

Neural signaling and network connectivity in the brainstem and spinal cord: an fMRI investigation of fixed-temperature (38 °C, 46 °C, 51 °C) thermal stimulation and sex differences in humans.

By

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Abstract

Pain is a multidimensional experience mediated by both peripheral input and central processing. While much of the existing research has focused on cortical mechanisms, the spinal cord and brainstem play a critical role in descending modulation of pain. Investigating how these subcortical systems respond to varying thermal stimulation offers insight into the physiological organization of pain networks, particularly with respect to stimulus intensity and sex-related variability. This thesis used functional magnetic resonance imaging (fMRI) to examine neural signaling and directional connectivity within brainstem and spinal cord regions during constant-temperature thermal stimulation at 38 °C, 46 °C, and 51 °C. The first study examined how temperature intensity influenced subjective pain ratings, pupil diameter, and functional connectivity using Structural and Physiological Modeling (SAPM). Higher temperatures were associated with significantly increased pain intensity and unpleasantness, larger pupil responses, and distinct patterns of blood oxygenation level dependent (BOLD) connectivity. The greatest number of significant connections emerged at 46 °C, while 51 °C produced less consistent engagement, indicating a non-linear response pattern. Building on these findings, the second study assessed sex differences in response to the same thermal conditions. Although males and females reported comparable pain intensity, females exhibited lower pain unpleasantness ratings at 51 °C and showed distinct connectivity patterns. Specifically, females demonstrated stronger engagement of top-down modulatory pathways, while males showed stronger sensory-related input to the spinal cord. Together, these findings advance understanding of how stimulus intensity and assigned sex modulate central pain networks. The results support the involvement of non-linear mechanisms in descending modulation and contribute to characterizing individual variability in pain perception.

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Table of Contents

Abstract.....	ii
Acknowledgements.....	iii
List of Tables	viii
List of Figures.....	ix
List of Abbreviations	xi
Chapter 1: Introduction.....	1
1.1 Opening.....	1
1.1 General Background	1
1.2 Peripheral Sensitization	3
1.3 Pain vs. Nociception	4
1.3.1 Encoding Nociception: Nociceptors	4
1.3.2 Spinal Cord Dorsal Horn	5
1.3.3 Ascending Pain Pathways	6
1.3.4 Descending Modulation of Pain.....	6
1.3.5 Gate Control Theory of Pain.....	7
1.4 MRI.....	8
1.4.1 T1 and T2 Relaxation.....	9
1.4.2 MRI Sequences and Parameters.....	10
1.4.3 fMRI.....	11
1.4.4 BOLD Signaling	12
1.4.5 Spinal Cord fMRI	13
1.5 Structural and Physiological Modelling (SAPM).....	15
1.6 Present Study	16
1.6.1 Objectives	17
1.6.2 Hypotheses.....	18

1.7 References.....	19
Chapter 2: Neural signaling and network connectivity in the brainstem and spinal cord: an fMRI investigation of fixed-temperature (38 °C, 46 °C, 51 °C) thermal stimulation in humans.....	
2.1 Abstract.....	22
2.2 Introduction.....	23
2.3 Materials and Methods.....	25
2.3.1 Procedure Overview.....	25
2.3.2 Participants.....	26
2.3.3 Sham Training.....	26
2.3.4 Thermal Stimulation	27
2.3.5 fMRI Data Acquisition	28
2.4 Data Analysis	28
2.4.1. fMRI Data Analysis	28
2.4.2 Eye-Tracking Analysis.....	29
2.4.3 Structural and Physiological Modelling (SAPM)	29
2.5 Results.....	31
2.5.1 Pain Ratings and Physiological Response	31
2.5.2 SAPM Connectivity Results	32
2.5.3 Eye-Tracking Results.....	36
2.5.4 Questionnaire Results	36
2.6 Discussion.....	37
2.7 Conclusions.....	39
2.8 Acknowledgements.....	40
2.9 Conflict of Interest Statement	40
2.10 Funding Information	40
2.11 References.....	41

Chapter 3: The correlation between sex and pain processing at innocuous to noxious temperatures (38 °C, 46 °C, 51 °C) by means of fMRI in the brainstem and spinal cord	43
3.1 Abstract.....	44
3.2 Introduction.....	45
3.3 Materials and Methods.....	47
3.3.1 Procedure Overview.....	47
3.3.2 Participants.....	47
3.3.3 Sham Training.....	48
3.3.4 Thermal Stimulation	48
3.3.5 Eye-Tracking.....	49
3.3.6 fMRI Data Acquisition	49
3.4 Data Analysis	50
3.4.1 fMRI Data Analysis	50
3.4.2 Eye-Tracking Analysis.....	50
3.4.3 Structural and Physiological Modelling (SAPM)	50
3.5 Results.....	52
3.5.1 Subjective Pain Ratings	53
3.5.2 SAPM Connectivity Differences by Sex	54
3.5.3 Eye-tracking Results	57
3.5.4 Behavioural Results	57
3.6 Discussion.....	58
3.7 Conclusion	61
3.8 Acknowledgements.....	62
3.9 Conflict of Interest Statement	62
3.10 Funding Information	62
3.11 References.....	63
Chapter 4: General Discussion.....	65

4.1 References.....	70
Appendix A.....	71
Appendix B.....	72

List of Tables

Table 2.1. Connectivity (DB) values for connections at 38 °C, 46 °C, and 51 °C. Values in bold are significant at a Bonferroni $p < 0.05$. The corresponding DB values for the same connection in other conditions are included for comparison purposes, although they may not be significant at Bonferroni $p < 0.05$	13
Table 2.2 Summary of results from questionnaires for all participants.....	16
Table 3.1. Descriptive statistics for pain intensity and pain unpleasantness ratings for males at each study condition (38 °C, 46 °C, 51 °C).	32
Table 3.2. Descriptive statistics for pain intensity and pain unpleasantness ratings for females at each study condition (38 °C, 46 °C, 51 °C).	32
Table 3.3. Connectivity (DB) values for connections separated by sex (males versus females). Values in bold are significant at $p < 0.05$. The corresponding DB values for the same connection in other conditions are included for comparison purposes.	33
Table 3.4. Table depicting p-values which reflect the main effect of sex, the main effect of stimulation condition, and the interaction term for each connection. These connections exhibited a p -value < 0.05 but did not remain statistically significant after Bonferroni correction ($\alpha = 0.00044$).	34
Table 3.5. Self-report questionnaire scores across all participants. Values represent mean, median, and standard deviation for the State-Trait Anxiety Inventory (STAI-Y1 and STAI-Y2) [14], Pain Catastrophizing Scale (PCS) [15], and Beck Depression Inventory-II (BDI-II) [13].	37

List of Figures

Figure 2.1. Thermal stimulation paradigm of a single run in the MRI. The temperature used (38 °C, 46 °C, or 51 °C) depended on the pattern selected according to participant number, but regardless, each participant received 4x 38 °C stimuli, 4x 46 °C stimuli, and 4x 51 °C stimuli. Thermal stimulation was applied for a period of 30 seconds, in 12 contacts, with 2.5 second onsets, and a duration of 1.5 seconds each.	7
Figure 2.2. The SAPM network model of 35 connections between 10 regions, plus three “latent inputs” signaling from the periphery.	10
Figure 2.3. Bar graph depicting the behavioural results across the different study conditions (38 °C, 46 °C, and 51 °C). Mean pain ratings for pain intensity and pain unpleasantness are shown for each condition, with error bars representing the standard error. The legend indicates the colours associated with each condition.	11
Figure 2.4. Connectivity diagrams of the three temperatures (38 °C, 46 °C, 51 °C) and connectivity between regions in the nociceptive processing network, which includes the hypothalamus, thalamus, periaqueductal gray (PAG), locus coeruleus (LC), parabrachial nuclei (PBN), nucleus raphe magnus (NRM), nucleus gigantocellularis (NGC), nucleus tractus solitarius (NTS), dorsal reticular nucleus of the medulla (DRt), and right dorsal region of the spinal cord in the 6th cervical segment (C6RD). The gray ovals indicate anatomical regions. Black arrows indicate excitatory connections (positive DB values), and red arrows indicate inhibitory connections (negative DB values). The width of each arrow corresponds with the significance (T-value) of each connection.	13
Figure 2.5. Anatomical regions that demonstrated significant differences in connectivity between regions involved in pain-processing at 38 °C, 46 °C, and 51 °C. A sagittal view of the brainstem and spinal cord, and sagittal views of each region are shown for visualization purposes.	14
Figure 2.6. Average pupil diameter over time for each thermal condition. The blue line represents pupil size at 38 °C, the green line represents pupil size at 46 °C, and the red line represents pupil size at 51 °C. The green shaded area shows when the thermal stimulus was applied. Lines reflect the group average, with shading showing standard error. The sudden increases in pupil diameter seen at the beginning and end of the plots occur due to the visual display in the MRI system presenting a white screen followed by a black screen, which helps ensure pupil size is calibrated for each participant, and that they start at a consistent baseline pupil diameter.	15
Figure 3.1. Thermal stimulation paradigm for a single MRI run. Each participant received four stimuli at three temperatures, 38 °C, 46 °C, and 51 °C, in an order determined by a pre-assigned pattern based on participant number. Thermal stimulation was delivered over 30 seconds using 12 contacts, each with a 2.5-second onset and lasting 1.5 seconds.	27
Figure 3.2. The SAPM network model of 35 connections between 10 regions, plus three “latent inputs” signaling from the periphery.	30
Figure 3.3. Connectivity diagrams for each sex (males versus females) of the nociceptive processing network consisting of the hypothalamus, thalamus, periaqueductal gray (PAG), locus coeruleus (LC), parabrachial nuclei (PBN), nucleus raphe magnus (NRM), nucleus gigantocellularis (NGC), nucleus tractus solitarius (NTS), dorsal reticular nucleus of the medulla (DRt), and right dorsal region of the spinal cord in the 6th cervical segment (C6RD). Black arrows are associated with excitatory connections, and red arrows are associated with inhibitory	

connections. The width (thicker vs. thinner) of each arrow corresponds with the relative strength of each connection.34

Figure 3.4. Bar graph displaying mean directional connectivity (DB) values (\pm standard error) for male and female participants across five brainstem and spinal cord connections that exhibited uncorrected $p < 0.05$ in ANCOVA analyses assessing the main effects of sex, condition, and their interaction. Although these connections demonstrated preliminary trends suggestive of sex-related differences in pain-modulatory network engagement, none remained statistically significant following Bonferroni correction for multiple comparisons (corrected $\alpha = 0.00044$).35

Figure 3.5. Average pupil size during thermal stimulation across all temperature conditions, separated by sex (males versus females). Males are represented in blue, and females are represented in red. The green shaded region represents the stimulation period, and the yellow region indicates the anticipation period, when participants are told the stimulus is coming. The sudden increases in pupil diameter seen at the beginning and end of these plots occur due to the visual display in the MRI system presenting a white screen followed by a black screen. This ensures pupil size is calibrated for each participant, and that they start at a consistent baseline pupil diameter.36

List of Abbreviations

ANCOVA	Analysis of covariance
ANOVA	Analysis of variance
B ₀	External magnetic field
BDI	Beck Depression Inventory
BOLD	Blood-oxygen-level-dependent
CBF	Cerebral blood flow
CIHR	Canadian Institutes of Health Research
CMRO ₂	Cerebral metabolic rate of oxygen
C6RD	Right dorsal region of the 6th cervical spinal cord segment
CSF	Cerebrospinal fluid
DB	Connectivity value
DH	Dorsal horn of the spinal cord
DICOM	Digital imaging and communication in medicine
DR _t	Dorsal reticular nucleus
EPI	Echo planar imaging
FM	Fibromyalgia
fMRI	Functional magnetic resonance imaging
FOV	Field-of-view
GE	Gradient echo
HASTE	Half-Fourier single-shot turbo spin echo
Hb	Deoxygenated hemoglobin
HbO ₂	Oxygenated hemoglobin
LC	Locus coeruleus
LT	Low threshold
MRI	Magnetic resonance imaging
N	Sample size
NGc	Nucleus gigantocellularis
NifTI	Neuroimaging informatics technology initiative
NRM	Nucleus raphe magnus
NSERC	Natural science and engineering research council
NTS	Nucleus tractus solitarius
PAG	Periaqueductal gray
PBN	Parabrachial nucleus
PCS	Pain catastrophizing scale
RVM	Rostroventromedial medulla
SAPM	Structural and physiological modelling
SE	Spin echo
STAI	State-trait anxiety inventory
T1	Longitudinal relaxation time
T2	Transverse relaxation time
T2*	Transverse relaxation time (observed)
WDR	Wide dynamic range

Chapter 1: Introduction

1.1 Opening

This thesis will discuss the influence of thermal stimuli at temperatures varying between innocuous and noxious (38 °C, 46 °C, 51 °C) on pain perception and the neural activity associated with this, in healthy individuals. Additionally, this thesis will review the influence of sex (males versus females) on pain perception, neural signaling and network connectivity in the brainstem and spinal cord. To better understand the complex relationship between variables such as differing stimuli or sex differences, it is crucial to understand the concept of peripheral sensitization. Peripheral sensitization is a key mechanism which plays a role in pain perception. It occurs when nociceptors, sensory neurons that detect harmful stimuli, become hypersensitive due to the presence of local inflammatory mediators. This results in a lowered activation threshold and an exaggerated response to stimuli. Due to this, primary afferent nociceptors exhibit increased firing rates. When this enhanced input reaches the dorsal horn (DH) of the spinal cord, the resulting pain signal is amplified, even if the original stimulus remains the same. In this thesis, functional magnetic resonance imaging (fMRI) will be used to examine regions within the brainstem and spinal cord to identify differences in neural connectivity associated with innocuous and noxious temperatures, as well as sex differences.

1.1 General Background

One of the first steps in understanding individual differences in pain perception is to look at what happens at the periphery. When tissue is damaged or inflamed, nociceptors in the area become more reactive due to the influence of chemical mediators. These mediators reduce the threshold for activation, meaning that even a light touch or minor heat stimulus can start to feel

painful [1, 2]. This process, known as peripheral sensitization, is involved in pain conditions like hyperalgesia and allodynia, and it also shapes how the brain and spinal cord respond to incoming sensory signals [1, 2]. As previous research has determined, pain perception is extremely individualized. For example, there are sex-based differences in pain experiences and perception, and some of these are thought to reflect differences in how peripheral input interacts with central processing [3, 4]. While earlier research focused on cortical regions, more recent work has begun to examine the role of the brainstem and spinal cord areas that help regulate pain through descending control pathways. These lower regions act as filters, modifying pain signals before they ever reach the brain. Because of that, changes in input, whether due to stimulus intensity or assigned sex, can significantly affect what a person feels [5, 6]. Although we know peripheral sensitization affects pain perception, there is still limited evidence on how it influences the brainstem and spinal cord in real time. Functional magnetic resonance imaging (fMRI) is one tool that helps address this gap. It can track blood oxygenation-level dependent (BOLD) signal changes during pain processing and show how communication between pain-related areas shifts depending on stimulus strength [7, 8]. Functional MRI also makes it possible to compare responses between males and females to examine whether the underlying mechanisms differ [9, 10]. The present study uses thermal stimulation at three temperatures, 38 °C (innocuous), 46 °C (moderately noxious), and 51 °C (noxious), to examine how subjective pain ratings and brainstem-spinal cord connectivity are affected. By comparing responses across male and female participants, this study aims to uncover whether the way peripheral input influences central pain systems differ by sex. Ultimately, the goal of this research is to better understand what causes the variability we see in pain responses across individuals, and to contribute to the broader work of exploring how pain is modulated throughout the nervous system.

