CHARACTERIZATION OF THE MECHANOSENSITIVE CATION CHANNEL PIEZO1 IN MICROGLIA

by

Heather J. Gerrie

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The following individuals certify that they have read, and recommend to the Faculty of Graduate and Postdoctoral Studies for acceptance, the thesis entitled:

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Examining Committee:

Brian MacVicar, Professor, Psychiatry, UBC
Supervisor

Terry Snutch, Professor, Psychiatry, UBC
Supervisory Committee Member

Annie Ciernia, Assistant Professor, Biochemistry & Molecular Biology, UBC
Supervisory Committee Member

Vanessa Auld, Professor, Zoology, UBC
Additional Examiner
Abstract

Microglia are the resident immune cells of the central nervous system. Following injury, infection, or disease, microglia rapidly mobilize to the site of damage and are critically involved in both launching and resolving an immune response. To detect insult and perform their immune functions, microglia interact chemically and mechanically with their surroundings. While the role of chemical signalling in modulating microglial behaviour and immune function has been extensively studied, the impact of mechanical force is less well established.

In 2014, a novel mechanosensitive channel was identified in the microglial transcriptome. Piezo1 is a cation channel with significant calcium (Ca$^{2+}$) permeability that activates in response to mechanical force. In addition to its mechanosensitive properties, it is now evident that Piezo1 channels are involved in mediating immune function in astrocytes and peripheral myeloid cells. As highly mechanosensitive and immune competent cells, microglia presented an interesting candidate for investigating Piezo1 expression and function.

In this thesis research we obtained evidence that Piezo1 channels are functionally expressed in primary microglia and upregulate in response to immune challenge. Western blot and RT-qPCR data revealed that Piezo1 protein and mRNA increase in response to the toxin lipopolysaccharide (LPS), an effect which is mediated by toll-like receptors 2 and 4. Using immunocytochemistry, we observed that LPS alters the pattern of Piezo1 expression in the peripheral cell membrane and microglial fine processes. Finally, we performed a series of Ca$^{2+}$ imaging experiments to elucidate the functional properties of Piezo1. We observed that the Piezo1 agonist Yoda1 induced an increase in cytosolic Ca$^{2+}$, an effect which was attenuated by blocking Piezo1 with gadolinium and...
by removing extracellular Ca\textsuperscript{2+}. This data suggests that Piezo1 channels directly mediate Ca\textsuperscript{2+} influx from the environment. Interestingly, acute LPS application abolished the Piezo1-mediated Ca\textsuperscript{2+} response, while blocking purinergic receptors potentiated the Ca\textsuperscript{2+} response. Lastly, we induced cell swelling to generate mechanical force on the cell membrane. Our results indicated that mechanical activation of Piezo1 robustly increases cytosolic Ca\textsuperscript{2+}. Overall, this thesis provides the first evidence that Piezo1 channels are expressed in primary microglia and are involved in microglial immune function.
Lay Summary

The brain has specialized immune cells called ‘microglia’. Microglia are highly active cells that constantly survey the health of their environment. In response to disease, injury, or infection in the brain, microglia mobilize to the site of damage and initiate the healing process. Microglia clear damaged and dying cells, and release signalling molecules that control inflammation and promote wound healing. One way that microglia monitor and respond to their surroundings is by sensing changes in mechanical forces, such as pressure from tissue swelling. Mechanical forces can be detected by cells through proteins called ‘mechanosensitive channels’. These channels convert external mechanical forces into signals that communicate to a cell how to best adapt or respond to its environment. In 2010, a mechanosensitive channel named ‘Piezo1’ was discovered. Interestingly, Piezo1 channels can both detect changes in mechanical force, as well as modulate immune response. The mechano-sensing and immune properties of Piezo1 channels made microglia an interesting type of cell to investigate Piezo1 expression. In this thesis, I provide the first evidence that Piezo1 channels are expressed in microglia and are involved in microglial immune function.
Preface

This thesis contains the original, unpublished work of the author, Heather Gerrie. All experiments were conceived by HG along with Drs. Hyun Beom Choi, Leigh Wicki-Stordeur, and Brian MacVicar. Experiments and data analysis were conducted by HG with the following exceptions: Jeff LeDue assisted with the data analysis pipeline and developed the MATLAB script used to analyze the Ca\(^{2+}\) imaging data (Chapter 2.12). HBC assisted with Western blot data acquisition (Chapter 3.2) and performed some of the Ca\(^{2+}\) imaging experiments (Chapter 3.6). LWS assisted with some of the sample preparation and image acquisition for immunostaining experiments (Chapter 3.4-5). Dr. Stefan Wendt performed the intracranial stab wound procedure and Ray Gopaul performed the intracardial perfusions used for immunohistochemistry (Chapter 2.6; Chapter 4.6). All experimental procedures presented in this thesis involving animals were performed with ethical approval from the UBC Animal Care Committee (Certificate # A17-0082).
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<table>
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<th>Description</th>
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<tbody>
<tr>
<td>Aβ</td>
<td>Amyloid beta</td>
</tr>
<tr>
<td>aCSF</td>
<td>Artificial cerebrospinal fluid</td>
</tr>
<tr>
<td>ASD</td>
<td>Autism spectrum disorder</td>
</tr>
<tr>
<td>ASIC</td>
<td>Acid-sensing ion channel</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
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<tr>
<td>AUC</td>
<td>Area under the curve</td>
</tr>
<tr>
<td>BSC</td>
<td>Biosafety cabinet</td>
</tr>
<tr>
<td>BMDMs</td>
<td>Bone-marrow derived macrophages</td>
</tr>
<tr>
<td>Ca&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>Calcium</td>
</tr>
<tr>
<td>CO&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Carbon dioxide</td>
</tr>
<tr>
<td>CD11b</td>
<td>Cluster differentiation molecule 11-b</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic AMP</td>
</tr>
<tr>
<td>Cryo-EM</td>
<td>Cryogenic electron microscopy</td>
</tr>
<tr>
<td>CED</td>
<td>Extracellular C-terminal loop domain</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CTD</td>
<td>Intracellular C-terminal domain</td>
</tr>
<tr>
<td>CX3CR1</td>
<td>C-X3-C motif chemokine receptor-1</td>
</tr>
<tr>
<td>DAMPs</td>
<td>Danger-associated molecular patterns</td>
</tr>
<tr>
<td>DAPI</td>
<td>4′,6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle Medium</td>
</tr>
<tr>
<td>EGTA</td>
<td>Ethylene glycol tetra-acetic acid</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<td>--------------</td>
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<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>GABA</td>
<td>Gamma-aminobutyric acid</td>
</tr>
<tr>
<td>Gd(^{3+})</td>
<td>Gadolinium</td>
</tr>
<tr>
<td>GLD</td>
<td>Generalized lymphatic dysplasia</td>
</tr>
<tr>
<td>HEK293</td>
<td>Human embryonic kidney 293 cell line</td>
</tr>
<tr>
<td>HIF1-(\alpha)</td>
<td>Hypoxia-inducible factor-1-alpha</td>
</tr>
<tr>
<td>HX</td>
<td>Hereditary xerocytosis</td>
</tr>
<tr>
<td>IL-1(\beta)</td>
<td>Interleukin-1-beta</td>
</tr>
<tr>
<td>IL-10</td>
<td>Interleukin-10</td>
</tr>
<tr>
<td>IN</td>
<td>Inner helix</td>
</tr>
<tr>
<td>IP(_3)</td>
<td>Inositol triphosphate</td>
</tr>
<tr>
<td>LPC</td>
<td>Lysophosphatidylcholine</td>
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<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
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<tr>
<td>LPS-EB</td>
<td>LPS-EB Ultrapure</td>
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<tr>
<td>Mg(^{2+})</td>
<td>Magnesium</td>
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<tr>
<td>myD88</td>
<td>Myeloid differentiation factor 88</td>
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<tr>
<td>Neuro2A</td>
<td>Neuroblastoma cell line-2A</td>
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<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>OH</td>
<td>Outer helix</td>
</tr>
<tr>
<td>P</td>
<td>Post-natal day</td>
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<tr>
<td>PAMPs</td>
<td>Pathogen-associated molecular patterns</td>
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<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>PDA</td>
<td>Pancreatic ductal adenocarcinoma</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>PPADS</td>
<td>Pyridoxal phosphate-6-azophenyl-2',4'-disulfonic acid</td>
</tr>
<tr>
<td>PSD-95</td>
<td>Postsynaptic density-95</td>
</tr>
<tr>
<td>ROI</td>
<td>Region of interest</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RT-qPCR</td>
<td>Real-time quantitative polymerase chain reaction</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>SNAP-25</td>
<td>Synaptosome associated protein-25</td>
</tr>
<tr>
<td>TGF-β1</td>
<td>Transforming growth factor-beta-1</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TMs</td>
<td>Transmembrane segments</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris buffered saline</td>
</tr>
<tr>
<td>TBST</td>
<td>Tris buffered saline + Tween20</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor-alpha</td>
</tr>
<tr>
<td>TRP</td>
<td>Transient receptor potentials</td>
</tr>
<tr>
<td>TRPV4</td>
<td>Transient receptor potential vanilloid 4</td>
</tr>
<tr>
<td>ZA</td>
<td>Zymosan-A</td>
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</table>
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This project was funded by the Canadian Institutes of Health Research.
For my family -

who has championed my education, always
Chapter 1: Introduction

1.1 Project overview

Known as the sentinels of the brain, microglia are the resident macrophages and primary immune cells of the central nervous system (CNS) (Kettenmann et al., 2011). Microglia colonize neural tissue early in development and subsequently migrate throughout the CNS to occupy discrete, non-overlapping spatial territories. As they mature, microglia take on a ‘resting’ or ‘ramified’ morphology, with small cell bodies and long, thin processes that extend throughout their territory (Nimmerjahn et al., 2005). Microglia maintain a ramified phenotype in healthy tissue but transform into an ‘activated’ morphology in response to immune challenge.

Once activated, microglia retract their processes and transform into a round, amoeboïd phenotype that enhances their phagocytic activity and enables them to mobilize to a site of damage. At a site of insult, microglia then perform their phagocytic operations, working to remove neuronal debris, clear dead cells, and drive cell death pathways in damaged or dying cells. Microglia further influence the outcome of a pathological event by controlling the release of cytokines, chemokines, and growth factors, acting to both initiate and resolve an immune response (Wolf et al., 2017). As the immune competent cells of the CNS, microglia are involved in virtually all neuropathologies - ranging from neurodegenerative diseases such as Alzheimer’s disease, traumatic brain injury such as concussion, psychiatric disorders such as schizophrenia, and neurodevelopmental disorders such as autism spectrum disorder (Wolf et al., 2017).
Historically, microglia have been entirely associated with pathology and immune response (Ransohoff & Perry, 2009). However, it is now evident that microglia play dynamic and important roles in the healthy brain as well. Recent use of in vivo imaging has revealed that microglia in a ‘resting’ state in the healthy brain are still very active, using their processes to constantly survey the health of their environment (Bernier et al., 2019; Nimmerjahn et al., 2005). During surveillance, microglia sense molecular cues within their local environment, contact neighbouring cells, and participate in homeostatic maintenance in the neural parenchyma (Nimmerjahn et al., 2005; Tremblay et al., 2011). In addition to homeostatic roles in the healthy adult brain, it is now well established that microglia are critical players in both the developing and ageing brain as well (Schafer & Stevens, 2015). As our understanding of these enigmatic cells increases, it is likely that more functions beyond the scope of microglia’s traditional immune role will be uncovered.

Many of the operations which characterize microglia – such as their migratory behaviour, motility, surveillance, and phagocytic activity – rely on microglia mechanically interacting with their environment (Bollmann et al., 2015; Velasco-Estevez et al., 2018). While it is known that microglia change their inflammatory profile and migrate in response to mechanical perturbations, the mechanisms of this remain largely un-examined (Ayata & Schaefer, 2020).

In 2010, a novel family of mechanosensitive channels was identified in mammals (Coste et al., 2010). Piezo channels are non-selective cation channels which have been identified in mechanically sensitive cells throughout the body, including in the vascular, respiratory, sensory, and renal systems (Wang & Xiao, 2018). Piezo1 in particular has galvanized much research over the past decade. Mechanical stimulation activates Piezo1 channels, inducing robust calcium (Ca^{2+})
influx into a cell and activation of downstream intracellular signalling cascades. Like all mechanosensitive ion channels, activation of Piezo1 enables a cell to sense changes to its mechanical environment and adapt or respond accordingly (Gottlieb & Sachs, 2012a).

Recent research has also identified and characterized Piezo1 in various cell types of the CNS. For example, in neurons, Piezo1 channels are reported to be involved in mechanically sensitive functions such as neuronal differentiation, migration, and axon guidance (Hung et al., 2016; Koser et al., 2016; Pathak et al., 2014). In addition to neurons, Piezo1 is also expressed by the brain’s highly mechanosensitive glial cells. Though a 2014 RNA-sequencing report revealed that Piezo1 is expressed in the transcriptome of all types of glia, to date only astrocytes have been examined (Zhang et al., 2014).

In astrocytes, Piezo1 is involved with detecting mechanical abnormalities in the surrounding environment, such as the presence of the stiff amyloid plaques which characterize Alzheimer’s disease (Velasco-Estevez et al., 2018). Beyond merely allowing astrocytes to detect pathological perturbations, recent data suggests that Piezo1 is also involved in mediating astrocytic response to immune challenge. Activation of Piezo1 following exposure to the inflammatory stimulus lipopolysaccharide (LPS) dampened pro-inflammatory cytokine release in cultured astrocytes (Velasco-Estevez et al., 2019). This presented the first indication that Piezo1 channels may be important contributors to immune function, a finding which has been further substantiated by a recent report that Piezo1 is critical for launching an immune response in peripheral myeloid cells (Solis et al., 2019).
Given their highly mechanosensitive properties and central role in immune function, microglia represent an interesting candidate to explore Piezo1 expression and function. Indeed, out of all the cell types in the CNS, microglia contain the highest expression of the gene encoding for Piezo1. However, to date no studies have examined Piezo1 in primary microglia (Zhang et al., 2014). Therefore, the purpose of this thesis was to establish Piezo1 channel expression in primary microglia and to characterize the functional properties of microglial Piezo1.
1.2 Discovery and definition of microglia

In 1856, Rudolf Virchow introduced the concept of ‘neuroglia’, describing a population of cells in the brain distinct from neurons (Virchow, 1856; Wolf et al., 2017). Despite a modern reputation as the “neglected cells of neuroscience”, following their discovery, glial cells were extensively studied by many of the prominent neuroanatomists and neuropathologists of this era. The observations of classical neuroscientists – including Santiago Ramón y Cajal, Camillo Golgi, and Heinrich Müller - laid the foundation for our current understanding of the role of glial cells in health and disease (Verkhratsky et al., 2018).

During the late 1800’s, important advancements in histological staining, most notably the Golgi stain, allowed for visualization and characterization of glial morphology in human brain tissue. Over the next several decades, widespread use of the Golgi stain resulted in the description of several different subtypes of glia, including the identification of ‘astroglia’ in 1895, and a ‘third element’ described by Cajal (Cajal, 1913; Lenhossék, 1895; Verkhratsky et al., 2018). However, in contrast to neurons, neuroglia are not electrically excitable. This presented a challenge in understanding their function and physiological properties, as glial cells could not be studied using traditional electrophysiological techniques (Navarrete & Araque, 2014). As a result, the function of glia became highly contested. Virchow and Carl Weigert led a camp of thought which asserted that neuroglia functioned as the connective tissue of the brain, a structural matrix in which neurons were embedded (Weigert, 1895; Wolf et al., 2017). Others went further, assigning homeostatic functions to glia. Cajal suggested that glial cells were involved in controlling blood flow, initiating sleep, and regulating information within neuronal networks (Cajal, 1925; Verkhratsky et al., 2018).
Decades later, it is now apparent that glia – particularly astrocytes - are involved in all of Cajal’s suggested operations and more.

At the same time that neuroanatomists were debating glial function, neuropathologists such as Franz Nissl and Alois Alzheimer began reporting stark morphological differences in glia between healthy and diseased tissue (Alzheimer, 1910; Nissl, 1899; Wolf et al., 2017). This led neuropathologists to suggest that glia played some kind of role in pathology of the CNS. At the turn of the century, William Ford Robertson described a subset of glial cells that he termed ‘mesoglia’, and proposed that it was the mesoglia which underwent a pathological transformation in the diseased brain (Robertson, 1900; Verkhratsky et al., 2018). These mesoglia correlated with the cells that Cajal had deemed the ‘third element’ of the brain. Mesoglia were later characterized in detail by Pio del Rio-Hortega, a Spanish neuroscientist who further differentiated mesoglia into oligodendrocytes and microglia. Rio-Hortega’s exhaustive histological and cytological studies of tumours in the nervous system provided him with keen insight into the role of microglia throughout development and during pathological states (Ramon y Cajal Agüeras, 2016).

Using a modified silver carbonate impregnation technique, Rio-Hortega labelled microglial cells and produced detailed images of microglia in various states. Based off of his anatomical findings, Rio-Hortega published a chapter titled ‘Microglia’ in the 1932 publication of Cytology and Cellular Pathology of the Nervous System, edited by Wilder Penfield. In this seminal work, Rio-Hortega made nine postulates: (1) Microglia appear early in the developing brain. (2) In the developing brain, microglia have a round or ‘amoeboid’ morphology and are of mesodermal origin. (3) During development, microglia use blood vessels and white matter tracts to guide their
migration, and ultimately appear in all brain regions. (4) Microglia transform into a branched, ramified phenotype in the mature the brain. (5) In the mature brain, microglia are evenly dispersed throughout the CNS. (6) Microglia rarely overlap, each occupying a defined territory. (7) Following a pathological event, microglia transform. (8) These transformed microglia appear amoeboid, similar to their morphology in early development, and (9) microglia can migrate, proliferate, and phagocytose neuronal debris (Del Rio-Hortega, 1932; Kettenmann et al., 2011). Remarkably, almost a century later, all of Río-Hortega’s postulates still hold.

1.3 Developing microglia

Microglia are not derived from the neural ectoderm, making them unique amongst cells in the brain. Originally, microglia were thought to originate from the bone marrow, similar to other mononuclear phagocytes, and later seed the brain to become organ-specific macrophages (Harry, 2013). However, a landmark fate-mapping study by Ginhoux et al. (2010) revealed that microglia arise from primitive myeloid progenitors in the embryonic yolk sac and colonize the brain very early in development. In mice, amoeboid cells expressing adult macrophage markers, such as C-X3-C motif chemokine receptor-1 (CX3CR1) and cluster differentiation molecule 11b (CD11b), appear in the brain rudiment as early as embryonic day 9.5 (Ginhoux et al., 2010). Microglial colonization of the brain occurs alongside embryonic vascularization, with primitive microglia relying on blood vessels and active blood circulation to migrate (Ginhoux et al., 2010). Though microglia primarily rely on communication with vascular sprouts for migration, the ventricular system is used as an alternative route of entry (Rymo et al., 2011). Once seeded in the brain, the number of microglia steadily increases during the first two postnatal weeks in mice, with 95% of microglia born during this period (Alliot et al., 1999).
In the human brain, microglia colonization occurs following a similarly orchestrated process. During the first trimester, amoeboid microglia appear in the developing brain via the meninges, choroid plexus, and ventricles, subsequently migrating along vasculature and white matter tracts (Rezaie & Male, 1999; Verney et al., 2010). The distribution of microglia along their migration paths is co-ordinated by spatially and temporally regulated expression of chemokines (Rezaie & Male, 1999). As they colonize the brain, microglia retain the same amoeboid morphology seen in yolk sac progenitors, which likely enables their migration and phagocytic properties at this stage. Microglia remain amoeboid as neurons begin to populate the brain, allowing microglia to phagocytose and clear excess neurons and synaptic connections during this stage (Perez-Pouchoulen et al., 2015).

