2017-12-14

Sexual Dimorphisms in P2X4R-evoked Pain Hypersensitivity

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by

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A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE

DEGREE OF MASTER OF SCIENCE

GRADUATE PROGRAM IN NEUROSCIENCE

CALGARY, ALBERTA

DECEMBER, 2017

Abstract

Neuropathic pain can arise from nerve injury caused by disease, infection or trauma. It is among the most debilitating chronic pain conditions and is refractory to the current available pharmacological treatments. Converging evidence suggests that ATP-gated P2X4Rs on spinal microglia are implicated in the development and maintenance of neuropathic pain. P2X4Rs are upregulated in the spinal dorsal horn following nerve injury, and stimulation of P2X4Rs drives p38-MAPK activation and BDNF release, which leads to aberrant nociceptive processing in the spinal cord. However, recent evidence suggests that the role of microglia in neuropathic pain is sexually dimorphic. In this study, I provide evidence that microglial P2X4Rs are a key cellular point of sexual divergence in the development of pain hypersensitivity following PNI. In cell cultures, I determined that ATP-stimulation upregulated P2X4Rs leading to p38-MAPK activation and SNARE-dependent release of BDNF only in microglia isolated from male postnatal rats. Additionally, I found that intrathecal injection of P2X4R-stimulated microglia derived from males, but not from females, induced mechanical allodynia in non-injured naïve rodents. I also show that P2X4R expression and function are increased in spinal microglia isolated from only male rats following PNI. Finally, my data provides novel evidence that transcription factor IRF5 differentially regulates *P2rx4* gene expression in males and females, providing a possible explanation for the dimorphisms underlying P2X4R-signalling. Together, the findings of this study suggest that the microglial P2X4R is a critical point of sexual divergence in pain hypersensitivity, and may provide a cellular basis for the development of more effective, sex-dependent treatment options for neuropathic pain.

Acknowledgements

This thesis has become a reality with the guidance and support of several individuals. I would like to extend a large thank you to everyone who has provided me assistance through my graduate program.

First and foremost, I am sincerely grateful to my supervisor, Dr. Tuan Trang, who has given me the necessary guidance and support to complete this project successfully. His exceptional mentorship has not only provided me the tools to develop as a graduate student, but has also been pivotal in building my confidence as a researcher. I am indebted to Dr. Trang for the numerous hours that he has spent editing my work, providing me with a constellation of new terminology and a greater appreciation for good writing, which I will carry forth in my career in science.

I would also like to extend sincere gratitude to my committee members, Dr. Hermann Schaetzl and Dr. Jeff Biernaskie, who have so graciously made time in their busy schedules to accommodate my committee meetings and have provided substantial insight into my project which has been extremely helpful for its completion. This also pertains to my external examiner, Dr. Deborah Kurrasch, who has taken the time to read this thesis and take part in my Masters defense.

This research could not be conducted without the funding received from Canadian Institute of Health Research, the Hotchkiss Brain Institute and the Faculty of Veterinary Medicine. I am eternally grateful for the travel grants that allowed me experience conferences in both Japan and Germany, and the funding that has provided me the resources I needed for the development of this project.

I owe a sincere thanks to the current and past members of the Trang lab, who have been instrumental for my success in graduate school. Their relentless support, friendship and constant willingness to play an active part in my education will never go unappreciated. A special thanks to HLP and NB for the coffee breaks, CF, CK and AP for the hours of time put into my project, and MM and NB for being so supportive through out my studies.

Last but not least, I would not have been able to complete my graduate degree without the undeniable support from my family and friends, who have always had faith in my abilities. I will always be grateful for their words of encouragement and understanding throughout my studies.

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List of Symbols, Abbreviations and Nomenclature

Symbol	Definition
°C	degrees Celsius
AMPA	α-amino-3-hydroxyl-5-methyl-4-isoxazolepropionic acid
ANOVA	analysis of variance
ATP	adenosine triphosphate
BDBD	5-3-Bromophenyl-1,3-dihydro-2-benzofuro-1,4-diazepin-2-one
BDNF	brain-derived neurotrophic factor
BoNT	botulinum toxin
Ca^{2+}	calcium
CAMKII	calmodulin-dependent protein kinase 2
CCI	constricted cuff injury
ChIP	chromatin immunoprecipitation
CIP	cognitive insensitivity to pain
Cl^-	chloride
CNS	central nervous system
CSF-1	colony stimulating factor 1
CSP-6	caspase 6
CO_2	carbon dioxide
DMEM	Dulbecco's modified eagle's medium
DNA	deoxynucleic acid
ECS	extracellular solution
EDTA	Ethylenediamine tetra-acetic acid
ELISA	enzyme-linked immunosorbent assay
EPSP	excitatory postsynaptic potential
ERK	extracellular signal-regulated kinase
FBS	fetal bovine serum
g	gram(s)
GABA	gamma-aminobutyric acid
GFAP	glial fibrillary acidic protein
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HBSS	Hanks balanced salt solution
Iba1	ionized calcium-binding adapter molecule 1
IRF	interferon regulatory factor
IFN-γ	interferon gamma
IL-6	interleukin 6
IL-1β	interleukin-1beta
IP	immunoprecipitate
KCC2	chloride-cotransporter potassium-chloride exporter 2
kDa	kilodalton
LPS	lipopolysaccharide

mM	millimolar
MAPK	mitogen-activated protein kinase
MCP	monocyte chemoattractant protein
MMP	matrix metalloproteinase
Na^+	sodium
NAP	NSF-associated protein
NMDA	N-methyl-D-aspartate
NSAID	nonsteroidal anti-inflammatory drugs
NSF	N-ethylmaleimide sensitive fusion protein
NTR	neurotrophin receptor
P2X7R	P2X7 receptor
P2X4R	P2X4 receptor
PAR2	protease-activated receptor 2
PBS	phosphate buffered saline
PI3K	phophoinositide 3-kinase
PKA	protein kinase A
PKC	protein kinase A
PLC	phospholipase C
PNI	peripheral nerve injury
PPAR	peroxisome proliferator activated protein
qPCR	quantitative polymerase chain reaction
RNA	ribonucleic acid
siRNA	short interfering ribonucleic acid
SEM	standard error of means
SNAP	soluble NSF attachment protein
SNARE	soluble NSF attachment protein receptor
SNI	spared nerve injury
TBST	tris-buffered saline
TLR	toll-like receptor
TNF-α	tumor necrosis factor-alpha
TrkB	tyrosine receptor kinase B
VAMP	vesicle-associated membrane protein
VAP	VAMP-associated protein
VNUT	vesicular nucleotide transporter
α	alpha
γ	gamma
β	beta
µg	microgram
µl	microlitre
µM	micromolar
µm	micrometer

CHAPTER 1: INTRODUCTION

1.1 Acute pain

Pain is an unpleasant sensory and emotional experience associated with actual or potential tissue damage.[1] Acute pain protects against tissue damage and resolves with removal of the noxious stimulus or resolution of the injury.[2] It motivates us to withdraw from damaging situations, protects the site of injury during the healing process and triggers complex emotional and cognitive responses that prevent subsequent pain-causing events.[3] The importance of feeling pain is especially apparent in patients who suffer from congenital insensitivity to pain (CIP), a debilitating condition caused by a genetic mutation in the sodium voltage-gated channel alpha subunit 9 (SCN9A) gene.[4] These individuals lack the ability to feel intense thermal and mechanical stimuli, and therefore, tend to engage in harmful activities at an early age leading to serious long-term health consequences or even death.[4]

The spinal dorsal horn is the primary point of integration and modulation of nociceptive information. Painful stimuli in the periphery is transmitted to the dorsal horn through a subpopulation of primary afferent fibres that contain specialized sensory receptors referred to as nociceptors.[5,6] Nociceptors are found in skin and various organs in the body and detect intense mechanical, chemical and thermal stimuli.[5] They are heterogenous in nature and are classified based on a variety of biochemical, physiological and anatomical properties.[6] They include C- and Aδ-fibres that have specific thresholds of detection, only firing when the stimulus reaches a noxious range.[6] C-fibers are unmyelinated small-diameter fibers that conduct at a low velocity.[7] They are polymodal, responding to a wider variety of intense stimuli that can be thermal, mechanical or chemical in nature.[7] C-fiber activation is associated with dull, deep tissue pain that is spread over an unspecific area.[5] Conversely, Aδ fibres are fast-transmitting, large diameter

myelinated fibers that fire in response to intense mechanical and thermal stimuli.[8] Aδ fibres constitute the afferent portion of the reflex arc and activation of these fibers usually results in rapid, sharp pain.[8]

Nociceptive afferents synapse primarily in lamina I, III, IV and V in the spinal dorsal horn.[9] Lamina II, although receiving little direct input from nociceptors, plays an important role for pain processing and integration through a complex relay system constructed of mostly interneurons.[9] Laminae III and IV constitute the nucleus proprius, receiving input from peripheral mechanoreceptors, C-fibres and Aβ fibres that carry sensations such as light touch, pain and temperature.[9] Lamina V contains wide dynamic neurons that process sensory information from muscle, joint, cutaneous and visceral tissue, making it an important processing center for proprioception.[9]

Lamina I is the first relay in the nociceptive pathway containing the majority of projection neurons in the posterior column. Here, primary afferent terminals of Aδ and C-fibers synapse with ascending projections that transmit information on pain to supraspinal regions.[9] Second-order neurons converge into bundles that constitute pain pathways such as the spinothalamic and spinoreticular tracts.[10] These pathways project to the thalamus, the brainstem and higher cortical brain regions such as the somatosensory cortex, the insula and the anterior cingulate cortex where pain is perceived.[8] Pain is derived from a complex processing of stimuli in the central nervous system (CNS) where factors such as emotions and cognition contribute to the sensation of 'feeling pain.'[11] Although the mechanisms controlling the sensation, integration and perception of painful stimuli have been well documented, how these systems become maladaptive in the context of chronic pain remain largely unknown.[8]

1.2 Chronic neuropathic pain

In some circumstances, acute pain may develop into chronic pain, where molecular changes in the CNS can lead to aberrant sensory processing.[12] Chronic pain is different from acute pain in that it is often attributed to a disease but does not always associate with an identifiable pathology.[1,2] In addition, chronic pain is long-lasting and serves no known biological function.[2] It can arise from an injury that has long since healed such as a surgical incision or an infection, but can also develop from disease states including diabetes mellitus, multiple sclerosis and arthritis.[1] Chronic pain affects 1 in 5 Canadians, a number that is continuously rising with the growing ageing population.[13] Aside from the detrimental effects that this disorder has on quality of life, it also poses a severe economical burden for society. It is estimated that chronic pain costs $43 billion a year which exceeds that of heart disease, cancer and human immunodeficiency virus (HIV) combined.[14]

Neuropathic pain is a subtype of chronic pain that occurs from damage to the central or peripheral nervous system. It is estimated to affect 4% of the global population, making it the most pervasive type of chronic pain.[15] Common conditions associated with this disorder include diabetic neuropathy, HIV, post-herpetic neuralgia, trigeminal neuralgia and amputation.[15] Neuropathic pain is particularly challenging to assess clinically because the manifestation may vary with the type of disease associated with the neuropathy, or ongoing pain may be the only manifestation, making it a heterogenous disorder that is complex in nature.[16] Additionally, the pain severity does not always correlate with the extent of injury, and those who suffer from neuropathic pain frequently experience comorbidities including sleep disturbances, depression and anxiety in addition to their pain.[15,17] Neuropathic pain often described as shooting, burning or itching and patients experience a distinct set of symptoms that include allodynia (a painful

response to a innocuous stimuli), hyperalgesia (an exaggerated response to a painful stimuli), and spontaneous pain (pain in response to an unknown stimulus).[18]

The mechanisms behind the development of neuropathic pain remain poorly understood. As a result, many treatment options are either ineffective or generate undesired side effects, resulting in only 40-60% of patients achieving partial pain relief.[18] First-line treatment options such as gabapentin (a gamma-amino butyric acid (GABA) analogue), pregabalin (a Ca^{2+} channel inhibitor) and duloxetine (a selective serotonin reuptake inhibitor) are among the most studied drugs used to treat neuropathic pain.[19] Capsaicin or lidocaine patches and tramadol (an opioid) are considered second-line treatment options and are used to treat peripheral neuropathy only, whereas patients show little improvement with non-steroidal anti-inflammatory drugs (NSAIDs), strong opioids and botulinum toxins.[19,20] Even among the first-line options, there is variability in responsiveness to these treamtents and they only provide symptomatic relief.[18] These factors substantially limit their clinical utility and make neuropathic pain an increasingly difficult syndrome to manage.[18] Despite the recent failures of new drugs in clinical trials, novel therapeutics have been developed preclinically that target Nav1.7,[21] angiotensin II[22] and stem cell therapies[3] that offer promise for human testing. The prevalence of neuropathic pain, together with the lack of effective treatment options, underlies a pressing need to understand the mechanisms behind this debilitating chronic condition.

1.3 Peripheral and central sensitization

Increasing evidence suggests that nerve injury alters the electrical properties of neurons at the peripheral and central levels, leading to an imbalance in sensory signalling that underlies neuropathic pain.[12] Sensitization is a form of plasticity that results in an enhancement in the function of neurons in the nociceptive pathway and is a key process involved in the transition

from acute to chronic pain.[6] Peripheral nociceptors are normally silent under homeostatic conditions.[12] After exposure to noxious stimuli, the somatosensory system becomes hyperexcitable and responds by lowering thresholds of activation and amplifying neuronal output in response to subsequent insult.[6,12] In acute pain, this heightened sensitization returns to a baseline level over time and serves a protective role for the nervous system, but this process becomes maladaptive in the context of chronic pain.[5] Sensitization at the peripheral and central levels provide mechanistic explanations for both the manifestation and chronification of neuropathic pain.

