEH}= 1.34, SD_{VEH}=.286) showed significantly higher overall **Δ** orbital AU scores than the experimental group (M_{CNO}= 1.30, SD_{CNO}= .395) after pain induction. The remaining **Δ** AU scores did not show a significant interaction between group and pain, **Δ** nose *(t (13*) = .1906, *p*=ns; M_{VEH}= .450, SD_{VEH}= .145, M_{CNO}= .543, SD_{CNO}= .235, Fig 3. E), **Δ** ears *(t (13*)=.0965, *p*=ns, M_{VEH}= .246, SD_{VEH}= .252, M_{CNO}= .108, SD_{CNO}= .126, Fig 3. H), **Δ** whiskers *(t (12*)=.3152, *p*=ns; M_{VEH}= 1.12, SD_{VEH}= .394; M_{CNO}= 1.01, SD_{CNO}= .416, Fig 3. F), **Δ** cheeks *(t (13*)=.1270, *p*=ns; M_{VEH}= .425, SD_{VEH}= .186; M_{CNO}= .279, SD_{CNO}= .271, Fig 3. G).

Sensory ROIs

Thalamus

A one-tailed t-test comparing pre-pain baselines to post-pain induction revealed a main effect of pain model in the following AU's and overall MGS scores for the control group ($t_{V\text{-}WHIS\text{-}BL}(2)$ =12.0, p<.01; $t_{V\text{-}CHKS\text{-}BL}(2)$ =11.8, p<.01; $t_{V\text{-}AVG\text{-}BL}(2)$ =4.71, p<.05) and the experimental group ($t_{C\text{-}ORB\text{-}BL}(3)$ = 2.94, p<.05; $t_{C\text{-}NOSE\text{-}BL}(3)$ =3.88, p<.05; $t_{C\text{-}WHIS\text{-}BL}(3)$=3.61, p<.05; $t_{C\text{-}CHKS\text{-}BL}(3)$ =4.45, p<.05; $t_{V\text{-}AVG\text{-}BL}(3)$ =3.89, p<.05) demonstrating that the acetic acid did in fact produce an effect on each of the action units of the thalamus mice in both groups. There was no main effect of pain model on the following AU's and overall MGS scores for the control group ($t_{V\text{-}ORB\text{-}BL}(2)$ = 2.00, p=.0921; $t_{V\text{-}NOSE\text{-}BL}(2)$ =1.87, p=ns; $t_{V\text{-}EARS\text{-}BL}(2)$ = 1.31, p=ns) and the experimental group ($t_{C\text{-}EARS\text{-}BL}(3)$ =1.61, p=ns).

There was not a significant interaction between group and pain *(t (22*) = .2249, *p*=ns, Fig 5. B) such that the control group (M_{VEH}=.6343, SD_{VEH}=.1750) did not show significantly higher

overall **Δ** MGS scores than the experimental group (M_{CNO}=.6927, SD_{CNO}= .1936) after pain induction. There was a significant interaction between group and pain *(t (18)* = .0525, *p=ns*, Fig 5. C) such that the control group (M_{VEH}= 13.0, SD_{VEH}= 5.48) showed significantly fewer writhes than the experimental group (M_{CNO}= 8.18, SD_{CNO}= 6.85).

There was not a significant interaction between group and pain for any of the **Δ** AU's; **Δ** orbital *(t (22)*=.4744, *p*=ns; M_{VEH}= 1.34, SD_{VEH}= .499; M_{CNO}= 1.33, SD_{CNO}= .439, Fig 5. D), **Δ** nose *(t (22)*=.2069, *p*=ns; M_{VEH}=.515, SD_{VEH}= .287; M_{CNO}= .605, SD_{CNO}= .245, Fig 5. E), **Δ** ears *(t (22)*=.4588, *p*=ns; M_{VEH}= .0956, SD_{VEH}= .156; M_{CNO}= .104, SD_{CNO}= .208, Fig 5. H), **Δ** whiskers *(t (22)*=.2356, *p*=ns; M_{VEH}= .893, SD_{VEH}= .521; M_{CNO}= 1.04, SD_{CNO}= .447, Fig 5. F), **Δ** cheeks *(t (22)*=.1615, *p*=ns; M_{VEH}= .841, SD_{VEH}= .489; M_{CNO}= .684, SD_{CNO}= .258, Fig 5. G) such that the control group and the experimental group did not show significantly different **Δ** AU scores after pain induction.

Primary Somatosensory Cortex (S1)

A one-tailed t-test comparing pre-pain baselines to post-pain induction revealed a main effect of pain model in all action units and overall MGS score for both the control group ($t_{V\text{-}ORB\text{-}BL}$ (14) = 8.57, p<.0001; $t_{V\text{-}NOSE\text{-}BL}$ (14) =4.78, p<.0001; $t_{V\text{-}WHIS\text{-}BL}$(10) =7.46, p<.0001; $t_{V\text{-}EARS\text{-}BL}$(14) = 2.64, p<.01; $t_{V\text{-}CHKS\text{-}BL}$ (10) =7.70, p<.0001; $t_{V\text{-}AVG\text{-}BL}$ (14) =7.66, p<.000); and the experimental group ($t_{C\text{-}ORBITAL\text{-}BL}$(11) = 9.96, p<.0001; $t_{C\text{-}NOSE\text{-}BL}$(11) =4.55, p<.001; $t_{C\text{-}WHIS\text{-}BL}$(8)=11.1, p<.0001; $t_{C\text{-}EARS\text{-}BL}$(11) =2.00, p<.05; $t_{C\text{-}CHKS\text{-}BL}$(8) =7.09, p<.0001; $t_{V\text{-}AVG\text{-}BL}$ (11) =11.4, p<.0001).

There was no significant interaction between group and pain (t (25) = .0962, p=ns, Fig 4. B) such that the control group (M_{VEH}= .599, SD_{VEH}= .303) did not show significantly higher overall **Δ** MGS scores than the experimental group (M_{CNO}= .609, SD_{CNO}= .186). There was a

significant interaction between group and pain for writhing behaviour *(t(*27) = 2.71, *p*<.01, Fig 4. C) such that the control group (M_{VEH}= 15.3, SD_{VEH}= 11.9) showed significantly fewer writhes than the experimental group (M_{CNO}= 5.00, SD_{CNO}= 7.96).

The pre-pain baseline and post-pain induction **Δ** MGS scores for all of the AU's in the control and experimental group were not significantly different from each other; **Δ** orbital *(t (*25)=.871, *p*=ns; M_{VEH} .937, SD_{VEH}= .424; M_{CNO}= 1.07, SD_{CNO}= .373, Fig 4. D), **Δ** nose *(t (*25) = .385, *p*=ns; M_{VEH}= .375, SD_{VEH}= .304; M_{CNO}= .421, SD_{CNO}= .320, Fig 4. E), **Δ** ears *(t (*25) = .319, *p*=ns; M_{VEH}= .153, SD_{VEH}= .224; M_{CNO}= .126, SD_{CNO}= .217, Fig 4. H), **Δ** whiskers *(t (*18) = 1.18, *p*=ns; M_{VEH}= .691, SD_{VEH}= .307; M_{CNO}= .836, SD_{CNO}=.226, Fig 4. F), **Δ** cheeks *(t (*18)=.875, *p=ns*; M_{VEH}= .373, SD_{VEH}= .161; M_{CNO}= .314, SD_{CNO}= .133, Fig 4. G).

Discussion

This set of studies examined the neuroanatomical underpinnings of pain-induced facial grimacing in mice, utilizing chemogenetic inhibition to probe the contributions of various brain regions associated with the sensory-discriminative (SD) and affective-motivational (AM) components of pain. Here we provided evidence that chemogenetic inhibition of known affective regions of the pain matrix attenuates the expression of pain-induced facial grimacing without altering reflexive pain behaviours. This reinforces previous evidence showing that facial grimacing predominantly reflects pain-related emotional suffering rather than the sensory aspects of pain (Langford et al., 2010)

The rAI integrates emotional and interoceptive information, making it crucial for affective processing (Craig, 2009); (Chang et al., 2013). Our findings align with previous studies demonstrating increased insular activation during pain and its role in the affective dimension of pain (Wiech & Tracey, 2009). The CeA's involvement in pain modulation and emotional

processing is well-documented (Neugebauer, 2015); (Johansen, 2010)). Our study confirms that CeA inhibition reduces facial grimacing, supporting its role in emotional responses to pain (Loggia et al., 2015). The rACC is associated with pain unpleasantness and anticipatory pain processing, which may explain why we did not observe any reduction in grimacing when the rACC was inhibited (Etkin, 2011; Rainville et al., 1997). In contrast, inhibition of regions predominantly associated with the SD component, such as the thalamus and primary somatosensory cortex (S1), did not significantly alter facial grimacing yet significantly decreases the amount of reflexive pain behaviour and in some cases almost completely ablating the behaviour. Thus, while these regions are critical for the sensory perception of pain, they do not appear to significantly influence the emotional expression of pain as captured by facial grimacing.

Our study primarily relied on facial grimacing and reflexive writhing as measures of pain. While these behaviors provide insights into the affective and sensory components of pain, they may not encompass the full spectrum of pain-related behaviors. Future studies could incorporate additional behavioural assays to provide a more comprehensive understanding of pain processing. Including other presumed measures of affect such as anxiety like behaviour (e.g., open field or EPM) or behavioural despair models such as forced swim or tail suspension may add to our understanding of what kind of relationship there is between facially expressed pain affect and other affective behaviours. Additionally, recapitulating our results using human-friendly techniques would serve to reinforce the translational utility of pain induced facial grimacing as our best measure of pain affect. Though of course, ethical considerations limit the feasibility of most direct experimental control of specific brain areas, except perhaps using tDCS for the more superficial cortical layers that we showed impact grimacing in mice (i.e., insular

cortex and anterior cingulate cortex). Importantly, the affective-motivational aspect of pain encompasses far more subtle and harder to measure aspects of non-sensory pain related behaviours such as the titular motivational aspects, as well as social or cognitive aspects. If future studies that examine the relationship between grimacing and other complex pain behaviours found a meaningful relationship between the two it could pave the way for use of the MGS in place of significantly more expensive, time-consuming, and resource-demanding motivational assays such as operant or Pavlovian tasks.

Our research contributes significantly to the understanding of pain processing by examining the roles of various brain regions in the emotional expression of pain as measured by the mouse grimaced scale. The validation of the MGS as a non-verbal measure of spontaneous pain enhances the toolkit available for preclinical pain research. This advancement facilitates the translation of animal findings to human pain conditions, which may help with the development of more effective pain management strategies. Additionally, highlighting the ability of the MGS to capture the emotional component of pain in mice will hopefully encourage researchers using other model organisms to consider incorporating it as a standard measure of pain affect alongside our classical sensory measures.