As the brain matures and neurons become established, the number of round and amoeboid microglia decreases, and a corresponding increase is seen in the number of mature, highly ramified microglia. In their mature form, microglia have small cell bodies (~10 µm diameter) and bear long, thin, branched processes. By 35 weeks of gestation in humans, microglia reach their terminal location and take on this fully ramified phenotype (Esiri et al., 1991; Monier et al., 2006). At birth, microglia are fully disseminated throughout all parts of the brain, occupying spatially discrete territories without substantial overlap, and comprising 10-15% of cells in the mature brain (Harry, 2013). Microglia are long-lived cells, renewing slowly at a median rate of ~30% per year. In humans, some microglia survive for over two decades without turnover (Réu et al., 2017).
1.4 Role of microglia in development

As the first glial cells to appear in the embryonic brain, microglia mature side by side with neurons. Thus, microglia are poised to heavily influence neuronal development and maturation (Bohlen et al., 2019). Despite this – and in contrast to the extensively documented role of microglia in etiology and disease course – it is only recently that the critical role of microglia during development has become well established.

Microglia initially influence brain development through their involvement in two key processes: neuron migration and axon guidance (Squarzoni et al., 2015). For example, a study by Squarzoni et al. (2014) reported that unlike the homogenous distribution of microglia in the adult brain, embryonic microglia cluster in ‘hotspots’ that correspond spatially to sites of axonal guidance and circuit formation during critical timepoints in neurodevelopment. Further, neurons release a chemokine called ‘fractalkine’, a signalling molecule that’s only known receptor in the brain, CX3CR1, is found on microglia. Squarzoni et al. found that in both CX3CR1−/− and microglia-depleted mutant mouse models, dopaminergic axonal growth in the forebrain and migration of neocortical interneurons was perturbed (Squarzoni et al., 2014). This provides evidence that microglia have important roles in modulating neuronal migration and axon guidance, processes which are critical in the proper formation of neural circuits and networks.

Microglia also influence circuit formation during development by phagocytosing extraneous synapses and apoptotic cells, as well as triggering cell death pathways in damaged neurons (Bilimoria & Stevens, 2015). Specifically, microglia preferentially engulf weak or less active synapses during development, resulting in refined neural circuits (Paolicelli et al., 2011). In part,
microglia achieve these operations by secreting a wide variety of signalling molecules, such as tumour necrosis factor alpha (TNF-α), reactive oxygen species, and glutamate which can trigger processes such as apoptosis (Bilimoria & Stevens, 2015). Though the role of microglia in regulating the number of synapses via synapse elimination and pruning is well known, increasing evidence suggests that microglia may also play a role in regulating the activity and overall function of synapses (Schafer et al., 2013). All of these operations enable microglia to play important and nuanced roles in patterning the landscape of the developing brain.

Proper synaptic pruning and axon guidance are essential for development, and impairments in these processes disrupts the balance of excitatory versus inhibitory synapses and results in inappropriate wiring of cortical circuits. It is postulated that dysfunction in synaptic pruning and axon guidance may be the root of many neurodevelopmental disorders (Koyama & Ikegaya, 2015). For example, the most extensively studied neurodevelopmental disorder in relation to microglia is autism spectrum disorder (ASD). Both mouse models of ASD and autopsied brains of individuals with ASD reveal an increase in microglial cell density and the number of activated microglia. Further, ASD is characterized at a molecular level by abnormalities in synaptic pruning – with contrasting studies reporting both attenuated and excessive synaptic elimination (Morgan et al., 2010; Pardo et al., 2005). Mouse models have provided much insight into the relationship between microglial abnormalities, synapse development, and neuropathologies. For instance, Rett Syndrome – a single gene X-linked disorder that is considered to be part of the autism spectrum – is caused by a mutation in the MeCP2 gene. In a mouse model of Rett Syndrome, microglia lacking MeCP2 release abnormal levels of neurotransmitters, inhibit the development of neurons, and exhibit impaired phagocytosis of synapses and dying cells (Bilimoria & Stevens, 2015; Maezawa
This underscores how important microglia function is during development, and the extent to which microglia can influence developmental outcomes.

1.5 Microglia in the healthy mature brain

Similar to our understanding of microglia in the developing brain, our understanding of microglial function in the healthy adult brain has only gained traction in recent years. This is due to the long-held belief that microglia are ‘quiescent’ or dormant in the healthy brain, only activating in response to immune challenge (Tremblay et al., 2011). However, it is now clear that following the developmental period, microglia continue to play important roles in the healthy adult brain – contributing to homeostatic function, surveillance, and synaptic maintenance.

As microglia mature, they transform from the amoeboid morphology seen during development into a highly ramified phenotype. Once fully mature, microglia establish themselves in a mosaic-like pattern throughout the CNS, occupying discrete, non-overlapping territories (Nimmerjahn et al., 2005). Ramified microglia display two types of processes: large processes that are continuously extended and retracted throughout the environment, and thin filopodia located at the tips of the larger processes. These thin filopodia move much faster than the large processes during surveillance, thus enabling microglia to more effectively monitor the cell-dense parenchyma (Bernier et al., 2019; Nimmerjahn et al., 2005). Strikingly, the processes of surveillant microglia are confined within each cell’s defined territory, suggesting that during homeostatic functioning an inhibition mechanism is in place to prevent contact between microglia. When injury or pathology occurs, this inhibition mechanism must be rapidly overridden, as microglia can quickly converge and overlap at sites of insult (Augusto-Oliveira et al., 2019; Hines et al., 2009).
The surveillant properties of microglia are conferred by a wide range of microglial receptors, including fractalkine receptors such as CX3CR1 and colony-stimulating factor 1 receptors (Augusto-Oliveira et al., 2019). These receptors enable communication between neurons and microglia and allow microglia to respond to a whole host of neurotransmitters such as glutamate, γ-aminobutyric acid (GABA), norepinephrine, cannabinoids, and acetylcholine (Augusto-Oliveira et al., 2019). Crosstalk between neurons and microglia is critical for maintaining CNS homeostasis, as it allows neurons to communicate their health status to microglia, thereby enabling microglia to respond appropriately. Based on the signalling molecules transmitted by neurons, microglia either maintain their non-inflammatory, surveillant state or launch a pro-inflammatory immune response (Liu, Leak, & Hu, 2016).

During development, microglia prune extraneous synapses to assist in cortical wiring. Mounting evidence suggests that microglia continue to selectively prune synaptic connections in the postnatal healthy brain as well – which may play a role in circuit maintenance. For example, Paolicelli et al. (2011) found elements of both the presynaptic protein Synaptosome-Associated Protein-25 (SNAP-25) and the postsynaptic protein Postsynaptic Density-95 (PSD-95) engulfed within microglia postnatally, suggesting that synaptic pruning by microglia continues in the healthy brain (Paolicelli et al., 2011). The ongoing phagocytic activity of microglia in the mature brain may contribute to important synaptic refinement processes, such as the elimination of weak synaptic connections during sleep (Choudhury et al., 2020). To underscore the importance of appropriate microglia synaptic maintenance in the adult brain, emerging evidence suggests that aberrant phagocytic activity in the adolescent and adult brain causes a stripping or over-pruning of synapses – which appears to be linked to onset of schizophrenia (Augusto-Oliveira et al., 2019).
The velocity at which the processes of ‘resting’ microglia in the healthy brain move can be altered by synaptic and neuronal activity (Nimmerjahn et al., 2005). Use of \textit{in vivo} two-photon imaging revealed that microglia in the resting state make brief (~5 min), direct contact with neuronal synapses. These contacts occur at an average rate of once per hour, though these contacts are activity dependent, as reductions in neuronal activity decrease the frequency of microglia contact and vice versa (Wake et al., 2009). Overall, this suggests that the dynamic motility of microglial processes during baseline surveillance is directed at synapses and enables microglia to respond to synaptic activity and functional status.

\section*{1.6 Microglia in pathology}
As the resident immune cells, microglia respond to any kind of pathologic insult in the brain. This includes trauma, stroke, neurodegenerative diseases, neuropsychiatric disorders, neurodevelopmental disorders, infection, and cancers. In order to be sensitive to this wide range of pathologies, microglia are highly attuned to their local microenvironment (Wolf et al., 2017). Any immune challenge in the environment triggers a rapid transformation in microglial phenotype and functional state, with microglia shifting from a ‘resting’, ramified morphology to an ‘activated’, ameboid morphology (Lull & Block, 2010). This amoeboid morphology closely resembles the amoeboid-like phenotype of primitive microglia seen during development. In both development and pathology, this ameboid phenotype likely enables microglial phagocytic activity and increases microglial mobility (Kettenmann et al., 2011; Wolf et al., 2017).

Classically, the phenotypic variation between ameboid and ramified microglia was viewed as an opposing dichotomy. Microglia were categorized as being in either the M1 or M2 state – with the
M1 state referring to an ameboid, ‘activated’, immuno-reactive state, and M2 related to a ramified ‘resting’, repair and immuno-resolution state (Tang & Le, 2016). However, this system of classification is no longer widely accepted. For one, advances in real-time imaging have revealed that microglia are never truly ‘resting’ in physiological conditions, as they are dynamically and actively surveying the environment and participating in homeostatic functioning. Moreover, microglia respond in a disease-dependent manner and ‘activation’ may look different depending on the context. Each disease in the CNS affects neurons, astrocytes, and oligodendrocytes to differing degrees, and often targets the brain in a region-specific manner (Wolf et al., 2017).

Microglia response can be regulated by biochemical and cellular composition, cellular subpopulation, neuronal circuitry, neurotransmitter release, and metabolic rate (Wolf et al., 2017). Thus, microglial activation is better understood along a spectrum, wherein the phenotype and functional response of microglia to insult is dynamic, nuanced, and can differ between neuropathologies.

Microglia are equipped to sense tissue injury or disease via a host of receptors that recognize neurotransmitters, danger-associated molecular patterns (DAMPs), pathogen-associated molecular patterns (PAMPs), or viral DNA and RNA (Benarroch, 2013). Upon sensing a signal from their environment that indicates a challenge, microglia react in order to maintain homeostasis or clear damaged cells. Morphologically, this reaction consists of a rapid transformation to an amoeboïd-like shape. Functionally, microglia activation is characterized by microglial release of cytokines and chemokines, such as interleukin-1-beta (IL-1β) and TNF-α (Biber et al. 2007).
Cytokines and chemokines are the primary immuno- and neuro-modulatory signalling molecules involved in microglial communication. Microglia can act as both the source and the target of these messenger molecules (Hanisch, 2002). As the source, microglia release these signalling molecules to act on resident and invading cells in the CNS, often triggering cell death pathways (Wolf et al., 2017). This communication involves regulating innate defense mechanisms, initiating and influencing immune responses, recruiting peripheral immune cells to the CNS for defense, and supporting tissue repair (Hanisch, 2002). Microglia can also receive signals from cytokines and chemokines released by cells such as astrocytes and neurons. Under influence from these signals, microglial behaviour, activation, and mobilization can be strongly influenced.

The cytokines, chemokines, and other immuno-signalling molecules microglia release can either be pro-inflammatory or anti-inflammatory. Pro-inflammatory signalling molecules promote an immune response and are aimed at driving cell death pathways or destroying invading pathogens. For example, low levels of adenosine triphosphate (ATP) and its degradation products released from neurons indicates healthy neuronal function to microglia. In contrast, high ATP levels signal cellular damage to microglia and can initiate microglial immune response, including potent release of TNF-α, a pro-inflammatory cytokine. TNF-α release then triggers death pathways in damaged cells (Hide et al., 2000). Microglia also release anti-inflammatory signalling molecules such as IL-10 or transforming growth factor-beta 1 (TGF-β1) that function to resolve immune responses through promoting wound healing and cell survival (Lull & Block, 2010).
1.7 Reactive microgliosis

As immune cells, microglia are typically thought of as playing a helpful role as the brain’s defense against disease and infection. However, accumulating evidence suggests that there are circumstances in which microglia can actually enhance and drive disease states in the brain. This phenomenon occurs when microglia become chronically activated, thus releasing sustained, high levels of pro-inflammatory signalling molecules. While an immune response requires cytokines to kill invading pathogens, if cytokine levels remain pathologically high it becomes neurotoxic and inappropriately promotes neuronal death (Lull & Block, 2010). The phenomenon of chronically activated microglia has been observed in both neurodegenerative diseases and neurodevelopmental disorders (Block & Hong, 2007; Rodriguez & Kern, 2011).

Further, prolonged microglia activation and sustained release of pro-inflammatory cytokines maintains the brain in a state of neuroinflammation. Chronic neuroinflammation is a hallmark of all neurodegenerative diseases, and it has been suggested that sustained neuroinflammation exacerbates the progressive death of neurons in diseases such as Alzheimer’s disease and Parkinson’s disease (Bachiller et al., 2018). For example, microglia become activated by the presence of disease-related proteins such as amyloid-beta peptide (Aβ), the protein which becomes misfolded and aggregates as plaques in Alzheimer’s disease. Aβ-plaques are toxic to neurons, and their presence both damages neurons as well as causes microglia to cluster around the plaque and activate in an attempt to clear the plaque from the extracellular space (Lull & Block, 2010). Once activated, microglia release pro-inflammatory cytokines and signalling molecules such as TNF-α and reactive oxygen species (ROS). However, if microglia continue to remain activated and release pro-inflammatory molecules in excess, the result is indiscriminate neurotoxicity – wherein healthy
neurons also become targets of reactive microglia (Lull & Block, 2010). This process is termed ‘reactive microgliosis’ and can be understood as a self-propelling cycle: microglia respond to neuronal damage, neuronal damage promotes further microglial activation, which then fuels more neurotoxicity (Block & Hong, 2005). In order for aberrant reactive microgliosis to occur, it is thought that homeostatic mechanisms either fail or are surpassed. Though the exact mechanism of this remains unclear – and is likely different between pathologies – it may be that an excessive inflammatory stimulus can overwhelm regulatory mechanisms and causes microglia to act in a deleterious way (Block & Hong, 2007). Thus, depending on the circumstances, microglia can either initiate, resolve, or exacerbate inflammatory responses.

1.8 Calcium signalling in microglia

Microglial operations are vast and varied. Microglial cells are involved in regulating development, disease, inflammation, and homeostatic maintenance of neural tissue. Accumulating evidence suggests that these functions are closely linked to and influenced by intracellular Ca\(^{2+}\) signalling.

Ca\(^{2+}\) signalling is the use of Ca\(^{2+}\) ions as a form of communication between cells or between cells and their environment. Moreover, Ca\(^{2+}\) ions can be understood as the essential mediators between extracellular signals and intracellular responses (Möller, 2002). Receptor-mediated changes to the free cytoplasmic Ca\(^{2+}\) concentration converts extracellular information into intracellular signalling cascades, thus exerting regulatory effects on many proteins and enzymes. These downstream events modulate nearly every aspect of cellular function, including motility, apoptosis, and transcription (Möller, 2002). When Ca\(^{2+}\) enters a cell and causes an increase in the intracellular Ca\(^{2+}\) concentration, it is known as a Ca\(^{2+}\) transient (Clapham, 2007). As non-electrically excitable
changes in the intracellular Ca\(^{2+}\) concentration represent a major pathway for signal transduction for microglia (Brawek & Garaschuk, 2013).

Broadly speaking, surveillant microglia in the healthy brain show low spontaneous Ca\(^{2+}\) transients, and express fewer functional receptors involved in Ca\(^{2+}\) signalling on the cell surface. However, in the presence of cell or tissue damage, microglia demonstrate a robust increase in Ca\(^{2+}\) transients with an accompanied increase in internal Ca\(^{2+}\) concentration (Brawek & Garaschuk, 2013). For example, Eichhoff et al. (2011) used *in vivo* two-photon imaging to investigate Ca\(^{2+}\) signalling in resting cortical microglia. They reported that the majority (80%) of microglial cells demonstrated no spontaneous Ca\(^{2+}\) transients at rest. However, large microglial Ca\(^{2+}\) transients could be reliably induced following neuronal damage from pipette-induced neuronal membrane rupturing. Notably, the same team reported that microglia imaged *in vitro* more readily demonstrate spontaneous Ca\(^{2+}\) transients at baseline (Eichhoff et al., 2011). This suggests that while microglia do not utilize somatic Ca\(^{2+}\) signalling during *in vivo* surveillance, the isolation of microglia in culture may result in slight activation even at baseline and therefore increase the likelihood of spontaneous Ca\(^{2+}\) transients at ‘rest’. It is important to be cognisant of the differences in Ca\(^{2+}\) signalling between *in vitro* and *in vivo* when drawing conclusions about the mechanisms of microglial Ca\(^{2+}\) signalling in cultured cells. That being said, the majority of studies investigating Ca\(^{2+}\) signalling in microglia are performed in culture due to difficulties in studying microglia *in vivo* without immediately causing microglial activation.

Purinergic receptors are heavily involved in microglial Ca\(^{2+}\) signalling. Two subclasses of purinergic receptors are expressed in microglia, namely the ionotropic P2X family and the
metabotropic P2Y family of receptors (Brawek & Garaschuk, 2013). P2X receptors are a family of homo/hetero-trimeric protein complexes that contain a transmembrane pore which upon opening functions as an ion channel (Wei et al., 2016). In contrast, P2Y receptors are metabotropic or G-protein-coupled receptors, meaning they are not inherently a channel. Rather, in response to the binding of extracellular nucleotides, P2Y receptors undergo a conformational change that facilitates the activation of G proteins, which in turn regulates the generation of second messengers and intracellular signalling cascades (Dubyak, 2013).

The primary link between P2 receptors and Ca\textsuperscript{2+} fluctuations is ATP and its derivatives. Most famous as a source of metabolic energy, ATP is also a robust signalling molecule in the CNS, and can act as either a primary transmitter or co-transmitter within the CNS (Kettenmann et al., 2011). In particular, extracellular ATP increases following pathologic events when damaged or dying cells release ATP. Microglia are attuned to this increase in extracellular ATP and become immune activated to respond to the pathology. Mechanistically, ATP binds to P2X receptors, causing the channel to open and Ca\textsuperscript{2+} to influx. One of the most well studied P2X receptors in microglia is P2X\textsubscript{7}, a channel which has been implicated in microglial activation and modulation of inflammatory response via influencing cytokine release (Brawek & Garaschuk, 2013; Inoue et al., 2009). Notably, microglia can also release ATP to act in an autocrine manner on P2X\textsubscript{7} channels. This is typically associated with toll-like receptor (TLR) activation from inflammatory stimuli such as LPS, wherein LPS stimulates TLRs, which in turn facilitate P2X\textsubscript{7} activation and thus Ca\textsuperscript{2+} influx (Hidetoshi et al., 2012a).
While P2X receptor activation causes direct Ca\textsuperscript{2+} influx into microglia, P2Y receptor activation from ATP or other nucleotides is associated with activation of downstream signalling cascades through G-proteins (Hidetoshi et al., 2012b). In microglia, the most highly expressed and well-studied P2Y receptors are P2Y\textsubscript{2}, P2Y\textsubscript{6} and P2Y\textsubscript{12} (Hidetoshi et al., 2012b). P2Y\textsubscript{2} and P2Y\textsubscript{6} receptors are linked to G\textsubscript{q} proteins, which activate the phospholipase C pathway and regulate intracellular Ca\textsuperscript{2+} levels through inositol triphosphate (IP\textsubscript{3}) mediated release of Ca\textsuperscript{2+} from endoplasmic reticulum (ER) stores. P2Y\textsubscript{2} and P2Y\textsubscript{6} receptor activation in microglia is usually associated with downstream modulation of inflammatory signalling molecules and phagocytotic activity (Hidetoshi et al., 2012b; Kim et al., 2011; Weisman et al., 2012). In contrast, P2Y\textsubscript{12} receptors are coupled to G\textsubscript{i} proteins, with P2Y\textsubscript{12} activation causing a decrease in levels of cyclic AMP (cAMP). P2Y\textsubscript{12}-mediated cAMP decreases in microglia are linked to large filopodia extension and chemotactic mobilization toward ATP (Haynes et al., 2006). In contrast, increased intracellular cAMP enhances the extension of microglial fine filopodia, which are involved in surveillance (Bernier et al., 2019). In general, P2Y receptor activation also influences microglial secretion of cytokine and chemokines to modulate inflammatory response (Hidetoshi et al., 2012a; Ogata et al., 2003).