At the peripheral level, inflammatory chemical mediators released in damaged tissue increase excitability of primary nociceptors or their central targets.[6] Persistent exposure to inflammatory mediators causes an upregulation of voltage-gated sodium (Na^+) (Nav1.3, Nav1.8, Nav1.9)[23] and calcium (Ca^{2+}) (N-type) channels in primary afferent fibers, which contributes to a heightened state of sensitivity.[12,24,25] Since increased responsiveness of nociceptors underlies peripheral sensitization, these properties are usually restricted to the site of injury and generally rely on ongoing peripheral pathology for its maintenance.[12] By contrast, chronic pain can manifest in the absence of injury or long after the initial injury has healed, suggesting that the pathology of persistent pain states cannot fully be explained by peripheral mechanisms alone.[2] Despite the contributions of peripheral sensitization, increasing evidence suggests that central sensitization is the pathophysiological mechanism underlying chronic pain.[12,26]

The spinal cord is the first level of ascending pain signal modulation in the CNS where lamina I in the dorsal horn is an important pain processing centre.[10] Here, peripheral nociceptors including Aδ-fibers, C-fibres, and newly recruited Aβ fibers[27] transmit information about noxious stimuli to second order lamina I projection neurons.[5,10] In response to PNI,

neurotransmitters including glutamate,[28] substance P,[29] prostaglandin,[30] and adenosine tri-phosphate (ATP)[31] are released from the terminals of primary afferents, resulting in dorsal horn network excitation.[6] Increased exposure to excitatory neurotransmitters enhances the functional status of lamina I neurons by altering the firing thresholds, kinetics and voltage dependencies of these cells.[12] This increase in neuronal excitability arises in part from phosphorylation of intracellular signalling molecules including protein kinase A (PKA), protein kinase C (PKC), extracellular signal-regulated kinase (ERK), and calmodulin-dependent protein kinase II (CAMKII) that alter the transcription, trafficking and phosphorylation of key excitatory ion channels including the N-methyl-D-aspartate (NMDA) receptor and voltage gated Ca^{2+} channels in second-order neurons.[12,32,33] The net effect of central sensitization mechanisms on lamina I neurons is an enlargement of sensory receptive fields, increased spontaneous activity, lower thresholds of activation and enhanced responses to peripheral noxious stimuli.[12] Since synaptic changes in the CNS underlie central sensitization, innocuous stimuli can now evoke nociceptive activity, causing pain in the absence of a triggering stimulus.[34] Central sensitization mechanisms are thought to contribute to neuropathic pain,[16] inflammatory pain,[35] migraine,[36] irritable bowel syndrome[37] and plays a fundamental role in fibromyalgia.[18] It provides a mechanistic explanation for pain hypersensitivity following nerve injury and underlies abnormal sensory responses elicited by peripheral stimuli.

1.4 Microglia and neuropathic pain

Microglia are resident immune cells in the CNS and account for 5-20% of the total glial cell population.[29] Although it was previously assumed that microglia play mainly supportive roles in the CNS, it has become recently apparent that they are instrumental in shaping healthy CNS structure during normal development and also contribute to several disease pathologies

including Alzheimers, multiple sclerosis, depression and chronic pain. [32,31,38–40] Microglia have highly motile processes that constantly survey the parenchymal environment for damage, debris or other threats to CNS homeostasis.[40] Under physiological conditions, microglia exist in a surveillance or 'resting' state and are generally characterized by a smaller soma and ramified processes. In response to challenge, microglia transition to more reactive phenotype where they transduce, integrate and respond to extracellular stimuli.[38] The morphological features of reactive microglia are hypertrophic cell bodies, retracted processes and an increase in cell number.[41] In addition, they upregulate a myriad of membrane-bound markers such as CD11b, ionized-calcium adapter binding molecule 1 (Iba1) and release inflammatory cytokines including BDNF, tumor necrosis factor-α (TNFα), interleukin-6 (IL-6), interleukin- β (IL-1β) and interferon-γ (IFNγ).[42–44] Although robust following CNS insult, the inflammatory response launched by microglia is fluid and depends on the type, duration and intensity of triggering stimuli.[38]

Microglia are implicated as key mediators for the development of chronic pain. Following PNI, neuroinflammatory mediators including ATP,[45] monocyte chemoattractant protein-1 (MCP-1),[46] caspase-6 (CSP-6),[47] matrix metalloproteinase-9 (MMP-9)[48] and colony-stimulating factor-1 (CSF-1)[49] are released from the terminals of injured nociceptive afferents into the spinal dorsal horn. These substrates act on microglial receptors and lead to increased microglial reactivity and proliferation ipsilateral to the site of injury.[39] Chronic activation of microglia in the spinal dorsal horn coincides with an upregulation of cell surface receptors (e.g. purinergic receptors), intracellular signalling molecules (e.g. kinases) and release of inflammatory cytokines (e.g. neurotrophins) that play crucial roles in lowering the activation thresholds of lamina I neurons and increasing dorsal network excitability.[51,52] Inhibiting microglial activation following PNI is sufficient to attenuate the development of pain

hypersensitivity, pinpointing spinal microglia as being key players for driving aberrant nociceptive processing following injury.[51]

1.5 P2X4 receptors and pain

A growing body of evidence suggests that purinergic signalling participates in the pathogenesis of neuropathic pain by acting through P2 purinergic receptors in the spinal cord.[53–55] Microglia express two types of P2 purinergic receptors; ionotropic P2X receptors that contain a Ca^{2+} permeable intrinsic pore, and metabotropic (P2Y) receptors that couple to intracellular messenger systems.[56] ATP, released in response to nerve injury, acts through P2XRs to drive microglial reactivity in the spinal dorsal horn.[45,55] P2XRs are oligomeric ion channels that have three membrane-spanning subunits with large extracellular loops and intracellular C- and N-terminal domains that participate in protein-protein interactions.[57] There are 7 types of P2X receptors (P2X1-7) although converging evidence pinpoints a specific subtype of P2X receptor, the P2X4 receptor (P2X4R), as being a key player in the development of neuropathic pain.[54]

P2X4Rs are upregulated in spinal dorsal horn microglia following nerve injury.[54,58,59] Exposure to cytokines released from injured neurons or glia including C-C chemokine ligand-21 (CCL21),[48] INF-γ,[60] protease-activated receptor-2 (PAR2),[61] or extracellular matrix proteins such as integrins or fibronectins,[62] and activation of Lyn tyrosine kinases[63] all lead to an upregulation of P2X4Rs on microglia. However, it is not yet clear how these substrates mediate P2X4R expression in the spinal dorsal horn, and which intracellular signalling pathways are involved in its upregulation. Using an immortalized microglial cell line (BV2s), it was previously shown that transcription factors interferon-regulatory factor 5 and 8 (IRF5 and IRF8) regulate P2X4R gene (*P2rx4*) expression.[60,64] IRFs play diverse roles in the immune system, regulating processes such as cell growth, differentiation and apoptosis.[65] IRF8 is critical for microgliogenesis in the

developing nervous system,[66] is an important modulator of microglial reactivity, and controls the expression of several pro-inflammatory cytokines in spinal microglia.[67] Interestingly, IRF5, which binds directly to the promoter region of *P2rx4* to regulate gene expression, is under the direct transcriptional control of IRF8.[35] Mice with depleted levels of spinal IRF5 do not upregulate P2X4Rs following PNI that coincides with higher pain thresholds.[67,68] Thus, the IRF5-IRF8 transcriptional axis may be necessary for the development of neuropathic pain following nerve injury.[64]

Microglial P2X4R activation is associated with pain related behaviours and hyperexcitability of dorsal horn neurons following nerve injury.[69–71] Evidence that microglial P2X4Rs are sufficient for pain hypersensitivity were shown using adoptive transfer experiments in which allodynia developed in naïve rats following intrathecal injection of P2X4R-stimulated primary microglia.[54] Furthermore, nerve injury-induced pain hypersensitivity was attenuated from pharmacological and genetic inhibition of spinal P2X4Rs.[54,58] To evoke pain behaviour, P2X4R activation on spinal microglia must initiate signalling cascades to communicate with dorsal horn neurons, which convey nociceptive information to the brain.[51] P2X4R activation drives BDNF release from microglia, and converging lines of evidence pinpoint microglial-derived BDNF acting on neuronal tyrosine kinase-B (TrkB) receptors as the key mechanism necessary for pain hypersensitivity following PNI.[71–74] It is now known that the secretion of BDNF by P2X4R activation depends on p38-mitogen activated protein kinase (p38-MAPK). p38-MAPK is phosphorylated upon activation, and spinal phospho-p38-MAPK is critical for the generation of neuropathic pain.[75] Following its phosphorylation, this kinase translocates into the nucleus to drive the transcription, translation and release of BDNF in the spinal dorsal horn[73] (See Figure 1). Attenuating key components in this pathway including P2X4R activation,[54,58]

p38-MAPK signalling,[75] or synthesis and release of BDNF[71] attenuates the development of pain hypersensitivity following PNI.

1.6 BDNF and pain

BDNF is a member of the neurotrophin family of growth factors and supports the neurogenesis, growth, survival and differentiation of neurons in the central and peripheral nervous systems.[76] It plays important roles in the modulation of learning, memory and higher thinking processes.[77] BDNF binds to two major neuronal receptors: TrkB and p75 neurotrophin receptors (p75NTR).[76] When TrkB binds its ligand, the receptor dimerizes and autophosphorylates, yielding docking sites for the Src homology 2 domain-containing adapter protein and phospholipase C-γ (PLC-γ).[76,78] This activates intracellular signalling cascades through PLC, phosphoinositide 3-kinase (PI3K) and ras that are linked to prosurvival and neurogenesis processes.[74]

BDNF acting through TrkB receptors plays an important role in modulating neuronal excitability in the spinal cord, which underlies the development and maintenance of neuropathic pain.[71,74] Activation of TrkB receptors by BDNF increases the neuronal excitability of lamina I projection neurons by facilitating Src-mediated NMDA receptor firing and lower thresholds for C-fibre-evoked responses.[12,79] In addition to increased glutamatergic transmission in the spinal dorsal horn, an important mechanism for pain hypersensitivity following PNI is disinhibition of lamina I neurons caused by BDNF acting on its cognate receptor.[70,71] TrkB activation leads to a reduction in the expression of potassium-chloride-cotransporter-2 (KCC2), which is responsible for maintaining low intracellular Cl^- levels through ion efflux.[70] KCC2 channels are the main chloride transporter expressed in lamina I neurons, so its downregulation causes an accumulation of intracellular chloride ions (See Figure 1). Under these conditions, opening of GABA and

glycine channels is much less effective at producing inhibition. Cl^- levels in some lamina I neurons are increased to such an extent that inhibitory synaptic transmission may cause chloride ions to leave the cell with their re-established gradient, shifting hyperpolarization events to depolarizing events.[67] Together, these cellular changes result in increased neuronal discharge, firing in response to innocuous stimuli and spontaneous activity of lamina I neurons.[80] Disinhibition of lamina I neurons through BDNF-TrkB-Cl^- signaling provides a mechanistic explanation for hyperexcitability leading to the three hallmark symptoms of neuropathic pain in humans: hyperalgesia, allodynia and spontaneous pain.[80]

1.7 Mechanisms of BDNF regulation and release

BDNF mRNA expression is found throughout the CNS and in peripheral tissues, including the thyroid, adrenal gland, spleen, lungs, gallbladder, heart muscle and reproductive organs.[81] In the CNS, BDNF is highly expressed in neurons, underlying the predominant neurocentric view on neurotrophins in CNS function.[81] In humans and rodents, BDNF transcription is tightly regulated in a tissue-specific manner by 9 different promoters that yield at least 18 transcript variants.[82,83] However, the translation of these variants ultimately produces the same BDNF protein, and the tight transcriptional regulation of *Bdnf* is thought to modulate the strict spatiotemporal expression patterns necessary for proper CNS development and function.[82] BDNF is initially synthesized in the endoplasmic reticulum as a 32 kDa proprecursor protein (pro-BDNF) that is cleaved intracellularly into a 28 kDa precursor (pre-BDNF) and secreted as 14kDa mature BDNF (mBDNF).[77,83] Although BDNF is predominately secreted in its 14 kDa form from microglia, regulated release of pro-BDNF occurs in both neuronal and microglial cultures and acts on different subsets of neuronal receptors to drive unique cellular processes.[82]

The docking of vesicles to their target membrane first requires the assembly of a ternary complex composed of soluble-NSF attachment protein receptors (SNAREs) at the plasma membrane.[84] SNAREs mediate the release of important modulators such as BDNF from neurons and are key components of the machinery required for protein secretion in the CNS.[85] Whereas the mechanisms underlying vesicular release in neurons have been well described, there is surprisingly little known on how exocytosis is regulated in microglia.[85,86] Over a decade ago, core components of the SNARE complex including synaptosomal-associated protein-23 (SNAP-23), syntaxin-1 and vesicle-associated membrane protein 3 (VAMP-3) were detected in glial cultures, suggesting that SNAREs mediate vesicular release in these cells.[84] These are homologous to core components of the trans-SNARE complex necessary for neurotrophin release from neurons (VAMP-1, VAMP-2 and SNAP-25), indicating that microglial BDNF release may occur by analogous mechanisms.[85,87] Indeed, a study published by Trang et al., (2012) showed that BDNF release from microglia was SNARE-mediated.[73] However, the presynaptic machinery expressed in microglia and the specific components that regulate BDNF release remain largely unexplored, despite the importance of this cytokine in pain processing. These gaps in knowledge have made it difficult to decipher the basic mechanisms of vesicular trafficking, recycling, packaging, docking and fusion of cargo-filled vesicles in microglia. Identifying key components of machinery that mediate vesicular release from these cells is therefore the first step in understanding aberrant microglia-neuronal signalling in the spinal cord.

1.8 Sex differences in P2X4R-evoked BDNF release

Mechanisms of microglial-neuronal signalling in neuropathic pain were established through studies using predominately male rodents.[88] Recent evidence suggests that, despite the development of robust hypersensitivity in both male and female rodents following PNI,

mechanisms of pain processing are disparate between sexes.[59] Sorge et al., (2015) show that microglia-neuronal signalling underlies the development of neuropathic pain in males, but females rely on a microglia-independent pathway that instead involves spinal infiltration of peripheral T-cells.[59] In addition, P2X4Rs are differentially upregulated in the spinal dorsal horn of male but not female rodents following PNI, and targeting key components of the microglial P2X4R-evoked BDNF release pathway using P2X4R antagonists, p38-MAPK inhibitors, BDNF sequestering peptides or TrkB receptor antagonists effectively reverses the development of allodynia exclusively in males.[59] Testosterone is necessary for the microglia-dependent development of allodynia following nerve injury, suggesting that sex differences underlying pain hypersensitivity may be hormonally mediated.[59] Together, these findings suggest that P2X4R-dependent signalling on microglia is engaged following PNI only in males, making it a key point of sexual divergence in neuropathic pain. Further examination into the basic mechanisms underlying the sex-dependency of microglia-neuron signalling in pain processing are needed to determine wherein lies the key point of divergence between males and females.