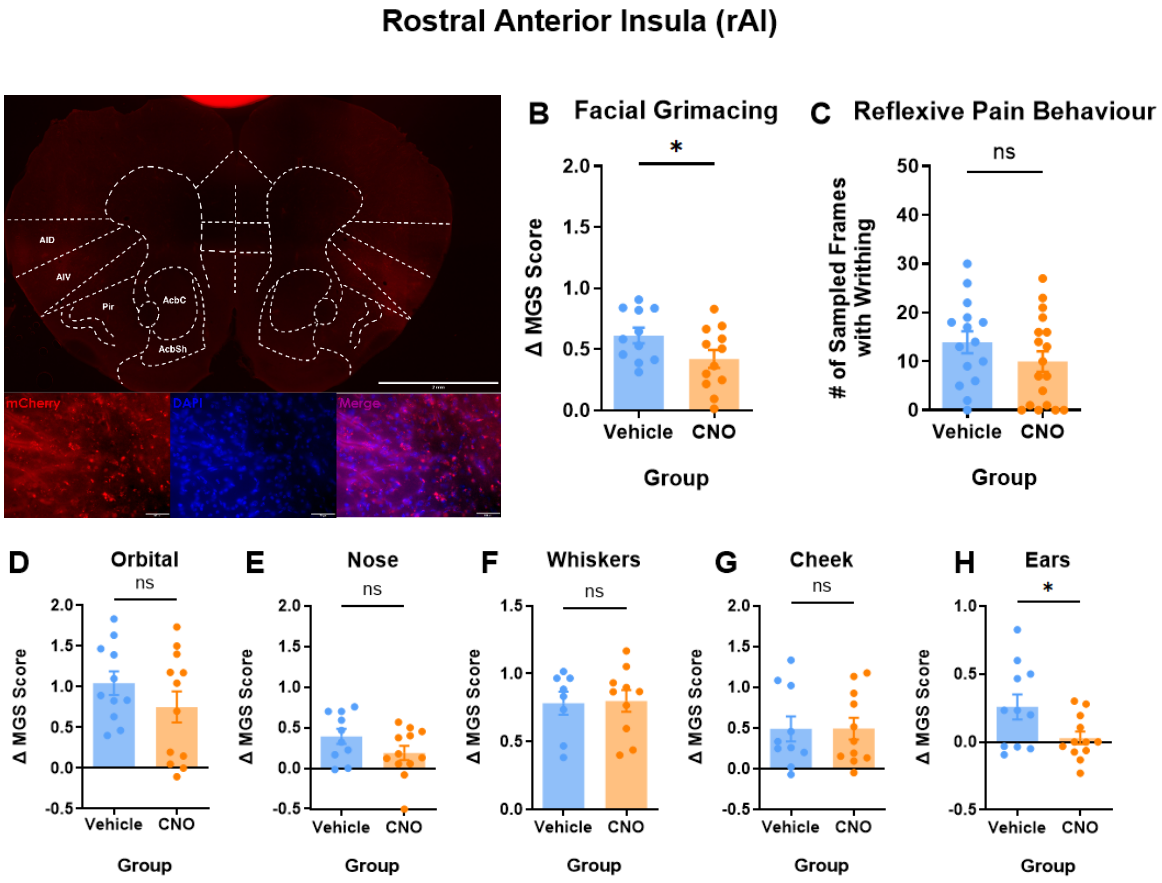


Figure 1: Change Score on Mouse Grimace Scale and Total number of Writhes for rostral anterior insula (rAI) **Chemogenetically Silencing the rAI Attenuates Pain Expression While Leaving Reflexive Pain Behaviour Unaltered - Fig 1. A** Representative image of DREADD fluorescence (mCherry) and co-stained with DAPI. **Fig 1. B** Mice that had their rAI chemogenetically inhibited (CNO group) showed lower facial grimacing then control mice after pain induction relative to baseline *(t (*21) =1.950, p<.05, M_{VEH}= .613, SD_{VEH}= .211, M_{CNO}= .422, SD_{CNO}=.255). **Fig 1. C** There was no significant difference in the number of writhes observed after pain induction between the control group and the CNO group (t (31) =1.289, p=ns; M_{VEH}= 13.93, SD_{VEH}= 8.689; M_{CNO}=9.944, SD_{CNO}=8.987). **Fig 1. D-H Individual Action Unit MGS Score.** Mice in the CNO group showed lower facial grimacing then control mice after pain induction relative to baseline. **Fig 1. D Δ** orbitals *(t* (21)=1.20, p=ns; M_{VEH}= 1.04, SD_{VEH}= .480; M_{CNO}= .751, SD_{CNO}= .664), **Fig 1. E Δ** nose (t (19)=1.94, p<.05; M_{VEH}= .435, SD_{VEH}= .275; M_{CNO}= .187, SD_{CNO}= .300), **Fig 1. F Δ** whiskers *(t (*16)= .1443, p=ns; M_{VEH}= .7819, SD_{VEH}=.2383; M_{CNO}= .7987, SD_{CNO}= .2515), **Fig 1. G Δ** cheeks (t (19)=.01650, p=ns, M_{VEH}= .4883, SD_{VEH}=.4831; M_{CNO}= .4916, SD_{CNO}= .4437), **Fig 1. H Δ** ears *(t (*21)=2.281, p<.05; M_{VEH}= .2586, SD_{VEH}= .3061; M_{CNO}= .02858, SD_{CNO}= .1620

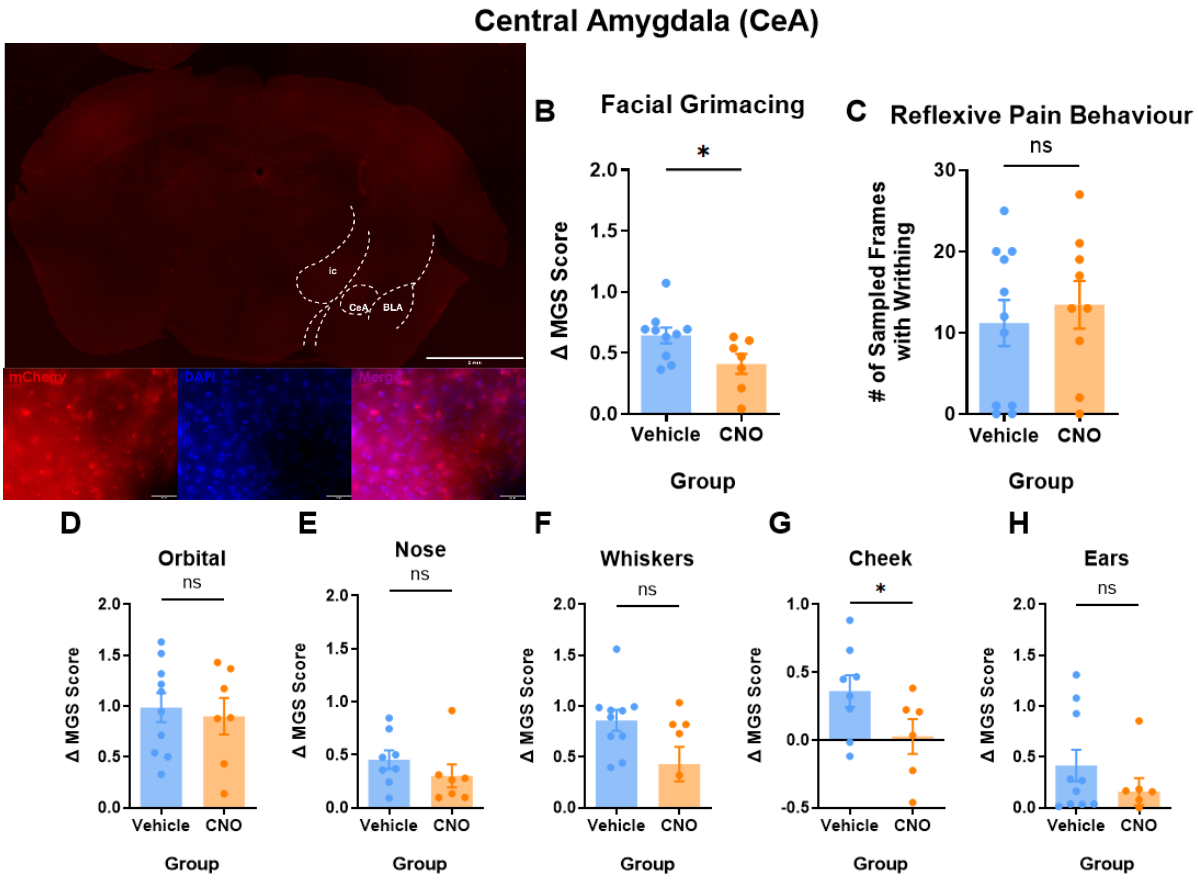


Figure 2: Change Score on Mouse Grimace Scale and Total number of Writhes for central amygdala (CeA) **Chemogenetically Silencing the CeA Attenuates Pain Expression While Leaving Reflexive Pain Behaviour Unaltered – Fig 2. A** Representative image of DREADD fluorescence (mCherry) and co-stained with DAPI. **Fig 2. B** Mice that had their CeA chemogenetically inhibited (CNO group) showed lower facial grimacing then control mice after pain induction relative to baseline *(t (*15) = 2.265, *p<.05*, M_{VEH}= .6431, SD_{VEH}= .2030, M_{CNO}= .4103, SD_{CNO}= .2167). **Fig 2. C** There was no significant difference in the number of writhes observed after pain induction between the control group and the CNO group *(t (*18) = .552, *p*=ns; M_{VEH}= 11.2, SD_{VEH}= 9.39; M_{CNO}= 13.4, SD_{CNO}=8.78). **Fig 2. D-H Individual Action Unit MGS Score.** Mice in the CNO group showed lower facial grimacing then control mice after pain induction relative to baseline. **Fig 2. D Δ** orbital (*t* (15)=.375, *p*=ns; M_{VEH}= .985, SD_{VEH}= .450; M_{CNO}= .900, SD_{CNO}= .478), **Fig 2. E Δ nose** (*t*(13)=1.11, *p*=ns; M_{VEH}= .453, SD_{VEH}= .249; M_{CNO}= .299, SD_{CNO}= .287), **Fig 2. F Δ** whiskers (*t* (3)=1.02, *p*=ns; M_{VEH}= .818, SD_{VEH}= .254; M_{CNO} .508, SD_{CNO}= .475), **Fig 2. G Δ** cheeks (*t* (12)=1.90, *p*<.05, M_{VEH}= .360, SD_{VEH}= .333, M_{CNO}= .0262, SD_{CNO}= .316), **Fig 2. H Δ** ears (*t* (15)=1.17, *p*=ns; M_{VEH}= .414, SD_{VEH}= .494; M_{CNO}= .159, SD_{CNO}= .354)

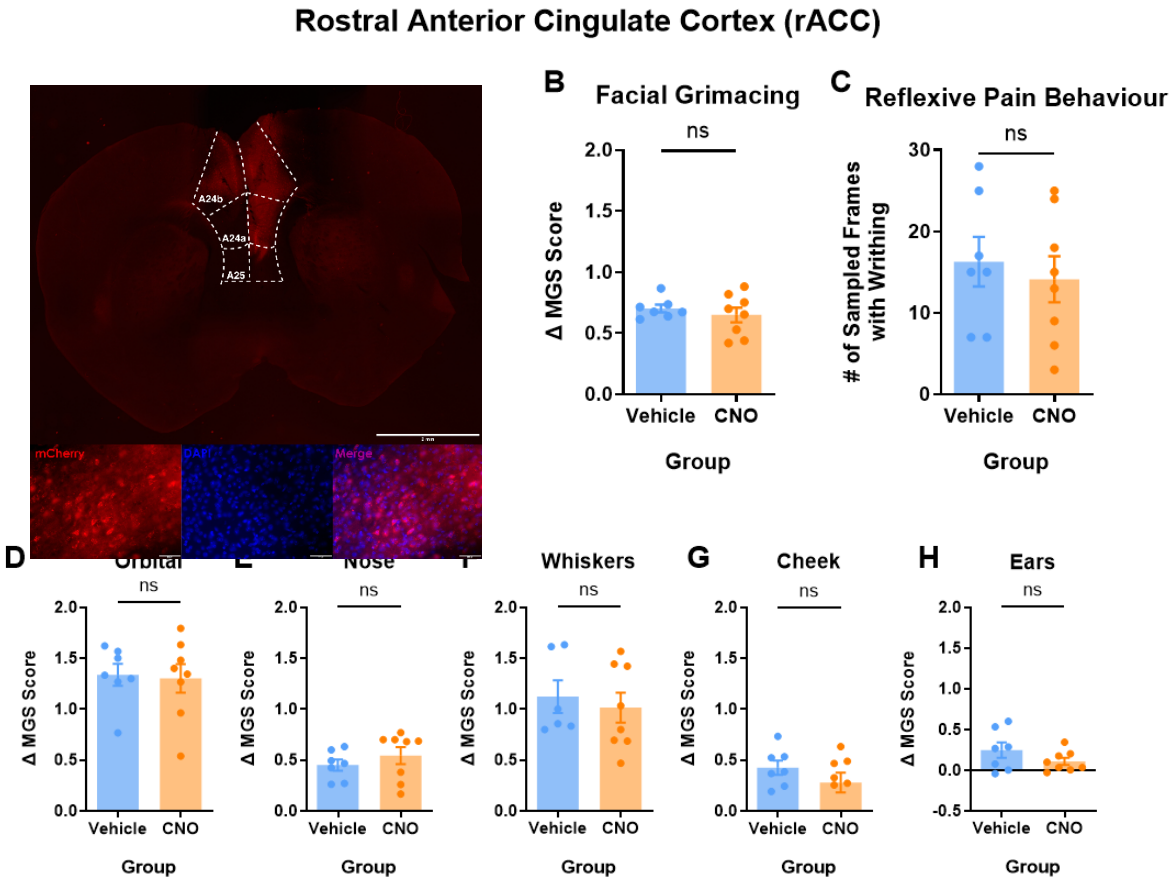


Figure 3: Change Score on Mouse Grimace Scale and Total number of Writhes for rostral anterior cingulate cortex (rACC) **Chemogenetically Silencing the rACC Attenuates Pain Expression While Leaving Reflexive Pain Behaviour Unaltered - Fig 3. A**. Representative image of DREADD fluorescence (mCherry) and co-stained with DAPI. **Fig 3. B** Mice that had their rACC chemogenetically inhibited (CNO group) showed lower facial grimacing then control mice after pain induction relative to baseline *(t (13)* = .2336, *p=ns*, M_{VEH}= .703, SD_{VEH}= .0834, M_{CNO}= .650, SD_{CNO}= .172). **Fig 3. C** There was no significant difference in the number of writhes observed after pain induction between the control group and the CNO group *(t (13)* =.3059, *p*=ns, M_{VEH}= 16.3, SD_{VEH}=8.06, M_{CNO}= 14.1, SD_{CNO}= 8.01). **Fig 3. D-H Individual Action Unit MGS Score.** Mice in the CNO group showed lower facial grimacing then control mice after pain induction relative to baseline. **Fig 3. D Δ** orbital *(t (13)* = .4269, *p=ns*, M_{VEH}= 1.34, SD_{VEH}=.286, M_{CNO}= 1.30, SD_{CNO}= .395), **Fig 3. E Δ** nose *(t (13)* = .1906, *p*=ns; M_{VEH}= .450, SD_{VEH}= .145, M_{CNO}= .543, SD_{CNO}= .235), **Fig 3. F Δ** whiskers *(t (12)*=.3152, *p*=ns; M_{VEH}= 1.12, SD_{VEH}= .394; M_{CNO}= 1.01, SD_{CNO}= .416), **Fig 3. G Δ** cheeks *(t (13)*=.1270, *p*=ns; M_{VEH}= .425, SD_{VEH}= .186; M_{CNO}= .279, SD_{CNO}= .271), **Fig 3. H Δ** ears *(t (13)*=.0965, *p*=ns, M_{VEH}= .246, SD_{VEH}= .252, M_{CNO}= .108, SD_{CNO}= .126).