Overall, Ca\textsuperscript{2+} dynamics are fundamental for signal transduction and executive functions in microglia. Purinergic receptors play a large role in microglial Ca\textsuperscript{2+} signalling, though they are not the exclusive mediators of microglial Ca\textsuperscript{2+} transduction. For example, microglia possess receptors for a host of signalling molecules such as glutamate, acetylcholine, and serotonin that all play a role in mediating microglial communication between microglia and neurons (Brawek & Garaschuk, 2013).
1.9 Mechanoception and mechanosensitive ion channels

While chemical signalling – such as Ca$^{2+}$ signalling – is critical for cell function, it is not the only way in which cells communicate with and adapt to their environment. Mechanical signalling, or ‘mechanoception’, is gaining traction as an important property in cell physiology and pathology. Cells can achieve mechanosensitivity through mechanotransduction: the process of converting mechanical stimuli into biological signals interpretable by cells (Volkers et al., 2014). Mechanoception is critical in mammalian systems for a variety of sensory processes, including proprioception and pain sensation, while also mediating physiological processes such as vascular tone, muscle homeostasis, and flow sensing in the respiratory system (Coste et al., 2010; Wang et al., 2016a). In the brain and nervous system mechanotransduction is necessary for axon guidance, neuronal differentiation, and cell migration (Hung et al., 2016; Koser et al., 2016; Pathak et al., 2014).

One mechanism for mechanotransduction is the activation of mechanosensitive ion channels that respond directly to cell membrane deformation (Hamill & Martinac, 2001). Cell deformation can arise from any compression, expansion/thinning, bending, or shear stress that occurs to the lipid membrane. A mechanosensitive protein thus needs to be sensitive to alterations in membrane properties and/or interact with extracellular and cytoskeletal tethers (Hamill & Martinac, 2001). Following mechanical perturbations, the activation of mechanosensitive proteins triggers an intracellular cascade that results in a response or adaptation of the cell to its environment.

Mechanosensitive channels are extensively characterized in lower organisms, but due to a lack of conservation, only a few mechanosensitive channels have been well described in mammals
In total, four families of mechanosensitive ion channels have been identified in mammals: transient receptor potential (TRP) channels, two-pore domain potassium channels, degenerin/epithelial sodium channels, and more recently the Piezo family of channels.

1.10 Immune cells are inherently mechanosensitive

The body’s immune cells are inherently mechanosensitive. This property enables immune cells to mobilize to sites of damage and detect external objects which have been inadvertently or purposefully (ex. pacemakers or other medical implants) placed in tissue. External objects in the body launch a foreign body response from the immune system, which drives immune cells towards the object and causes an immune response to eliminate or segregate the foreign body from the tissue (Ayata & Schaefer, 2020). Peripheral immune cells use their mechanosensitive capabilities to differentiate foreign objects from tissue by detecting alterations in stiffness or changes in mechanical forces. Detection of mechanical alterations to the cellular environment induces conformational changes in macrophage morphology to allow cells to mobilize and respond accordingly (Ayata & Schaefer, 2020).

As the resident macrophages of the brain, microglia are also mechanosensitive and highly attuned to the mechanical forces within their microenvironment (Moshayedi et al., 2014). Indeed, many of the functions which characterize microglia are mechanical processes which require microglia to be exquisitely sensitive to mechanical perturbations. Mechanosensitivity is involved in the motility of microglial processes during surveillance, mobilization of microglia through the parenchyma to sites of damage, assisting with axon guidance during development, as well as microglial phagocytotic activity (Ayata & Schaefer, 2020). In addition to phagocytosis, microglia also use
their mechanosensitive capabilities to initiate healing and repair processes. For example, Liu et al. (2016) used a multi-photon laser to induce a cerebrovascular rupture in a zebrafish model, a procedure which resulted in a vascular lesion with two endothelial ‘ends’. In vivo time-lapse imaging revealed that microglia migrated to the site of damage, used their filopodia to physically adhere to the endothelial ends, and then used mechanical traction to pull the endothelial ends towards each other and ligate them (Liu et al., 2016).

Mechanosensation also contributes to microglia’s ability to detect and mobilize to the stiff plaques that form during the course of Alzheimer’s disease. Both in vitro and in vivo, microglia display a phenomenon termed ‘dermotaxis’, meaning that they will preferentially migrate towards stiffer materials if given the option. (Bollmann et al., 2015). Thus, the stiff and brittle amyloid plaques present in brains with Alzheimer’s disease likely register as a stark mechanical difference from the surrounding soft CNS tissue. Remarkably, the core of Aβ plaques can reach a stiffness comparable to that of bone, making them $10^6$-fold stiffer than brain tissue (Fitzpatrick et al., 2013). Notably, environmental stiffness is directly correlated with the inflammatory activity of peripheral macrophages as well as glia, including microglia and astrocytes (Moshayedi et al., 2014).

### 1.11 Mechanosensitive channels implicated in immune function

While the underlying molecular mechanisms involved in mechanically induced peripheral macrophage and microglia activation are not well understood, it is clear that mechanically gated ion channels contribute to this process. For example, TRP-vanilloid 4 (TRPV4) channels have been shown to directly regulate innate immune function in peripheral macrophages. Scheraga et al. (2016) reported that TRPV4 channels were required for macrophage activation following
alterations in matrix stiffness in a murine model of lung injury, which mimicked inflamed or fibrotic lungs. Active TRPV4 channels mediated macrophage phagocytosis and played a role in the release of anti-inflammatory cytokines for immuno-resolution (Scheraga et al., 2016).

TRPV4 channels are not the only mechanosensitive channels implicated in immune function. The Piezo family of ion channels, which consists of Piezo1 and Piezo2, is the most recently discovered family of mechanosensitive channels. Recent studies have revealed that Piezo1 channels can play a role in modulating immune response. For example, Velasco-Estevez et al. (2018) reported that Piezo1 expression upregulated in plaque-associated astrocytes in a model of Alzheimer’s disease. In 2019, the same team reported that Piezo1 activation was linked to cytokine release following application of LPS in cultured astrocytes (Velasco-Estevez et al., 2019).

Furthermore, Solis et al. (2019) reported that Piezo1 is critical for launching an immune response in bone-marrow derived macrophages (BMDMs). First, they established that in BMDMs, Piezo1 RNA is highly expressed, with negligible expression of other mechanosensitive ion channels. BMDMs were then placed in a bio-reactive pressure chamber, which mimics the pressure conditions of a lung. Upon exposure to cyclical hydrostatic changes in pressure, BMDMs upregulated an array of pro-inflammatory genes, most of which were targets of hypoxia-inducible factor-1-alpha (HIF1α). HIF1α is a regulating factor involved in cellular adaptation to hypoxia, and is known to mediate pro-inflammatory gene expression, influence macrophage migration and innate immunity, as well as promote bacterial killing (Imtiyaz & Simon, 2010). However, when Piezo1 was selectively deleted, cells no longer showed this pro-inflammatory transcriptional re-programming. Overall, the authors reported that Piezo1 can alter immune cell function and that
Piezo1 may represent a novel therapeutic target for controlling pulmonary inflammatory diseases, such as fibrosis (Solis et al., 2019).

1.12 Piezo channel identification

While the role of Piezo1 mechanosensitive channels in modulating immune response is a recent discovery that still requires further investigation, Piezo1 channels in general have garnered significant attention since their identification by Coste et al. in 2010. Using siRNA screening to select candidate genes, followed by RNA interference knockdown of selected genes, Coste et al. were able to identify the gene *FAM38A* as responsible for encoding a mechanically activated cation current in a mouse neuroblastoma cell line (Neuro2A). Knockdown of *Fam38A* resulted in a reduction of pressure- and stretch-induced currents in Neuro2A cells, and thus the authors renamed *Fam38A* to *Piezo1* as an homage to the Greek word *píesi* meaning ‘pressure’ (Coste et al., 2010).*

Coste et al. also identified a related gene, *Piezo2*, in dorsal root ganglia neurons. Overexpression of the *Piezo1* and *Piezo2* genes resulted in large, mechanically activated cation currents that were inhibited by application of the non-selective mechanosensitive blockers ruthenium red and gadolinium (Gd$^{3+}$). Overall, this suggests that the Piezo1 and Piezo2 proteins are sensitive to mechanical force and capable of generating currents in the presence of a mechanical force. Since their identification, Piezo2 channels have been exclusively reported in sensory systems, while Piezo1 channels have been identified broadly throughout the body and modulate mechanical responses in a variety of organ systems (Volkers et al., 2014).

* The *Piezo1* gene has many aliases, including *Fam38A, KIAA0233*, and *Mib.*
Notably, while Coste et al. were the first to identify that the *Piezo* genes encoded for mechanically gated ion channels, they were not the first to identify the *Piezo1* gene. In 2006, Satoh et al. reported that a gene they deemed ‘*Mib*’ was transcriptionally upregulated in plaque-associated astrocytes in a rodent model of Alzheimer’s disease. They reported that while *Mib* expression increased in plaque-associated astrocytes, it was virtually undetectable in quiescent astrocytes in healthy brain tissue (Satoh et al., 2006). While their study exclusively focused on the genetic findings, Satoh et al. hypothesized that the protein encoded for by *Mib* must be involved somehow in astrocyte immune response. Thus, since the discovery of the gene encoding for Piezo1, these channels have been implicated in immune response – though it would take several years before Piezo1 channels were identified as the protein encoded by *Mib*, and another decade before the first direct immune functions of Piezo1 channels were identified.

### 1.13 Piezo1 channel structure

Piezo1 is an extremely large protein, made of over 2500 amino acids and spanning between 30-40 transmembrane domains – the largest number of transmembrane segments recorded to date in a mammalian protein (Bagriantsev et al., 2014). Further underscoring the size of Piezo1 is its ~900 kDa weight and the fact that its homo-oligomeric structure, meaning that the channel is comprised of a single protein complex (Zhao, Zhou, Li, & Xiao, 2019). Apart from its remarkable size, Piezo1 is also distinctive due to its lack of sequence homology with any other known class of ion channels, its conservation across organisms, and its presence in a wide variety of cell types (Gottlieb & Sachs, 2012b).
Technical breakthroughs in the use of cryogenic electron microscopy (cryo-EM) provided powerful insight into the 3D structure of membrane proteins like Piezo1. Using cryo-EM, Ge et al. (2015) determined the architecture of mouse Piezo1 to be a striking propeller-shaped channel made of three extracellular blades, an extracellular cap, and intracellular beams which serve to anchor the propeller-blades to a transmembrane ion-conducting pore (Figure 1-1).

**Figure 1-1. Structure of mouse Piezo1.**
A) Extracellular, B) side, and C) intracellular views of mouse Piezo1 structure. 3D renderings based on Piezo1 structural images acquired using cryogenic electron microscopy. Adapted from Zhao et al., 2018.

The two main components of Piezo1 are an ion-conducting pore and the extracellular blades. The pore is enclosed by three pairs of transmembrane segments (TMs) – with each pair comprised of an outer helix (OH) and an inner helix (IH) – as well as an intracellular C-terminal domain (CTD) and an extracellular C-terminal loop domain (CED) (Zhao et al., 2018). To validate that these components form a functional ion-conducting pore, Zhao et al. (2016) replaced the CTD and CED from mouse Piezo1 with the analogous sequence from *Drosophila* Piezo1. The result was a chimeric channel that maintained the properties of fly Piezo1 and revealed that the C-terminal
region of Piezo1 critically determines Piezo1 conductance, ion selectivity, and the inactivation kinetics of the channel (Zhao et al., 2016).

While the pore-containing region of Piezo1 is responsible for ion conductance, the extracellular blade modules are responsible for the mechanosensitive property of the channel. Each Piezo1 propeller blade is comprised of three distinct components: 1) peripheral TMs, 2) an anchor domain that penetrates into the membrane and connects the peripheral TMs to the OH of the transmembrane pore, and 3) a long (~90 Å) intracellular beam that supports and further connects the blade to the central pore (Zhao et al., 2016). The three blades are twisted in a clockwise manner to form the propeller shape, with each blade demonstrating flexibility and mobility. When reconstituted in a small liposome, Piezo1 blades locally deformed the lipid bilayer and responded to movement or conformational changes of the membrane (Guo & MacKinnon, 2017). Taken together, these properties indicate that the blade structures are the mechanosensitive module of Piezo1.

1.14 Inherent mechanosensitivity of Piezo1 conferred by structure

To further examine the role of the triskelion blades in mediating Piezo1 mechanosensitivity, Zhao et al. generated a series of deletion mutants. They reported that selective deletion of the sequences encoding for the Piezo1 blades ablated the mechanically-induced currents (Zhao et al., 2018). Moreover, replacing the Piezo1 pore with the pore region from the mechano-insensitive acid-sensing ion channel (ASIC) resulted in a channel that responded to both pH and mechanical force. This further establishes that the pore region of Piezo1 is not involved in mechanotransduction, and rather that it is the blades which are sensitive to mechanical force (Zhao et al., 2016). Taken
together, these structural studies suggest that Piezo1 channels contain a discrete, mechanosensitive region and do not require the presence of additional cellular components for mechano-sensing. Indeed, Piezo1 is considered as an inherently mechanosensitive channel (Syeda et al., 2016; Zhao et al., 2018).

To investigate whether Piezo1 channels are inherently mechanosensitive, Syeda et al. (2016) reconstituted Piezo1 channels into an asymmetric, purified lipid bilayer. They reported that mechanical perturbation of the lipid bilayer alone was sufficient to generate a mechanosensitive cationic current. While movement of the lipid bilayer is one way in which mechanosensitive channels sense mechanical forces, the cytoskeleton and extracellular matrix represent other potential factors involved in the transduction of mechanical forces. To elucidate whether Piezo1 responded to alterations in either the cell membrane, cytoskeleton, or extracellular matrix, Cox et al. (2016) generated blebs from a human embryonic kidney 293 (HEK293) cell line that were uncoupled from the cytoskeleton. When these blebs were patched and the currents were recorded, Cox et al. reported that Piezo1 activity persisted. As well, Piezo1 activity was preserved in cells that had been pretreated with drugs that disrupt microtubule assembly and actin depolymerization. Thus, Piezo1 channels sense and respond directly to conformational changes and force applied to the lipid membrane (Syeda et al., 2016).

1.15 Piezo1 pharmacology

In addition to the structural and functional evidence, the mechanosensitive properties of Piezo1 are substantiated by pharmacological approaches as well. For example, Coste et al. (2010) were the first to apply ruthenium red and Gd$^{3+}$ in HEK293 cells overexpressing Piezo1, and they
reported that both drugs blocked the Piezo1-induced mechanically activated current. While ruthenium red acts as a pore blocker, Gd$^{3+}$ alters membrane curvature by binding to negatively charged headgroups of membrane phospholipids. Gd$^{3+}$ binding generates lateral pressure on the membrane, which effectually “squeezes” the channel and increases the likelihood that a channel will shift toward a closed conformation (Ermakov et al., 2010). Ruthenium red and Gd$^{3+}$ are known blockers of cationic mechanically activated currents, and their effective blocking of Piezo1 currents provides further support that Piezo1 is mechanically gated. However, ruthenium red and Gd$^{3+}$ are general inhibitors of cationic channels – including voltage-gated Ca$^{2+}$ channels – and thus do not exclusively block mechanosensitive channels (Boland et al., 1991). Thus, their application is somewhat limited for elucidating the contribution of Piezo1 in a physiological context.

In recent years, pharmacological advancements have increased our understanding of Piezo1 function. In 2015, Syeda et al. identified the first synthetic agonist, termed Yoda1, which acts on both human and mouse Piezo1. Yoda1 affects the sensitivity and slows the inactivation kinetics of Piezo1 response to mechanical stimulation. Reconstituting Piezo1 channels in an artificial membrane also revealed that Yoda1 treatment induced single-channel currents with increased open time. Intriguingly, Yoda1 also induces channel activation in the absence of any mechanical perturbation, suggesting that Piezo1 can be chemically activated as well (Syeda et al., 2015). While this raises the possibility that an endogenous Piezo1 agonist might exist, to date no such molecule has been identified. Since the identification of Yoda1, two more chemical agonists of Piezo channels have been synthesized – Jedi1 and Jedi2 – though Yoda1 is still the most commonly used Piezo1 agonist (Botello-Smith et al., 2019).
Following the identification of the synthetic agonist Yoda1, a synthetic antagonist was engineered in 2018. Evans et al. generated a Yoda1 analogue by altering the pyrazine ring on Yoda1. This analogue, referred to as Dooku1, lacks agonist activity and reversibility antagonized the effects of Yoda1. Evans et al. reported that Dooku1 successfully blocked Yoda1-induced Piezo1 Ca²⁺ entry in HEK293 cells, while having no effect on store-operated Ca²⁺ entry or Ca²⁺ entry via TRPV4 channels (Evans et al., 2018). Dooku1 has been used to block Yoda1-induced Piezo1 activation in a variety of cell types, including pancreatic β-cells and smooth muscle cells (Deivasikamani et al., 2019; Evans et al., 2018). However, it is important to note that Dooku1 is not a direct antagonist of Piezo1, but rather competes against Yoda1.**

### 1.16 Piezo1 expression and physiology

The physiological importance of mechanotransduction in mammalian systems is highlighted by the wide range of cell types and organs in which Piezo1 is expressed. For example, in the seminal 2010 study by Coste et al., mouse Piezo1 mRNA was identified in stomach, lung, intestine, bladder, kidney, and skin tissue. In the ensuing decade, Piezo1 has been further identified in the nervous, renal, and lymphatic systems, as well as a range of cell types such as chondrocytes, osteoblasts, and adipocytes (Koser et al., 2016; Li, Zhang, Chen, Cui, & Zhang, 2017; Liu et al., 2018; Sun et al., 2019; Wang et al., 2016a; Zhao et al., 2019).

Given that Piezo1 is widely expressed in various cell types, it is unsurprising that Piezo1 function is also diverse and involved in a wide range of cellular operations. One of the most well-studied

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** The name ‘Dooku1’ was selected because Dooku1 does not act directly on the Piezo1 protein, but rather competes with the agonist Yoda1. The research team at the University of Leeds who synthesized Dooku1 chose this name as an homage to the battle between Dooku and his former mentor Yoda in Star Wars Episode 2. Of note, the supervisor of this research team has never seen Star Wars.
roles of Piezo1 is its function in endothelial cells. Endothelial cells line blood and lymphatic vessels, forming the barrier between vessels and the adjacent tissue, and serving to control the flow of fluid into and out of tissue (Aman et al., 2016). In terms of the lymphatic system, mouse models lacking endothelial Piezo1 have revealed that Piezo1 is essential for the formation of lymphatic valves. Lymphatic valves are involved in proper circulation of lymph by preventing the backflow of fluid in vessels (Nonomura et al., 2018).