Several examples of sex differences exist in both human and rodent studies of pain.[40] In humans, prevalent chronic pain conditions such as headache, lower back pain, neck pain, fibromyalgia and interstitial cystitis occur predominantly in women.[89,90] In rodents, females are more vulnerable to the long-term consequences of neonatal injuries,[91] and there is a sex-specific role for toll-like receptor-4 (TLR-4) in inflammatory and neuropathic pain models.[92] Meta-analysis of studies conclude that females are more susceptible to chronic pain syndromes, have lower nociceptive thresholds and experience pain at a greater severity compared to males.[93–96] The large male-oriented bias in preclinical pain research has made it difficult to decipher the precise mechanisms underlying increased pain hypersensitivity in female rodents, although it is

now clear that spinal microglia may play key roles in mediating these sex differences.[59,96]

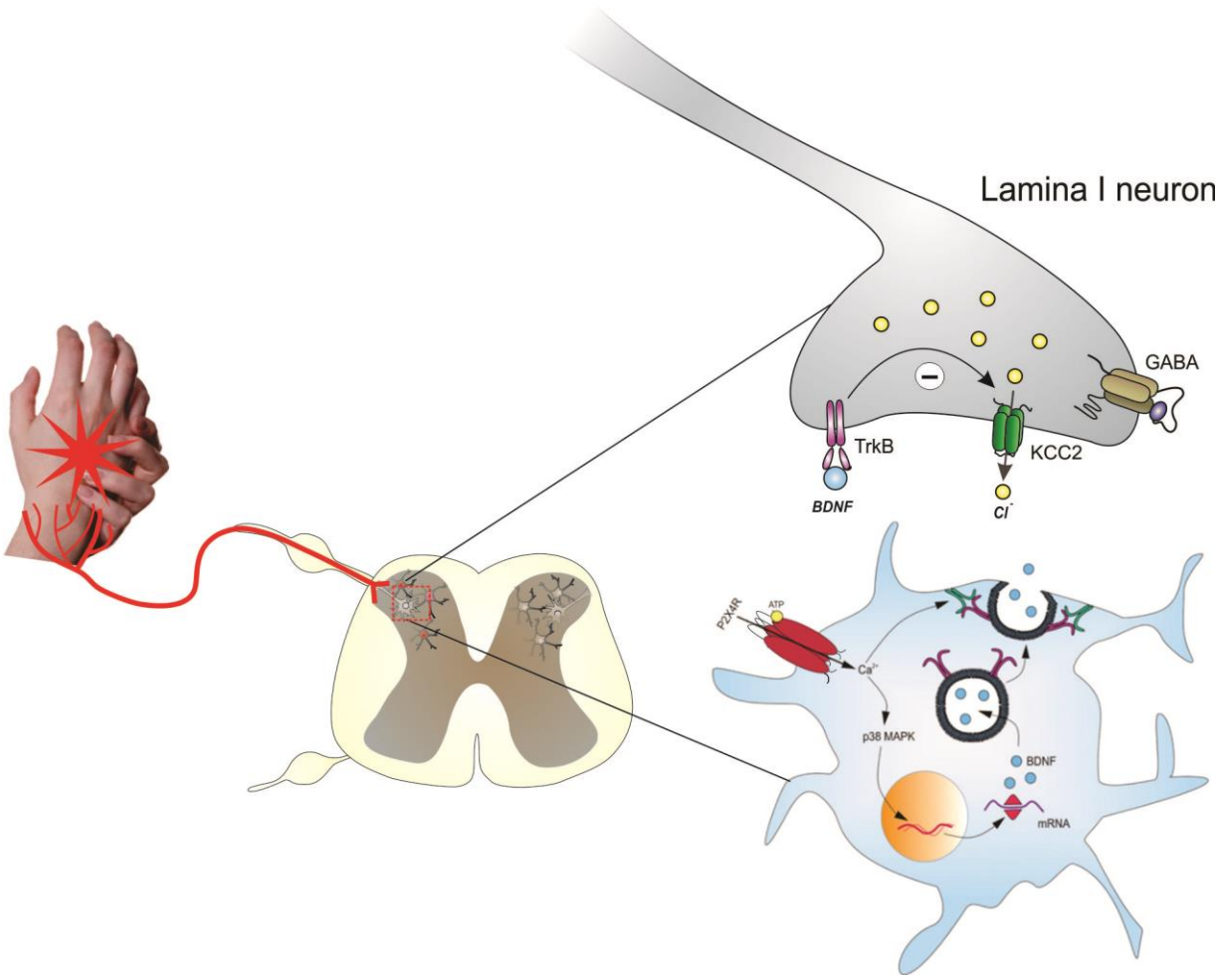

Figure 1: P2X4R-evoked BDNF release from microglia is implicated in pain hypersensitivity.

1.9 Research Statement

In rodents, PNI causes robust pain hypersensitivity and comparable levels of microgliosis in the spinal dorsal horn of both males and females. There is, however, evidence to suggest that microglia are not necessary for the development of pain hypersensitivity in females. P2X4R-signalling is engaged in males but not females following nerve injury, making it a key point of sexual divergence. Here, I investigated the cellular and molecular mechanisms underlying P2X4R-evoked BDNF release in both sexes to determine where the key point of divergence lies. Below, I will provide an overview of the key objectives and hypotheses that were tested in primary microglia.

1.10 Research Objectives

1.11 Determine the mechanisms required for P2X4R-mediated BDNF signalling and release

I first investigated whether key components of P2X4R-signalling were convergent or divergent between sexes using primary microglia cultures isolated from male or female postnatal rats (P1-P3). In male-derived microglia, I found that ATP-stimulation increased P2X4R expression and function, p38-MAPK activation, and synthesis and release of BDNF. These ATP-evoked responses were sex-dependent, in that they did not occur in microglia isolated from female postnatal pups. To test whether this sexual divergence had consequences for pain hypersensitivity, I intrathecally administered ATP or PBS-treated primary microglia into naïve rats and measured the development of mechanical hypersensitivity over the course of 3 hours. In these microglia adoptive transfer experiments, I found that only transfer of stimulated male-derived microglia was sufficient to induce allodynia in naïve rats, providing further evidence that ATP-evoked BDNF signalling is impaired in female-derived microglia.

BDNF is released from microglia downstream of P2X4R activation.[73] Several studies suggest that this process relies on an exocytotic mechanism involving SNARE proteins, although the machinery necessary for vesicular release in microglia remains largely unknown. To investigate this process, I first determined the expression profile of mammalian SNAREs in microglia by analyzing online microarray data. This led us to identify t-SNAREs and v-SNARES as putative targets for BDNF release. Of particular interest were subfamilies VAMPs and SNAPs that participate in BDNF release from neurons. I confirmed the expression of these SNAREs in primary microglia cultures and selectively targeted them *in vitro* using botulinum toxin and siRNA treatments. By measuring BDNF levels in the supernatant following targeted knockdown, I concluded that SNAP-23 and VAMP-3 are core components of the SNARE complex mediating BDNF release from microglia.

1.12 Determine the impact of PNI on spinal P2X4R expression and function

I next asked whether nerve injury impacts P2X4R expression and function on spinal microglia. I first isolated microglia from the spinal dorsal horn of sham or nerve injured rats, and used flow cytometry to measure mean P2X4R expression on two distinct cell populations: CD11b-positive cells (microglia) and CD11b-negative cells (neurons and astrocytes). I found that nerve injury increased P2X4R expression on microglia isolated from males but not females, whereas receptor levels were not different between surgery groups in the non-microglial population. Additionally, I measured changes in CD11b expression on the microglial population as a correlate for reactive microgliosis in the spinal dorsal horn. I next made use of calcium imaging approaches to test whether nerve injury impacts P2X4R function, and found that Ca^{2+} responses were potentiated following PNI only in males. Together, these findings show that

P2X4R expression and function is increased on spinal microglia from male but not female nerve-injured rats.

1.13 Examine the role of transcription factor IRF5 in the sex-dependent upregulation of P2X4R

Dimorphic P2X4R upregulation on microglia is a primary point of sexual divergence between males and females. Since *P2rx4* mRNA levels are increased only in males following ATP-stimulation, I investigated the regulation of the *P2rx4* gene at the transcriptional level. Transcription factor IRF5 directly binds to the promoter region of *P2xr4*, yet the loci identified previously are specific to mouse immortalized cell lines and do not reflect potential sex differences.[64] I characterized IRF5-mediated *P2rx4* upregulation in male and female-derived primary microglia. By using IRF5-targetted knockdown in cultures, I first show that IRF5 expression is necessary for ATP-evoked *P2rx4* upregulation. Next, I identified novel IRF binding sites using on the rat *P2rx4* promoter region using Genomatix software, and tested these sites using chromatin immunoprecipitation coupled with quantitative PCR (ChIP-qPCR). Lastly, I investigated the binding affinity of IRF5 at the *P2rx4* promoter in the spinal cord of males and females following injury. These experiments identified a sexually divergent role for IRF5 in *P2rx4* expression, providing novel evidence that differences in transcriptional regulation may underlie sexually dimorphic pain processing.

Collectively, the findings of this study provide further insight into the role of spinal microglial P2X4Rs in the development of neuropathic pain. Understanding P2X4R-evoked pain hypersensitivity at the cellular level is key to developing effective therapies that target persistent pain states in both males and females.

CHAPTER 2: METHODS

2.1 Animals

All experiments were approved by the University of Calgary Animal Care Committee and are in accordance with the guidelines of the Canadian Council on Animal Care. Adult male (200-250g) and female (150-200g) Sprague Dawley rats 6-8 weeks of age, or untimed pregnant females (E_{12-14}), were purchased from Charles River (Sherbrooke, QC, Canada) and housed under a 12/12 hour light/dark cycle with *ad libitum* access to food and water.

2.2 Primary microglia isolation

2.21 Primary microglia culture from postnatal rats

Postnatal day 1-3 (P1-P3) rats were sexed to obtain male- or female-only primary cell cultures. Microglia cultures were prepared as previously described by Trang et al., (2009).[73] In brief, mixed glial culture was isolated from postnatal Sprague Dawley rat brains and maintained for 10-14 days in Dulbecco's Modified Eagles Medium (DMEM) containing 10% fetal bovine serum (FBS) (Invitrogen) and 1% Penicillin-Streptomycin at 37˚C with 5% carbon dioxide ($CO_{2)}$. Microglia were separated from the mixed culture by gentle shaking and replated onto 100 mm plastic dishes for western blot analysis or coverslips for live-cell imaging.

2.22 Mixed neuron-glia isolation from adult rat spinal cords

On day 7 post-surgery, male or female rats were anaesthetized, the spinal cord was isolated by hydraulic extrusion and the dorsal ipsilateral lumbar region was placed in Hanks Balanced Salt Solution (HBSS). Following blunt dissociation using a Miltex surgical blade, the spinal cord was filtered through a 70 μm cell strainer into DMEM containing 10 mM HEPES and

2% FBS. Isotonic Percoll (density 1.23 g/mL) was added to the cell suspension, followed by a 1.08 g/mL Percoll underlay. Samples were spun at 3,000 rpms for 30 min at 20°C. Following centrifugation, myelin debris was removed, and the interface between Percoll gradients was collected and transferred into fresh medium. Samples were centrifuged again at 1,400 rpm. for 10 min at 4°C, and the pellet was reconstituted in PBS containing 10% FBS for flow cytometry or DMEM containing 10% FBS and 1% penicillin–streptomycin for calcium imaging.

2.3 Western blotting

Following microglia isolation and treatment, cells were harvested in cell lysis buffer containing 50 mM TrisHCl, 150 mM NaCl, 10 mM EDTA, 0.1% Triton-X, 5% glycerol, protease inhibitors (Sigma) and phosphatase inhibitors (GBiosciences). Samples were left on ice for 30 min prior to centrifugation at 10,000 rpms at 4˚C for 30 min. Total protein was measured using a BCA Protein Assay Kit (Thermo Scientific). Samples were heated at 95°C for 5 min in loading buffer (350 mM Tris, 30% glycerol, 1.6% SDS, 1.2% bromophenol blue, 10% β-mercaptoethanol), electrophoresed on a 12% sodium dodecyl sulfate gel and transferred onto a 2μm nitrocellulose membrane. The membrane was blocked with 5% milk for 1 h prior to overnight incubation with rabbit antibody to P2X4R (1:250, Alomone), mouse antibody to β-actin (1:2,000, Sigma-Aldrich), rabbit antibody to p38-MAPK (1:1,000, Cell Signalling), rabbit antibody to p-p38-MAPK (1:500, Cell Signalling), rabbit antibody to BDNF (1:500, Abcam), rabbit antibody to IRF5 (1:500, Abcam), rabbit antibody to SNAP-23 (1:1,000, Abcam) or rabbit antibody to VAMP-3 (1:1000, Abcam). Membranes were washed with TBST and incubated for 1 h at room temperature in anti-mouse and anti-rabbit fluorophore-conjugated secondary antibodies (1:5,000, Mandel Scientific). Membranes were imaged using the LICOR Odyssey CLx Infrared imaging system and the band intensity was quantified using ImageJ.

2.4 P2X4R cell surface biotinylation

Primary microglia in culture were plated onto 100 mm dishes prior to cell surface biotinylation. Adherent cells were incubated with 1 mg/mL EZ-link Sulfo-NHS-SS-Biotin (Thermo Scientific) in HBSS on ice for 1 hour. The reaction was quenched by incubation with 100 mM glycine. Cell surface protein samples were normalized to total protein content and incubated with High Capacity Neutravidin Agarose Resin (Thermo Scientific) for 1.5 hours at 4˚C with rotation. Beads were washed and suspended in loading buffer. Surface P2X4R protein levels were measured by western blotting.