1.2 Peripheral Sensitization

Peripheral sensitization happens when nociceptors at the site of injury or inflammation increase in sensitivity. Essentially, they start reacting more easily and more intensely than is typical under normal conditions [1, 11]. This shift plays a large role in phenomena such as how acute pain sometimes develops into long-term or chronic pain, and it is especially relevant in both inflammatory and neuropathic pain scenarios [1, 11]. After tissue damage occurs, a wide variety of inflammatory substances are released, including prostaglandins, bradykinin, ATP, serotonin, cytokines like TNF- α and IL-1 β , as well as nerve growth factor (NGF), and these come from multiple sources such as immune cells, damaged tissue, as well as the neurons themselves [1, 11]. These mediators bind to receptors on the peripheral ends of nociceptors, triggering internal responses which affect the way the neurons behave. Specifically, they influence ion channels, major ones being TRPV1, Nav1.7, Nav1.8, and ASICs, making the neurons more excitable and easier to activate [2]. This change explains why something that normally would not hurt at all, such as a light touch, can suddenly cause discomfort (allodynia), or why mildly painful input can become harder to tolerate (hyperalgesia). Furthermore, environmental factors at the injury site, such as low oxygen or increased acidity, can increase pain sensitivity [2]. Additionally, neurogenic inflammation takes place, where neurons themselves release neuropeptides like CGRP and substance P, which increase blood flow and inflammation in the area, and maintain the sensitization [2]. The longer this continues, the more likely it is to start affecting the central nervous system too, resulting in a state where the brain and spinal cord amplify pain signals. This process is known as central sensitization. It is well known that this interaction between peripheral and central mechanisms often leads to persistent pain, even after the original injury has healed. Stopping this process early has been shown to be effective in the management and treatment of

pain [2]. Pain medications, including NSAIDs and COX inhibitors, block the chemical messengers involved in peripheral sensitization. Newer therapies, such as TRPV1 antagonists, try to prevent this sensitization from happening in the first place without interfering with normal pain signaling. Ongoing research continues to investigate the molecular mechanisms of this process, with the goal of designing more targeted and effective treatments [2].

1.3 Pain vs. Nociception

Although the terms 'pain' and 'nociception' are often used synonymously, these words refer to distinct processes in the nervous system. The IASP defines pain to comprise both sensory and emotional components, therefore making it a subjective experience. Pain is associated with literal or potential injury to tissues, but it is also influenced by an individual's context, past experiences, and how the individual interprets the situation [12]. Thus, understanding pain requires more than just understanding biological signals; it also requires the interpretation of neural and psychological components. Conversely, nociception refers to the detection of potentially dangerous or harmful stimuli by the nervous system. Such processes can take place without awareness and may produce involuntary autonomic changes, for example, changes in heart rate, or reflexes such as moving a limb. Interestingly, the sensation of pain is not necessary for these reactions to occur. Essentially, nociception has to do with the processing and transmitting of harmful signals, whereas pain has to do with the perception of such signals and the influence of biopsychosocial components [13].

1.3.1 Encoding Nociception: Nociceptors

Nociceptors are specialized receptors that are triggered when potentially harmful stimuli are detected. They are found throughout the skin and other tissues and respond to chemical,

mechanical, and thermal inputs. C polymodal nociceptors are the most prevalent, and they are known to respond to all three types of noxious stimuli [1, 14]. These nociceptors are associated with slow conducting unmyelinated C fibers that are responsible for dull, lingering pain long after an injury. Conversely, other nociceptors have a narrower focus on thermal and mechanical damage. These are associated with thinly myelinated A δ fibers, which conduct more quickly and are responsible for the sharp pain felt immediately after an acute injury [1, 14].

1.3.2 Spinal Cord Dorsal Horn

Noxious stimuli, which activate nociceptors, produce impulses that travels along either unmyelinated C fibers or thinly myelinated A δ fibers, to the dorsal horn of the spinal cord. This locality plays a central role in the early reception and structuring of sensory stimuli [14]. Afferent input in the dorsal horn has synapses with both projection neurons and local interneurons, where they are integrated before further transmission [14]. Three main classes of projection neurons participate in this process. Nociceptive-Specific (NS) neurons, located predominantly within Lamina I, respond exclusively to noxious inputs. Further, Low-Threshold (LT) neurons are found in Laminae III and IV and are provoked by noxious stimulation. Finally, Wide Dynamic Range (WDR) cells in mediolaterally-located Lamina V can respond to both noxious and innocuous input from a variety of fiber-types. Conjointively, these projection neurons enable the dorsal horn to process and interpret complex sensory input before transmitting it through ascending pathways to the brain [14].

1.3.3 Ascending Pain Pathways

Following initial processing in the dorsal horn, several distinct pathways carry nociceptive signals to the brain. Among the most studied is the spinothalamic tract, which transmits important pain and temperature information from the spinal cord to the thalamus. The tract has two principal subdivisions. The lateral, or neospinothalamic, tract primarily projects to the ventral posterolateral (VPL) nucleus of the thalamus and is mainly responsible for the somatosensory-discriminative aspects of pain, such as identifying its location and intensity [15]. The medial spinothalamic tract, or paleospinothalamic tract, projects to the medial thalamic nuclei and is more closely related to the emotional and motivational components of the pain experience. Other ascending systems also play a role in the perception and control of pain. The spinoreticular tract projects to the brainstem reticular formation, and modulates arousal and alertness in response to pain, predominantly via the Reticular Activating System. Moreover, the spinomesencephalic projection reaches the periaqueductal gray (PAG), a midbrain area essential for triggering descending pain inhibitory pathways [15]. Collectively, these two parallel pathways make it possible for the CNS to not only pinpoint the sites of painful stimuli but also to sort out the kinds of painful stimuli it experiences.

1.3.4 Descending Modulation of Pain

Pain perception is shaped both by ascending nociceptive input, and descending modulation from the brain, which can suppress or amplify pain signals. Descending pain pathways serve a very important role, in that they can initiate nociceptive processing, which heightens the sensitivity of nociceptors so that even unharmed, innocuous stimuli feel painful [6]. This is the case in several chronic pain conditions, such as fibromyalgia and complex regional pain syndrome. Conversely, nociceptive sensitivity can be reduced through inhibitory modulation, a mechanism which

functions as a protective mechanism during the presence of acute stress. This phenomenon is important when the suppression of pain is crucial to survival [6]. The main descending pain modulatory circuit is comprised of the periaqueductal gray (PAG), the rostroventromedial medulla (RVM), and the dorsal horn of the spinal cord [16]. The integrated input that the PAG receives comes from cortical and limbic structures and from ascending nociceptive signals that reach it mainly through the parabrachial nucleus. The PAG communicates in both directions with the RVM. The RVM serves as the final relay that the pain signal reaches before it descends to the spinal cord [16]. In the RVM, important subdivisions like the nucleus raphe magnus (NRM) and nucleus gigantocellularis (NGC) contain unique sets of neurons that oppose each other in pain modulation. Neurons in these subdivisions, but not limited to these subdivisions, have been designated as "on" or "off" cells in terms of their relationship to nociceptive transmission. Opioids are known to suppress on-cell activity and enhance the firing of off-cells [5, 16]. In chronic pain conditions, such as fibromyalgia, suggested to affect descending inhibition, this system is suspected to be disrupted. For this reason, the PAG-RVM-spinal cord pathway remains a focus in identifying neural mechanisms underlying chronic pain and in developing targeted therapeutic interventions [17].

1.3.5 Gate Control Theory of Pain

The gate control theory of pain, first introduced by Melzack and Wall in 1965 [18], provided a foundational shift in how pain transmission and modulation are understood. In this model, there is a gate-like formation located in the dorsal horn of the spinal cord, particularly within the substantia gelatinosa, which can either permit or inhibit the transmission of nociceptive signals to the brain. If considering the substantia gelatinosa as containing 'gates' that can open and close, then

when they are open, nociceptive signaling from small-diameter fibers (such as unmyelinated C fibers and thinly myelinated A δ fibers) can be transmitted to the brain [18]. When the 'gates' are closed, however, the neural signals carried by these small-diameter fibers are less likely to be transmitted to the brain [18]. Moreover, when A δ fibers are stimulated, the 'gates' are closed more than when the 'gates' are open. With the components just described, this model can explain a number of important processes. Firstly, it can account for the 'Rubbing Effect', which occurs when an individual is experiencing pain and rubs the area, A δ fibers become activated, and this inhibits the nociceptive input to the brain [18]. The importance of this theory goes beyond neurophysiology; it also explains why the experiences of pain can vary so dramatically between individuals, even with comparable injuries. Emotional factors and states, such as anxiety, stress, or past trauma, might facilitate the 'gate opening' in the spinal cord and thereby intensify perception of the noxious stimulus [18]. Conversely, one individual might perceive the same noxious stimulus as less intense than another, simply due to more effective "gate control" in their spinal cord. In more recent years however, this theory has been updated and expanded to better explain how descending pain regulation also contributes to how nociceptive signals are relayed from the spinal cord to the brain [18].

1.4 MRI

MRI, a non-invasive and powerful imaging technique, is used extensively to investigate internal anatomy and functions. It is especially valued in neuroscience for imaging the brain, brainstem, and spinal cord at high spatial resolution without using ionizing radiation [19]. The technique uses the principles of nuclear magnetic resonance by changing the magnetic properties of hydrogen nuclei, which are abundant in biological tissues due to high water content. When an

individual is placed in a strong static magnetic field (B_0), the magnetic dipoles of hydrogen protons tend to align with the field, producing a net magnetization vector in the longitudinal direction. This alignment signifies an equilibrium state. A radiofrequency (RF) pulse at the Larmor frequency temporarily tips this magnetization away from the longitudinal axis into the transverse plane [19]. After the RF pulse is terminated, the protons gradually return to their original alignment through the process of relaxation, resulting in signals that can be detected and spatially encoded using magnetic field gradients. The strength and timing of these signals vary depending on the molecular environment, which forms the basis for contrast in MRI. By collecting signal data at specific time points during relaxation, image contrast between tissues can be optimized [19]. High magnetic field strengths, such as those used in 3 Tesla (3T) MRI systems, increase the signal-to-noise ratio, and improve anatomical detail relative to lower field strengths. These systems are advantageous in research environments where submillimeter structural resolution is needed, such as for visualizing cortical and tissues structures and deep brain nuclei. In the context of pain research, MRI offers the ability to image critical structures involved in nociceptive processing and descending modulation, including the spinal cord and brainstem, which are difficult to assess with other imaging modalities [19].

1.4.1 T1 and T2 Relaxation

Once a radiofrequency (RF) pulse has tipped the net magnetization vector into the transverse plane, the return to equilibrium occurs via two simultaneous but distinct relaxation processes. Longitudinal relaxation, or spin-lattice relaxation, is described by the time constant T_1 and involves the recovery of the magnetization vector along the direction of the external magnetic field (B_0) [19]. Transverse relaxation, or spin-spin relaxation, is described by the time constant T_2 and

accounts for the dephasing of spins in the transverse plane due to microscopic magnetic interactions between neighboring protons. These processes are a result of the mobility and interaction of water molecules and macromolecules within tissues [19]. T1 recovery occurs through energy transfer from excited spins to the surrounding lattice, while T2 decay occurs due to loss of phase coherence caused by spin interactions without energy exchange [19]. T1 and T2 are intrinsic tissue properties, and their values vary across different tissues, forming the basis for image contrast in MRI. T1 tends to be shorter in fat-rich tissues and longer in fluid-rich environments such as cerebrospinal fluid. T2 is generally shorter than T1 and is influenced by molecular complexity and interactions. Additionally, field inhomogeneities introduce a third relaxation parameter, T2*, which incorporates both spin-spin effects and dephasing caused by magnetic field variations [19]. T2* is shorter than T2 and is relevant in functional MRI where subtle changes in susceptibility, such as those caused by blood oxygenation levels, are detected. T2*-weighted images provide the foundation for blood oxygenation level-dependent (BOLD) contrast, making the understanding of these relaxation times essential in both structural and functional imaging contexts. The variation in these parameters based on molecular environment allows for precise tissue characterization using carefully timed pulse sequences [19].

1.4.2 MRI Sequences and Parameters

The contrast and spatial properties of an MR image are heavily influenced by acquisition parameters and the pulse sequences applied. These include the repetition time (TR), echo time (TE), and flip angle [19]. TR is defined as the time interval between successive RF excitations, while TE is the delay between the RF pulse and the peak of the echo signal that is received. Adjusting these values determines whether the resulting image is T1-, T2-, or T2*-weighted. A

short TR and TE will yield a T1-weighted image, while a long TR and TE result in T2 contrast. Flip angle, the degree to which the magnetization vector is tipped from the longitudinal axis, also influences signal intensity and image contrast [19]. Most sequences fall into two general categories: spin-echo (SE) and gradient-echo (GE). SE sequences use a 180° refocusing pulse to correct for magnetic field inhomogeneities and produce high-contrast, high-quality images, which are typically T2-weighted. GE sequences use gradient reversals and a lower flip angle, which enables faster data acquisition but increases sensitivity to magnetic field distortions. These distortions are particularly problematic in anatomically complex areas such as the brainstem and spinal cord. For functional imaging, GE sequences are often chosen due to their enhanced sensitivity to T2* contrast, which is crucial for detecting BOLD signal changes. However, GE sequences are more prone to geometric distortion and signal dropout [19]. To overcome these limitations, hybrid sequences like the half-Fourier single-shot turbo spin-echo (HASTE) have been developed. HASTE sequences offer a balance between speed, spatial resolution, and artifact resistance. In the current study, a HASTE sequence, rather than echo planar imaging (EPI) was selected to obtain functional images in the brainstem and spinal cord, minimizing geometric distortion while preserving sensitivity to physiologically relevant signal changes. The ability to tailor pulse sequence parameters makes MRI a versatile tool for assessing both anatomical structure and physiological function [19].

1.4.3 fMRI

Functional MRI (fMRI) expands on the capabilities of structural MRI by allowing for the imaging of neural activity based on localized changes in physiology [19]. The most commonly used mechanism for functional contrast is Blood Oxygenation Level Dependent (BOLD) contrast.

Neuronal activity demands high energy consumption, primarily through oxidative metabolism. When a brain region becomes active, it consumes oxygen more rapidly, prompting a localized increase in cerebral blood flow that typically exceeds metabolic demand [19]. This overcompensation results in a relative increase in oxygenated hemoglobin and a reduction in deoxygenated hemoglobin within the capillary bed of the active region. Deoxygenated hemoglobin is paramagnetic and creates local magnetic field inhomogeneities that accelerate $T2^*$ decay, reducing signal intensity in $T2^*$ -weighted images [19]. When its concentration decreases due to increased perfusion, the local magnetic field becomes more homogeneous, leading to an increased $T2^*$ signal. Thus, fluctuations in $T2^*$ -weighted signal can indirectly indicate localized neural activity [19]. Although fMRI does not measure electrical activity, the close coupling between synaptic activity, metabolic demand, and hemodynamic response makes BOLD MRI a reliable tool to study brain activation. Echo-planar imaging is typically used due to its speed and compatibility with time-series acquisition, though it is vulnerable to distortion, especially in regions like the brainstem [19]. Spatial resolution and temporal sampling can be adjusted based on experimental needs. Functional imaging data are collected over time, often during alternating periods of task and rest, allowing comparison of signal intensity across conditions. The ability to visualize and quantify neural activity in real time has made fMRI an essential tool for studying sensory, motor, and cognitive processes, as well as for mapping the circuits involved in pain processing and modulation [19].