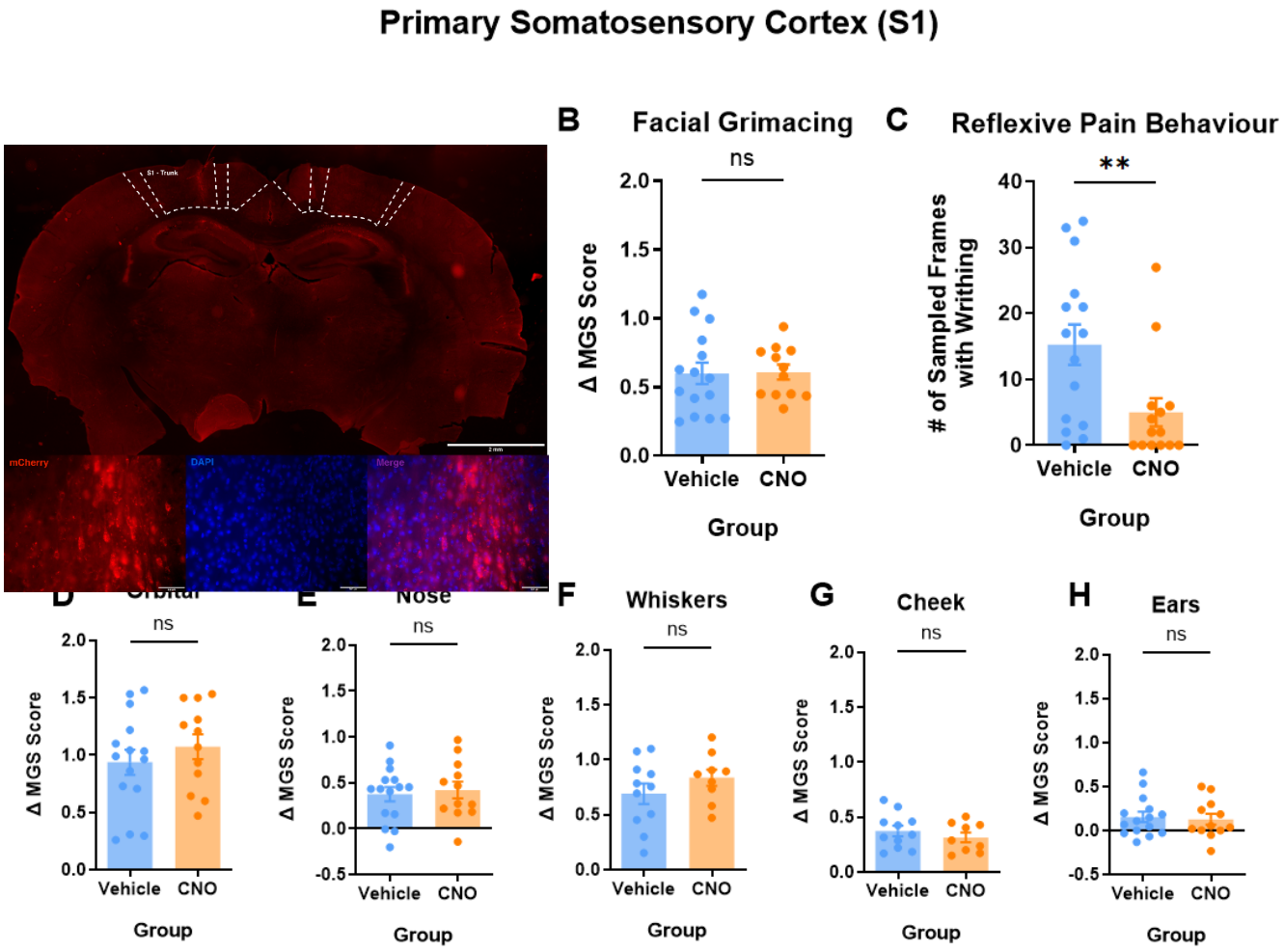


Figure 4: Change Score on Mouse Grimace Scale and Total number of Writhes for primary somatosensory cortex 1 (S1) **Chemogenetically Silencing the S1 Attenuates Pain Expression While Leaving Reflexive Pain Behaviour Unaltered - Fig 4. A** DREADD virus expression. Fluorescent image is representative brain showing viral vector transfection in the S1 with DAPI. **Fig 4. B** Mice that had their S1 chemogenetically inhibited (CNO group) showed lower facial grimacing then control mice after pain induction relative to baseline (t (25) = .0962, p=ns, M_{VEH}= .599, SD_{VEH}= .303, M_{CNO}= .609, SD_{CNO}= .186). **Fig 4. C** There was no significant difference in the number of writhes observed after pain induction between the control group and the CNO group (t (27) = 2.71, p<.01, M_{VEH}= 15.3, SD_{VEH}= 11.9, M_{CNO}= 5.00, SD_{CNO}= 7.96). **Fig 4. D-H Individual Action Unit MGS Score.** Mice in the CNO group showed lower facial grimacing then control mice after pain induction relative to baseline. **Fig 4. D Δ** orbital (t (25)=.871, p=ns; M_{VEH} .937, SD_{VEH}= .424; M_{CNO}= 1.07, SD_{CNO}= .373), **Fig 4. E Δ** nose (t (25) = .385, p=ns; M_{VEH}= .375, SD_{VEH}= .304; M_{CNO}= .421, SD_{CNO}= .320), **Fig 4. F Δ** whiskers (t (18) = 1.18, p=ns; M_{VEH}= .691, SD_{VEH}= .307; M_{CNO}= .836, SD_{CNO}=.226), **Fig 4. G Δ** cheeks (t (18)=.875, p=ns; M_{VEH}= .373, SD_{VEH}= .161; M_{CNO}= .314, SD_{CNO}= .133), **Fig 4. H Δ** ears (t (25) = .319, p=ns; M_{VEH}= .153, SD_{VEH}= .224; M_{CNO}= .126, SD_{CNO}= .217).

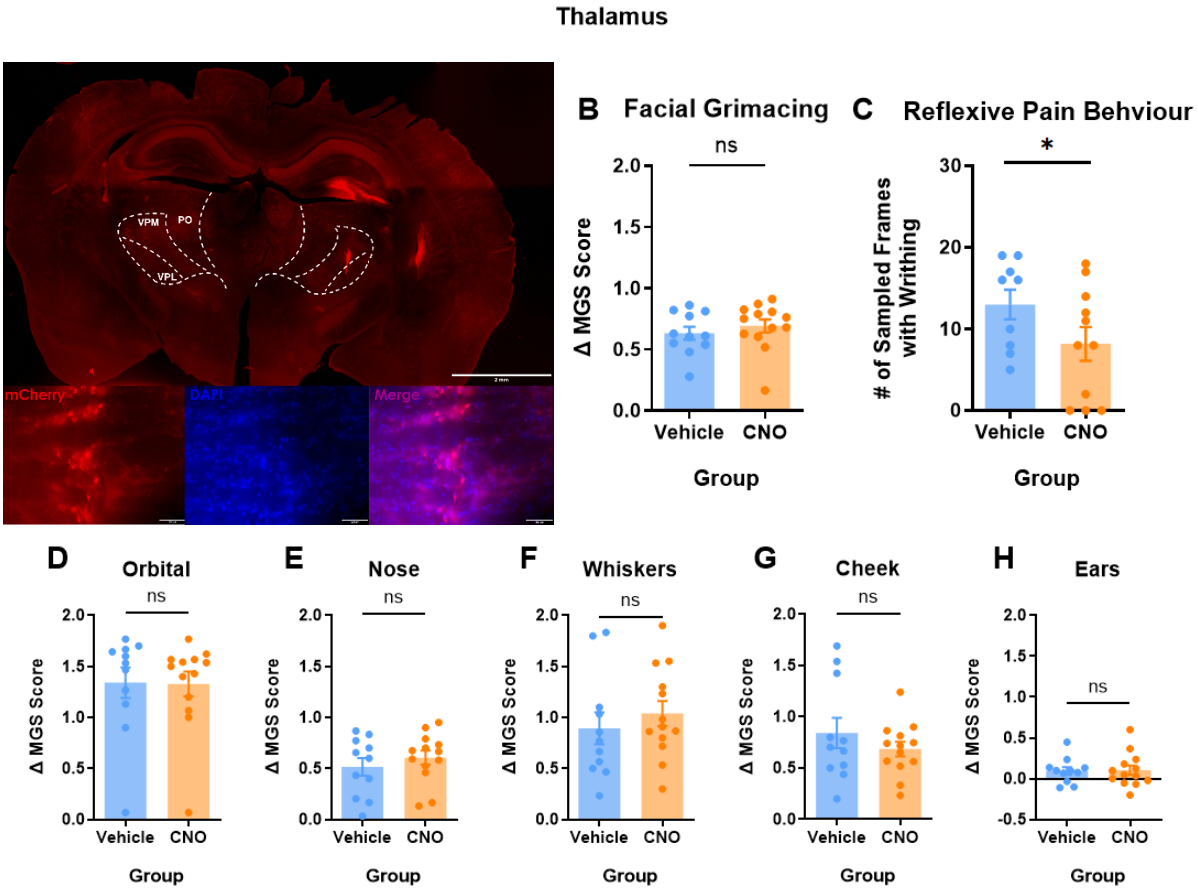


Figure 5: Change Score on Mouse Grimace Scale and Total number of Writhes for thalamus **Chemogenetically Silencing the Thalamus Attenuates Pain Expression While Leaving Reflexive Pain Behaviour Unaltered - Fig 5. A** DREADD virus expression. Fluorescent image is representative brain showing viral vector transfection in the thalamus with DAPI. **Fig 5. B** Mice that had their thalamus chemogenetically inhibited (CNO group) showed lower facial grimacing then control mice after pain induction relative to baseline *(t (22)* = .2249, *p*=ns, M_{VEH}=.6343, SD_{VEH}=.1750, M_{CNO}=.6927, SD_{CNO}= .1936). **Fig 5. C** There was no significant difference in the number of writhes observed after pain induction between the control group and the CNO group *(t (18)* = .0525, *p=ns*, M_{VEH}= 13.0, SD_{VEH}= 5.48, M_{CNO}= 8.18, SD_{CNO}= 6.85). **Fig 5. D-H Individual Action Unit MGS Score.** Mice in the CNO group showed lower facial grimacing then control mice after pain induction relative to baseline. **Fig 5. D Δ** orbital *(t (22)*=.4744, *p*=ns; M_{VEH}= 1.34, SD_{VEH}= .499; M_{CNO}= 1.33, SD_{CNO}= .439), **Fig 5. E Δ** nose *(t (22)*=.2069, *p*=ns; M_{VEH}=.515, SD_{VEH}= .287; M_{CNO}= .605, SD_{CNO}= .245), **Fig 5. F Δ** whiskers *(t (22)*=.2356, *p*=ns; M_{VEH}= .893, SD_{VEH}= .521; M_{CNO}= 1.04, SD_{CNO}= .447), **Fig 5. G Δ** cheeks *(t (22)*=.1615, *p*=ns; M_{VEH}= .841, SD_{VEH}= .489; M_{CNO}= .684, SD_{CNO}= .258), **Fig 5. H Δ** ears *(t (22)*=.4588, *p*=ns; M_{VEH}= .0956, SD_{VEH}= .156; M_{CNO}= .104, SD_{CNO}= .208).

Bridging Text between Chapter 3 and Chapter 4

Chapter 3 presents evidence that pain-induced facial grimacing in mice can be modulated by inhibiting specific brain regions associated with pain affect, such as the insular cortex and central amygdala. These findings suggest that the Mouse Grimace Scale (MGS) primarily reflects the affective component of pain, offering a more comprehensive and translatable measure for preclinical pain research. Chapter 4 shifts focus to explore the lateralization of pain-induced facial grimacing-uncharted territory in pain research. The lateralization of facial expressions of emotion, where the left side of the face often expresses emotions more strongly, is well-documented in the literature. However, whether this asymmetry extends to pain-induced expressions remains undetermined. Chapter 4 aims to fill this gap by systematically examining the lateralization of pain-induced facial grimacing in mice. That is, if pain-induced grimacing is similarly lateralized to other emotions, it would further validate the MGS as a measure of pain affect, aligning it with other well-recognized emotional expressions. This chapter employs a variety of pain models, including acute, chronic, and inflammatory pain, to assess whether the left side of the face exhibits more pronounced grimacing than the right. By comparing left-face and right-face grimacing across different pain modalities and durations, Chapter 4 seeks to determine the extent to which pain-induced facial expressions are lateralized and how this lateralization is influenced by the central amygdala. The findings from this chapter offer novel insights into the neural and behavioral dynamics of pain, further establishing the MGS as a robust and multidimensional tool for affective pain assessment. In summary, Chapter 4 moves from the general neuroanatomical underpinnings to the specific phenomenon of lateralization.