2.5 Detection of BDNF release from cultured microglia by enzyme-linked immunosorbent assay (ELISA)

Male or female-derived microglia were treated with ATP (50 μM, Sigma) or PBS for 1 hour. Samples were centrifuged at 3000 rpms for 10 min at 4°C to pellet cells. The cell supernatant was transferred to a 10 kDa micron centrifugal filter unit (Millipore) and centrifuged at 13000xg for 15 min at 4°C. Measurement of BDNF in the supernatant was performed using a total BDNF ELISA kit (R&D Systems). Samples were considered BDNF positive if their signal was higher than the background signal and within the range of the standard curve. Each assay was run in the presence of PBS and ATP-only internal control groups.

2.6 Calcium imaging

Primary microglia in culture or adult isolated mixed glia were prepared as described above and plated on coverslips. Cells were incubated for 30 min with the fluorescent Ca^{2+} indicator dye Fura-2 AM (2.5 μM, Molecular Probes) in extracellular solution (ECS) containing 140 mM NaCl, 5.4 mM KCl, 1.3 mM $CaCl_2$, 10 mM HEPES and 33 mM glucose (pH

7.35). Following fluorophore loading, cells were washed with ECS lacking Fura-2 AM. To block P2X4R, 5-BDBD (10μM) in ECS was bath applied following Fura-2 AM loading. Cells were incubated in ECS or ECS containing 5-BDBD at room temperature for 30 min prior to ATP stimulation (50 μM). All experiments were conducted using an inverted microscope (Nikon Eclipse Ti C1SI Spectral Confocal), and the fluorescence of individual microglia was recorded using EasyRatioPro software (PTI). Excitation light was generated from a xenon arc lamp and passed alternatingly through 340- or 380-nm band-pass filters (Omega Optical). The 340/380 fluorescence ratio was calculated after baseline subtraction.

2.7 Flow cytometry

Mixed glia culture was isolated from the spinal dorsal horn of adult rats as described above. Cells were stained with fluorophore-conjugated CD11b/c–PE (1:500, eBioscience) and rabbit antibody to extracellular P2X4R (1:250, Alomone) preincubated with fluorophore-conjugated anti-rabbit secondary antibody. Cell fluorescence was measured by an Attune Acoustic Focusing Cytometer (Applied Biosystems). Live single-cell population was gated using forward and side scatter plots. CD11b- and P2X4R-positive staining were gated using BL2 and RL1 intensities, respectively, in single stained cells compared to unstained cells.

2.8 Spared nerve injury model

Under anesthesia, an incision was made on the left thigh through the skin and bicep femoris muscle to expose the sciatic nerve and its three terminal branches (sural, common peroneal and tibial nerves). The common peroneal and tibial nerves were tightly ligated and transected, while the sural nerve was left intact. The muscle and skin were sutured and the rat was left undisturbed to recover from anaesthesia. As control, sham surgery was performed without ligation and transection of the nerves.

2.9 Microglia Transfer Experiments

2.91 Intrathecal injection of microglia

Male- or female-derived primary microglia cultures were prepared as described above. In preparation for the microglia transfer experiments, microglia in culture were rinsed with PBS, harvested using a cell scraper and transferred into eppendorf tubes (see Ferrini et al., 2013).[74] Cells were counted using a haemocytometer and adjusted to a final concentration of 100 cells/μL. Primary microglia were treated with ATP (50 μM) or PBS as a control, incubated at 37˚C for 1 hour, and then 10 μL of this preparation was injected via lumbar puncture in anesthetized naïve male or female rats. Mechanical paw withdrawal threshold was assessed using von Frey filaments before injection (baseline), and after 30, 60, 90, 120, 150, and 180 min.

2.92 Mechanical paw withdrawal threshold (von Frey Filament test)

Rats were placed in a transparent Plexiglas chamber with an opaque flat-surfaced platform that contained holes in a grid pattern. A series of von Frey filaments of incremental forces (0.6-26.0 g) were applied to the plantar surface of the hind paw through the holes using a modified up-down method.[97] Each hind paw was assessed alternatively with the filaments. A positive response to a filament evoked a paw withdrawal response, which involved lifting, flinching and/or licking of the paw. When a positive response to a filament was observed, the next lower filament was applied four times. If a positive response occurred at least once in response to the lower filament, the force of that filament was recorded as the paw withdrawal threshold. Data is represented as an average of the paw withdrawal thresholds of the left and right hind paws.

2.10 Suppression of mRNA expression with siRNA

Primary microglia cultures were transfected with short-interfering RNA (siRNA) targeted to VAMP-3, SNAP-23, IRF5 or non-targeted siRNA (control) that does not share sequence homology to any known mammalian gene. In brief, Lipofectamine reagent 2000 (Sigma) and siRNA (30nM) were diluted in 100 μL Optimem media, incubated for 10 min, and added to microglia cultures containing serum/antibiotic free media for 6 hours. Cells were harvested and knockdown efficiency was assessed by western blot.

2.11 Chromatin Immunoprecipitation

Chromatin immunoprecipitation (IP) was performed using the SimpleChIP Enzymatic Chromatin IP Kit (Cell Signaling Technology) according to the manufacturers instructions with rabbit antibody to IRF5 (1:150, Abcam) and normal rabbit IgG (Cell Signaling). ChIP signals were quantified with qPCR using a real-time PCR system (Applied Biosystems). Values obtained for immunoprecipitated samples (% Input DNA) were normalized to their respective 2% input samples. Primer pairs for the *P2rx4* promoter region are as follows:

Forward Primer: TTCCTCTCAGGTTTCCGACA

Reverse Primer: GCCCTGTGCAGTATAAATCTAGC

2.12 Statistics

All data are shown as mean ± standard error of the mean (SEM). Tests of statistical differences were performed with GraphPad Prism 6 software using an unpaired t-test, one-way analysis of variance (ANOVA) (Sidak's post-hoc test), two-way ANOVA (Sidak's or Tukey's post-hoc test) or two way repeated measures ANOVA (Sidak's post-hoc test).

CHAPTER 3: RESULTS AND FIGURES

3.1 P2X4R-mediated BDNF signalling and release is sex-dependent and SNARE-mediated.

P2X4R activation is initiated by ATP. I therefore investigated the cellular mechanisms underlying P2X4R-signalling by treating primary microglia cultures from male or female postnatal rats with ATP (50 μM) or PBS as a control for 1 hour. This concentration of ATP has been shown to preferentially activate P2X4Rs as opposed to P2X7Rs.[54] I found that ATP-stimulation increased total P2X4R protein (**Fig. 3.1a**), cell-surface P2X4R (**Fig. 3.1b**) and *P2rx4* mRNA levels **(Fig. 3.1c)** in primary microglia. However, this occurred by a sex-dependent mechanism specific to cultures isolated from male postnatal rats, since no significant differences were observed between control and treatment groups in females. I next assessed P2X4R-p38-BDNF pathway activation in primary microglia cultures. Application of ATP increased expression of phospho-p38-MAPK (**Fig. 3.2a**), the active form of the kinase in males but not females, whilst total levels of p38-MAPK remained unchanged in both sexes (**Fig. 3.2b**). Furthermore, treatment with ATP evoked a male-specific increase in both the release of BDNF (**Fig. 3.2c**) and its synthesis (**Fig. 3.2d**). Together, these findings indicate that a sex-dependent mechanism underlies ATP-induced P2X4R-p38-MAPK-BDNF signalling in primary-derived microglia cultures.

I next examined whether differences in P2X4R function on microglia could account for the dimorphisms underlying P2X4R-signalling. In primary microglia cultures, I assessed baseline cation channel function using Ca^{2+} imaging and found that ATP (50 μM) stimulation evoked transient Ca^{2+} responses; these responses were not different between male and female-derived microglia. Calcium influx was abolished by pre-treatment with selective P2X4R antagonist, 5-

BDBD (10 μM) **(Fig. 3.3a),** suggesting that P2X4R-mediated Ca^{2+} responses are intact in both sexes. Since my previous findings show that application of ATP causes sex-dependent upregulation of P2X4Rs, I next investigated whether calcium influx was different between males and females following a 1 hour incubation with ATP (50 μM). Upon a second stimulation, Ca^{2+} responses were significantly higher in males when compared to females (**Fig. 3.3b**), indicating that a male-specific upregulation of P2X4Rs following ATP-stimulation corresponds to increased receptor function.

Sufficiency of P2X4R stimulation on microglia for the development of allodynia has been previously shown using 'adoptive transfer experiments.' Mechanical hypersensitivity developed in naïve rats following intrathecal administration of ATP-stimulated primary microglia, whereas injection of PBS-treated controls or ATP alone did not result in pain hypersensitivity.[54] However, these experiments were done using mixed microglia cultures isolated from both male and female postnatal rats. Here, I asked whether transfer of microglia derived from male vs. female rats stimulated with ATP (50 μM) was sufficient to cause allodynia. I found that mechanical hypersensitivity developed over the course of 3 hours in male (**Fig. 3.4a)** and female (**Fig. 3.4b)** naïve rats following administration of stimulated male-derived primary cultures. In contrast, injection of female-derived microglia had no effects on mechanical thresholds in males (**Fig. 3.4c**) and females (**Fig. 3.4d**). These findings further demonstrate that sexual dimorphisms underlie ATP-evoked BDNF release from microglia, and therefore, only transfer of male-derived microglia stimulated with ATP was sufficient to produce allodynia.

In neurons, vesicular release of BDNF is SNARE-mediated.[85] In microglia, the core presynaptic machinery regulating this release has yet to be determined. I first characterized the basal expression levels of SNAREs in primary microglial cultures by analyzing online

microarray data collected from Zawadzka et al., (2012).[98] Based on mRNA levels, primary microglia possess a full complement of synaptobrevins (**Fig. 3.5a**), syntaxins (**Fig. 3.5b**) and miscellaneous SNARE machinery (**Fig. 3.5c**). I further confirmed the protein expression of several t- and v-SNAREs in BV2 and primary microglia cultures using western blotting techniques (**Fig. 3.5d**). From these analyses, I narrowed down VAMP-3 and SNAP-23 as potential targets for vesicular release from microglia, and determined that homologues VAMP-2, VAMP-1 and SNAP-25 are likely unfit targets because of their comparably low expression levels *in vitro* (**Fig. 3.5d**).

I further investigated the role of SNAPs and VAMPs in vesicular release of BDNF by targeting subsets of SNAREs using botulinum toxins (BoNTs). I pre-treated primary-derived microglia cultures with BoNT-A and BoNT-B (100 pM), which target SNAPs and VAMPs respectively,[99] and found that ATP-evoked BDNF release was attenuated following pre-treatment only with BoNT-B (**Fig. 3.6a**). This experiment identified VAMP-3 as a potential target for BDNF release whilst indicating that SNAP-25 is likely not involved in regulated release of BDNF from microglia. I then used siRNAs to knockdown SNARE expression prior to stimulation with ATP (50 μM) or PBS as a control and found that ATP-evoked BDNF release from microglia was attenuated following knockdown of VAMP-3 (**Fig. 3.6b**) and SNAP-23 (**Fig. 3.6c**). From these experiments, I pinpointed specific subsets of t-SNAREs and v-SNAREs as core components of the trans-SNARE complex necessary for BDNF release from microglia.

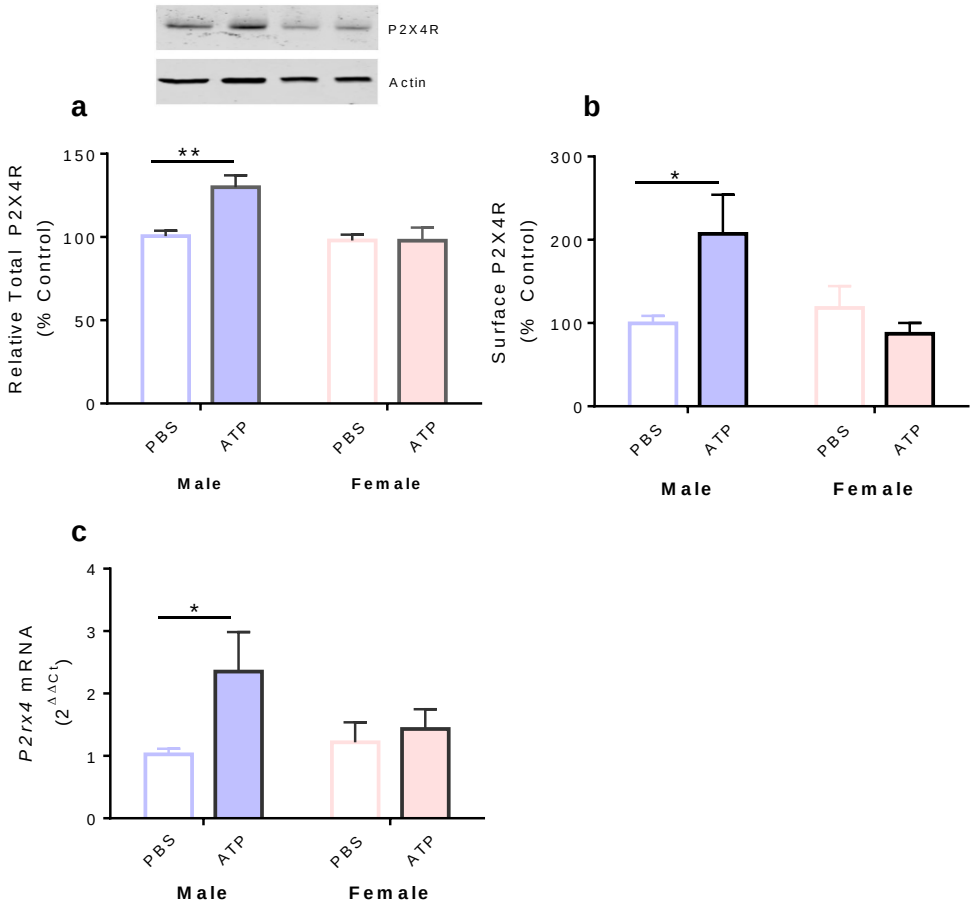

Figure 3.1

Primary microglia cultures isolated from male or female postnatal rats were stimulated with ATP (50 μM) or PBS (control) for 1 hour. Total and cell-surface levels of P2X4R were measured by western blot. mRNA levels were measured using qPCR. Quantification of **a)** total P2X4R protein levels (*n* =7-9 per treatment /sex) **b)** cell surface biotinylation of P2X4R (*n* =6-7 per treatment /sex) and **c)** mRNA expression levels of *P2rx4* (*n* =8-9 per treatment /sex) in primary microglia. Data are normalized to male PBS. Two-way ANOVA and Sidak's *post-hoc* test. *p<0.05, **p<0.01