1.4.4 BOLD Signaling

The Blood Oxygenation Level Dependent (BOLD) signal is the foundation for most functional MRI studies. It is based on the principle that neural activity is associated with changes

in local blood oxygenation [19]. Increased neuronal firing raises the demand for oxygen, resulting in an increase in cerebral blood flow. This vascular response generally delivers more oxygenated blood than is metabolically required, resulting in a temporary decrease in deoxygenated hemoglobin concentration [19]. Oxygenated hemoglobin is diamagnetic and does not disrupt the magnetic field, whereas deoxygenated hemoglobin is paramagnetic and shortens T2 and T2* relaxation times by introducing magnetic field inhomogeneities [19]. Thus, an increase in oxygenation leads to a more homogeneous magnetic field and an increase in T2*-weighted signal intensity. BOLD signals are measured by collecting images repeatedly over time to track these fluctuations. Experimental designs typically include either block paradigms, where task and control conditions are alternated in sustained intervals, or event-related paradigms, which involve brief, discrete stimuli. BOLD contrast does not provide direct measures of neuronal firing, but because it provides insights into neural activity, metabolic demand, and hemodynamic response, it is an important measure used in pain research [19]. The magnitude of the BOLD response is influenced by several physiological factors including vascular reactivity, baseline blood flow, and local tissue properties. In summary, BOLD MRI has been shown to be a reliable and widely used method for evaluating localized neural function in both healthy and clinical populations, thus being useful in pain research [19].

1.4.5 Spinal Cord fMRI

Functional MRI of the spinal cord introduces a set of technical challenges that are not present in standard brain imaging. The anatomical location of the cord within the vertebral column, surrounded by bone, cerebrospinal fluid, and other tissues, causes magnetic field inhomogeneities that lead to geometric distortions and signal loss [19]. Additionally, the small cross-sectional area

of the spinal cord limits the achievable spatial resolution. Physiological motion presents another complication: cerebrospinal fluid pulsation, driven by cardiac cycles and respiratory pressure changes, causes constant motion of the cord and surrounding structures. These fluctuations introduce temporal noise and make it difficult to detect small changes in signal intensity. Gradient-echo imaging is particularly sensitive to these effects and prone to signal dropout. To address these issues, the current study used a half-Fourier single-shot turbo spin-echo (HASTE) sequence, which is based on spin-echo principles and therefore less affected by artifacts [19]. This sequence provides a balance between spatial resolution, functional sensitivity, and artifact resistance. For this study, a 3 Tesla MRI system was used with a TR of 6.75 seconds and TE of 75 milliseconds, based on previous work showing these parameters optimize spinal cord BOLD signal detection. However, acquisition alone is insufficient; specialized data processing is also required. Unlike brain imaging pipelines, spinal fMRI processing must account for unique anatomy and physiological noise [10]. This includes spatial normalization to a spinal cord template, motion correction, and co-registration tailored to individual cord geometry. These steps are essential for accurate localization of functional responses and interpretation of BOLD signal changes in the spinal cord. As spinal cord MRI continues to develop, these combined acquisition and analysis strategies remain essential for studying sensorimotor pathways and nociceptive modulation within the cord. All of these methods have been optimized for use in the brainstem and spinal cord and are included in the software package called “Pantheon” (<https://github.com/stromanp/pantheon-fMRI>) [19].

1.5 Structural and Physiological Modelling (SAPM)

To investigate how neural signaling changes during pain, it is essential to use an analysis method capable of capturing detailed physiological activity across both the spinal cord and brainstem. Functional connectivity can be analyzed using specialized methods such as SAPM, which stands for Structural and Physiological Modeling, an advanced method of fMRI data analysis [20]. SAPM was developed by Dr. Patrick W. Stroman, and it is most effective when investigating predefined networks containing several regions of interest and makes it possible to quantify the directional interactions (i.e., how one region affects another) that occur between them. Unlike traditional correlation-based methods, which assess whether regions co-activate over time, SAPM estimates the signal each region receives (input) and sends (output), and whether these influences are excitatory or inhibitory in nature. SAPM begins with a predefined model network composed of anatomical regions in the brainstem and spinal cord known to be involved in pain processing and modulation [20]. For the present study, the modeled network included the right dorsal horn of the sixth cervical spinal segment (C6RD), dorsal reticular nucleus (DRt), hypothalamus, locus coeruleus (LC), nucleus gigantocellularis (NGc), nucleus raphe magnus (NRM), nucleus tractus solitarius (NTS), periaqueductal gray (PAG), medial and lateral parabrachial nuclei (PBN), and the medial thalamus. In addition to these defined regions, the model incorporates latent inputs. These are hypothetical sources of modulation that represent external influences not directly observed in the imaging data. Such influences could be cognitive, emotional, or autonomic in nature. Together, with these additional components, the model can provide a more comprehensive understanding of how various direct and indirect factors shape neural communication [20]. SAPM is based on two main ideas. The first is that the BOLD signal in any given region is the integrated result of all the influences coming into that region. The second

is that the region, in turn, sends out a signal that is a weighted sum of all those influences. If increased input corresponds to increased output, the effect is classified as excitatory. Conversely, if greater input results in reduced output, it is considered inhibitory. These relations of directionality allow us to figure out which parts influence which other parts in the network [20]. Because each anatomical region may consist of a variety of physiologically distinct subregions, testing every possible configuration for each participant is too much to compute. Thus, SAPM uses a gradient-descent search algorithm to find the best set of subregions that fit the observed time-series data [20]. In this context, a subregion consists of a group of connected pixels in the image data, and SAPM uses a combination of subregions to estimate the connectivity values. Using a gradient-descent search implies that, starting with a poorly fitting configuration, the algorithm will reliably identify a better set of subregions with improvements on each iteration until no further improvements can be found. Averaging across participants yields the average connectivity values, and for each value a standard error is calculated [20]. When the mean DB is divided by its standard error, we get a T-value that is a measure of the strength of that connection and its reliability, or statistical significance, across the group. Using this method, connectivity diagrams can be generated for the experimental conditions and can also be related to behavioural measures (e.g., pain intensity or pain unpleasantness). Overall, SAPM provides a description of functional connectivity in networks within the brainstem and spinal cord that is more specific and detailed than what is usually obtained with other methods [20].

1.6 Present Study

The present study examined the relationship between varying intensities of thermal stimuli, ranging from innocuous to noxious, as well as associated subjective pain perception, physiological

reactions, and the neural signaling within in the brainstem and spinal cord. The data were obtained from 42 healthy adult participants (21 females, 21 males), 20 to 57 years in age. Each subject received thermal stimulation at three fixed temperatures (38 °C, 46 °C, and 51 °C) applied to the right palm, while spinal cord and brainstem fMRI scans were acquired. For each condition, participants provided pain intensity and unpleasantness ratings, and physiological responses, including pupil diameter, were recorded through eye-tracking. This study was divided into two separate analyses, and thus two projects. Project 1 examined the relationship between stimulus intensity and both subjective (verbal pain ratings) and physiological pain measures (via fMRI). Analyses involved evaluating changes in verbal pain reports across temperature conditions and investigating peripheral sensitization. Project 2 expanded further on this analysis to evaluate potential differences between sexes in pain perception and neural connectivity. More specifically, it examined whether there were differences between males and females in pain ratings, physiological markers, and modeled network connectivity under each thermal condition. All together, these experiments provide insight into the intensity of stimuli and its interaction with peripheral and central mechanisms of the pain processing system. They also contribute to a deeper understanding of how those processes might be influenced by assigned sex, providing a more distinct view of individual variations in the experience and regulation of pain.

1.6.1 Objectives

Our objectives were to identify 1) the effect of innocuous versus noxious temperatures on neural signaling and network connectivity in the brainstem and spinal cord, shown by means of fMRI, and 2) the difference in neural signaling and network connectivity in the brainstem and spinal cord between males and females.

1.6.2 Hypotheses

Hypothesis 1) Higher temperatures, compared to lower temperatures, increase pain ratings in all healthy participants.

Hypothesis 2) An increase in temperature results in an increase in connectivity between brainstem and spinal cord regions.

Hypothesis 3) There is a difference in regional connectivity and neural signaling to the brainstem and spinal cord with males compared to females at all three fixed temperatures.

Previous research has demonstrated that increasing thermal stimulus intensity is associated with higher pain ratings as well as changes in neural signaling within brainstem and spinal cord circuits [1,8]. This evidence led to the hypothesis that higher temperatures would result in greater perceived pain, and related changes in network connectivity in the brainstem and spinal cord, consistent with mechanisms of peripheral sensitization [2]. While sex differences in pain perception and modulation are well-documented, particularly surrounding pain sensitivity and affective processing, their role within subcortical pain-modulatory networks still lacks understanding [3, 4]. By using a combination of fMRI scans and SAPM analyses [20], this present study sought to examine how variations in stimulus intensity and assigned sex influence pain-related connectivity across spinal cord and brainstem regions in healthy individuals.

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**Chapter 2: Neural signaling and network connectivity in the brainstem and spinal cord:
an fMRI investigation of fixed-temperature (38 °C, 46 °C, 51 °C) thermal stimulation in
humans**

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Data Availability Statement:

Data are available upon reasonable request from the corresponding author and are not publicly available. The analysis software, Pantheon, is freely available on GitHub at <https://github.com/stromanp/pantheon-fMRI>

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2.1 Abstract

Pain is a complex phenomenon involving both peripheral sensitization and central modulation. While much of the existing literature has focused on cortical mechanisms, a deeper understanding of pain processing requires investigation of subcortical structures, particularly the brainstem and spinal cord, where descending regulatory circuits are engaged. This study examined how thermal stimulation influences pain perception, network connectivity, and physiological markers within these regions. Forty-two healthy adults underwent fMRI scanning while receiving thermal stimuli at 38 °C, 46 °C, and 51 °C. Subjective pain ratings and pupil diameter were recorded to assess both perceptual and autonomic responses. Directed network modeling was used to evaluate input and output signaling across defined brainstem and spinal cord regions. Findings revealed that pain perception and connectivity patterns varied as a function of stimulus intensity. The highest number of significant directional connections was observed at 46 °C, while fewer connections emerged at 51 °C, suggesting potential adaptive modulation at higher intensities. Pupil diameters increased as participants experienced the noxious stimuli, indicating a link between autonomic arousal and nociceptive processing. These results demonstrate temperature-dependent shifts in subcortical network dynamics and expand current models of pain by incorporating brainstem contributions to sensory, affective, and autonomic regulation.

Keywords: pain; fMRI; brainstem; spinal cord; peripheral sensitization

2.2 Introduction

Sensation, emotion, and cognition coincide in the intricate and multidimensional experience of pain. Pain begins at the periphery with the activation of nociceptors. These specialized afferent neurons can sense noxious thermal, mechanical, or chemical stimuli. They are connected by vast and intricate networks of neurons. These signals travel up the spinal cord and along the dorsal horn of the spinal cord to several, more complex, supraspinal structures, where the signals are processed. The nervous system component mediating the sensation and discrimination of painful stimuli is responsible for localizing the stimulus in space and for assessing its intensity and quality. This part is known as the spinothalamic tract. Affective and motivational parts of the brain also work with limbic and paralimbic areas that impact the range of emotional responses associated with pain and drive behavioural responses towards actions that may reduce pain [1, 2]. Furthermore, modulatory processes influence pain perception at multiple points along the neuroaxis. Among these, descending control systems starting in the brainstem are responsible for regulating spinal nociceptive transmission. These descending circuits can inhibit or facilitate nociceptive output depending on physiological and contextual factors. At the peripheral level, intense or repeated stimulation can cause peripheral sensitization. In this condition, nociceptors display enhanced excitability and lowered thresholds, which makes them more likely to respond to signals they normally would not [3]. This mechanism is well characterized in chronic pain states, but its immediate impact on subcortical circuits during acute noxious stimulation is not fully established.

The relay and regulation of nociceptive information critically involve the spinal cord and brainstem. Several brainstem structures are part of a distributed network that modulates ascending

and descending pain signals. These structures include the periaqueductal gray (PAG), nucleus raphe magnus (NRM), parabrachial nucleus (PBN), hypothalamus, and locus coeruleus (LC). These areas contain inputs from both the cortical and limbic structures, as well as from peripheral sensory pathways, and influence nociceptive output based on threat level, emotional state, attention, and homeostatic need [4, 5, 6]. Functional connectivity among these regions is thought to reflect the balance of excitatory and inhibitory influences which shape how pain is perceived under different physiological conditions, yet relatively few studies have characterized these interactions within the brainstem and spinal cord under varying levels of acute nociceptive input. Researchers often use thermal stimulation in pain studies because it can deliver reliable, controlled, repeatable, and painful stimuli that do not damage tissue. By altering skin temperature from an innocuous range to noxious levels, researchers can examine the effects of escalating nociception on neural responses [7, 8]. Past research has demonstrated that higher stimulus intensities correspond with elevated subjective pain ratings and increased activation in brain areas known to be involved in pain [8]. However, information is lacking about how such stimuli influence functional and connectivity within brainstem and spinal circuits, especially regarding network reorganization and modulation at different temperatures.

The present study addresses this gap in knowledge by examining the effects of fixed temperatures thermal stimulation at 38 °C (innocuous), 46 °C (moderately noxious), and 51 °C (noxious), on subjective pain ratings, pupil-linked autonomic arousal, and connectivity between pre-defined brainstem and spinal cord regions. Functional MRI data were analyzed using Structural and Physiological Modeling (SAPM), a technique developed in our lab, that estimates the signaling input to and output from a set of predefined regions in a way that is both directional

and physiologically interpretable [9]. Using this method, one can detect the temperature-dependent changes that occur in connectivity and gain insight into the pain-modulatory networks that reorganize in response to increased noxious input. We hypothesized that increasing thermal intensity would lead to corresponding increases in pain intensity and unpleasantness ratings, greater pupil dilation, and systematic changes in functional connectivity. Particularly, we expected that moderately noxious (46 °C), and noxious (51 °C) stimulation would involve descending pathways engaged in pain modulation, whereas innocuous stimulation (38 °C) would not. By exploring connectivity changes across this range of stimulation, this study aims to advance current understanding of how subcortical structures contribute to the sensory, affective, and autonomic components of acute pain processing.

2.3 Materials and Methods

2.3.1 Procedure Overview

This study involved a single visit to our MRI Facility, divided into two parts. First, participants underwent a "sham" training phase in an MRI simulator. During this phase, they became familiar with the study procedures, including the MRI environment and pain rating scale usage. Following this, participants proceeded to the MRI system for the second part of the visit, where their brainstem and spinal cord were imaged, and eye-tracking was conducted while they experienced periods of noxious thermal stimulation on their hand. Each participant then used the pain rating scales, as introduced earlier, to quantify their pain experienced from the thermal stimulation, as practiced during the training session. Further details regarding each component of the study are outlined below.