Chapter 4

Lateral Asymmetry of Pain-Induced Facial Grimacing in Mice

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Abstract

Pain serves an essential adaptive function, despite generally being a mostly aversive experience. The International Association for the Study of Pain recognizes pain as a complex experience characterized by both sensory and emotional elements. Despite this recognition, most pain assessment tools focus predominantly on the SD component (e.g., von Frey filaments, cold pressor test), with fewer measures adequately capturing the AM component. Lesion studies indicate that measures of pain-induced facial grimacing such as that which is measured by the Mouse Grimace Scale (MGS) captures pain affect as damage to emotion-related brain areas attenuates pain-induced facial grimacing (Langford et al., 2010). Comparing pain-induced facial grimacing to facial expressions of emotion is one way to determine the degree to which the Mouse Grimace Scale reflects pain affect over pain sensation. Extensive research demonstrates that facial expression of other emotions (e.g., fear, joy) are stronger on the left side of the face. Here we examine differences in pain-induced facial grimacing on the left and right side of the face by analyzing left and right side only images of mouse faces while they experience various pain modalities and locations on the body. We hypothesized that grimacing would be lateralized to the left side of the face in alignment with existing non-pain facial expression findings. We found that pain is expressed predominantly on the right side of the face, contrary to other emotions. This is the only investigation of lateralization of pain-induced facial grimacing that we are aware of. The results here have important implications for clinical treatment of pain in non-verbal human and in veterinary contexts as well as for translational validity in preclinical pain testing.

Introduction

Given the ubiquitous nature of pain and its significant aversive characteristics, it is crucial to have validated, translational methods to objectively measure all aspects of the pain experience. Pain has sensory and emotional components, which pain research refers to as the sensory-discriminative (SD) and affective-motivational (AM) experience of pain. The SD component has many validated preclinical (e.g., von Frey, Hargreaves) and clinical measures (e.g., cold pressor), most of which are incapable of measuring the motivational-affective part of pain in a parsimonious and translationally relevant way. Pain-induced facial grimacing is one exception to this translational gap as evidence from mouse models suggest it is a measure of pain affect (Langford et al., 2010). Longstanding research on non-pain facial expressions show that emotional expression is lateralized such that the left side of the face exhibits facial expressions more strongly than the right side (Lindell et al., 2013, 2018). If pain-induced facial grimacing is also lateralized it would further confirm that the Mouse Grimace Scale reflects pain affect over pain sensation.

Lateralization of Facial Expressions of Emotion

Charles Darwin first observed the asymmetry of facial expressions, despite the face's apparent symmetry. Subsequent research has shown that emotional expressions (e.g., fear, anger) are predominantly displayed on the left side of the face (Borod et al., 1997; Sackeim et al., 1978). This left-side bias is evident in both humans and non-human animals and is reflected in the neural circuitry governing emotion expression. Studies have demonstrated a stronger left-side display for spontaneous versus posed emotions, suggesting an innate aspect of emotional expression asymmetry (Kowner, 1995; Mandal & Singh, 1990). However, it remains unknown whether pain-induced facial expressions exhibit similar lateralization.

Though there is currently no other existing research on lateralization of pain-induced facial grimacing, there is a substantial body of literature that demonstrates asymmetry in certain area of the pain matrix. Though this is the case for several brain areas, the one with the most likely involvement in expression of pain affect is the amygdala. The right amygdala is responsible for negative emotions and responding to threat and reacts more to fearful stimuli (Morawetz C, 2017) (Sergerie et al., 2008) (Adolphs, 2001). In contrast, the left amygdala is involved in processing both positive and negative emotions and plays a more sophisticated role in the conscious evaluation and interpretation of emotional stimuli. This includes verbal and cognitive aspects of emotional labeling and contextualization as well as cognition and processing emotion-related social cues (Burklund et al., 2014) (Adolphs, 2001; Phelps & LeDoux, 2005; Sergerie et al., 2008; Calder et al., 2002).

Regarding pain more specifically, the amygdala retains its asymmetrical contributions. The right amygdala responds more strongly to painful heat stimuli and to anticipation of pain (Ploner et al., 2010) (Baliki et al., 2006). Furthermore chronic pain appears to alter amygdala structure and function, particularly on the right (Apkarian et al., 2005). The left amygdala appears to capture and convey the more cognitive-evaluative aspects of pain processing. For instance, during a task where participants had to label and evaluate their pain experience there was more activity in the left amygdala (Simons et al., 2014). Additionally, when recalling and contextualizing pain-related memories participants showed greater left-amygdala activation (Liberzon et al., 2000).

Given the substantial body of evidence showing lateralization of emotional facial expressions and asymmetry of pain affect related neuroanatomy (i.e., amygdala), we wanted to see if mouse pain expression as measured by the MGS was similarly lateralized and attempt to

gain chemogenetic control over facial lateralization by inhibiting the central amygdala (CeA). Here, we examined facial grimacing in mice across five pain models: zymosan (ZYM), complete Freund's adjuvant (CFA), acetic acid (AA), carrageenan (CARR), and spared nerve injury (SNI), representing neuropathic, inflammatory, and reflexive pain types, respectively. Additionally, though large-scale normative data studies have demonstrated there is no evidence for lateralization of withdrawal threshold in any of the canonical preclinical SD measures of pain (i.e., Hargreaves, von Frey), we also measured the SD component of pain using von Frey. We also used Designer Receptors Exclusively Activated by Designer Drugs (DREADDs) to chemogenetically inhibit either the left or right CeA and reevaluated MGS scores of mice in pain before and after DREADD activation. Lastly, we sought to probe if there was evidence for lateralization of the other pertinent areas of the pain matrix (e.g., somatosensory cortex, thalamus) as well as the CeA by quantifying cFos expression on the left and right sides of the brain in mice who experienced left side or right-side pain. We hypothesized that pain-induced facial grimacing would exhibit lateralization, similar to other emotional expressions, with a stronger display on the left side of the face. Given the strong evidence for both the left and right amygdala contributions to pain affect we made no ad-hoc hypotheses as to which side would attenuate lateralization. We did predict greater cFos expression on the right side of the brain, which controls the left side of the face.

Materials and Methods

Video Capture and Pain Model Induction

Overview

Adult male and female CD-1 mice on a 12/12-hour light-dark cycle were subject to one of several possible pain assays and video recorded for 1hr. These assays were left or right intraplantar injection of complete Freund's adjuvant, zymosan, or carrageenan; left or right spared nerve injury; or intraperitoneal injection of acetic acid (non-lateral pain). Symmetrical still images of the mouse face were taken every 3 minutes. In each 3-min epoch, we collected an unaltered centre-front-facing as well as a left and right-side version of each of several image variants which were scored using the Mouse Grimace Scale (MGS). Post-pain video recordings were taken following a window of time respective to each algogen used: Acetic acid (unilateral visceral pain), immediately after injection; CFA, 2hrs post-injection; Zymosan 30-45 minutes post injection; Spared Nerve Injury, 4 days post surgery [21]; and Carrageenan, 3 hours post-injection.

Video Capture

Facial grimacing and other pain behaviour relevant to the model (i.e., reflexive writhing in AA and nocifensive licking or biting of the affected hind paw in CFA) were recorded in high definition (HD). Mice were placed in custom stainless steel and Plexiglas observation cubicles (9 × 5 × 5 cm high) with HD digital video cameras positioned perpendicular to the cubicle facing the mouse.

Image Generation and Standard MGS Scoring

We grabbed frames from video taken before and after model induction at ~3-min intervals. Image captures were cropped so the resultant JPEG file did not reveal body position. Blind scoring of the pictures will be done as previously described (Langford et al., 2010) to get an average baseline and post-induction MGS score for each of the five action units (cheek, whiskers, nose, ears, eyes) as well as an overall score. A separate observer also analyzed the videos to count the number and duration of abdominal constrictions (writhes). Writhing is a specific behaviour characterized by the lengthwise stretching of the torso and concave dorsiflexion of the back, like lordosis posture observed in sexually receptive female mice (Langford et al., 2010)

Facial Chimera Image Generation for Grimace Lateralization Scoring

Seven image types were used to evaluate the lateral symmetry of a mouse face during each of the pain models (Fig. 1). They were unaltered front-facing, left and right profile (sideview), left and right hemiface, and left and right composite (chimera). To generate these images symmetrical still captures of the mouse face were sampled every ~3 min and were cut and mirrored about the y-axis to create left-left and right-right facial chimeras. Additionally, we took still captures of a side profile when the mouse is facing approximately 90° to the left and the right of the centre-front where the camera will be located. Lastly the same images that were cut and used to create the facial chimera composites were presented singly without mirroring (hemiface).

Scoring Using the Mouse Grimace Scale

The MGS applies a 5-action unit (AU) scoring system that includes the degree of orbital squinting, nose and cheek bulge, as well as ear and whisker position change (Fig 2; Langford et

al., 2010). Rating of AU's is on a scale from 0 to 2 depending on the degree of expression (e.g.: 0=AU not present, 1=moderate presence of the AU, 2=severe presence of the AU).

Mechanical Hypersensitivity

Von Frey (vF) was used on all mice except AA to capture the sensory component of pain to ensure that any MGS lateralization observed was not related to the lateralization of nociception. All vF measurements were taken by the same research assistant who remained blind to the conditions using manual fiber application and the up-down method. Mice were habituated for 30 minutes before vF measurements were taken.

Chemogenetic Inhibition of Central Amygdala to Alter Lateralization

Stereotaxic Surgery

Adult male and female CD-1 mice were anesthetized with ~2% isoflurane in oxygen (0.8-1L/min flow rate) before being head-fixed into stereotaxic apparatus (Stoelting, Woodlane, IL). After removing the hair and disinfecting the area a ~1-cm sagittal incision was made in the scalp along the midline and the incision was held open by bulldog clamps (Fine Science Tools). We verified the horizontality of the skull on the rostral-caudal axis by ensuring that the DV coordinate ~2 mm anterior to bregma was within ± 0.2 µm range of the DV coordinate ~2 mm posterior to lambda and on either side of the mid-sagittal cranial suture along the medial-lateral axis. Using a dental drill (Foredom, MH-1 70) with a 0.2-mm burr, holes were drilled through the skull dorsal to the appropriate target area after which we lowered Hamilton microsyringes (33 G) through the skull hole to the appropriate DV coordinate (see table XX). Once the needle was in place we injected.4µl of the inhibitory DREADD AAV-hSyn-hM4D(Gi)-mCherry (NeuroPhotonics, Quebec City). Coordinates were CeA (5° angle; AP: -1.1; ML: ± 2.7; DV: -4.6

to -3.8 from bregma). Virus was delivered unilaterally to either the left or the right CeA by gradually injecting .1µl at the initial DV coordinates and then subsequently, the needle was retracted by 0.1 to 0.2 µm and a further 0.1 µl was injected at each stop until all the viruses had been delivered. At that point, the needle was left in place for 10 min to allow for the virus to diffuse. After diffusion, the needle was slowly removed by retracting 0.1µm every minute until the needle was full out of the brain tissue. The scalp was then sutured with a 1-0 cutting needle pre-threaded with dissolvable suture material. The mice were monitored on a heating source until recovery, at which point the mouse was returned to its home cage.

cFos Methods

Free-floating sections (30–40 µm) were blocked in normal goat serum containing 30% Triton in 0.1M PBS and put on the rocker for an hour. Sections were then incubated in the primary antibody (1:5000, Synaptic Systems #226308) diluted in TTBS for 2 nights at 4 °C. Sections were then washed three times in 0.1M PBS and incubated with the anti-guinea pig IgG, made in goat, biotinylated secondary antibody (1:500, Vector Laboratories #BA-7000) diluted in TTBS and put on the rocker for two hours. Sections were again washed three times in 0.1M PBS and incubated with the ABC solution from the ABC-Peroxidase Kit (Vector Laboratories #PK-6100) for another hour on the rocker, the ABC solution having been combined on the rocker for at least 30 min prior to usage. Sections were washed three times again in 0.1M PBS. The DAB Peroxidase (HRP) Substrate Kit (2/4/2 per 5 µl of dH2O, Vector Laboratories #PK-6100) was used to visualize cFos-marked cells. Sections were stained in the DAB solution for 3:30 to 4 minutes and placed in dH2O to stop the staining process. Sections were then stored in 0.01M PBS for later mounting. Slides were finally mounted, air-dried, washed, and cover slipped with DPX mountant.

Results

Overview

All analyses were conducted, and graphs made using GraphPad Prism (version 10). To test our hypothesis that pain-induced facial grimacing is lateralized to the left side of the face, we analyzed raw MGS scores and compared them to the average MGS scores for each AU. This analysis was done for each picture orientation: front-facing (unaltered), left and right composite (chimera), left and right side only (hemiface), and left and right profile view (profile). Baseline (pre-pain induction) MGS scores were subtracted from post (post-pain induction) MGS scores to obtain a change/delta score (D MGS). We analyzed these scores using a one-tailed t-test comparing mean scores to zero. Scores greater than zero indicated a right bias, whereas scores below zero indicated a left bias.

MGS Lateralization

We observed a significant effect of lateralization of pain-induced facial grimacing such that MGS scores were more strongly biased to the right side of the face for all image types; composite ($t(162)=2.757$, $p<.01$); hemiface ($t(162)=3.685$, $p<.001$); profile ($t(162)=8.486$, $p<0.001$); average ($t(162)=6.682$, $p<0.001$).

A one-tailed t-test comparing the delta MGS scores for each action unit revealed a significant right side bias for orbitals ($t(161)=2.418$, $p<.05$), nose bulge ($t(161)=3.836$, $p<.001$), and cheek bulge ($t(161)=2.912$, $p<.01$). The ears ($t(161)=0.6841$, p=ns) and whiskers ($t(154)=1.666$, $p<.05$) AU scores were not significantly biased to any side.

A one-tailed t-test showed a significant effect of pain side on MGS scores such that pain-induced facial grimacing was right-biased regardless of pain location (left, right or unilateral).

Pain injection on the right (t (73)=4.370, $p<0.001$), left (t(72)=4.236, $p<0.001$), and i.p. (t(15)=2.831, $p<.05$).