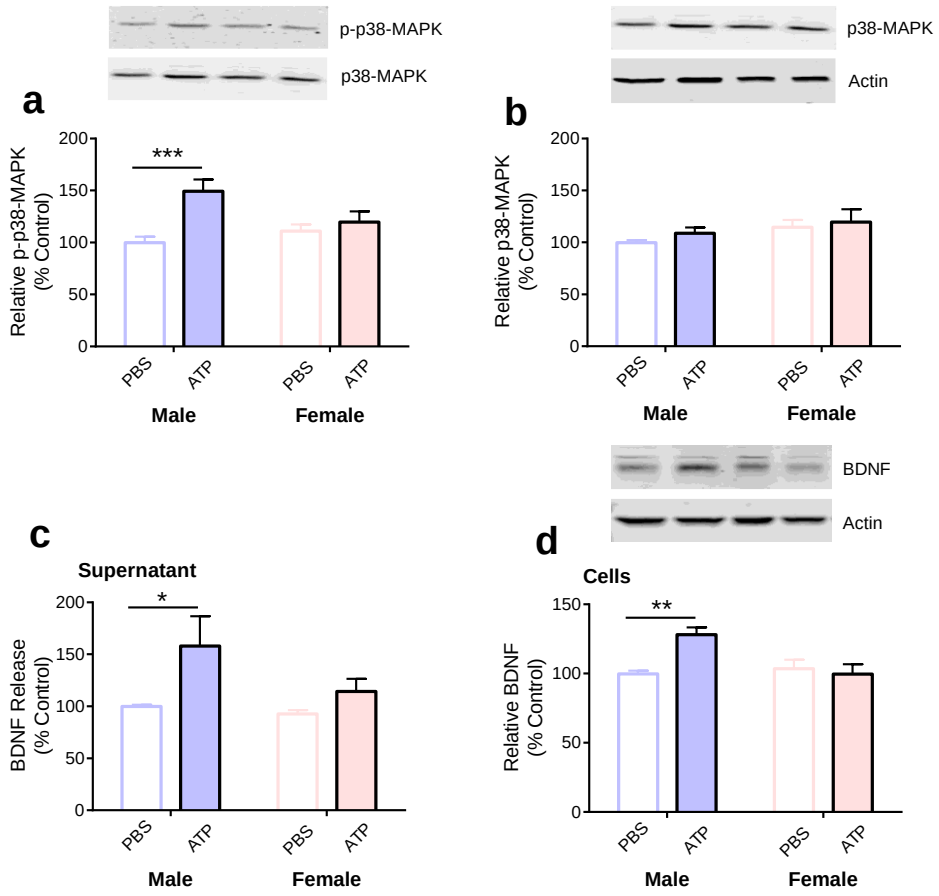

Figure 3.2

Primary microglia cultures isolated from male or female postnatal rats were stimulated with ATP (50 μM) or PBS (control) for 1 hour. Protein levels were measured by western blot. Quantification of **a)** phospho-p38-MAPK (*n* =6-7 per treatment /sex) **b)** total p38-MAPK (*n* =6-7 per treatment/sex) **c)** ELISA-based measurement of BDNF release (n=6-8 per treatment/sex) and **c)** total BDNF (*n* =7-9 per treatment group/sex) in primary microglia. Data are normalized to male PBS. Two-way ANOVA and Sidak's *post-hoc* test. *p<0.05, **p<0.01, ***p<0.001

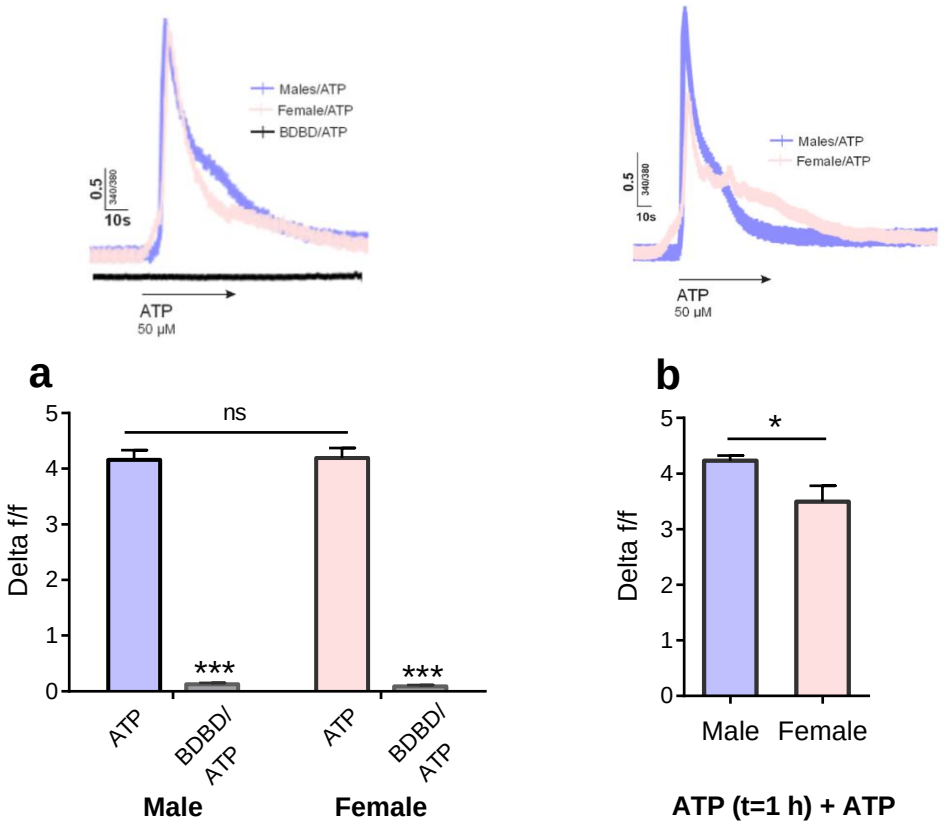

Figure 3.3

Primary microglia cultures isolated from male or female postnatal rats were pre-treated with PBS, 5-BDBD (10 µM) or ATP (50 µM) prior to measuring ATP-evoked calcium responses by Ca^{2+} imaging. **a)** Quantification of peak rise intracellular Ca^{2+} levels evoked by ATP in primary microglia cultures (*n* =31-37 cells per treatment group/sex). Ca^{2+} rises were blocked by pre-treatment with selective P2X4R antagonist, 5-BDBD (10 µM) for 30 min (*n* =6-10 cells/sex). Two-way ANOVA Tukey's *post-hoc* test. **b)** Quantification of peak rise intracellular Ca^{2+} levels evoked by ATP following 1 hour of ATP pre-treatment in primary microglia (*n* =8-12 cells/sex). Unpaired t-test. *p<0.05, ***p<0.001, ns- no significance

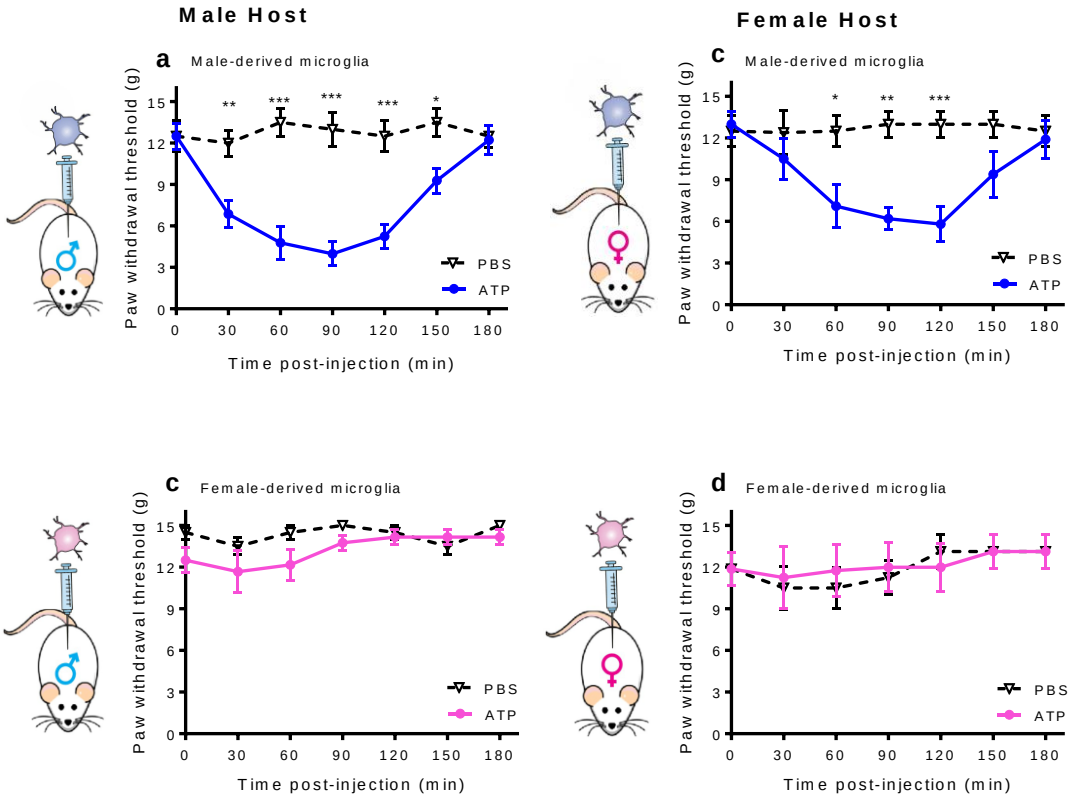

Figure 3.4

Primary microglia isolated from male or female postnatal rats were stimulated with ATP (50 μM) or PBS for 60 min prior to intrathecal administration. Mechanical paw withdrawal thresholds were measured at 30 minute intervals post-injection. Mechanical withdrawal thresholds of **a)** male rats (*n*=5-7 per treatment group/sex) and **b)** female rats (*n*=5 per treatment group/sex) following administration of male-derived primary microglia. Mechanical withdrawal thresholds of **c)** male rats (*n*=5-6 per treatment group/sex) and **d)** female rats (*n*=4 per treatment group/sex) following administration of female-derived primary microglia. Two-way repeated measures ANOVA with Sidak's *post-hoc* test. *p<0.05, **p<0.01, ***p<0.001.

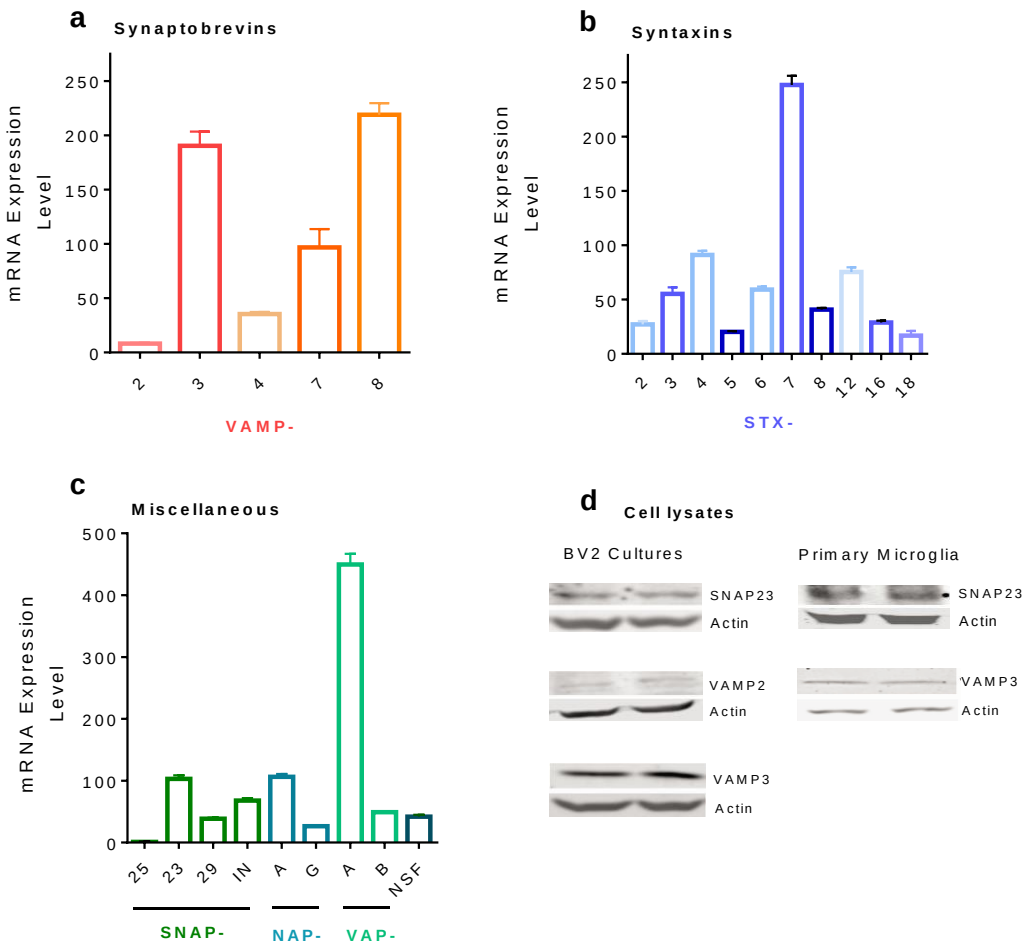

Figure 3.5

Microarray data from primary microglia in culture was used to determine the gene expression profile of several known mammalian SNARE proteins. Quantification of mRNA Expression levels of **a)** VAMP proteins **b)** Syntaxins and **c)** miscellaneous SNARE proteins including SNAPs, N-ethylmaleimide sensitive fusion protein (NSF), NSF-associated proteins (NAPs) and VAMP-associated proteins (VAPs). Samples were normalized to their respective housekeeping gene (actin) and averaged among 5 biological replicates. **d)** Presence or absence of SNARE proteins were confirmed by western blot in BV2 and primary isolated microglia cultures.

