Dynamic regulation of the cellular prion protein in microglia following histamine-induced stimulation

by

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ABSTRACT

Although the cellular prion protein (PrP^C) has been evolutionarily conserved throughout evolution, its precise role remains elusive. Recent findings suggest that PrP^C may be involved in neuroinflammation and immune responses in the brain, and its expression can be modulated through various mechanisms. Additionally, the role of mast cells in the central nervous system is a growing topic of interest, with evidence that histamine released by mast cells in mice can stimulate various cells, including microglia, an innate immune cell of the central nervous system. This thesis investigates the expression and regulation of PrP^C in immune and neurological cell models, with a focus on human microglial cells and their response to histamine.

We first established a reliable method to measure total and surface PrP^C in various immune, neurological, and microglial cell models using flow cytometry and western blot. This included confirmation that PrP^C was present on the human microglial cell line, HMC3, which was then used for the remaining studies to examine the effects of histamine on microglia.

Western blotting and quantitative reverse transcription-polymerase chain reaction were used to measure protein and gene expression levels of histamine receptors HRH1-4. HMC3 cells expressed all but HRH4. Histamine-induced responses, including metabolic activity, cytokine release, and intracellular calcium levels were assessed. Metabolic activity was measured via XTT assay and calcium flux was assessed following histamine stimulation. Cytokine production was monitored by enzyme-linked immunosorbent assay (ELISA), with IL-6 and IL-8 levels increasing following histamine treatment.

We then examined how HMC3 PrP^C levels were affected by different doses and durations of histamine treatment. Histamine-induced stimulation for 6 and 24 hours increased HMC3 surface PrP^C expression, with a subsequent decrease after 48 and 72 hours. Antagonist studies

confirmed that these changes in surface PrP^C were mediated via stimulation of the HRH2 receptor. Total PrP^C levels were unchanged, suggesting the surface PrP^C changes were secondary to a change in localization. Fluorescence microscopy of the cells further supported this hypothesis, as more surface PrP^C was seen on the cells after 24 hours of histamine stimulation. Notably, while the HRH2 antagonist blocked the histamine-induced increase in surface PrP^C, it did not inhibit IL-8 release and only partially reduced IL-6 release.

In conclusion, we demonstrated that the HMC3 human microglial cell line expresses PrP^C and histamine receptors 1-3. HMC3 cells can be stimulated by histamine to release IL-6 and IL-8 and undergo intracellular calcium influx. More notably, the surface levels of PrP^C on HMC3 cells are altered by histamine exposure, primarily mediated by HRH2 in a time- and dose-dependent manner. While histamine exposure also leads to the release of IL-6 and IL-8 in these cells, this cytokine release is not fully dependent on PrP^C levels, as IL-6 release is only partially reduced and IL-8 release is unchanged under the conditions of HRH2 blockade that prevent PrP^C changes. Overall, this suggests that PrP^C may play a role in modulating microglial responses to histamine but does so through pathways other than cytokine release.

PREFACE

A version of Chapter 3 of this thesis is currently in revision with the academic journal *Scientific Reports* titled “Histamine stimulates human microglia to alter cellular prion protein expression via the HRH2 histamine receptor,” with the following authors: Marcus Pehar, Melissa Hewitt, Ashley Wagner, Jagdeep K. Sandhu, Aria Khalili, Xinyu Wang, Jae-Young Cho, Valerie L. Sim, and Marianna Kulka. M.P., V.S., and M.K. designed the study. M.P. performed all non-microscopy experiments. A.W. designed calcium flux experiments. M.H. performed fluorescence microscopy. M.P., A.K. and X.W. performed Cryo-SEM. M.P., V.S., and M.K. carried out the data analysis. M.P., V.S., and M.K. wrote and edited the manuscript with editorial input from M.H., A.W., A.K., X.W., J-Y.C., and J.K.S..

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LIST OF ABBREVIATIONS

ALS: Amyotrophic lateral sclerosis

BBB: Blood-brain barrier

CNS: Central nervous system

Cryo-SEM: Cryo-scanning electron microscopy

DAMP: Damage-associated molecular pattern

ELISA: Enzyme-linked immunosorbent assay

FSC: Forward-scatter (cell size measurement)

GABA: Gamma-aminobutyric acid

GluR: Glutamine Receptor

GPCR: G-protein coupled receptor

GPI: Glycosylphosphatidylinositol

HMC1.1: Human mast cell line 1.1

HMC1.2: Human mast cell line 1.2

HMC3: Human microglia clone 3

HRH: Histamine receptor

IFN: Interferon

IL-1β: Interleukin-1 beta

IL-6: Interleukin-6

IL-8: Interleukin-8

LAD2: Laboratory of Allergic Disease 2

mAChR: muscarinic acetylcholine receptor

MFI: Mean fluorescent intensity

Myd88: Myeloid differentiation primary response 88

NF-κB: Nuclear factor kappa-light-chain-enhancer of activated B cells

NK cells: Natural killer cells

PAMP: Pathogen-associated molecular pattern

PrP^C: Cellular prion protein

Prnp: Mouse gene encoding PrP^C

PRNP: Human gene encoding PrP^C

PSD: postsynaptic density

P2RY12: Purinergic Receptor P2Y

SSC: Side-scatter (cell complexity measurement)

THP-1: Human monocytic cell line derived from acute monocytic leukaemia patient

TLR: Toll-like receptor

TMEM119: Transmembrane protein 119

TNF: Tumour necrosis factor

U-87 MG: human malignant glioma cell line 87

VMAT-2: Vesicular monoamine transporter 2

XTT: 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-carboxanilide-2H-tetrazolium

CHAPTER 1: General Introduction

1.1 Chapter 1 Abstract

The cellular prion protein (PrP^C) is implicated in the pathogenesis of prion diseases, however, the protein in its normally folded state is thought to play important roles in cellular signaling, neuroprotection, cellular stimulation, and neuroinflammation. This introductory chapter discusses the multifaceted roles and various functions of PrP^C with a focus on its involvement in neuroinflammatory regulation and signaling in the central nervous system. Current literature indicates that PrP^C may mediate neuroinflammation through specific and dynamic expression on the cell surface. One such cell that expresses PrP^C are microglia, which are the innate immune cell of the central nervous system. This chapter introduces the concepts of neuroinflammation and outlines the significance of PrP^C in cells that participate in neuroinflammation such as microglia.

Ultimately, this chapter aims to set the stage for investigations into the role of PrP^C within human-derived microglial cells while exploring the overarching function of PrP^C in neuroinflammation.

1.2 Introduction

The cellular prion protein (PrP^C) plays a vital role in the pathogenesis of prion diseases in humans and various other mammals. Despite this, the physiological role of this protein remains unclear; there are numerous proposed functions, including the regulation of cellular transport and cellular signaling[1], participating in immune signaling[2], modifying synaptic transmission and signal transduction[3], and mediating neuroprotection and neuroinflammation[4,5]. PrP^C interacts with a diverse range of partners, including copper ions[6], various molecular chaperones[7,8], proteins involved in signal transduction[9,10], and various cellular receptors[9,11-13] on a plethora of cells throughout the central nervous system (CNS)[9,14,15] and immune system[16,17]. As the protein has widespread distribution throughout the CNS and an extensive number of interaction partners, PrP^C likely has many physiological roles.

One such role that has gained particular interest recently is that PrP^C may be involved in regulating neuroinflammation, both in healthy and pathological cases. PrP^C has been detected in various immune cells and other cells within the CNS, including microglia, the resident immune cell of the brain[14]. In microglia and neuroinflammatory cells, PrP^C is thought to play a role in cellular stimulation[18] alongside cell signaling[9] and cytokine modulation[18]. Notwithstanding, the physiological role of PrP^C remains elusive, and research has aimed to understand how this protein may mediate neuroinflammation.

Due to the growing body of evidence tying PrP^C to neuroinflammation, one primary goal of the present study was to investigate the role of PrP^C in neuronal cells and immune cells, with a particular interest in microglial cells, the primary neuronal cell type responsible for immunity in the CNS.

1.3 Neuroinflammation

Broadly, neuroinflammation is a localized immune response by which the innate immune system attempts to remove an injury or stimuli within the brain or spinal cord and initiate the necessary processes required to repair and heal the CNS. Causes of neuroinflammation include, but are not limited to: aging, pathogens, traumatic brain injury, spinal cord injury, and autoimmunity[19,20]. Following the initiation of the inflammatory response, there are various physiological and biochemical consequences depending on the context and duration of the stimuli[19]. Neuroinflammation is mediated by various cells, including neuro-immune cells such as microglia, astrocytes, and oligodendrocytes[21], as well as endothelial cells and peripheral immune cells[19]. These cells act to modulate their local milieu directly (through phagocytosis) and indirectly (through cell signaling and CNS maintenance) and can produce cytokines and chemokines to direct signaling in order to mediate neuroinflammation.

Neuroinflammation is an integral component of maintaining CNS innate immunity. Although this process plays a heavy role in mediating harmful stimuli and neural tissue, persistent neuroinflammation can become harmful and damaging to neural cells and tissue. Notably, neuroinflammation is considered one of the most common pathologies in CNS disease[20]. Neuroinflammation is a complex homeostatic process with a plethora of components and factors that mediate it, and accordingly, research into this topic is crucial to further understand how this system is physiologically regulated.

1.3.1 Immune cells in the central nervous system

Glial cells are the primary cells responsible for mediating inflammation, and these cells are the most abundant cell type of the CNS[20]. Glial cells, including astrocytes, oligodendrocytes, and microglia, not only interact with each other, but also neurons, immune cells, and blood vessels to mediate neuroinflammation. Collectively, glial cells work in tandem with other cells in their

environment to mediate homeostasis within the CNS. Although astrocytes and oligodendrocytes are critical to regulating this homeostasis, and communication of these cells with not only their local environment but other cells, including microglia, is imperative to maintaining and regulating neuroinflammation, the details of how these cells directly contribute to neuroinflammation are outside the scope of this thesis.

Additionally, a subgroup of mast cells that reside on the brain side of the blood-brain barrier (BBB) are thought to also be involved in neuroinflammation and neuroimmune communication through signaling with neurons, astrocytes, and microglia[22]. Although mast cells are typically found in the bloodstream and immune organs such as blood vessels, lungs, and the gut, a small subset of these cells are found in the CNS, where they can cross the BBB and orient themselves close to glial cells in the meninges and hypothalamus[23]. Here, they play a sentinel role, and accordingly, mast cells are thought to be one of the first responders to pathogenic events[24]. Following the detection of stimuli, mast cells can initiate cell signaling and communicate with other immune effector cells to initiate innate and adaptive immune responses[24]. Notably, mast cells in the CNS that have been activated degranulate to release histamine which can mediate phenotypic changes and stimulation of microglia[25].

1.3.2 Cytokines and chemokines in neuroinflammatory signaling

Cytokines and chemokines utilize chemical signals to mediate cell signaling, and these molecules participate in neuroinflammation while regulating the immune response. Whereas cytokines are a broader category of proteins and molecules released by inflammatory cells to mediate the immune response, chemokines are a subset of cytokines heavily involved in directing the location of immune cells to sites of inflammation. Cytokines can act on cells via autocrine, paracrine, and endocrine signaling pathways to enact their functions[26]. These substances are

released by immune and inflammatory cells and facilitate the type of inflammatory response, as well as its strength and duration[27]. In cases of neuroinflammation, cytokines and chemokines can directly and indirectly signal microglia and astrocytes through various signaling pathways; for instance, interleukin-6 (IL-6), a proinflammatory cytokine heavily focussed on in our work, is known to regulate astrocytic expression of inflammatory proteins via the JAK/STAT3 pathway to regulate transcription of genes involved in either pro- or anti-inflammatory action[28].

In the present thesis, we focus on two vital proinflammatory cytokines: IL-6 and IL-8. IL-6 is a key mediator in the immune cascade, and it utilizes the gp130 functional receptor complex to initiate proinflammatory gene transcription in cases of inflammation[29]. IL-6 is highly detected in chronic inflammatory diseases, and this compound is upregulated in microglial cells during inflammation and is thought to stimulate these cells in proinflammatory conditions[30,31]. Astrocytes and microglia are known to both react to and release IL-6 following stimulation, and this mediator is known to play an important role in neuroinflammatory signaling[32,33]. On the other hand, IL-8 is another key mediator in the neuroinflammatory response, and various cell types secrete this chemokine to act as a chemoattractant in proinflammatory conditions[34]. IL-8 effects are mediated by two G-protein coupled receptors: CXCR1 and CXCR2[35]. This chemokine acts via the Akt/PKB, MAPK, and Protein Kinase C signaling pathways to induce inflammation, and IL-8 signaling promotes transcription of various inflammatory genes via these signaling cascades[36]. During proinflammatory signal transduction, research has indicated high levels of IL-8[37], and research by Ryu et al. has indicated that IL-8 and its receptor, CXCR2, are responsible for chemotactic responses in Alzheimer's disease-induced inflammation[38]. Ultimately, IL-6 and IL-8 act in tandem in the neuroinflammatory signaling, upregulating gene expression of inflammatory genes to induce immune signaling cascades.

1.4 Microglia

One key mediator in CNS homeostasis is a tissue-specific macrophage called microglia. These cells comprise roughly 80% of brain immune cells[39], and microglia are largely denoted as the resident immune cells of the CNS. Microglia are incredibly dynamic, and in the healthy brain, these cells survey their local surroundings for pathogens, apoptotic cells, amyloid fibrils, and other debris[40]. Via the release of cytokines and signaling molecules, microglia mediate healthy CNS function and homeostasis through various mechanisms, including modulating synaptic transmission, remodeling the extracellular matrix, and contributing to synaptic pruning and long-term potentiation[41].

1.4.1 Microglial states

Microglia exist in dynamic, multifaceted states depending on the context of their local milieu[41,42]. Direct RNA sequencing has revealed unique microglial transcriptomic features that make these cells capable of sensing endogenous ligands and microbes, otherwise known as the microglial "sensome"[43]. To monitor changes in their environment, microglia use surface receptors to detect various stimuli within their sensome, which thereby dictates the 'state' of the microglial cell; this microglial state is thus influenced by their epigenome, transcriptome, proteome, and metabolome, which in turn leads to a range of morphological outputs (**Figure 1.1**)[42]. Notwithstanding, the functional implications of these different morphologies are not uniform or static. Whereas previous work has sought to connect specific morphologies with specific functions (such as "amoeboid" shaped microglia indicating microglial cells in a phagocytic state[44] or "rod-shaped" microglia indicative of microglia participating in neuronal repair[45]), recent work has highlighted that these previously defined morphological states can be detected in other contexts[42]. Accordingly, while it is important to note that microglia have various observable morphologies,

these structural shapes may not indicate the role of these cells; rather these various morphological outputs highlight just how dynamic microglial cells can be.

Microglial cell bodies are typically static within the body, whereas microglial processes regularly survey their environment[46]. Following the detection of stimuli by their processes, microglia can adopt a corresponding functional state depending on various factors such as the location, strength, and length of the stimuli[47]. These cells can then dynamically alter their function and state, contributing to the mediation of neuroinflammation through direct physiological actions such as phagocytosis and communication with other neuroinflammatory cells or indirect physiological actions such as cell signaling.

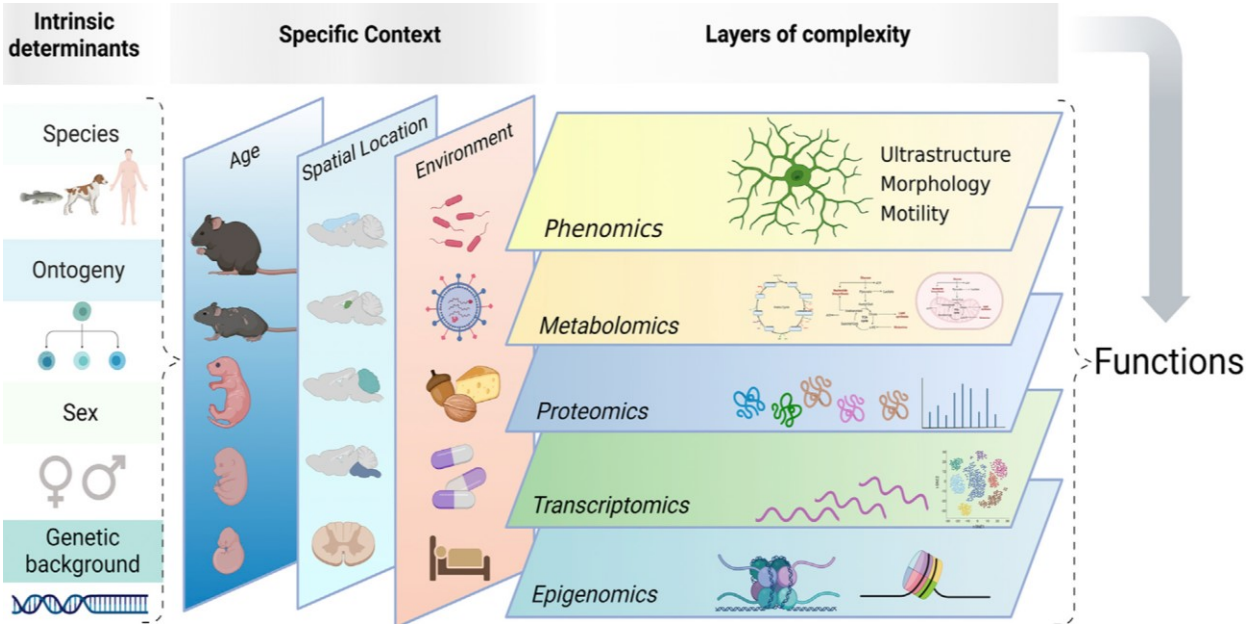


Figure 1.1. Microglial cells adopt various "states" depending on contextual cues within their environment. Intrinsic factors ranging from species to the genetic background of the host, alongside contextual determinants (age, spatial location, local microenvironment) impact microglia on various levels such as their phenomics, metabolomics, proteomics, transcriptomics, and epigenomics, which in turn determines microglial function. Figure adapted from Paolicelli et al. (2023)[42].

1.4.2 Microglia-mediated neuroinflammation

Microglia have an extensive repertoire of signaling pathways that act to play a role in neuroinflammation. The key pathways that drive microglial-induced pro-inflammation include the MAPK pathway, the JAK-SAT pathway, the PI3K/AKT pathway, and Wnt/ β-catenin mediated signaling[48], which ultimately activate the NF-κB transcription factor to synthesize proinflammatory cytokines and factors[49]. These cells also express numerous pattern recognition receptors that detect damage-associated molecular patterns (DAMPs) and pathogen-associated molecular patterns (PAMPs)[50]. Stimulation of microglia by DAMPs and PAMPs can also induce various signaling pathways that upregulate proinflammatory transcription genes through signaling by transcription factors such as NK-κB and AP-1[48].

Microglia can both release cytokines and react to cytokine signaling in response to numerous stimuli depending on their local microenvironment. The key proinflammatory cytokines released by these cells following their stimulation include IL-1β, IL-6, interferons (IFNs), and IL-8[26,48,51]. In particular, IL-1β and IL-6 play crucial roles in amplifying and initiating the microglial proinflammatory signaling response[50] as these cytokines are highly generated in the early stages of the innate immune response and participate in the activation of TLR, Myd88, and NF-κB pathways involved initiating inflammation[48,52]. IFN-α, IFN-β, and IFN-γ are the three most common IFNs in the CNS, with microglia and astrocytes being the key cells responsible for their production[48]. These molecules utilize Jak-STAT signaling pathways to drive innate and adaptive immune responses[53] and drive inflammation via upregulation of proinflammatory mediators such as IL-1β and IL-6[48].

In addition to their various functional states and ability to mediate neuroinflammation via cytokine regulation, microglia can also directly aid in mediating neuroinflammation via phagocytosis and interactions with other immune cells in the CNS. The phagocytic function of

microglia mediates tissue homeostasis via their recognition, engulfment, digestion, and response following microglial stimulation by cell signaling molecules[54]. These signals are appropriately named ‘find me’ and ‘eat-me’ signals; ‘find me’ signals are released by debris, apoptotic cells, and unwanted synapses, which, when detected by microglia, results in the migration of these cells towards the stimuli[55]. ‘Eat me’ signals are exposed on damaged cells and unwanted synapses. Following the detection and recruitment of microglial cells to stimuli, these cells may then engulf this material via phagocytosis and degrade them via endolysosomal processing[55]. Lastly, microglia initiate a signaling response by activating transcription factors that induce lipid clearance and release anti-inflammatory mediators to mediate neuroinflammation in the local milieu[56]. This cell signaling may also induce cytokine release and recruitment of other neuroimmune cells, such as astrocytes or other infiltrating immune cells, to assist in mediating neuroinflammation at sites of detected stimuli[57].

1.5 Histamine as a neurotransmitter and neuromodulator

In the brain, histaminergic neurons are most frequently found in the tuberomammillary nucleus of the posterior hypothalamus[58], and these neurons innervate the cortex, thalamus, cortex, and basal ganglia[58,59]. Histamine is a monoamine signaling molecule that can act as an excitatory neurotransmitter and, following release into the synaptic cleft, it can bind histamine receptors located on both pre- and post-synaptic terminals[59]. There are four known G-protein coupled histamine receptors (HRH1-HRH4). HRH1-HRH3 are highly expressed in the CNS, whereas HRH4 is primarily expressed in peripheral tissue[58,60]. HRH1 is coupled to the G_q-protein[58], which is typically associated with phospholipase C and inositol triphosphate pathways involved in cellular excitability and stimulation via the facilitation of calcium ion release[61]. HRH2 is coupled to the $G_S\alpha$ protein[58] which is associated with the activation of protein kinase A, a key regulator of

neuronal and glial physiology and plasticity via the regulation of cyclic AMP[62.63]. HRH3 engages with $G_{i/o}$ and utilizes $G_{q/11}$ signaling to modulate axonal and synaptic plasticity within the CNS[58], and this receptor also regulates the release of neurotransmitters including gamma-aminobutyric acid (GABA)[64,65], glutamate[65], and acetylcholine[66]. Lastly, HRH4 acts via Gi/o proteins to regulate neuronal excitability, particularly in the frontal cortex, hippocampus, and striatum[67,68].

In the CNS, histamine is typically stored in the cell somata and axons[69,70], and it utilizes the vesicular monoamine transporter 2 (VMAT-2) to transport into vesicles and is released during action potentials[71]. Histamine turnover in the brain is notably fast[72], and this compound is thought to be dynamically involved with neuromodulation, locomotion, memory, and sleep[58,73,74]. In neuroinflammation, histamine has been linked to the regulation of proinflammatory cytokines[75], primarily via the HRH1[76], HRH2[77], and HRH3[78] receptors, although HRH4 has been linked to other non-neuroinflammatory disorders such as allergy and autoimmune disease[79]. One key source of histamine in the CNS is mast cells, and microglia-mast cell crosstalk is a growing area of interest in neuroinflammation[24]. Notably, histamine release following mast cell degranulation is thought to signal a variety of ligands and cells, including microglia[80]. Interestingly, histamine and its receptors have been identified as potential therapeutic targets for multiple neuroinflammatory disorders, including amyotrophic lateral sclerosis (ALS)[81,82] and Parkinson's disease[83], and accordingly, research into this compound and neuroinflammation is crucial to understand its implications in the pathology of these cognitive disorders.

1.6 The cellular prion protein and the central nervous system

Genomic and structural research on PrP^C has indicated that it is highly conserved among vertebrates suggesting that it has an evolutionary advantage[84]. Notwithstanding, mouse models that are knockout for the PrP^C gene (*Prnp*) typically show a wildtype phenotype with no significant

abnormalities[85]. PrP^C is encoded by the *PRNP* gene in humans, which is located on exon 2 of chromosome 20[86].

Mature PrP^C is attached to the cell surface via a glycosylphosphatidylinositol (GPI) anchor[87], and the protein consists of a flexible N-terminal domain from amino acid residues 23-120 and a C-terminal domain from amino acid residues 121-131[88]. *PRNP* is highly expressed throughout the central and peripheral nervous systems[89,90] **(Figure 1.2)**. Certain cell types with particularly high PrP^C protein expression are largely involved in neuroinflammation and immune system regulation; this includes glial cells[91], dendritic cells[92,93], T cells[93], and peripheral blood leucocytes[94].