When examining the data according to pain assay we observed a significant bias to the right for SNI (t (29)=4.088, $p<.001$), AA (t(15)=2.831, $p<.05$), ZYM (t(32)=3.075, $p<.01$), and CFA (t(59)=3.860, $p<.001$). For CARR the bias was not statistically significant, but trended towards being right biased like all the other measures (t (23)=1.407, $p=ns$).

For the pain assays in which pain was lateralized to one foot or the other (i.e., all except acetic acid), there was no significant difference between vF withdrawal thresholds between the left side and the right side for either the ipsilateral or contralateral side (Figure XX; all p's ns).

Chemogenetic Inhibition of Central Amygdala

A comparison of mice who had either their right or left CeA infused with an inhibitory DREADD (Fig 9) showed that those who right CeA was inhibited has a significant reduction in MGS bias before and after DREADD activation with CNO Right CeA Pre vs. right CeA-post (Fig. 8 t (34)=2.091, p<.05, $M_{\text{right CeA-pre}}$=.1999, $M_{\text{right CeA-post}}$=.06000). Left CeA-pre vs. left CeA-post (t (34)=.8356, p=ns, $M_{\text{left CeA-pre}}$=.1757, $M_{\text{left CeA-post}}$=.1179) were not significantly different.

Discussion

The studies here show that pain-induced facial grimacing was stronger on the right side of the face than on the left. This finding is directly opposite to all existing literature on lateralization of facial expression of emotion and our hypothesis. Mechanical withdrawal thresholds as measured by von Frey were not lateralized in congruence with previous evidence (Zumbusch et al., 2023). Though the right-side bias is contradictory to our hypothesis and in conflict with existing facial lateralization research, the face that pain faces in mice are lateralized so significantly no matter the type of pain or its location suggests that it is still similar to other

facial emotion expression. Additionally, our within-subject analyses showed incongruent lateralization in the SD measure of pain and the AM aspect of pain. That is, we observed no asymmetry in von Frey withdrawal threshold and consistent right side bias in the MGS scores. This incongruency reaffirms that MGS and von Frey capture different components of the pain experience.

Facial expressions, including those associated with emotions such as fear, anger, and happiness, are generally lateralized to the left side of the face, suggesting dominance of the right hemisphere in emotional processing (Indersmitten & Gur, 2003; (Sackeim et al., 1978). This phenomenon is thought to be rooted in the contralateral control of facial muscles, where the right hemisphere predominantly governs the left side of the face (Lindell, 2013). However, our findings indicate that pain-induced facial grimacing in mice is more pronounced on the right side of the face, hinting at a possible deviation from this pattern. Additionally, when we chemogenetically inhibited the left or right CeA we found that only right-CeA inhibition ameliorated the right-side bias. At face value, this appears contradictory to existing neuroanatomical explanations of the lateralization of facial emotions, which typically say that they are left-biased due to being controlled by the right side of the brain. However, there is precedence in the literature for both contralateral *and* ipsilateral processing of sensory and motor information related to pain.

The predominant belief is that somatosensory stimuli are processed mainly in the contralateral hemisphere to the stimuli. However, brain imaging and lesion studies of pain processing suggest this might not always be the case. For instance, during painful heat stimuli on the forearms there was more right hemisphere activation in the thalamus, inferior parietal lobule, and dorsolateral and dorsal prefrontal cortex (Coghill, 2001). Additionally, noxious electrical

stimulation of the fingertips yielded more right hemispheric activation in the ACC (BA 32), middle frontal gyrus (BA 9/46/10), medial and superior frontal gyri (BA 6/8), ventrolateral prefrontal cortex, and inferior parietal lobule (Symonds, 2006).

Perhaps most importantly for synthesizing the perplexing results that the right amygdala (aka the ipsilateral side to our observed facial grimacing bias) is the notion that facial expressions are mediated by both ipsilateral and contralateral pathways (Rinn, 1984). This may explain the observed right-side dominance in pain-induced grimacing and our ability to abolish this bias by inhibiting the right CeA. Furthermore, the facial motor nucleus receives bilateral cortical inputs, which allows for some degree of ipsilateral control (Schmidt et al., 2011). For example, individuals with lesions in the left hemisphere may still exhibit some degree of movement on the right side of the face, suggesting that the intact right hemisphere can partially compensate through ipsilateral pathways (Boll, 1974).

Additionally, though there is vast evidence for the right amygdala's role in affective pain processing in and of itself, we also know that the interaction of the right amygdala and various cortical regions is crucial for regulating pain-related emotions. This connection appears to modulate the immediate emotional response to pain and influences pain-related behaviours. For instance, Bushnell et al. (2013) found that the right amygdala-prefrontal cortex pathway is involved in downregulating pain-related anxiety. Their study showed that individuals with higher connectivity between these regions reported lower pain-related anxiety.

Our study provides novel insights into the lateralization of pain-induced facial grimacing, highlighting the unique neural mechanisms involved in pain processing. The ipsilateral processing of nociceptive signals and the distinct roles of the SD and AM components of pain contribute to the observed lateralization patterns. These findings have important implications for

the measurement of pain in non-verbal populations and the development of more effective pain management strategies.

Figure 1

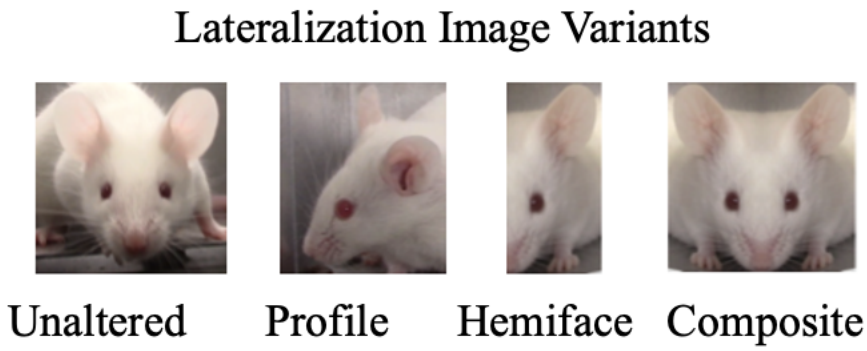

Fig 1. Lateralization Image Variants: 4 image types of mice faces were collected from video recordings; unaltered front-facing view images, both left and right side-profile images, both left and right hemiface/half face images, and both left, and right composite images made from two identical left or right hemiface images mirrored to make a symmetrical face.

Figure 2

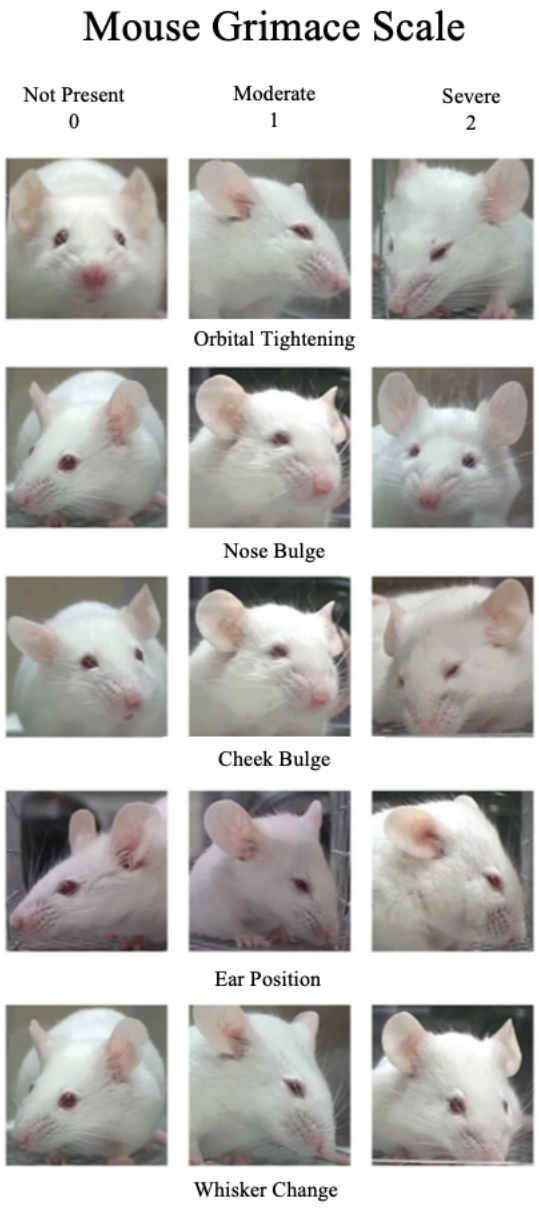

Fig 2. Mouse Grimace Scale: The Mouse Grimace Scale assesses the absence, moderate or severe presence of five action units; (1) orbital tightening, (2) nose bulge, (3) cheek bulge, (4) ear position, (5) whisker position.

Figure 3

Delta MGS Scores by Image Type

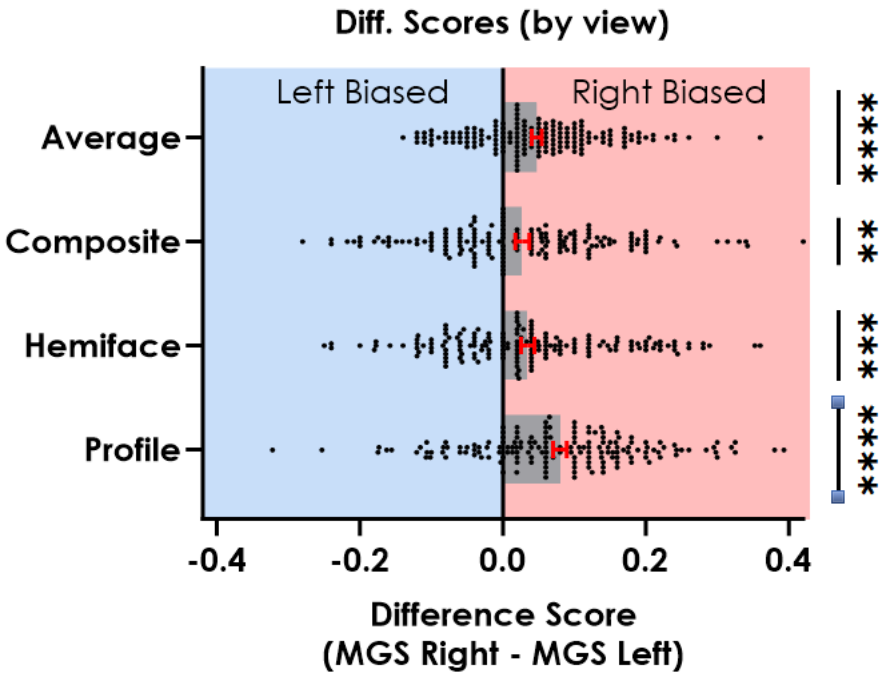

Fig 3. MGS Difference Scores Shown by Face Side: *Pain induced facial grimacing was expressed more strongly to the right side of the face from every facial angle. Composite (t (162)=2.757, p<.01, M=.02727, SD=.1262); hemiface (t(162)=3.685, p<.001, M=.03461, SD=.1199); profile (t(162)=8.486, p<.0001, M=.07984, SD=.1201); average (t(162)=6.682, p<.0001, M=.04730, SD=.09037). *p≤0.01, ***p≤0.001, ****p≤0.0001.*

Figure 4

Delta MGS Scores Separated by Action Unit

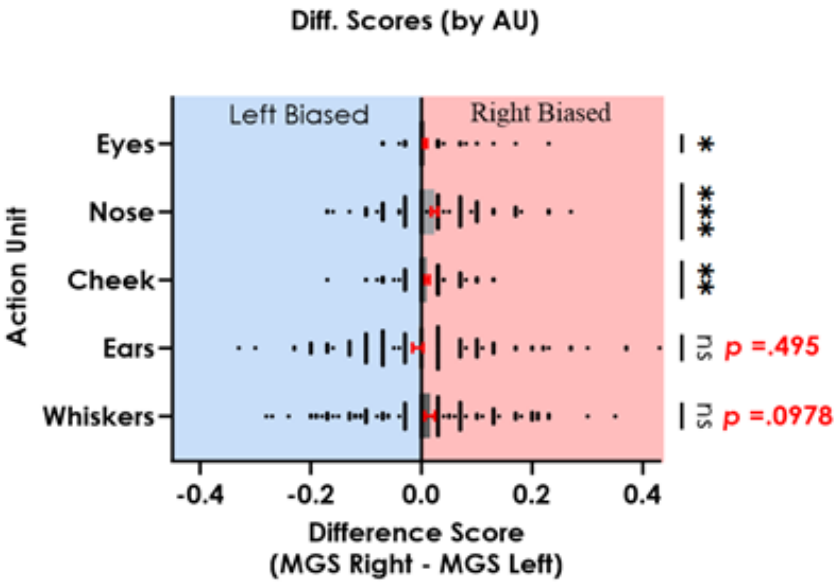

Fig 4. MGS Difference Scores by Action Unit: *Mice showed a change in pain induced facial grimacing to the right in the eye.s (t(161)=2.418, p<.05, M=.006790, SD=.03575), nose (t(161)=3.836, p<.001, M=.01056, SD=.1217), and cheeks (t(161)=2.912, p<.01, M=.01056, SD=.04613) action units. The ears (t (161)=.6841, p=ns, M=-.006543, SD=.1217) and whiskers (t(154)=1.666, p<.05, M=.01523, SD=.1138), although trending towards right-biased lateralization, did not reach significance, which indicates differential lateralization of grimacing AU's. *p≤0.05, **p≤0.01, ***p≤0.001, ns=nonsignificant.*

