INVESTIGATING THE EFFECTS OF CALRETICULIN AND β-GALACTOSIDASE ON MICROGLIAL FUNCTIONS AND NEURONAL LOSS

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DECLARATION

This thesis is the result of my own work and includes nothing which is the outcome of work done in collaboration unless specified in the text. This work is not substantially the same as any work that has already been submitted before for a degree or another qualification. This dissertation does not exceed the prescribed word limit of 60,000 for the Biology Degree Committee.

Emily Jessica Ann Kitchener
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RESEARCH IMPACT STATEMENT

My research is lab based and so I was unable to conduct research for approximately 4 months during the first COVID-19 lockdown in 2020. Following this, the department was reopened but ran at a reduced capacity for a further six months, meaning that there was limited access to the lab. It also took several months to get animal work setup again and to have access to consumables that were on backorder due to limited supplies across the country. This made it difficult to work during that period.

Investigating the effects of calreticulin and β-galactosidase on microglial functions and neuronal loss

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ABSTRACT

Microglia are the primary immune cells of the central nervous system (CNS); they have dynamic roles in brain development, maintaining homeostasis, and responding to insult and injury, thereby contributing to CNS health. However, increasing evidence highlights a role for microglial dysfunction in many brain pathologies, like Alzheimer's disease (AD) and Parkinson's disease (PD). Understanding how and when microglia are beneficial, detrimental or both, is an active area of research and, elucidating the underlying mechanisms involved in neurodegeneration could uncover novel therapeutic avenues. In this work, I investigated how microglial functions and neuronal loss were affected by calreticulin and β-galactosidase, two proteins associated with ageing, chronic inflammation, and neurodegeneration.

The aggregation of amyloid-β to form oligomers and insoluble amyloid plaques in the brain is a hallmark of AD. Amyloid-β can be directly neurotoxic and induce pro-inflammatory activation of microglia, which may contribute to neurodegeneration. Molecular chaperone proteins are commonly found intracellularly, where they interact with proteins to prevent their aggregation and facilitate proper folding. The endoplasmic reticulum-resident chaperone protein, calreticulin, can be released from microglia and has been found to bind amyloid-β. I investigated whether exogenous calreticulin affects amyloid-β aggregation and amyloid-β-induced neurotoxicity. *In vitro* assays revealed inhibition of amyloid-β fibrillisation by calreticulin, and transmission electron microscopy showed that calreticulin promoted formation of larger amyloid-β oligomers. Furthermore, exogenous calreticulin was protective in the context of amyloid-β-induced neuronal loss in primary mixed neuronal-glial cultures. Together this data suggests that calreticulin might act as an extracellular chaperone for amyloid-β and be neuroprotective, hence treatments increasing extracellular calreticulin in the brain might be beneficial for AD.

Glycohydrolase enzymes, including neuraminidase 1 (Neu1) and β-galactosidase, play a fundamental role in the degradation of glycoproteins and glycolipids, to maintain cellular constituent turnover and glycosylation homeostasis. Neu1 hydrolyses terminal sialic acid residues to expose galactose residues, which can then be hydrolysed by β-galactosidase. Previously it was thought that these enzymes were exclusively located in lysosomes, but recent evidence has found Neu1 activity also associated with the external surface of the

plasma membrane. As Neu1 can be structurally and functionally coupled to β-galactosidase in a lysosomal multienzyme complex, I investigated whether BV-2 microglia and primary rat microglia have increased extracellular β-galactosidase activity when activated by a variety of inflammatory stimuli, including lipopolysaccharide (LPS) and adenosine triphosphate (ATP). Inflammatory activation of microglia increased β-galactosidase activity at the cell surface and increased β-galactosidase protein levels extracellularly. Extracellular β-galactosidase might remove galactose residues from the surface of microglia and neurons, potentially disrupting homeostasis. I found that addition of β-galactosidase to primary mixed neuronal-glial cultures caused a significant loss of neurons and promoted microglial activation. Whereas inhibition of β-galactosidase in LPS-stimulated cultures reduced LPS-induced neuronal loss and microglial activation, suggesting that β-galactosidase may activate microglia in a way that promotes neuronal loss.

Together, this work elucidates novel effects of calreticulin and β-galactosidase on microglial function and neuronal loss, which may contribute to understanding the roles of these proteins in neurodegeneration and disease.

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Emily Jessica Ann Kitchener

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LIST OF ABBREVIATIONS

Table 1. List of frequently used abbreviations.

Aβ	Amyloid-β
AD	Alzheimer's disease
ANOVA	Analysis of variance
APP	Amyloid precursor protein
ATP	Adeonise-5'-triphosphate
Bleo	Bleomycin
C1q	Complement component 1q
C3	Complement component 3
CGCs	Cerebellar granule cells
CNS	Central nervous system
CR3	Complement receptor 3
CRT	Calreticulin
CSF	Cerebrospinal fluid
DA	Dopaminergic
DAMP	Damage-associated molecular pattern
DGJ	1-Deoxygalactonojirimycin
DIV	Days *in vitro*
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulphate
DOX	Doxorubicin
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
FBS	Foetal bovine serum
HRP	Horse radish peroxidase
HS	Hoechst
IB_4	Isolectin B4
Iba1	Ionized calcium binding adaptor molecule 1
IFNγ	Interferon gamma
IL-6	Interleukin 6
iPSC	Induced pluripotent stem cell
LPS	Lipopolysaccharide
LRP1	Low density lipoprotein receptor-related protein 1
MBL	Mannose-binding lectin
MUB	4-methylumbelliferone
MUG	4-methylumbelliferyl-β-D-galactopyranoside
NADPH	Nicotinamide adenine dinucleotide phosphate

Neu	Neuraminidase
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate buffered saline
PD	Parkinson's disease
PI	Propidium iodide
PMA	Phorbol 12-myristate 13-acetate
PPCA	Protective protein cathepsin A
PRR	Pattern recognition receptor
ROS	Reactive oxygen species
SA-β-galactosidase	Senescence-associated β-galactosidase
SIGLEC	Sialic acid-binding immunoglobulin-type lectin
TEM	Transmission electron microscopy
ThT	Thioflavin T
TLR	Toll-like receptor
TNFα	Tumour necrosis factor α
TREM2	Triggering receptor expressed on myeloid cells 2
(c) DNA	(complementary) Deoxyribonucleic acid
(q) PCR	(quantitative) Polymerase chain reaction
(si) RNA	(small interfering) Ribonucleic acid

1 INTRODUCTION

1.1. General introduction and scope

Neurodegenerative diseases are characterised by the progressive and irreversible loss of neurons in the central nervous system (CNS). While genetic and environmental factors strongly affect disease risk, age is widely recognised as the most significant risk factor for the development of neurodegenerative diseases, including Alzheimer's disease (AD) and Parkinson's disease (PD).