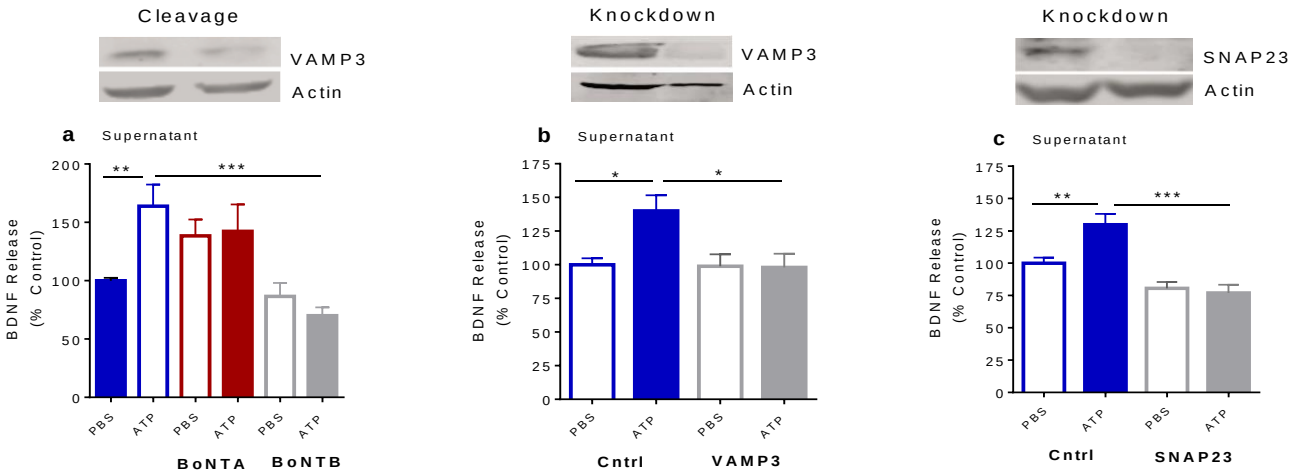

Figure 3.6

Subsets of SNAREs in primary microglia were targeted using botulinum toxins or selective siRNAs. BDNF levels in the supernatant was measured by BDNF ELISA and SNARE knockdown was confirmed by western blot. Quantification of BDNF release following treatment with **a)** BoNTA or BoNTB (100 pM) (*n*=3-9 replicates/treatment) **b)** VAMP-3 targeting siRNA (30 nM) (*n*=4-6 replicates/treatment) or **c)** SNAP-23 targeting siRNA (30 nM) (*n*=4-7 replicates/treatment). Data are normalized to control PBS. One-way ANOVA and Sidak's *post-hoc* test. *p<0.05, **p<0.01, ***p<0.001

3.2 P2X4R expression and activity are increased on spinal microglia following PNI only in male rats.

Having formed a basis for dimorphic P2X4R-signalling in primary microglia cultures, I next examined the cellular consequences of P2X4R-mediated pain processing in rats. It has been previously shown that PNI causes a sex-dependent increase in spinal *P2rx4* gene expression.[92] However, the cell type-specificity of this upregulation, and whether this translated to protein expression, had not previously been examined. I measured changes in P2X4R levels using flow cytometry on cells isolated from the spinal dorsal horn 7 days following sham or spared nerve injury (SNI) surgeries. Using fluorophore-conjugated antibodies to CD11b and P2X4R, I quantified mean fluorescence intensities within two discrete populations of cells: CD11b-positive (microglia) and CD11b-negative (neurons and astrocytes) (**Fig. 3.7a-d**). I found that P2X4R labeling was significantly increased in CD11b-positive cells from male but not female rats following nerve injury **(Fig. 3.7e).** In the CD11b-negative population, there were no significant differences in mean P2X4R expression between sham and nerve-injured animals within sex groups (**Fig. 3.7f**). As a correlate for reactive microgliosis, I quantified CD11b staining within the microglial population and found that CD11b expression was increased following PNI in both sexes. Furthermore, there were no significant differences in CD11b staining between nerve-injured male and female groups (**Fig. 3.7g**). These data provide evidence that despite comparable levels of microgliosis between sexes, P2X4Rs are upregulated on spinal microglia isolated from male but not female rats following PNI.

I next tested whether nerve injury impacts P2X4R function on spinal dorsal microglia isolated from male and female rats. Using calcium imaging, I measured ATP-evoked calcium responses as a correlate for P2X4R function. I found that ATP-stimulation induced a rise in

intracellular calcium levels, and this response was potentiated following nerve injury in males (**Fig. 3.8a**) but not females (**Fig. 3.8b**). Transient responses were blocked by pre-treatment with selective P2X4R antagonist, 5-BDBD (10 μM), demonstrating that the calcium influx was dependent on P2X4Rs. Collectively, these findings indicate that nerve injury causes a sex-dependent increase in P2X4R expression and function on spinal microglia.

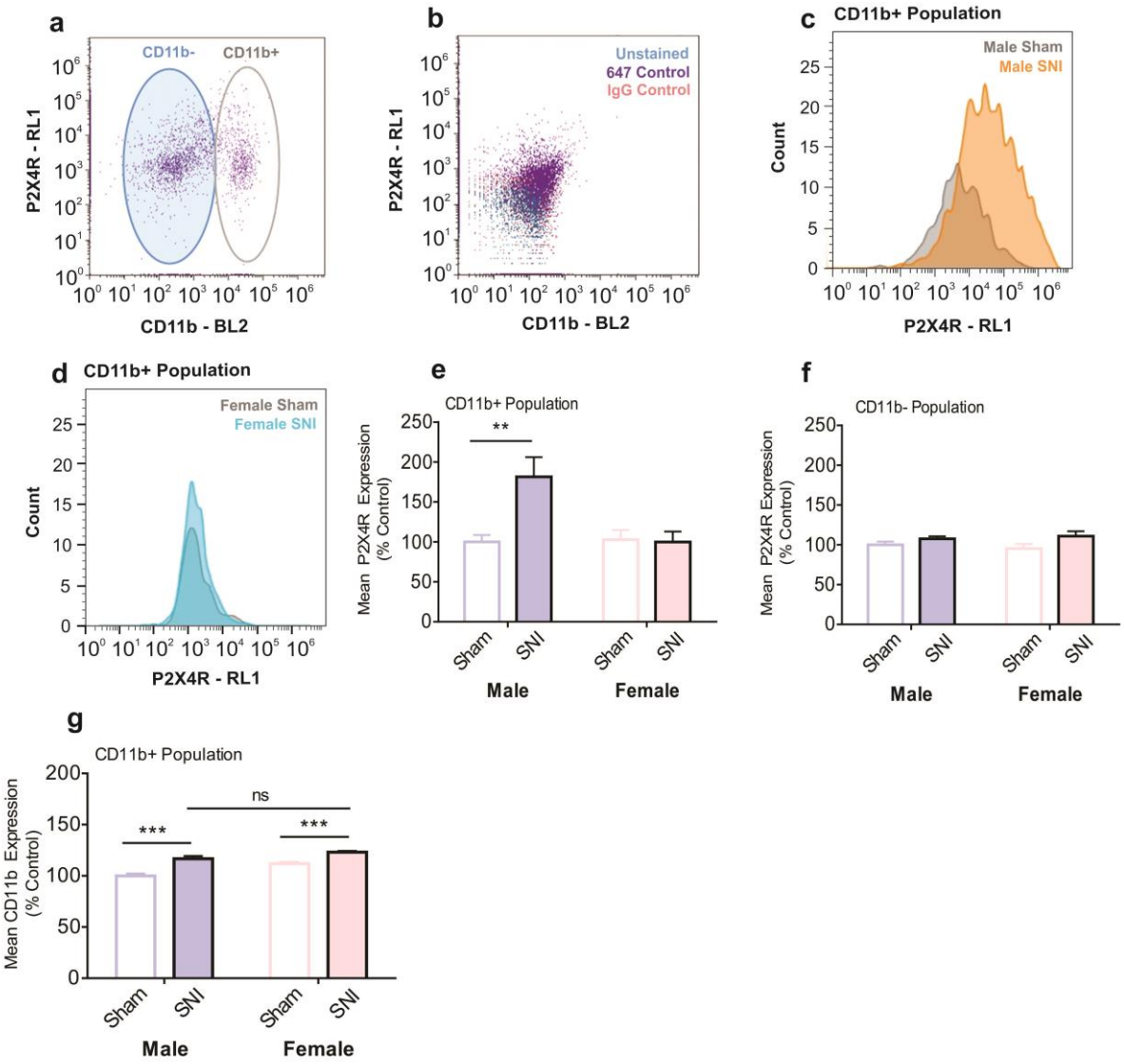

Figure 3.7

Adult spinal microglia were acutely isolated from the dorsal horn of male rats (*n* =7/group) and female rats (*n* =8/group) 7 days following sham or SNI surgeries. Cells were stained with fluorescent-conjugated antibodies to CD11b and P2X4R, and imaged using flow cytometry. **a)** Representative dot plot of adult isolated spinal cord lysates labeled for CD11b (BL2 area, x-axis) and P2X4R (RL1 area, y-axis) with gates around the CD11b+ population (microglia) and CD11b- population (neurons and astrocytes). **b)** Representative dot plot of overlapping

unstained, anti-rabbit IgG Fab2 647 (RL1) and anti-rat IgG2b κ Isotype controls (PE). Representative histograms of P2X4R fluorescence (RL1) in the CD11b positive population of a **c)** male sham vs. SNI rat or **d)** female sham vs. SNI rat. Quantification of mean P2X4R fluorescence in the **e)** CD11b+ population of cells (*n*=7-9 replicates per treatment group/sex) or **f)** CD11b- population of cells (*n*=7-9 replicates per treatment group/sex). **g)** Quantification of mean CD11b fluorescence in the CD11b+ population of cells (*n*=7-9 replicates per treatment group/sex). Data are normalized to male sham. Two-way ANOVA and Sidak's *post-hoc* test. **p<0.001, ***p<0.0001, ns- no significance

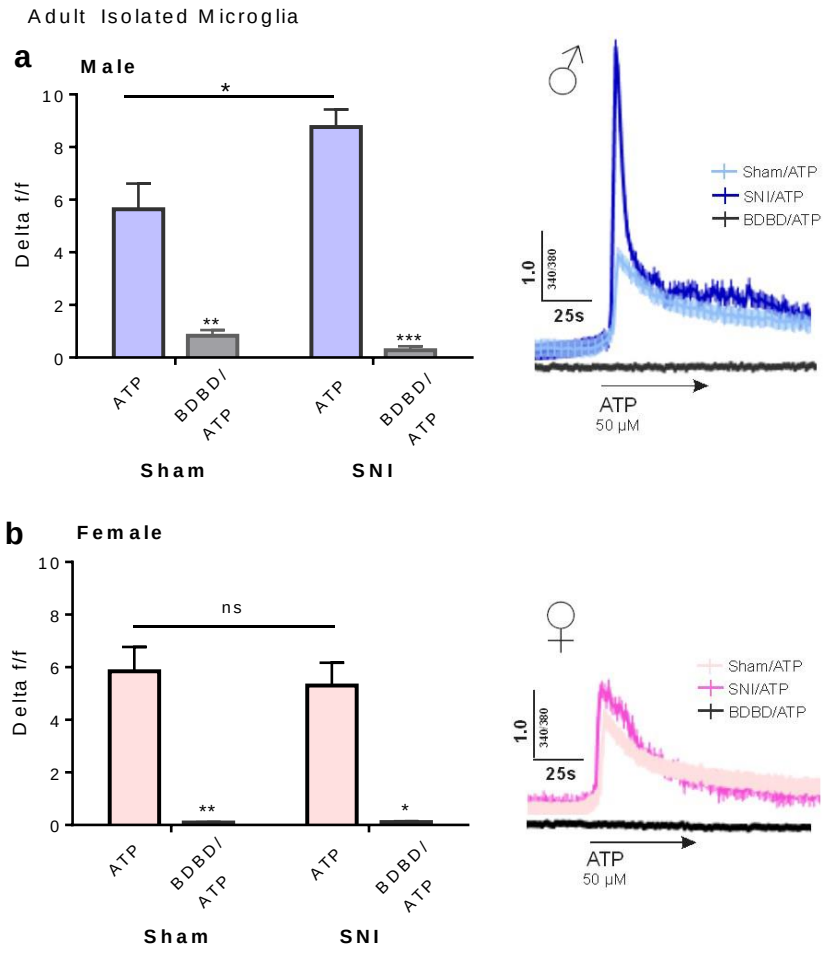

Figure 3.8

Adult spinal microglia were acutely isolated from the dorsal horn of male rats (*n* = 4/group) and female rats (*n*= 4/group) 7 days following surgery. ATP-evoked calcium responses were measured by calcium imaging. Quantification and representative traces of single-cell responses from microglia isolated from **a)** male rats (*n*=16 cells/treatment group) or **b)** female rats (*n*=8-16 cells/treatment group). Pre-treatment with 5-BDBD (10 µM) for 30 min blocked ATP-evoked rises in intracellular calcium in both sexes. (*n*=4-9 cells per treatment group/sex). Two-way ANOVA and Tukey's *post-hoc* test. *p<0.05, **p<0.01, ***p<0.001

3.3 Transcription factor IRF5 differentially regulates P2X4R expression in male and female rats.

P2rx4 mRNA levels are increased only in male-derived primary microglia cultures following ATP-stimulation, and IRF5 is a known regulator of *P2rx4* gene expression[68] (**Fig. 3.1c**). To elucidate the mechanisms underlying dimorphic P2X4R upregulation, I first characterized the role of transcription factor IRF5 in receptor expression. I treated male or female-derived microglia with IRF5-targetted or control siRNAs **(Fig. 3.9a)** prior to application of ATP (50 μM) or PBS as a control and measured P2X4R expression levels using western blot. P2X4R was upregulated following stimulation in male but not female-derived primary microglia treated with control siRNA **(Fig. 3.9b)** and this effect was blocked by interfering with IRF5 **(Fig. 3.9c)**. These data show that IRF5 is necessary for the male-specific upregulation of P2X4Rs in primary cultures.