Figure 5

Delta MGS Scores Relative to Pain Assay

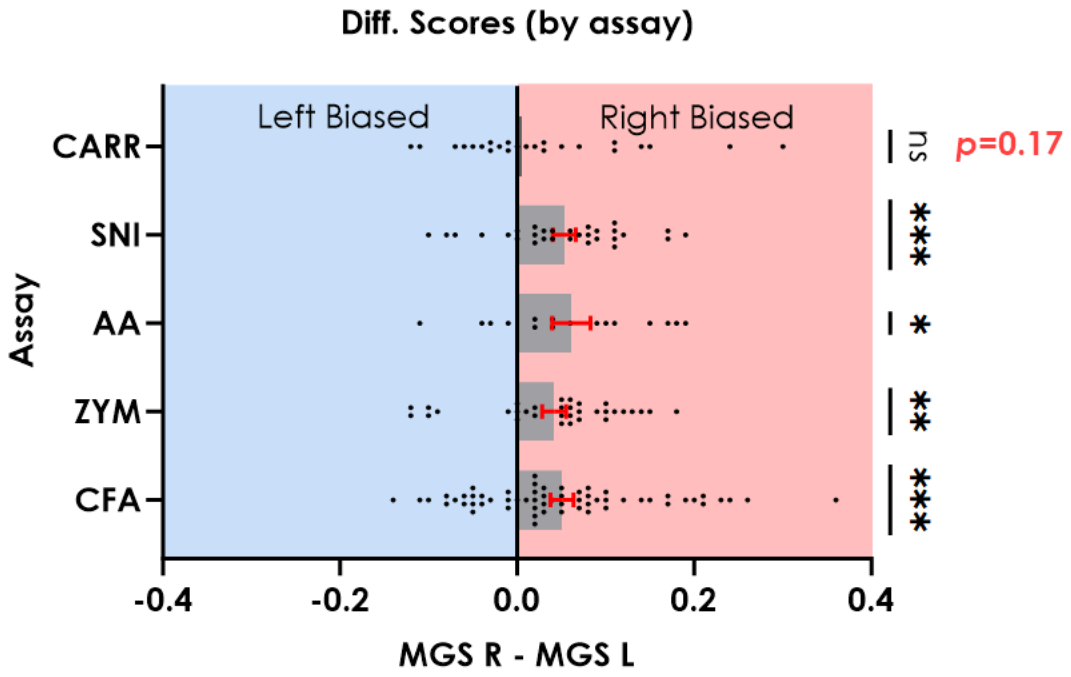

Fig 5. **MGS Difference Scores by Assay:** *Mice showed a stronger facial grimacing response to pain assays. (t(29)=4.088, p<.001, M=.05333, SD=.07145), AA (t(15)=2.831, p<.05, M=.06125, SD=.08655), ZYM (t(32)=3.075, p<.01, M=.04182, SD=.07812), and CFA (t(59)=3.860, p<.001, M=.05067, SD=.1017) than to CARR (t(23)=1.407, p<=ns, M=.02958, SD=.1030). *p≤0.05, **p≤0.01, ***p≤0.001.*

Figure 6

Delta MGS Scores Relative to Site of Injection of Pain Assay

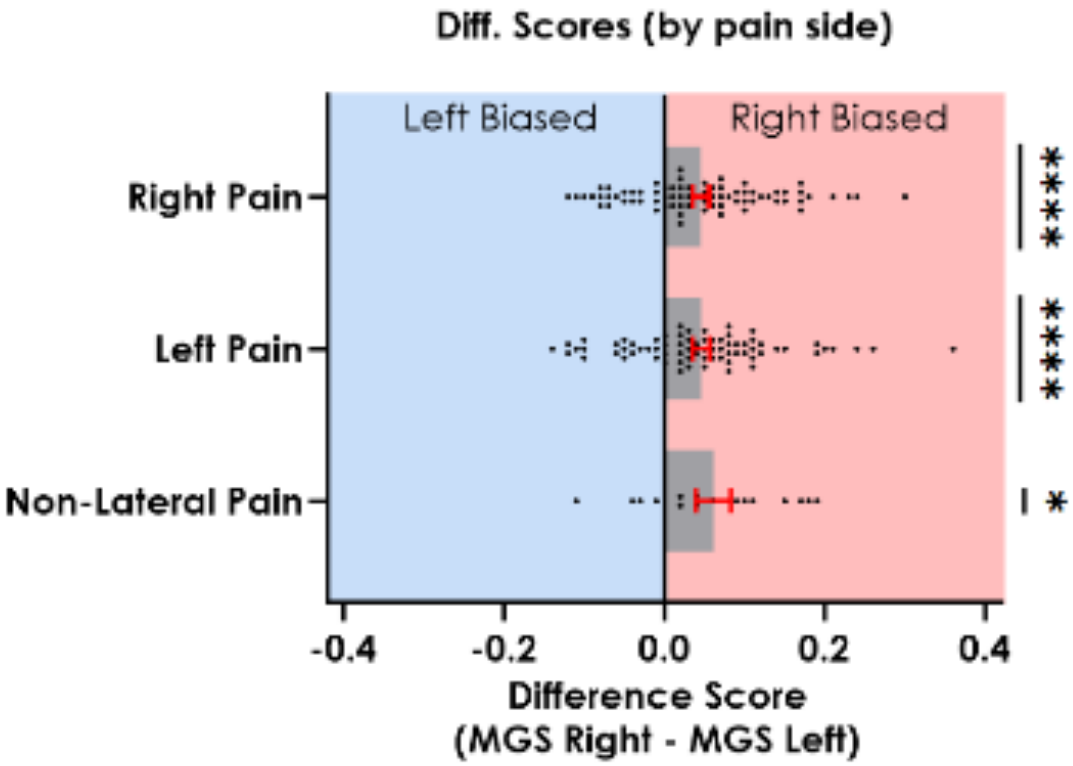

Fig 6. MGS Difference Scores Shown by Pain Location. *Pain induced facial grimacing was predominantly expressed on the right side of the face regardless of pain location; right pain (t(73)=4.370, p<.0001, M=.04554, SD=.08964), left pain (t(72)=4.236, p<.0001, M=.04603, SD=.09284), non-lateral pain (t(15)=2.831, p<.05, M=.06125, SD=.08655).*p≤0.05, ****p≤0.0001.*

Figure 7

von Frey Withdrawal Thresholds

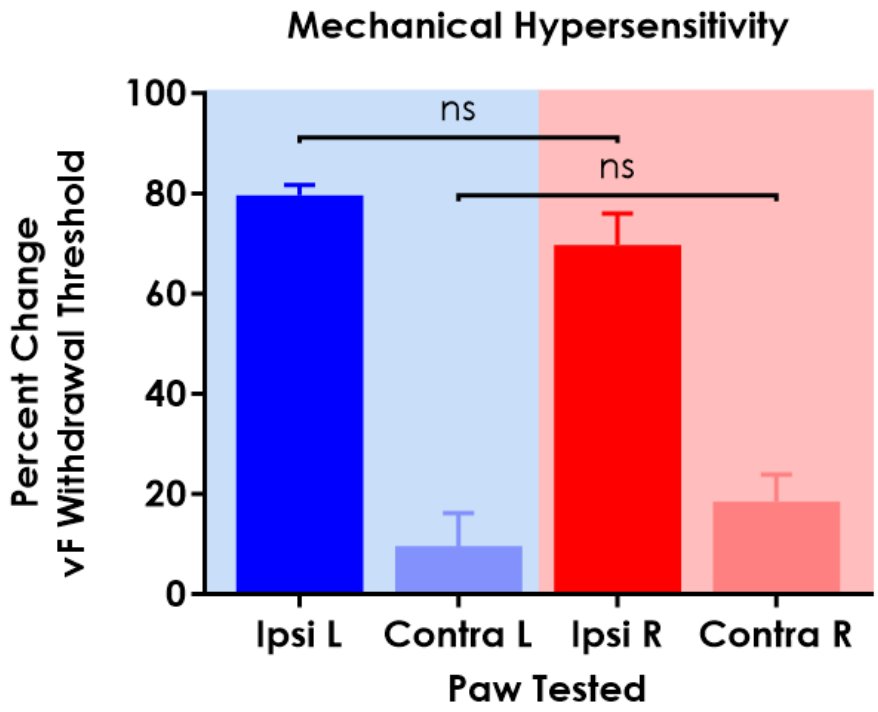

Fig 7. von Frey Withdrawal Thresholds and Site of Injury: *Variance in withdrawal thresholds were congruent with the site of injury. Left side injury would predict lower von Frey withdrawal thresholds on the ipsilateral side and higher von Frey withdrawal thresholds on the contralateral side suggesting that mechanical hypersensitivity is not lateralized. Ipsi-L vs. ipsi-R (t (218)=1.303, p<ns, $M_{ipsi\text{-}L}$= 79.64, $M_{ipsi\text{-}R}$= 69.69); contra-L vs. Contra-R (t(218)=1.160, p<ns, $M_{contra\text{-}L}$= 9.604, $M_{contra\text{-}R}$= 18.47). ns=nonsignificant.*

Figure 8

MGS Difference Scores Pre and Post Central Amygdala DREADDs Inhibition

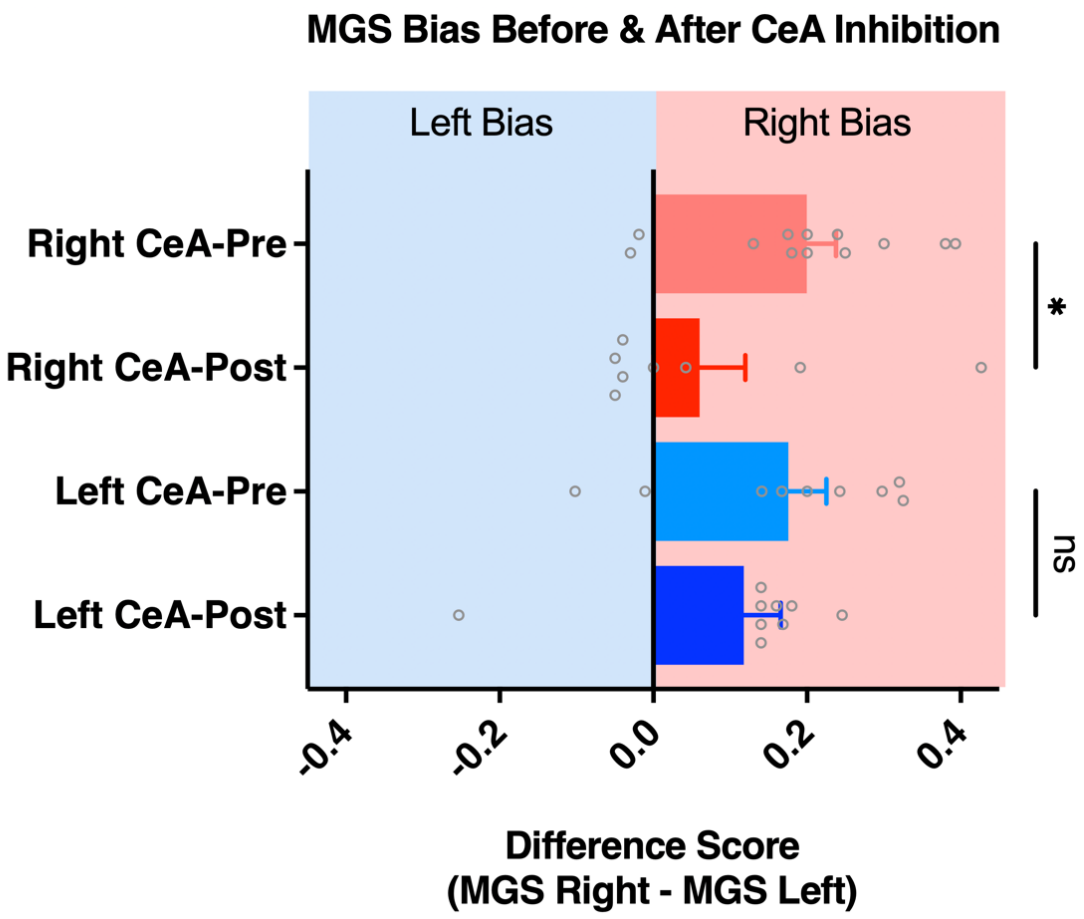

Fig 8. MGS Difference Scores by Pain Side Pre and Post Central Amygdala DREADDs Inhibition: Right CeA-pre *vs. right CeA-post (t (34)=2.091, p<.05, $M_{right\ CeA\text{-}pre}$=.1999, $M_{right\ CeA\text{-}post}$=.06000) were significantly different. Left CeA-pre vs. left CeA-post (t (34)=.8356, p=ns, $M_{left\ CeA\text{-}pre}$=.1757, $M_{left\ CeA\text{-}post}$=.1179) were not significantly different. *p≤0.05, ns=nonsignificant.*