2.3.2 Participants

The present study was comprised of 42 healthy participants (21 males, 21 females), aged between 20 and 57 years. These individuals were recruited using community posters and through social media platforms, such as LinkedIn and Reddit. Certain criteria were required to be met, including being free of chronic pain, and having no serious past or present neurological injuries or diseases. These criteria classified participants as healthy in this study. All participants provided informed consent. Self-report questionnaires were administered to assess affective and cognitive factors known to influence pain perception. These included the Beck Depression Inventory-II (BDI-II) for depressive symptoms, the State-Trait Anxiety Inventory (STAI-Y1 and STAI-Y2) for state and trait anxiety, and the Pain Catastrophizing Scale (PCS) for maladaptive pain-related cognitions. Each measure was scored according to standardized protocols, with higher scores indicating greater symptom severity or catastrophizing. These questionnaires were included to characterize differences in psychological state, which can modulate both subjective pain ratings and neural responses to nociceptive stimuli.

2.3.3 Sham Training

Participants underwent an initial one-hour training session in a “sham” MRI (an MRI simulator), where they became familiar with the MRI environment, pain rating scales, and thermal stimuli to be used during the MRI session. During this training, participants were introduced to the experimental pain stimulus and study design. They were trained how to rate their pain using a standardized numerical pain intensity scale (NPS) with a 0-10 range (0 = no pain, 5 = moderate pain, 10 = worst possible pain) and rated their pain intensity and pain unpleasantness. This process helped participants acclimate to the stimulus, study procedures, and pain rating scale usage. The

scales were also used to adjust the temperature to yield a pain rating of 5 ± 1 NPS units, representing moderate pain. Each participant completed this training/familiarization, and the temperature needed to evoke moderate pain was determined; however, this temperature was not used for the fMRI portion of the study.

2.3.4 Thermal Stimulation

Noxious thermal stimulation was applied to the thenar eminence of the right hand using an MRI-compatible robotic contact-heat thermal stimulator (RTS-2 device). The RTS-2 device delivered heat stimuli repeatedly on the right hand at the specified timing and temperature under software control (MATLAB, version R2021b, Natick, MA, United States). Stimulation was delivered in blocks, with onsets every 2.5 seconds and contact duration of 1.5 seconds. For the sham portion of the study, a stimulation temperature determined to be moderately painful for each individual participant was delivered. For the fMRI portion, stimulation temperatures of 38 °C, 46 °C, and 51 °C were delivered (Fig 2.1).

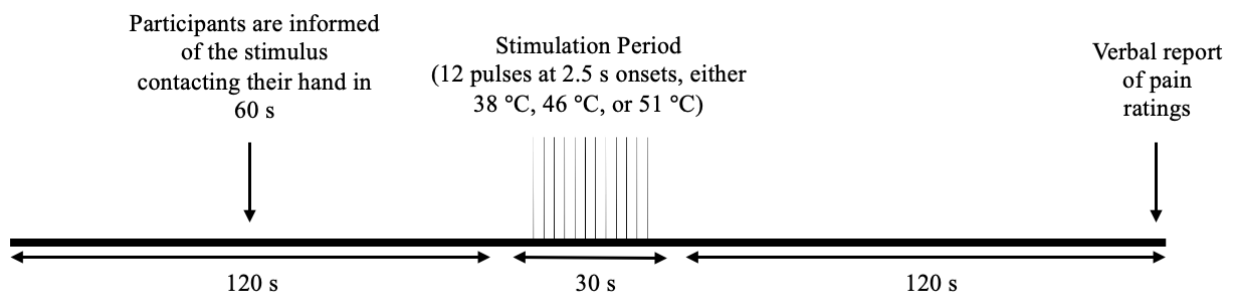


Figure 2.1. Thermal stimulation paradigm of a single run in the MRI. The temperature used (38 °C, 46 °C, or 51 °C) depended on the pattern selected according to participant number, but regardless, each participant received 4x 38 °C stimuli, 4x 46 °C stimuli, and 4x 51 °C stimuli. Thermal stimulation was applied for a period of 30 seconds, in 12 contacts, with 2.5 second onsets, and a duration of 1.5 seconds each.

2.3.5 *fMRI Data Acquisition*

Functional MRI data were acquired at 3 Tesla using a Siemens Prisma system at the MRI Facility at Queen's University. The fMRI data were obtained using our established method with a T2-weighted half-Fourier single-shot fast spin-echo (HASTE) sequence, as echo planar imaging (EPI) distortion may be problematic in regions such as the brainstem and spinal cord [10]. Images covered a 3D volume from the first thoracic vertebra to above the thalamus in nine contiguous sagittal slices. Data were collected with a repetition time (TR) of 6.75 seconds per volume, an echo time (TE) of 76 milliseconds to optimize T2-weighted BOLD sensitivity, and a field-of-view (FOV) of 28×21 cm with a resolution of $1.5 \times 1.5 \times 2$ mm³. The entire session included 12 short fMRI runs, with one of the selected temperatures applied in each run. The order of runs was pseudo-randomized. Data from runs at the same temperature were combined to ensure sufficient statistical power while avoiding effects of participant fatigue. This approach allowed participants to verbally report their pain ratings for each stimulation period and take brief rests between runs.

2.4 Data Analysis

2.4.1 *fMRI Data Analysis*

Our lab's software, "Pantheon" (available on GitHub at <https://github.com/stromanp/pantheon-fMRI>), was used to process the fMRI data. Pantheon includes methods that were specifically designed for analyzing fMRI data acquired from the spinal cord and brainstem [9]. The fMRI data were first converted from DICOM to NIfTI format. Other pre-processing steps included correction for body movement and slice timing adjustment (co-registration), followed by slice-timing correction, and then spatial normalization which consists of

interpolation to 1 mm³ resolution and alignment to an anatomical reference template spanning the brainstem and spinal cord. Anatomical maps have been defined from various sources to create a unified reference. Noise from physiological sources, including cardiac motion and bulk motion, was identified, and removed as part of the pre-processing as well. The first two volumes in each sequence were replaced by the third volume to maintain consistent T1-weighting. These pre-processing steps and removal of physiological noise have been previously validated and shown to be highly effective [9]. Finally, voxel time-series data were expressed as a percent signal change from the mean for subsequent analyses.

2.4.2 Eye-Tracking Analysis

Eye-tracking data were analyzed using custom-written software in Python. The results provided dynamic measures of pupil diameter during each run in the MRI.

2.4.3 Structural and Physiological Modelling (SAPM)

Structural and Physiological Modeling (SAPM) is a novel fMRI connectivity analysis technique developed in our lab, combining anatomical, physiological, and BOLD signal information to model coordinated signaling across networks of regions implicated in pain processing [9]. SAPM estimates directional input and output signaling across a network derived from known anatomical pathways involved in pain processing, and accounts for latent inputs to each region (Fig 2.2). Each anatomical region is subdivided into five time-series-based subregions via k-means clustering, enabling selection of subregion combinations that maximize model fit (i.e., R²) for each participant. The modeling is constrained by a predefined network structure developed based on neuroanatomy and known connectivity patterns, with connections included only where

physiological relevance in pain processing has been demonstrated [9]. In SAPM, the BOLD signal from each region is modeled as a combination of output signals from other connected regions, weighted by D values (input contributions), and DB values (reflecting both the input weight and its effect on output signaling). DB values indicate excitatory (positive) or inhibitory (negative) influence of input signals on a region's output, representing effective connectivity. The latent inputs model unobserved drivers of signal variability, such as afferent sensory input or top-down modulatory influences [9]. SAPM solves for optimal D and DB matrices using a gradient descent algorithm to minimize error between modeled and observed BOLD time-series data. To ensure robustness, the fitting process is repeated across multiple initializations, and regularization is used to minimize parameter magnitude without sacrificing fit [9]. To apply SAPM in this study, one subregion per anatomical region was selected to form a 10-region network model. A gradient-descent subregion search was conducted to identify combinations with the highest average R^2 . Once a subregion set was established, SAPM was run for each participant individually. The resulting DB values were then evaluated at the group level [9]. Connections were deemed significant if DB values differed from zero, based on comparison with null-model simulations that defined statistical thresholds while correcting for multiple comparisons. This enabled identification of directional, physiologically relevant signaling during pain processing in the brainstem and spinal cord. SAPM thus provided interpretable measures of excitatory and inhibitory connectivity dynamics associated with nociceptive stimuli, beyond what is captured from solely BOLD amplitude [9].

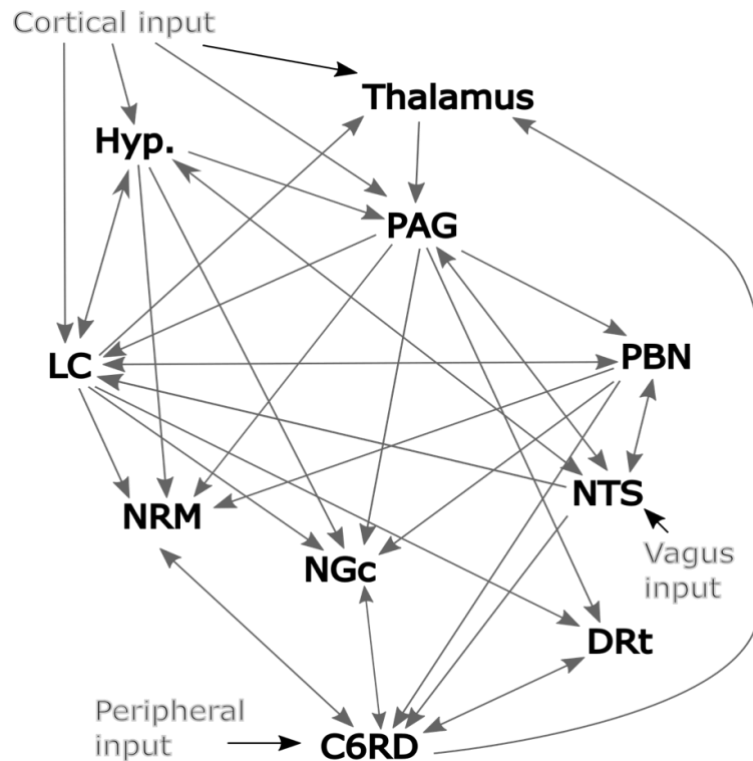


Figure 2.2. The SAPM network model of 35 connections between 10 regions, plus three “latent inputs” signaling from the periphery.

2.5 Results

2.5.1 Pain Ratings and Physiological Response

Pain intensity and unpleasantness ratings increased as the stimulus temperature rose (Fig 2.3). At 38 °C, the reported pain intensity was lowest ($M = 1.25$, $SD = 1.33$), increased at 46 °C ($M = 2.44$, $SD = 1.72$), and reached its highest level at 51 °C ($M = 4.77$, $SD = 2.31$). Pain unpleasantness exhibited a similar pattern, with mean ratings increasing from 0.57, $SD = 1.08$ at 38 °C to 3.74, $SD = 2.50$ at 51 °C. Pairwise comparisons done through t-tests revealed significant temperature-dependent increases in both pain intensity and unpleasantness ratings. Pain intensity was significantly higher at 46 °C compared to 38 °C ($p = 0.0298$), and further increased at 51 °C, with significant differences observed relative to both 38 °C ($p = 0.0001$) and 46 °C ($p = 0.0007$).

Pain unpleasantness followed a similar trend, with a non-significant increase from 38 °C to 46 °C ($p = 0.0645$), but significant differences emerged between 38 °C and 51 °C ($p = 0.0001$) and between 46 °C and 51 °C ($p = 0.0009$). These results confirm that both subjective pain ratings, intensity and unpleasantness, increased in a temperature-dependent manner, with the steepest rise occurring between 46 °C and 51 °C.

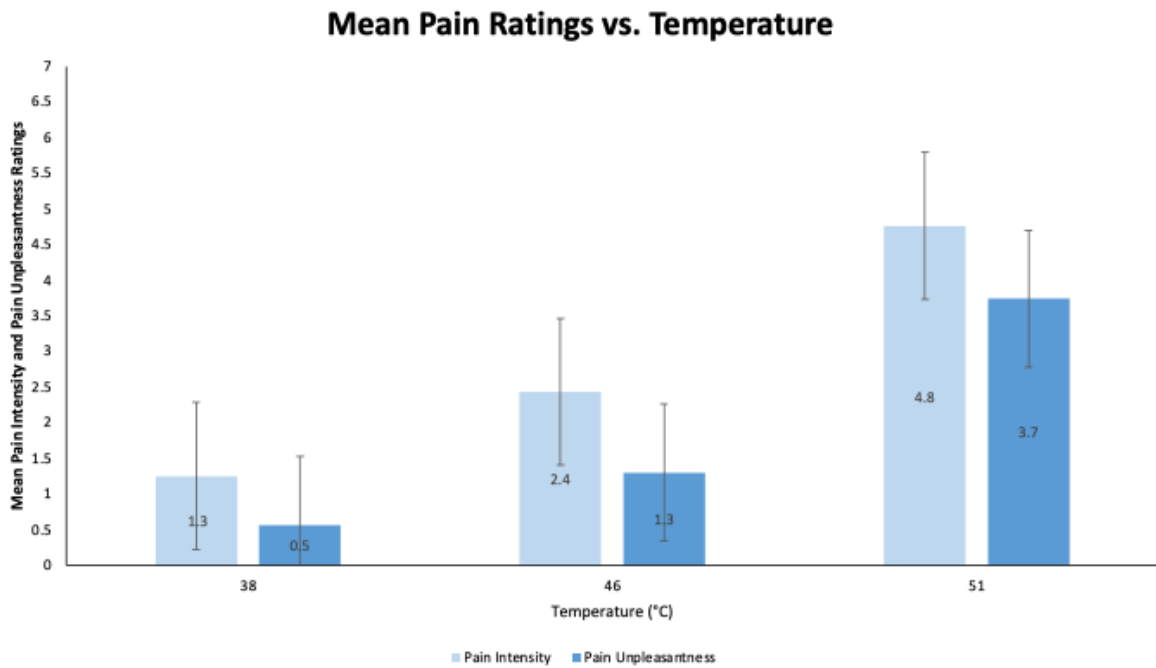


Figure 2.3. Bar graph depicting the behavioural results across the different study conditions (38 °C, 46 °C, and 51 °C). Mean pain ratings for pain intensity and pain unpleasantness are shown for each condition, with error bars representing the standard error. The legend indicates the colours associated with each condition.

2.5.2 SAPM Connectivity Results

SAPM revealed temperature-dependent variations in connectivity within the brainstem-spinal cord network associated with nociceptive processing. The 46 °C condition elicited the highest number of statistically significant connections, as defined by Bonferroni-corrected thresholds, including strong excitatory connectivity from the periaqueductal gray (PAG) to the

hypothalamus (DB = 0.311, SD = 0.061, T = 4.91), and from the nucleus tractus solitarius (NTS) to the hypothalamus (DB = 0.193, SD = 0.092, T = 3.72). At 38 °C, while fewer connections reached significance, the LC-thalamus pathway showed the highest overall magnitude (DB = 0.550, SD = 0.097, T = 4.81), and the PAG-hypothalamus connection also met statistical significance (DB = 0.259, SD = 0.048, T = 5.13). In contrast, the 51 °C condition demonstrated reduced connectivity strengths across most pathways. For example, the PAG-hypothalamus connection weakened (DB = 0.091, SD = 0.043, T = 1.87) and was not statistically significant. The locus coeruleus (LC) to thalamus pathway remained robust across all temperatures, with sustained significance at 46 °C (DB = 0.517, SD = 0.146, T = 2.96) and 51 °C (DB = 0.454, SD = 0.090, T = 4.09). Inhibitory connectivity was observed in select pathways, including PAG to parabrachial nuclei (PBN) at 46 °C (DB = -0.142, SD = 0.039, T = -4.00). Notably, the NRM to hypothalamus connection was only significant at 38 °C (DB = 0.104, SD = 0.031, T = 3.82) and was non-significant at higher temperatures. The connection between the nucleus gigantocellularis (NGC) and the C6RD was significant at 46 °C (DB = 0.220, SD = 0.084, T = 3.38) but was not significant at 38 °C or 51 °C. Overall, SAPM results indicated differential patterns of connectivity across conditions, with the 46 °C stimulus yielding the broadest set of significant pathways in this network (Table 2.1, Fig 2.4, Fig 2.5).