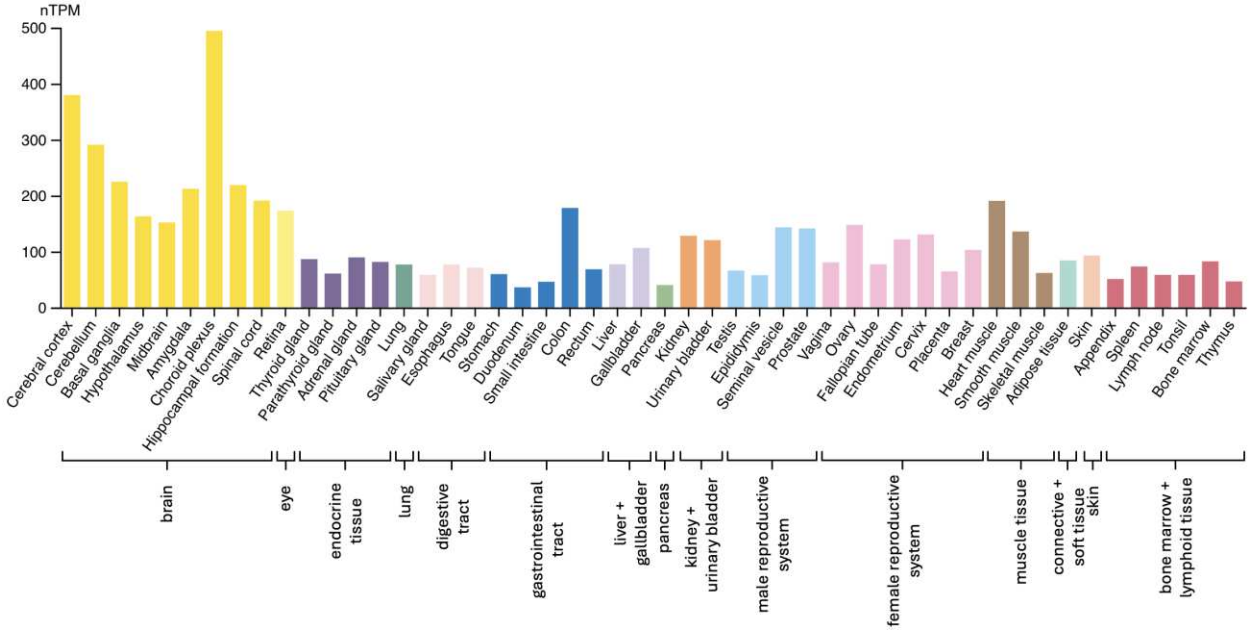


Figure 1.2. Normalized expression of *PRNP* gene across 55 human tissue types. Normalized transcripts per million (nTPM) of *PRNP* among different tissue types in various regions of the human genome. Colours indicate organ location. Data generated using the Human Protein Atlas (https://www.proteinatlas.org/).

CNS distribution of PrP^C is relatively high, with evidence supporting a high degree of expression in neurons[95,96] and neuroinflammatory cells, including astrocytes[97], microglia[14,91], and oligodendrocytes[91]. As previously described, PrP^C is notably abundant in regions of the brain

affiliated with higher cognitive functions, including the cerebral cortex, where it is believed to play an important role in neuronal function and CNS communication[98-100].

One key proposed role for PrP^C is that of a mediator for cellular signaling as the protein is located on the extracellular surface, and accordingly, it may modulate and participate in extracellular transmembrane signaling in its local milieu[10]. GPI-anchored proteins are known to act as molecular scaffolds for cellular signaling[101,102], and proteins containing GPI-anchors play key roles in signal transduction, immune response, and disease pathogenesis[103]. Due to these commonalities in GPI-anchored proteins, alongside the high expression of PrP^C in various immune cells, the involvement of PrP^C in cellular signaling in neuroinflammation is a topic of broad and current interest.

1.7 Rationale, Hypothesis, and Aims

Rationale:

Given that histaminergic neurons and mast cells in the CNS can release histamine which causes phenotypic changes and stimulation of microglia in mouse models[25], we questioned whether similar changes might occur in human microglia exposed to histamine. Furthermore, we sought to explore whether PrP^C may be involved in modulating any effect seen, given its probable role in many facets of neuroinflammation.

Hypothesis and Aims:

In the present study, we hypothesized that human-derived microglia could be stimulated by the neurotransmitter and endogenous immune mediator histamine and that this stimulation would alter the expression of PrP^C. The study aims are presented in **Figure 1.3** and are as follows: **Aim 1**: To determine a reliable, consistent approach towards measuring total and surface PrP^C expression in neuroimmune cells with a focus on microglia.

Aim 2: To identify histamine receptors on human microglia cells and investigate histamine as a potential mediator to stimulate microglia.

Aim 3: To explore PrP^C as a potential mediator of neuroinflammation in microglia and measure PrP^C expression changes following microglial stimulation.

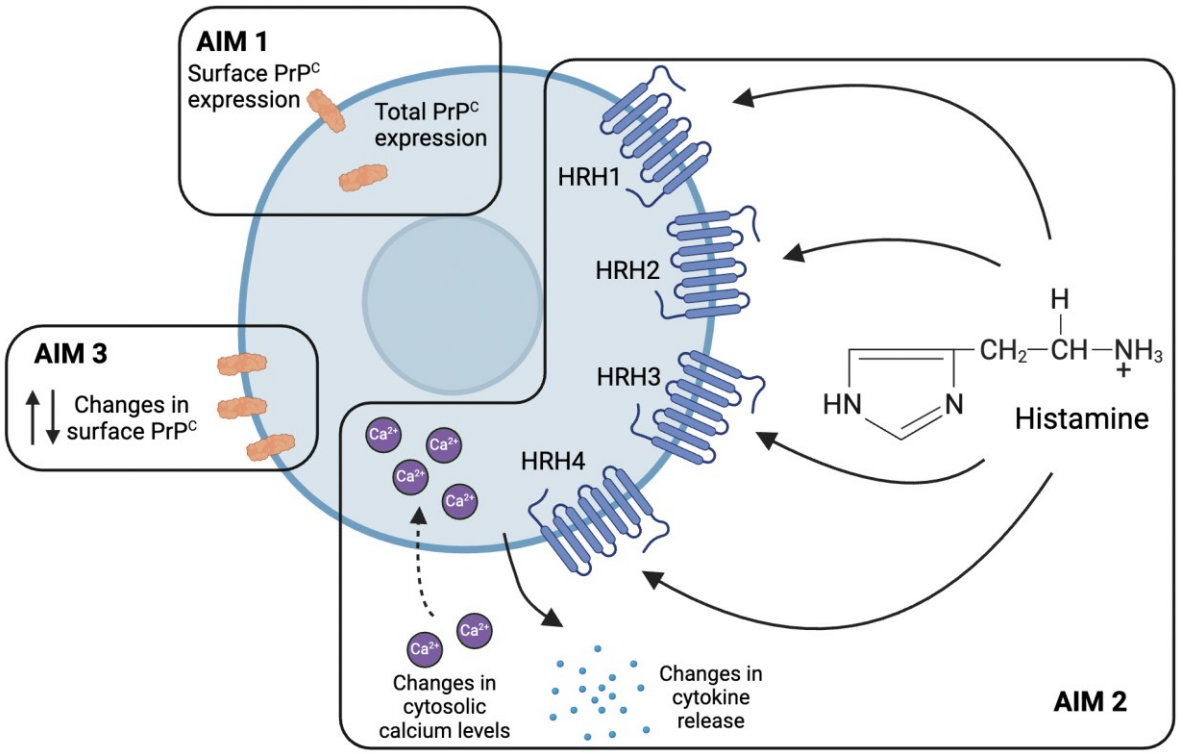


Figure 1.3. Study aims. The initial aim was to test various immune, neurological, and neuroimmune cell models to establish an approach towards determining relative levels of PrP^C expression. The second aim was to explore histamine receptors on human microglial cells and to test how histamine may induce stimulation of these cells to modify their reactivity. Lastly, the final aim was to identify how PrP^C expression levels may be modified in microglial cells following histamine-induced stimulation. Overall, these aims looked to explore how PrP^C on microglial cells may be influenced by histamine-induced stimulation to contribute to neuroinflammation.

CHAPTER 2: Comparative measurements of total and surface prion protein expression on central nervous system cells and cell lines

2.1 Chapter 2 Abstract

This chapter further discusses the various roles of the cellular prion protein (PrP^C) in the immune and central nervous systems. First, we discuss relative gene expression levels of PrP^C throughout the brain before discussing the role of this protein in immune cell regulation and cellular signaling. Here, we highlight how PrP^C plays critical roles in neuronal signaling and synaptic transmission, interacting with key pathways that influence neuronal survival and plasticity.

Next, this chapter focusses on the expression of PrP^C in various cell models. We identify a reliable and consistent approach towards measuring relative total and surface PrP^C levels via western blotting and flow cytometry, respectively. Our results confirm that we can accurately measure relative levels of PrP^C on various cell models including human-derived mast cells, monocytes, glioblastoma cells, and microglial cells. Our findings introduce the potential significance of surface PrP^C expression levels in these cell models, providing potential insights into its potential role in neuroinflammation and immune signaling. This groundwork sets the stage for further exploration of the dynamic expression of PrP^C in response to stimulation in microglial cells, which will be elaborated on in the ensuing chapter.

2.2 Background

The cellular prion protein (PrP^C) has been identified in a vast number of cells involved in immune responses including human lymphocytes[104], dendritic cells[105], and T cells[93]. Human RNA-sequencing analysis has identified PrP^C distribution in nearly all immune cell types with particularly high expression in basophils, monocytes, T cells, B cells, dendritic cells, and natural killer (NK) cells[106]. The function of PrP^C in the innate immune system is still largely contested; however, literature suggests the protein is involved in the differentiation and activation of immune cells and immunoregulatory cellular interactions and communication networks between different cell types. Additionally, human RNA-sequencing in the CNS has demonstrated diverse expression of PrP^C across the brain, with particularly high expression in the choroid plexus, cerebral cortex, basal ganglia, thalamus, and white matter[107] (**Figure 2.1**). In the brain, PrP^C has been found to play a role in neuronal and synaptic transmission and cellular signaling. The diverse and nearly ubiquitous expression of PrP^C across the immune system and the brain may hint at an extensive and vast number of physiological functions in healthy and diseased states.

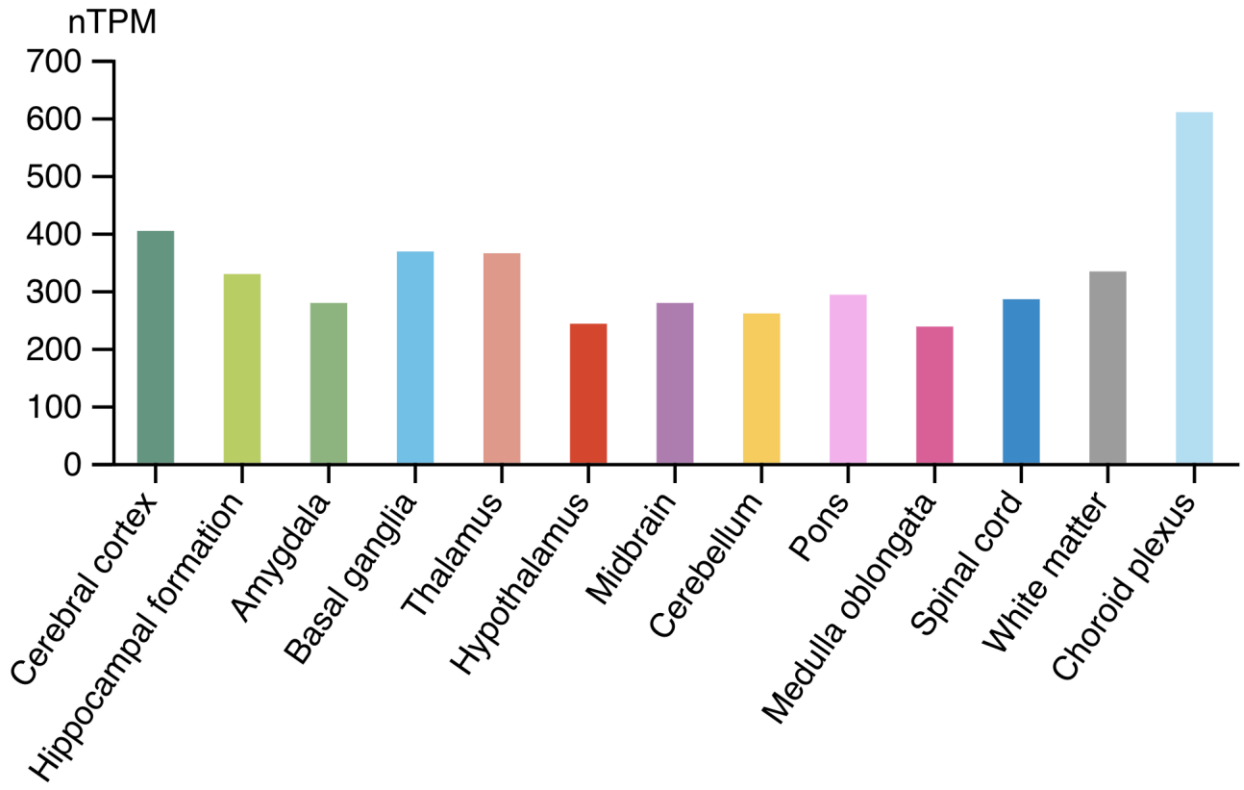


Figure 2.1. Normalized expression of *PRNP* in the brain. Normalized transcripts per million (nTPM) of *PRNP* among different tissue types in (**A**) immune cells and (**B**) different regions of the brain. Data generated using the Human Protein Atlas (https://www.proteinatlas.org/).

2.2.1 PrP^C in development and activation of immune cells

PrP^C is expressed in numerous types of cells that participate in immune regulation, and relative expression levels vary significantly between different cell types[108,109]. Generally, immune cells develop from pluripotent stem cells and differentiate into a variety of cells, including granulocytes, mast cells, monocytes, macrophages, dendritic cells, NK cells, and lymphocytes[110]. These cells play a physiological role in the clearance of pathogens and protection from disease. Research in dendritic cells and monocytes has demonstrated that not only do these cell types express PrP^C, but this protein is upregulated following the maturation of these cells[93,94]. Upregulation of PrP^C has also been observed in human NK cells undergoing differentiation and functional maturation[94], and activated lymphocytes increase their expression of PrP^C as well[104].

Ultimately, current literature suggests PrP^C may mediate inflammation in immune cells, likely in an anti-inflammatory capacity[111]. PrP^C has also been identified in human mast cells[112,113]. These cells play an essential role in modulating inflammation and regulating both innate and adaptive immune responses in the body through the potentiation of signaling mediators following their activation.

2.2.2 PrP^C as a signaling molecule

Evidence that PrP^C can act as a signaling molecule is extensively reviewed by Hirsch et al.[114], Liebert et al.[115], and Bakkebø et al.[111]. Due to the intrinsically disordered nature of its N-terminal domain[116], PrP^C has promiscuous binding tendencies leading to a vast range of signaling interactions[117]. Notably, PrP^C binding has been identified to interact with more than 45 different ligands[115], including proteins in the extracellular matrix[95], molecules on the plasma membrane[10,11], intracellular membrane domains[118], transmembrane proteins[10] and ion channels[119], and numerous scaffolding proteins[120] and chaperones[7,121]. The propensity of PrP^C to engage with molecules and signaling complexes on the cellular surface is crucial in PrP^C-mediated cell signaling. As PrP^C is GPI-anchored, the protein typically localizes in lipid rafts within cells[114], or in postsynaptic densities (PSDs)[122]. Due to the location and composition of these rafts and PSDs, PrP^C signaling is facilitated by the spatial segregation of receptors[114] which provides a favourable environment for the protein to act effectively as a signaling molecule.

2.2.3 PrP^C in immunoregulatory signaling

Looking specifically at PrP^C signaling within immunoregulatory pathways, PrP^C has been found to participate as a signaling molecule in the T cell receptor signaling pathway[104] in addition to extracellular and transmembrane signaling pathways involved in immunoregulation[115,123]. Although PrP^C signaling has been identified in an abundance of immunoregulatory pathways, the

unequivocal role of the protein in cellular signaling remains elusive. Broadly, the protein is thought to participate in cell signaling on patrolling immune cells to interact with other signaling molecules and facilitate immunoregulatory signaling both extracellularly and intracellularly[111].

2.2.4 PrP^C in neuronal signaling and synaptic transmission

Within the brain, PrP^C signaling is key in the differentiation of human embryonic stem cells into neurons, oligodendrocytes, and astrocytes[15]. In neurons, PrP^C is hypothesized to participate in nearly every aspect of neuronal physiology[114]. It has been found to target and activate Src family kinases Fyn and Lyn to participate in neurite outgrowth[10,124], protein kinase A family kinases and ERK1/2 kinases to assist in neuroprotection[9], and the phosphatidylinositol 3-kinase/protein kinase B/Akt (PI3K/Akt) pathway to modulate neuronal survival[6]. PrP^C is thought to mediate anterograde and retrograde axonal transport in both pre- and post-synaptic compartments of axon terminal endings[98,125].

Molecules that reside within the lipid raft of neurons, such as neurotransmitter receptors, interact with PrP^C[114]. Extracellular and intracellular interactions between PrP^C and G-protein coupled neurotransmitter receptors have been observed in serotonergic epithelial cells by Mouillet-Richard et al.[12], and the mediated effects of PrP^C were thought to be dependent on G-protein coupled receptor (GPCR) pathways and the presence of the plasma membrane protein caveolin. Additionally, signaling by PrP^C in metabotropic glutamate GPCRs has been observed in several glutamate receptor subtypes including mGlu1 and mGluR5[13], two key receptors in synaptic plasticity and synaptic transmission[126]. An extensive analysis of PrP^C and its interactions with GPCRs will be discussed in section 3.5 of this thesis.

Expression of PrP^C has been identified in various human cell lines across the brain (**Figure 2.2A**) and numerous neuroblastoma cell lines (**Figure 2.2B**). Notably, RNA expression of *PRNP*

can be found in the U-87 MG human glioblastoma cell line[127], and, in the present thesis, we measured surface PrP^C protein expression via flow cytometry in these cells.

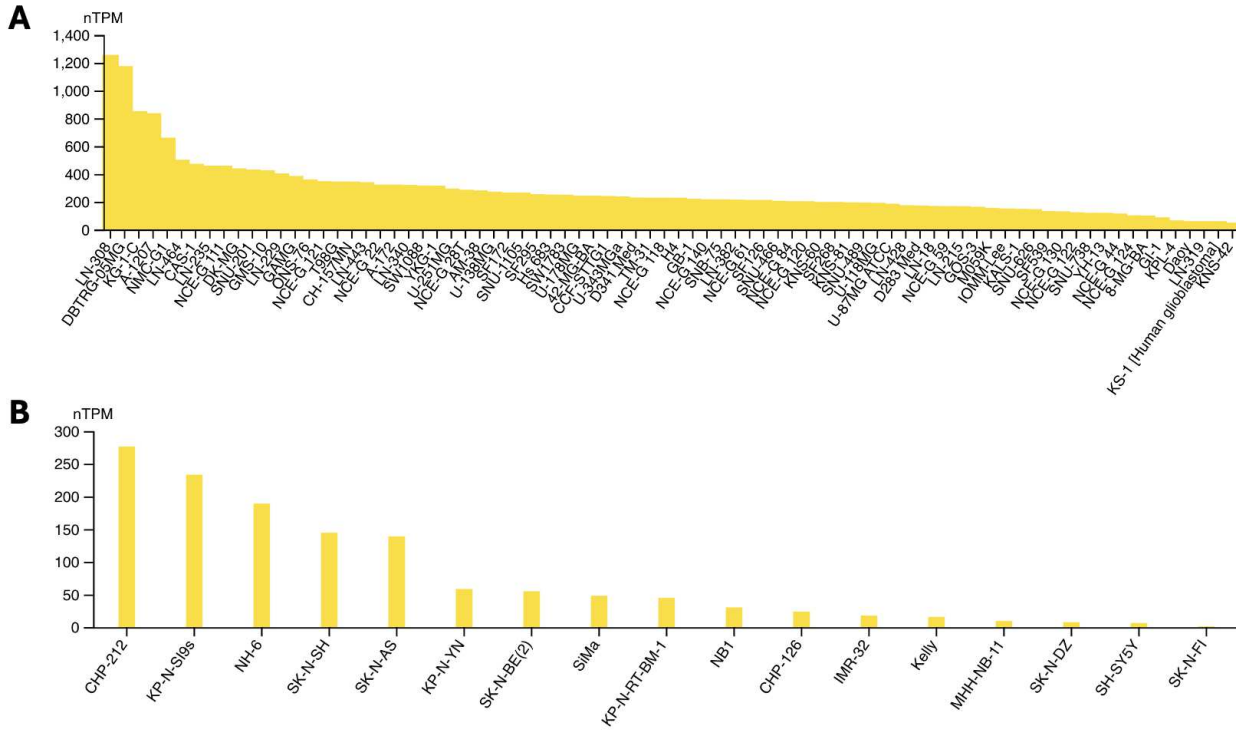


Figure 2.2. Normalized transcripts per million (nTPM) of *PRNP* in central nervous system cell lines. (**A**) PrP^C expression has been detected in numerous human cell lines of the brain. (**B**) PrP^C expression in neuroblastoma cell lines. Data is reported as normalized transcripts per million (nTPM). Data generated via the Human Protein Atlas (https://www.proteinatlas.org/).

2.2.5 PrP^C in neuro-immune cells

The neuroimmune system is a complex network connecting the nervous system to the immune system. Fundamentally, this network protects neurons and the nervous system from pathogens and harm. As previously discussed in section 1.3, the neuroimmune system is made up primarily of glial cells including astrocytes, oligodendrocytes, and microglia. PrP^C was initially identified in neurons, astrocytes, and oligodendrocytes by Moser et al. in 1995[91], and since this discovery, the role of PrP^C in glial cells has been an area of interest. Neuron-glial interactions are essential for the development of neural circuits and synapses[128,129], and PrP^C in astrocytes has been

observed to modulate cell-cell interactions and neuronal survival in order to promote neuritogenesis through engagements with stress-inducible protein-1 and laminin[97]. In microglia, PrP^C has been implicated in modulating apoptosis on top of mediating the balance between pro- and anti-inflammatory signaling[130,131]. More background on PrP^C and microglial interaction is provided in sections 3.2 and 3.5 of this thesis.

In the present section, we aimed to test PrP^C expression on various cell lines via flow cytometry and western blotting to establish a reliable, consistent approach towards quantifying relative expression levels of this protein.

2.3 Methods

2.3.1 Cell culture

Laboratory of Allergic Diseases 2 (LAD2) human-derived mast cells were cultured in StemPro-34 serum-free medium (Fisher Scientific) supplemented with 20 mM L-glutamine (Fisher Scientific), 100 U/ml penicillin, 100 µg/ml streptomycin, and 100 ng/ml of stem cell factor (PeproTech). LAD2 cells were gifted by Dr. Arnold Kirschenbaum and Dr. Dean Metcalfe at the National Institute of Allergy and Infectious Diseases, National Institutes of Health.

The human mast cell line 1.1 (HMC1.1) and human mast cell line 1.2 (HMC1.2) cell lines were cultured in Iscove's Modified Dulbecco's Medium (Lonza, Mississauga, ON, Canada) supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin. The THP-1 human monocyte cell line (ATCC, Manassas, VA, USA) was cultured in RPMI-1640 (Sigma, St. Louis, MO, USA) supplemented with 10% heat-inactivated FBS and 0.05 mM 2-mercaptoethanol. U-87 MG human glioblastoma cells (ATCC) were cultured in EMEM supplemented with 10% heat-inactivated FBS.

Human microglia cell 3 (HMC3) cells (ATCC) were cultured in complete culture media consisting of minimum essential medium (MEM) supplemented with 10% heat-inactivated FBS (HyClone, Logan, UT, USA), 100 U/mL penicillin (Thermo Scientific, Waltham, MA, USA), and 100 µg/ml streptomycin (Gibco, Waltham, MA, USA). Twenty-four hours prior to the experiments, the complete culture media was replaced with MEM supplemented with 1% heat-inactivated FBS to limit the basal proliferation and cytokine secretion of cells and maintain a less stimulated microglial state. All cells were maintained at the confluency recommended by ATCC and other publications, cultured in the appropriate media at 37°C and 5% CO_2, and used within eight weeks of thawing from cryopreservation.

2.3.2 Flow cytometry

We first measured surface PrP^C expression on various cell types via flow cytometry. Specifically, we tested three human mast cell models: LAD2 cells, HMC1.1 cells, and HMC1.2 cells. We also tested a human monocyte cell model, THP-1 cells, and a glioblastoma cell line, U-87 MG cells. Lastly, HMC3 cells were used to test surface PrP^C in a microglial cell model.

We isolated 5.0×10^5 cells which were washed in sterile PBS and blocked for 10 minutes on ice with 3% bovine serum albumin (BSA) in PBS (Marlborough, MA, USA) to prevent nonspecific interactions with the test antibodies. Cells were incubated with primary antibody, mouse anti-PrP^C IgG1κ (POM2, Millipore Sigma), or a mouse IgG1κ isotype control antibody (eBioscience), for 30 minutes at 4°C on a plate shaker. The cells were washed twice with 0.1% BSA in PBS and then the cells were incubated with anti-mouse APC (Invitrogen) for 1 hour at 4°C on a plate shaker. The cells were washed three times with 0.1% BSA in PBS and resuspended in 0.1% BSA in PBS for analysis on a Cytoflex flow cytometer (Beckman Coulter). Cells and debris

were separated by gating on an FSC-A vs. SSC-A plot while single cells were selected by gating on an FSC-A vs. FSC-H dot plot. Changes in fluorescence were observed following gating.