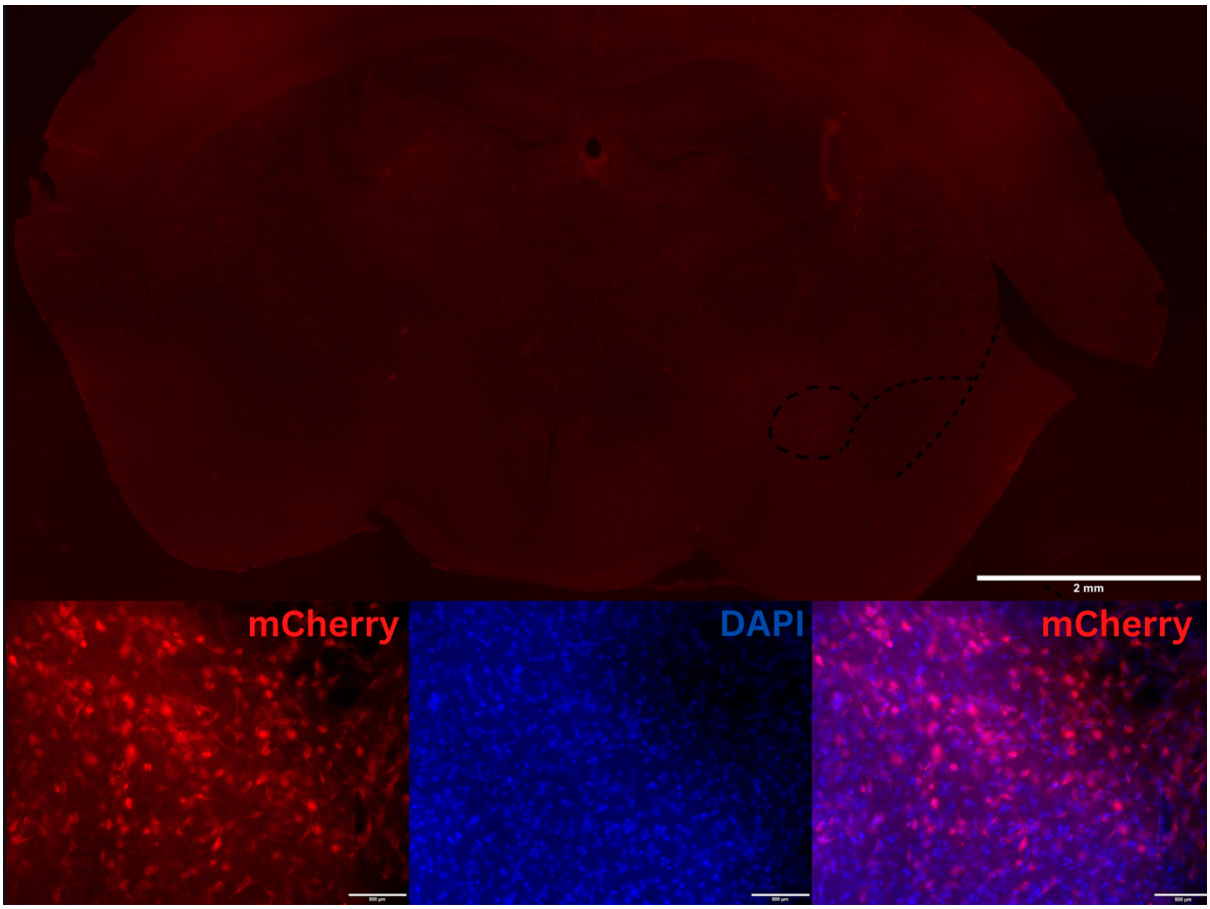

Figure 9 Representative of Mouse Brain showing DREADD expression Co-stained with DAPI

C-Fos Results

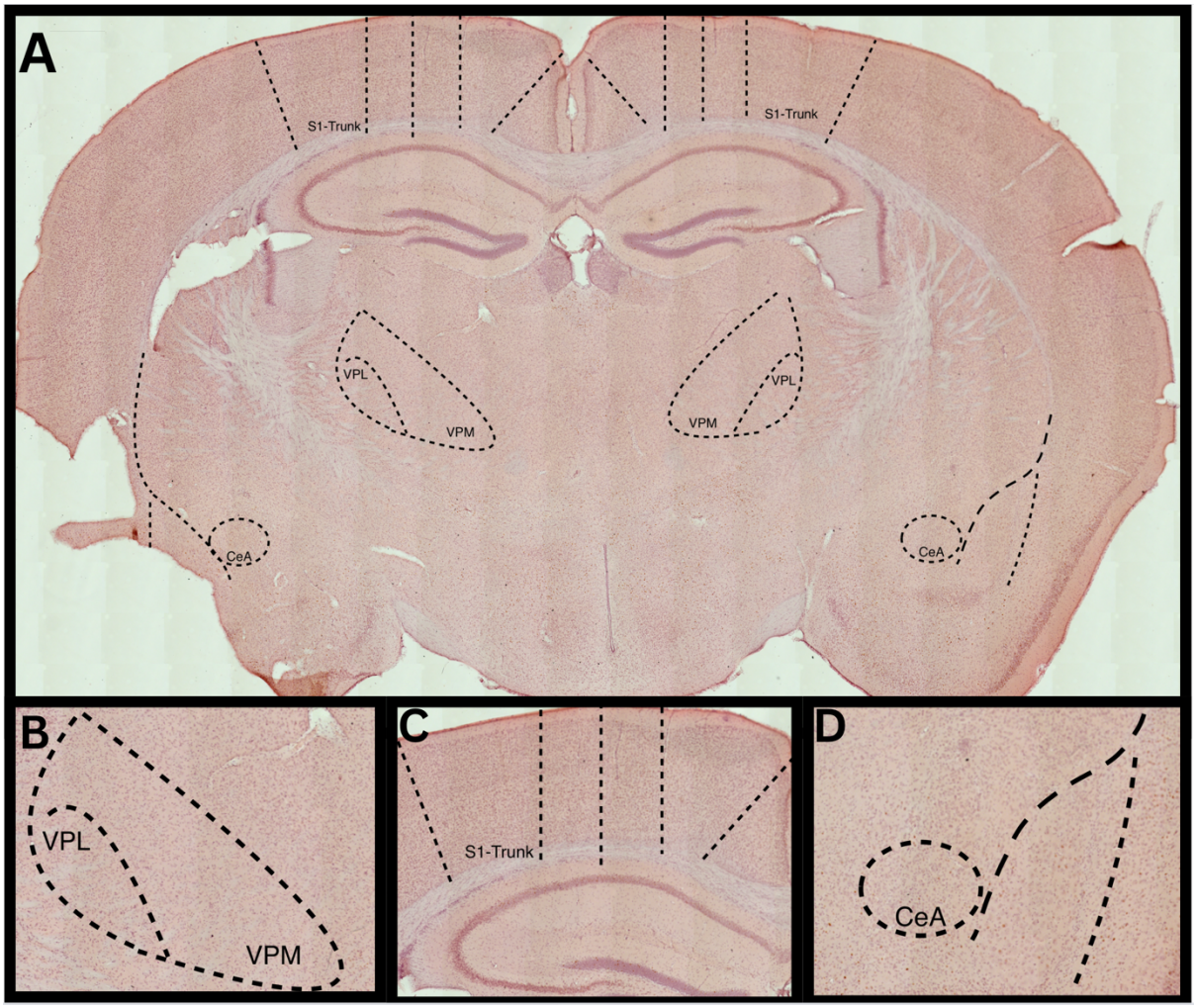

Figure 10 Representative of Mouse Brain Stained for c-Fos Expression in Candidate Regions- Fig 10 A- Whole Brain. **Fig 10 B-** Ventroposteromedial and ventroposterolateral nucleus of the thalamus. **Fig 10 C** – Trunk Region of the Primary Somatosensory Cortex. **Fig 10 D** – Central Nucleus of the Amygdala

Chapter 5: Discussion

Pain is a multifaceted experience characterized by both sensory-discriminative (SD) and affective-motivational (AM) components, reflecting the complexity of pain as both a sensory input and an emotional experience (Melzack & Casey, 1968; Raja et al., 2020). This dissertation explores these components through a series of studies with three main focuses, including first, the insufficiency of sensory models for capturing spontaneous, affective pain; second, looking at the neuroanatomical substrates of pain-induced facial grimacing; and lastly examining lateralization of pain-related facial expressions and their neuroanatomical correlates. These studies had the broad purview of highlighting a regrettable and longstanding gap in preclinical pain measurement and providing evidence that the Mouse Grimace Scale (MGS) is the ideal measure to fill that gap.

The previous sections of this dissertation began in Chapter 1 with a review of the existing literature regarding algesiometry and our current ability to adequately measure *all* aspects of pain. Crucially, there is a lack of validity and translatability in most of the classic preclinical measures of pain. Existing preclinical measures that rely on withdrawal thresholds (e.g., von Frey, Hargreaves, tail flick, etc) or quantification of nocifensive behaviour (e.g., licking a paw that has had an algogen administered) are inadequate at modelling the type of spontaneous pain that is most clinically valuable. Additionally, in Chapter 2, I show that in a large-scale normative data set, many of our field's default algesiometry methods show startling variability—further, models such as these measures only one aspect of the pain experience, the sensory component. Critically, the affect component of pain conveys the aversive aspects of pain, which engender the suffering that makes most pain unpleasant. Thus, it is imperative to be able to not only measure pain affect, but to do so in a highly translational manner. Of course, in the clinical sphere of pain

research and treatment, we can question participants/patients and administer inventories and surveys *ad nauseum.* While these methods yield exquisite detail of an individual's pain experience these methods exclude individuals incapable of verbal communication and lack any ability to back translate to preclinical model organisms. The necessity for a translational measure of spontaneous pain for studying and treating pain is vital, and to my knowledge, there are few well-established measures that can satisfy that necessity, one exception being grimace scales.

While there is evidence to suggest that grimacing is a measure of the affective aspect of pain (Langford et al., 2010; Rainville et al., 1997), the research examining the degree to which this is the case is sparse. The studies presented here confirm that pain-induced facial grimacing is indeed primarily a measure of affect as opposed to sensation. Additionally, these data are a crucial first step towards precisely characterizing pain-induced facial grimacing by using known neuroanatomical correlates of the SD and AM parts of pain. I showed that inhibiting affective regions of the pain matrix led to a decrease in pain-induced facial grimacing while not altering reflexive pain behaviour. Reciprocally, I showed that inhibiting sensory regions of the pain matrix led to a decrease and, in some cases, almost obliteration of reflexive writhing behaviour while leaving grimacing unaltered. I also provide additional evidence for facial grimacing as a measure of pain affect by examining the degree to which pain-related facial expressions are similar to other emotional facial expressions which are lateralized to the left side of the face. I showed that pain-induced facial grimacing in mice *is* lateralized but to the opposite side of all other facial emotions. I then used chemogenetics to show that the right CeA is a key neuroanatomical underpinning of this right face bias, given that only right CeA inhibition ameliorated grimacing asymmetry.

Chapter 2: Normative Preclinical Algesiometry Data

Overview and Impact

Chapter 2 presents an analysis of normative preclinical algesiometry data from over 8,000 mice, examining mechanical (von Frey) and thermal (Hargreaves') paw withdrawal tests across various conditions. This comprehensive dataset highlights significant interindividual variability influenced by factors such as tester genotype, mouse sex, tester sex, replicate order, and injury type. The data in Chapter 2 underscore the complexity of preclinical pain testing and the myriad factors that can influence results. The observed variability emphasizes the need for standardized testing protocols and careful consideration of biological and experimental variables, as well as accentuating the drawbacks of a sensory-focused approach to preclinical pain measurement. The findings in Chapter 2 align with previous research highlighting the influence of genetic and environmental factors on pain sensitivity (Mogil, 2012; Chesler et al., 2002a, 2002b). The variability in withdrawal latencies and thresholds suggests that while these tests are useful for detecting pain hypersensitivity, they certainly cannot fully capture the spontaneous and affective dimensions of pain, necessitating complementary measures such as the MGS for a more holistic assessment.

Critical Analysis, Remaining Questions & Further Research

One pertinent critique of the data presented in Chapter 2 is that though the dataset is large enough to represent normative values, there is no way to prove that the results are not idiosyncratic to the conditions of the lab they were generated in (i.e., the Mogil Lab). That said, there is established precedent in the peer-reviewed literature for all of the sources of variability presented in the manuscript (e.g., tester sex, strain, etc.), so there is equally no reason to believe

that they would not be generalizable outside the original context. Furthermore, though the most salient takeaway of the data presented in this paper is that there are many varied and considerable sources of variability and potential error in our mainstay preclinical algesiometry methods, what is more valuable, albeit less exciting, is that data at this scale may serve as a benchmark for the expected amount of variability and what is within normative expectations when using these methods in any setting. Another important consideration for this particular manuscript which this dissertation belabours is that these are only sensory measures, and it would be very valuable to have a representative sample of expected variation for affective measures like that observed in the MGS. Of course, the data provided in subsequent chapters is a step towards having such values, though hardly in the realm of thousands of mice. Additionally, the large-scale analysis of affective measures is a task better relegated to automated scoring. Indeed, the manual method used for subsequent chapters takes a considerable amount of work that would stymie any attempt to deliberately obtain the amount of data necessary to generate normative values.

Of course, given the major critiques above, it would be ideal to have other research groups that use these types of measures to analyze and compare their values to ours. Additionally, it would be useful to see if there are similar degrees of variability in large data sets from other model organism or even humans, though again, data at this scale was obtained over 20 years and thus it would be a substantial undertaking.