As well, Piezo1 is critical for proper development of blood vasculature and function of the endothelial cells which line blood vessels. Ranade et al. (2014) demonstrated that Piezo1 activates in response to shear stress from blood flow, which is the primary mechanical force in vasculature. Knockdown of Piezo1 in endothelial cells resulted in abnormal endothelial cellular orientation and an inability of endothelial cells to adjust their alignment when subjected to shear stress (Ranade et al., 2014). Shear force also modulates the release of nitric oxide (NO) from endothelial cells, a signalling factor heavily involved in inducing vasodilation. Mechanical stimulation on endothelial cells from shear force triggers endothelial release of ATP. In turn, ATP activates metabotropic P2Y2 receptors and leads to downstream stimulation of NO formation from endothelial cells (Wang et al., 2016b). Wang et al. (2016) demonstrated that Piezo1 was the ion channel responsible for endothelial flow-induced ATP release, and that mice with endothelium-specific Piezo1 deficiency lack the ability to produce NO and regulate vascular tone, ultimately leading to hypertension. The critical role of Piezo1 in vasculature is further emphasized by: 1) Global Piezo1 knockout in mice is embryonically lethal by mid-gestation, with mutant mice demonstrating extensive vascular defects, and 2) In humans, both loss-of-function and gain-of-function mutations in the gene
encoding for Piezo1 result in diseases associated with abnormalities in blood and lymphatic vasculature (Andolfo et al., 2013; Fotiou et al., 2015; Ranade et al., 2014).

In addition to endothelial cells, Piezo1 activation is also associated with ATP release in other cell types. For example, following mechanical stretch, Piezo1 activation leads to Ca\(^{2+}\) influx in bladder urothelial cells. Piezo1-induced Ca\(^{2+}\) influx results in downstream ATP release that regulates bladder constriction (Miyamoto et al., 2014). Piezo1 activation from shear stress is also important in regulating erythrocyte cell volume. In red blood cells, shear stress triggers robust Ca\(^{2+}\) influx and subsequent release of ATP, a process which is attenuated with treatment from ruthenium red and Gd\(^{3+}\) (Cinar et al., 2015). Moreover, in mesenchymal stem cells, Yoda1-induced Piezo1 Ca\(^{2+}\) influx results in P2X\(_7\) receptor activation and ATP release, which ultimately influences cell migration (Wei et al., 2019).

Increasingly, Piezo1 is implicated in CNS physiology as well. Indeed, a 2014 RNA-sequencing report revealed that Piezo1 is expressed in the transcriptome of all cell types in the CNS (Zhang et al., 2014). In neurons, Piezo1 channels are implicated in key processes such as neuronal differentiation, migration, and axon guidance (Hung et al., 2016; Koser et al., 2016; Pathak et al., 2014). Aside from neurons, Piezo1 expression has also been characterized in the brain’s highly mechanosensitive glial cells. As mentioned previously, Piezo1 is upregulated in plaque-reactive astrocytes and mediates cytokine release in cultured astrocytes (Velasco-Estevez et al., 2018; Velasco-Estevez et al., 2019). More recently, Velasco-Estevez et al. (2020) demonstrated that overactivation of Piezo1 promotes neuron-driven demyelination in murine slice culture, while application of the mechanosensitive channel blocker GsMTx4 attenuated demyelination (Velasco-
Estevez et al., 2020). The same study also reported that intracortical injection of lysophosphatidylcholine (LPC), a toxin known to induce demyelination in vivo, increased the number of reactive microglia and astrocytes around the site of injection. Both microglia and astrocyte reactivity was attenuated by co-treatment of GsMTx4 (Velasco-Estevez et al., 2020). While indirect, the findings of Velasco-Estevez et al. (2020) suggest that Piezo1 function is involved in microglia and astrocyte response to pathology.

In the CNS, Piezo1 expression and function has only been investigated in neurons and astrocytes. However, microglia contain the highest transcriptomic expression of Piezo1 of cells in the CNS (Zhang et al., 2014). Given the highly mechanosensitive nature of microglia and the mounting evidence that Piezo1 can regulate immune function, microglia represent an interesting and important cell to further investigate Piezo1 function (Moshayedi et al., 2014; Solis et al., 2019).

1.17 Pathologies associated with Piezo1 dysfunction

In humans, both loss-of-function and gain-of-function mutations in the gene encoding for Piezo1 have been reported (Alper, 2017). The most well-characterized disease associated with a loss-of-function Piezo1 mutation is generalized lymphatic dysplasia (GLD). GLD is a rare, congenital autosomal recessive disease that results in lymphatic dysfunction and widespread lymphoedema. In addition to edema, individuals with GLD can present with seizures and microencephaly (Fotiou et al., 2015; Lukacs et al., 2015). In a 2015 study by Lukacs et al., functional analysis of red blood cells from patients with GLD demonstrated a significantly reduced Piezo1 response to both mechanical stimuli and Yoda1 application. As well, patch clamp analysis of a HEK293 cell line
containing a Piezo1 loss-of-function mutation showed diminished expression of Piezo1 on the cell surface and attenuated mechanosensitive current amplitudes (Lukacs et al., 2015).

The most common Piezo1 gain-of-function mutations in humans also result in abnormal red blood cell function. Autosomal dominant hereditary xerocytosis (HX), also known as dehydrated stomatocytosis, is an anemic disorder characterized by erythrocyte dehydration and mild hemolysis (Andolfo et al., 2013; Rotordam et al., 2019). Approximately 11 different heterozygous Piezo1 missense mutations are associated with different clinical presentations of HX (Andolfo et al., 2013). Mechanistically, HX is a result of cation and osmotic imbalances in red blood cells that occur from increased Ca\(^{2+}\) influx, subsequent K\(^+\) channel activation, and K\(^+\) efflux. This chain of events ultimately results in osmotically driven dehydration of red blood cells (Alper, 2017).

Interestingly, not all mutations associated with the Piezo1 gene are detrimental. In fact, a common gain-of-function Piezo1 variant (E756DEL) is found in one-third of the African population and has shown to be protective against infection from Plasmodium falciparum – the causative parasite for malaria (Ma et al., 2018). Though the exact mechanisms of how the E756DEL variant confers resistance against malaria has yet to be elucidated, red blood cells from patients with HX show a delayed infection rate to the Plasmodium parasite in vitro (Tiffert et al., 2005).

### 1.18 Rationale and hypotheses

Since its identification as a mechanosensitive ion channel in 2010, Piezo1 has emerged as a significant contributor to many important cellular and physiological functions in a range of mechanically sensitive cell types. Piezo1 has been characterized in cells of the renal, respiratory,
and lymphatic systems – and more recently, the nervous system as well. A 2014 transcriptomic report from the Barres lab revealed that Piezo1 is expressed in virtually all cell types of the CNS (Zhang et al., 2014). In neurons, Piezo1 has been linked to mechano-sensitive operations such as neuronal migration, differentiation, and axon guidance. Aside from neurons, Piezo1 has also been examined in astrocytes. For example, evidence from the MacVicar lab indicated that Piezo1 mediates Ca\(^{2+}\) transients in astrocyte fine processes (Ko, 2018). Additionally, Piezo1 was shown to influence astrocyte cytokine release and be upregulated in plaque-associated astrocytes (Velasco-Estevez et al., 2018; Velasco-Estevez et al., 2019). The contribution of Piezo1 to astrocyte cytokine release and plaque reactivity was the first evidence that beyond mechanosensation, Piezo1 channels are also involved in immune function. Supporting this is the recent report that Piezo1 activation is essential for immune response in peripheral myeloid cells (Solis et al., 2019).

As highly mechanosensitive and immune competent cells, microglia represent an interesting candidate for investigating Piezo1 expression and function. Indeed, though microglia contain the highest transcriptomic expression of Piezo1 out of all cell types in the CNS, to date no studies have examined the role of Piezo1 in primary microglia. Therefore, we hypothesize that:

1. Piezo1 channels are expressed in microglia and upregulate in response to immune challenge.
2. Piezo1 channels in microglia are functional and respond to chemical and mechanical stimulation.
Objective 1: Establish Piezo1 channel expression in microglia in vitro.

To examine the expression of Piezo1 in microglia and assess the effects of immune challenge on Piezo1 expression, I performed Western blots and real-time quantitative polymerase chain reaction (RT-qPCR) to quantify Piezo1 protein and mRNA levels at baseline and in response to immune challenge from LPS treatment. Additionally, immunocytochemistry and confocal microscopy was utilized to visualize Piezo1 expression and localization in microglia.

Objective 2: Investigate the functional properties of Piezo1 channels in microglia.

Microglia were pre-loaded with the Ca$^{2+}$ dye Fluo-4 AM and then imaged with confocal microscopy to elucidate the functional properties of Piezo1 channels. Using this Ca$^{2+}$ imaging technique, I assessed the effects of pharmacological Piezo1 agonist and antagonist activity on Piezo1 channel activation and Ca$^{2+}$ dynamics. In addition to chemical activation, I also investigated the effects of microglial Piezo1 mechanical activation using an osmotic stress model.
Chapter 2: Material and Methods

2.1 Preparation of primary microglia culture

Rat microglia were obtained from embryonic day 18 Sprague-Dawley rats of either sex. Pups were harvested from timed-pregnant Sprague-Dawley rats (*Rattus norvegicus*, Jackson Laboratories, Sacramento, CA) into 150-mm Petri dishes. Pup brains were removed, and cortices were dissected and placed in pre-chilled Hanks’ Balanced Salt Solution (StemCell Technologies) on ice. Tissue for primary microglia culture was provided by Dr. Shernaz Bamji’s lab.

The dissociated cortices were gently agitated via pipette to manually separate the tissue, and then filtered through a 75 μm nylon mesh. Tissue was then resuspended in Dulbecco’s Modified Eagle Medium (DMEM/F12; Gibco) supplemented with 10% fetal bovine serum (FBS; Gibco), and 1% each of penicillin and streptomycin (Gibco). Cells were plated at a density of 2-3 x 10^6 cells per Petri dish and grown for 10-14 days until confluent in a 37°C incubator with 5% CO₂ in DMEM/F12 media.

To dislodge and isolate microglia from the culture, Petri dishes were shaken at 80 rpm for 2 hrs (Digital Shaker; Southwest Science) in the incubator. Supernatant was collected into a 10 mL Falcon tube in the biosafety cabinet (BSC) and spun down at 2400 rpm for 6 mins using the Universal 320 R centrifuge (Hettich). Next, the supernatant was removed in the BSC via suction while leaving the pellet of microglial cells intact at the bottom. 500 mL of fresh media was immediately added into the Falcon tube and microglia were resuspended via gentle agitation using a pipette. Microglia were then plated according to the experiment. To minimize microglial non-
specific activation during experiments, FBS serum was reduced to 2% in the DMEM/F12 media after microglia were plated.

For Western blotting and RT-qPCR experiments, resuspended microglia were plated directly onto the bottom of a 96 well-plate. Cells were transferred to the incubator for 1 hr to allow for adhesion to the well-plate, and then flooded with 100 μL fresh media and left overnight. For Ca^{2+} imaging, cells were plated onto 10 mm diameter glass coverslips (poly-D-lysine-coated; MatTek) placed within 24 well-plates, and then transferred to the incubator for 1 hr to allow for cell adhesion to the coverslip. Wells were then flooded with 1 mL fresh media and left overnight.

2.2 Immune activation in primary microglia culture

To evaluate protein and mRNA levels of Piezo1 in microglia following immune activation, LPS, LPS-EB Ultrapure (LPS-EB), and Zymosan-A (ZA), were applied to microglia culture. LPS is an endotoxin and general macrophage-activator, while LPS-EB and ZA are agonists of the immune receptors TLR-4 and -2, respectively (Hirschfeld et al., 2000; Sato et al., 2003).

To incubate plated microglia with the immune activating compounds, media was discarded and then replaced with fresh media containing either LPS (100 ng/mL; Sigma-Aldrich), LPS-EB (1 μg/mL; Invitrogen), or ZA (10 μg/mL; Cayman). Cells were treated for either 2 or 24 hrs depending on the experiment. All treatment conditions were compared against untreated controls from the same culture. Controls had fresh media added at the same timepoints as experimental conditions.
2.3 Western blots

Western blotting was performed to assess the effects of immune activation on Piezo1 protein levels in cultured microglia. Cells were treated with either LPS, LPS-EB, or ZA for 2 or 24 hrs. Media was removed via suction and cells were collected and homogenized on ice in RIPA lysis buffer (Tris Buffered Saline (TBS); Cedarlane, with 0.1% Tween20; VWR). Cells were centrifuged at 4°C for 20 min at 12,000 rpm to remove debris. Protein concentration was determined using a Protein Standard Assay (Bio-Rad) and equal protein amounts were then incubated at room temperature overnight in 2x Laemmli Sample buffer with 5% β-mercaptoethanol.

The next day, samples were loaded into 4-20% graded pre-cast gels (Mini-PROTEAN TGX Gels; Bio-Rad). Gels were run for 30 mins at 120 V in 1x running buffer (192mM glycine, 25mM Tris and 0.1% SDS; pH8.3) and then transferred to a polyvinylidene fluoride membrane using the Trans-Blot Turbo semi-dry transfer system (Bio-Rad). Membranes were blocked for 30 mins in blocking buffer (5% skim milk powder in TBS with 0.1% Tween20). Following this, membranes were incubated with blocking buffer containing primary antibodies and 5% normal donkey serum overnight at 4°C with gentle rocking. Primary antibodies used were: 1:500 anti-Piezo1 (rabbit; Alomone Labs) and 1:500 β-actin (rabbit; Santa Cruz Biotechnology). The following day, membranes were washed 3x for 10 mins each in 1x TBS buffer with 0.1% Tween20 (TBST) before secondary antibody incubation. Secondary antibodies in blocking buffer were applied for 2 hrs at room temperature with gentle rocking. Secondary antibodies used were 1:1000 horseradish peroxidase-conjugated donkey anti-rabbit (Jackson Immunoresearch). Membranes were washed again 3x for 10 mins in TBST, with a final 10 min wash in TBS. A chemiluminscent pen (Thermo
Fisher Scientific) was used to visualize the protein bands. Blots were imaged using a C-DiGit western blot scanner (LI-COR) with Image StudioTM Lite software.

2.4 Real-time quantitative polymerase chain reaction

RT-qPCR was performed to assess the effects of immune activation on Piezo1 mRNA levels in microglia culture. Cells were treated with either LPS, LPS-EB, or ZA for 2 or 24 hrs. To collect the cells for RT-qPCR, samples in the well-plates were washed twice with phosphate buffered saline (PBS; ThermoFisher), then PBS was removed via suction. 200 μL TRIzol (Invitrogen) was added to each well and samples were shaken on ice for 20 mins to dislodge cells.

Samples were collected and incubated for 5 mins at room temperature. Next, samples were vortexed briefly, 20 μl BCP (1-bromo-3-chloropropane) was added, and then samples were vortexed briefly again. Samples were then centrifuged at 12,000 rpm and 4°C for 10 minutes. 100 μl of the upper aqueous phase from each sample was collected and added to a 96 well-plate with a conical bottom for processing. 50 μL isopropanol was added to each sample, briefly mixed mechanically via pipette, and then shaken for 1 min using a SkyLine Shaker-Thermostat (Rose Scientific Ltd.). Following this, 10 μL of RNA nanomag-D magnetic binding beads (Micromod Partikeltechnologie) was added to each sample, mixed mechanically via pipette, and then shaken for 3 mins. Beads were magnetically captured for 1-2 mins and supernatant was discarded. To wash the beads, 150 μL of magnesium (Mg^{2+}) wash solution was applied twice using the following procedure: Mg^{2+} solution was added to the wells, samples were shaken for 1 min, beads were magnetically captured for 1 min, and then supernatant was discarded. Beads were dried for 2 mins.
by shaking. To resuspend the beads, 55 µL of elution buffer was added and samples were shaken for 2 mins. Beads were magnetically captured with the resulting supernatant containing total RNA.

The RNA concentration of each sample was assessed using a nanophotometer (Implen). cDNA synthesis was performed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) with quantities of each reagent determined using the kit template. cDNA tubes were then spun down and samples were run using a SpectraCount (Neuromed Technologies Inc.).

Gene-specific RT-qPCR was performed using the KAPA Probe Fast Universal qPCR Kit (KAPA Biosystems). Samples were mixed with a combination of 2x Mix Master and endonuclease free water according to kit template. Samples then were combined with a gene-specific primer and TaqMan probe to amplify the gene of interest and run in triplicate using a CFX384 Real-Time PCR System (Bio-Rad) with BioRad CFX Mangaer 3.1 software. The TaqMan probes used were PIEZO1 (rPiezo1-1 Rn.PT.58.12896870; rPiezo1-2 Rn.PT.58.46120084; both IDT), and GAPDH (rGAPDH Rn.PT.58.35727291; IDT) for the internal control.

2.5 Immunocytochemistry

Immunocytochemistry was performed to visualize Piezo1 in cultured microglia. Microglia were either remained untreated as controls or were treated for 24 hrs with LPS to visualize differences in the pattern of channel expression following immune activation.

To fix cells for immuno-staining, coverslips with plated microglia were transferred into 4% paraformaldehyde (PFA; FD NeuroTechnologies Inc.) for 10 mins to fix cells. Following fixation,
cells were washed 3x with PBS. To permeabilize the cells, coverslips were placed into a permeabilization solution comprised of PBS and 0.05% Tween20 for 30 mins. Permeabilization solution was removed via suction and 50 μL blocking solution was applied to each coverslip for 1 hr. Blocking solution contained PBS with 0.05% Tween20 and 5% normal goat serum (Cedarlane). Fixation, washes, permeabilization, and blocking all were performed at room temperature.

Blocking solution was then removed via suction and 50 μL of the primary antibody solution was applied to each coverslip. Primary antibody solution consisted of PBS with 0.05% Tween20, 5% normal goat serum, and primary antibody. Primary antibodies used were: 1:200 Piezo1 (rabbit; Alomone) and 1:200 CD11b (rat; Invitrogen). Coverslips were left in primary antibody solution overnight in 4°C. The following day, coverslips were washed 3x in PBS and 50 μL of the secondary antibody solution was applied. Secondary antibody solution consisted of PBS with 0.05% Tween20, 5% normal goat serum, 1:2000 4′,6-diamidino-2-phenylindole (DAPI; Thermo Fisher), and secondary antibody. Secondary antibodies used were: 1:1000 goat anti-rabbit Alexa Fluor-568 and 1:1000 goat anti-rat Alexa Fluor- 488 (both from Cedarlane). Coverslips were incubated in secondary antibody solution for 2 hrs at room temperature. Finally, coverslips were washed 3x with PBS and mounted on slides with FluorSave Reagent (EMD Millipore).

2.6 Stab wound procedure in Piezo1-tdTomato mice

All in vivo procedures were performed according to protocols approved by the University of British Columbia Animal Care Committee. Preliminary data was collected from the tissue of Piezo1-tdTomato mice expressing Piezo1 fused to the fluorescent marker tdTomato (Ranade et al.,
To examine the effects of immune challenge on Piezo1 expression in microglia *in vivo*, a cortical stab wound assay was performed on post-natal day (P) 59 mice.

Mice were deeply anesthetized with 5% isoflurane in an incubation chamber and transferred to a surgical stage. Anesthesia was maintained with 1-2% isoflurane throughout the procedure. Mice were kept on a warming pad for the duration of the surgery to maintain a body temperature of ~37ºC. Temperature was monitored throughout the surgery via rectal probe. To prepare for the surgery, fur was shaved off of the scalp with a small beard trimmer and eye ointment was applied to prevent eyes from drying during the procedure. Mice were then fixed in a stereotaxic frame. A local anesthetic was injected intradermally on the scalp at the point of incision (Bupivicaine 2.5 mg/kg). The surgical area was sterilized with chlorhexidine, wiped with 70% alcohol three times, and then covered in a sterile fenestrated drape.