A major hallmark of neurodegenerative diseases is the accumulation of protein aggregates, for example the aggregation of amyloid-β in AD. Extracellular amyloid-β aggregates can exert toxic effects on neighbouring cells, promoting inflammation, and activating immune responses, ultimately contributing to cellular dysfunction, degeneration, and death. As the resident immune cells of the CNS, microglia are considered the primary mediators of inflammation in the brain and play a pivotal role in maintaining CNS homeostasis, supporting neuronal function, and safeguarding against potential threats. However, with ageing and disease, microglia can contribute to chronic inflammation and subsequent tissue damage. As microglia are equipped to sense and respond to changes in their environment, increasing interest is placed on understanding mediators of their functions and how these might impact neurodegeneration. Cell surface glycosylation is important in cell-to-cell communication, influencing neuronal development, synaptic plasticity, and microglial immune responses. Indeed, aberrant biosynthesis and degradation of glycosylated cellular components, like gangliosides, have been linked to neurodegenerative diseases, such as AD and PD, highlighting their significance in pathology.

Despite research efforts, the intricate molecular mechanisms underlying neurodegeneration are poorly understood. Consequently, most neurodegenerative diseases lack effective treatment, and preventive strategies remain elusive. As protein aggregation and microglial neuroinflammation are implicated in neurodegeneration, preventing neurodegeneration requires further investigation of these two processes. On the one hand, by understanding the process of protein aggregation, and subsequently targeting the production or clearance of these aggregates, the hope is to mitigate the subsequent cellular dysfunction and toxicity, and potentially slow or halt disease progression. On the other hand, understanding how microglia respond to various signals and how they contribute to neuroinflammation, neurodegeneration, and brain repair may be important for developing treatments that target these processes and promote brain health.

In this thesis, I aimed to further our understanding of two proteins, calreticulin and β-galactosidase and their functions in microglial-mediated neurodegeneration. I first present findings on the effects of calreticulin, a potential extracellular chaperone, on amyloid-β

aggregation and the subsequent effects of these aggregates on neuronal loss in mixed neuronal-glial co-cultures. Then, I show evidence that β-galactosidase, a key glycohydrolase enzyme, is released from microglia under certain conditions and investigate the effects of extracellular β-galactosidase on neuronal loss. Hence, this introduction will discuss key microglial functions, focussing particularly on phagocytosis and inflammation before outlining microglial contributions to neurodegenerative disorders, including AD and PD. I introduce the concept of extracellular chaperones, before outlining known functions of calreticulin. Then, I discuss the importance of glycosylation in the brain, with a particular focus on gangliosides, the major glycosylated species in the CNS, and introduce known functions of glycohydrolase enzymes, including β-galactosidase. The objective of this literature review is not to provide an exhaustive examination of the pathogenesis and biology associated with individual neurodegenerative diseases, nor to describe the entirety of microglial biology found in the literature. Rather I review the biology relevant to the experimental work of this thesis, which is at the intersection of microglia, neurodegeneration, chaperones, and glycosylation.

1.2. Microglia in development and homeostasis of the CNS

1.2.1. Microglial origins

Microglia were first described in 1919 by neuroscientist Pío del Río-Hortega (Rio-Hortega, 1919). At first, they were believed to be passive spectators of CNS physiology; grouped together with other glial cells, including astrocytes and oligodendrocytes, they were thought to function solely as structural support for neurons. Microglia have since gained recognition as the primary resident macrophage-like cells of the brain, capable of a myriad of context-dependent immune functions. In fact, many of the risk genes associated with neurodegenerative disorders, identified by genome-wide association studies (GWASs), are preferentially expressed by microglia (Villegas-Llerena *et al.*, 2016; Efthymiou and Goate, 2017; Wightman *et al.*, 2021), further raising their profile in the field of neuroimmunology.

Widespread throughout the brain, retina and spinal cord, microglia make up 5 – 12 % of the total cells in the healthy CNS (Lawson *et al.*, 1990). Microglia originate from the yolk sac, invading the CNS during embryonic development until the blood-brain barrier is formed (Ginhoux *et al.*, 2010). Thereafter, the microglial population is sustained by a process of self-renewal (Ajami *et al.*, 2007; Hashimoto *et al.*, 2013; Bruttger *et al.*, 2015). Microglia are characterised by their longevity, exhibiting a notably low turnover rate in the healthy brain (Réu *et al.*, 2017). Their prolonged survival is linked to the continuous stimulation of the colony-stimulating factor 1 receptor (CSF-1R) present on their cell surface. The ligands responsible for maintaining CSF-1R signalling in microglia are interleukin-34 (IL-34) and CSF-1 itself, both of which are produced within the CNS. The interplay between CSF-1R, IL-34, and CSF-1 is crucial for establishing a balance between microglial survival and function (Lawson, Perry and Gordon, 1992; Erblich *et al.*, 2011; Elmore *et al.*, 2014).