Table 2.1. Connectivity (DB) values for connections at 38 °C, 46 °C, and 51 °C. Values in bold are significant at a Bonferroni $p < 0.05$. The corresponding DB values for the same connection in other conditions are included for comparison purposes, although they may not be significant at Bonferroni $p < 0.05$.

Connections	38 °C		46 °C		51 °C	
	DB Values	T-values	DB Values	T-values	DB Values	T-values
PAG-Hypo	0.259 ± 0.048	T = 5.13	0.311 ± 0.061	T = 4.91	0.091 ± 0.043	T = 1.87
LC-Thal	0.550 ± 0.097	T = 4.81	0.517 ± 0.146	T = 2.96	0.454 ± 0.090	T = 4.09
Hypo-PAG	0.193 ± 0.046	T = 4.55	0.299 ± 0.067	T = 4.69	0.083 ± 0.072	T = 1.38
NRM-Hypo	0.104 ± 0.031	T = 3.82	0.011 ± 0.059	T = 0.41	0.008 ± 0.053	T = 0.40
PAG-PBN	-0.060 ± 0.036	T = -2.08	-0.142 ± 0.039	T = -4.00	0.057 ± 0.048	T = 0.88
NTS-Hypo	0.033 ± 0.065	T = 2.76	0.193 ± 0.092	T = 3.72	0.078 ± 0.072	T = 3.14
NGC-C6RD	0.045 ± 0.066	T = 1.65	0.220 ± 0.084	T = 3.38	0.060 ± 0.076	T = 1.63

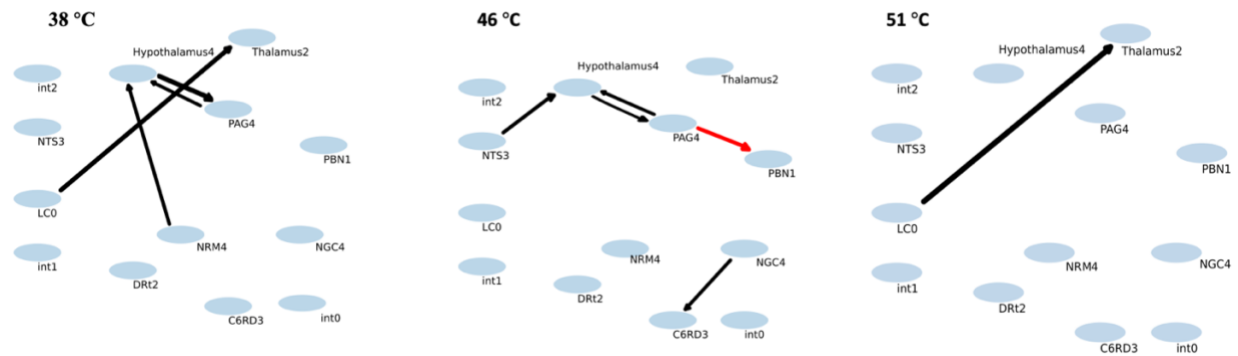
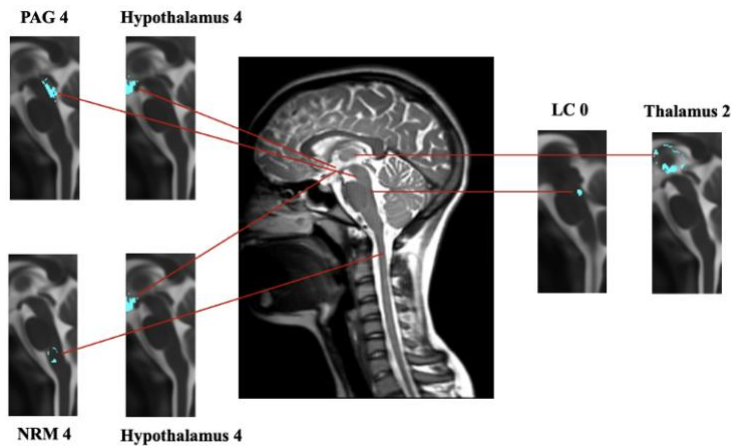
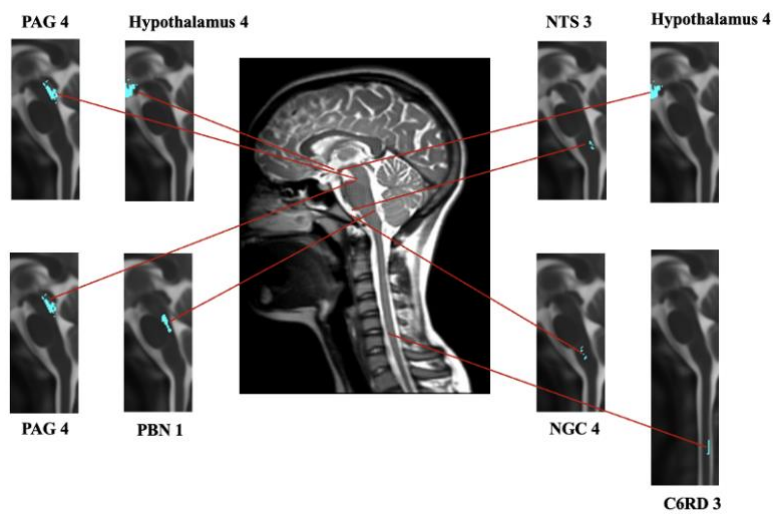


Figure 2.4. Connectivity diagrams of the three temperatures (38 °C, 46 °C, 51 °C) and connectivity between regions in the nociceptive processing network, which includes the hypothalamus, thalamus, periaqueductal gray (PAG), locus coeruleus (LC), parabrachial nuclei (PBN), nucleus raphe magnus (NRM), nucleus gigantocellularis (NGC), nucleus tractus solitarius (NTS), dorsal reticular nucleus of the medulla (DRt), and right dorsal region of the spinal cord in the 6th cervical segment (C6RD). The gray ovals indicate anatomical regions. Black arrows indicate excitatory connections (positive DB values), and red arrows indicate inhibitory connections (negative DB values). The width of each arrow corresponds with the significance (T-value) of each connection.

Interaction Effect (38°C x Intensity)



Interaction Effect (46°C x Intensity)



Interaction Effect (51°C x Intensity)

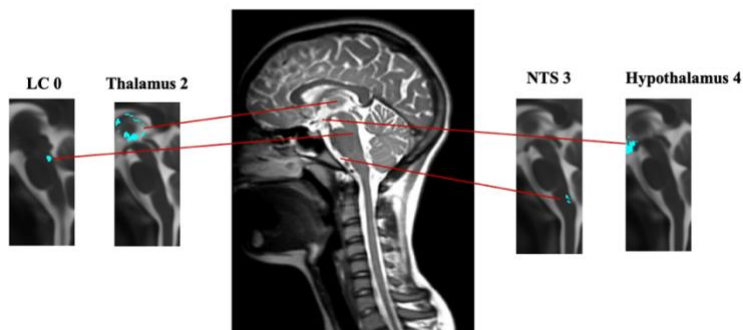


Figure 2.5. Anatomical regions that demonstrated significant differences in connectivity between regions involved in pain-processing at 38 °C, 46 °C, and 51 °C. A sagittal view of the brainstem and spinal cord, and sagittal views of each region are shown for visualization purposes.

2.5.3 Eye-Tracking Results

Measures of pupil diameters were used as an index of physiological arousal and were obtained under the three temperature conditions. As shown in Fig 2.6, there was a noticeable increase in pupil size following the onset of thermal stimulation, marked by the shaded green region. All three temperatures provoked a dilation response, but there were no significant differences between pupil size, and thus, autonomic responses across temperatures.

Average pupil size during thermal stimulation at 38 °C, 46 °C, and 51 °C

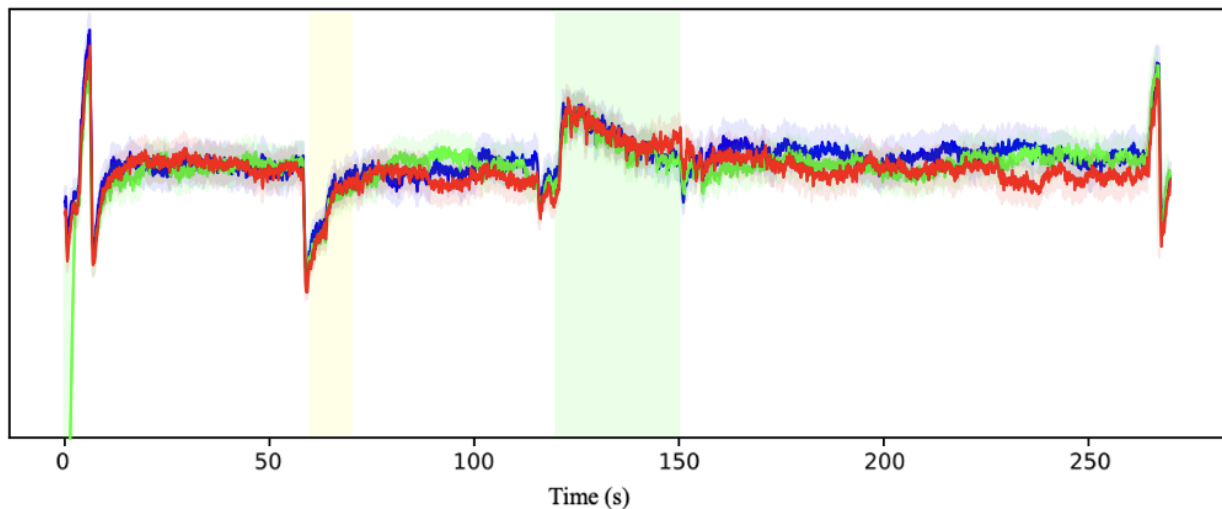


Figure 2.6. Average pupil diameter over time for each thermal condition. The blue line represents pupil size at 38 °C, the green line represents pupil size at 46 °C, and the red line represents pupil size at 51 °C. The green shaded area shows when the thermal stimulus was applied. Lines reflect the group average, with shading showing standard error. The sudden increases in pupil diameter seen at the beginning and end of the plots occur due to the visual display in the MRI system presenting a white screen followed by a black screen, which helps ensure pupil size is calibrated for each participant, and that they start at a consistent baseline pupil diameter.

2.5.4 Questionnaire Results

The neural and physiological response patterns we observed across temperature conditions (38 °C, 46 °C, 51 °C) are best understood when looked at with validated behavioural questionnaire. Thus, we collected behavioural questionnaire data on depression (BDI) [11], anxiety (STAI Y-1,

STAI Y-2) [12], pain catastrophizing (PCS) [13], and widespread pain (WPI). On average, participants reported minimal depressive symptoms ($M = 3.93$, $SD = 4.91$) and low pain catastrophizing ($M = 6.05$, $SD = 6.01$), suggesting a psychologically healthy sample. Scores for state and trait anxiety were in the mild-to-moderate range ($M = 39.93$ for STAI Y-1; $M = 46.68$ for STAI Y-2), which suggests that mood factors were likely not having significant influence on pain perception. These low-to-moderate questionnaire scores suggest that any observed responses to thermal stimulation are not influenced by psychiatric conditions (Table 2.2). Analyses of correlations between pain ratings and questionnaires were carried out using Excel. The aim was to determine any relationships that might exist between depression, anxiety, pain catastrophizing, and self-reported widespread pain. Moderate positive correlations were observed between trait and state anxiety (STAI Y-1 and STAI Y-2), and both were found to be positively associated with pain catastrophizing. However, no strong correlations were found between depression scores and either PCS or WPI, suggesting that while affective states like anxiety may influence cognition, depressive symptoms were largely independent in this sample.

Table 2.2 Summary of results from questionnaires for all participants.

Questionnaire	Mean	Median	Standard Deviation
STAI-Y1	40.7	40	5.33
STAI-Y2	47.6	47	5.10
PCS	6.07	5	6.22
BDI-II	3.95	2	5.07

2.6 Discussion

This study investigated how brainstem and spinal cord network connectivity changes in response to varying levels of thermal stimulation, using Structural and Physiological Modeling

(SAPM) applied to functional magnetic resonance imaging (fMRI) data. The application of SAPM, which integrates structural pathways and physiological regions to detect directional connectivity changes enabled detailed evaluation of nociceptive processing across thermal intensities [9]. Our findings show a distinct temperature-dependent modulation of connectivity strength and significance across the evaluated network. The 46 °C condition elicited the highest number of statistically significant connections, consistent with a state of moderate noxious stimulation. These included strong excitatory pathways such as PAG-hypothalamus (DB = 0.311, SD = 0.061, T = 4.91) and hypothalamus-PAG (DB = 0.299, SD = 0.067, T = 4.69), suggesting robust connectivity between these regions during intermediate nociceptive states. This aligns with prior work highlighting the PAG's central role in pain modulation, particularly during active engagement of descending inhibitory mechanisms [14]. Notably, the connection from the NTS to the hypothalamus (DB = 0.193, SD = 0.092, T = 3.72) was significant at 46 °C only, indicating ascending visceral sensory integration at moderate temperature. Additionally, inhibitory connectivity from the PAG to the PBN (DB = -0.142, SD = 0.039, T = -4.00) was uniquely observed at 46 °C, pointing to a temperature-specific shift in PAG-mediated signaling. In contrast, the 51 °C condition exhibited fewer significant connections, despite resulting in the highest average pain ratings (mean pain intensity = 4.8, mean pain unpleasantness = 3.7). For example, connectivity in the PAG-hypothalamus pathway declined substantially at 51 °C (DB = 0.091, SD = 0.043, T = 1.87), and no longer reached statistical significance. Similarly, connections such as hypothalamus-PAG and NTS-hypothalamus, both of which were significant at 46 °C, were not significant at 51 °C. This attenuation suggests that increased stimulus intensity does not lead to a linear increase in functional engagement within the nociceptive network, but may instead reflect network desensitization or altered modulation at higher thermal intensities. Interestingly, the LC-thalamus

pathway was robust across all conditions, with significance at both 38 °C (DB = 0.550, SD = 0.097, T = 4.81) and 51 °C (DB = 0.454, SD = 0.090, T = 4.09). While the connection was not significant at 46 °C (DB = 0.517, SD = 0.146, T = 2.96), its relatively high DB magnitude and standard deviation point to greater inter-subject variability at this intermediate thermal level. This may represent an important transition point between innocuous and noxious thermal stimulation, where network recruitment is less uniform across participants [15]. Such variability could explain the lack of statistical significance at 46 °C despite a high average DB value and a signal pattern consistent with nociceptive engagement. The LC's consistent engagement at the lower and upper ends of the thermal intensities underscores its role in both arousal and pain processing [9, 16]. The NRM-hypothalamus connection was only significant at 38 °C (DB = 0.104, SD = 0.031, T = 3.82), with a marked drop in connectivity strength at higher temperatures. This pattern suggests its involvement may be limited to lower-intensity or anticipatory processing stages. The occurrence of the NGC-C6RD pathway at 46 °C (DB = 0.220, SD = 0.084, T = 3.38) further emphasizes the concept that this moderate temperature represents a unique threshold where spinal-level integration becomes functionally significant. Neither 38 °C nor 51 °C produced significant NGC-C6RD connectivity, indicating that this interaction is not only a linear function of thermal intensity, but reflects a more nuanced state of sensitization and pain modulation [17, 18].