2.3.3 Western blot

LAD2 and HMC3 cells were washed with PBS and lysed by resuspending the cell pellets in RIPA lysis buffer (100 mM Tris HCL pH 8.0, 10 mM EDTA pH 8.0, 100 mM NaCl, 0.5% sodium deoxycholate, 0.5% NP-40, EDTA-free cOmplete™ protease inhibitor cocktail (Roche), EDTA-free SIGMAFAST™ Protease Inhibitor Cocktail, and 6-10 U/mL benzonase). Lysates were boiled and loaded onto a 12% acrylamide gel for SDS–polyacrylamide gel electrophoresis. After transfer to 0.2 μm PVDF membranes (Millipore Sigma), the membranes were blotted with mouse anti-prion protein antibody IgG1κ (POM2, Millipore Sigma). All immunoblots in a single subfigure are from a single membrane that was stripped and re-probed between blots. The membranes were imaged with an Odyssey CLX Imaging System (LI-COR).

2.4 Results

2.4.1 PrP^C expression in immune cells

To further understand PrP^C as a potential mediator of immune signaling and inflammation, we first wanted to measure PrP^C expression on immune cell models. Using western blot analysis with the anti-PrP^C POM2 antibody, we confirmed that we could detect total PrP^C in LAD2 mast cell lysates (**Figure 2.3A**). Next, as PrP^C is typically localized on the cell surface where it is thought to participate in cellular signaling, we used the same antibody to measure surface PrP^C via flow cytometry. In addition to LAD2 cells, we identified cell-surface PrP^C expression in two other mast cell models, HMC1.1, and HMC1.2 cells, and one monocytic cell model, THP-1 cells via flow cytometry (**Figure 2.3**). Notably, LAD2 and THP-1 cell models had higher surface expression of PrP^C relative to HMC1.1 and HMC1.2 cells.

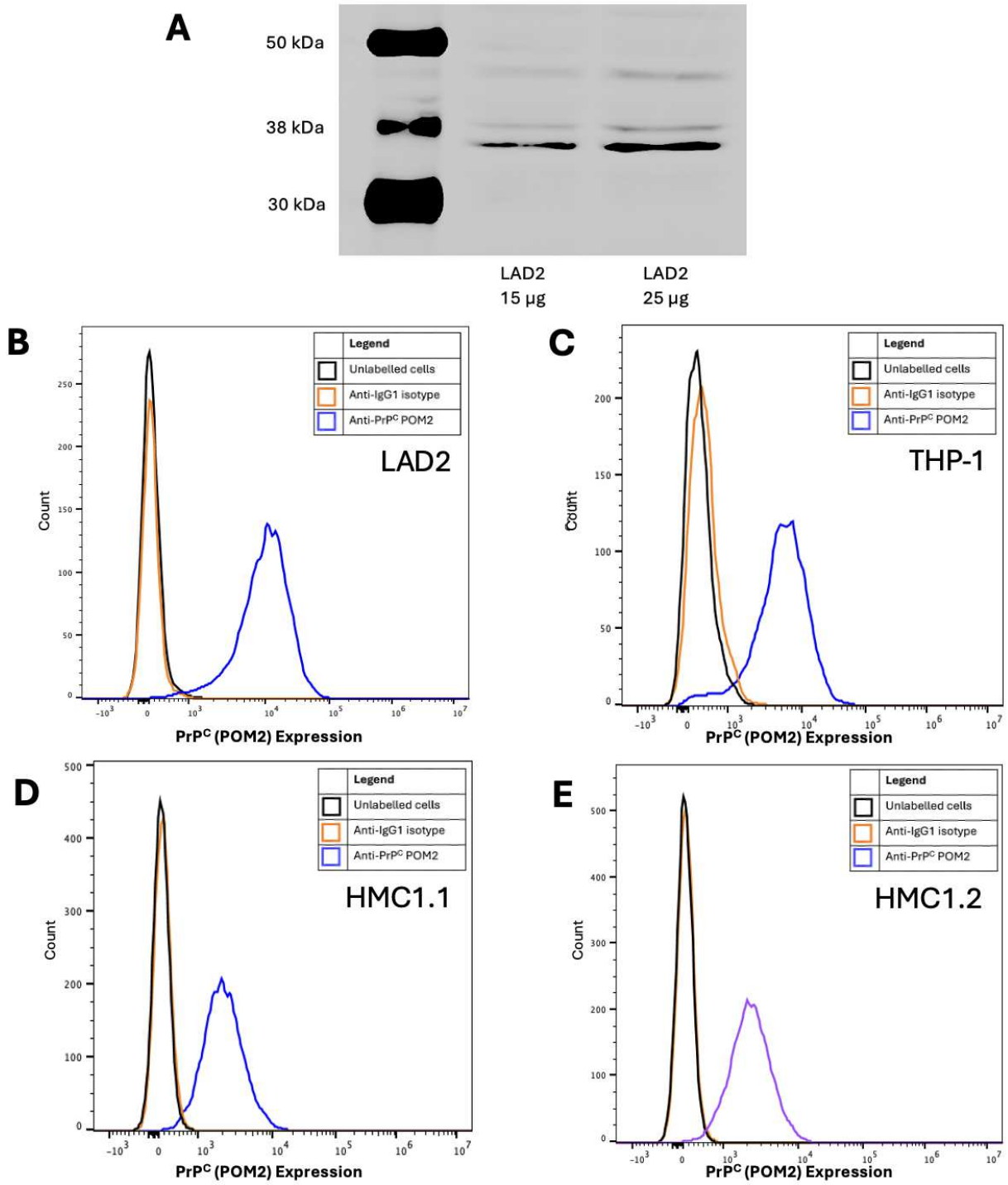


Figure 2.3. PrP^C is expressed in human immune cell lines. (**A**) Total PrP^C in LAD2 cells via western blot with anti-PrP^C POM2. A representative image of three independent experiments is shown. (**B-E**) Surface PrP^C expression was measured by flow cytometry with the anti-PrP^C POM2 antibody (blue). PrP^C expression was identified in (**B**) LAD2, (**C**) THP-1, (**D**) HMC1.1, and (**E**) HMC1.2 cell lines. Unlabelled cells (black) and cells labeled with the anti-IgG1 isotype (orange) were included as controls. N=3.

2.4.2 PrP^C expression in the U-87 MG neurological cell model

We next wanted to measure surface PrP^C expression in a neurological cell model to compare relative levels of expression in neurological cells with that of immune cells and microglial cells. We utilized the human U-87 MG glioblastoma cell line and repeated the flow cytometry experiments previously described and identified surface PrP^C expression levels similar to that of LAD2 and THP-1 cells (**Figure 2.4**).

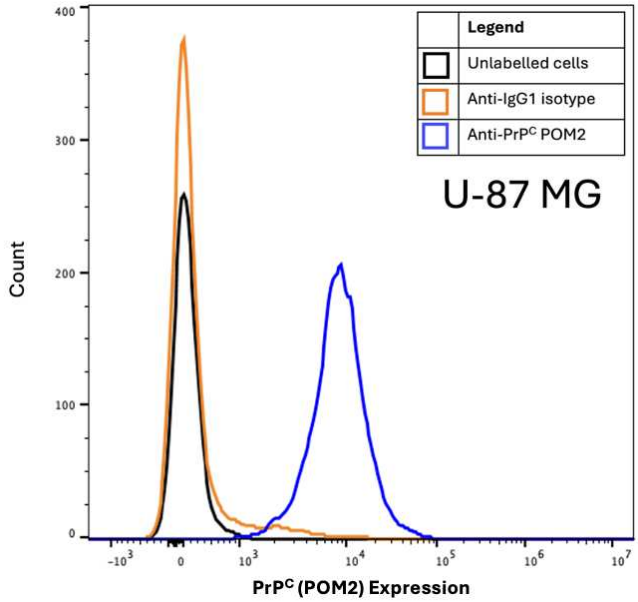


Figure 2.4. Surface PrP^C is expressed in the U-87 MG human neurological cell line. Surface PrP^C expression was identified using the anti-PrP^C POM2 antibody (blue). PrP^C expression was identified in the U-87 MG cell line. Unlabelled cells (black) and cells labelled with the anti-IgG1 isotype (orange) were included as controls. N=3.

2.4.3 PrP^C expression in the HMC3 microglial cell model

Upon establishing that models of neurological cells and immune cells express surface PrP^C, and confirming our methods in detecting this expression, we shifted our focus to a cell type that represents both of these classifications as neuro-immune cells: microglia. We sought to understand PrP^C as a regulator of neuroimmune cells and further explore the role of this protein in neuroinflammation, and accordingly, we used the HMC3 human-derived microglia model which

is further described in Chapter 3 of this thesis. First, we measured total PrP^C expression via western blot on HMC3 microglial cells (**Figure 2.5A**) and cell-surface PrP^C expression in HMC3 microglial cells via flow cytometry. (**Figure 2.5B**).

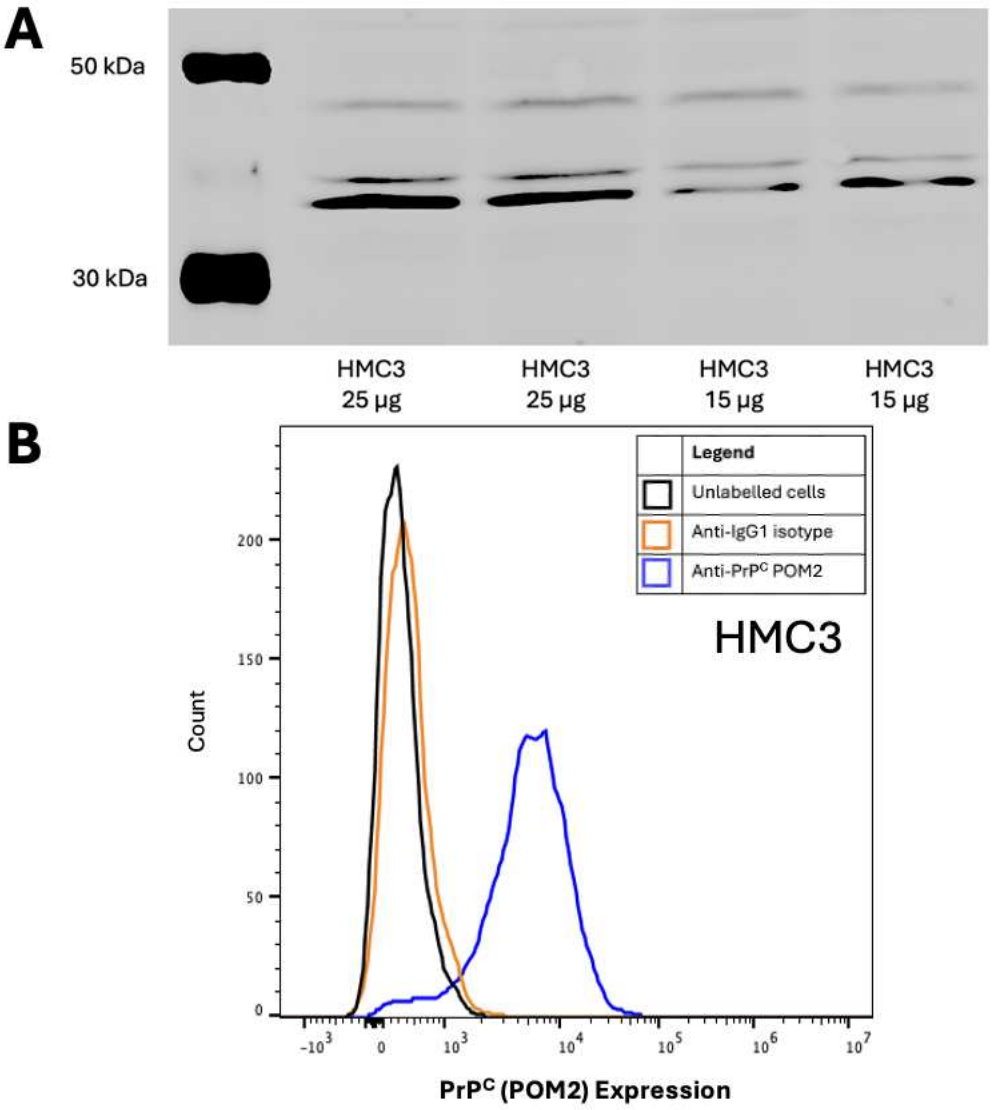


Figure 2.5. Surface PrP^C is expressed in the HMC3 human microglial cell line. (**A**) Total PrP^C can be identified in HMC3 via western blot with anti-PrP^C POM2. N=3. (**B**) Surface PrP^C expression was identified in HMC3 using the anti-PrP^C POM2 antibody (blue). Unlabelled cells (black) and cells labelled with the anti-IgG1 isotype (orange) were included as controls. N=6.

2.5 Discussion

In order to explore the relative expression of surface PrP^C in various cell models and establish a reliable, consistent approach towards measuring this expression, we screened various cell types and models via flow cytometry and western blot. As PrP^C is typically localized on the cellular surface attached via a GPI-anchor, we primarily focussed on measuring PrP^C via flow cytometry. We identified surface PrP^C on three human mast cell models (LAD2, THP1.1, THP1.2), a human monocyte cell model (THP-1), a human glioblastoma model (U-87 MG), and a human microglial cell model (HMC3). In establishing this detection, we confirmed a consistent technique that could be used to detect relative surface PrP^C expression levels. We also established that total PrP^C can be detected in LAD2 and HMC3 cells using the anti-PrP^C POM2 antibody via western immunoblotting.

In mast cell models, previous reports validate our findings and have identified high amounts of PrP^C in LAD2 cells and lower amounts of PrP^C in HMC1.1 and HMC1.2 cells[113]. PrP^C in mast cells is thought to mediate the activation of these cells[132], and interestingly, mast cells have been found to express PrP^C while resting and alter PrP^C expression during degranulation[133]. PrP^C expression on these cells is speculated to play a role in the balancing of inflammatory signaling via paracrine interactions[134].

PrP^C expression in monocytes is usually maintained throughout the differentiation of these cells[135], and PrP^C expression is typically increased during maturation[94]. We performed our experiments on undifferentiated THP-1 cells and our findings demonstrating the expression of PrP^C substantiates previous reports that identified PrP^C in these cells[8]. In monocytes, PrP^C is thought to play a role in mediating signaling cascades in the ERK1/2 signaling pathway which is

responsible for a variety of cellular and signaling processes such as cell proliferation, survival, and apoptosis[136].

Our data demonstrating surface expression of PrP^C in U-87 MG cells validates previously reported data by Lopes et al. which showed U-87 MG cells express high amounts of PrP^C within the cell membrane[137], though we are the first to show surface expression via flow cytometry. Research by this group not only demonstrated PrP^C expression on the U-87 MG glioblastoma model but also that PrP^C signaling could be important in the ERK1/2 signaling pathway. PrP^C in glioblastoma cell-signaling also plays a role in glioma-programmed cell death[138].

Although previous research in human microglial cells has identified PrP^C expression in these neuroimmune cells[5,131,139], we are the first to show PrP^C in HMC3 cells. We established that our flow cytometry and western blotting techniques could be utilized to detect relative PrP^C expression levels and that HMC3 microglial cells have a relatively high amount of surface PrP^C. Accordingly, since we have established these techniques and can quantify the basal level of PrP^C expression on unstimulated microglia, we next wanted to test how microglial cells may dynamically alter the expression of this protein following stimulation. More details of PrP^C and microglia – including the rationale behind the use of these cells, the expression of PrP^C in them, and observed changes in surface PrP^C expression levels following microglial stimulation – will be discussed in Chapter 3.

CHAPTER 3: Histamine stimulates human microglia to alter cellular prion protein expression via the HRH2 histamine receptor

Note: A version of this chapter is currently in revision with Scientific Reports as follows: Pehar M., Hewitt M., Wagner A., Sandhu J.K., Khalili A., Wang X., Cho J-Y., Sim V.L., Kulka M. "Histamine stimulates human microglia to alter cellular prion protein expression via the HRH2 histamine receptor".

3.1 Chapter 3 Abstract

Although the cellular prion protein (PrP^C) has been evolutionarily conserved, the role of this protein remains elusive. Recent evidence indicates that PrP^C may be involved in neuroinflammation and the immune response in the brain, and its expression may be modified via various mechanisms. Histamine is a proinflammatory mediator and neurotransmitter that stimulates numerous cells via interactions with histamine receptors 1-4 (HRH1-4). Since microglia are the innate immune cells of the central nervous system, we hypothesized that histamine-induced stimulation regulates the expression of PrP^C in human-derived microglia.

The human microglial clone 3 (HMC3) cell line was treated with histamine, and intracellular calcium levels were measured via a calcium flux assay. Cytokine production was monitored by enzyme-linked immunosorbent assay (ELISA). Western blotting and quantitative reverse transcription-polymerase chain reaction were used to determine protein and gene expression of HRH1-4. Flow cytometry and western blotting were used to measure PrP^C expression levels. Fluorescence microscopy was used to examine Iba-1 and PrP^C localization.

HMC3 cells stimulated by histamine exhibited increased intracellular calcium levels and increased release of IL-6 and IL-8, while also modifying PrP^C localization. HMC3 stimulated

with histamine for 6 and 24 hours exhibited increased surface PrP^C expression. Specifically, we found that stimulation of the HRH2 receptor was responsible for changes in surface PrP^C. Histamine-induced increases in surface PrP^C were attenuated following inhibition of the HRH2 receptor via the HRH2 antagonist ranitidine. These changes were unique to HRH2 activation, as stimulation of HRH1, HRH3, or HRH4 did not alter surface PrP^C. Prolonged stimulation of HMC3 decreased PrP^C expression following 48 and 72 hours of histamine stimulation.

HMC3 cells can be stimulated by histamine to undergo intracellular calcium influx. Surface expression levels of PrP^C on HMC3 cells are altered by histamine exposure, primarily mediated by HRH2. While histamine exposure also increases release of IL-6 and IL-8 in these cells, this cytokine release is not fully dependent on PrP^C levels, as IL-6 release is only partially reduced and IL-8 release is unchanged under the conditions of HRH2 blockade that prevent PrP^C changes. Overall, this suggests that PrP^C may play a role in modulating microglial responses.

3.2 Introduction

Microglia are ubiquitously found throughout the brain and are considered the resident innate immune cells of the central nervous system (CNS). In healthy adult brains, these cells dynamically monitor their local milieu, mediating homeostasis and regulating immune function[40]. Microglia enrich and support healthy brain function and CNS regulation via the release of cytokines, chemokines, and various trophic factors in response to damage- and pathogen-associated molecular patterns presented by harmful stimuli and debris[140]. These proinflammatory mediators can then accumulate in specific tissue regions, inducing an inflammatory cascade and stimulating more microglia to participate in inflammation and immune regulation.

Histamine is a neurotransmitter and neuromodulator in the CNS that participates in numerous brain functions. This mediator is involved in neuroinflammation and contributes to both innate and adaptive immune responses[141]. The sources of histamine in the body and CNS include but are not limited to, mast cells, which release histamine during degranulation[24], and the tuberomammillary nucleus, which consists of various histaminergic neurons responsible for immune function, cognition, wakefulness, and neurotransmission[59]. Histamine acts via interaction with four G protein-coupled histamine receptors (GPCRs): HRH1, HRH2, HRH3, and HRH4. The HRH1 and HRH2 receptors are primarily involved in proinflammatory actions[142,143], whereas physiological actions such as cytokine release and neuroinflammation regulation are mediated by HRH2 and HRH3[141,142]. The HRH4 receptor was the last to be discovered, and although the function of this receptor is not yet fully understood, HRH4 is thought to be involved in mediating mast cell chemotaxis[144] and stimulating the immune response in asthma, potentially through its neuroinflammatory capacity[145].

Recently, studies on microglia have shown that histamine alters microglial reactivity and stimulates these cells in neurological disease[146]. Microglia can be exposed to histamine via various mechanisms, as this molecule is frequently found in the CNS as a neurotransmitter. Recent studies have shown that mast cells can interact with microglia via the release of histamine[147], potentially resulting in changes in microglial phenotypes[148].

Studies have indicated that murine microglia react to histamine by increasing the release of the inflammatory mediators IL-1β[149], TNF[141], IL-6[150], and various others[83]. Bader et al. were the first to demonstrate that histamine activates microglia[151]; this work used calcium imaging to highlight that primary rat microglia were reactive to histamine and increased their intracellular calcium concentrations after exposure. There is limited and contradictory evidence pertaining to the expression of histamine receptors on microglia; for example, functional HRH1 has been demonstrated in murine N9 microglia[83] but not in primary mouse microglia[152]. Notwithstanding, microglial reactivity to histamine has been reported via all four histamine receptors in some capacity[83,152,153].

The cellular prion protein (PrP^C) is a small cell-surface glycoprotein vastly spread throughout the CNS[131,154]and immune system[113,155,156]. A range of physiological functions have been reported for PrP^C, and this protein functions as an important signaling coreceptor in the brain that may form signaling complexes with other receptors, such as gangliosides or microdomains common in immune cell activation[156]. Genomic and structural analysis of PrP^C has indicated that this protein is highly conserved among vertebrates[84], suggesting that PrP^C has an evolutionary advantage. PrP^C is expressed at various levels in immune cells, and accordingly, there have been numerous proposed physiological roles for it, including mediating inflammation[132] and immune cell activation[15]. The gene that encodes PrP^C, *PRNP*, is expressed in neurons and glial cells of the

CNS[2], and PrP^C is thought to mediate inflammation and protection against pathological stresses such as oxidative damage[157]. PrP^C is expressed in mouse microglia, where it may play a role in the maintenance of these cells in their quiescent state and regulate microglial reactivity by modifying cellular homeostasis and promoting inflammation[131]. However, there is currently little understanding of PrP^C in human microglia and immune cells; the physiological role of this protein in human glial cells remains poorly understood.

In previous studies, stimulating BV-2 microglia with neurotoxins resulted in changes in *Prnp* gene expression[139] and PrP^C protein expression[5]; PrP^C expression may be necessary for microglial stimulation and inflammation induction[14]. As histamine is a key regulator of neuroinflammation, we wanted to test the effects of histamine on microglial reactivity and investigate potential changes in PrP^C expression. In the present study, we hypothesized that human-derived microglia could be stimulated by the neurotransmitter and immune mediator histamine to alter the expression of PrP^C, potentially revealing important new insights into PrP^C functions in the brain and neuroinflammation.

3.3 Materials and methods

3.3.1 Microglial research and the human microglial clone 3 (HMC3) cell line

Microglia have been observed to participate in neuroinflammation and neuropathology in both beneficial and harmful ways. As such, microglial cells are an area of valiant research efforts aiming to fully understand how these cells may be physiologically important in both healthy and diseased models, and how they may be a target for pharmacological intervention in disease. Notwithstanding, the research of these cells has proven difficult for a variety of reasons; firstly, *in vivo* microglia dynamically interact with their environment, interacting with neurons, astrocytes, immune cells, and other microglia. Signals to and from these cells within their environment dictate

their physiological role at any given point in time, and this role can be modified should cell signaling be altered or if a stimulus is introduced. Studying these cells in an *in vitro* model essentially removes them from their milieu and, accordingly, *in vitro* microglia are phenotypically and characteristically different from *in vivo* cells. Additionally, as there is such a high research interest in the glial field, nomenclature and classifications of microglia are dynamically evolving and changing at a rapid rate[41,158]. As such, while research in the field of microglia is crucial due to their broad and significant role in health, development, and disease, it comes with difficult and significant challenges related to maintaining and establishing their functional properties *in vitro* and *in vivo*.

There is limited availability of primary sources of human microglia, and many such models are cost-ineffective for experiments requiring high amounts of cells and extensive replication of experiments. As most of the research performed in the present thesis was novel, with limited preliminary experiments performed in human microglial models, we elected to utilize the human microglia clone 3 (HMC3), a human immortalized microglial cell line derived from glioma tissue via SV-40-dependent immortalization.

3.3.2 Cell culture

HMC3 cells (ATCC, Manassas, VA, USA) were cultured in complete culture media consisting of minimum essential medium (MEM) (#11095080, Thermo Scientific, Waltham, MA, USA) supplemented with 10% heat-inactivated FBS (HyClone, Logan, UT, USA), 100 U/mL penicillin (Thermo Scientific), and 100 µg/mL streptomycin (Gibco, Waltham, MA, USA). Cells were maintained in a humidified atmosphere at 37ºC and 5% CO_2. HMC3 cells were cultured at 2 × 10^4 cells/cm^2 and, once reaching 80% confluency, were detached with a 5-minute treatment of 0.25% trypsin EDTA (Gibco) before the addition of complete culture media to deactivate the

enzyme. Notably, microglia are known to react to serum[159], and we have demonstrated that microglia in high-serum conditions secrete higher amounts of IL-8 and IL-6 cytokines (**Supplementary 3.1**), which may indicate a stimulated state. Accordingly, 24 hours prior to experiments, the complete culture media was replaced with MEM supplemented with 1% heat-inactivated FBS to limit the basal proliferation and cytokine secretion of cells and maintain a less stimulated microglial state.