As cells of the innate immune system, microglia monitor their environment to detect and respond to potential insults. Microglia are sensitive to their environment, owing to an array of receptors distributed along highly dynamic and ramified processes that continuously survey the brain parenchyma (Davalos *et al.*, 2005; Nimmerjahn, Kirchhoff and Helmchen, 2005). Upon detection of signals, microglia may execute two main functions: 1) phagocytosis, i.e., the engulfment and digestion of particles, such as pathogens, synapses, debris, and protein aggregates, and 2) initiation of inflammation, involving the secretion of pro-inflammatory factors and recruitment of peripheral immune cells. These functions allow microglia to contribute to the development, survival, and overall homeostasis of the CNS.

1.2.2. Microglia in development

Microglia play a vital role in brain development; their active participation in sculpting neural circuits and supporting neuronal survival ensures that functional neural networks are established. Microglia help to fine-tune neural circuits through synaptic pruning; whereby they phagocytose excess synapses to promote efficient communication between neurons. Microglia mediate synaptic pruning through various signalling pathways and receptors, including fractalkine (or CX3CL1) and complement component 1q (C1q), complement 3 (C3) and complement receptor 3 (CR3) (Stevens *et al.*, 2007; Schafer *et al.*, 2012; Hong *et al.*, 2016). Indeed, mice deficient in these proteins are found to have aberrant synaptic pruning and connectivity (Paolicelli *et al.*, 2011; Schafer *et al.*, 2012). Microglia can also contribute to neurogenesis and synapse maturation by secreting molecules, including brain-derived neurotrophic factor (BDNF) and transforming growth factor-β (TGF-β), that promote the formation of functional connections between neurons and facilitate neuronal proliferation, differentiation, plasticity, and survival (Parkhurst *et al.*, 2013; Bathina and Das, 2015; Meyers and Kessler, 2017). Collectively, microglial roles in brain development highlight their ability to support neuronal health and fine-tune neural circuits, making them indispensable players in sculpting the intricate architecture of the developing brain.

1.2.3. Microglial activation states and role in homeostasis

Microglia have the capacity to resolve damage within the CNS and are thought to enter distinct activation states upon encountering certain stimuli. Until about ten years ago, the classification of microglial activation *in vitro* drew parallels with non-CNS macrophages, resulting in the nomenclature of microglial activation states: M0, M1, and M2. Upon exposure to lipopolysaccharide (LPS), homeostatic ‘M0’ microglia transition to a classically activated ‘M1’ state, characterised by increased secretion of inflammatory molecules (including cytokines, chemokines and, reactive oxygen and nitrogen species) and increased phagocytic capacity. M1 microglia are considered important for cellular immunity against pathogens but may also mediate neurotoxicity (Orihuela, McPherson and Harry, 2016). Conversely, a shift towards an alternatively activated 'M2' state occurs when exposed to cytokines like IL-4 and IL-13, which are released by activated T cells. This M2 state is associated with some anti-inflammatory responses and may promote tissue repair (Orihuela,

McPherson and Harry, 2016). However, over the past decade, the idea of microglial polarisation into three distinct states has evolved substantially, partly driven by RNAseq expression profiling of individual microglia. Instead, the prevailing notion is that microglia exist within a spectrum of multiple activation states, influenced by diverse environmental factors. Indeed, several subsets of microglia have been identified depending on the brain region, age and disease context (Xue *et al.*, 2014; Grabert *et al.*, 2016; Hammond *et al.*, 2019)

Microglial mediated removal of various insults, including pathogens, apoptotic cells, neuronal debris, and protein aggregates, is crucial for maintaining homeostasis in the CNS. Upon detection of a foreign or damaging agent, microglia undergo rapid proliferation, transitioning from a highly branched and surveillant state to a more amoeboid morphology, enabling them to migrate quickly toward the site of damage or infection (Ransohoff, 2007). By mounting an inflammatory and phagocytic response, microglia can eliminate such threats and resolve inflammation through secretion of an array of anti-inflammatory cytokines, including IL-10 (Lobo-Silva *et al.*, 2016; Laffer *et al.*, 2019). Through targeted removal of damaging and infectious agents, microglia contribute to the restoration of CNS integrity and maintenance of tissue homeostasis.

1.3. Regulation of microglial inflammatory and phagocytic pathways

Neuroinflammation refers to the inflammatory response of the CNS. Microglia, in particular, play a key role as the resident immune cells of the brain. During neuroinflammation, microglia become activated changing their morphology and function. They release various signalling molecules, including cytokines and chemokines, which attract other immune cells to the affected site. Microglia can also clear cellular debris, aggregated proteins, pathogens, and damaged cells through phagocytosis. Phagocytosis involves the recognition of targets, via their binding to receptors on the cell surface, then engulfment of the targets into phagosomes, which subsequently fuse with lysosomes where the targets are degraded. Phagocytosis serves to eliminate harmful or excess agents. While neuroinflammation and phagocytosis are generally considered protective, aimed at restoring tissue homeostasis and repairing damage, they can also have detrimental effects. Chronic inflammation can contribute to neuronal damage and excessive microglial phagocytosis has been implicated in the loss of stressed-but-viable neurons (Neher *et al.*, 2011; Neniskyte, Neher and Brown, 2011; Fricker, Oliva-Martín and Brown, 2012).

Neuroinflammation is closely intertwined with various neurological disorders, including neurodegenerative diseases like AD and PD. In these conditions, chronic inflammation can exacerbate tissue damage and contribute to disease progression. Researchers are studying the intricate mechanisms of neuroinflammation and phagocytosis to develop strategies that balance their protective and detrimental effects.