A midline incision was made along the dorsal surface of the scalp in order to expose the landmark bregma. A hole was drilled using a dental drill to gain access to the cortex. To perform the stab wound assay, a borosilicate pipette was pulled and the tip was broken off. The pipette was lowered 2 mm into the brain into one hemisphere, with the other hemisphere serving as the control. No substance was injected during this procedure. Following the stab wound, skin over the scalp was sutured together.

### 2.7 Immunohistochemistry

24 hrs after the stab wound surgery was performed, Piezo1-tdTomato mice were perfused for tissue collection. Mice were anesthetized with 5% isoflurane and given an intraperitoneal injection of
urethane (0.1 mL of 30% urethane per 10 g body weight). Mice were then transcardially perfused with 0.1 M PBS, followed immediately by 4% PFA. Brains were extracted and immersed in a post-fix sucrose solution (10% sucrose in 4% PFA) at 4°C overnight. The next day, brains were immersed in a cryoprotection solution (30% sucrose in 0.1 M PBS) and left overnight at 4°C. The following day, brains were immersed for the final time in fresh cryoprotection solution and left overnight at 4°C.

After the final solution change, brains were placed on a 150 mm Petri dish, covered with optimal cutting temperature compound (Fisher Scientific) and frozen overnight at -80°C to prepare for slicing on the cryostat. Using a cryostat (Leica), 20 μm coronal sections around the stab wound were collected onto glass slides and stored at -20°C.

Tissue sections were rehydrated by immersing the slides in PBS for 10 mins with gentle shaking. Sections were then blocked for 1 hr at room temperature with PBS containing 3% BSA and 0.3% Triton X. During blocking, slides were placed in a humid chamber and covered with a small piece of parafilm to prevent tissue drying. Blocking solution was removed and 75 – 100 μL of primary antibodies diluted in buffer (PBS with 3% BSA, 0.3% Triton X, and 5% normal goat serum) was then pipetted on top of the sections and slides were left overnight at 4°C. Sections were then rinsed in PBS and incubated with secondary antibodies diluted in PBS buffer for 1 hr at room temperature. Finally, slides were rinsed again in PBS and coverslipped with FluorSave Reagent. Primary antibodies used were: 1:200 Piezo1 (Alomone) and 1:200 CD11b (Invitrogen). Secondary antibodies used were: 1:300 donkey anti-rabbit Alexa Fluor 568; 1:300 donkey anti- rat Alexa Fluor 488; and 1:1000 DAPI (all from Invitrogen).
2.8 **Confocal image acquisition**

Following immuno-staining, images were acquired using a TCS SP8 upright confocal laser scanning microscope (Leica) with a 60x oil-immersion objective lens. The acquisition parameters, such as the laser power and gain, were set using the control samples for every experiment and kept consistent throughout imaging experimental replicates.

2.9 **Ca\(^{2+}\) Imaging of microglia culture**

For Ca\(^{2+}\) imaging, microglia were plated on glass coverslips as outlined in section 2.1. Prior to imaging, microglia were incubated with the Ca\(^{2+}\) indicator Fluo-4 AM (Thermo Fisher) for 30-40 mins and left in the incubator until ready to image. Fluo-4 AM was pre-dissolved in DMSO at a 1:10 dilution, and then added to the culture media at a 1:2000 dilution. Coverslips were then transferred from the incubator to a bath containing artificial cerebrospinal fluid (aCSF) for imaging.

Experiments were performed at room temperature using HEPES-buffered aCSF containing (in mM): NaCl, 140.4; HEPES, 10.8; KCl, 2.5; glucose 10.0; MgCl\(_2\), 2.0; CaCl\(_2\), 2.0. HEPES buffer was used in place of NaHCO\(_3\) buffer to prevent precipitation with Gd\(^{3+}\). For experiments in hypotonic aCSF, the NaCl concentration was reduced to 60.0 mM to reduce the osmolarity from 300 mOsm to 150 mOsm. Experiments performed in Ca\(^{2+}\)-free conditions omitted CaCl\(_2\) from the aCSF, but contained an additional 2.0 mM MgCl\(_2\) and 2.0 mM of the Ca\(^{2+}\) chelator ethylene glycol tetra-acetic acid (EGTA; Sigma) instead. All experiments were performed with continuous perfusion (1.5 mL/min) of aCSF solution. HEPES-buffered aCSF and experimental procedure for Ca\(^{2+}\) imaging adapted from Ko (2018).
For experimental manipulations, the following chemicals were used: Yoda1 (1, 5, 10, or 20 µM; Tocris), Gd³⁺ (10 µM; Tocris), pyridoxal phosphate-6-azophenyl-2',4'-disulfonic acid (100 µM; PPADS; Tocris), ATP (100 µM; Sigma), and LPS. Stock solutions of Yoda1 were prepared in dimethyl sulfoxide, while all others were prepared in water. All chemicals were diluted in aCSF immediately prior to use. For experiments involving LPS, microglia were incubated with 100 ng/mL LPS for 2 hrs prior to imaging.

Microglia Ca²⁺ signals were imaged with a confocal microscope (Zeiss), using a 40x water immersion lens. Microglia loaded with Fluo4-AM were excited at 488 nm and the emission was detected by a photomultiplier tube after passing through a 500 – 550 nm filter. Transmitted light was also collected to identify cells. Images were acquired as a time series of 350 x 600 pixels using Zen 2009 (Zeiss) software at a frame rate of 2.6 frames/sec and 8-bit depth.

2.10 Western blot analysis

Western blot images were analyzed in Fiji (ImageJ; NIH). Images were colour-inverted, and the rectangular selection tool was used to highlight bands of interest and background. Mean intensity of each band and the adjacent background was acquired, and these measurements were transferred to Excel (Microsoft). Each band of interest was processed by subtracting the adjacent background intensity to control for noise and each experimental condition was normalized to a control lane from the same blot. Control intensity levels were given the value 1.0 and experimental samples were calculated as relative to controls and portrayed in terms of fold-difference.
2.11 RT-qPCR analysis

RT-qPCR data was analyzed by importing the mRNA quantification results obtained from the BioRad CFX Manager 3.1 software into Excel. Each sample was run in triplicate and then values were averaged together for analysis. Piezo1 mRNA expression levels were normalized to the internal control GAPDH. Experimental conditions with either 2 hr or 24 hr LPS treatment were compared against untreated controls from the same cell culture. Untreated control mRNA levels were assigned the value 100.0 and experimental mRNA levels were calculated as percent relative to controls.

2.12 Ca\(^{2+}\) imaging processing and analysis

Time series acquired from microglial Ca\(^{2+}\) imaging were processed using Fiji. The fluorescent channel was separated from the transmitted light channel, and then an initial threshold was set to remove as much background noise as possible from the data set while still maximizing detection of the cells. To binarize the pixels associated with cells from the background, images were passed through a second filter - the automated Otsu threshold filter. Otsu applied a single-intensity threshold to the image series in order to create a binary mask, wherein the background was assigned a value of zero and each cell that passed the intensity threshold was assigned a value of one. This mask was then multiplied over the original fluorescent series, so that the background remained at the assigned value of zero, while each cell above the threshold retained its raw fluorescent intensity value. Following this, regions of interest (ROIs) were manually drawn around individual cells with the rectangular selection tool. An additional ROI was drawn that contained only background. This was used to subtract the average background intensity per slide from each ROI containing a cell. Cells were excluded from further analysis based on the following criteria:
poor dye loading with Fluo-4 AM, saturated fluorescent intensity, no response to the positive control ATP, moved out of the frame or ROI, located on the edge of the field of view, did not appear to be microglial cells, or were clustered with other cells and therefore could not be individually isolated. ROIs for selected cells were then converted to TIFF stacks and imported into MATLAB (MathWorks). A custom MATLAB script was used to record changes in the fluorescent intensity for each pixel in the ROI over time. Changes in intensity were normalized relative to the assigned baseline, which was defined as the frames recorded five minutes prior to application of Yoda1 ($\Delta F/F_0$). Output from the MATLAB analysis contained the normalized fluorescent values for each frame over the time series per cell. These values were then exported to Excel for further analysis. The pipeline for Ca$^{2+}$ imaging analysis is summarized in Figure 2-1.

Ca$^{2+}$ imaging data was quantified in two ways: 1) The peak Ca$^{2+}$ response during Yoda1 and/or ATP application, which was calculated as the difference in fluorescence intensity between the maximum fluorescence and the average baseline intensity. The baseline was defined as the average fluorescence during the five minutes of imaging prior to Yoda1 application. 2) The cumulative Ca$^{2+}$ response over time when Yoda1 was applied, which was calculated as the increase in amplitude of the Ca$^{2+}$ response above the baseline, ($\Delta F/F_0$)$(\text{min})$, and indicates the area under the curve (AUC). Values for peak Ca$^{2+}$ response and AUC were calculated in Excel. Each data set with an experimental manipulation was compared relative to the control condition for statistical analysis.
2.13 Statistical analysis

All data values are expressed as means ± standard error of the mean (SEM). Raw data handling and calculation was performed in Excel and then imported into GraphPad Prism (version 8.0) for statistical analysis. Statistical significance was determined using one-way analyses of variance (ANOVA) with Dunnett’s multiple comparison post-hoc test, or unpaired t-test with Welch’s correction, as indicated in figure legends. Statistical significance was defined as p<0.05. P-values are indicated in figures as: * = p<0.05, ** = p<0.01, *** = p<0.001, and **** = p<0.0001.
Figure 2-1. Flowchart of Ca\textsuperscript{2+} imaging analysis.
Chapter 3: Results

3.1 Piezo1 gene is expressed in the microglial transcriptome

In 2014, the Barres lab published an extensive RNA-seq report on cells isolated from the mouse cerebral cortex. Using cell type-specific purification, cells were differentiated into neurons, astrocytes, microglia, oligodendrocytes, and endothelial cells, followed by bulk RNA sequencing to characterize differential gene expression in each cell type (Zhang et al., 2014). Findings from this RNA-seq report indicated that Piezo1 is expressed in virtually all cell types in the brain, but most robustly expressed in microglia (Figure 3-1). Further, preliminary immuno-blot data from the MacVicar lab (data not shown; conducted by Dr. Leigh Wicki-Stordeur) suggested that microglial Piezo1 protein levels could be modulated by immune challenge through treatment with the inflammatory stimulus LPS. Therefore, our first objective was to establish Piezo1 expression in microglia, and then examine how immune challenge alters microglial Piezo1 protein expression.

Figure 3-1. Piezo1 gene is robustly expressed in microglia isolated from mouse cortex.
Transcriptomic report of Piezo1 gene expression in various cell types isolated from the mouse cerebral cortex. Piezo1 is most highly expressed in microglial cells, though it is expressed in endothelial cells, astrocytes, neurons, and oligodendrocyte precursor cells as well. Units of quantification expressed in fragments per kilobase of exon model per million reads mapped. RNA-seq conducted by Zhang et al. (2014) and Piezo1-specific gene expression results accessed from https://www.brainrnaseq.org/.
3.2 Microglial Piezo1 protein expression increases following immune challenge

To both establish Piezo1 expression in microglia and investigate how immune challenge modulates Piezo1 expression, we performed Western blot analysis on cultured microglia (Figure 3-2). Microglia were incubated for either 2 or 24 hrs with LPS, or with the TLR-4 and 2 agonists LPS-EB and ZA, respectively. All experimentally treated microglia were compared against untreated controls from the same culture. An example Western blot of the protein expression of Piezo1 compared to β-actin across all conditions is illustrated in Figure 3-2 (A).

First, we treated microglia with LPS, an endotoxin found on the outer membrane of gram-negative bacteria. LPS is commonly used to induce sterile inflammatory responses (Bertani & Ruiz, 2018). As shown in Figure 3-2 (A), Piezo1 protein levels were low in controls and remained low after 2 hrs of treatment with LPS. However, Piezo1 protein expression increased after 24 hrs of incubation with LPS. Quantification of Piezo1 expression for LPS treated microglia (B) revealed a significant 0.613 ± 0.103 (n = 14; p<0.0001) increase of Piezo1 protein expression in 24 hr LPS treated compared to control. Average intensity for 24 hr LPS = 1.613 ± 0.114 (n = 14). There was no significant change after 2 hrs (difference = 0.158 ± 0.146; n = 6) compared to control. Average intensity for 2 hr LPS = 1.158 ± 0.258; n = 6.

LPS acts on microglia to induce an immune response via a range of receptors and pathways (Qin et al., 2005). To elucidate which receptors were involved in mediating Piezo1 upregulation in response to LPS, we incubated microglia with agonists for either TLR-4 or TLR-2 (Hirschfeld et al., 2000; Sato et al., 2003). TLRs are a crucial component of innate immunity. Members of the family of TLR receptors recognize exogenous pathogenic ligands (PAMPs), or endogenous...
molecules that signal host tissue injury (DAMPs). Activation of TLRs leads to downstream signaling pathways and influences the production of inflammatory cytokines (Goulopoulou et al., 2016). We chose to examine TLR-4 and TLR-2 due to their relatively high level of expression in microglia and their known involvement in mediating LPS-induced microglial activation (Fernandez-Lizarbe et al., 2013; Qin et al., 2005). As shown in Figure 3-2 (A), when incubated with the TLR-4 agonist LPS-EB or the TLR-2 agonist ZA, microglial Piezo1 protein expression increased compared to control at the 24 hr timepoint. Quantification of immunoblot band intensity for LPS-EB (C) revealed a significant $0.752 \pm 0.140$ (n = 8; p < 0.01) increase of Piezo1 protein expression after 24 hrs compared to controls (control n = 8). Average band intensity for 24 hrs LPS-EB = $1.751 \pm 0.157$ (n = 8). In LPS-EB treated microglia, quantification of the average band intensity at 2 hrs treatment also revealed a significant increase of $0.289 \pm 0.154$ (n = 6; p < 0.05) compared to controls. Average band intensity for 2 hrs LPS-EB = $1.289 \pm 0.203$ (n = 6). Quantification of microglia treated with ZA for 24 hrs (D) demonstrated a $0.558 \pm 0.125$ (n = 9; p < 0.001) increase in Piezo1 expression compared to controls. Average band intensity for 24 hrs ZA = $1.558 \pm 0.132$ (n = 9). 2 hrs ZA treatment demonstrated a significant $0.208 \pm 0.0525$ decrease (n = 6; p < 0.01) in band intensity compared to controls. Average band intensity for 2 hrs ZA = $0.791 \pm 0.069$. Our findings indicate that microglial Piezo1 protein expression robustly increases in response to immune challenge from LPS at 24 hrs. Further, this effect could be mediated in part by activation of TLR-4 and TLR-2, as Piezo1 protein expression increases 24 hrs after LPS-EB and ZA treatment as well. Overall, this suggests that Piezo1 channels are 1) expressed in microglia, 2) increase expression in response to immune challenge, and 3) are likely involved in microglial immune response.
Figure 3-2. Piezo1 protein expression increases by 24 hrs in cultured microglia following immune challenge.

(A) Representative Western blot of whole cell lysate showing relative protein expression of Piezo1 in cultured rat microglia. Cells were treated with LPS, LPS-EB, or ZA for either 2 or 24 hrs and compared to an untreated control. Western blot demonstrates an increased band intensity for all 24 hr conditions, relative to control and 2 hrs. (B-D) Quantification of Western blots from (A) LPS, (B) LPS-EB, and (C) ZA treated microglia shows relative Piezo1 protein expression at 2 and 24 hrs compared to untreated controls. (B) Microglia treated with LPS showed a significant 0.613 ± 0.103 (n = 14; p<0.0001) increase in Piezo1 protein expression compared to control at 24 hrs. There was no significant difference (n.s.) between 2 hr LPS and control (0.158 ± 0.146; n = 6). Average Piezo1 expression for 2 hr LPS treated microglia = 1.158 ± 0.258 (n = 6). Average Piezo1 intensity for 24 hr LPS = 1.613 ± 0.114 (n = 14). (C) Microglia treated with LPS-EB showed a significant increase at 2 hrs treatment (fold difference = 0.289 ± 0.154; n = 6; p < 0.05) and 24 hrs (fold difference = 0.752 ± 0.140 (n = 8; p < 0.01) compared to control. Average Piezo1 expression for 2 hrs LPS-EB treatment = 1.289 ± 0.203 (n = 6), and 24 hrs LPS-EB treatment = 1.751 ± 0.157 (n = 8). (D) Microglia treated with ZA showed a significant decrease in Piezo1 expression of 0.208 ± 0.052 (n = 6; p < 0.01) at 2 hr treatment and a significant increase of 0.558 ± 0.125 (n = 9; p < 0.001) at 24 hr treatment compared to control. Average Piezo1 expression for 2 hrs ZA treatment = 0.791 ± 0.069 (n = 6), and 24 hrs ZA treatment = 1.557 ± 0.132 (n=9). Each treated condition
was normalized and compared to untreated controls which were from the same culture and run on the same blot. Y-axis represents relative Piezo1 protein expression as fold-change compared to untreated control. The number of different cultures used in each condition was 8, 5, and 4 for LPS, LPS-EB, and ZA, respectively. Results were analyzed with one-tailed, un-paired t-test with Welch’s correction; * = p<0.05; ** = p<0.01; *** = p<0.001; **** = p<0.0001; error bars show SEM.

3.3 Microglial Piezo1 mRNA expression increases following immune challenge

Next, we quantified Piezo1 mRNA expression in primary microglia using RT-qPCR. To induce immune challenge in microglia, we incubated microglia for 2 or 24 hrs with LPS and then quantified Piezo1 mRNA compared to untreated controls. Results are displayed as percent change compared to control ± SEM. As shown in Figure 3-3 (A), Piezo1 mRNA levels significantly increased by 154.30 ± 28.88 (n = 7; p < 0.001) after 2 hrs of treatment with LPS compared to control. Average Piezo1 mRNA expression at 2 hr LPS = 254.257 ± 35.022 (n = 7). After 24 hrs of treatment with LPS, Piezo1 mRNA expression significantly decreased by 19.08 ± 6.929 (p < 0.01) compared to control. Average Piezo1 mRNA expression at 24 hrs LPS = 80.922 ± 7.808 (n = 8).

Following this, we incubated microglia with LPS-EB and ZA to explore the effects of TLR-4 and TLR-2 activation on Piezo1 mRNA levels, respectively. As demonstrated in (B), treatment with LPS-EB for 2 hrs resulted in a significant Piezo1 mRNA expression increase of 83.34 ± 24.99 (n = 7; p < 0.01) compared to control. Average Piezo1 mRNA expression at 2 hr LPS-EB = 183.341 ± 26.625 (n = 7). Treatment with LPS-EB for 24 hrs resulted in a significant 34.290 ± 5.620 (n = 7; p < 0.001) decrease in Piezo1 mRNA expression compared to control. Average Piezo1 mRNA expression at 24 hrs LPS-EB = 65.718 ± 6.439 (n = 7).
As demonstrated in (C), treatment with ZA for 2 hrs resulted in a significant 40.62 ± 10.42 (n = 10; p < 0.01) increase of Piezo1 mRNA expression compared to control. Average Piezo1 mRNA expression at 2 hrs ZA = 140.624 ± 10.42 (n = 10). Treatment with ZA for 24 hrs resulted in a significant decrease of 47.84 ± 3.080 (n = 8; p < 0.0001) compared to control. Average Piezo1 mRNA expression at 24 hrs ZA = 52.157 ± 3.079 (n = 8). Piezo1 mRNA levels across all conditions were normalized to the internal control GAPDH. Each sample was subsequently analyzed relative to a control sample taken from the same culture.