I next tested whether IRF5 differentially regulates the promoter region of *P2rx4* in males and females. I first identified IRF binding motifs upstream of the *P2rx4* start codon using Genomatix MatInspector Software (**Fig. 3.10a**). I pulled down IRF5-bound DNA fragments in primary microglia cultures treated with ATP (50 μM) and amplified *P2rx4* gene loci using qPCR. I found that enrichment of *P2rx4* by IRF5 was 4 times greater than that of the non-specific *Gapdh* promoter in microglia isolated from male and female postnatal rats **(Fig. 3.10b)**. Interestingly, IRF5 has a significantly higher binding affinity at the *P2rx4* promoter region of males as compared to females, suggesting that a dimorphism in the transcriptional regulation of the *P2x4r* gene may underlie its sex-specific upregulation in cells.

I next investigated the regulation of P2X4R in the rat spinal cord following nerve injury. Despite an upregulation of spinal IRF5 protein in both sexes (**Fig. 3.11a**), IRF5 binds to the *P2rx4* promoter region at a significantly greater affinity in males than females following PNI (**Fig. 3.11b**), suggesting that differences in transcriptional regulation of *P2rx4* may underlie the male-specific increase in spinal P2X4R.

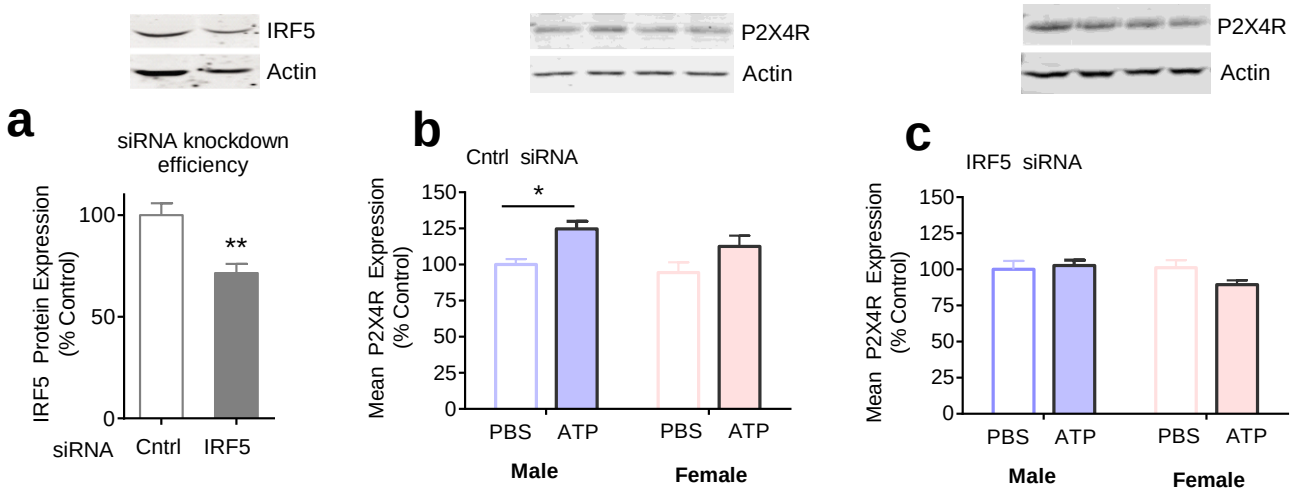

Figure 3.9

Primary cultures were isolated from male or female postnatal rodents. P2X4R protein levels were measured by western blot following siRNA knockdown with control or IRF5-targetted siRNAs. **a)** IRF5 protein was knocked down in cultures by approximately 30% following siRNA treatment. Quantification of P2X4R protein levels in primary microglia cultures treated with 50 nM of **b)** non-targeted or **c)** IRF5-targeted siRNA prior to treatment with ATP (30 μM) or PBS (control) for 1 hour (*n*=7-8 per treatment/sex). Data are normalized to male PBS. Two-way repeated measures ANOVA with Sidak's *post-hoc* test. *p<0.05

a

Genomatix MatInspector Data			
i) Strand	**ii) Binding location**	**iii) Matrix Similarity**	**iv) Sequence**
(+)	424-448	0.889	agatttggaaaaatGAAAagaactg
(-)	749-773	0.948	gcctctgagaaaagGAAAcagccag

b

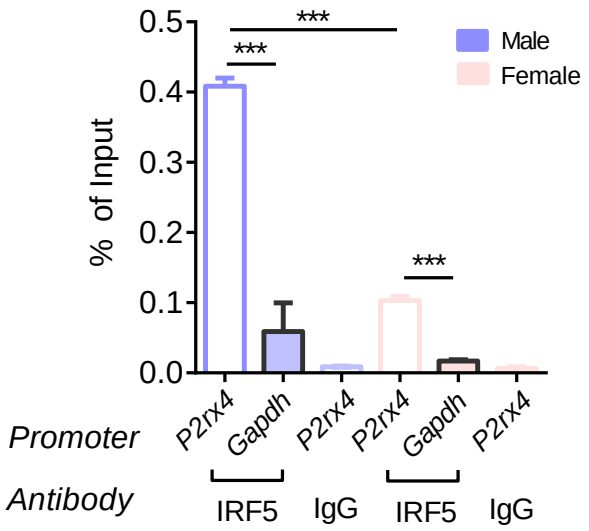

Figure 3.10

Primary microglia cultures isolated from male or female postnatal rats were stimulated with ATP (50 μM) for 1 hour. **a)** Putative IRF5 binding sites on the rat *P2rx4* promoter region were identified using Genomatic MatInspector Software. Data table specifies the i) strand sense (sense=(+), antisense=(-)) ii) binding location upstream of the start codon iii) matrix similarity and iv) binding site sequence. **b)** Quantification of (ChIP)-qPCR data analyzed using the % input method. DNA was pulled down in male- or female-derived primary cultures using rabbit antibody to IRF5 or non-specific IgG. Primers were specific to *P2rx4* or *Gapdh* promoter regions. Data are presented as average of *n*=2 technical replicates. One-way ANOVA with Sidak's *post-hoc* test. ***p<0.001

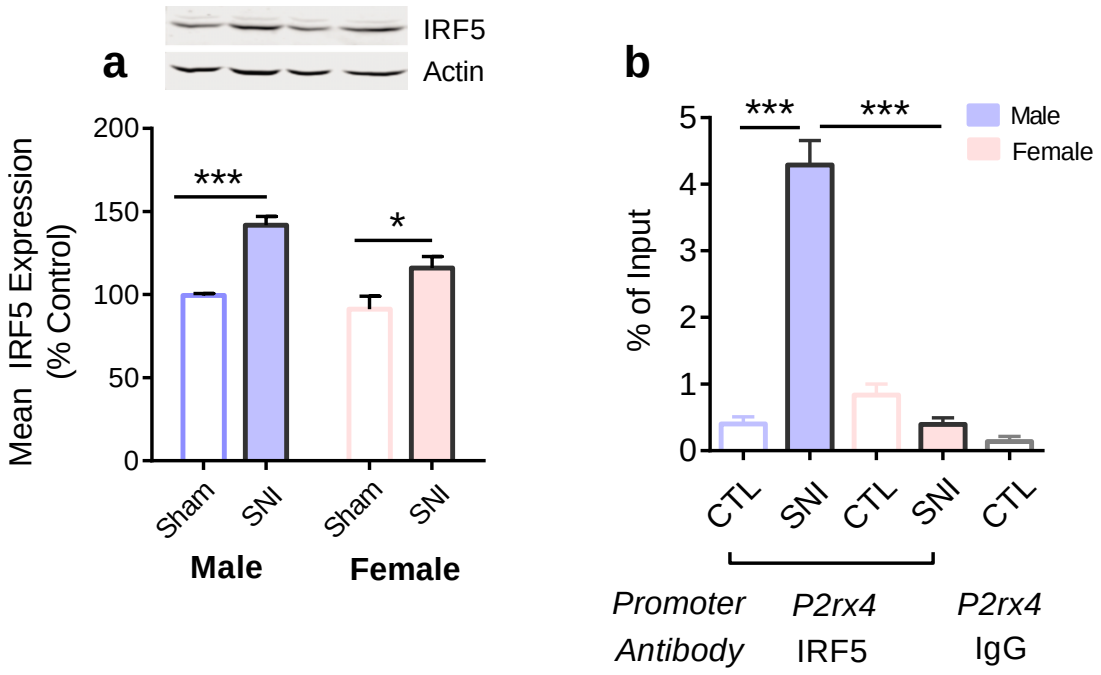

Figure 3.11

Adult spinal microglia were acutely isolated from the spinal cord of naïve male and female rats or 7 days following sham or SNI surgeries. **a)** Quantification of western blot analysis of total IRF5 in spinal lysates isolated from the dorsal horn (*n*=7 per group/sex). Data are normalized to male sham. Two-way ANOVA and Sidak's *post-hoc* test. **b)** Adult spinal cells were isolated from the lumbar region of naïve (CTL) or SNI rats. IRF5-binding affinities at the *P2rx4* promoter were measured using (ChIP)-qPCR data and analyzed by the % input method (*n*=2 technical replicates from *n*=5 rats per group/sex). One-way ANOVA and Sidak's *post-hoc* test. *p<0.05, ***p<0.001

CHAPTER 4: DISCUSSION

P2X4R-mediated release of BDNF from microglia is a core mechanism implicated in mechanical allodynia following PNI. The realization that this pathway is differentially required for mechanical allodynia in male, but not female rodents, has shifted the focus toward sex-dependent mechanisms involved in the development of neuropathic pain.[92] In the present study, I examined the detailed steps controlling P2X4R-mediated release of BDNF from microglia, and identified key cellular points of sexual divergence. In primary microglia cultures, I showed that P2X4R-evoked BDNF release occurs in male, but not female, derived microglia. Specifically, in male-derived microglia, ATP-stimulation upregulated P2X4Rs leading to p38-MAPK activation and SNARE-dependent release of BDNF. Intrathecal administration of P2X4R-stimulated male-derived microglia induced mechanical allodynia in naïve rats, an effect not seen following intrathecal injection of P2X4R-stimulated female derived microglia. Thus, microglia from male, but not female postnatal rats, are sufficient to induce mechanical allodynia.

In my second Aim, I confirmed these findings in a model of neuropathic pain by showing that even though nerve injury induces microgliosis in both sexes, P2X4R expression and function are increased on spinal microglia isolated from only male rats. Based on these findings, dimorphic P2X4R upregulation is a primary point of divergence between sexes.

Finally, in Aim 3, I elucidated the cellular underpinnings of P2X4R upregulation. I showed that *P2rx4* gene expression is differentially mediated by transcription factor IRF5 in male and female rats, providing a mechanism that accounts for the discrepancies in receptor upregulation. Together, these findings pinpoint the P2X4R-evoked BDNF release pathway as a key point of sexual divergence in pain hypersensitivity. For this discussion, I will describe how

these findings fit into the scope of the field. I will also address the limitations of this study, and propose further experiments to answer questions raised by my results.

4.1 P2X4R upregulation on microglia is sexually dimorphic

The first lines of evidence implicating P2X4Rs in PNI-induced tactile allodynia were reported by Tsuda et al., (2003).[54] They showed that pharmacological blockade of spinal P2X4Rs reversed allodynia, and intrathecal transfer of primary microglia in which P2X4Rs had been stimulated lowered mechanical thresholds, demonstrating both the necessity and sufficiency of these receptors for pain hypersensitivity.[54] It has recently become apparent that microglia-neuronal signalling underlies pain hypersensitivity only in males.[59] Inhibition of spinal P2X4Rs abolishes pain behaviours in male but not female mice, suggesting that P2X4R-signalling is mechanistically different between sexes. In this study, I analyzed cellular changes in P2X4R expression and function, as well as the upstream and downstream consequences of P2X4R activation in microglia. ATP is an endogenous ligand for the P2X4R, and serves as an important signalling molecule between neurons and microglia.[31] In response to PNI, ATP release is significantly increased in the spinal dorsal horn, and elevated levels of extracellular ATP are critical for the development of neuropathic pain.[100] Until recently, the cell type responsible for releasing ATP into the spinal horn was unclear. There is now evidence that nerve-injury induced allodynia relies on the expression of a vesicular nucleotide transporter (VNUT) in dorsal horn neurons.[100] This finding, alongside several others, implicate spinal ATP as a crucial substrate for neuropathic pain, the effects through which are mediated by P2X receptors on microglia.[45,54] My project focuses on deriving the mechanisms of how ATP modulates P2X4R expression, activity and signalling in microglia to better understand the aetiology of neuropathic pain.

Several molecules released into the spinal dorsal horn following nerve injury are capable of upregulating P2X4Rs on microglia. These include extracellular bioactive factors such as fibronectin,[101] INF-γ[60] and PAR2.[61] While ATP plays an important role in mediating microglial reactivity in the spinal dorsal horn,[45] whether this substrate could also cause P2X4R upregulation was previously unknown. I found that direct stimulation of primary microglia cultures with ATP induced a significant increase in P2X4R expression. An unexpected finding in this present study, however, was that this upregulation was specific to microglia isolated from male postnatal rats. Stimulation with ATP caused an increase in total P2X4R protein, surface P2X4R and *P2rx4* mRNA in males, whereas no such alterations were observed in females. In addition to total P2X4R levels, I found that application of ATP increased P2X4Rs on the plasma membrane of male- but not female-derived microglia. This effect has been shown previously using extracellular signalling molecules CCL2,[102] CCL12[102] and lipopolysaccharides (LPS),[103] although no sex-dependent mechanisms were reported. Aside from evidence showing that P2X4Rs are localized to lysosomes in microglia,[104] and that treatment with ionomycin induces P2X4R exocytosis,[104] the cellular mechanisms regulating P2X4R trafficking remain enigmatic. Further experiments to elucidate whether increased levels of P2X4Rs on the cell-surface has implications for receptor function, or how endosomal compartments are trafficked within microglia, may have broader applications outside of P2X4Rs and pain.