1.3.1. Pattern recognition receptors

Pattern recognition receptors (PRRs) are a class of proteins that are an essential part of the innate immune system. They play a crucial role in detecting molecules known as “pathogen-associated molecular patterns" (PAMPs), which are either released by or displayed on the surface of various microorganisms. PRRs may also recognise "damage-associated molecular patterns" (DAMPs), which are molecules released from damaged or stressed host cells. The recognition of these molecules triggers an immune response aimed at neutralising the threat and maintaining tissue integrity. Examples of PRRs expressed by microglia include: 1) toll-like receptors (TLRs), for example TLR2 and TLR4, which recognise bacterial components like LPS and initiate an immune response against potential infections (Olson and Miller, 2004); 2) nucleotide-binding oligomerisation domain (NOD)-like receptors (NLRs), like NLRP3, which contribute to the detection of intracellular pathogens and cellular stress (Franchi *et al.*, 2009; Xie *et al.*, 2021); 3) C-type lectin receptors (CLRs), that recognise a variety of pathogens, particularly those with carbohydrate components on their surfaces, for example Dectin-1 primarily recognises β-glucans, which are components found in the cell walls of fungi (Koutsouras, Ramos and Martinez, 2017); 4) scavenger receptors, such as CD36, involved in recognising a variety of ligands, including those associated with both pathogens and damaged cells (Coraci *et al.*, 2002; Grajchen *et al.*, 2020) and; 5) triggering receptor expressed on myeloid cells 2 (TREM2), which is not regarded as a classical PRR, but plays a role in microglial sensing of cellular debris and apoptotic cells, helping to maintain tissue homeostasis (Takahashi, Rochford and Neumann, 2005; Cignarella *et al.*, 2020).

Another category of molecular patterns, known as "self-associated molecular patterns" (SAMPs), has been proposed to function in distinguishing between self and non-self. Rather than triggering the innate immune inflammatory response, SAMPs interact with intrinsic inhibitory receptors, thus suppressing the immune response and maintaining the baseline non-activated state of innate immune cells (Varki, 2011). Typically present at the end of glycan chains on mammalian cells, sialic acid has been proposed as a SAMP. Sialic acid-containing glycans, can function as ligands for receptors like sialic acid-binding immunoglobulin-type lectins (SIGLECs), which deliver inhibitory signals to immune cells to prevent overstimulation (Maverakis *et al.*, 2015).

1.3.2. “Find-me” signals

“Find-me” signals are chemoattractants for phagocytes; these molecules are released from cells to recruit phagocytes that then phagocytose the cells releasing these signals (Figure 1.1). Examples include nucleotides adeonise-5’-triphosphate (ATP) and adeonise-5’-diphosphate (ADP). In the brain, ATP and ADP are released from stressed, damaged or activated neurons and can activate P2Y12 purinergic receptors on microglia to chemoattract microglia to the neurons (Honda *et al.*, 2001; Cockram *et al.*, 2021). Lysophosphatidylcholine (LPC) is a find-me signal recognised by microglial G2A receptors. Activation of G2A receptors initiates intracellular signalling pathways that lead to changes in cell morphology

and motility, promoting microglial migration toward areas of LPC release (Sheikh *et al.*, 2009). Another find-me signal, is the ubiquitously expressed membrane protein, fractalkine, that can be cleaved to a soluble form by cells undergoing apoptosis. As mentioned previously, fractalkine is implicated in brain development; soluble fractalkine can be released from neurons and through interactions with its receptor (CX3CR1) on microglia may mediate synaptic pruning by phagocytosis of excess synapses (Paolicelli *et al.*, 2011).

1.3.3. "Eat-me" signals, opsonins and "don't eat-me" signals

An "eat-me" signal is a molecular cue, displayed on the surface of a cell, that marks it as a target for phagocytosis (Figure 1.1). Eat-me signals may exist as either membrane-anchored or soluble molecules and originate from the target cell itself (Cockram *et al.*, 2021). Conversely, opsonins are extracellular proteins that facilitate phagocytosis by binding to both the target and the phagocyte; they differ from eat-me signals in that they do not originate from the target cell (Cockram *et al.*, 2021). Phagocytic cells, including microglia, have specific receptors that detect these signals. Upon recognition, the process of phagocytosis is initiated, leading to the internalisation and subsequent breakdown of the target.

Phosphatidylserine is the most well characterised example of an eat-me signal; it is typically found on the inner leaflet of the plasma membrane but becomes exposed on the outer leaflet during cellular stress or apoptosis (Krahling *et al.*, 1999; Segawa and Nagata, 2015). Phosphatidylserine can bind directly to phagocytic receptors, including TREM2 (Takahashi, Rochford and Neumann, 2005; Scott-Hewitt *et al.*, 2020) but can also be bound by opsonins, like milk fat globule factor E8 (MFG-E8) which bridges with vitronectin receptors to promote phagocytosis (Hanayama *et al.*, 2002). Asialoglycans can also be eat-me signals; they lack the sialic acid residue typically found at the terminus of glycan chains. Asialoglycans have been shown to promote the phagocytosis of apoptotic lymphocytes (Meesmann *et al.*, 2010). Although they can directly interact with phagocytic receptors, asialoglycans can also be bound by opsonins. Galectin-3 can bind to cell-surface galactose residues which, on mammalian cells, are normally concealed by sialic acid. Desialylation (i.e. the removal of sialic acid residues) promotes galectin-3 deposition on neuroendocrine cells, which prompts their phagocytosis by microglia via MERTK (Nomura *et al.*, 2017). Moreover, desialylation enhances C1q binding to neurites, resulting in their phagocytosis by microglia (Linnartz *et al.*, 2012), which may be mediated by C1q binding to exposed galactose (Païdassi *et al.*, 2008). Removal of further sugar residues by glycohydrolase enzymes including β-galactosidase and β-hexosaminidase exposes N-acetylglucosamine and mannose residues. Exposed N-acetylglucosamine on aged platelets can directly trigger their phagocytosis through the lectin domain of the phagocytic complement receptor 3 (CR3) (Hoffmeister *et al.*, 2003). Furthermore, both N-acetylglucosamine and mannose can be recognised by mannose-binding lectin (MBL) and ficolins, which can initiate immune responses by activating the complement system or directly interacting with immune cells, including

microglia (Matsushita and Fujita, 2002; Krarup *et al.*, 2004; Saevarsdottir, Vikingsdottir and Valdimarsson, 2004).