The Laboratory of Allergic Disease 2 (LAD2) mast cell line, which was donated by Dr. Arnold Kischenbaum and Dr. Dean Metcalfe from the National Institute of Allergy and Infectious Disease, was cultured according to previously described protocols[113]. Brain tissue homogenates were obtained from the National Prion Disease Pathology Surveillance Centre (NPDPSC) at Case Western Reserve University (CWRU; Cleveland, OH) and prepared as previously described[160].

3.3.3 Treatments

Stock concentrations of histamine (0.1-1000 µM, Sigma, St. Louis, MO, USA) and LPS (1 µg/mL, Sigma) were prepared in sterile phosphate-buffered saline (PBS, Gibco) at pH 7.4 without calcium or magnesium and stored at -20ºC. Histamine receptor agonists (HRH1: HTMT, HRH2: amthamine, HRH3: R-(-)-α-methylhistamine, and HRH4: 4-methylhistamine, Cayman Chemical Company, Ann Arbor, MI, USA) and antagonists (HRH1: clemastine, HRH2: ranitidine, HRH3: JNJ-5207852, and HRH4: JNJ-7777120, Cayman Chemical Company) were prepared in sterile phosphate-buffered saline (PBS, Gibco) at pH 7.4 without calcium or magnesium. For all experiments that included histamine receptor antagonists, HMC3 cells were seeded at relevant densities for 2 hours prior to antagonist treatment and then left for an additional hour prior to histamine treatment.

3.3.4 Cryo-scanning electron microscopy (Cryo-SEM)

HMC3 cells were cultured on a poly-D-lysine treated coverslip for 72 hours and collected in their homeostatic state. The coverslip was then soaked in PBS buffer and gently blotted with filter paper to remove excess solution. The coverslip was rapidly plunged into liquid nitrogen and allowed to stabilize. Sublimation at -90°C, followed by platinum sputter-coating for 120 seconds, was performed using a Leica ACE 600. The sample was then transferred using the VCT100 (Leica) to a ZEISS NVision 40 for imaging at an accelerating voltage of 2 kV and a temperature of -140°C, utilizing a secondary electron detector.

3.3.5 Quantitative reverse-transcription-polymerase chain reaction (qRT-PCR)

HMC3 cells were pelleted, and RNA was isolated using a combined TRIzol (Invitrogen) and column method as follows. First, the pellets were resuspended in TRIzol at 1.5×10^6 cells/mL before the addition of chloroform (the ratio of TRIzol to chloroform was 1:0.167). The mixture was then spun at 12,000 × g for 5 minutes at 4°C, after which the RNA-containing layer was removed. This layer was then added to an RNeasy Mini Kit (Qiagen) and an equal volume of 70% ethanol was added. The RNA and ethanol were vigorously mixed and washed according to the RNeasy® protocol before the RNA was eluted and RNA quality was assessed via NanoDrop One UV-Vis spectrophotometer (Thermo Scientific).

RNA (1 µg) was reverse transcribed with M-MLV reverse transcriptase (Invitrogen) according to manufacturer protocol (Invitrogen). Thirty nanograms of RNA per reaction was analysed by qRT–PCR using PrimeTime Gene Expression Master Mix (IDT, Newark, NJ, USA) on a StepOnePlus real-time PCR instrument (Applied Biosystems). The primers used were human HRH1 (Hs.PT.58.39265294, IDT), HRH2 (Hs.PT.58.27972838, IDT), HRH3 (Hs.PT.58.38998576, IDT), and HRH4 (Hs.PT.58.4403324, IDT). GAPDH PrimeTime Probes (Mm.PT.58.30458786 and Mm.PT.39a.1, IDT) were used according to manufacturer protocol

(IDT). The results are presented as the ΔCT relative to the GAPDH level as previous studies with HMC3 have utilized GAPDH as an internal control[161-163].

3.3.6 Metabolic analysis via XTT

HMC3 cells were seeded at a density of 1.0×10^4 cells/mL in a 96-well plate and incubated in a humidified atmosphere of 5% CO_2 at 37ºC for 2 hours to adhere to the plate. The cells were then treated with histamine (0.1 μM-1000 μM) or LPS (0.1 μg/mL-50 μg/mL) and incubated for 24 hours at 37ºC and 5% CO_2. Metabolic activity was assessed via a Cell Proliferation Kit II (XTT Assay, Roche, Basel, Switzerland) according to the manufacturer's instructions, and the results are presented as the percent metabolic activity relative to that of untreated cells.

3.3.7 Cytokine enzyme-linked immunosorbent assays (ELISA)

HMC3 cells were seeded at a density of 5.0×10^4 cells/cm^2 in a 6-well plate and incubated in a humidified atmosphere of 5% CO_2 at 37ºC for 2 hours to adhere to the plate. The cells were then treated with histamine (10 μM-1000 μM) and incubated for 24, 48, or 72 hours at 37ºC and 5% CO_2. Cell-free supernatants were isolated and stored at -20ºC. ELISA analysis for human IL-8 and IL-6 was performed using commercial ELISA kits (R&D Systems, Minneapolis, MIN, USA), and the plates were read using a VarioSkan Lux plate reader (Thermo Scientific). Additional cell-free supernatant samples were analysed via electrochemiluminescence as previously described[164] to measure for the production of IL-8, IL-6, IL-10, IL-4, IFN-γ, and IL-12p70.

3.3.8 Intracellular calcium flux assay

HMC3 cells were seeded at a density of 5.0×10^4 cells/cm^2 in a 96-well plate and incubated in a humidified atmosphere of 5% CO_2 at 37ºC overnight to adhere to the plate. The media was then removed, and the cells were washed with HEPES-buffered saline (HBS, pH 7.4) before the

addition of fura-2 AM (5 μM) and Pluronic F-127 (0.05%) in HBS for 1 hour at room temperature in the dark. To allow for fura-2 AM de-esterification, the cells were washed twice with HBS before the addition of probenecid (2.5 mM) and BSA (0.1%) for 30 minutes at room temperature in the dark. Calcium flux was measured after treatment with histamine (100 μM) or non-fluorescent 4-bromo-A23187 (1 μM, Sigma) at 30 seconds via alternating fluorescent excitation wavelengths 340/380 at 510 nm emission at 3-second intervals over the course of 5 minutes on the BioTek Synergy H1 microplate reader (Agilent Technologies, Santa Clara, CA, USA). The results are presented as the ratio of 340/380 excitation wavelengths normalized to the first 30 seconds of reads prior to treatments.

3.3.9 Fluorescence microscopy

HMC3 cells were seeded at a density of 5×10^4 cells/cm^2, grown on poly-D-lysine coated glass coverslips, and then treated with lipopolysaccharide (LPS, 1 μg/mL) or histamine (100 μM) for 24 h. Following treatment, the cells were rinsed twice with PBS and fixed with 4% paraformaldehyde for 20 min at room temperature. For immunofluorescence staining, the cells were rinsed twice with PBS and nonspecific binding was blocked by incubating the cells with an Agilent X0909 serum-free protein block (Dako, Santa Clara, CA, USA) for 20 min at room temperature. The excess block was removed, and the cells were incubated in a humidified chamber overnight at 4ºC with anti-Iba-1 (Wako 019-19741) diluted 1:1000 in Dako antibody diluent (Agilent Dako) or anti-PrP^C POM2 IgG1κ (Millipore Sigma, Burlington, MA, USA) diluted 1:100 in Dako antibody diluent. After the cells were rinsed (2-min each), they were incubated with a goat anti-rabbit Alexa 568 secondary antibody (Invitrogen A11036) diluted 1:500 in Dako antibody diluent. Omission of the primary antibody was used as a negative control. Following secondary incubation, the cells were rinsed three times with PBS and once with distilled water to remove

salts and then mounted on Superfrost glass slides with ProLong Glass Antifade mounting media containing NucBlue (Invitrogen P36981). Images were acquired using an Olympus IX81 inverted microscope with a 20× objective and a Leica Stellaris 5 laser scanning confocal microscope with a 60× oil objective.

3.3.10 Flow cytometric analysis of surface PrP^C

HMC3 cells were seeded at a density of 5.0×10^4 cells/cm^2 in a 6-well plate and incubated in a humidified atmosphere of 5% CO_2 at 37 ºC for 2 hours to adhere to the plate. The cells were then treated with histamine (10 μM-1000 μM) or LPS (1 mg/mL) and incubated for 6, 24, 48, or 72 hours at 37ºC and 5% CO_2. For experiments involving antagonists, cells were treated with 50, 100, or 500 μM of clemastine, ranitidine, JNJ-5207852, or JNJ-7777120 and incubated for 1 hour before treatment with 100 μM histamine. The cells were then detached with 0.25% trypsin-EDTA, washed with PBS, and blocked for 10 minutes on ice with 3% bovine serum albumin (BSA) to prevent nonspecific interactions (Marlborough, MA, USA) in PBS. Primary antibodies included mouse anti-PrP^C IgG1κ (POM2, Millipore Sigma) and mouse IgG1κ isotype antibodies (eBioscience). For unconjugated antibodies, cells were washed twice in 0.1% BSA in PBS and exposed to anti-mouse APC (Invitrogen) for 1 hour at 4°C on a plate shaker. The cells were then washed three times in 0.1% BSA in PBS and resuspended in 0.1% BSA in PBS before being analysed on a Cytoflex flow cytometer (Beckman Coulter). Cells and debris were separated by gating on an FSC-A vs. SSC-A plot, while single cells were selected by gating on an FSC-A vs. FSC-H diagram. Changes in fluorescence were observed following gating.

3.3.11 Western blot

HMC3 cells were seeded at a density of 5.0×10^5 cells/mL in a 6-well plate and incubated in a humidified atmosphere of 5% CO_2 at 37ºC for 2 hours to adhere to the plate. The cells were

then treated with histamine (10 µM-1000 µM) or LPS (1 µg/mL) and incubated for 24 hours at 37ºC and 5% CO_2. The cells were then removed with 0.25% trypsin-EDTA, washed with PBS, and lysed by resuspending the cell pellets in RIPA lysis buffer (100 mM Tris HCL pH 8.0, 10 mM EDTA pH 8.0, 100 mM NaCl, 0.5% sodium deoxycholate, 0.5% NP-40, EDTA-free cOmplete™ protease inhibitor cocktail (Roche), EDTA-free SIGMAFAST™ Protease Inhibitor Cocktail, and 6-10 U/mL benzonase (Millipore Sigma)). Human brain homogenates from patients with sporadic Creutzfeldt–Jakob disease of the MM1 subtype or from healthy donors were used as positive controls. Bone marrow-derived mast cells from *Prnp* knockout mice were used as negative controls. After transfer to 0.2 µm PVDF membranes (Millipore Sigma), the membranes were blotted with anti-prion protein antibody, a.a. 109-112 (3F4, Millipore Sigma), or anti-histamine receptor antibodies: anti-HRH1 A82454 (antibodies.com), anti-HRH2 A83284 (antibodies.com), anti-HRH3 A98094 (antibodies.com), or anti-HRH4 A101422 (antibodies.com). After washing, the membranes were blotted with secondary antibodies diluted 1:15000: donkey anti-mouse 680RD (LI-COR) and donkey anti-rabbit IRDYE800CW (LI-COR). All immunoblots in a single subfigure are from a single membrane that was stripped and re-probed between blots. The membranes were imaged with an Odyssey CLX Imaging System (LI-COR).

Western blot images were analysed using ImageJ (https://imagej.nih.gov/ij/). The images were inverted, and the brightness/contrast was adjusted by altering the minimum value. Band intensities were obtained from the original scan files using ImageJ2 version 2.14.0/1.54F. To quantify band intensities, images were converted to grayscale and the background was subtracted before generating a ratio of the band with the protein of interest to the loading control.

3.3.12 Statistical Analysis

For flow cytometry experiments, three or more separate experiments were independently performed. For all other experiments, three or more separate experiments were independently performed, each containing at least three technical replicates. The standard error of the mean (SEM) or the standard deviation (SD) is indicated. The graphs were either plotted as bar graphs ± SEM or SD, or as violin plots with the median indicated via dotted line. *p* values for statistical significance were determined by one-way ANOVA with Dunnett's multiple comparisons or Tukey *post hoc* analysis for multiple comparisons, $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***), $p \leq 0.0001$ (****). GraphPad Prism software version 9.5.1 was used to generate the figures and perform the statistical analysis (GraphPad Software, San Diego, CA, United States).

3.4 Results

3.4.1 Human-derived HMC3 microglia express histamine receptors

Microglia are physiologically dynamic and modify their morphology actively[35]. Cryo-SEM of HMC3 cells show a heterogenous morphology with a very rough surface and several processes that appear to be pseudopodia (**Figure 3.1**). Cells that interact or were in close proximity to each other appeared to have increased pseudopodia and we observed cells with various morphologies.

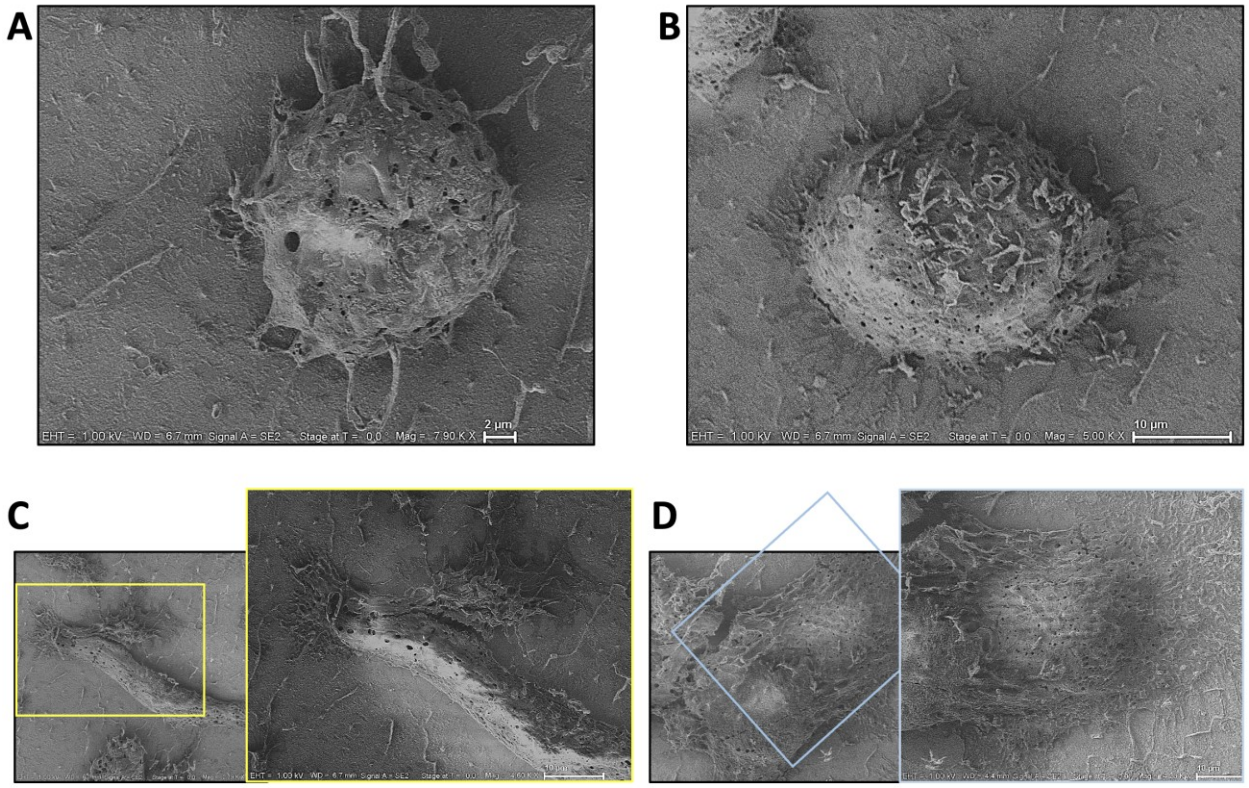


Figure 3.1. Cryo-SEM images of homeostatic HMC3 cells. (**A-D**) HMC3 were cultured on coverslips treated with poly-D-lysine and Cryo-SEM images were collected. Cells were observed in various morphological states. In panels (**C**) and (**D**), boxes represent fields of view following increased magnification individual microglia.

There are four histamine receptors, each associated with different G proteins and with different signaling pathways dictating various functional outcomes[165]. To determine which of the four known histamine receptors were expressed by HMC3 microglia, HRH1, HRH2, HRH3, and HRH4 expression was analysed by qRT–PCR. LAD2 cells were used as a positive control because these cells are known to express mRNA for all four histamine receptors[166]. HMC3 expressed HRH1, HRH2, and HRH3 mRNA but no HRH4 transcripts were detected (**Figure 3.2A**). To measure the histamine receptor protein levels, we performed western blotting for the HRH1, HRH2, HRH3, and HRH4 receptors (**Figure 3.2B-E**). Because the human frontal lobe contains all four histamine receptors[167,168], we included human frontal lobe brain homogenates taken from

healthy donors as positive controls. As expected, we detected measurable protein levels of HRH1, HRH2, and HRH3, but not HRH4 in HMC3 cells **(Figure 3.2B-E)**.

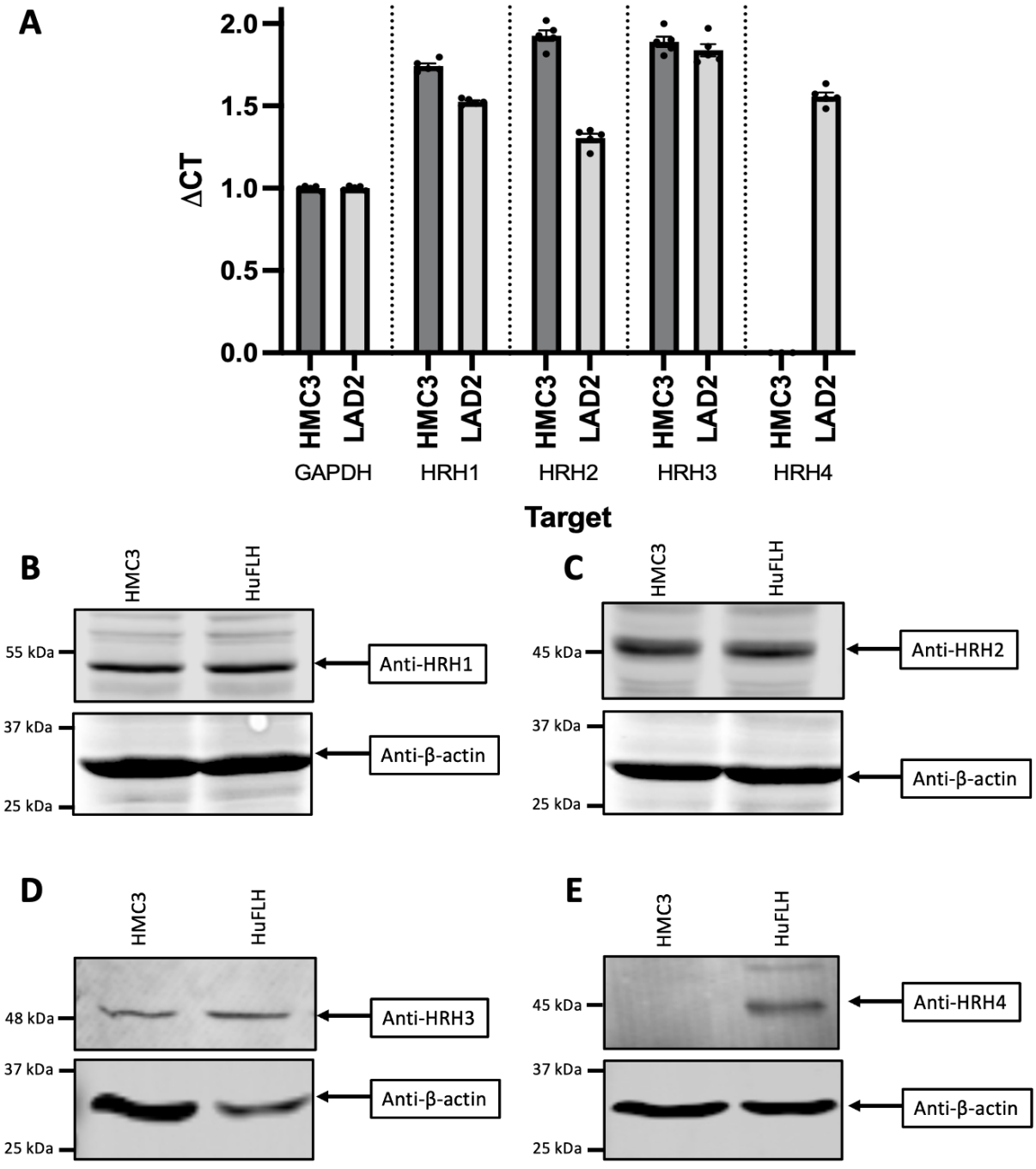


Figure 3.2. Human-derived HMC3 microglia express three of the four known histamine receptors. (A) RNA from HMC3 or LAD2 cells was isolated, and the expression of HRH1, HRH2, HRH3, and HRH4 was analysed. The data are presented as the average change in cycle threshold (ΔCT) for four independently isolated RNA samples; the expression of mRNA for each gene is relative to the GAPDH mRNA expression for each cDNA sample. (N=5, bars indicate ± SD). **(B-**

E) HMC3 or human frontal lobe homogenates (HuFLH) were isolated, and lysates were analysed via western blotting to test for protein expression of **(B)** HRH1, **(C)** HRH2, **(D)** HRH3, or **(E)** HRH4. Representative images of three independent experiments are shown.

3.4.2 Histamine stimulates human-derived microglia

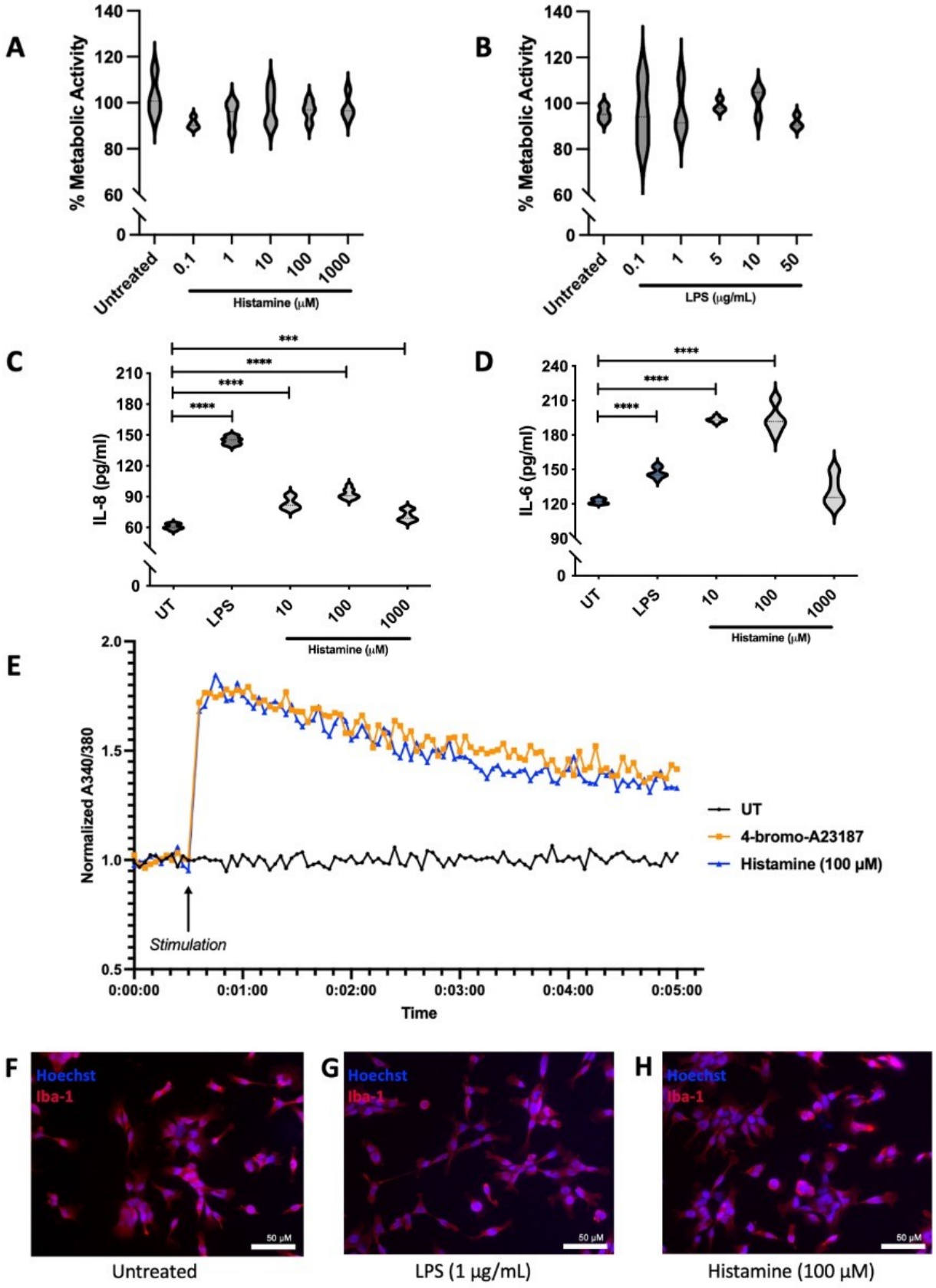
As the metabolic activity of microglia may be altered following stimulation, we tested potential metabolic changes in HMC3 microglia following histamine stimulation using a formazan reduction assay. This method utilizes XTT (2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-carboxanilide-2H-tetrazolium), which is reduced by NADH produced by microglial mitochondria to form a soluble orange-yellow formazan salt. Stimulation of HMC3 cells with histamine at various concentrations (0.1–1000 μM) did not alter the metabolic activity of these cells (**Figure 3.3A)**. We also demonstrated that stimulation of HMC3 cells with LPS at various concentrations (0.1–50 μg/mL) did not alter HMC3 metabolic activity **(Figure 3.3B)**. To ensure that any observed alterations in the XTT assay were indeed due to metabolic changes within cells as opposed to changes in cell death or cell number, cellular viability and cell quantification were performed using a hemacytometer (**Supplementary 3.2A-B**).