Overall, our findings suggest that microglial Piezo1 mRNA is transiently upregulated in microglia following either immune challenge with LPS or immune receptor activation via LPS-EB or ZA. In line with our finding that microglial Piezo1 protein levels are influenced by immune challenge, our findings in Figure 3 suggest that Piezo1 mRNA expression is also modulated following immune challenge to microglia. Taken together, our results suggest a timeline in which Piezo1 mRNA levels increase within 2 hrs of immune challenge, followed by an increase in Piezo1 protein levels by 24 hrs.
Figure 3. Piezo1 mRNA expression increases in cultured microglia at 2 hrs following immune challenge.

Quantification of RT-qPCR analysis of Piezo1 mRNA expression in cultured rat microglia treated with either LPS (A), LPS-EB (B), or ZA (C). (A) Microglia with 2 hrs LPS treatment showed a significant 154.30 ± 28.88 (n = 7; p < 0.001) increase in Piezo1 mRNA expression compared to control. After 24 hrs LPS treatment, Piezo1 mRNA expression significantly decreased by 19.08 ± 6.929 (p < 0.01) compared to control. Average Piezo1 mRNA expression at 2 hr LPS = 254.257 ± 35.022 (n = 7), and at 24 hrs LPS, average Piezo1 mRNA expression = 80.922 ± 7.808 (n = 8). (B) Microglia with 2 hrs LPS-EB treatment resulted in a significant Piezo1 mRNA expression increase of 83.34 ± 24.99 (n = 7; p < 0.01) compared to control. After 24 hrs LPS-EB treatment, Piezo1 mRNA expression significantly decreased by 34.29 ± 5.620 (n = 7; p < 0.001) compared to control. Average Piezo1 mRNA expression at 2 hr LPS-EB = 183.341 ± 26.625 (n = 7) and at 24 hrs LPS-EB, average Piezo1 mRNA expression = 65.718 ± 6.439 (n = 7). (C) Microglia with 2 hrs ZA treatment resulted in a significant Piezo1 mRNA expression increase of 40.62 ± 10.42 (n = 10; p < 0.01) compared to control. After 24 hrs ZA treatment, average Piezo1 mRNA expression significantly decreased by 47.84 ± 3.080 (n = 8; p < 0.0001). Average Piezo1 mRNA expression at 2 hr ZA = 140.624 ± 10.42 (n = 10, and at 24 hrs ZA, average Piezo1 mRNA expression = 52.157 ± 3.079 (n = 8). The number of different cultures used in each condition was 8, 5, and 3 for LPS, LPS-EB, and ZA, respectively. Y-axis represents relative Piezo1 protein expression as percent (%) increase compared to untreated control. Results were analyzed with one-way ANOVA and Dunnett’s multiple comparison’s test; ** = p<0.01; *** = p<0.001; **** = p<0.0001; error bars show SEM.
3.4 Microglia demonstrate different patterns of Piezo1 expression following immune challenge

To visualize the expression pattern of Piezo1 in microglia, we performed immunostaining on cultured microglia. First, we stained untreated microglia to explore Piezo1 expression at baseline. Next, we stained microglia which had been incubated for 24 hrs with LPS to explore whether immunoreactive microglia demonstrated a different pattern of Piezo1 expression.

Figure 3-4 demonstrates Piezo1 immunofluorescent patterns in untreated microglia compared to 24 hr-LPS treated microglia. We observed that in untreated microglia, Piezo1 immunofluorescence showed a characteristic expression pattern (A) that was dramatically altered by LPS treatment (B). In untreated microglia (A), Piezo1 immunofluorescence was clustered close to the DAPI-stained nucleus. Although further analyses are required, it is possible that at least some of Piezo1 immunostaining around the nucleus could be due to Piezo1 co-localization with the ER. Indeed, a previous study reported that Piezo1 was localized to the ER based on immunostaining and overexpression of reporter-tagged constructs in an ovarian cell line (McHugh et al., 2012). Following immune challenge via LPS, microglia demonstrated an increased as well as an altered pattern of Piezo1 immunofluorescence staining. The immunofluorescent staining around the nucleus was no longer observed whereas now there was a distinctive pattern of staining at the peripheral edges of the microglia (B). This is congruent with previous reports that Piezo1 is located in the cell membrane (Cox et al., 2016). Overall, our results show that Piezo1 expression is altered in immunoreactive microglia. Further, the increase in global Piezo1 immunofluorescence between untreated and LPS treated microglia suggests that there is an overall upregulation of Piezo1 in response to immune challenge.
Figure 3-4. LPS treated microglia demonstrate changes in the pattern of Piezo1 expression compared to untreated controls.

Confocal microscopy was used to acquire single-plane images of cultured microglia. Acquisition settings and laser intensities were kept the same during imaging of (A) untreated controls and (B) 24 hr LPS treated microglia from the same culture. Untreated controls demonstrate an overall lower Piezo1 immunofluorescent intensity compared to LPS treated cells. In untreated controls, Piezo1 staining overlaps with the nuclear stain DAPI and also forms a cluster-like pattern around the nuclear area (indicated by dotted white line). In LPS treated microglia, Piezo1 is localized to the peripheral edge of the cell membrane. Replicates of this experiment; n = 3; taken from 3 different cultures. Scale bars = 10 µm.

3.5 Piezo1 is differentially expressed in microglial fine processes following immune challenge

Next, we co-stained cultured microglia with Piezo1 and CD11b. CD11b is a part of a heterodimer integrin complex that is commonly used as a marker of macrophages and microglia. As an integrin, CD11b is a transmembrane protein that functions to link the extracellular matrix to the actin cytoskeleton of the cell (Roy et al., 2006). Co-staining microglia with Piezo1 and CD11b was
performed to investigate Piezo1 expression in the cell membrane. We investigated Piezo1 expression in untreated control cells, as well as microglia treated for 24 hrs with LPS. This provided insight as to whether Piezo1 expression patterns differ in immunoreactive microglia.

In untreated microglia co-stained with Piezo1 and CD11b antibodies, we observed that Piezo1 expression was overall dim, but especially low in the peripheral cell membrane and fine processes (Figure 3-5). However, following incubation with LPS for 24 hrs, immunoreactive microglia demonstrated a different pattern of Piezo1 expression (Figure 3-6). Beyond a global increase in Piezo1 immunofluorescence, we observed an increase in Piezo1 expression along the edge of the cell membrane and in the fine processes (Figure 3-6). The increase in Piezo1 expression along the peripheral membrane in immunoreactive microglia may contribute to an increase in microglial mobility following immune challenge (Bollmann et al., 2015). Additionally, the increased Piezo1 expression in the fine processes may confer a heightened sensitivity of microglia to their environment when an immune challenge is detected.
Figure 3-5. Untreated microglia demonstrate low Piezo1 expression in cell membrane and fine processes.
Confocal microscopy was used to acquire single-plane images of untreated cultured microglia. The microglial marker CD11b demonstrated robust expression in the peripheral cell membrane and in microglial fine processes. In contrast to CD11b, untreated microglia demonstrated low Piezo1 localization and expression in microglial fine processes. Arrows indicate location of fine processes. Replicates of this experiment = 4; taken from 4 different cultures. Scale bars = 10 µm.
Figure 3-6. LPS treated microglia demonstrate increased Piezo1 expression in cell membrane and fine processes.

Confocal microscopy was used to acquire single-plane images of cultured microglia treated for 24 hrs with LPS. The microglial marker CD11b is robustly expressed in the peripheral cell membrane and the fine processes. Additionally, Piezo1 immunofluorescence is increased at the edge of the cell membrane in the fine processes of LPS treated microglia. Arrows indicate fine processes. Replicates of this experiment = 4; taken from 4 different cultures. Scale bars = 10 µm.
### 3.6 Piezo1 channel functional properties

After establishing Piezo1 expression in microglia, we next investigated the functional properties of Piezo1 channels and the role of Piezo1 in mediating changes in cytosolic Ca$^{2+}$ levels through a series of Ca$^{2+}$ imaging experiments. We incubated microglia with Fluo-4 AM, a Ca$^{2+}$-sensitive fluorescent dye, to monitor cytoplasmic Ca$^{2+}$ levels, and imaged microglia via confocal microscopy. In all conditions the Piezo1 agonist, Yoda1, was applied to activate Piezo1. In most experiments ATP was used as a positive control because ATP causes a rapid, transient increase in intracellular Ca$^{2+}$ levels in microglia (Choi et al., 2003). The exception was experimental protocol performed in Ca$^{2+}$ free aCSF, in which case aCSF containing Ca$^{2+}$ was used as a positive control.

In regular aCSF (300 mOsm), we performed Ca$^{2+}$ imaging on microglia under five different conditions: 1) control, 2) with the mechanosensitive channel blocker Gd$^{3+}$, 3) in Ca$^{2+}$ free solution, 4) following acute LPS treatment, and 5) with the P2 receptor antagonist PPADS. A schematic of the different pharmacological agents and their effects are displayed in Figure 3-7. Figure 3-8 shows example images acquired during Ca$^{2+}$ imaging in the following conditions: (A) control, (B) with Gd$^{3+}$ in the imaging bath, and (C) with PPADS in the imaging bath. Images show the fluorescent and merged (transmitted light + fluorescent) channels at various time points in the experiment: at baseline and during Yoda1 and ATP application. Ca$^{2+}$ traces demonstrating changes in fluorescence compared to the baseline ($\Delta F/F_0$) over time for microglia in each condition were generated from the fluorescent channel. Representative Ca$^{2+}$ traces for each condition are presented in Figure 3-9. Ca$^{2+}$ traces on the left for each condition are the average $\Delta F/F_0 +$ SEM over time for all microglia in a single imaging experiment. On the right, superimposed Ca$^{2+}$ traces from 5
single cell examples are shown from the same experiment to show individual patterns. Figure 3-10 displays the peak Ca$_2^+$ response and the AUC during Yoda1 application across all conditions.

**Figure 3-7. Schematic of pharmacological agents and their cellular impact used to investigate Piezo1 channel properties in microglia.**

The Piezo1 agonist Yoda1 causes Piezo1 channel activation and subsequent Ca$_2^+$ influx, while Piezo1 channel activity is blocked by the mechanosensitive channel antagonist Gd$_3^+$. LPS is a bacterial endotoxin that induces Piezo1 upregulation via the immune receptors TLR-2 and -4 after 24 hrs, but at 2 hrs LPS attenuates Piezo1 activity. Blocking P2 receptors with the antagonist PPADS increases the Piezo1-mediated Ca$_2^+$ response, suggesting that P2 signalling attenuates Piezo1 activity under homeostatic conditions. Schematic adapted from Liu et al. (2018).
3.6.1 Yoda1-induced Piezo1 activation increases cytosolic Ca\textsuperscript{2+} in microglia

First, we investigated the effects of the Piezo1 agonist, Yoda1, on microglial Ca\textsuperscript{2+} response to determine whether Piezo1 channels could be activated in microglia by this agent. We observed that Yoda1 induced increases in cytosolic Ca\textsuperscript{2+} in microglia exhibited sustained as well as transient components, with some variability in magnitude of the Ca\textsuperscript{2+} response between cells. Figure 3-9 (A) displays the average Ca\textsuperscript{2+} response ± SEM from a single imaging experiment (n = 16 cells), as well as single cell examples taken from the same experiment. Quantification of the maximum Ca\textsuperscript{2+} response during Yoda1 (10 µM) application revealed a 0.396 ± 0.085 (n = 10) increase in fluorescence compared to the baseline (ΔF/F\textsubscript{0}), shown in Figure 3-10 (A). Additionally, we analyzed the cumulative Ca\textsuperscript{2+} response over time during Yoda1 application. As shown in Figure 3-10 (B), the average AUC of the Ca\textsuperscript{2+} response when Yoda1 was applied was 1.918 ± 0.407 (ΔF/F\textsubscript{0})*(min); n = 10. Peak Ca\textsuperscript{2+} response and AUC values were calculated from the average responses of 10 different Ca\textsuperscript{2+} imaging experiments (n = 94 cells total; 7 different cultures). The results of these experiments indicate that microglial Piezo1 channels are functionally expressed and that chemical activation of Piezo1 with Yoda1 increases cytosolic Ca\textsuperscript{2+} levels.
Figure 3-8. Sample images of microglia acquired during Ca\(^{2+}\) imaging.
Microglia were loaded with the Ca\(^{2+}\) dye Fluo-4 AM prior to imaging. Images acquired during Ca\(^{2+}\) imaging are displayed as the fluorescent channel as well as the merged channel (transmitted light + fluorescent) for a subset of experimental conditions. Images were taken at three time points: baseline, when Yoda1 was applied, and when the positive control ATP was applied. (A) control microglia show increased cytosolic Ca\(^{2+}\) in response to Yoda1 compared to baseline (B) Gd\(^{3+}\) application attenuated the Ca\(^{2+}\) response to Yoda1 (C) PPADS application potentiated the Ca\(^{2+}\) response to Yoda1. All conditions (A-C) demonstrated an increase in cytosolic Ca\(^{2+}\) in response to ATP.
Figure 3-9. Ca\(^{2+}\) traces demonstrate the changes in cytosolic Ca\(^{2+}\) levels in microglia over time compared to the baseline.

Ca\(^{2+}\) imaging of microglia in vitro generated Ca\(^{2+}\) traces indicating changes in cytosolic Ca\(^{2+}\) levels over time, as compared to the baseline. For these experiments, the baseline was defined as the first 5 minutes of imaging before Yoda1 application. Y-axis indicates ΔF/F\(_0\) and x-axis indicates time (min). Yoda1 was washed in to induce Piezo1 activation and subsequent Ca\(^{2+}\) response, with ATP used as a positive control in every condition, except for the experimental series using Ca\(^{2+}\) free aCSF – in which case regular aCSF containing Ca\(^{2+}\) was used as a positive control. Ca\(^{2+}\) imaging was performed on microglia in the following conditions: (A) control, (B) with application of the mechanosensitive channel blocker Gd\(^{3+}\), (C) in Ca\(^{2+}\) free aCSF, (D) immediately following 2 hr incubation with LPS, and (E) with application of the purinergic receptor antagonist PPADS. As displayed above, Yoda1 application induced a Ca\(^{2+}\) response in controls (A), which was attenuated by Gd\(^{3+}\) (B), removing Ca\(^{2+}\) from the bath (C), and pre-incubating microglia for 2 hrs with LPS.
(D). In contrast, application of PPADS potentiated the Ca\(^{2+}\) response (E). All conditions are displayed as both the average ± SEM Ca\(^{2+}\) response calculated from all cells in a single imaging experiment, as well as examples of single cell Ca\(^{2+}\) traces generated from the same imaging experiment. The number of cells displayed in the average Ca\(^{2+}\) responses for each condition are 16, 8, 9, 10, and 17 for (A-E), respectively. All experiments displayed were imaged in regular aCSF (300 mOsm).
Quantification of Yoda1-induced changes in cytosolic Ca\(^{2+}\) levels in microglia.

Quantification of Ca\(^{2+}\) responses generated from Yoda1-induced Piezo1 activation. All measurements are quantifications of changes in fluorescence relative to the baseline, where fluorescence is indicative of changes in Ca\(^{2+}\) levels, and shown as the average response from all imaging experiments in a condition + SEM. (A) displays peak Ca\(^{2+}\) response during Yoda1 application. Application of Yoda1 in the control condition resulted in a 0.396 (± 0.085) fold-increase in Ca\(^{2+}\) compared to baseline (n = 10). Application of the mechanosensitive channel blocker Gd\(^{3+}\), removal of Ca\(^{2+}\) from the extracellular solution, and acute LPS significantly decreased (p < 0.05) the maximum Ca\(^{2+}\) response compared to control by 0.326 ± 0.113, 0.332 ± 0.105, and 0.268 ± 0.088, respectively. The average peak Ca\(^{2+}\) responses for Gd\(^{3+}\), Ca\(^{2+}\) free, and 2 hrs LPS primed microglia were 0.074 ± 0.045 (n = 4), 0.292 ± 0.154 (n = 5), and 0.128 ± 0.040 (n = 9), respectively. In contrast, application of the purinergic receptor antagonist PPADS resulted in a significant increase of 0.290 ± 0.105 (n = 5; p < 0.05) in peak Ca\(^{2+}\) response compared to control (PPADS average peak response = 0.687 ± 0.106; n = 5). (B) displays the quantification of the AUC during the Yoda1-induced Ca\(^{2+}\) response for the same conditions as (A). AUC was calculated as (ΔF/F\(_0\))^*(min)*, indicating the cumulative Ca\(^{2+}\) response over time. AUC quantification for the control condition was 1.918 ± 0.407 (n = 10). AUC significantly decreased (p < 0.05) compared to control in the Gd\(^{3+}\), Ca\(^{2+}\) free, and 2 hrs LPS conditions, by 1.779 ± 0.587, 1.627 ± 0.542, and 1.27 ± 0.456, respectively. Average AUC responses for Gd\(^{3+}\), Ca\(^{2+}\) free, and 2 hrs LPS were 0.139 ± 0.0485 (n = 4), 0.292 ± 0.154 (n = 5), and 0.647 ± 0.193 (n = 9), respectively. PPADS application resulted in a significant 2.486 ± 0.543 increase in the AUC (n = 5; p < 0.001) compared to control (PPADS average AUC response = 4.404 ± 0.686; n = 5). Peak Ca\(^{2+}\) response and AUC quantifications for control, Gd\(^{3+}\), Ca\(^{2+}\) free, 2 hr LPS, and PPADS conditions were calculated from the average responses of 10, 4, 5, 9, and 5 imaging experiments and a total of 94, 27, 46, 50, and 53 cells, respectively. Statistical significance was determined for all conditions with one-way ANOVA and Dunnett’s multiple comparisons test. * = p < 0.05; ** = p < 0.01; *** = p < 0.001.
3.6.2 Gd$^{3+}$ attenuates Yoda1-induced Ca$^{2+}$ response in microglia

Next, we tested whether application of the non-selective mechanosensitive channel antagonist Gd$^{3+}$ would inhibit the Ca$^{2+}$ increases triggered by Yoda1. Gd$^{3+}$ is a non-selective, potent inhibitor of mechanosensitive channels, such as Piezo1, but is not reported to alter P2 receptor activity or inhibit P2X receptor activated channels (Ermakov et al., 2010). Thus, we applied Gd$^{3+}$ (10 µM) and monitored microglial Ca$^{2+}$ levels to elucidate whether blocking Piezo1 would reduce the Yoda1-induced cytosolic Ca$^{2+}$ increase. This was done to investigate whether the Ca$^{2+}$ response we observed was mediated from direct entry through Piezo1.