Calreticulin can serve as both an eat-me signal and an opsonin, aiding in the recognition and phagocytosis of target cells. As an eat-me signal, surface calreticulin can bind the low density lipoprotein receptor-related protein 1 (LRP1) receptor on phagocytes, which is implicated in the phagocytosis of healthy, stressed or apoptotic cells including leukocytes and neurons (Gardai *et al.*, 2005; Martins *et al.*, 2010; Fricker, Oliva-Martín and Brown, 2012; Garg *et al.*, 2012). Calreticulin may also bind to C1q (Verneret *et al.*, 2014), which can itself activate LRP1 to induce phagocytosis (Duus *et al.*, 2010). Furthermore, calreticulin can be secreted as a soluble protein and act as an opsonin by binding asialoglycans on target cells (Feng *et al.*, 2018). Calreticulin, released by activated microglia, has also been found to bind to bacteria and induce their phagocytosis by microglia via LRP1 (Cockram, Puigdellívol and Brown, 2019). Together, eat-me signals and opsonins play a critical role in initiating and orchestrating the immune response, to ensure the timely removal of potential threats and cellular waste, thus contributing to the overall resilience and proper functioning of the immune system.

As opposed to eat-me signals, "don't-eat-me" signals are surface molecules that suppress the phagocytosis of a target (Figure 1.1). As mentioned previously, sialic acid residues can be classed as SAMPs, but have also been described as don’t eat-me signals (Cockram *et al.*, 2021). The terminal sialic acid residue on sialoglycans can interact with inhibitory SIGLEC receptors (e.g. CD33), on phagocytes, which supress phagocytic uptake (Claude *et al.*, 2013; MacAuley, Crocker and Paulson, 2014). Factor H also binds to sialic acid residues and inhibits the alternative pathway and the amplification loop of the complement system (Ferreira, Pangburn and Cortés, 2010), thereby preventing the potential detrimental effects of uncontrolled complement activation and aberrant targeting of self-components, which could lead to tissue damage and excessive inflammation.

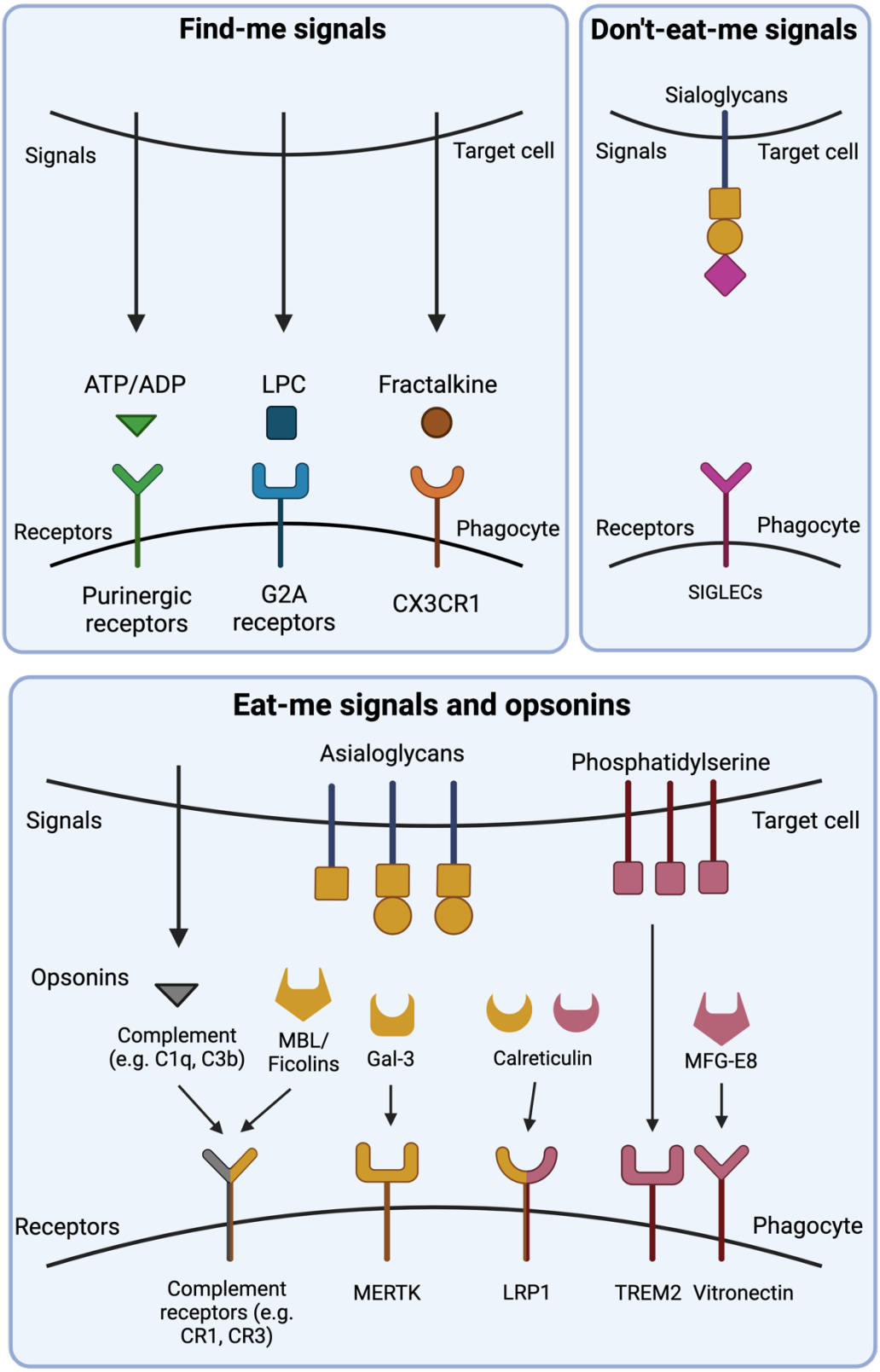

Figure 1.1. Overview of signals that regulate phagocytosis. Phagocytosis is regulated by the recognition of signals by receptors on the phagocytic cell. Find me signals first serve as attractants to induce migration of the phagocyte towards the target. Find-me signals include ATP/ADP, LPC and fractalkine which signal via purinergic receptors, G2A receptors and CX3CR1. Eat-me signals can then mark the target for phagocytosis. Eat-me signals include asialoglycans, i.e., desialylated glycan