Chapter 3: Affective and Sensory Neuroanatomical Substrates

Overview and Impact

Chapter 3 investigates the neuroanatomical substrates of pain-induced facial grimacing, focusing on the roles of regions associated with SD (thalamus, primary somatosensory cortex)

and AM (anterior insula, central amygdala, rostral anterior cingulate cortex) components of pain. Using chemogenetic inhibition, this study demonstrated that facial grimacing is predominantly linked to the affective dimension of pain, with significant reductions in grimacing following inhibition of AM-associated regions. The primary somatosensory cortex (S1) and thalamus, crucial for sensory discrimination, showed minimal impact on facial grimacing when inhibited, highlighting their previously identified role in the sensory aspect of pain (Jones, 2007; Kaas, 2012). In contrast, inhibition of the anterior insula and central amygdala significantly reduced grimacing, underscoring their involvement in the emotional processing of pain (A. D. Craig, 2009; Langford et al., 2010). These findings are consistent with neuroimaging studies in humans showing increased activation of the insular cortex and amygdala during pain experiences (Wiech & Tracey, 2009) Kong et al., 2010). The study also aligns with the theoretical framework proposed by Melzack and Casey (1968), reinforcing the concept that pain is not merely a sensory experience but also an emotional one. The distinct roles of the anterior insula and central amygdala in integrating interoceptive and emotional information make them critical targets for interventions aimed at alleviating pain-related suffering (Gasquoine, 2014) Augustine, 1996).

Critical Analysis, Remaining Questions & Further Research

At the outset of these experiments, we hypothesized that there would be a clear separation between the AM and SD regions of interest such that inhibition of all the AM regions would lead to attenuated grimacing and not reflexive pain behaviour and vice versa for the sensory. Thus, we were surprised when inhibiting the rACC, a key affective node of pain circuitry, did not lead to any significant differences to facial grimacing. One potential reason why the rACC was an exception in the affective ROIs I examined is due to its role in descending pain modulation. For example, the rACC contains high concentrations of opioid receptors, which

are involved in endogenous pain relief mechanisms Bliss et al. (2016). This is of course, complementary to the myriad findings that placebo analgesia greatly involves the rACC (Zubieta et al, 2005; Eippert et al., 2009; Tracey & Mantyh, 2007). Studies using functional magnetic resonance imaging (fMRI) have shown that placebo-induced pain relief is associated with increased activity in the rACC (Wager et al., 2004). This indicates that the rACC is involved in the cognitive modulation of pain. Marrying the existing knowledge of the rACCs top-down control of the pain experience and the fact that the mechanisms appear to be mostly opioidergic leads me to conclude that the rACCs apparent lack of involvement in pain-induced facial grimacing is not because it is *not* involved in the AM aspect of pain, but that it likely reflects more of the motivational than the affective aspect of non-sensory dimensions of pain. This is supported by evidence showing that chemogenetic inhibition of the ACC opioidergic cells reduced non-grimace measures of the AM component of pain (James et al., 2024).

This leads to a more theoretical consideration relating to this thesis as a whole, that the AM versus SD dichotomy is an oversimplification. The affective and motivational components of pain are similar in that they are more abstract than sensory-discriminative components, but they are distinct enough aspects of existence in general that they both motivation and emotional are their own gigantic subfields in neuroscientific research. Thus, the results here seem to demand a more nuanced view of the non-sensory aspects of pain both for the lived pain experience and for research of pain neuroanatomy.

Regarding all the brain areas that I chose, another opportunity for further research is to examine connectivity of each of the regions and begin to understand the contribution so various brain circuits to pain-induced facial grimacing versus reflexive pain behavior. For example, one could use Cre-dependent mutant mouse lines to achieve circuit specific control of grimacing and

writhing. Another level of granularity that could be useful, particularly for finding novel therapeutics, is to focus on receptor-level specificity. This type of investigation could use the above techniques or pharmacological studies to investigate the roles of certain receptors and ligands that contribute to grimacing. The following section is a brief overview of some candidate circuits, receptor and/or cell types suitable for these types of investigations.

Circuit Level Contributions to Grimacing

The medial thalamic nuclei, including the central medial nucleus (CM) and the parafascicular nucleus (PF) receive inputs from brainstem structures such as the PAG and the RVM, which are involved in pain modulation (Li et al., 2019). Activation of CM and PF neurons elicits emotional responses to noxious stimuli and facilitate pain-related behaviors, underscoring their role in the affective dimension of pain (Jensen et al., 2017). The involvement of these nuclei in emotional and motivational aspects of pain is supported by studies demonstrating their activation during affective pain states (Borsook et al., 2010). Lateral thalamic nuclei, including the posterior thalamic nucleus (Po) and the ventrolateral nucleus (VL), contribute to the integration of nociceptive information with other sensory modalities. Po neurons receive inputs from the spinothalamic tract (STT) and project to the somatosensory, insular, and cingulate cortices, where they participate in the processing of nociceptive and non-nociceptive stimuli (Zhang et al., 2020). VL neurons, on the other hand, play a role in sensorimotor integration and motor responses to noxious stimuli, highlighting their involvement in pain-related behaviors (Shyu et al., 2017).

The primary somatosensory cortex (S1) integrates nociceptive information that of other sensory modalities, such as proprioception and touch facilitating the discrimination between

nociceptive and non-nociceptive stimuli (Chudler & Dong, 1995). Additonally, S1 interacts with other cortical regions such as the insula and cingulate cortices to influence cognitive appraisal of pain and pain perception (Iannetti & Mouraux, 2010). S1 also exhibits remarkable plasticity in response to pain, such as alterations in neuronal excitability, synaptic connectivity, and cortical reorganization (Flor et al., 2006). Chronic pain states lead to maladaptive changes within S1, resulting in sensitization to noxious stimuli and alterations in sensory discrimination thresholds (Woolf & Salter, 2000). Moreover, interventions targeting S1 plasticity, such as cortical stimulation and neurofeedback training, hold promise for alleviating chronic pain symptoms and restoring sensory function (Moseley et al., 2018).

The affective processing of pain within the insular cortex relies on complex neurophysiological mechanisms, including neurotransmitter systems, synaptic plasticity, and oscillatory activity. Glutamatergic and GABAergic neurotransmission play crucial roles in mediating excitatory and inhibitory signaling within the insula, modulating neuronal activity and synaptic plasticity (Gogolla, 2017). Furthermore, oscillatory activity within the insular cortex, particularly in the theta and gamma frequency bands, facilitates the synchronization of neuronal ensembles and the integration of affective information (Boll et al., 2021). These mechanisms are critical for the insula's role in processing the emotional aspects of pain (Duerden & Albanese, 2013).

The central amygdala (CeA) is intricately interconnected with a plethora of brain regions, particularly those involved in motivation and reward such as the nucleus accumbens (NAc) and ventral tegmental area (VTA) as well as intra-amygdalar connection to the basolateral amygdala (BLA). CeA projections to the NAc regulate reward-seeking behaviors and motivational states in response to pain and aversive stimuli (Johansen, 2010). Additionally, CeA inputs to the VTA

modulate dopaminergic signaling and incentive salience, influencing the motivational aspects of pain perception (Han et al., 2017). Within the amygdala itself, there is extensive evidence for the role of the BLA, a highly connected neighboring subnuclei of the CeA, in pain affect and pain related emotion. Evidence shows that the BLA houses neuronal ensembles that are directly involved in encoding pain valence (Corder et al., 2019). The CeA also interacts with descending pain modulatory pathways through connections to regions such as the RVM (Heinricher et al., 2009; McNally et al., 2011).

The above sections are hardly an exhaustive list of potential targets for further research on pain affect as measured by the MGS, but certainly studies probing these circuits and cell types would be a boon to further our understand of grimacing as an affective readout of pain.

Chapter 4: Lateral Asymmetry of Pain-Induced Facial Grimacing

Overview and Impact

Chapter 4 explores the lateral asymmetry of pain-induced facial grimacing in mice, finding that pain is predominantly expressed on the right side of the face, contrary to other emotions that are typically lateralized to the left. This novel finding has significant implications for understanding the neurobiological basis of pain expression and its distinction from other emotional expressions. The lateralization of pain-induced facial grimacing to the right side of the face challenges existing notions about the lateralization of emotional expressions. While most facial expressions of emotion are stronger on the left side, reflecting right hemisphere dominance in emotional processing (Borod, 1992; Davidson, 1992), pain appears to deviate from this pattern. This discrepancy suggests that pain, as an aversive and motivationally significant experience, may engage different neural circuits than other emotions.

The right-sided predominance of pain grimacing could be related to the asymmetric organization of pain pathways in the brain. The central amygdala (CeA), a key region in pain affect, shows lateralized activation patterns, which might contribute to the observed asymmetry in facial expressions (Gao et al., 2021). Additionally, the involvement of the insular cortex and its connections to limbic structures may play a role in this lateralization, given its extensive network for integrating sensory and emotional information (Craig, 2009) (Gasquoine, 2014). The findings from Chapter 4 also have practical implications for the use of the MGS in pain research. Recognizing the lateralization of pain grimacing can refine the application and interpretation of the MGS, enhancing its sensitivity and specificity as a measure of pain affect. This insight can also inform the development of more targeted analgesic interventions that address the affective dimension of pain more effectively.

Critical Analysis, Remaining Questions & Further Research

The lateralization of facial expressions, particularly emotions, is well-documented, with the right hemisphere typically associated with more intense emotional expressions (Davidson, 1992). This phenomenon is particularly evident in spontaneous, genuine expressions of emotion, as opposed to voluntary, posed expressions (Borod, 1992). However, the right-side dominance in pain-induced facial grimacing observed in the studies suggests a different neuroanatomical basis for pain expression. This finding aligns with research indicating that the right hemisphere, particularly the right amygdala, plays a prominent role in the emotional processing of pain (Symons et al., 2014). The right amygdala's involvement in immediate and automatic emotional responses to pain, as well as its structural and functional changes in chronic pain patients, supports the observed lateralization.

The expression of pain may involve both ipsilateral and contralateral pathways. For instance, research by Symons et al. (2014) found differential activation of the amygdalae in response to pain, with the right amygdala playing a more prominent role in emotional processing. This suggests that pain-related facial expressions could be influenced by the ipsilateral connections of the right amygdala to the facial motor nucleus.

The exact mechanisms underlying ipsilateral control of facial expressions, especially in the context of pain, are not fully understood. Motor control of facial expressions and sensory perception of facial stimuli involve complex neural pathways that undergo decussation, where nerve fibers cross from one side of the body to the other. This process occurs at various levels of the nervous system, including the spinal cord, brainstem, and higher brain structures.

At the spinal cord level, sensory input from the face is primarily transmitted by the trigeminal nerve (cranial nerve V), which enters the brainstem at the level of the pons. The trigeminal nerve carries sensations of touch, temperature, and pain from the face (Nolte, 2010). Motor commands for facial movements originate in the facial motor nucleus within the brainstem and are carried by the facial nerve (cranial nerve VII) (Nolte, 2010). Sensory fibers carrying information from the face synapse with second-order neurons in the spinal cord. These neurons then cross over to the opposite side of the spinal cord before ascending through the brainstem to higher brain centers (Basbaum & Jessell, 2000). Similarly, motor commands from the facial motor nucleus decussate in the brainstem before exiting via cranial nerve VII to innervate the facial muscles (Nolte, 2010).

At the brainstem level, sensory fibers from the trigeminal nerve synapse in the trigeminal nucleus. Some of these fibers ascend ipsilaterally, while others decussate and ascend

contralaterally to higher brain structures (Basbaum & Jessell, 2000). Motor fibers from the facial motor nucleus also decussate in the brainstem before traveling through the facial nerve to innervate facial muscles (Nolte, 2010). In higher brain structures, sensory information from the face, after crossing over in the brainstem, ascends to areas such as the thalamus and somatosensory cortex for further processing and perception (Craig, 2003). Motor commands for facial expressions, originating from the facial motor nucleus, ascend to higher brain regions for integration with emotional and cognitive processing (Nolte, 2010). This intricate organization makes it extremely difficult to dissect the underlaying mechanism of lateralization of pain-induced facial grimacing.

One additional possible explanation as to why pain and emotion expression are not congruently lateralized is in their evolutionary underpinnings. Pain is the only emotional experience with an explicitly physical location and a direct tie to immediate threat of survival. Emotions communicate socially meaningful information (e.g., social norms), while pain communicates vital information that likely pertains to survival even if only indirectly which may be the basis for segregated information processing pathways for pain related emotions versus non pain related ones.

Summary and Conclusion

The collective findings from Chapters 2, 3, and 4 contribute to a nuanced understanding of pain processing, emphasizing the importance of both sensory and affective components. The normative data in Chapter 2 provide a foundational reference for preclinical pain testing, highlighting the variability inherent in these measures and the need for complementary assessments like the MGS. Chapter 3's exploration of the neuroanatomical substrates of pain-

induced facial grimacing underscores the distinct pathways involved in sensory and emotional pain processing. The clear link between facial grimacing and the affective dimension of pain supports the use of the MGS as a translationally relevant tool for assessing spontaneous pain. Chapter 4's novel finding of right-sided lateralization in pain-induced facial grimacing differentiates pain from other emotional expressions and suggests unique neural mechanisms underlying pain affect. This lateralization insight enhances the application of the MGS and underscores the need for a holistic approach to pain assessment that considers both sensory and emotional dimensions.

Together, these studies advance our understanding of pain as a complex, multidimensional experience. They highlight the need for comprehensive pain assessment tools that capture both the sensory and emotional aspects of pain, paving the way for more effective pain management strategies. The integration of these findings into preclinical and clinical research can enhance our ability to translate animal findings to human pain conditions, ultimately improving pain treatment and patient outcomes.

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