As displayed in Figure 3-9 (B), applying Gd$^{3+}$ potently inhibited the Ca$^{2+}$ signal generated from Yoda1-induced Piezo1 activation. Figure 3-9 (B) displays the average Ca$^{2+}$ response + SEM from a single imaging experiment (n = 8 cells), as well as single cell examples taken from the same experiment. Quantification of the peak Ca$^{2+}$ response during Yoda1 application revealed an average maximum response of 0.074 ± 0.045, as shown in Figure 3-10 (A), which was a significant decrease of 0.326 ± 0.113 in peak response compared to the control condition (n = 4; p<0.05). As shown in Figure 3-10 (B), quantification of the AUC during Yoda1 application demonstrated an average AUC magnitude of 0.139 ± 0.048 ($\Delta F/F_0$)*(min). This was a significant decrease of 1.779 ± 0.587 (n = 4; p<0.05) compared to the control condition. Peak Ca$^{2+}$ response and AUC values were calculated from the average responses of 4 different Ca$^{2+}$ imaging experiments (n = 27 cells total; 2 different cultures). The attenuation of the Ca$^{2+}$ response in the presence of Gd$^{3+}$ suggests that in microglia, Piezo1 activation may result in direct Ca$^{2+}$ influx through Piezo1 channels.
3.6.3 Removing extracellular Ca\(^{2+}\) attenuates Yoda1-induced Ca\(^{2+}\) response in microglia

There is extensive evidence that Piezo1 activation induces Ca\(^{2+}\) influx from the extracellular space in various cell types (Gottlieb & Sachs, 2012b). Though it is less substantiated, there is also evidence that Piezo1 activation can increase cytosolic Ca\(^{2+}\) by evoking Ca\(^{2+}\) release from ER stores through P2Y receptor activation (Santana et al., 2019; Wei et al., 2019). Thus, to investigate whether the Ca\(^{2+}\) response generated from Piezo1 activation in microglia was a result of Ca\(^{2+}\) influx from the extracellular space or Ca\(^{2+}\) release from intracellular stores, we applied Yoda1 in Ca\(^{2+}\) free solution (aCSF containing 0 mM Ca\(^{2+}\) + 2 mM EGTA).

As displayed in Figure 3-9 (C), we observed that removing extracellular Ca\(^{2+}\) attenuated the Yoda1-induced Ca\(^{2+}\) response. Figure 3-9 (C) displays the average Ca\(^{2+}\) response + SEM from a single imaging experiment (n = 9 cells), as well as single cell examples taken from the same experiment. As shown in Figure 3-10 (A), quantification of the maximum Ca\(^{2+}\) response during Yoda1 application revealed a peak Ca\(^{2+}\) response of 0.064 ± 0.023 compared to baseline, which was a significant decrease of 0.332 ± 0.105 compared to the control condition (n = 5; p<0.05). Quantification of the AUC during Yoda1 application is shown in Figure 3-10 (B). Quantification revealed an AUC magnitude of 0.292 ± 0.154 (ΔF/F\(_0\))*(min), which was a significant decrease of 1.627 ± 0.543 compared to the control condition (n = 5; p<0.05). Peak Ca\(^{2+}\) response and AUC values were calculated from the average responses of 5 different Ca\(^{2+}\) imaging experiments (n = 46 cells total; 2 different cultures). Overall, our findings suggest that Piezo1 activation may cause Ca\(^{2+}\) influx from the extracellular environment, as removal of Ca\(^{2+}\) from the imaging solution attenuated the Yoda1-induced Piezo1 Ca\(^{2+}\) response. This is in line with previous studies
demonstrating that Piezo1 activation can directly regulate Ca²⁺ influx (Gottlieb & Sachs, 2012b; Volkers et al., 2014).

### 3.6.4 Acute LPS treatment attenuates Yoda1-induced Ca²⁺ response in microglia

Next, we primed microglia with the endotoxin LPS (100 ng/mL) for 2 hrs immediately prior to Ca²⁺ imaging to investigate the effects of acute LPS treatment on Piezo1 activation. Of note, in line with our previous findings that Piezo1 protein levels upregulate 24 hrs post-LPS treatment, we also performed Ca²⁺ imaging experiments on microglia at the 24 hr timepoint (data not shown). However, due to concerns about cell toxicity, we did not include data from this timepoint. In the future, it would be beneficial to prime microglia with LPS for 2 hrs, and then perform Ca²⁺ imaging 24 hrs later to investigate Piezo1 channel upregulation and Piezo1-dependent Ca²⁺ response.

As displayed in Figure 3-9 (D), incubating microglia with LPS for 2 hrs attenuated the Yoda1-induced Piezo1 Ca²⁺ response. Figure 3-9 (D) displays the average Ca²⁺ response + SEM from a single imaging experiment (n = 10 cells), as well as single cell examples taken from the same experiment. As shown in Figure 3-10 (A), quantification of the maximum Ca²⁺ response during Yoda1 application revealed a significant decrease of 0.268 ± 0.088 (n = 9; p < 0.05) in Ca²⁺ signal compared to control (mean peak response = 0.128 ± 0.040). Quantification of the AUC during Yoda1 application, displayed in Figure 3-10 (B), revealed a significant decrease of 1.271 ± 0.456 (n = 9; p < 0.05) in cumulative Ca²⁺ response compared to control, with an average AUC response of 0.647 ± 0.193 (ΔF/F₀)*(min). Peak Ca²⁺ response and AUC values were calculated from the average responses of 9 different Ca²⁺ imaging experiments (n = 53 cells total; 5 different cultures). The reduced Piezo1 activity following acute LPS treatment was an unexpected finding of our
study. While further investigation is required to determine the causal mechanism, it is possible that acute LPS application may result in Piezo1 channel internalization or act via second messenger pathways to block Piezo1 activity. Indeed, there is evidence that under certain conditions LPS triggers internalization of immune receptors, such as TLR-4. This can occur rapidly, with TLR-4 internalization occurring within 30 minutes of LPS application (Ciesielska et al., 2020; Tan et al., 2015).

Interestingly, acute LPS treatment was the only condition in which the Ca\(^{2+}\) response induced by ATP application differed significantly from controls (Figure 3-11). 2 hr LPS priming resulted in a significant 0.471 ± 0.027 (n = 9; p < 0.05) increase in peak ATP-evoked Ca\(^{2+}\) response. Average control ATP response = 0.636 ± 0.118 (n = 10), and average acute LPS ATP response = 1.108 ± 0.169 (n = 9). This is in line with previous reports that acute LPS exposure (starting at 1 hr LPS treatment) can modulate microglial Ca\(^{2+}\) signalling (Hoffmann et al., 2003). Though the mechanisms of this are not well understood, acute LPS exposure can promote immediate intracellular Ca\(^{2+}\) responses – as opposed to sustained increases in cytosolic Ca\(^{2+}\) – a process which may be mediated by glutamate or purinergic receptor activation (Hoffmann et al., 2003; Liu et al., 2014).
Figure 3-11. Maximum Ca\(^{2+}\) response during Yoda1 and ATP application in microglia following 2 hrs LPS incubation.

Quantification of the peak Ca\(^{2+}\) response induced by Yoda1 and ATP application in microglia with acute (2 hr) LPS treatment. Results displayed as the average Ca\(^{2+}\) response from all imaging experiments in each condition + SEM (control n = 10; LPS n = 9). Maximum Yoda1-induced Ca\(^{2+}\) responses were previously shown and discussed in Figure-10. Acute LPS treatment resulted in a significant 0.471 ± 0.027 (p<0.05) increase in the ATP-evoked Ca\(^{2+}\) response compared to controls. The average ATP response for controls = 0.636 ± 0.118 (n = 10). The average ATP response for the LPS condition = 1.108 ± 0.169 (n = 9). Statistical significance analyzed with a one-tailed, unpaired t-test with Welch’s correction. * = p < 0.05.

3.6.5 PPADS potentiates Yoda1-induced Ca\(^{2+}\) response in microglia

The purinergic signalling system, which consists of P2X and P2Y receptors, is a significant regulator of microglial activation and behaviour (Calovi et al., 2019). ATP activates both P2X and P2Y receptors, which causes increases in cytosolic Ca\(^{2+}\). While P2X channel activation directly mediates Ca\(^{2+}\) influx into cells, activation of P2Y receptors coupled to \(G_q\) proteins can initiate Ca\(^{2+}\) release from the ER (Wei et al., 2019). P2X receptors with high expression in microglia include P2X\(_1\), P2X\(_4\), and P2X\(_7\), while P2Y receptors with high expression in microglia include P2Y\(_6\) and P2Y\(_{12}\) (Zhang et al., 2014). Interestingly, Piezo1 activation has been shown to induce ATP release.
into the extracellular space, which can subsequently activate both P2X and P2Y receptors to increase cytosolic Ca\(^{2+}\). This has been demonstrated in a variety of cell types, including urothelial, endothelial, and red blood cells (Cinar et al., 2015; Miyamoto et al., 2014; Wang et al., 2016b).

Thus, we investigated whether the Yoda1-induced Ca\(^{2+}\) response we observed in microglial cells was a direct result of Ca\(^{2+}\) entry through Piezo1 channels or a result of Piezo1-mediated ATP release and subsequent Ca\(^{2+}\) response via P2 receptors. To investigate the source of Ca\(^{2+}\) entry, we applied the generic P2 receptor antagonist PPADS to microglia during Ca\(^{2+}\) imaging. PPADS is a non-selective P2 antagonist that differentially blocks various P2X and P2Y receptors across species. Of the highly expressed P2 receptors in rat microglia, PPADS blocks P2X\(_1\) and P2Y\(_6\), but not P2X\(_4\) or P2Y\(_{12}\). In certain conditions, PPADS can also block P2X\(_7\) channels. (Abbracchio et al., 2019; Buell et al., 1996; Di Virgilio et al., 2020).

As displayed in Figure 3-9 (E), we observed that blocking P2 receptors with PPADS (100 µM) did not abolish the Yoda1-induced Ca\(^{2+}\) response. Figure 3-9 (E) shows the average Ca\(^{2+}\) response ± SEM from a single imaging experiment (n = 17 cells), as well as single cell examples taken from the same experiment. Unexpectedly, application of PPADS significantly increased the Yoda1-induced peak Ca\(^{2+}\) response compared to controls by 0.290 ± 0.105 (n = 5; p<0.05). In the presence of PPADS, the average peak Ca\(^{2+}\) response during Yoda1 application was response = 0.687 ± 0.106 (n = 5; Figure 3-10 (A)). Similarly, AUC quantification during Yoda1 application demonstrated a significant 2.486 ± 0.543 increase (n = 5; p<0.001) in cumulative Ca\(^{2+}\) response over time compared to controls. The average AUC response during Yoda1 application with PPADS, as shown in Figure 3-10 (B), was 4.404 ± 0.686 ((ΔF/F\(_0\))*(min); n = 5. Peak Ca\(^{2+}\) response and AUC
values were calculated from the average responses of 5 different Ca\(^{2+}\) imaging experiments (n = 53 cells total; 2 different cultures).

An unexpected finding of our study was that in the presence of PPADS, the Yoda1-induced Ca\(^{2+}\) response was potentiated. This raises the possibility that under homeostatic conditions, P2 receptor activity may partially attenuate Piezo1 Ca\(^{2+}\) influx, and thus blocking P2 receptors via PPADS removes this inhibition and increases Piezo1 mediated Ca\(^{2+}\) entry. Additionally, an unusual property of rat P2X\(_4\) is its insensitivity to PPADS, even at concentrations as high as 100 – 500 µM. Indeed, there have been reports that PPADS application actually potentiates ATP-evoked currents through rat P2X\(_4\), though the mechanisms of this are unknown (Gever et al., 2006). Further investigation with selective P2X and P2Y antagonists is required to elucidate which P2 receptor is involved in the potentiated Yoda1-induced Ca\(^{2+}\) response in the presence of PPADS.

### 3.7 Piezo1 channels activate in response to mechanical force induced by osmotic stress

Piezo1 channels are mechanosensitive ion channels that respond to changes in mechanical force on the membrane (Gottlieb & Sachs, 2012b). Therefore, in addition to chemical activation with Yoda1, we investigated the impact of mechanical force on Piezo1 activation and Ca\(^{2+}\) response. To stimulate a mechanical force, we induced cell swelling by superfusion of hypotonic aCSF (150 mOsm) into the imaging bath. Using an osmotic stress model has been used previously used to activate Piezo1 channels in a variety of cell types (Nourse & Pathak, 2017).

**Figure 3-12 (A)** shows example images of microglia acquired during Ca\(^{2+}\) imaging in hypotonic aCSF. Images of microglia dye loaded with Fluo4-AM were taken at baseline and following Yoda1
and ATP application. **Figure 3-12 (B)** displays the Ca$^{2+}$ traces generated from imaging microglia in hypotonic aCSF. Traces demonstrated a decrease in Ca$^{2+}$ signalling after the hypotonic solution was washed in, indicating cell swelling and thus dilution of the Fluo4-AM signal, followed by a robust increase in intracellular Ca$^{2+}$ when Yoda1 was applied. In hypotonic solution, we applied different concentrations of Yoda1 (10 and 20 µM), and observed robust whole cell increases in Ca$^{2+}$ at both concentrations. Traces are presented as the average Ca$^{2+}$ response + SEM from a single imaging experiment ($n = 11$ cells for 10 µM Yoda1; $n = 10$ cells for 20 µM Yoda1), as well as single cell examples taken from the same experiments.

**Figure 3-12 (C)** demonstrates the quantification of the peak Ca$^{2+}$ response generated by application of 10 and 20 µM Yoda1 in hypotonic aCSF. At 10 and 20 µM Yoda1, the peak Ca$^{2+}$ response was $0.976 \pm 0.111$ and $0.870 \pm 0.178$ compared to baseline, respectively. For experiments conducted in hypotonic aCSF, the baseline was defined as the average Ca$^{2+}$ signal after the hypotonic aCSF was washed in, prior to Yoda1 application. At both Yoda1 concentrations, the peak Ca$^{2+}$ responses generated in hypotonic aCSF were significantly larger compared to the Yoda1-induced Ca$^{2+}$ response in regular aCSF. Hypotonic + 10 µM Yoda1 demonstrated a $0.579 \pm 0.156$ -fold increase ($n = 6$; $p < 0.01$) increase in peak response compared to control, while hypotonic + 20 µM Yoda1 demonstrated a $0.4782 \pm 0.168$ -fold increase ($p < 0.05$) increase compared to control. As shown in **Figure 3-12 (D)**, quantification of the AUC during Yoda1 application demonstrated an average AUC magnitude of $1.997 \pm 0.230$ and $2.968 \pm 0.916$ ($\Delta F/F_0$)*(min) for hypotonic + 10 µM Yoda1 and hypotonic + 20 µM Yoda1, respectively. AUC values calculated for data sets in hypotonic aCSF were not compared to controls in regular aCSF due to differences in duration of Yoda1 application. For the hypotonic + 10 µM Yoda1 condition, peak Ca$^{2+}$ response
and AUC values were calculated from the average responses of 6 different Ca$^{2+}$ imaging experiments (n = 60 cells total; 4 different cultures), while for the hypotonic + 20 µM Yoda1 condition values were calculated from 5 different Ca$^{2+}$ imaging experiments (n = 63 cells total; 4 different cultures). The results of these experiments indicate that Piezo1 channels respond to mechanical force in microglia, and that the Ca$^{2+}$ response generated from chemical activation of Piezo1 with Yoda1 is amplified in the presence of mechanical stress on the cell membrane.
A

Baseline

10 µM Yoda1

100 µM ATP

Merge

Fluo-4 AM

B

Regular aCSF

Hypotonic aCSF

Regular aCSF

Hypotonic aCSF

C

Fold Change in Fluorescence from Baseline

* **

D

Area Under the Curve

Hypotonic (10 µM Yoda1)

Hypotonic (20 µM Yoda1)
Figure 3-12. Microglial cell swelling induced by osmotic stress increases Yoda1-induced Ca\^{2+} response in microglia.

Ca\^{2+} imaging of microglia *in vitro* generated Ca\^{2+} traces indicating changes in cytosolic Ca\^{2+} levels over time, as compared to the baseline. For these experiments, the baseline was defined as the 5 minutes of imaging before Yoda1 application, once the hypotonic solution (150 mOsm) was washed into the bath. Y-axis indicates ΔF/F\textsubscript{0} and x-axis indicates time (min). Yoda1 was washed in at either 10 or 20 µM to induce Piezo1 activation and subsequent Ca\^{2+} response, with ATP used as a positive control. Hypotonic solution was used to induce cell swelling, and therefore create a mechanical force on the membrane. (A) shows samples images taken during Ca\^{2+} imaging at baseline and during Yoda1 and ATP application. (B) displays Ca\^{2+} traces, presented as the average + SEM Ca\^{2+} response for a single imaging experiment, as well as examples of single cell Ca\^{2+} traces generated from the same imaging experiment. The number of cells displayed in the average Ca\^{2+} traces for hypotonic + 10 µM Yoda1 and hypotonic + 20 µM Yoda1 is 11 and 10, respectively. (C) displays the peak Ca\^{2+} response compared to baseline. The control values presented here are the same as shown in Figure 3-10. In the hypotonic conditions, the average maximum response for 10 µM Yoda1 was 0.976 ± 0.111, which was a significant increase of 0.579 ± 0.156 (n = 6; p < 0.01) compared to control. At 20 µM Yoda1, the average peak response was 0.870 ± 0.178, which was a significant increase of 0.4782 ± 0.168, compared to controls (n = 5; p < 0.05). (D) displays the AUC quantifications. For 10 µM Yoda1, the average AUC was 1.997 ± 0.230 (n = 6), and for 20 µM Yoda1, the average AUC was 2.968 ± 0.916 (n = 5). Statistical significance was determined with a one-tailed, unpaired t-test with Welch’s correction. * = p < 0.05; ** p < 0.01.
Chapter 4: Discussion

4.1 Piezo1 channels are functionally expressed in primary microglia

This study produced the novel discovery that Piezo1 channels are functionally expressed in primary microglia. The main findings of the present study are: 1) in response to immune challenge, Piezo1 expression is upregulated, specifically along the peripheral edge of microglia and in the fine processes extending from the peripheral edges, and 2) activation of Piezo1 channels via the chemical agonist, Yoda1, increases cytosolic Ca$^{2+}$, an effect which is further enhanced by inducing swelling with hypo-osmotic solution. These findings suggest that Piezo1 may be an important component of microglial physiology and immune function.

We demonstrated that Piezo1 mRNA and protein levels significantly increased following immune challenge with LPS. Further, we demonstrated using selective agonists that the immune receptors TLR-2 and TLR-4 can both contribute to mediating this effect (Figures 3-2; 3-3). In addition to the general increase in Piezo1 protein expression observed in western blots, immunostaining revealed that LPS altered the pattern of Piezo1 expression in microglia. Activated microglia demonstrated a robust increase in Piezo1 immunofluorescence at the peripheral edges of the cell membrane and in the microglial fine processes (Figures 3-4; 3-5; 3-6). Together, this data suggests that microglial Piezo1 expression is sensitive to immune challenge and that increased Piezo1 expression may be involved in modulating microglial immune response.

In terms of channel properties, we demonstrated that application of the agonist Yoda1 increased intracellular Ca$^{2+}$ levels in microglia. This increase was likely due to Piezo1 activation and Ca$^{2+}$ entry via Piezo1 channels in microglia because the Yoda1-induced Ca$^{2+}$ response was attenuated...
by the mechanosensitive channel blocker Gd^{3+} and by removing extracellular Ca^{2+} (Figures 3-9 and 3-10). Interestingly, additional data indicated that there may be multiple pathways that can modulate Piezo1-mediated Ca^{2+} entry. For example, acute LPS treatment attenuated the Yoda1-induced Ca^{2+} response, while also increasing the ATP-evoked Ca^{2+} response (Figures 3-9; 3-10; 3-11). In addition, application of the P2 receptor antagonist PPADS potentiated the Yoda1-induced Ca^{2+} response. These additional data suggest that the increase in cytosolic Ca^{2+} following Piezo1 activation may be a result of direct Ca^{2+} entry via Piezo1 channels, and that there are potential interactions between Piezo1 activation and P2 receptor activity (Figures 3-9 and 3-10).

We also demonstrated that Piezo1 channels respond to mechanical stimulation. We performed Ca^{2+} imaging in a hypotonic solution to generate a mechanical force on the cell membrane from osmotic stress. The combined chemical and mechanical activation of Piezo1 with Yoda1 and osmotic stress robustly increased intracellular Ca^{2+} (Figure 3-12). This data suggests that Piezo1 channels contribute to microglial mechanosensitivity.