chains exposing galactose (yellow circle) or N-acetylglucosamine residues (yellow square)) or phosphatidylserine. Eat-me signals are then recognised by corresponding opsonins and phagocytic receptors. Opsonins are soluble factors that induce phagocytosis of the target by bridging between the target and the phagocytic cell. For example, calreticulin and galectin-3 (Gal-3) bind asialoglycans and signal via LRP1 or MERTK, respectively. MFG-E8 and calreticulin bind to exposed phosphatidylserine on the target and signal via the vitronectin receptor or LRP1 on the phagocyte. Exposed phosphatidylserine can also bind directly to TREM2. When bound to target cells, complement components including MBL, ficolins, etc. are recognised by complement receptors on the phagocytic cell. Finally, healthy cells expose don't-eat-me signals to prevent their uptake by phagocytes. These signals include sialoglycans (containing terminal sialic acid residues (pink diamond)), which are recognised by SIGLECs to suppress phagocytosis. Figure created using BioRender.com.

1.3.4. Complement

The complement system is an integral part of the immune system. When activated, a cascade of enzymatic reactions is initiated that enhance the immune response through a range of functions, including opsonisation of targets, activation of inflammation and cell lysis. However, as mentioned above, some complement components, like factor H, can prevent excessive or unwanted complement activation on host cells.

The complement system can be activated through three main pathways: the classical pathway, the alternative pathway, and the MBL (or lectin) pathway. Each pathway has distinct triggers but converge, upon cleavage of C3. The proteolytic fragments of C3, particularly C3b, aid in stimulation of proinflammatory responses. In the context of the brain, C3b, along with inactivated (i)C3b, binds to neurons, stimulating phagocytosis via microglial CR3 (Stevens *et al.*, 2007). Research has demonstrated the essential role of C3 in synaptic pruning as C3 knockout in mice leads to an abundance of synapses due to decreased microglial pruning activity (Stevens *et al.*, 2007). Moreover, disrupting microglia-specific C3/CR3 signalling in postnatal mice results in aberrant synaptic connectivity (Schafer *et al.*, 2012).

C1q is a multifunctional protein that serves as a vital component of the classical complement system. C1q can opsonise target cells by binding to phosphatidylserine, calreticulin, or galactose residues and can also interact directly with phagocytic receptors like LRP1 on phagocytes (Cockram *et al.*, 2021). In the brain, C1q has been found to bind neurons, resulting in CR3-mediated microglial phagocytosis of neurites (Linnartz *et al.*, 2012). C1q is also known to tag synapses for degradation during development and ablation of C1q results in reduced synaptic pruning and increased synaptic deficits (Stevens *et al.*, 2007; Schafer *et al.*, 2012). In AD, both C1q and C3b have been implicated in microglia-mediated synapse loss and linked with cognitive decline (Hong *et al.*, 2016).

Factor H is a serum glycoprotein that regulates the formation and function of complement C3 convertase enzymes, suppressing the downstream action of the classical and alternative pathways. Regulatory activity is attributed to its ability to recognise and bind C3b fragments, thus inhibiting the formation of the C3 convertase (Zipfel *et al.*, 1999). Factor H also binds to preformed C3 convertases and causes rapid release of the catalytic subunit Bb in a process known as decay acceleration (Hourcade, Mitchell and Medof, 1999; Kopp *et al.*, 2012).

Factor H can discriminate self from non-self by recognising sialylated structures on self-cells and deposited C3b, thereby inhibiting complement activation on host surfaces by suppressing the downstream action of the alternative complement cascade (Kazatchkine, Fearon and Austen, 1979; Carreno *et al.*, 1989). In neural cells, the induction of stress by metal sulphates has been shown to reduce expression of factor H (Pogue *et al.*, 2009). This might affect the inflammatory response observed in neurodegenerative diseases, like AD, due to reduced suppression of complement activation by factor H (Pogue *et al.*, 2009).

MBL is a protein that plays a critical role in the immune system; it can initiate the lectin pathway of the complement system via mannan-binding lectin serine protease (MASP) which promotes the production of C3b and other complement components (Cockram *et al.*, 2021). MBL can also bind to phosphatidylserine (Kilpatrick, 1998) and glycans exposing N-acetylglucosamine, mannose or fucose residues (Lu *et al.*, 2002), which may mediate binding to apoptotic cells, altered host cells and pathogens. It has been suggested that elevated MBL levels following ischemic stroke may contribute to brain damage, due to lectin pathway activation and sustained inflammation. Indeed, MBL deficient mice present with smaller infarctions and better functional outcomes than wild-type mice following acute stroke (Cervera *et al.*, 2010).

Thus, although a critical part of the innate immune response against infection, accumulating evidence supports a role for the complement system in neurodegenerative disorders.

1.3.5. Cytokines and chemokines

Cytokines and chemokines are pivotal mediators of neuroinflammation, orchestrating interactions between microglia, other glial cells, and neurons. These small signalling molecules can modulate both protective and detrimental responses within the CNS, profoundly influencing physiological and pathological processes.