2.7 Conclusions

SAPM analysis of MRI data revealed temperature-dependent modulation of functional connectivity within the brainstem-spinal cord nociceptive network [9]. The 46 °C condition consistently engaged the broadest set of significant connections, indicating that moderate thermal stimulation may represent a functional threshold for coordinated descending and ascending

signaling. Notably, the LC-thalamus pathway remained robust across temperatures but showed individualized variability at 46 °C, suggesting variances in the recruitment of pain modulatory mechanisms. These results emphasize the complex, non-linear nature of nociceptive processing and demonstrate the efficacy of SAPM in analyzing network connectivity dynamics which would not be detected through analyzing BOLD activation alone [9].

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2.9 Conflict of Interest Statement

All listed authors have reviewed and approved of this manuscript. None of the authors have any conflicts of interest to report.

2.10 Funding Information

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Chapter 3: The correlation between sex and pain processing at innocuous to noxious temperatures (38 °C, 46 °C, 51 °C) by means of fMRI in the brainstem and spinal cord

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Data Availability Statement:

Data are available upon reasonable request from the corresponding author and are not publicly available. The analysis software, Pantheon, is freely available on GitHub at <https://github.com/stromanp/pantheon-fMRI>

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Total number of figures: 5

Total number of tables: 5

3.1 Abstract

Well-documented sex differences exist in the perception of pain and its processing. Despite this, the neural mechanisms that underlie these differences, especially in the brainstem and spinal cord, lack full understanding. This study examined whether males and females differ in pain ratings, brainstem–spinal cord connectivity, and autonomic responses during acute thermal stimulation. Forty-two healthy adults (21 males, 21 females) underwent functional MRI scanning while receiving thermal stimuli at 38 °C, 46 °C, and 51 °C. Ratings of pain intensity and unpleasantness were obtained for each condition, along with measurements of pupil diameter via eye tracking to assess autonomic arousal. A directional network analysis method, Structural and Physiological Modeling (SAPM) was used to model input and output signaling between regions in the brainstem, and spinal cord known to be involved in pain processing. Results showed no significant sex differences in pain ratings or brainstem-spinal connectivity across temperatures. However, SAPM analyses revealed non-significant trends suggesting sex-specific patterns of network engagement. Females showed stronger connectivity in circuits like PAG-hypothalamus and NRM-hypothalamus, while males showed greater connectivity in hypothalamus-PAG and NTS-hypothalamus pathways. Both sexes exhibited similar pupil responses, indicating comparable autonomic engagement. These findings suggest that while subjective and physiological responses to acute thermal pain are similar, subtle sex differences in pain modulation may exist and should be explored in future studies.

Keywords: pain; fMRI; brainstem; spinal cord; sex differences

3.2 Introduction

Pain is a multidimensional process that has both a sensory-discriminative and an affective-motivational component [1]. The sensory aspect identifies the location, intensity, and quality of a noxious stimulus. The affective component varies according to the emotional and cognitive interpretation of the noxious sensation, including how unpleasant or distressing it is perceived to be [2]. A distributed system of cortical and subcortical regions supports these components, and they include the somatosensory cortex, the anterior cingulate cortex, the insula, and several brainstem structures that are important in descending pain modulation [3]. In both clinical populations and experimental studies, sex differences in pain perception have been consistently explored and documented. Females frequently display greater sensitivity to unpleasant stimuli, and a higher reported incidence of chronic pain conditions than males do [4, 5]. These disparities stem from a combination of biological and psychosocial influences. From a biological perspective, the influence of sex hormones like estrogen and progesterone on nociceptive processing has been investigated in previous research [6]. Furthermore, psychosocial aspects, which also account for the difference in pain reporting between the sexes, may include variations in how each sex manages stress, regulates affects, and communicates their pain in accordance with social norms [7, 8].

Cortical activation patterns during pain processing may demonstrate the presence of sex-related differences, as identified by neuroimaging studies. Key areas involved include the anterior cingulate cortex and insula [9, 10]. These results have broadened comprehension of sex differences in pain at the cortical level. Despite this, the extent to which these differences exist in subcortical structures, particularly in the brainstem and spinal cord, remains unclear. Brainstem regions such as the periaqueductal gray (PAG), nucleus raphe magnus (NRM), and locus coeruleus (LC) are

key components of the descending pain modulatory system, and they play a central role in adjusting spinal nociceptive input through inhibitory and excitatory signaling [11]. It is not well characterized, however, whether males and females engage these systems differently during acute pain. This study examines male-female differences in subcortical pain modulation during brief, fixed-temperature thermal stimulation. Although group differences in individual's subjective ratings might be subtle, measures of directional connectivity may reveal sex-specific group differences in how the brainstem and spinal cord regions respond to nociceptive input.

In this experiment, we used Structural and Physiological Modeling (SAPM), a connectivity approach that estimates both input and output signaling across defined anatomical regions, to analyze the functional MRI data. Connectivity was compared between males and females across all three thermal stimulus levels (38 °C, 46 °C, and 51 °C) at once, alongside pain intensity, pain unpleasantness ratings, and autonomic responses measured via pupil diameter. The aim of this study is to determine whether functional connectivity within the pain-modulatory network differs by sex under controlled stimulation. Circuits involving arousal (e.g., LC-thalamus) and descending inhibition (e.g., PAG-NRM, hypothalamus-PAG) were especially focused on. This study builds on previous neuro imaging studies, concentrating on subcortical neural network responses, and broadens the understanding of the potential influence of sex on the central regulation of pain. Findings from this study may help characterize why males and females differ in their pain experiences and inform future studies aiming to develop sex-specific approaches to pain assessment and treatment.

3.3 Materials and Methods

3.3.1 Procedure Overview

The study involved a single, two-component visit to our MRI facility. The first phase occurred in an MRI simulator and acted as a "sham" training session. During this time, participants were introduced to the study procedures, including the MRI environment and how to use the pain rating scales. In the second phase, participants were put in the MRI scanner, where we acquired functional images of the brainstem and spinal cord while they received thermal stimulation on the right hand. Eye-tracking was also carried out during scanning. Participants provided pain ratings using the scales they had practiced with during the training portion. Additional procedural details are outlined in the sections that follow.

3.3.2 Participants

This study obtained 42 healthy adults' participation, all of whom were aged between 20 to 57 years. Out of these 42 participants, there were 21 males and 21 females. Recruitment was done via local advertisements and through social media platforms such as Reddit and LinkedIn. Individuals who had chronic pain or any significant neurological condition or injury (e.g., spinal cord trauma, brain injury) were excluded from being considered for participation. All participants read a detailed consent form and had the opportunity to ask questions before signing the informed consent document. Participants also completed standardized self-report questionnaires to evaluate psychological variables relevant to pain processing. The Beck Depression Inventory-II (BDI-II) measured depressive symptoms, the State-Trait Anxiety Inventory (STAI-Y1 and STAI-Y2) assessed current and general anxiety levels, and the Pain Catastrophizing Scale (PCS) captured pain-related catastrophic thinking. Scores were calculated using established guidelines, with

higher values reflecting greater symptom severity. These assessments provided context for individual variability in pain ratings and neural responses observed during the experiment.

3.3.3 Sham Training

Before scanning, participants took part in a one-hour familiarization session using an MRI simulator. This session introduced them to the MRI environment, thermal stimulation, and pain rating procedures. Participants were trained to use a 0-10 numerical pain scale (NPS) for both intensity and unpleasantness ratings. The purpose of this session was to allow participants to acclimate to the procedures and become consistent in using the pain rating tools. During this phase, thermal stimuli were adjusted to elicit a moderate pain level. Although this individualized temperature was recorded, it was not used in the scanning session.

3.3.4 Thermal Stimulation

A contact heat stimulator (RTS-2), which is compatible with MRI, was used to apply heat stimuli to the thenar region of the right hand. The device delivered controlled thermal pulses under software control (MATLAB, R2021b, Natick, MA, USA), following a block design. Every 2.5 seconds, the stimuli were administered, with each pulse administered lasting 1.5 seconds. (Fig 3.1). The fMRI portion of the study involved the use of consistent temperatures of 38 °C, 46 °C, and 51 °C. The sham training involved a similar design, with individualized temperatures aimed at inducing moderate pain.

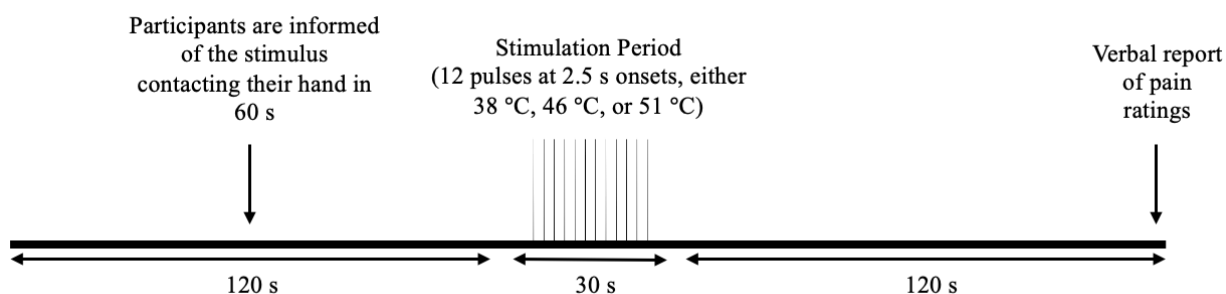


Figure 3.1. Thermal stimulation paradigm for a single MRI run. Each participant received four stimuli at three temperatures, 38 °C, 46 °C, and 51 °C, in an order determined by a pre-assigned pattern based on participant number. Thermal stimulation was delivered over 30 seconds using 12 contacts, each with a 2.5-second onset and lasting 1.5 seconds.

3.3.5 Eye-Tracking

Pupil diameter was recorded using the SR-Research Eyelink 1000 system, sampled at 500 Hz. Eye-tracking provided continuous measurements of pupil dynamics.

3.3.6 fMRI Data Acquisition

Imaging was carried out on participants at Queen's University with a Siemens Prisma 3T MRI scanner. A T2-weighted half-Fourier single-shot fast spin-echo (HASTE) sequence was employed to capture functional data from both the brainstem and spinal cord, as the distortion from echo planar imaging (EPI), for example, may be problematic in these regions. Every scan captured nine sagittal slices, stretching from the thalamus to the upper thoracic spinal cord. Key parameters for image acquisition included a repetition time (TR) of 6.75 seconds, an echo time (TE) of 76 milliseconds, and a field of view measuring 28 by 21 centimetres, with spatial resolution set at $1.5 \times 1.5 \times 2$ mm. Each participant completed 12 short scanning sessions. Between these sessions, short rest periods were provided, during which participants gave verbal pain intensity and pain unpleasantness ratings.

3.4 Data Analysis

3.4.1 fMRI Data Analysis

All functional data were processed using our lab's software, Pantheon (<https://github.com/stromanp/pantheon-fMRI>), developed specifically for analysis of spinal cord and brainstem fMRI. Pre-processing included DICOM to NIfTI conversion, motion correction, and slice-timing adjustments. The images were resampled to 1 mm³ isotropic resolution and aligned to a combined brainstem and spinal cord template. Anatomical references were used to guide registration. Physiological noise (e.g., pulse and motion) was identified and removed. To maintain consistent contrast across volumes, the first two timepoints of each scan were replaced with the third. Finally, time-series signals were normalized to percent signal change for statistical analysis.

3.4.2 Eye-Tracking Analysis

Python scripts were used to process the outputs of eye tracking. Pupil diameter was continuously recorded during each scan, producing measures of pupil response variability, which was used in this study as an index of autonomic nervous system engagement.

3.4.3 Structural and Physiological Modelling (SAPM)

To continue interpreting the fMRI data, we used Structural and Physiological Modeling (SAPM). This is a method for directional connectivity analysis that was developed in our lab by Dr. Patrick W. Stroman [12]. With SAPM, researchers can account for dynamic signaling to and from each region of the model and capture the variability that can come from either within the modeled network or from outside the network (Fig 3.2). SAPM estimates the net effect on each

region, determining whether the effect is mostly excitatory (positive) or mostly inhibitory (negative) [12]. Using SAPM, this study assessed the interconnectivity among important spinal cord and brainstem areas which are known to process thermal pain. One subregion was assigned to each of the ten anatomical regions in the network model by each iteration of the analysis. A gradient descent algorithm was used to find the combination of subregions that fit best with the BOLD time courses we observed. After identifying a best-fit configuration, that subregion set was applied across all participants for the purpose of conducting individual-level SAPM analyses. To assess the strength of the connection, modeled connectivity values were tested across participants. Additionally, to examine sex differences in pain-related connectivity, an ANCOVA was conducted using SAPM-derived directional connectivity (DB) values as the dependent variable. Connectivity values were compared between males and females across each pairwise connection in the network model. Sex was included as a fixed variable, and thermal stimulation condition was included as a covariate to control for any temperature-related effects. The analysis was conducted using Python, and ANCOVA p-values were calculated for the main effect of sex, the main effect of condition, and the interaction term. Only connections with statistically significant p-values ($p < 0.05$) were visualized. Overall, SAPM results provided insight into the directional nature of information flow in brainstem and spinal regions, deepening our understanding of how these networks contribute to pain signalling during noxious thermal stimulation [12].

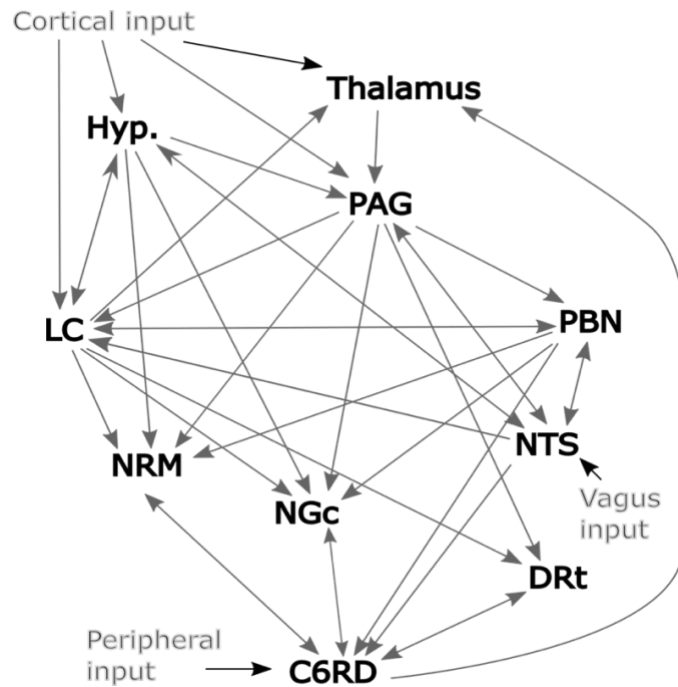


Figure 3.2. The SAPM network model of 35 connections between 10 regions, plus three “latent inputs” signaling from the periphery.