In rodents, microglia respond to histamine by increasing the production of the inflammatory mediators IL-6[141], TNF, IL-1β, and IL-10[146]. To test the response of HMC3 microglia to histamine, a neuroinflammatory mediator, we stimulated HMC3 cells with histamine and LPS and measured the release of the proinflammatory mediators IL-8 (**Figure 3C**) and IL-6 (**Figure 3D**) via sandwich ELISA. Histamine-induced stimulation of HMC3 cells increased the release of IL-8 at 10 μM, 100 μM, and 1000 μM ($F(4,36) = 397.5$, $R^2 = 0.9779$, $p = <0.0001$; 10 μM: 83.48 pg/mL, SEM ± 2.538, $p = <0.0001$; 100 μM: 92.78 pg/mL, SEM ± 2.159, $p = <0.0001$; 1000 μM: 71.65 pg/mL, SEM ± 2.381, $p = 0.0008$) relative to the release of IL-8 by the untreated controls (60.72 pg/mL). LPS also increased the amount of IL-8 (144.6 pg/mL SEM ± 1.767, $p =$

<0.0001) released from HMC3 cells compared to that released from untreated controls. Additionally, histamine-induced stimulation of HMC3 cells increased the release of IL-6 at 10 μM and 100 μM, but not 1000 μM (F (4,37) = 142.8, R^2 = 0.9392, *p* = <0.0001; 10 μM: 193.4 pg/mL, SEM ± 1.114, *p* = <0.0001; 100 μM 193.9 pg/mL, SEM ± 4.628, *p* = <0.0001; 1000 μM: 131.4 pg/mL, SEM ± 5.225, *p* = 0.2241) relative to the release of IL-6 by the untreated controls (122.2 pg/mL). LPS also increased the amount of IL-6 released from HMC3 cells (147.1 pg/mL SEM ± 1.114, *p* = <0.0001) compared to that released from untreated controls. To substantiate our data, we also observed that HMC3 cells regulated the release of IL-8, IL-6, IL-10, IL-4, IFN-γ, and IL-12p70 release following histamine and LPS stimulation via electrochemiluminescence (**Supplementary 3.3**).

Intracellular calcium ions are highly regulated in microglia and changes in cytosolic calcium levels can be indicative of cellular stimulation[119]. Our work demonstrated that, following histamine (100 μM) stimulation, HMC3 increased calcium influx resembling the changes in intracellular calcium observed in HMC3 cells stimulated with the calcium ionophore 4-bromo-A23187 **(Figure 3.3E)**.

Iba-1 is a cytoplasmic protein that indicates microglial motility and is considered a microglial marker that may change expression levels following microglial stimulation[169,170]. We performed fluorescence microscopy to measure the expression of Iba-1 in HMC3 cells following histamine or LPS stimulation for 24 hours and found that these cells did not exhibit altered expression of this marker **(Figure 3.3F-H)**.


A
% Metabolic Activity
140
120
100
80
60
0
Untreated
0.1
1
10
100
1000
Histamine (μM)
B
% Metabolic Activity
140
120
100
80
60
0
Untreated
0.1
1
5
10
50
LPS (μg/mL)
C
IL-8 (pg/ml)
210
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Histamine (μM)
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0:00:00
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Time
UT
4-bromo-A23187
Histamine (100 μM)
F
Hoechst
Iba-1
50 μM
Untreated
G
Hoechst
Iba-1
50 μM
LPS (1 μg/mL)
H
Hoechst
Iba-1
50 μM
Histamine (100 μM)

Figure 3.3. Histamine stimulates HMC3 cells to alter cytokine production and intracellular calcium levels, but not metabolic activity or Iba-1 expression. (A-B) HMC3 cells were stimulated with **(A)** histamine (0.1 μM – 1000 μM) or **(B)** LPS or left untreated for 24 hours, and metabolic activity was measured by a reduction in XTT to formazan. The data are presented as the means ± SEMs (N=6). **(C-D)** HMC3 cells were stimulated by histamine (10 μM, 100 μM, or 1000 μM), LPS (1 μg/mL), or left untreated for 24 hours, and **(C)** IL-8 and **(D)** IL-6 were measured by sandwich ELISA. Data are presented as the mean ± SEM (N=9), and statistical significance was measured via one-way ANOVA and Dunnett's multiple comparison post-hoc analysis relative to untreated (UT) cells. $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***), $p \leq 0.0001$ (****). (N=4). **(E)** HMC3 cells were stimulated by histamine (100 μM) (blue) or 4-bromo-A23187 (1 μM) (orange) and intracellular calcium was measured via fura-2 AM dye which increases the 340/380 fluorescence ratio when bound to calcium ions. **(F-H)** Fluorescence microscopy images of HMC3 cells labelled with Hoechst 33342 (Hoechst) (blue) and Iba-1 (red) under **(F)** untreated conditions and following 24 hours treatment with **(G)** LPS and **(H)** histamine. Images are representative of four independent experiments using a 20× objective and a Leica Stellaris 5 microscope.

3.4.3 HMC3 microglia express PrP^C

To test the expression of PrP^C by HMC3 microglia, we performed flow cytometry and western blotting. The expression of PrP^C by HMC3 microglia has not yet been described. We detected surface PrP^C via flow cytometry using an anti-PrP^C POM2 antibody at various concentrations (1 μg/mL to 10 μg/mL) (**Figure 3.4A-B).** Total cellular PrP^C was measured via western blot (**Figure 3.4C),** where PrP^C appeared as several bands representing di-, mono-, and unglycosylated forms of the protein, as well as C-terminal fragments. Human brain homogenates taken from a patient with sporadic Creutzfeldt-Jakob disease of the MM1 subtype (sCJDMM1) were used as a positive control[33]. Of note, the variations in fragment sizes observed across the different sample types are due to different glycosylation profiles and cleavage products of the protein in these different cell types. PrP^C is known to vary in its immunoblot banding appearance depending on cell type. Bone marrow-derived mast cells (BMMCs) from *Prnp* knockout mice, denoted as $PrP^{C-/-}$, served as a negative control.

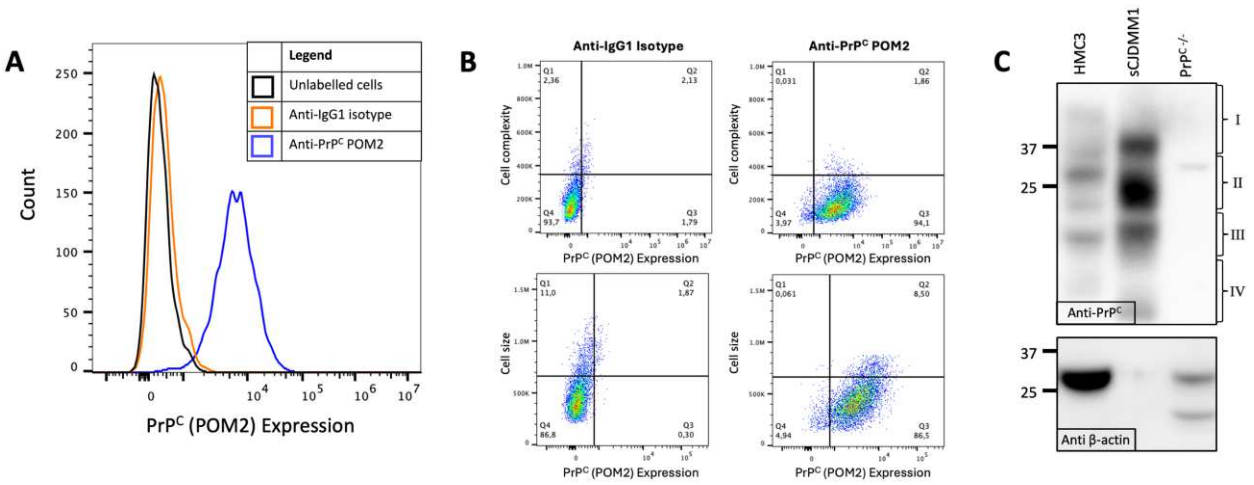


Figure 3.4. HMC3 cells express PrP^C. (A) Flow cytometry immunolabelling of HMC3 cells with anti-PrP^C POM2. Black line: unlabelled HMC3 cells; orange line: HMC3 cells labelled with an IgG1κ isotype control; blue line: HMC3 cells labelled with the POM2 anti-prion protein antibody. **(B)** Flow cytometry scatterplots showing side-scatter (cell complexity, top) or forward-scatter (cell size, bottom) relative to PrP^C expression (x-axis). **(C)** Immunoblot of total PrP^C expression in HMC3 cells. Top: The anti-PrP^C 3F4 antibody indicated I. di-, II. mono-, and III. unglycosylated forms, as well as IV. PrP^C C-terminal fragments. sCJDMM1 from human brain homogenates was included as a positive control. PrP$^{C-/-}$ BMMCs were included as a negative control. Bottom: Anti-β-actin was used to indicate protein loading. (N=3).

3.4.4 Histamine stimulation alters HMC3 expression of surface PrP^C

To measure how microglia alter their expression of PrP^C following stimulation with histamine, we measured surface protein and total protein by flow cytometry and western blot, respectively. Following 6 hours of stimulation, 10 μM, 100 μM, and 1000 μM histamine treatment led to similar increases in surface PrP^C ($F(4,14) = 8.403$, $R^2 = 0.7060$, $p = 0.0011$; 10 μM mean MFI = 5594, SEM ± 239.9, $p = 0.0018$; 100 μM mean MFI = 5462, SEM ± 257.8, $p = 0.0048$; 1000 μM mean MFI = 5416, SEM ± 205.8, $p = 0.0069$) compared to those of the untreated controls (mean MFI = 4465) **(Figure 3.5A)**. Scatter plot analysis also showed that individual cells expressed an increase in surface PrP^C while slightly increasing complexity **(Supplementary 3.4A)** and size **(Supplementary 3.5A)**. Following 24 hours of stimulation, 10 μM and 100 μM histamine treatment led to similar increases in surface PrP^C ($F(4,17) = 21.47$, $R^2 = 0.8348$, $p = <0.0001$; 10

μM mean MFI = 6009, SEM ± 148.1, p = 0.0003; 100 μM mean MFI= 5818, SEM ± 312.2, p = 0.0007), while 1000 μM led to the greatest increase (mean MFI = 6927, SEM ± 274.0, p = <0.0001) compared to that of the untreated controls (mean MFI = 4498) **(Figure 3.5B)**. Scatter plot analysis also showed that individual cells expressed increased surface PrP^C and slightly increased cell complexity **(Supplementary 3.4B)** and size **(Supplementary 3.5B)**.

To test the persistence of PrP^C changes over time following prolonged histamine exposure, we stimulated HMC3 microglia with histamine for 48 hours (F (4,12) = 10.15, R^2 = 0.7719, p = 0.0008) and 72 hours (F (4,19) = 15.03, R^2 = 0.7599, p = <0.0001) and repeated these assays. At a 10 μM dose of histamine for 48 hours, HMC3 cells had similar levels of surface PrP^C expression (mean MFI = 4903, SEM ± 122.9, p = 0.1572) as the untreated controls (mean MFI = 4539) **(Figure 3.5C)**, whereas 72 hours of stimulation decreased surface PrP^C (mean MFI = 3508, SEM ± 274.2, p = 0.0273) relative to that of the untreated controls (mean MFI = 4458) **(Figure 5D)**. At a 100 μM dose of histamine for 48 and 72 hours, HMC3 cells had significantly decreased surface PrP^C (mean MFI = 3809, SEM ± 134.8, p = 0.0034, untreated mean MFI = 4539; mean MFI = 2951, SEM ± 288.5, p = 0.0006, untreated mean MFI = 4458, respectively). Similarly, at a 1000 μM dose of histamine, HMC3 slightly decreased surface PrP^C (mean MFI= 4227, SEM ± 78.01, p = 0.2537, untreated mean MFI = 4539) following 48 hours of stimulation and significantly decreased surface PrP^C (mean MFI= 2383, SEM ± 305.8, p = <0.0001, untreated mean MFI = 4458) following 72 hours of stimulation. Scatter plot analysis also revealed that individual cells exhibited a concentration-dependent decrease in surface PrP^C without altering complexity following 48 and 72 hours of histamine stimulation **(Supplementary 3.4C-D)** or size **(Supplementary 3.5C-D).**

To assess whether decreases in PrP^C over time were due to prolonged culture conditions, we plotted PrP^C surface protein expression over time and compared its expression levels with those of untreated cells **(Supplementary 3.6).** We found that in untreated HMC3 cells, PrP^C expression did not significantly change over time **(Supplementary 3.6A)**, whereas in response to histamine stimulation, PrP^C expression first increased, then decreased over extended exposure times **(Supplementary 3.6B-D)**.

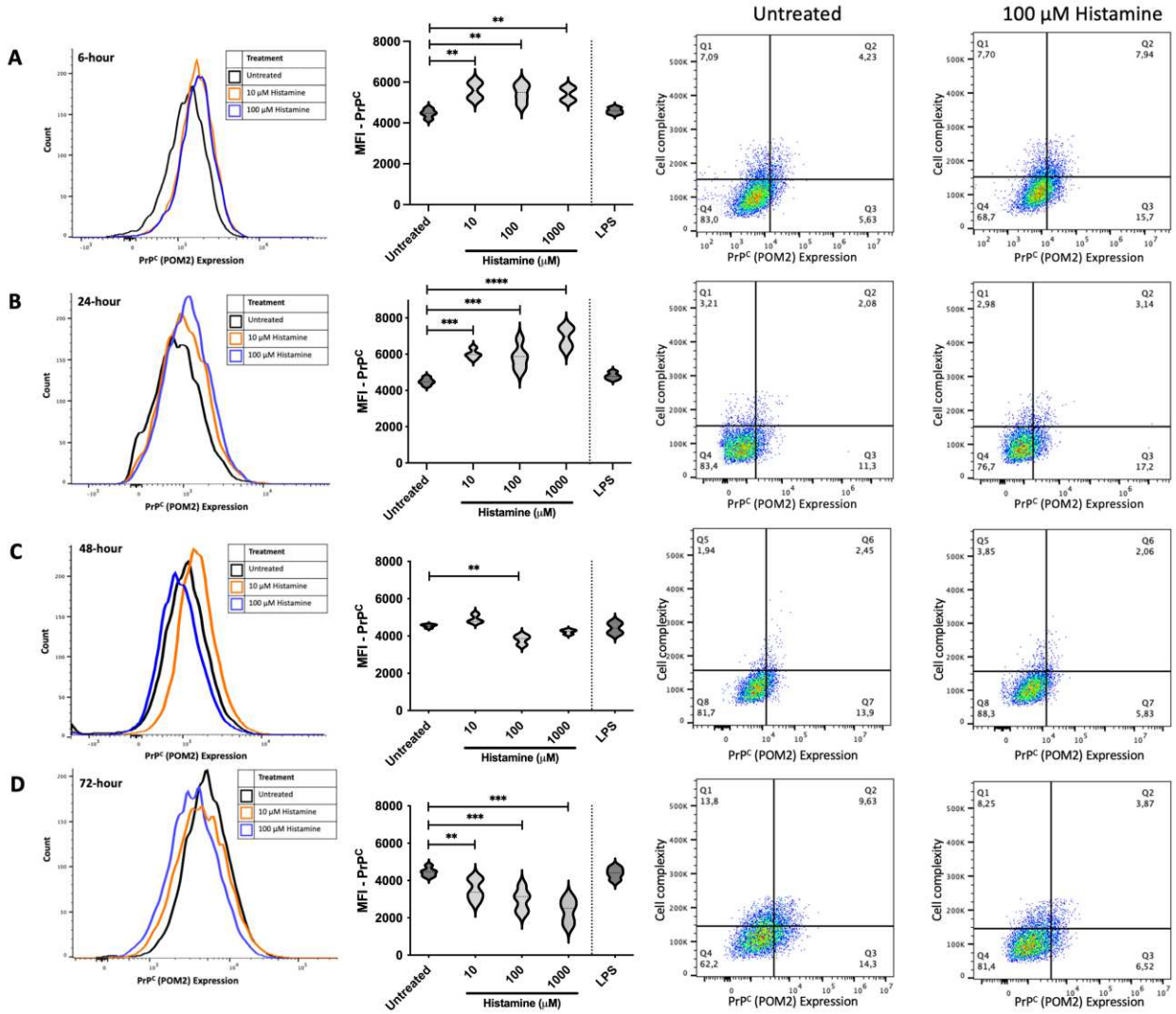


Figure 3.5. Histamine alters surface PrP^C expression on HMC3 cells. Flow cytometry analysis of cell surface PrP^C following exposure to 10, 100, or 1000 µM histamine for **(A)** 6, **(B)** 24, **(C)** 48, or **(D)** 72 hours. MFI = Mean Fluorescent Intensity. Statistical significance was calculated using One-way ANOVA with Tukey's post hoc analysis, $p \leq 0.01$ (**), $p \leq 0.001$ (***), $p \leq 0.0001$

(****). Individual scatterplots were included to show side-scatter against PrP^C. Graphs plot side-scatter (y-axis) as a measurement of cell complexity against PrP^C expression (x-axis). The percentages in each corner reflect the population of cells in each quadrant. (N=4).

We next tested whether the changes in surface PrP^C levels were due to changes in the total amount of cellular PrP^C present or changes in the localization of PrP^C. HMC3 cells were treated with histamine for 24 hours before they were lysed, and total PrP^C was quantified via western blotting. We found that total PrP^C expression was not significantly altered following histamine or LPS stimulation **(Figure 3.6A-B)**, indicating that changes in surface PrP^C expression were likely due to changes in PrP^C localization on the cell surface.

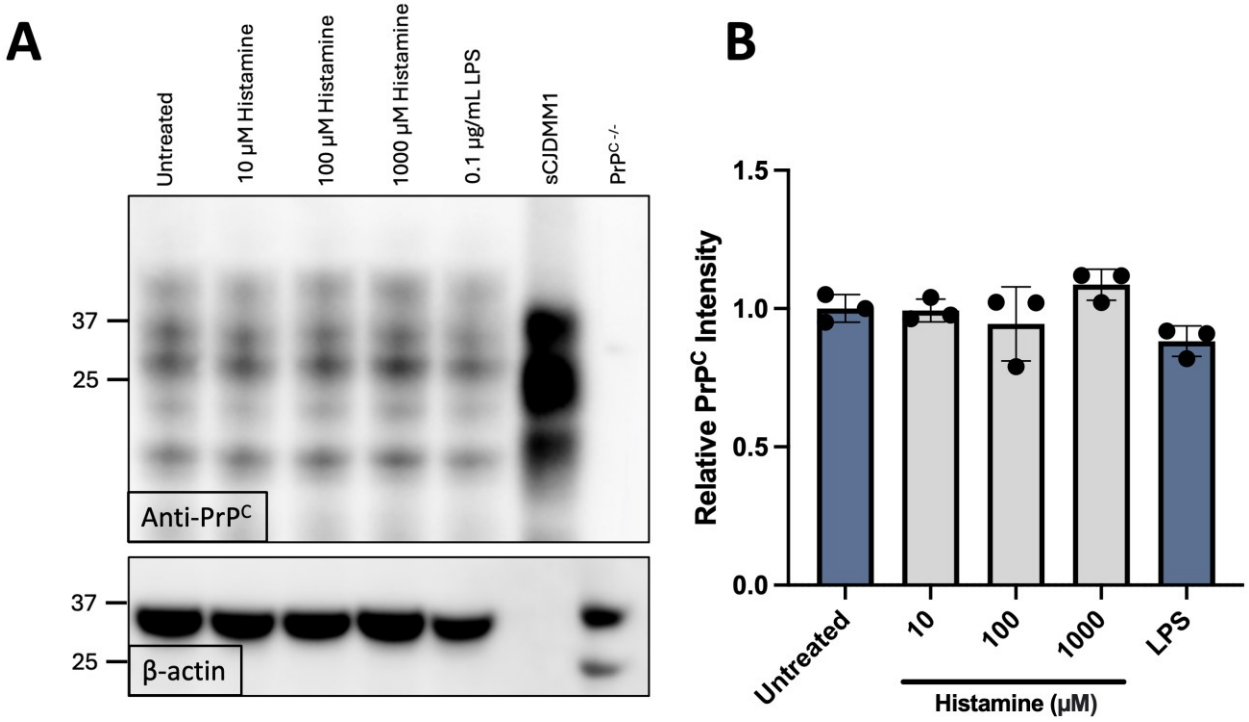


Figure 3.6. Histamine does not change total PrP^C expression in HMC3 cells. (**A**) A representative western blot image of HMC3 cells treated with various concentrations of histamine or LPS for 24 hours. *Top:* Anti-PrP^C 3F4 antibody. *Bottom:* Anti-β-actin was used to indicate protein loading. Human sCJDMM1 was included as a positive control. $PrP^{-/-}$ BMMC were included as negative control. **(B)** Quantification of western blot band intensity. The relative intensity was quantified against that of untreated controls. (N=3 bars indicate ± SEM).

3.4.5 Fluorescent microscopy of HMC3 morphology and PrP^C expression

To further visualize any changes in PrP^C surface localization in HMC3 cells, we used fluorescence microscopy to assess the surface expression of PrP^C following histamine and LPS stimulation using mouse monoclonal anti-PrP^C POM2 antibody. HMC3 cells stimulated with histamine and LPS exhibited notable increases in the expression of PrP^C **(Figure 3.7B and 3.7C)** relative to untreated cells (**Figure 3.7A**). Specifically, HMC3 cells stimulated with histamine increased PrP^C expression slightly more than cells stimulated with LPS. Neither histamine nor LPS treatment appeared to modify HMC3 morphology.

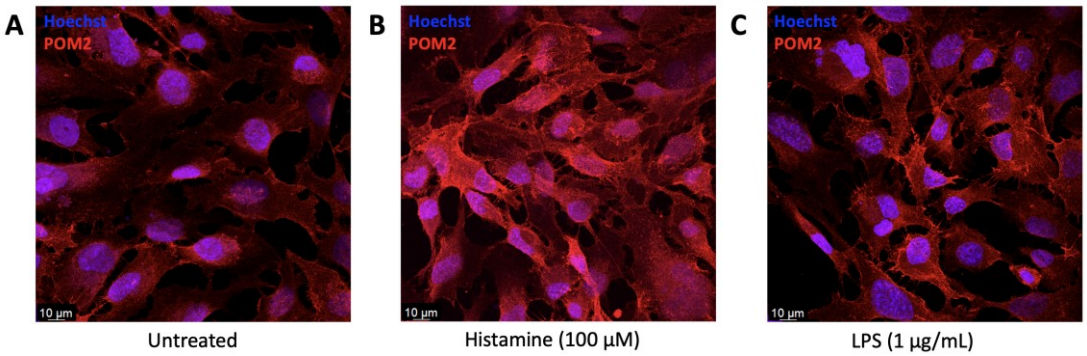


Figure 3.7. Immunofluorescence detection of surface PrP^C expression in HMC3 cells treated with histamine and LPS. Representative fluorescence microscopy images of HMC3 labelled with Hoechst 33342 (Hoechst) (blue) and anti-PrP^C POM2 (red) under **(A)** untreated conditions or after 24 hours of stimulation by **(B)** histamine or **(C)** LPS. Images are representative of three independent experiments using a 20× objective and a Leica Stellaris 5 microscope.

3.4.6 Histamine-induced changes in surface PrP^C are due to signaling by the HRH2 receptor

Having demonstrated that 24-hour histamine stimulation increases surface PrP^C expression, we next investigated which histamine receptor may be responsible for these changes. Accordingly, we treated HMC3 cells with histamine receptor agonists for 24 hours: we used 100 µM each of HTMT, Amthamine, R-(—)-α-methylhistamine, or 4-methylhistamine which activate HRH1, HRH2, HRH3, or HRH4, respectively. We then measured surface PrP^C levels via flow cytometry **(Figure 3.8)** and demonstrated that activation of the HRH2 receptor increased

PrP^C expression (F (5,31) = 15.43, R^2 = 0.7133, p = <0.0001; mean MFI = 5064, SEM ± 156.8, p = 0.0012) relative to that in untreated controls (mean MFI = 4511).