Microglia are key contributors to cytokine and chemokine production. When activated, microglia release cytokines like IL-1β, TNFα and IL-6 (Ramesh, Maclean and Philipp, 2013). These cytokines stimulate inflammation, and amplify the immune response. However, if left unchecked, excessive pro-inflammatory cytokine release can escalate into chronic neuroinflammation, perpetuating tissue damage and neuronal degeneration. Indeed, it is widely documented that expression of pro-inflammatory cytokines and their cognate receptors are upregulated in neurodegenerative diseases (Smith *et al.*, 2012; Wang *et al.*, 2015). IL-6 and TNFα can induce neurotoxicity through a variety of mechanisms, including elevated glutamate production that results in neuronal excitotoxic death (Ye *et al.*, 2013). Moreover, dopaminergic neurons appear to be particularly sensitive to TNFα insult *in vitro* (McGuire *et al.*, 2001) and, IL-1 has been shown to promote neuronal cell death when combined with either IFN-γ or TNFα (Chao *et al.*, 1995; Hu, Peterson and Chao, 1997). Conversely, transforming growth factor (TGF)-β can reduce microglial activation by

suppressing inflammation following injury (Suzumura *et al.*, 1993; Lodge and Sriram, 1996). Furthermore, *Tgfb1*$^{-/-}$ mice, which lack TGF-β1, have increased neuronal cell death and microgliosis, whereas transgenic overproduction of TGF-β1 can prevent degeneration after excitotoxic injury (Brionne *et al.*, 2003). Hence, TGF-β is proposed to suppress neuronal loss by dampening microglial activation.

Upon activation, microglia also release chemokines like CXCL10 and fractalkine. These chemokines can guide immune cell migration to damaged areas, containing threats and aiding their removal. However, excessive chemokine production can promote chronic neuroinflammation and perpetuate damage. Indeed, CXCL10 has been shown to induce apoptosis of neurons by elevating intracellular calcium levels (Sui *et al.*, 2006). By contrast, fractalkine and its receptor CX3CR1 have been shown to be neuroprotective, by reducing the levels of nitric oxide produced by activated microglia during neuroinflammation (Mattison *et al.*, 2013).

Given the central role of cytokines and chemokines in mediating inflammation, they have been the focus of intense investigation to identify effective therapeutics. However, the challenge lies in achieving a balance between suppressing harmful cytokine/chemokine-driven inflammation while not compromising their normal immune functions.

1.3.6. Interplay between microglial phagocytosis and inflammation in neurodegeneration

As outlined above, microglia have diverse functions in phagocytosing targets and mediating inflammation. Activated microglia can elicit neuronal cell death through production of pro-inflammatory cytokines and chemokines, glutamate, and reactive oxygen species (ROS) or by phagocytosing stressed-but-viable neurons (Neher *et al.*, 2011; Fricker, Oliva-Martín and Brown, 2012; Takaki *et al.,* 2012; Ramesh, Maclean and Philipp, 2013). However, microglial-mediated phagocytosis and inflammation are not distinct processes. Indeed, activation of TLRs by internal or external ligands leads to the generation of ROS by microglia through the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase pathway (Chéret *et al.*, 2008). Elevated levels of ROS may then promote exposure of phosphatidylserine, on neurons, promoting their phagocytosis by microglia (Brelstaff *et al.*, 2018; Cockram *et al.*, 2021). TLR activation also upregulates production of microglial opsonins, including galectin-3, which can enhance the phagocytic response (Nomura *et al.*, 2017). Therefore, understanding the intricate interactions between neuroinflammatory and phagocytic processes is important in identifying potential avenues for therapeutic intervention.

1.4. Microglia in ageing and neurodegenerative disease

Ageing results in a progressive loss of normal functions, with cellular-level changes thought to contribute to this phenomenon (López-Otín *et al.*, 2013). Age-related diseases are increasingly prevalent and have become a significant public health concern, necessitating a

deeper understanding of their underlying mechanisms and effective strategies for prevention and management. Despite ongoing research, our understanding of the underlying causes of ageing in the brain remains limited. Neuronal survival and function may be affected by cellular changes that are neuron-specific (Morrison *et al.*, 2002), however, neurons are also highly dependent on microglia, therefore microglial ageing may have a significant impact on neurons and brain ageing as well (Luo, Ding and Chen, 2010).

1.4.1. Microglia in ageing

Microglial cells that display altered morphology or function with age have been described as senescent or dystrophic (Streit *et al.*, 2004), and these terms are often used interchangeably. It is not known if these are separate states; it is more likely that they form part of a spectrum of aged phenotypes, similar to that proposed for activated microglial states mentioned previously. Generally, microglial changes observed in ageing include increased production of secretory factors including pro-inflammatory cytokines and ROS and a decrease in phagocytosis (Ye and Johnson, 1999; Floden and Combs, 2011; Harry, 2013; von Bernhardi, Eugenín-von Bernhardi and Eugenín, 2015). A closer look at the interplay between microglial senescence, ageing, and their effects provides valuable insights into the complexities of brain health during the ageing process.

The accumulation of senescent microglia with age has been demonstrated in rodents, both *in vivo* (Stojiljkovic *et al.*, 2019) and *ex vivo* (Flanary *et al.*, 2007; Stojiljkovic *et al.*, 2019). In ageing rodent brains, an increase in pro-inflammatory cytokines and ROS, and a decline in anti-inflammatory cytokines and antioxidants have been observed (Frank *et al.*, 2006; Sierra *et al.*, 2007). Elevated production of pro-inflammatory TNFα and IL-6 have been reported in aged microglia (Sierra *et al.*, 2007; Njie *et al.*, 2012), while reduced levels of anti-inflammatory IL-4 have been associated with increased inflammation and disrupted synaptic plasticity in aged rodents (Maher, Nolan and Lynch, 2005). Furthermore, increased IL-6 and lipid peroxidation have been shown to correlate with psychomotor dysfunction in 12-month-old mice, with these deficits mitigated by antioxidant-rich diets (Richwine *et al.*, 2005). It has also been reported that increased neuroinflammation associated with age may be attributed to diminished cellular responsiveness to anti-inflammatory cytokines (Fenn *et al.*, 2012). Hence, aged microglia may lose their ability to return to a quiescent state resulting in prolonged inflammation and subsequent cognitive impairment.