4.2 Impact of Piezo1 activation on immune function and cytokine release

In 2019, Velasco-Estevez et al. published a report that investigated the role of Piezo1 activation on primary astrocyte immune response. Similar to our findings, Velasco-Estevez et al. reported that Piezo1 protein levels are upregulated in astrocytes following LPS-stimulation. Beyond observing the change in Piezo1 expression following immune challenge, Velasco-Estevez et al. also demonstrated that Piezo1 activation inhibited the release of pro-inflammatory cytokines, specifically IL-1β and TNF-α, following LPS stimulation. Recently, Liu et al. (2020) reported that Piezo1 activation modulates immune response in the BV2 microglial cell line. Of note, BV2 cells
are an immortalized cell line that demonstrate a different gene expression profile than primary microglia. These differences in gene expression are largely because – unlike primary microglia – BV2 cells are designed to rapidly proliferate. Further, BV2 cells demonstrate an ovoid morphology that is not present in primary microglia or microglia in vivo. This could indicate that BV2 cells are ‘pre-activated’ and thus may react differently upon stimulation (Stansley et al., 2012). Overall, primary microglia more accurately reflect the genotypes and morphologies that characterize microglia in vivo. Bearing these limitations in mind, the findings of Liu et al. still provide insight as to how Piezo1 activation may affect microglial immune response. For example, in line with our findings, Liu et al. reported that Piezo1 mRNA and protein upregulated in BV2 cells following LPS stimulation, and that Piezo1 activation from Yoda1 increased cytosolic Ca\(^{2+}\). Liu et al. also reported that following LPS stimulation, Piezo1 activation altered cytokine expression by inhibiting the release of IL-1β and TNF-α (Hailin Liu et al., 2020). Our study is limited in that we did not investigate how Piezo1 activation alters immune response via cytokine release in primary microglia. However, based on the findings of Velasco-Estevez et al. (2019) and Liu et al. (2020), it is possible that Piezo1 activation in primary microglia might similarly act to inhibit the release of pro-inflammatory cytokines like IL-1β and TNF-α.

4.3 Piezo1 activation and purinergic signalling

The release of intracellular ATP into the extracellular space is an important part of the purinergic signalling system. Extracellular ATP then acts in an autocrine or paracrine manner to activate ionotropic P2X receptors or metabotropic P2Y receptors. Activation of either P2X or P2Y receptors results in increased cytosolic Ca\(^{2+}\), albeit through different mechanisms. P2X activation opens ion channels that can directly lead to Ca\(^{2+}\) entry. In contrast P2Y receptors are G-protein
coupled receptors whose activation, if linked with Gq subunits, can cause release of Ca^{2+} from intracellular stores via IP_3-dependent Ca^{2+} release from the ER (Verkhratsky & Burnstock, 2014; Wei et al., 2019). In microglia, purinergic signalling is linked to phagocytosis, chemotaxis, and modulation of inflammatory factors (Hidetoshi et al., 2012a).

One of the mechanisms long known to induce extracellular ATP release and subsequent P2 receptor activation, both in vitro and in vivo, is mechanical force (Petruzzi et al., 1994; Riddle et al., 2007). However, the mechano-sensor involved in coupling mechanical stimuli to P2 receptor activity has remained elusive. Intriguingly, in the past few years, accumulating evidence points to a relationship between Piezo1-dependent mechanosensation, P2 receptor activation, and downstream Ca^{2+} regulation of cell functions and physiological processes. While the mechanisms of how Piezo1 activation may trigger ATP-release remain unknown, evidence from red blood cells, urothelial cells, mesenchymal stem cells, and endothelial cells suggests that Piezo1 activation is coupled to ATP release – which then acts on P2 receptors to increase cytosolic Ca^{2+}. (Cinar et al., 2015; Miyamoto et al., 2014; S. P. Wang et al., 2016a; Wei et al., 2019). Thus, as we investigated the functional properties of Piezo1 in primary microglia through a series of Ca^{2+} imaging experiments, we endeavoured to understand whether Piezo1 activation resulted in Ca^{2+} influx directly through Piezo1 channels, and whether P2-mediated increases in Ca^{2+} were also involved.

In the present study, we reported that chemical activation of Piezo1 with the agonist Yoda1 increased intracellular Ca^{2+} levels (Figure 3-9 (A) and 3-10). This effect was attenuated by blocking Piezo1 channels with Gd^{3+}, a potent mechanosensitive channel antagonist (Figures 3-9 (B) and 3-10). Gd^{3+} is not reported to alter P2Y receptor activity or block P2X channels (Ermakov
et al., 2010). This data suggests that Piezo1 activation in microglia may directly cause Ca$^{2+}$ influx through Piezo1 channels.

Removing extracellular Ca$^{2+}$ also eliminated the Yoda1-induced Ca$^{2+}$ response (Figures 3-9 (C) and 3-10). This data could suggest that increases in intracellular Ca$^{2+}$ following Piezo1 activation are dependent on extracellular Ca$^{2+}$ influx, as opposed to release of Ca$^{2+}$ from internal stores. However, data from the MacVicar lab (data not shown) has demonstrated that Yoda1 stimulation of Piezo1 in astrocytes can cause ATP release. Therefore, another possibility is that in the absence of external Ca$^{2+}$, Piezo1 stimulation with Yoda1 is not sufficient to induce ATP release into the extracellular space. Under these conditions, P2Y receptors would remain inactive and thus not promote Ca$^{2+}$ release from internal stores. Interestingly, P2Y receptor activation in microglia has been shown to inhibit the release of TNF-α and IL-1β in LPS-activated microglia following ATP stimulation (Hidetoshi et al., 2012b; Ogata et al., 2003). Given the previous reports that Piezo1 activation in glia is linked to inhibition of TNF-α and IL-1β release following LPS treatment, this could suggest a role of P2Y receptors in mediating this effect.

To further investigate the role of P2 receptor signalling in modulating Piezo1 activity, we applied the P2 antagonist PPADS. Unexpectedly, the results of these experiments indicated that PPADS potentiates the Yoda1-induced Ca$^{2+}$ response (Figures 3-9 (E) and 3-10). While further investigation is required to elucidate the causal mechanism, our findings suggest that under homeostatic conditions, P2 receptor activity may reduce Piezo1-mediated Ca$^{2+}$ influx. Thus, when P2 receptors are blocked with PPADS, this inhibitory mechanism on Piezo1 channels is removed, thereby increasing the Piezo1-mediated Ca$^{2+}$ response.
The effect of PPADS on purinergic signalling is complex with different effects depending on the species. Of the P2 receptors most highly expressed in microglia and based on the activity of PPADS in the rat, PPADS blocks P2X_1 and P2Y_6, but not P2X_4 or P2Y_{12} in rat microglia (Abbracchio et al., 2019; Di Virgilio et al., 2020). PPADS also has antagonistic effects on P2X_7 channels under certain conditions, but P2X_7 channels require a high concentration of ATP to activate, and are also not highly expressed in microglia until after P30 (Alves et al., 2014; Xiang & Burnstock, 2005). Our experiments were conducted on primary microglia cultured from embryonic tissue and also in conditions lacking sufficient ATP to activated P2X_7. Thus, P2X_7 channels were likely not involved in the effect we observed. The effect of PPADS application on intracellular [Ca^{2+}] we observed was also unlikely to be mediated by either P2Y_6 or P2Y_{12}. P2Y_6 is coupled to G_q proteins, which activate IP_3 mediated Ca^{2+} release from the ER. Since PPADS blocks P2Y_6 activity, yet we still observed an increase in intracellular [Ca^{2+}], P2Y_6 was likely not responsible for this effect. Additionally, even though PPADS does not block P2Y_{12} receptors, P2Y_{12} is coupled to G_i protein activity and is involved in regulating cAMP, as opposed to Ca^{2+} levels.

P2X_1 and P2X_4 receptors are two candidates that might be involved in mediating increased Ca^{2+} responses in the presence of PPADS, albeit through quite different mechanisms. PPADS blocks P2X_1, which is most highly expressed in embryonic microglia, although the role of P2X_1 in microglial physiology is not well-defined (Crain & Watters, 2010; Xiang & Burnstock, 2005). For PPADS to enhance Piezo1 response to Yoda1, then P2X_1 activation would have to normally inhibit Piezo1 activation, thus resulting in disinhibition when PPADS is applied. At present, there are no data supporting this scenario. Alternatively, another possible candidate for mediating the increase
in intracellular [Ca$^{2+}$] we observed during PPADS application is the P2X$_4$ channel, which is linked to microglial mobility and chemotaxis (Ohsawa et al., 2007). Intriguingly, though the mechanisms of this are unknown, P2X$_4$ activation in the presence of PPADS has been shown to actually increase ATP-evoked currents. Thus, it is possible that PPADS exacerbates P2X$_4$ activity, and is responsible for the increased Ca$^{2+}$ response we observed.

Overall, the results of our study suggest that while Ca$^{2+}$ influx may occur directly through Piezo1 channels in primary microglia, P2 receptor activity may also be involved and contribute to the magnitude of the changes in intracellular [Ca$^{2+}$]. Our study is limited in that we were not able to draw specific conclusions about the extent of P2X or P2Y receptor activation impact on Piezo1-mediated Ca$^{2+}$ influx. Future studies should aim to further elucidate the role of purinergic signaling on Piezo1 activation in microglia by the comparison of the efficacy of selective agonists and antagonists that target the purinergic receptors expressed in rat microglia.

4.4 Acute LPS treatment alters microglial evoked-Ca$^{2+}$ responses

An unexpected finding of our study was that acute LPS treatment reduced Yoda1-mediated Ca$^{2+}$ responses in microglia (Figure 3-9 (D) and 3-10). While determining the underlying mechanism of this effect was beyond the scope of the present study, it is possible that acute LPS treatment induces Piezo1 channel internalization or acts via second messenger pathways to block Piezo1 activity. In terms of channel internalization, there is evidence that acute LPS stimulation can cause receptor internalization from the cell surface via endosomes (Ciesielska et al., 2020; Tsukamoto et al., 2018). In immune cells, an example of this phenomenon is seen with TLR-4 receptors. LPS binding to TLR-4 receptors triggers the myeloid differentiation 88 (MyD88)-dependent signalling
pathway, which further initiates downstream inflammatory signalling cascades (Kagan & Medzhitov, 2006). Following this, TLR-4 receptors are internalized in endosomes, a process which terminates MyD88-dependent signalling pathway activity. Tan et al. (2016) showed that within 30 minutes of LPS stimulation, TLR-4 receptor expression at the surface of the membrane decreased by up 60%. This demonstrates that LPS can rapidly internalize surface proteins. In terms of our study, it is possible that acute LPS stimulation of microglia causes Piezo1 internalization along a similar time course, contributing to the attenuated Ca\(^{2+}\) response we observed following Yoda1 stimulation. As well, we observed differences in Piezo1 expression patterns following 24 hr LPS treatment (Figures 3-4; 3-5; 3-6). Thus, it is also possible that LPS stimulation causes Piezo1 channels to be internalized for redistribution at the peripheral edges of microglia. Increased Piezo1 expression in the membrane and in the microglial fine processes may assist with phagocytic activity or enhance mobility in activated microglia.

Another finding of our study was that acute LPS treatment increased the ATP-evoked Ca\(^{2+}\) response compared to controls (Figure 3-11). There is evidence that LPS stimulation can increase P2X receptor mRNA in immune cells (Guerra et al., 2003). Given that P2X receptors are robustly expressed in microglia, it is possible that LPS upregulated P2X channels in microglia, which contributed to the robust ATP-evoked Ca\(^{2+}\) response we observed (Bhattacharya & Biber, 2016).

4.5 Piezo1 links mechanical force with immune response

The results of our study also indicate that Piezo1 can be activated by mechanical stimulation in primary microglia (Figure 3-12). This is in line with a growing body of literature that suggests Piezo1 channels serve as an important link between mechanical force and immune regulation in
various immune cells. As discussed briefly in Chapter 1.11, Solis et al. (2019) reported that Piezo1 is critical for modulating immune response in peripheral myeloid cells in an in vitro model of the lung. While the lung is subjected to cyclical changes in mechanical force under healthy conditions – as tissue is compressed during breathing – these mechanical forces are altered when tissue is swollen from infection or inflammation. By comparing wild-type and Piezo1 deficient myeloid cells in models of lung inflammation, the authors concluded that Piezo1 activation is necessary for inducing a pro-inflammatory gene expression profile and downstream production of immune signalling molecules, such as endothelin-1 – which is known to be involved in mediating immune response to inflammation and fibrosis (Elisa et al., 2015; Solis et al., 2019).

While the increase in pro-inflammatory gene expression suggests that Piezo1 activation can be protective against infection or bacteria by launching an immune response, it is interesting to consider the beneficial effects of blocking Piezo1 activity as well. For instance, Solis et al. also reported that loss of Piezo1 was protective in a mouse model of pulmonary fibrosis – a disease which causes chronic inflammation and tissue scarring in the lungs – due to the decrease in pro-inflammatory activity. Taken together, these contrasting pieces of evidence show how both Piezo1 activation and Piezo1 inhibition can, in different ways, have a positive impact on disease outcome.

The broader implication of the study by Solis et al. is that mechanosensation is directly related to immune outcomes, and that Piezo1 is a critical link between mechanical stimuli and immune function. In light of this, our finding that Piezo1 in microglia responds to mechanical stimulation and is modulated by immune challenge raises the possibility that Piezo1 may similarly link mechanotransduction and immune response in the CNS.
Another example of Piezo1 serving as the link between mechanotransduction and immune response is a recently published study by Aykut et al. (2020). Aykut et al. reported that Piezo1 is the primary sensor of mechanical force in mouse leukocyte cells and is involved in modulating immune response to cancer. Piezo1-deficient leukocytes from a mouse line that lacked Piezo1 in myeloid cell lineages revealed that leukocytes are unable to transduce mechanosensory signals in the absence of Piezo1. Aykut et al. then investigated Piezo1 involvement in regulating leukocyte immune response in a mouse model of pancreatic ductal adenocarcinoma (PDA). The authors reported that activation of Piezo1 accelerated tumour growth, while in contrast, inhibition of Piezo1 was protective against tumour growth. Cancerous cells are known to increase the stiffness of the extracellular matrix, leading to increased tissue stiffness around the tumour (Wullkopf et al., 2018). Thus, it is possible that Piezo1 enables leukocytes to detect the mechanical differences in the tumour compared to healthy tissue, and subsequently modulate leukocyte immune response. Overall, this suggests that Piezo1 is an important link between mechanical force and immunity.

While it is of general interest to investigate how Piezo1 mechanotransduction contributes to immune cell regulation, the outcome of such studies may have a profound impact on human health. For example, in addition to the mouse model work, Aykut et al. also investigated Piezo1 involvement in human PDA by using surgically resected tumour specimens and analyzing Piezo1 expression in intertumoral myeloid-derived cells. The results of their study were striking. Low gene expression of Piezo1 was associated with extended survival of PDA patients, with approximately ~20% of patients with low Piezo1 expression surviving five years post-diagnosis. In contrast, there were no long term PDA survivors in the high Piezo1 expression group (Aykut et al., 2020). Overall, these studies highlight the importance of further investigation into how Piezo1
mechanotransduction regulates immune function, while also suggesting that Piezo1 could serve as an effective therapeutic target.

4.6 Final remarks and future directions

Though the results of our study reported in Chapter 3 were exclusively obtained from primary culture, we also performed preliminary *in vivo* immunostaining in tissue from Piezo1-tdTomato mice (P59) to investigate whether Piezo1 expression in microglia was altered *in vivo* following immune challenge. To induce an immune challenge and microglial activation, we performed a cortical stab wound procedure in the right hemisphere, while the untreated hemisphere served as a within-subject control. 24 hrs after the stab wound procedure was conducted, mice were perfused for tissue collection (procedure and immunostaining methodology outlined in Chapter 2.7). **Figure 4-1** demonstrates a coronal section of the Piezo1-tdTomato cortex, and outlines the stab wound and control areas that were used for further imaging.
Figure 4-1. Coronal section of Piezo1-tdTomato brain demonstrating location of stab wound. Single-plane coronal section of brain acquired with confocal microscopy from a Piezo1-tdTomato (P59) mouse. Tissue was fixed 24 hrs after stab wound procedure was performed and then sliced via cryostat into 20 µm thick sections. Sections were stained with the nuclear marker DAPI (A) and the microglial marker P2Y\textsubscript{12} (B). Boxes demonstrate locations where further imaging was acquired above the hippocampus in the cortex – in either the left hemisphere, which served as a control, or the right hemisphere where the stab wound injury was located. Scale bar is 1 mm.

We observed in the control hemisphere that overlap between Piezo1 and the microglial marker P2Y\textsubscript{12} was low, though there were sites along the microglial membrane where puncta of Piezo1 immunostaining were present (Figure 4-2). In contrast, around the stab wound we observed a general increase in Piezo1 immunofluorescence, with notable overlap between Piezo1 and P2Y\textsubscript{12}-expressing cells (Figure 4-3). However, it is important to note that following insult in the CNS, peripheral macrophages can be recruited to the site of damage and acquire microglial markers – including P2Y\textsubscript{12} (Corraliza, 2014; Grassivaro et al., 2020). Therefore, the overlap between Piezo1 and P2Y\textsubscript{12}-expressing cells in Figure 4-3 does not necessarily indicate that Piezo1 expression is increased in microglia specifically. Use of a microglia-specific Piezo1 knockout model or staining
with a more microglial-specific marker like triggering receptor expressed on myeloid cells 2, is required to validate and expand upon our preliminary in vivo observations (Grassivaro et al., 2020).

Figure 4-2. Microglia in control hemisphere of Piezo1-tdTomato mouse demonstrate little overlap with Piezo1 immunofluorescence.
Single plane image of control hemisphere (no stab wound) acquired with confocal microscopy from a Piezo1-tdTomato (P59) mouse. Tissue was sliced into 20 µm thick sections via cryostat and stained with the nuclear marker DAPI, the microglial marker P2Y12, and Piezo1. Hollow arrows demonstrate robust Piezo1 staining along a blood vessel, likely due to high expression of Piezo1 in endothelial cells. White arrows demonstrate points where Piezo1 immunofluorescent puncta co-localizes with P2Y12 staining. Co-localization between Piezo1 and P2Y12 staining is low. Scale bars = 25 µm and 10 µm for the top and bottom row of images, respectively.
Figure 4-3. Piezo1 immunofluorescence is upregulated around the stab wound in Piezo1-tdTomato mouse cortex.

Single plane image of tissue around stab wound acquired with confocal microscopy from a Piezo1-tdTomato (P59) mouse. Tissue was sliced into 20 µm thick sections via cryostat and stained with the nuclear marker DAPI, the microglial marker P2Y₁₂, and Piezo1. Stab wound area demonstrates ‘hotspots’ of robust Piezo1 immunofluorescence. White arrows indicate sites where Piezo1 co-localizes with P2Y₁₂-expressing cells. Scale bars = 25 µm and 10 µm for the top and bottom row of images, respectively.
Overall, our study has provided evidence that the mechanosensitive ion channel Piezo1 is expressed in primary microglia. Moreover, our results indicate that Piezo1 channels in microglia are functionally expressed, as they are activated in response to both chemical and mechanical modulation and are upregulated following immune challenge. While much work remains to be done to validate our findings in vivo and explore the mechanisms by which Piezo1 regulates microglial immune function, the results presented in this thesis provide foundational evidence that Piezo1 channels contribute to microglia physiology. Given the vast array of physiological and pathological processes that Piezo1 is implicated in throughout the body, a more comprehensive understanding of Piezo1 channel expression and function in microglia could have widespread impact on our knowledge of brain health and disease.
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