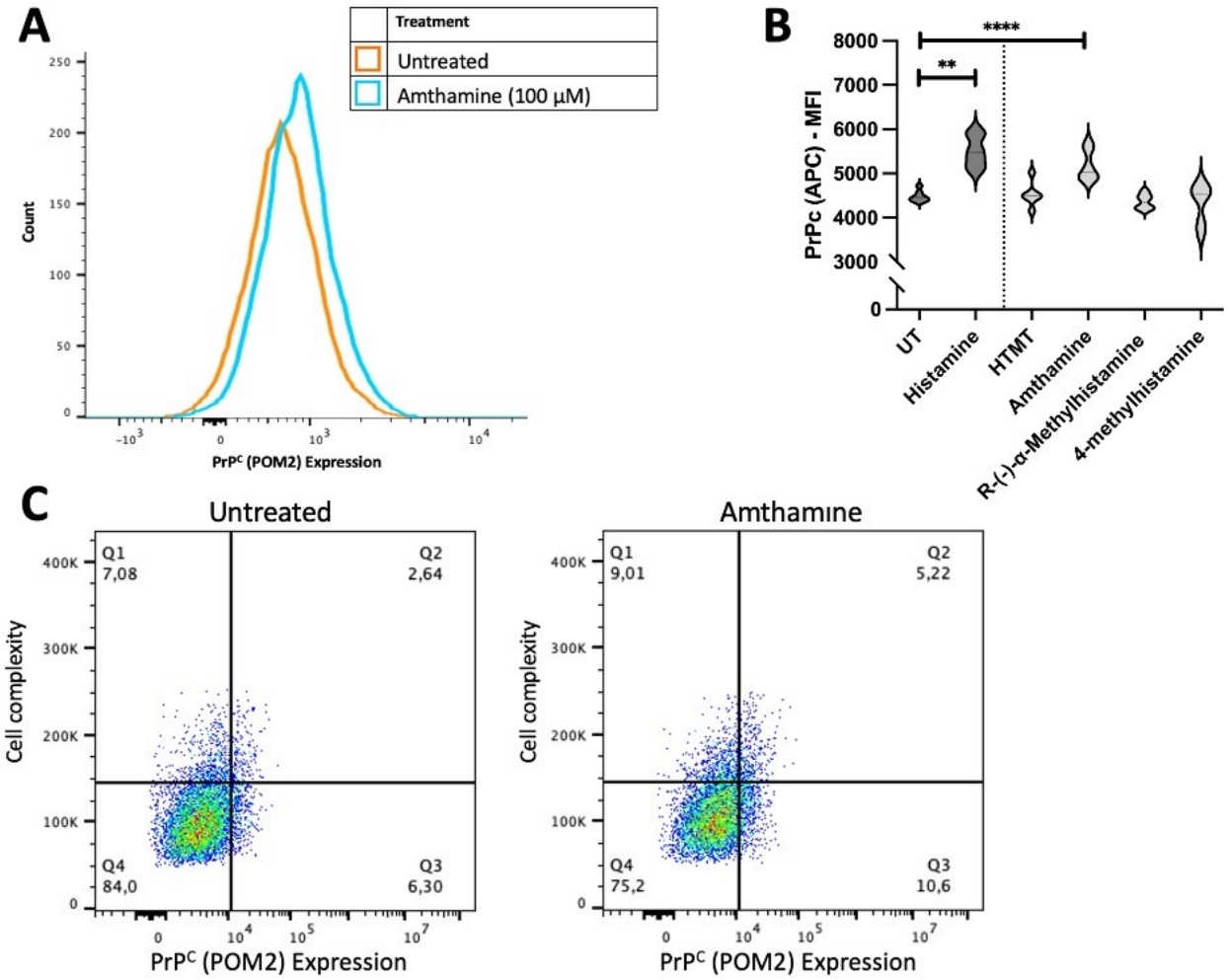


Figure 3.8. PrP^C expression following activation of histamine receptors. (A) HMC3 cells were stimulated with the HRH2 agonist amthamine for 24 hours and surface PrP^C levels were measured via flow cytometry. **(B)** HMC3 cells were treated with 100 µM of histamine, HTMT (an HRH1 agonist), amthamine (an HRH2 agonist), R-(–)-α-methylhistamine (an HRH3 agonist), or 4-methylhistamine (an HRH4 agonist) for 24 hours and surface PrP^C levels were measured via flow cytometry. The mean fluorescent intensity (MFI) was quantified, and statistical significance was calculated using one-way ANOVA with Tukey's post hoc analysis, $p \leq 0.01$ (**), $p \leq 0.0001$ (****). **(C)** Individual scatterplots show side-scatter against PrP^C. Graphs plot side-scatter (y-axis) as a measurement of cell complexity against PrP^C expression (x-axis). The percentages in each corner reflect the population of cells in each quadrant. (N=4).

To determine whether the HRH2 receptor was the sole contributor to changes in PrP^C expression, we treated HMC3 cells with histamine receptor antagonists for 1 hour prior to

stimulating them with 100 μM histamine and repeated experiments to measure PrP^C expression changes and cytokine release. We first measured cellular viability and cell proliferation following treatments of 50, 100, or 500 μM of histamine receptor antagonists. We used clemastine, ranitidine, JNJ-5207852, and JNJ-7777120 to block HRH1, HRH2, HRH3, and HRH4, respectively. Using a hemacytometer, we indicated that HMC3 cells treated with 50 or 100 μM of each of these compounds did not significantly alter their cellular viability (**Supplementary 3.7A-C)** or proliferation (**Supplementary 3.7E-G)**, whereas 500 μM significantly decreased both viability (**Supplementary 3.7D)** and cell proliferation (**Supplementary 3.7H).**

Accordingly, we then measured surface PrP^C via flow cytometry following 1 hour of 100 μM histamine receptor antagonist treatments and 24 hours of 100 μM histamine-induced stimulation **(Figure 3.9)**. As expected, blocking the HRH2 receptor prior to histamine treatment prevented the increase in surface PrP^C ($F(3,14) = 40.95$, $R^2 = 0.8977$, $p = <0.0001$; mean MFI= 4242, SEM ± 180.6, $p = <0.0001$; histamine treatment alone: mean MFI= 5635). In contrast, the inhibition of HRH1, HRH3, and HRH4 did not prevent the histamine-induced changes in surface PrP^C expression HRH1 inhibition: ($F(3,14) = 21.15$, $R^2 = 0.8193$, $p = <0.0001$, mean MFI = 5579, SEM ± 253.5, $p = 0.9914$; HRH3 inhibition: ($F(3,14) = 12.96$, $R^2 = 0.7353$, $p = <0.0001$, mean MFI = 5092, SEM ± 301.8, $p = 0.1082$; HRH4 inhibition: ($F(3,14) = 18.09$, $R^2 = 0.7950$, $p = <0.0001$, mean MFI = 5477, SEM ± 230.2, $p = 0.8195$; histamine treatment alone: mean MFI = 5635). Scatter plot analysis supported these data (**Supplementary 3.8**), and these results support HRH2 as the receptor likely involved in the observed surface PrP^C expression changes.

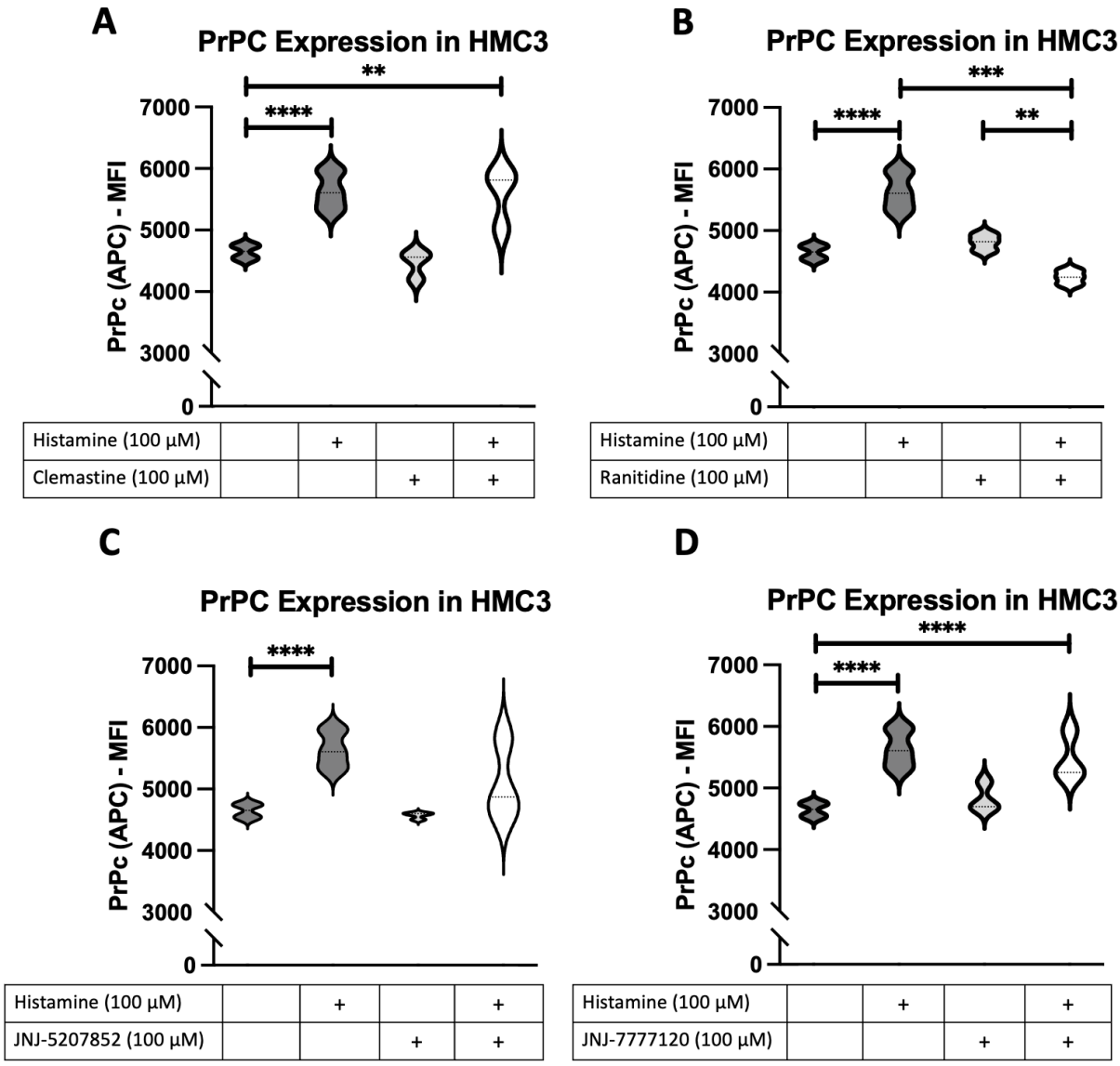


Figure 3.9. Histamine acts via the HRH2 receptor to upregulate surface PrP^C expression. HMC3 cells were treated with 100 µM (**A**) clemastine (an HRH1 antagonist), (**B**) ranitidine (an HRH2 antagonist), (**C**) JNJ-5207852 (an HRH3 antagonist), or (**D**) JNJ-7777120 (an HRH4 antagonist) for 1 h, and then treated with 100 µM histamine for 24 hours, after which PrP^C expression was measured by flow cytometry. Statistical significance was calculated using one-way ANOVA with Tukey's post hoc analysis, $p \leq 0.01$ (**), $p \leq 0.001$ (***), $p \leq 0.0001$ (****). (N=4).

We additionally measured PrP^C surface expression following 1-hour treatments of 50 and 500 µM histamine receptor antagonism followed by 24 hours of histamine treatment to compare these results with our 100 µM antagonist results and create a dose-response curve of various doses of histamine receptor antagonist compared to surface PrP^C expression (**Supplementary**

3.9). These results highlighted that 100 μM of ranitidine antagonism of HRH2 was sufficient to block histamine-induced increases in PrP^C surface expression and increased doses of histamine receptor antagonists caused various changes in PrP^C expression, likely due to aforementioned changes in cellular viability and proliferation.

Following confirmation that HRH2 was the primary receptor involved in PrP^C expression changes, we next tested whether the HRH2 receptor was responsible for the histamine-induced changes in cytokine release. After a 1-hour pretreatment with the histamine receptor antagonists, we stimulated HMC3 with 100 μM histamine and measured IL-8 and IL-6 release (**Supplementary 3.10**). We found that histamine-induced IL-8 released following blocking of HRH1 (91.33 pg/mL, SEM ± 4.024, $R^2 = 0.04584$, $p = 0.5802$), HRH2 (80.00 pg/mL, SEM ± 4.140, $R^2 = 0.4030$, $p = 0.0663$), HRH3 (90.33 pg/mL, SEM ± 3.823, $R^2 = 0.01708$, $p = 0.7376$), and HRH4 (94.67 pg/mL, SEM ± 4.041, $R^2 = 0.2193$, $p = 0.2036$) was not significantly different from that of cells treated with histamine alone (89.00 pg/mL). Interestingly, histamine-induced IL-6 release following blocking of HRH2 (165.3 pg/mL, SEM ± 7.189, $R^2 = 0.6109$, $p = 0.0128$) was significantly lower than that of cells treated with histamine alone (189.2 pg/mL), whereas histamine-induced changes in IL-6 following inhibition of HRH1 (185.7 pg/mL, SEM ± 7.452, $R^2 = 0.03055$, $p = 0.6529$), HRH3 (187.3 pg/mL, SEM ± 8.069, $R^2 = 0.007321$, $p = 0.8268$), and HRH4 (192.3 pg/mL, SEM ± 7.762, $R^2 = 0.02322$, $p = 0.6955$) was not significantly different from that of histamine treatment alone (189.2 pg/mL).

3.5 Discussion

In this study, we asked whether the cellular prion protein would (PrP^C) play a role in modulating microglial responses to stimuli, with a particular focus on histamine as a stimulus. To our knowledge, this work is the first to demonstrate that histamine stimulates the human-derived

microglial cell line HMC3, leading to the release of proinflammatory cytokines and increases in intracellular calcium ions but no significant changes in metabolic activity. Additionally, we are the first to show that HMC3 cells express PrP^C, and following histamine stimulation, these cells alter the surface levels but not the total expression of PrP^C via stimulation of the HRH2 receptor.

HMC3 cells were utilized for this study and are admittedly an imperfect model for primary human brain-derived microglial cells. Currently, there are limited options for human microglial cell lines available for *in vitro* research. HMC3 have been utilized in proinflammatory conditions and these cells are frequently used in both healthy and neurodegenerative disease models[164,171-174]. HMC3 are physiologically similar to primary microglia because they express numerous biomarkers of microglia and macrophages such as CD11b, CD45, CD68, and Iba-1[174].

Our team and others have previously demonstrated that HMC3 cells modify cytokine release following stimulation via various mechanisms and treatments[161,164]. Our current data show that histamine stimulation alters microglial release of IL-8 and IL-6, two key inflammatory mediators involved in neuroinflammation and microglial inflammatory signaling[57,175]. IL-8 is a chemokine largely involved in immune cell regulation, and this chemokine plays a key role in cell signaling as it recruits neutrophils and various other immune and inflammatory cells, such as monocytes and macrophages, to sites of tissue damage and infection in the CNS[175,176]. Walker et al. indicated that this inflammatory mediator is the most highly upregulated factor following $A\beta_{1-42}$-induced stimulation of human microglia, highlighting the importance of this chemokine as a neuroinflammatory mediator[177]. Notably, as microglia express the IL-8 receptors CXCR1[178] and CX3CR1[179], which are known to be involved in the recruitment of microglia to sites of neuroinflammation, the release of IL-8 by stimulated microglia in the brain may further promote neuroinflammation and the accumulation of additional immune cells. On the other hand, IL-6 is

produced by various cells in the CNS, and it plays an important role in both homeostatic states[29] and in states of tissue injury and neuroinflammation[180]. IL-6 is a key indicator of microglial stimulation[32], as it is thought to play an important role in neuroinflammatory signaling. Studies have indicated that following stimulation of primary cultures of human CNS cells, microglial IL-6 release is precisely and dynamically modified[29,181]. Our data showed that histamine induces human microglia to dynamically increase IL-8 and IL-6 levels, further suggesting that histamine is a key regulator of neuroinflammatory signaling in microglia.

Studies by Xia et al., Rocha et al., and Zhang et al. have indicated that murine microglia respond to histamine by increasing intracellular Ca^{2+} and phagocytosis[83,146,152]. Our data showed that histamine altered calcium flux in HMC3 cells similarly to that of 4-bromo-A23187, a calcium ionophore that mediates the transport of calcium ions intracellularlly[182]. Previous studies have suggested that stimulated microglia alter intracellular calcium as a means to regulate cellular proliferation, cellular motility, ramification, and the release of proinflammatory cytokines[183].

Our data indicated that histamine may modify how microglia produce inflammatory mediators. This work demonstrated that histamine-induced stimulation did not alter the expression of Iba-1. This molecule is a cytoplasmic protein that is typically used to identify microglia and macrophages; however, there is conflicting evidence regarding whether Iba-1 levels are altered following microglial stimulation[169,170,184]. Furthermore, recent research on microglia and histamine has demonstrated that histamine-induced stimulation of murine microglia alters motility and modifies the release of the proinflammatory cytokines IL-1β and TNF[149], increases microglial ROS production[83] and modifies the release of proinflammatory mediators by these cells[149]. These studies substantiated our electrochemiluminescence data which demonstrated that microglia altered their release of IL-8, IL-6, IL-10, IL-4, IFN-γ, and IL-12p70 following histamine- and LPS-

induced stimulation, although a more comprehensive analysis of mediator release is necessary to fully understand the transcriptional pathways that are engaged and the regulatory networks that may control how these mediators are secreted.

Previous studies in murine models have indicated ambiguity in the expression of histamine receptors in murine models, with some studies suggesting that murine microglial cell lines express all four known histamine receptors (HRH1, HRH2, HRH3, and HRH4)[149], while others suggest that primary murine models only express certain receptors on certain cell types[153,185]. There has been little research on histamine receptor expression in human microglia. To the best of our knowledge, we are the first to show that human-derived microglia express HRH1, HRH2, and HRH3 mRNA and protein, but not HRH4. The absence of HRH4 in HMC3 cells in the present study concurs with previously reported transcriptomic and proteomic data, which indicates that there is limited expression of HRH4 in the central nervous system[152,186], which is typically found in neurons in the deep laminae of the cortex in humans[167].

There is growing evidence for the role of PrP^C in the immune system, and this protein is variably expressed by numerous immune cells, including haemopoietic stem cells[187], dendritic cells, T cells[93], and leukocytes[94], suggesting that physiological PrP^C may be linked to inflammatory and immune responses. Murine microglia express PrP^C, and Shi et al. demonstrated that this protein is likely involved in the maintenance of microglia in homeostatic and reactive states[131]. Although many studies have indicated that microglia are involved in prion disease[188-190], very few studies have investigated PrP^C expression in human microglia; we are one of the first to directly show PrP^C protein expression in human-derived microglia.

Microglial stimulation induces changes in the expression of genes and proteins involved in the immune response and activation[191,192]. Although PrP^C expression on activated murine

microglia has been examined[193], our data are the first to suggest that human microglial stimulation may alter cell-surface PrP^C expression, suggesting that PrP^C may function as a mediator of inflammation and the immune response in the human brain. Our data showing that PrP^C expression is altered in a time- and concentration-dependent manner support the findings of Izzy et al., who suggested that unique changes in cellular protein expression occur at different time points following modifications in microglial microenvironments[194]. More specifically, Izzy et al. reported that microglia alter inflammatory gene expression in a time-dependent manner following cerebral contusion in mice, leading to changes in microglial sensitivity to tissue damage. Our data support this concept of dynamic changes in microglial inflammatory gene and protein expression following stimulation of these cells. Our work expands upon this evidence of histamine-induced changes in PrP^C by demonstrating that this stimulation is primarily induced by HRH2-receptor signaling.

The four histamine receptors are GPCR receptors, characterized by their seven transmembrane α-helices, three intracellular loops, and three extracellular loops[195]. In the CNS, GPCRs have the highest expression of any receptor type[196], and these receptors modulate the CNS to precisely and dynamically respond to endogenous and exogenous stimuli[197]. Our data indicated that HRH2 was the primary receptor responsible for histamine-induced changes in PrP^C surface expression in microglia, however this inhibition of histamine-induced changes in PrP^C did not block IL-8 release and only partially blocked IL-6 release. This was an interesting observation, and these results suggest that modulation of surface PrP^C is dynamic and receptor-specific, as HRH2 does not appear to be involved in cytokine release. The HRH2 receptor acts via the Gαs subunit which promotes 3'-5'-cyclic adenosine monophosphate (cAMP) generation[198]. cAMP signaling via the phosphoinositide 3-kinase/AKT (PI3K/AKT) pathway is crucial for inflammatory signaling in Alzheimer's and other neurodegenerative diseases[198], and this cellular

messenger is a key regulator of microglial function and stimulation[199]. Notably, cAMP signaling tightly regulates calcium levels and dynamics in neuronal cells and astrocytes[200], and accordingly, the changes in calcium flux observed in the present study may indeed support the notion of HRH2 stimulation via this mechanism. Additionally, histamine and its receptors are known to promote and regulate wakefulness[201], and PrP^C is also thought to be crucial for circadian rhythms and sleep[202]. Since our data suggests the dynamic regulation of PrP^C via histamine-induced stimulation, it is possible that histamine receptors may regulate the accumulation and stimulation of microglia at sites of inflammation, promoting an increase in inflammatory cytokines. The exact role of PrP^C as a neuroinflammatory regulator is not clear, but this protein may be involved in facilitating this intercellular communication and regulating the release of cytokines at the microglial membrane[18]. The expression of surface PrP^C appears to be linked to cytokine release and secretion in other cells[113,203], suggesting that this link between secretion and PrP^C shuttling may be a common phenomenon. As PrP^C has previously been identified as a scaffolding adaptor for other receptors[117,204], our data suggests that PrP^C may localize to the cell surface to promote cytokine release immediately following histamine stimulation. However, as PrP^C gradually decreases following extended histamine stimulation, it may in turn gradually decrease cytokine production thereby attenuating the inflammatory response. In this way, PrP^C may act as a control dial that tightly modulates microglial function. Although the molecular mechanisms are unclear, when this balance is interrupted, chronic inflammation may occur, and accordingly, this mechanism may prove as a useful drug target in treating neuroinflammation.

PrP^C is a membrane-bound protein typically located on the cell surface[205] where it regulates cellular transport and participates in cellular signaling[1]. The protein has been found in pre- and postsynaptic compartments of nerve terminals[3], the outer membrane of neurons[206], and the

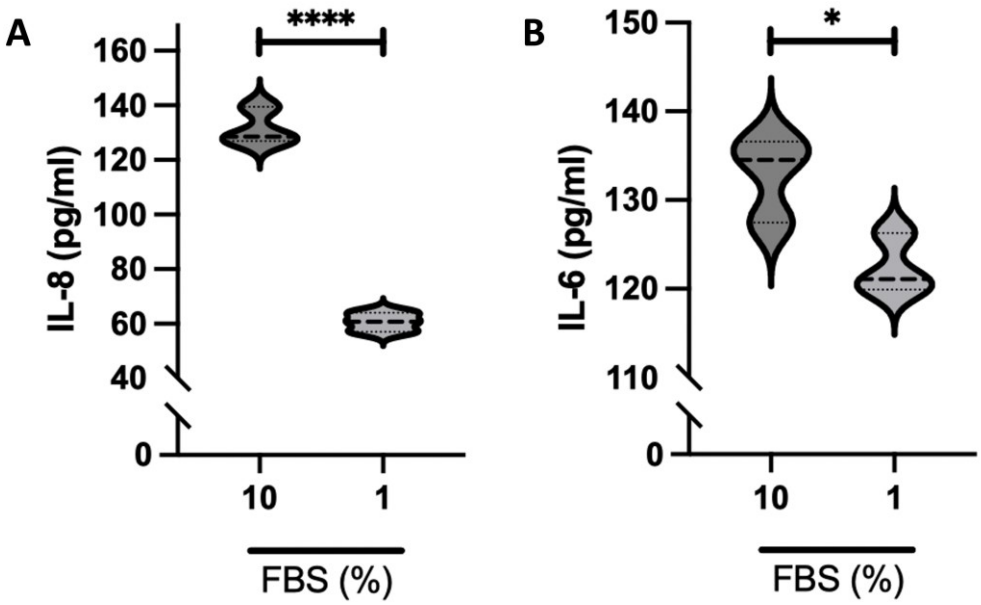
cytoplasm and nucleus of both neurons and glial cells of the CNS[1,96,207]. The localization of PrP^C is dynamic, and the protein is known to change location in neurodegenerative disease[208]. Our microscopy results demonstrated that following 24 hours of stimulation by both histamine and LPS microglia modify the localization of PrP^C, which is trafficked to the cell surface, depending on the duration of stimulation. These microscopy data are particularly interesting as our flow cytometry results demonstrated that histamine stimulation, but not LPS stimulation, increased surface PrP^C. These discrepancies may be due to differences in signaling pathways; whereas LPS stimulates the TLR4 receptor[209], histamine acts via G-coupled protein receptors[210]. As PrP^C varies in localization in different cellular compartments depending on various physiological conditions[101,208,211], these differences in histamine- and LPS-induced stimulation may highlight the separate, distinct signaling pathways that induce microglial regulation of PrP^C expression. Though the molecular pathway remains unclear, our data highlighting the different mechanisms responsible for discrepancies in histamine- and LPS-induced stimulation of microglia could prove a useful pharmacological target in treating and regulating neuroinflammation.