3.5 Results

This study examined whether there are sex-based differences in pain perception and neural signaling in response to thermal stimulation across the range of three temperatures: 38 °C (innocuous), 46 °C (moderately noxious), and 51 °C (highly noxious). We gathered pain intensity along with unpleasantness ratings and measured pupil diameters to capture both subjective and physiological responses. Functional connectivity was evaluated using values obtained from SAPM [12]. We conducted analyses on a sample of 42 healthy adults, with an equal division of male and female participants (21 per group).

3.5.1 Subjective Pain Ratings

Analysis of variance (ANOVA) revealed no statistically significant main effect of sex on pain intensity ($F(1, 466) = 0.78, p = 0.377$) or pain unpleasantness ($F(1, 466) = 2.20, p = 0.139$). Additionally, there was no significant interactions between sex and stimulus temperature. The expected outcome of increasing pain ratings with increasing temperatures was shown by both sexes; however, their average ratings were closely aligned. For example, at 51 °C, females reported a mean pain intensity of 4.67 (SD = 2.48), while males reported a similar mean of 4.86 (SD = 2.17) (Table 3.1, Table 3.2). Mean unpleasantness ratings were also close, with females averaging 3.43 (SD = 2.64) and males 4.05 (SD = 2.37) (Table 3.1, Table 3.2). Although these differences did not achieve statistical significance, the data indicate a slight trend toward males rating the stimuli as more unpleasant, especially with the higher stimulation temperature.

Table 3.1. Descriptive statistics for pain intensity and pain unpleasantness ratings for males at each study condition (38 °C, 46 °C, 51 °C).

Measure	Temperature (°C)	Mean	Median	Standard Deviation
Pain Intensity	38 °C	1.3	1	1.18
	46 °C	2.64	2	1.62
	51 °C	4.77	5	2.11
Pain Unpleasantness	38 °C	0.55	0	0.92
	46 °C	1.46	1	1.4
	51 °C	4.01	4	2.4

Table 3.2. Descriptive statistics for pain intensity and pain unpleasantness ratings for females at each study condition (38 °C, 46 °C, 51 °C).

Measure	Temperature (°C)	Mean	Median	Standard Deviation
Pain Intensity	38 °C	1.22	1	1.46
	46 °C	2.23	2	1.79
	51 °C	4.76	5	2.48
Pain Unpleasantness	38 °C	0.59	0	1.22
	46 °C	1.18	1	1.62
	51 °C	3.51	3	2.57

3.5.2 SAPM Connectivity Differences by Sex

SAPM was used to assess directional connectivity strength (DB values) across key regions implicated in descending pain modulation, comparing males and females during noxious thermal stimulation. A subset of seven region-to-region connections was examined in greater detail: PAG-hypothalamus, LC-thalamus, hypothalamus-PAG, NRM-hypothalamus, PAG-PBN, NTS-hypothalamus, and NGC-C6RD (Figure 3.5). Although DB values depicted in Table 3.3 revealed apparent sex-based differences in several connections, such as PAG-hypothalamus and NRM-hypothalamus, these trends did not reach statistical significance in an ANCOVA analysis. This suggests that any observed group differences in DB values are not robust after accounting for covariates. Nonetheless, mean connectivity values across these connections showed some divergence. For instance, females exhibited higher PAG-hypothalamus connectivity (DB = 0.259, SD = 0.048) compared to males (DB = 0.199, SD = 0.048), while the reverse was true for hypothalamus-PAG (males: DB = 0.263, SD = 0.073; females: DB = 0.193, SD = 0.046). Notably, NRM-hypothalamus connectivity appeared higher in females (DB = 0.104, SD = 0.031) than in males (DB = 0.032, SD = 0.027) (Table 3.3). Connectivity diagrams further depict these trends, with both shared and subtle sex-specific patterns of connectivity (Fig 3.3). While most excitatory pathways were conserved across sexes, the NTS-hypothalamus connection exhibited relatively stronger connectivity in males (DB = 0.101, SD = 0.073) than females (DB = 0.010, SD = 0.063), though the connection did not surpass Bonferroni-corrected significance (Table 3.4, Fig 3.4).

Table 3.3. Connectivity (DB) values for connections separated by sex (males versus females). Values in bold are significant at $p < 0.05$. The corresponding DB values for the same connection in other conditions are included for comparison purposes.

Connections	Males		Females	
	DB Values	T-values	DB Values	T-values
PAG-Hypo	0.199 ± 0.048	T = 3.90	0.259 ± 0.048	T = 5.13
LC-Thal	0.647 ± 0.143	T = 3.94	0.550 ± 0.097	T = 4.81
Hypo-PAG	0.263 ± 0.073	T = 3.82	0.193 ± 0.046	T = 4.55
NRM-Hypo	0.032 ± 0.027	T = 1.64	0.104 ± 0.031	T = 3.82
PAG-PBN	-0.011 ± 0.061	T = -0.42	-0.012 ± 0.047	T = -0.56
NTS-Hypo	0.101 ± 0.073	T = 3.42	0.010 ± 0.063	T = 2.51
NGC-C6RD	0.067 ± 0.096	T = 1.37	0.053 ± 0.116	T = 1.01

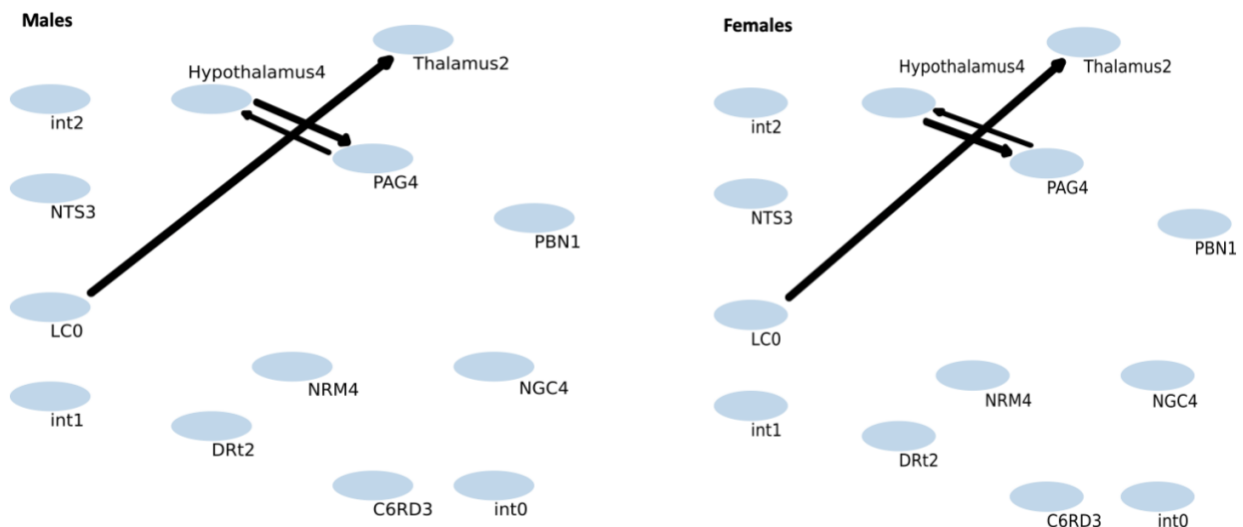


Figure 3.3. Connectivity diagrams for each sex (males versus females) of the nociceptive processing network consisting of the hypothalamus, thalamus, periaqueductal gray (PAG), locus coeruleus (LC), parabrachial nuclei (PBN), nucleus raphe magnus (NRM), nucleus gigantocellularis (NGC), nucleus tractus solitarius (NTS), dorsal reticular nucleus of the medulla (DRt), and right dorsal region of the spinal cord in the 6th cervical segment (C6RD). Black arrows are associated with excitatory connections, and red arrows are associated with inhibitory connections. The width (thicker vs. thinner) of each arrow corresponds with the relative strength of each connection.

Table 3.4. Table depicting p-values which reflect the main effect of sex, the main effect of stimulation condition, and the interaction term for each connection. These connections exhibited a p-value<0.05 but did not remain statistically significant after Bonferroni correction ($\alpha = 0.00044$).

Connection	p-value (Sex)	p-value (Condition)	p-value (Interaction)
lat2-NTS	0.9164	0.0101	0.1561
NTS-PAG	0.19	0.0152	0.0283
C6RD-NRM	0.0643	0.0207	0.6732
NRM-NGC	0.0391	0.9967	0.2405
PBN-LC	0.9057	0.0833	0.0397

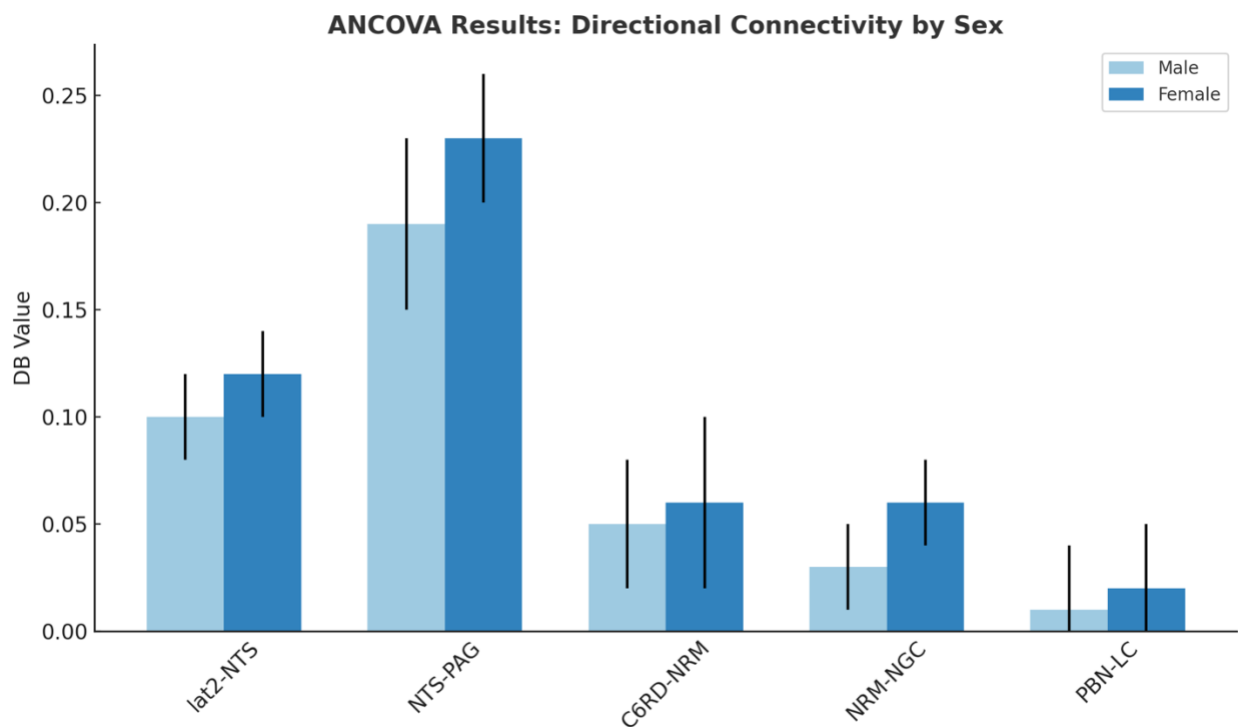


Figure 3.4. Bar graph displaying mean directional connectivity (DB) values (\pm standard error) for male and female participants across five brainstem and spinal cord connections that exhibited uncorrected $p < 0.05$ in ANCOVA analyses assessing the main effects of sex, condition, and their interaction. Although these connections demonstrated preliminary trends suggestive of sex-related differences in pain-modulatory network engagement, none remained statistically significant following Bonferroni correction for multiple comparisons (corrected $\alpha = 0.00044$).

3.5.3 Eye-tracking Results

The average pupil size over time during thermal stimulation at all temperature levels is displayed in Figure 3.5 for male and female participants. In both sexes, pupil size increased during the stimulation period, highlighted in green. The changes seen in average pupil size, indicating changes in autonomic responses, was not significantly difference between sexes.

Average pupil size for males versus females during thermal stimulation

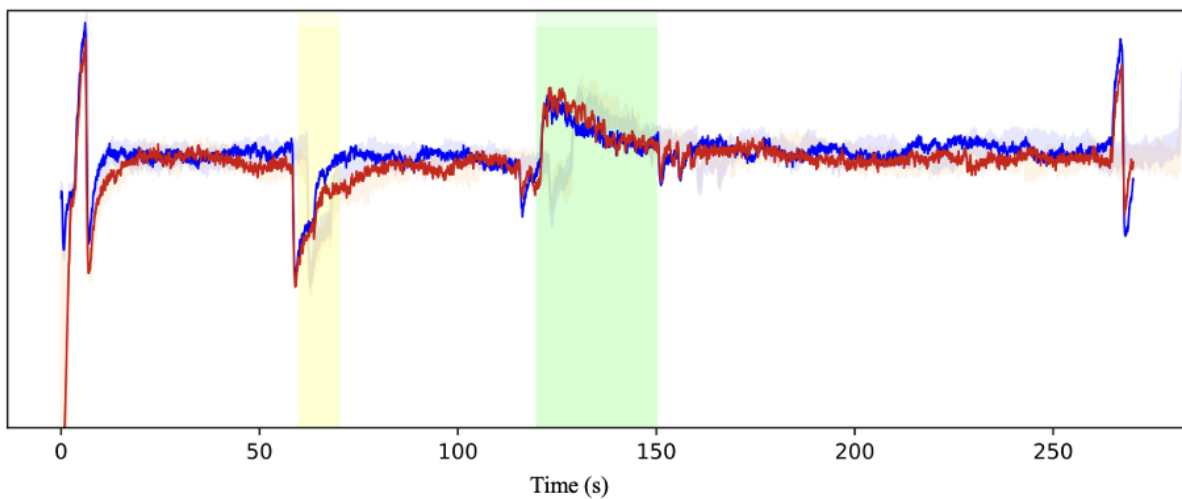


Figure 3.5. Average pupil size during thermal stimulation across all temperature conditions, separated by sex (males versus females). Males are represented in blue, and females are represented in red. The green shaded region represents the stimulation period, and the yellow region indicates the anticipation period, when participants are told the stimulus is coming. The sudden increases in pupil diameter seen at the beginning and end of these plots occur due to the visual display in the MRI system presenting a white screen followed by a black screen. This ensures pupil size is calibrated for each participant, and that they start at a consistent baseline pupil diameter.

3.5.4 Behavioural Results

Behavioural questionnaire results were used to account for possible psychosocial influences on pain modulation across sexes. Female and male participants reported comparable levels of depression, anxiety, and pain catastrophizing, with no indications of clinically elevated

scores in any domain (Table 3.5). These findings support the interpretation that sex differences in pain perception and connectivity are not attributable to confounding psychological variables, but instead likely reflect biological or neurophysiological mechanisms. The inclusion of WPI scores further confirmed that widespread somatic pain was not a factor in the present cohort. Therefore, the sex-related findings in BOLD signaling and SAPM connectivity modeling can be attributed with greater confidence to differences in pain system processing rather than to variance in affective state or pain-related beliefs. While analysing sex differences across behavioural metrics, no statistically significant differences were observed in depression (BDI) [13], anxiety (STAI Y-1, Y-2) [14], pain catastrophizing (PCS) [15], or WPI scores between male and female participants. These findings help reinforce that the sex-based variations in pain response observed in the fMRI and behavioural pain ratings are unlikely to be due to psychological or affective confounds. Instead, the results support the hypothesis that biological or physiological factors underlie observed sex differences in pain modulation and network connectivity.