Having established that ATP-stimulation caused a sex-specific upregulation of P2X4Rs in primary culture, I next examined the impact of nerve injury on P2X4R expression and function in microglia isolated from the spinal dorsal horn of male and female rats. Other studies have shown that *P2rx4* gene expression is increased in the spinal dorsal horn following PNI,[59] but whether this translated to protein expression on microglia specifically was unclear. I used

flow cytometry to analyze P2X4R fluorescent intensity on CD11b-positive and CD11b-negative (neurons and astrocyte) populations of cells isolated from the spinal dorsal horn. Consistent with my results in primary cultures, I found that P2X4R expression and function was increased following nerve injury on spinal microglia derived only from male rats, whereas no changes in receptor expression were observed in the CD11b-negative population. By analyzing levels of CD11b staining within the microglial population, I provide further evidence that males and females display comparable levels of microgliosis in the spinal dorsal horn following PNI, which was previously described in mice.[59] Despite a lack of sex differences observed in nerve injury-induced microgliosis, these data suggest that dimorphic P2X4R upregulation on spinal microglia is a key point of sexual divergence in pain hypersensitivity. My analyses, however, do not rule out the possibility that discrete changes of P2X4R expression may be occurring within the CD11b-negative population. Staining with NeuN and glial fibrillary acidic protein (GFAP)-specific markers, to label neurons and astrocytes respectively, would more concisely show whether PNI affects P2X4R expression levels within these cell types, providing a more holistic view on how the CNS reacts to nerve injury.

4.2 The sex-dependent transcriptional regulation of P2rx4 by IRF5

A major conclusion from this study is that in response to ATP-stimulation or PNI, P2X4Rs are upregulated in male but not female rats. Given that *P2rx4* mRNA is increased only in males following ATP-stimulation, I next asked whether differences in regulation at the transcriptional level of *P2rx4* could explain these findings. In a microglia-like cell line (BV2s), it has been shown that fibronectin-mediated P2X4R upregulation relies on the transcription factor IRF5, which binds directly to the *P2rx4* promoter to regulate gene transcription.[68] I provide novel evidence that IRF5 binds to the promoter loci of *P2rx4* in rat-derived cells stimulated by

ATP. Furthermore, I showed that IRF5 expression in primary cultures is necessary for P2X4R upregulation in male-derived microglia. In the spinal cord, I observed that IRF5 binds with four-times greater affinity to the *P2rx4* promoter loci in nerve injured males compared to females, suggesting that IRF5 preferentially activates receptor transcription in males. How this transcription factor differentially modulates P2X4R upregulation between sexes is an important open question, the answer to which potentially underlies why sex differences occur in microglia-neuron pain signalling. The most parsimonious explanation is that IRF5 function is influenced by testosterone, driving *P2rx4* transcription in males but not females. Treating primary microglia with dihydrotestosterone, the endogenous agonist of the androgen receptor,[105] and measuring subsequent P2X4R levels following ATP-treatment would be the first step to answer this question. Alternatively, patterns of gene regulation could be influenced by epigenetic modifications at the *P2rx4* loci, promoting sex-dependent transcription factor binding through DNA methylation or histone modifications. Mechanisms underlying epigenetic modifications, an emerging area of research, are more difficult to decipher, but could involve measuring levels of methylation at the *P2xr4* promoter loci in males and females using bisulfite conversion.

4.3 Sexual dimorphisms underlie p38-MAPK-BDNF signalling

p38-MAPK activation is an important downstream signalling molecule of the P2X4R.[73] For my next lines of investigation, I asked whether dimorphic P2X4R upregulation has consequences for downstream p38-MAPK-BDNF signalling in primary microglia cultures. Mechanisms of p38-MAPK activation in microglia were first elucidated using mixed cultures isolated from male and female postnatal rats.[73] Levels of phosphorylated p38-MAPK were significantly increased upon stimulation with ATP, and these effects were blocked by either pre-treating cells with a P2X4R antagonist or when conducting experiments using calcium-free ECS,

indicating that p38-MAPK activation relies on P2X4R-mediated calcium influx.[73] Furthermore, extensive findings show that the synthesis and release of BDNF from microglia is mediated by p38-MAPK activation, providing a mechanistic explanation for how P2X4R stimulation leads to BDNF release from microglia. I investigated whether ATP-treatment evokes sex-dependent changes in key components of this pathway. Concomitant with a dimorphic increase in P2X4R expression, my findings show increased levels of phosphorylated p38-MAPK, intracellular BDNF and BDNF release only in male-derived microglia following application of ATP. I ruled out the possibility that these findings are caused by differences in baseline P2X4R function on microglia by showing that ATP-evoked calcium responses are comparable between males and females. In addition, upon a second exposure to ATP, calcium responses were higher in males, suggesting that increased P2X4R function coincides with increased receptor expression. I therefore propose that the sex differences in p38-MAPK activation and BDNF release are a direct result of P2X4R upregulation in primary microglia, driving downstream signalling in males but not females.

My findings in primary microglia cultures are cohesive with emerging evidence in the field. Spinal p38-MAPK is a key player for nerve-injury induced allodynia, and has been studied in the context of inflammatory, neuropathic pain and cancer-induced bone pain.[75,103,106] However, converging lines of evidence suggest that p38-MAPK activation is not necessary for nerve-induced pain hypersensitivity in females.[106] Consistent with higher levels of p38-MAPK phosphorylation in the spinal dorsal horn of males versus females, intrathecal administration of selective p38-MAPK inhibitors was sufficient to alleviate neuropathic (constricted cuff injury (CCI) or SNI) and inflammatory (formalin) pain behaviour only in male rodents.[106] This sex-dependent effect translated to an attenuation of excitatory post-synaptic currents (EPSCs) in

lamina II neurons only in males, suggesting that pain hypersensitivity following PNI in females develops by a p38-MAPK independent mechanism.[106] Sex-dependent p38-MAPK signalling in the spinal cord is also consistent with the male-specific requirement for BDNF in PNI-induced allodynia.[59] Sorge et al., (2015) show that intrathecal administration of neurotrophin inhibitors, BDNF sequesters and genetic depletion of BDNF in microglia is sufficient to attenuate the development of allodynia in male but not female mice.[59] Since p38-MAPK activation and BDNF synthesis are both downstream targets of the P2X4R, it is possible that these findings may be a direct result of dimorphic P2X4R upregulation in spinal microglia.

4.4 SNARE-mediated mechanisms of BDNF release from microglia

BDNF release from microglia is an important substrate for nerve-injury induced pain hypersensitivity.[71,74] Whereas secretion of BDNF from neurons has been well characterized, the basic mechanisms regulating this process in microglia remain poorly understood.[77] The identification of VAMP-3 and SNAP-23 in cultured glia raised the possibility that SNARE proteins may mediate exocytosis in non-neuronal cells.[87] In astrocytes, there is now substantial data on the expression profile of SNAREs in the plasma membrane and on secretory organelles.[107] Since astrocytes play important roles for neuromodulation through release of small molecules, peptides and protein factors, the pattern of core-SNARE proteins involved in vesicular fusion have also been identified.[108] These mechanisms in microglia have yet to be elucidated. This is surprising, considering that vesicular release of cytokines from microglia contributes to neuroinflammation in several pathologies, and could therefore be exploited as a promising therapeutic target.[42] To derive mechanisms of BDNF secretion from microglia, I initially focused on determining the expression profile of SNAREs, since key pieces of evidence published by Trang et al., (2012) suggested that regulated release of BDNF from microglia is

SNARE-mediated.[73] I examined reports of basal mRNA levels in primary cultures provided by an online microarray database and found that microglia possess a full complement of synaptobrevins, SNAPs, NAPs and VAPs.[98] Of particular interest, I identified non-neuronal SNARE isoforms VAMP-3 and SNAP-23. By targeting these SNAREs *in vitro* using siRNA knockdowns and botulinum toxins, I showed that vesicular release of BDNF from microglia is dependent on SNAP-23 and VAMP-3, but not SNAP-25. Whether attenuation of these SNAREs at the spinal level is sufficient to reduce pain hypersensitivity has yet to be determined, and is an important next step in my investigation. In humans, peripheral administration of BoNTs is efficacious for treating symptoms of post-herpetic neuralgia, trigeminal neuralgia, peripheral neuralgia and diabetic neuropathy.[18,20] Although BoNTs act to relieve pain by inhibiting release of neurotransmitters from the synaptic terminals of sensory nerves, there is some evidence to suggest that they also act on glia to prevent cytokine release and to inhibit microglial reactivity following PNI.[109] By identifying subsets of SNAREs expressed specifically in glial cells, it may be possible to selectively target these homologues without affecting neuronal transmission and thus limit the negative side effects that are associated with current BoNT treatments. For example, the BoNT light chain has been shown to have a higher affinity and catalytic activity for the non-neuronal isoform VAMP-3 relative to VAMP-1 and VAMP-2, and therefore could be a potential therapeutic agent to treat neuropathic pain.[99] However, further analysis on the substrate specificity of BoNTs, potential dosing paradigms, and effective routes of administration is needed prior to using this toxin for widespread neuropathic pain treatment.

4.5 Overlapping mechanisms of dimorphic TLR-4 and P2X4R-signalling

In addition to dimorphic P2X4R-signalling in microglia, the role of innate immune receptors, TLR-4s, in pain also depends on sex.[92] Interestingly, TLR-4 and P2X4R-signalling pathways share many similarities. TLR-4s are expressed primarily by microglia in the spinal dorsal horn and activation of spinal TLR-4s by LPS induces robust hypersensitivity in male but not female mice.[92] In addition, pharmacological blockade of TLR-4s attenuates allodynia only in males, and these sex differences appear to be restricted to the spinal cord.[92] Recent evidence suggests that mechanisms of TLR-4 and P2X4R-signalling in microglia may overlap, with p38-MAPK activation being a converging target.[103,110] *In vitro* studies show that LPS upregulates P2X4R expression on microglia,[110] and that BDNF release is partially dependent on TLR-4-p38-MAPK signalling.[106] Whether these pathways interact in spinal microglia following PNI, or how these interactions are affected by sex, remains unknown but would be important next lines of investigation. Together, the data from these studies combined with my findings, affirms the notion that PNI is likely eliciting responses from several signalling pathways on microglia that may converge to drive aberrant microglia-neuronal signaling in males, but not females.

4.6 Microglia-independent mechanisms underlie pain hypersensitivity in females

Our findings demonstrate that P2X4R-mediated release of BDNF from microglia does not contribute to mechanical allodynia in nerve-injured female rodents. PNI, however, results in comparable mechanical allodynia in both male and female rodents.[59] Therefore, we reason that females must use an alternate mechanism that plays an analogous role to males. Adaptive immune cell infiltration into the spinal cord following PNI is a key mediator of pain hypersensitivity.[111–113] It has been recently shown that instead of microglia, females preferentially use T-cells to produce allodynia following nerve injury.[59] Although the exact

mechanisms for this process have yet to be elucidated, the dependency on microglia for pain seems to rely on testosterone.[59] In short, there is some evidence to suggest that androgens increase the expression of peroxisome proliferator activated receptors (PPARα), a group of transcription factors that repress the transcription of pain-associated cytokines including IFNγ.[114] One hypothesis is that through this dimorphic gene regulation, infiltrating T-cells in males are less able to mediate pain hypersensitivity, thus resulting in the adoption of the microglia-dependent pathway. This was illustrated in female mice that 'switch' to the microglia-dependent pathway following testosterone administration.[59]

Although microglia play a divergent role for pain processing in males and female rodents, pain circuitry seems to converge downstream of microglia at the level of pain transmitting neurons. It is currently unknown whether TrkB receptor activation, Src-family kinases, or downregulation of KCC2 channels on lamina I neurons play a role for pain hypersensitivity in females. In my study, I show that females develop comparable levels of allodynia to males following intrathecal administrating of stimulated male but not female-derived microglia. This is consistent with my data in primary cultures showing that ATP-evokes BDNF release only in males, suggesting that BDNF release from microglia is key for producing pain hypersensitivity in both sexes. However, we have not excluded the possibility that other substrates released from microglia could be mediating this effect. Further lines of investigation to determine whether BDNF in itself is sufficient to cause pain hypersensitivity in female rats could clarify whether downstream converging mechanisms in pain processing exist between males and females. It is important to continue looking into these divergent pathways to understand why males and females use alternate lines of pain processing, and to develop drugs that effectively target convergent mechanisms between sexes.

4.7 Significance

Neuropathic pain is a debilitating disorder that severely impacts quality of life and is resistant to medical intervention. Current treatment options for neuropathic pain mainly target neuronal pathways, reducing their effectiveness and limiting their use due to adverse effects. Several studies have recently highlighted the role of spinal microglia in chronic pain, and it is now widely accepted that aberrant microglia-neuronal signalling underlies the development of hypersensitivity.[54,58,71] The focus of this research was therefore to investigate the role of a key microglial signalling pathway necessary for pain hypersensitivity following PNI: P2X4R-evoked BDNF release. The overwhelming majority of preclinical studies deciphering these mechanisms were performed in male rodents and my research has drawn attention to key sex differences that underlie this pathway.[88] I have found that P2X4R upregulation is a primary point of divergence between males and females, and have pinpointed a potential mechanism controlling its dimorphic upregulation. My research, alongside other findings in the literature, emphasizes the importance of including both sexes in preclinical pain research.

Whether microglia play a role for pain processing in humans is currently under investigation. Although females typically report more pain and higher pain intensities than males in a clinical setting,[115] whether it is divergent pain processing pathways or a complex interaction of biological, psychological and sociocultural factors that underlie these sex differences has yet to be determined. There has been some indication that microglia are engaged following nerve injury in humans.[116] Preliminary studies show that microgliosis occurs in the spinal cord of females with complex regional pain syndrome,[117] and that increased microglia reactivity occurs in the thalamus of males and females that suffer from chronic lower back pain.[118] However, a broad microglial inhibitor, propentofylline, had little success for treating post-herpetic neuralgia

in clinical trials.[119] Since the authors did not address possible sex-dependent effects or the translatability of their findings to a model of PNI, this finding does not rule out microglia as key mediators of neuropathic pain in humans.[119] In general, the recent literature supporting sex differences in pain underlies the pressing need for equal representation of male and female subjects in preclinical pain research so that more sex-specific treatments can be developed for chronic pain sufferers. My research has added to our understanding of the mechanistic framework of neuropathic pain, and provides a cellular basis for the development of more effective, individualized treatment options for neuropathic pain.

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