Taken together, these data suggest that histamine stimulates human-derived microglia to increase the release of inflammatory cytokines, increase intracellular calcium levels, and alter PrP^C surface expression, likely via the HRH2 receptor. Future studies are needed to further understand the role of histamine and PrP^C in inflammatory signaling in human models. Specifically, experiments using primary human microglia should be conducted and tests on physiologically relevant microglial mediators such as P2RY12 should be performed, as others have shown that microglial responses to histamine are primarily dependent on this receptor in microglia[152].

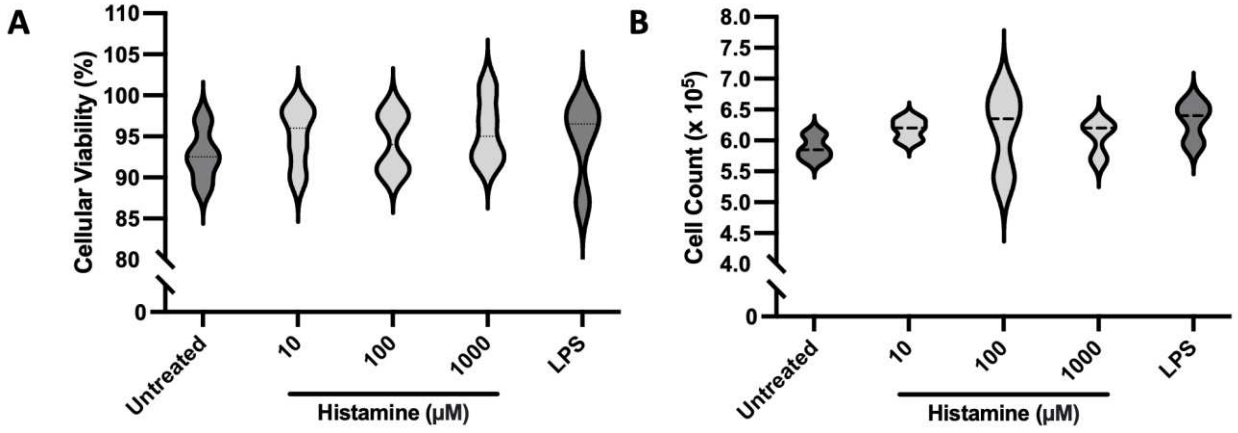
In conclusion, our work highlighted that the HMC3 human microglial cell line express relatively high amounts of PrP^C which can be detected on the surface via flow cytometry. These

cells also express histamine receptors and can be stimulated by histamine to alter their release of inflammatory cytokines while modifying cytosolic calcium levels. Interestingly, this histamine-induced stimulation also dynamically modified surface PrP^C expression in a time- and dose-dependent manner, primarily through the HRH2 histamine receptor. While histamine-induced changes in PrP^C expression was found to be mediated via the HRH2 receptor, changes in IL-8 and IL-6 release following histamine-induced stimulation is likely due to signaling via other pathways. Overall, our findings suggest that PrP^C likely contributes to microglia action physiologically, potentially participating in neuroinflammatory and immune signaling following microglial stimulation.

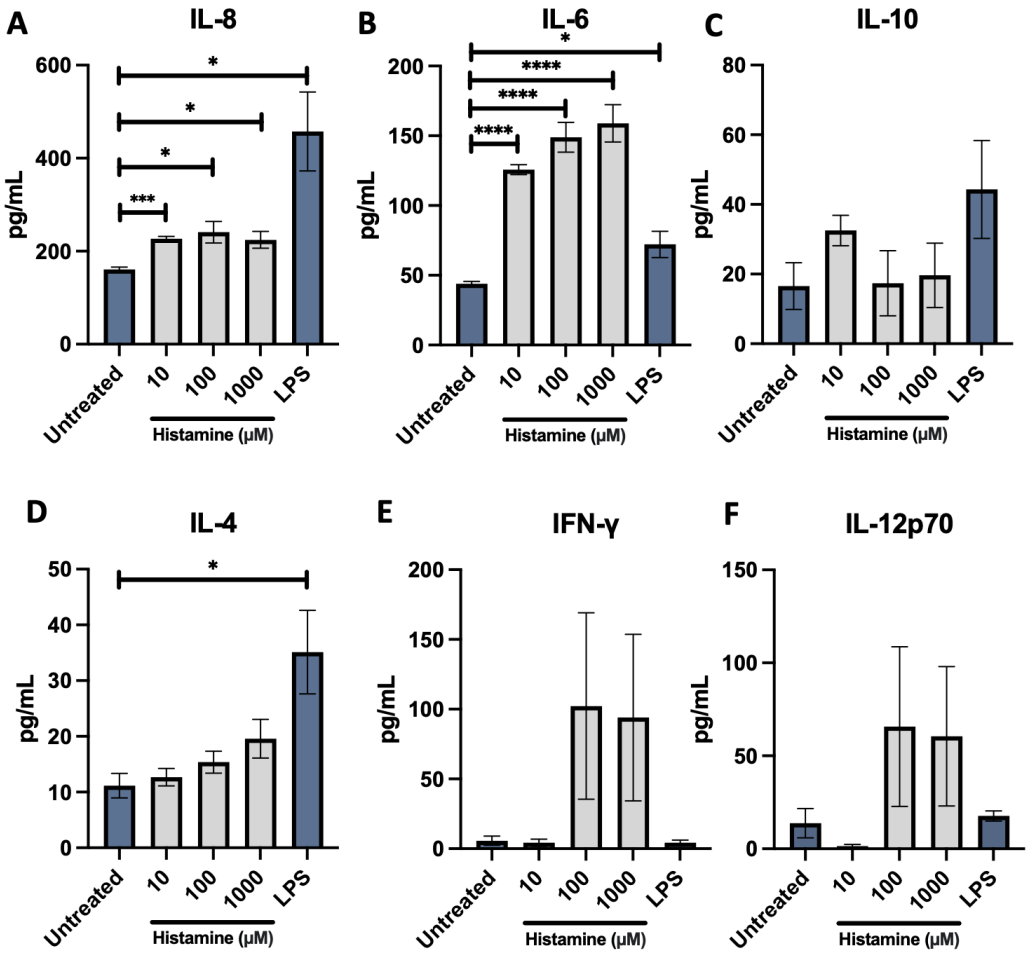
3.6 Supplementary data



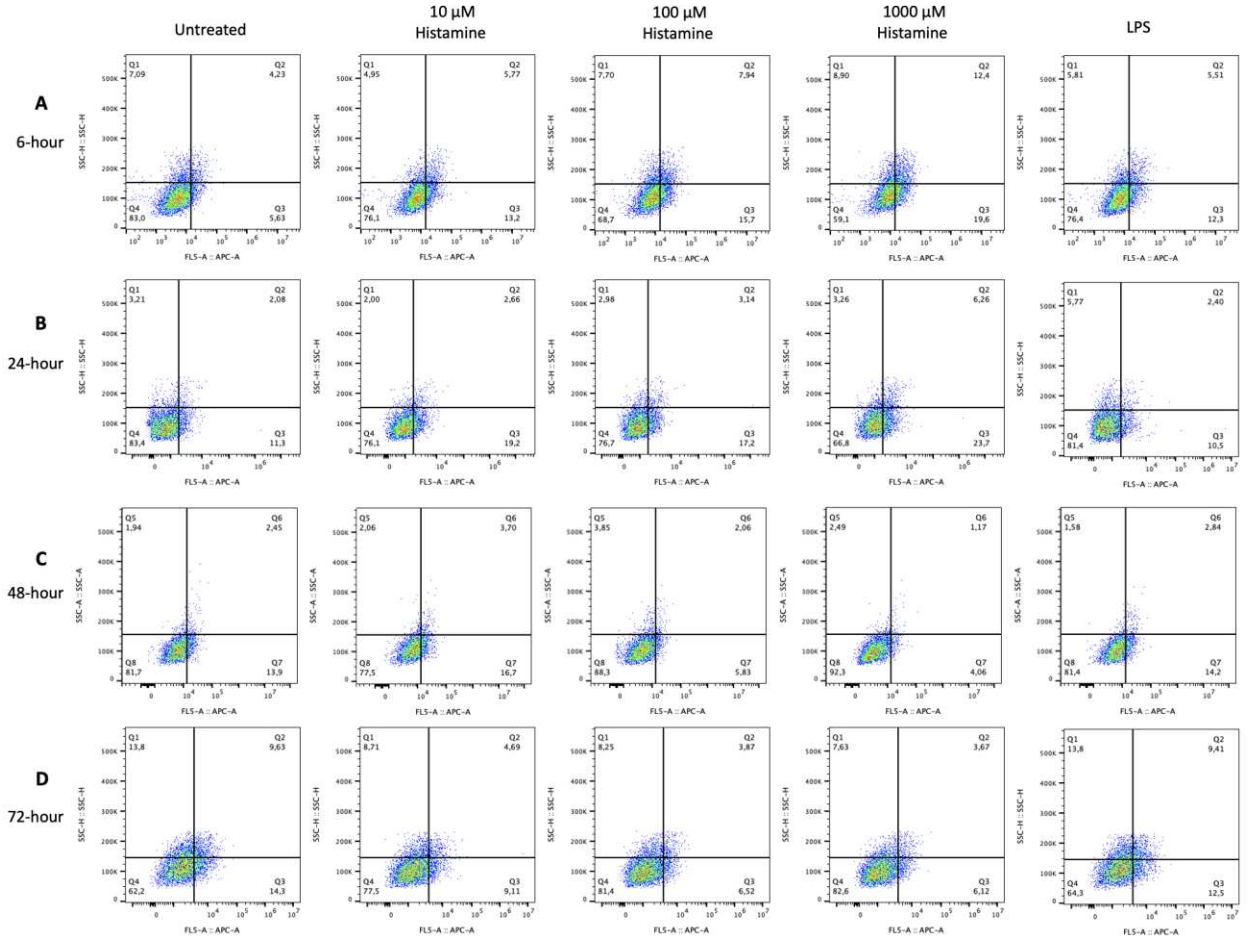
Supplementary 3.1. HMC3 cells grown in media with lower serum concentrations release less IL-8 and IL-6. HMC3 were cultured in complete media consisting of MEM, 10% heat-inactivated FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin. Twenty-four hours prior to supernatant collection, complete MEM media was replaced with media containing 1% heat-inactivated FBS or left unchanged. (**A**) IL-8 and (**B**) IL-6 release were measured by sandwich ELISA. Data are presented as the mean ± SEM and statistical significance was measured via Student's t-test, $p \leq 0.05$ (*), $p \leq 0.0001$ (****). (N=3).



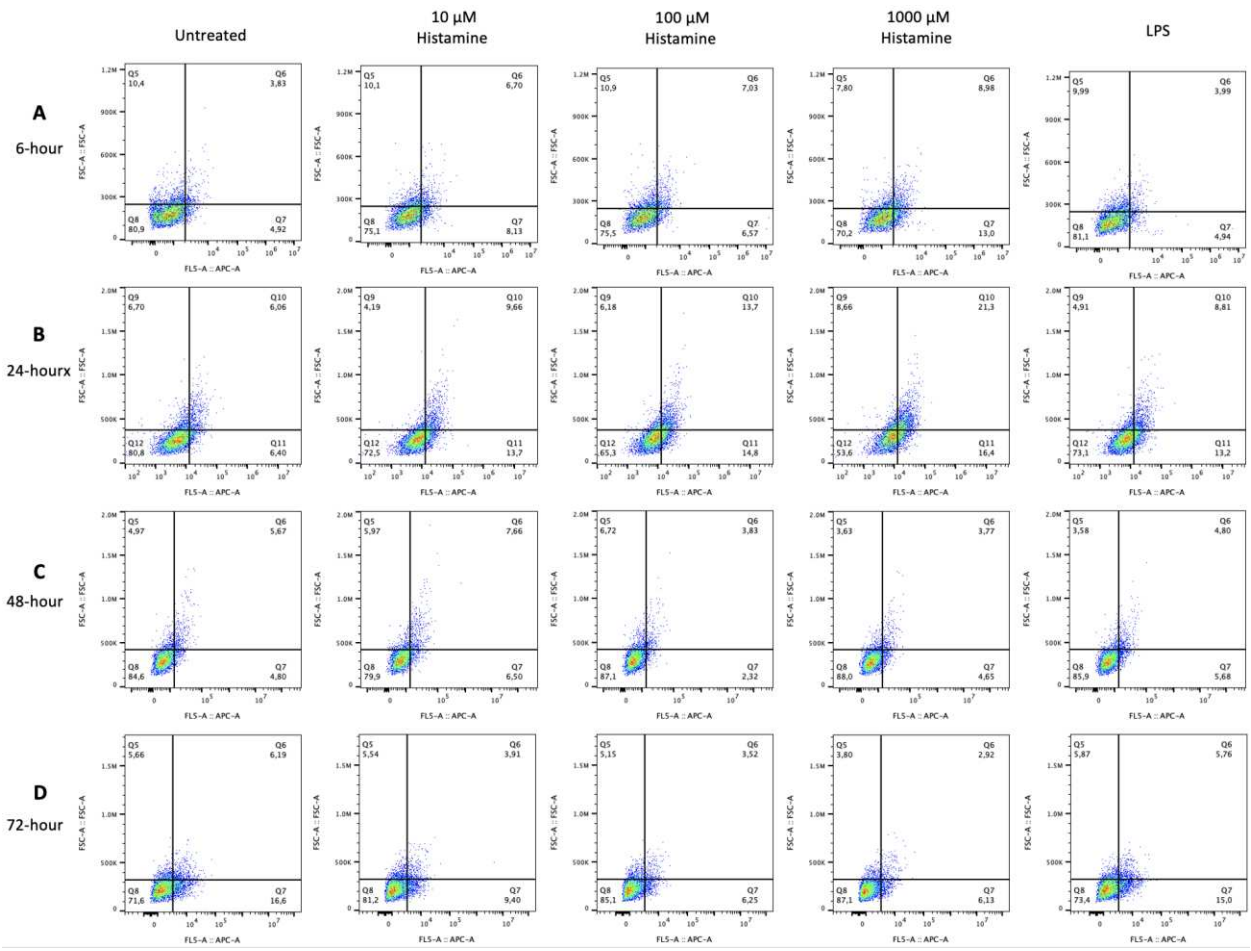
Supplementary 3.2. HMC3 cells do not alter cellular viability or cell proliferation following histamine or LPS stimulation. Cells were treated with histamine (10, 100, or 1000 µM) or LPS (1 µg/mL) for 24 hours and (**A**) cellular viability and (**B**) cell proliferation were measured using trypan blue staining and a hemacytometer. Counts were performed via a single-blinded method. (N=4).



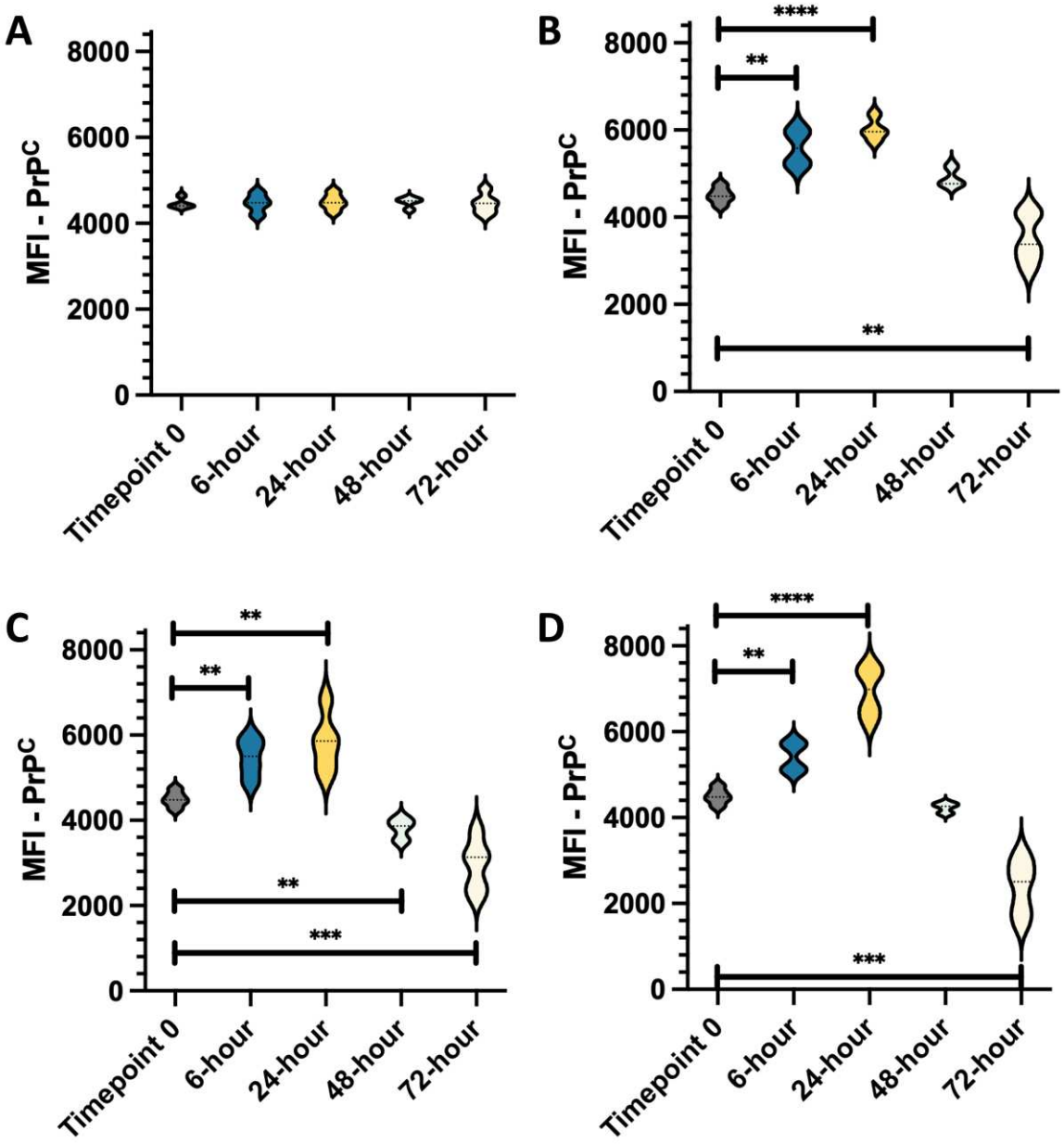
Supplementary 3.3. HMC3 cells alter cytokine production following stimulation by histamine and LPS. An electrochemiluminescence assay performed to measure cytokine release following histamine-induced stimulation showed that HMC3 altered the production of (**A**) IL-8, (**B**) IL-6, (**C**) IL-10, (**D**) IL-4, (**E**) IFN-γ, and (**F**) IL-12p70. (N=2, bars indicate ± SEM).



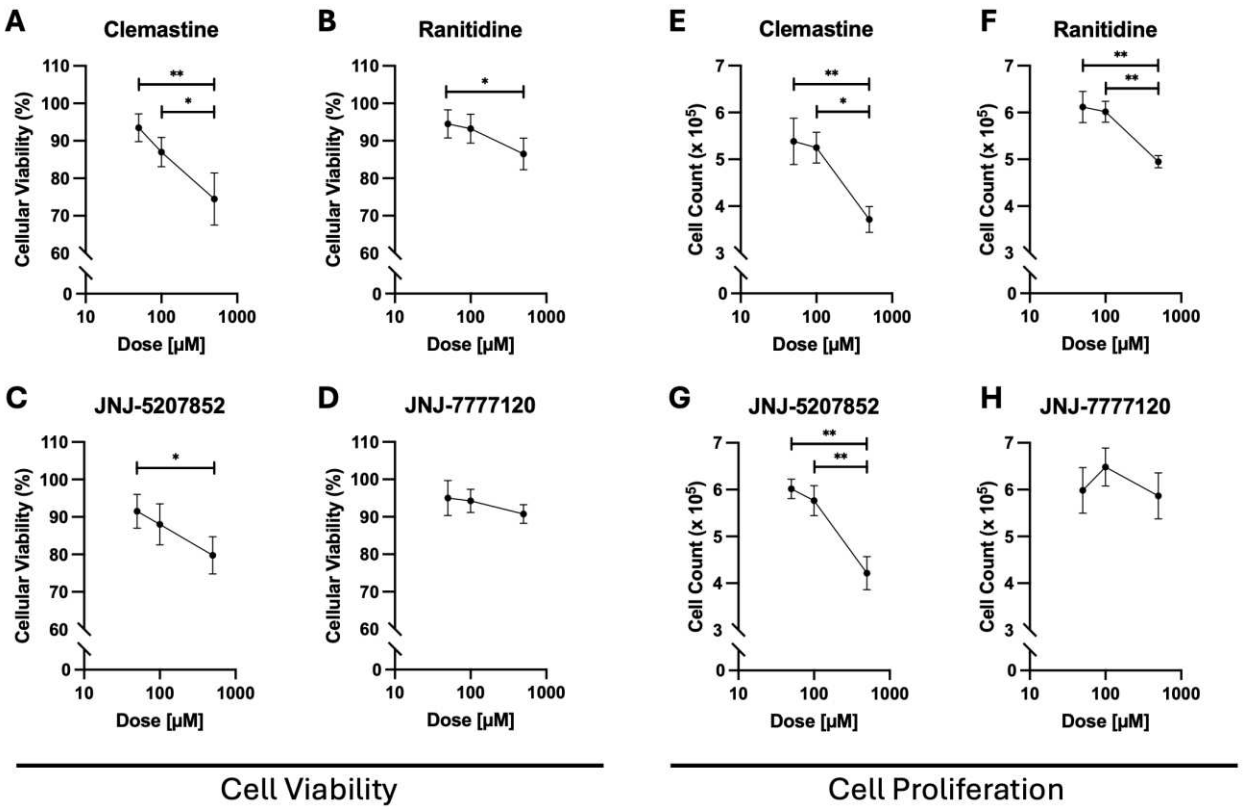
Supplementary 3.4. **Scatterplot representation of HMC3 cell complexity relative to PrP^C following histamine treatment for various periods of time.** Flow cytometry of HMC3 following treatment with 10, 100, or 1000 µM of histamine, 1 µg/mL of LPS, or no treatment. HMC3 were treated with histamine or LPS for (**A**) 6 hours, (**B**) 24 hours, (**C**) 48 hours, or (**D**) 72 hours. Graphs plot side-scatter (y-axis) against PrP^C expression (x-axis). The percentages in each corner reflect the population of cells in each quadrant. Side-scatter (SSC) indicates cell complexity. (N=4).



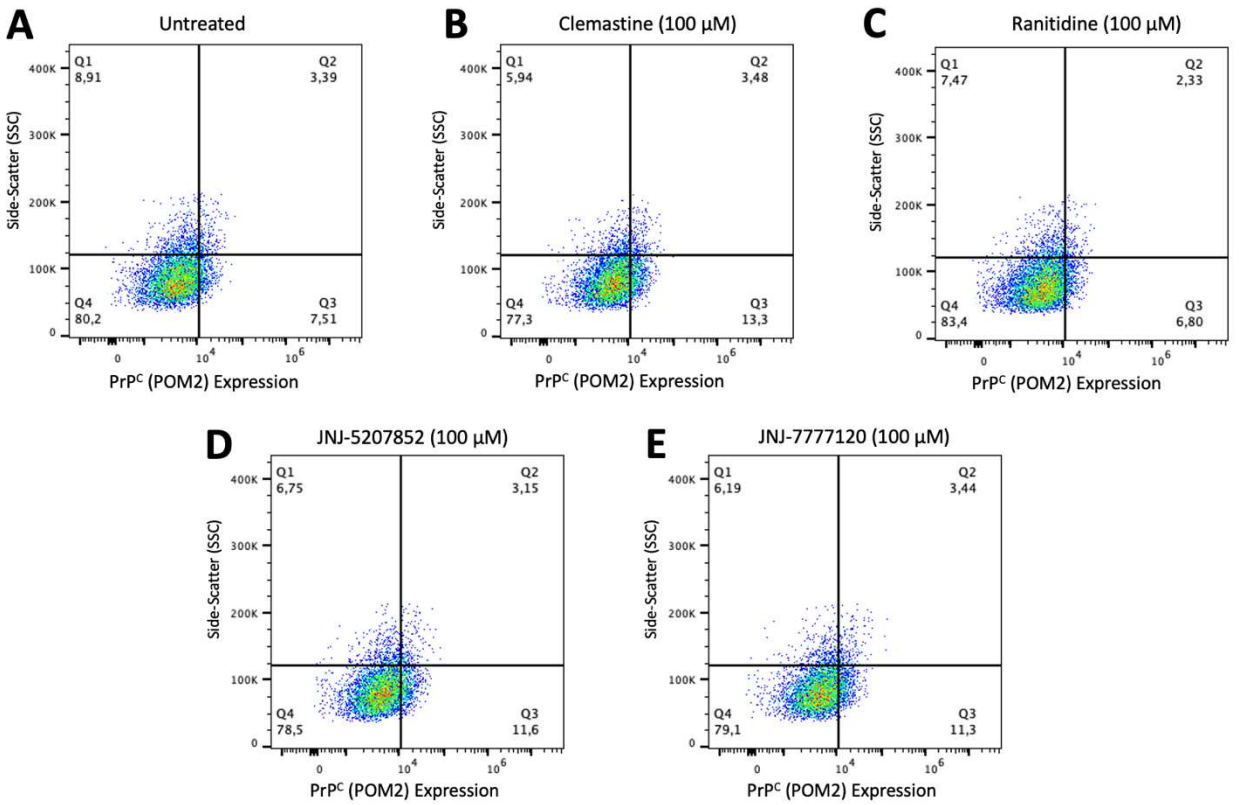
Supplementary 3.5. Scatterplot representation of HMC3 cell size relative to PrP^C following histamine treatment for various periods of time. Flow cytometry of HMC3 following treatment with 10, 100, or 1000 µM of histamine, 1 µg/mL of LPS, or no treatment. HMC3 were treated with histamine or LPS for (**A**) 6 hours, (**B**) 24 hours, (**C**) 48 hours, or (**D**) 72 hours. Graphs plot forward-scatter (y-axis) against PrP^C expression (x-axis). The percentages in each corner reflect the population of cells in each quadrant. Forward-scatter (FSC) indicates cell size. (N=4).