Table 3.5. Self-report questionnaire scores across all participants. Values represent mean, median, and standard deviation for the State-Trait Anxiety Inventory (STAI-Y1 and STAI-Y2) [14], Pain Catastrophizing Scale (PCS) [15], and Beck Depression Inventory-II (BDI-II) [13].

Questionnaire	Mean	Median	Standard Deviation
STAI-Y1	40.7	40	5.33
STAI-Y2	47.6	47	5.1
PCS	6.07	5	6.22
BDI-II	3.95	2	5.07

3.6 Discussion

The second part of this study investigated how assigned sex influences brainstem and spinal cord connectivity during thermal pain processing. Using Structural and Physiological Modeling (SAPM), observed several directional differences in connectivity between males and females

across the network, although these were not statistically significant. Although males and females provided similar subjective pain ratings, females showed stronger connectivity between key connections that are involved in both ascending nociceptive transmission and descending pain modulation, including the periaqueductal gray (PAG) and hypothalamus, as well as the nucleus raphe magnus (NRM) and hypothalamus. These patterns align with the hypothesis that sex differences in pain modulation exist, although not statistically confirmed in this study. For example, female greater connectivity from the PAG to the hypothalamus was observed, suggesting that a top-down regulatory influence over neuroendocrine or homeostatic responses during painful stimulation was more robust in females. In contrast, connectivity in males between the hypothalamus and PAG was heightened, an indication of sex-specific recruitment and regulation of descending modulatory circuits. In contrast, connectivity in males between the hypothalamus and PAG was heightened, an indication of sex-specific recruitment and regulation of descending modulatory circuits. Although directional differences were observed in several connections such as PAG-hypothalamus and NRM-hypothalamus, these effects did not reach statistical significance after Bonferroni correction. The variances in connectivity, however, may suggest that in females, key serotonergic brainstem structures exert more control over certain brain regions or systems that are affected by or receive signals from the hypothalamus under nociceptive conditions. Although speculative, these trends may imply that males and females vary in connectivity in brainstem and spinal cord regions during pain processing. Interestingly, the connection between the nucleus tractus solitarius (NTS) and the hypothalamus was stronger in males. Traditionally, the NTS is known for integrating visceral and autonomic input, and the connection between NTS and hypothalamus may indicate that the sexes are different in terms of how sensory-autonomic feedback loops are activated [16, 17]. This trend, though speculative, might show how males and

females are different in the way they monitor and respond to internal physiological states during pain. In addition, the stronger PAG-hypothalamus connection seen in females may indicate a more vigorous operation of the top-down modulatory system, whereby the PAG integrates the input from the limbic system and sensory organs and sends projections to the hypothalamus and various brainstem structures that control autonomic and pain mechanisms [17]. Additionally, NRM-hypothalamus connectivity in females is greater and may indicate stronger engagement of the serotonergic system during pain [18]. This study did not find statistically significant sex differences in connectivity using ANCOVA, however, the results did reveal some trends, from which we can hypothesize that males and females may engage subcortical pain-modulatory circuits differently. As shown in the results, female subjects had a higher average connectivity in pathways such as the PAG-hypothalamus and NRM-hypothalamus, while male subjects showed greater connectivity in pathways hypothalamus-PAG and NTS-hypothalamus. These directional differences allude to the possibility of sex-specific modulation within the descending pain system [19]. One potential explanation for these differences involves how sex hormones influence brainstem circuitry [20]. Previous studies involving animal models have indicated that estradiol has the potential to change the binding of opioid receptors and can modulate GABA signaling in the PAG and the NRM, potentially influencing descending pain modulation [20, 21, 22]. Moreover, the NRM's serotonergic and opioid systems are known to operate differently between sexes [22]. This finding suggests that both hormonal and neurochemical factors may help explain the patterns observed in the present study. It is important, however, to emphasize that these trends were not statistically confirmed. The ANCOVA results indicated that neither the main effect of sex, the main effect of condition, nor the interaction term, reached significance at Bonferroni correction $\alpha = 0.00044$. This implies that even though connectivity patterns appeared to diverge

between males and females, these differences were not significant enough to rule out the influence of participant variability. These findings highlight the difference between males and females in the way they modulate pain at the central level. They support the inclusion of sex as an important variable in both clinical pain research and neuroimaging study design. In addition, this study also validates SAPM as an analytical method that can model subtle sex differences in the way key components of the nociceptive system are connected directionally [12]. Despite these non-significant findings, this study still offers valuable contributions to the complex field of sex differences in pain processing. The observed trends support the notion that pain modulation might be organized in a different way between the sexes, even when ratings of subjective pain are similar. These results highlight the need for studies that are larger and more statistically powerful. Such studies can better characterize sex differences that exist in connectivity within the brainstem and spinal cord.

3.7 Conclusion

Overall, there were no significant sex differences found in the pain intensity or unpleasantness ratings during thermal stimulation. Although males and females reported comparable pain ratings, SAPM analyses showed that they had differences in connectivity between regions that are involved in both ascending and descending pain pathways. These trends suggest that males and females might carry out pain modulation pathways differently, which may be attributed to hormonal or neurochemical influences. These findings emphasize the importance of including sex as a key variable in pain research, despite statistical significance not being achieved. SAPM was shown to be effective in identifying these differences, supporting its value for studying subcortical pain mechanisms [12]. This study's results highlight the necessity of treating sex as a

biological variable in pain research, particularly in studies investigating the pathways involved in pain modulation.

3.8 Acknowledgements

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3.9 Conflict of Interest Statement

All listed authors have reviewed and approved of this manuscript. None of the authors have any conflicts of interest to report.

3.10 Funding Information

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Chapter 4: General Discussion

This thesis investigated how thermal stimuli of varying intensities (38 °C, 46 °C, and 51°C) influence pain perception, physiological arousal, and functional connectivity within the brainstem and spinal cord. This work also looked at sex-based differences in pain-related responses across two projects. These results improve our understanding of pain-related networks in subcortical regions. They suggest that stimulus intensity and assigned sex may be important determinants of network dynamics during acute pain processing. Across both Project 1 and Project 2, the observed rise in subjectively reported pain with the temperature of the stimulus confirms that thermal stimulation is a valid model for studying nociceptive responses. Notably, increases in pain intensity and unpleasantness occurred in a temperature-dependent manner. This finding is consistent with previous studies that have shown escalating peripheral input recruits progressively more central processing resources [1, 2].

As discovered in Project 1, however, the most extensive pattern of significant network connectivity emerged not at the highest noxious temperature (51 °C), but at the moderately noxious level (46 °C). This non-linear relationship indicates a possible adaptive modulation of pain processing at greater stimulus intensities. Compared to 38 °C or 46 °C, the relative decrease in connectivity at 51 °C may be indicative of a ceiling effect in network recruitment or the engagement of inhibitory mechanisms that limit further excitatory signaling. These results align with the idea of stimulus-dependent reorganization of pain networks, especially in the descending pain control systems [3, 4]. Structural and Physiological Modeling (SAPM) showed that connectivity can be affected by temperature-related changes across a series of regions linked to nociceptive processing. These regions include the periaqueductal gray (PAG), hypothalamus,

nucleus tractus solitarius (NTS), and locus coeruleus (LC) [5]. The connection from the PAG to the hypothalamus was strongest at 46 °C, and it was weakened at 51 °C. This is an example of temperature-sensitive modulation within descending pathways. This trajectory may indicate that with high amounts of nociceptive input, descending pathways either saturate or switch from a facilitatory to an inhibitory function, preventing overactivation of central circuits [6]. Inhibitory connections, such as those from the PAG to the parabrachial nuclei (PBN), further support the notion that systems are recruited to modulate pain and downregulate nociceptive output when the nervous system is intensely stimulated [7]. The LC-thalamus connection was robust and remained present across all temperatures, demonstrating its consistent role in pain processing. The LC is known to modulate attention and vigilance in response to stimuli, and its consistent connectivity with the thalamus suggests that pain-related pathways remain engaged regardless of stimulus intensity [8]. These findings are further supported by eye-tracking data, which showed increased pupil diameter when the stimulus was applied to participants' hands, regardless of the stimulus temperature. These results indicate the presence of greater autonomic arousal, even though no significant differences were found in pupil size across the three temperatures. Furthermore, the finding that 46 °C stimulation produced the most extensive connectivity pattern may also reflect an optimal level of arousal and network recruitment, where facilitatory and inhibitory circuits are both highly active. Reduced connectivity at 51 °C, despite the higher subjective pain ratings, raises important questions about the limits of network plasticity in acute pain processing. Prior research has identified similar patterns between stimulus intensity and cortical activation, and the findings in the present study extend this idea to subcortical networks [9]. A potential limitation to this present study, however, is that functionally distinct subregions were identified using k-means clustering applied to BOLD time series within anatomically defined regions, but optimal clusters

were derived from the 46°C condition only. This makes it unclear whether different thermal intensities would yield different clustering outcomes or network patterns.

In Project 2, sex differences in subjective pain ratings and connectivity were examined. Although male and female participants reported similar pain ratings overall, connectivity patterns varied across several brainstem-spinal cord regions. Females showed higher average connectivity in connections including PAG-hypothalamus and NRM-hypothalamus, although these trends did not reach statistical significance. These patterns may reflect greater top-down regulatory engagement during nociception. Conversely, males showed stronger connectivity in hypothalamus-PAG and NTS-hypothalamus pathways, potentially reflecting different strategies for engaging descending pain control or integrating sensory-autonomic feedback. These trends may be indicative of underlying sex-specific mechanisms of descending pain modulation. However, it is important to note that none of the ANCOVA results survived Bonferroni correction for multiple comparisons (adjusted $\alpha = 0.00044$), and therefore, all observed sex differences in connectivity should be interpreted as exploratory. Previous studies have shown that hormonal factors such as estrogen can influence opioid receptor binding and GABAergic transmission within the PAG and NRM, which may help explain the greater connectivity observed in females [10, 11]. Additionally, sex-related differences in serotonergic and opioid systems within the NRM have been reported, supporting the possibility that neurochemical and hormonal influences contribute to these connectivity trends [11]. Although these patterns did not reach statistical significance, they demonstrate the importance of considering sex as a variable in pain research. This study demonstrates that directional modeling methods like SAPM can detect potential subcortical connectivity differences which may vary by sex. Including latent inputs in SAPM modeling makes

it possible to consider unmeasured modulatory influences that are potentially hormonal, cognitive, or emotional in nature [5]. These influences may act across different participants to differentially affect signaling. Therefore, future research should investigate the specific modulatory factors that contribute to the apparent sex-based differences observed in Project 2. For example, hormone assays or phase-controlled testing could be used to investigate the contribution of estradiol and progesterone in pain perception and subsequent connectivity patterns. Additionally, the inclusion of affective or cognitive modulators (e.g., anxiety levels, emotional valence) may help to more generally explain the individual variability in pain processing.

Methodologically, throughout Project 1 and Project 2, the use of HASTE sequences and Pantheon software optimized MRI data quality in the brainstem and spinal cord, minimizing geometric distortions often encountered with EPI sequences [12]. The application of SAPM allowed for a physiologically accurate analysis of directional connectivity, allowing us to interpret input and output relationships between regions, and identify both excitatory and inhibitory influences [5]. This modeling approach offers a more detailed understanding of pain-related signaling compared to other methods and highlights the influence of subcortical networks in the pain experience [5]. In conclusion, the findings from this thesis highlight the complex and non-linear nature of pain network dynamics in response to thermal stimulation. The study demonstrates that subcortical circuits may be differently engaged depending on stimulus intensity and assigned sex. Moderate noxious input (46 °C) appears to result in the most extensive connectivity, suggesting a critical range of input where both facilitatory and inhibitory mechanisms are maximally engaged. Differences in average connectivity between males and females suggest possible variances in the way pain is modulated, highlighting the need for further investigation

into sex-specific mechanisms. Together, these findings underscore the importance of examining spinal and brainstem circuitry in pain research and support the use of directional modeling approaches, such as SAPM, to advance our understanding of nociceptive processing [5].

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Appendix A



Queen's University Health Sciences & Affiliated Teaching Hospitals Research Ethics Board (HSREB)

HSREB Initial Ethics Clearance

January 17, 2023

Dr. Patrick Stroman
Centre for Neuroscience Studies
Queen's University

TRAQ #: 6037552

Department Code: CNS-029-22

Study Title: "CNS-029-22: Pilot study to investigate alterations in nociceptive processing related to chronic widespread muscle pain and fibromyalgia"

Co-Investigators: Dr. Christopher Haley, Dr. Caroline Pukall, Dr. Scott S Duggan, Dr. Tracy Cupido

Review Type: Delegated

Date Ethics Clearance Issued: January 17, 2023

Ethics Clearance Expiry Date: January 17, 2024

Dear Dr. Stroman:

The Queen's University Health Sciences & Affiliated Teaching Hospitals Research Ethics Board (HSREB) has reviewed the application and granted ethics clearance for this study as of the date noted above.

Document Name	Comments	Version Date
Poster	poster/flyer for bulletin boards and posting on social media	2023/01/16
Other document	linking log template	2023/01/09
Letter of Information/Consent Form (combined document)	information and consent form	2023/01/10
Protocol	CIHR grant application	2022/09/13
Questionnaire	Study questionnaires	2022/10/25
Other document	On-line information and screening form	2022/10/25

Documents Acknowledged:

- Ethics Training Certificates
- Principal Investigator's CV
- Peer Review documents (CIHR, NSERC)
- Study budget

Amendments: No deviation from, or changes to the protocol, informed consent form and conduct of study should be initiated without prior written clearance or an appropriate amendment event from the HSREB, except when necessary to eliminate immediate hazard(s) to study participants or when the change(s) involves only administrative or logistical aspects of the study.

Appendix B



QUEEN'S UNIVERSITY HEALTH SCIENCES & AFFILIATED TEACHING HOSPITALS RESEARCH ETHICS BOARD (HSREB)

HSREB Delegated Amendment to Ethics Clearance

February 21, 2023

Dr. Patrick Stroman
Centre for Neuroscience Studies
Queen's University

TRAQ #: 6037552

Department Code: CNS-029-22

Study Title: "CNS-029-22: Investigating the neurobiological basis of heightened pain and dysfunction in fibromyalgia, by means of functional magnetic resonance imaging"

Review Type: Delegated

Date Ethics Clearance Issued: February 21, 2023

Dear Dr. Stroman:

The Queen's University Health Sciences & Affiliated Teaching Hospitals Research Ethics Board (HSREB) has reviewed the amendment event form and is granting ethics clearance for the changes listed below:

Document Name	Comments	Version Date
Letter of Information/Consent Form (combined document)	revised letter of information and consent form, clean copy	2023/02/21
Poster	revised recruitment poster	2023/02/16

Regards,

Dean A. Tripp, PhD
Chair, Queen's University Health Sciences and Affiliated Teaching Hospitals Research Ethics Board (HSREB)
Professor, Dept of Psychology, Anesthesiology & Urology
Queen's University
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The HSREB operates in compliance with, and is constituted in accordance with, the requirements of the Tri-Council Policy Statement: Ethical Conduct for Research Involving Humans (TCPS 2); the international Conference on Harmonisation Good Clinical Practice Consolidated Guideline (ICH GCP); Part C, Division 5 of the Food and Drug Regulations; Part 4 of the Natural Health Product Regulations; Part 3 of the Medical Devices Regulations,