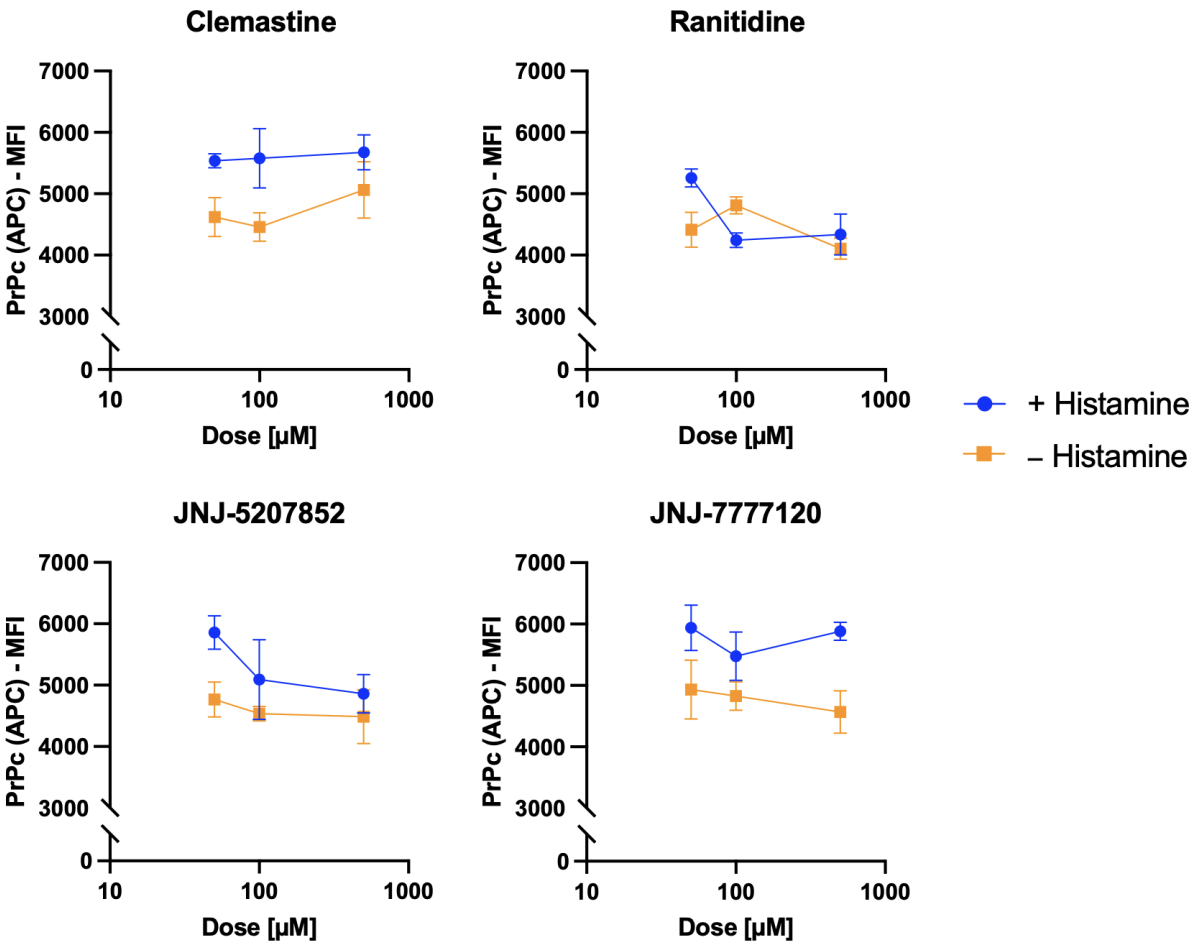
Supplementary 3.6. HMC3 cells altered PrP^C expression following histamine-induced stimulation. (**A**) Untreated HMC3 cells do not alter cell surface PrP^C over time. HMC3 decrease cell surface PrP^C following in a time-dependent manner following (**B**) 10 μM, (**C**) 100 μM, and (**D**) 1000 μM histamine treatment. Statistical significance was calculated using Student's t-test, $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.0001$ (****). (N=4).



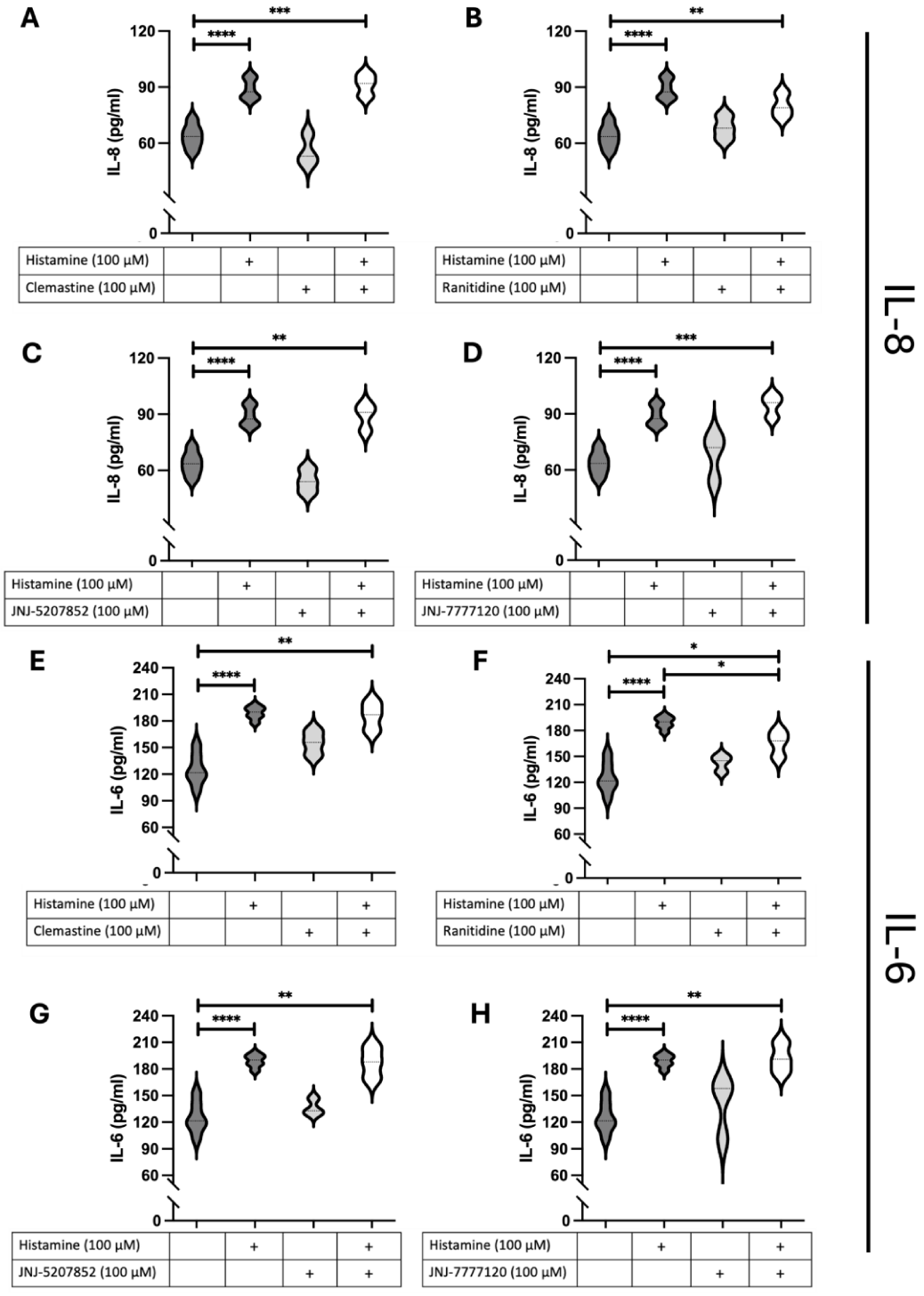
Supplementary 3.7. HMC3 cells alter cellular viability and proliferation following treatment with high doses of histamine receptor antagonists. Cells were treated with 50, 100, or 500 μM of clemastine, ranitidine, JNJ-5207852, or JNJ-7777120 for 24 hours and (**A**) cellular viability and (**B**) cell proliferation were assessed manually. Counts were performed via a single-blinded method. Data are presented as a dose-response curve with the mean ± SEM and statistical significance was measured via Student's t-test, $p \leq 0.05$ (*), $p \leq 0.01$ (**). (N=4).



Supplementary 3.8. Scatterplot representation of HMC3 cell complexity relative to PrP^C. HMC3 cells were treated with individual histamine receptor antagonists for 1 hour followed by 100 µM histamine for 24 hours. Cell complexity (y-axis) was plotted relative to PrP^C expression (x-axis). (**A**) Untreated HMC3. (**B**) HRH1 inhibition by Clemastine. (**C**) HRH2 inhibition by Ranitidine. (**D**) HRH3 inhibition by JNJ-5297852. (**E**) HRH4 inhibition by JNJ-7777120. Side-scatter (SSC) indicates cell complexity. (N=4).



Supplementary 3.9. Dose-response curves of histamine receptor antagonists. HMC3 cells were treated with 50, 100, or 500 µM (**A**) clemastine, (**B**) ranitidine, (**C**) JNJ-5207852, or (**D**) JNJ-7777120 for 1 hour and then either treated with 100 µM histamine (blue) or left untreated (orange) for 24 hours. Subsequently, PrP^C expression was measured by flow cytometry. Graphs are plotted as a dose-response curve. (N=3).



Supplementary 3.10. Cytokine release of HMC3 cells following histamine receptor inhibition. HMC3 cells were treated with individual 50, 100, or 500 µM of histamine receptor antagonists for 1 hour and then either treated with 100 µM histamine or treated with vehicle (PBS) for 24 hours. IL-8 and **(D)** IL-6 were measured by sandwich ELISA. Data are presented as the mean ± SEM (N=3) and statistical significance was measured via one-way ANOVA and Dunnett's multiple comparison post-hoc analysis relative to untreated (UT) cells. $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***), $p \leq 0.0001$ (****).

CHAPTER 4: General Discussion and Conclusions

4.1 Chapter 4 Abstract

This final and concluding chapter synthesizes the findings of our research into the role of the cellular prion protein (PrP^C) in neuroinflammation and immune signaling. Here, we contextualize our research on PrP^C protein expression levels in human-derived microglia and discuss the implications of the dynamic regulation of this protein in HMC3 cells.

Ultimately, our work established a reliable methodology for detecting total and surface PrP^C protein expression levels and this chapter further discusses how histamine may influence microglial behaviour and induce relative changes in PrP^C expression in human microglia. Our data demonstrated that histamine-induced stimulation results in time- and concentration-dependent changes in surface PrP^C expression, likely mediated by the HRH2 histamine receptor. This novel discovery suggests that PrP^C may play a critical role in microglial signaling pathways influenced by histamine.

Lastly, this chapter highlights the necessity for future investigations into microglia, prion protein, and histamine to further understand how these neuroimmune mediators may contribute to neuroinflammatory signaling. Notably, we suggest research using a more physiologically relevant cellular model such as primary microglial cells or microglia grown in co-culture conditions with other factors frequently found to interact with microglia *in vivo.* Finally, we discuss the complexities of histamine signaling and histamine receptor interactions which further suggests future work be necessary to elucidate the mechanisms driving microglial responses and PrP^C dynamics in neuroinflammatory signaling.

4.2 Summary of results

In order to establish a potential role for PrP^C in neuroinflammation and immune signaling, we sought to test numerous immune cells for PrP^C surface expression, and specifically focus on expression in microglia. As PrP^C is not well-studied in human cells, our initial experiments aimed to identify total and surface PrP^C expression in human-derived neurological, immune, and neuroimmune cells. We established that we could detect and compare total PrP^C as well as PrP^C located on the cell surface in different cell types. Following this proof-of-concept, we focussed on microglia as these cells are the crux of neuroinflammation and neuroimmune regulation, and our HMC3 microglial model expressed relatively high surface PrP^C levels.

As the role of histamine in neuroinflammation is a growing topic of interest, we wanted to focus on histamine-induced stimulation of microglia and understanding potential changes in PrP^C expression following this stimulation. There are two key sources of histamine in the CNS: histaminergic neurons typically found in the posterior hypothalamus[58], and mast cells which are spread throughout the CNS but are most commonly found in the meninges, perivascular space, and choroid plexus[23,24]. Histaminergic neurons primarily function to release histamine as a neurotransmitter where it contributes to cognition, wakefulness, and immune signaling[58]. Histamine-induced signaling by these neurons has been indicated in microglia and neuroinflammation[212] where histamine may be released by neurons and directly act on microglial cells[83,150].

Mast cell-microglia communication is a growing topic in understanding neuroinflammation[24,80,148]. While microglia are commonly labeled as the sentry cell of the brain, mast cells are typically considered the sentry cell of the body. Bidirectional communication between these cells can be conducted by numerous cytokines and signaling molecules (**Figure 4.1**)

and stimulation by one of these cells can induce stimulation of the other to mediate immune signaling via cytokine release, neurotransmission, direct signaling, or protease release. Following their stimulation, mast cells rapidly release proinflammatory mediators including histamine into their environment. This histamine can induce *in vivo* changes to microglia, reducing their ramifications and modifying their neuroprotective effects[25]. Mast cell-microglia interactions in the brain are not currently well understood, however, due to mast cells being a key source of histamine in the CNS, alongside the growing evidence that histamine is a mediator of microglial reactivity, further research on how these cells may communicate and mediate neuroinflammation is necessary. Our work introduces histamine-induced stimulation in human microglia as a possible means by which PrP^C signaling may participate in neuroinflammatory conditions. As our results were conducted in an *in vitro* model system, future work is necessary to further understand the physiological implications of our findings in a more relevant human model, while also exploring mast cell-microglia communications as a source of histamine-induced microglial stimulation.

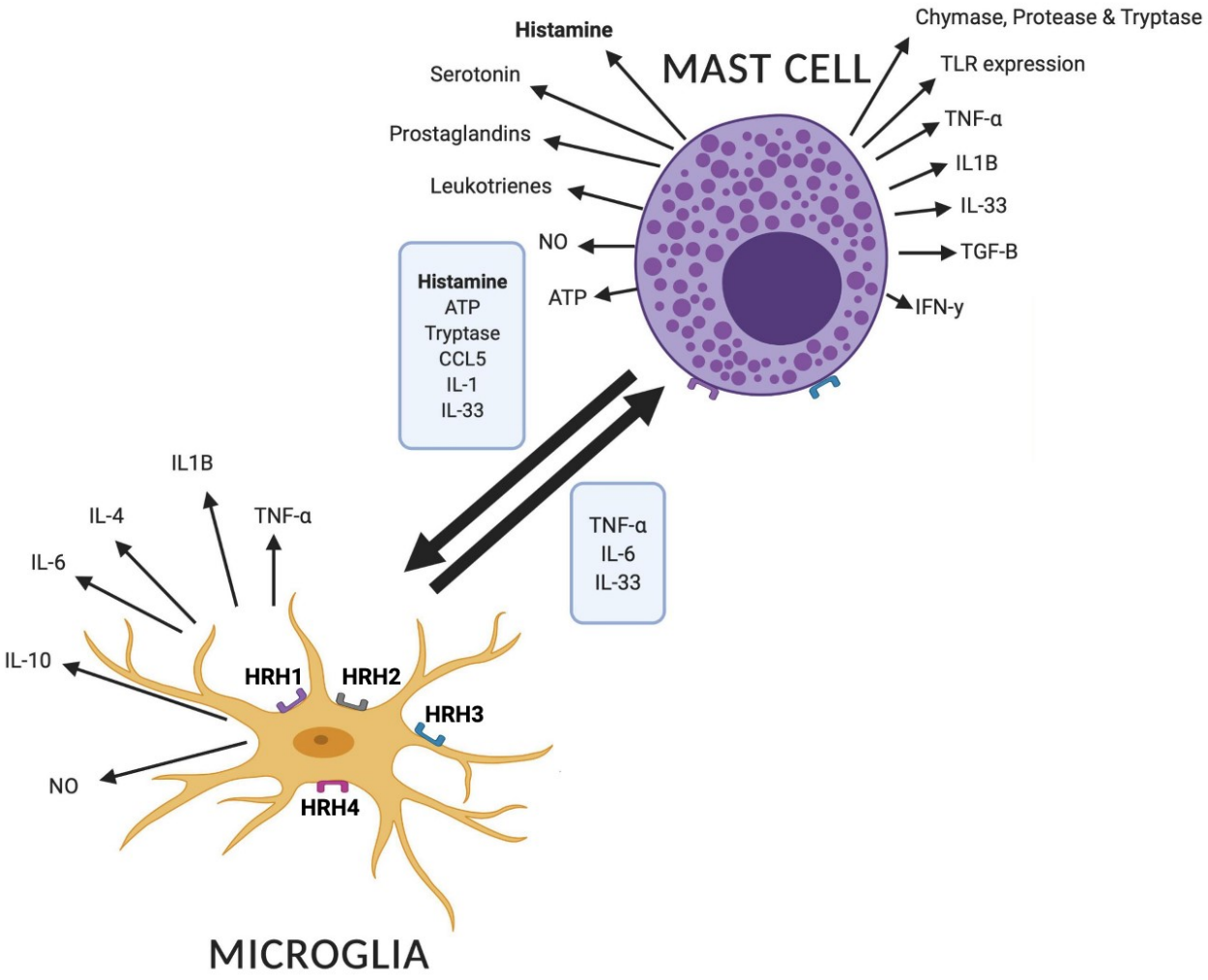


Figure 4.1. Mast cell-microglia interactions in the brain. Bidirectional feedback and signaling between mast cells and microglia can be mediated by numerous cytokines and signaling molecules. Following signaling from one cell type, other cells can be stimulated to perform various physiological actions such as neuroinflammatory regulation, cytokine release, phagocytosis, or initiating other neuroimmune pathways. (Figure adapted from Carthy and Ellender (2021) *Front. Neurosci*. 15: 680214)[215].

In the present study, following the identification of histamine as a means to stimulate microglia, inducing increases in cytokine release and cytosolic calcium ion levels, our results demonstrated that histamine-induced simulation of these cells resulted in time- and concentration-dependent changes in surface, but not total PrP^C protein expression. In order to inquire about potential mechanisms by which these changes in surface PrP^C were taking place, we used

histamine receptor agonists and antagonists to demonstrate that the HRH2 GPCR is responsible for mediating surface PrP^C expression changes. This demonstration of HRH2 as the plausible pathway of histamine-induced changes in PrP^C surface expression could be a potential mechanism by which mast cells influence microglial behaviours, potentially through histamine signaling. Our findings, highlighting HRH2 as a key receptor in mediating dynamic PrP^C expression, may have implications in the clinical field; ranitidine is a common medication prescribed in patients with stomach acid diseases. Our results suggest that blocking the HRH2 receptor long-term may have implications in microglial function and signaling, although the implications of this dynamic regulation of PrP^C must be explored further.

Notably, HRH2 has garnered particular interest for its potential involvement in neuropathic pain and neuroinflammation[213]. One particular study conducted by Rudick et al. indicated that, in mice, one subset of neurogenic pain was mediated by histamine released by degranulating mast cells, and the mechanism by which this pain was exerted was via the HRH2 receptor[214]. Additionally, another study in humans conducted by Thilagarajah et al. found that blocking the HRH2 receptor with antagonists decreased neuropathic pain associated with interstitial cystitis, a chronic condition affiliated with bladder pain and inflammation. Taken together, these findings substantiate our results that suggest HRH2 as a key receptor in the mediation of neuroinflammation.

4.3 Future directions, limitations, and conclusions

Our findings are the first to demonstrate a dynamic modification of cell-surface cellular prion protein expression following microglial stimulation. Although these results are novel and exciting as they indicate PrP^C as a potential mediator of neuroinflammatory signaling and as a

potential signaling molecule in microglia, it is important to contextualize our findings and highlight potential avenues that can be expanded upon.

Our data was generated utilizing the human-derived HMC3 microglial cell line. In the context of the limited microglial cell lines that are human-derived, highly proliferative, and physiologically relevant, these cells were the best possible candidate for the desired experiments. Previous research utilizing this cell model have looked into their release of cytokines[164,216], phagocytic properties[217], and intracellular signaling pathways that play a role in immune signaling[162,218]. There has been limited research into the cellular signaling molecules present in these cells, and our work, alongside others, have indicated that unstimulated HMC3 cells do not possess microglial expression markers, such as CD45 and CD86, typically found in primary microglial cells[164,174]. Recent evidence suggests that iPSC-derived microglia have notably high homology to primary microglia[219,220], and, alongside experiments with primary human microglia, these more physiologically relevant models should be tested to explore the role of PrP^C in neuroimmune regulation. Additionally, future work should look to understand the direct role of PrP^C expression on histamine-induced microglial changes through the usage of mouse knockout models. Notwithstanding, utilizing *in vitro* microglial cell culture assays limits physiological relevance regardless of how the cells were derived; *in vivo* microglia are dynamic and in constant communication with their environment, including interactions with astrocytes, neurons, cell debris, a plethora of cell signaling molecules such as cytokines, and other factors within its local milieu. These limitations hinder the physiological relevance of microglial cell culture assays, suggesting that future research be conducted *in vivo* where possible.

Physiologically, within its environment, the activation state of microglia is tightly regulated by a variety of signals. Following their stimulation these cells may become responsible for

participating in phagocytosis to clear debris and harmful toxins or material from the CNS[161], alter their morphology[41], and may alter their release of inflammatory signaling materials[216]. Our results demonstrated clear, rapid changes in calcium ion influx and changes in the release two key inflammatory cytokines, which are expected phenotypic changes in microglia following stimulation[221,222]. In order to strengthen our results and fully understand the extent of histamine-induced stimulation of these cells, more assays testing various physiological and morphological changes in these cells should be conducted; for instance, experiments measuring changes in phagocytosis via the pHrodo phagocytosis assay[223] and quantifying potential morphology changes via well-established methods[224,225] should be conducted to develop a well-rounded profile of histamine-induced microglial stimulation. Additionally, testing microglial cell-surface indicators such as P2RY12 and TMEM119, two highly specific microglial markers that alter expression levels following microglial stimulation[226], should be considered to build on the impact of the present study.

One additional limitation to the findings presented in this paper is regarding histamine receptors in microglial cells. Although the primary mechanism of histamine-induced stimulation in the vast majority of cells is indeed through the histamine receptors[58,142], histamine has been found to stimulate muscarinic acetylcholine receptors (mAChRs)[227], a receptor that microglia possess[228]. Accordingly, the histamine-induced activation outcomes we observed in our research may not solely be resulting from histamine-histamine receptor interactions, as histamine may be acting through other receptors to induce these microglial changes. Indeed, non-specificity between ligand and receptor has been observed in histamine receptors before: compounds such as VU6021625, which typically exclusively bind mAChRs, have been observed to interact with histamine receptors[229]. As histamine receptors belong to the large, rhodopsin (class A) family of

GPCRs – of which microglia possess many[230] – homology between and within this class of receptors may cause off-target allosteric interactions between histamine and other receptors. For instance, promiscuous binding by human HRH2 receptors has recently been observed by Jacob et al. who indicated that the vasoconstricting adrenergic agonist ergotamine binds and stimulates HRH2 in the heart[231]. Accordingly, additional work exploring these potential nonspecific interactions between histamine and other receptors should be conducted to confirm that the findings observed in our study are not impacted by outstanding variables.

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