Dystrophic or senescent microglia also demonstrate reduced phagocytic capacity and motility, affecting their ability to migrate towards sites of damage and clear insults. For example, primary microglia cultured for 16 days are less capable of migrating towards and phagocytosing amyloid-β oligomers and fibrils than those only cultured for 2 days (Caldeira *et al.*, 2017). Njie *et al.,* (2012) also showed that microglia from aged mice internalise less amyloid-β peptide, demonstrating a reduction in phagocytic capacity with age.

The accumulation of senescent cells in neurodegenerative diseases, including AD and PD (Baker and Petersen, 2018; Hu *et al.*, 2021) suggests a contribution of senescence to the pathophysiology of these disorders. But little is known about the contribution of senescent cells in actively driving neurodegenerative processes. The importance of senescent and aged microglia in neurodegeneration and cognitive decline has been highlighted by research that has assessed the targeted removal of such cells. Indeed, removal of p16-expressing senescent microglia reduced deposition of neurofibrillary tangles and levels of phosphorylated and insoluble tau, preventing tau-dependent cognitive decline in a mouse model of neurodegenerative disease (Bussian *et al.*, 2018). Furthermore, senolytic removal of p16-expressing cells in aged mice resulted in reduced microglial activation and significantly improved cognitive function (Ogrodnik *et al.*, 2021). Interestingly, a targeted approach involving the removal of microglia followed by their repopulation in aged mice resulted in the restoration of microglial physical attributes to those observed in young mice, including cell density and morphology. This intervention also yielded improvements in spatial memory (Elmore *et al.*, 2018). Therefore, targeting senescent or aged microglia may be beneficial in preventing neurodegeneration and further investigation into the microglial senescent phenotype will provide insight into their contribution towards pathology.

1.4.2. Amyloid-β, tau and microglia in Alzheimer's disease

In 2019, an estimated 57 million people were suffering with dementia worldwide. Due to increases in population growth and population ageing, this number is anticipated to reach over 152 million by 2050, posing a substantial burden to global healthcare systems (Nichols *et al.*, 2022). AD is the most common cause of dementia, characterised by progressive cognitive decline, memory loss, and altered behaviour (Knopman *et al.*, 2021). AD is generally split into two categories, late-onset AD (LOAD) is the most common form and typically manifests in individuals aged 65 and older. Unlike early-onset AD (EOAD), which is relatively rare and usually has a strong genetic basis, LOAD is believed to result from a combination of genetic, environmental, and age-related factors (Panegyres and Chen, 2013; Reitz, Rogaeva and Beecham, 2020).

Hallmarks of AD include the deposition of extracellular amyloid plaques and intracellular tau neurofibrillary tangles and the progressive loss of synapses and neurons (Hamos, DeGennaro and Drachman, 1989; Gómez-Isla *et al.*, 1996, 1997; Hardy and Selkoe, 2002; Knopman *et al.*, 2021). Despite concerted efforts to understand the underlying causes of AD, the full complexity of these mechanisms remains elusive.

Amyloid-β is a protein fragment that plays a central and intricate role in the pathogenesis of AD. The amyloid hypothesis was first proposed in the early 1990s (Hardy and Allsop, 1991; Selkoe, 1991; Hardy and Higgins, 1992). It gained prominence as researchers observed the presence of amyloid plaques in the brains of individuals with AD and speculated that these might play a central role in disease development and progression. The hypothesis posits that the accumulation of amyloid-β peptides in the brain is the causative agent in AD; with

amyloid-β aggregation leading to plaque formation and initiating a cascade of events that cause neuroinflammation, neuronal damage, cognitive decline, and ultimately AD. Since its inception the amyloid hypothesis has guided much AD research and drug development.

Amyloid-β is produced naturally in the brain, but its balance is disrupted in neurodegenerative diseases. When amyloid-β production exceeds its clearance, soluble amyloid-β aggregates into oligomers and further assembles into insoluble amyloid fibrils and plaques (Chen *et al.*, 2017).

In the non-amyloidogenic pathway, amyloid precursor protein (APP), from which amyloid-β is derived, is processed through sequential cleavage by enzymes called secretases (Figure 1.2). APP is cleaved by α-secretase, generating soluble APP alpha (sAPPα) and the alpha C-terminal fragment (α-CTF) (Chen *et al.*, 2017). sAPPα is neuroprotective and neurotrophic, promoting the survival of cultured neurons (Mattson *et al.*, 1993; Mucke, Abraham and Masliah, 1996) and, sAPPα levels in CSF correlate with spatial memory abilities in rats and AD patients (Almkvist *et al.*, 1997; Anderson *et al.*, 1999). Hence, the non-amyloidogenic pathway is considered beneficial for neuronal health as APP processing produces neuroprotective sAPPα and avoids the production of amyloidogenic amyloid-β.

Conversely, the amyloidogenic pathway involves cleavage of APP by β-secretase and γ-secretase. β-secretase cleaves APP at its N-terminus, generating sAPPβ and CTF-β (Chen *et al.*, 2017). Subsequently, γ-secretase cleaves CTF-β within its transmembrane domain, releasing amyloid-β peptides of varying lengths, including amyloid-β 1-40 and 1-42 (Wang *et al.*, 1996; Zhao *et al.*, 2007; Schieb *et al.*, 2011) (Figure 1.2). It has been shown that amyloid-β 1-40 may actually confer protective effects by inhibiting amyloid-β deposition *in vivo* (Kim *et al.*, 2007) whereas, amyloid-β 1-42 has a greater propensity to aggregate and is considered particularly neurotoxic and prone to form plaques in AD (Jarrett, Berger and Lansbury, 1993; Iwatsubo *et al.*, 1996). Dysregulation of APP processing, often characterised by an increased ratio of amyloidogenic to non-amyloidogenic cleavage, can result in the accumulation of amyloid-β aggregates. Amyloid-β processing is influenced by several proteins, including presenilin-1 and -2, which are core components of the γ-secretase complex (De Strooper, Iwatsubo and Wolfe, 2012). Mutations in the genes encoding

presenilin-1 and -2 cause EOAD, highlighting the role of amyloid-β processing in disease pathogenesis (Chen *et al.*, 2017).

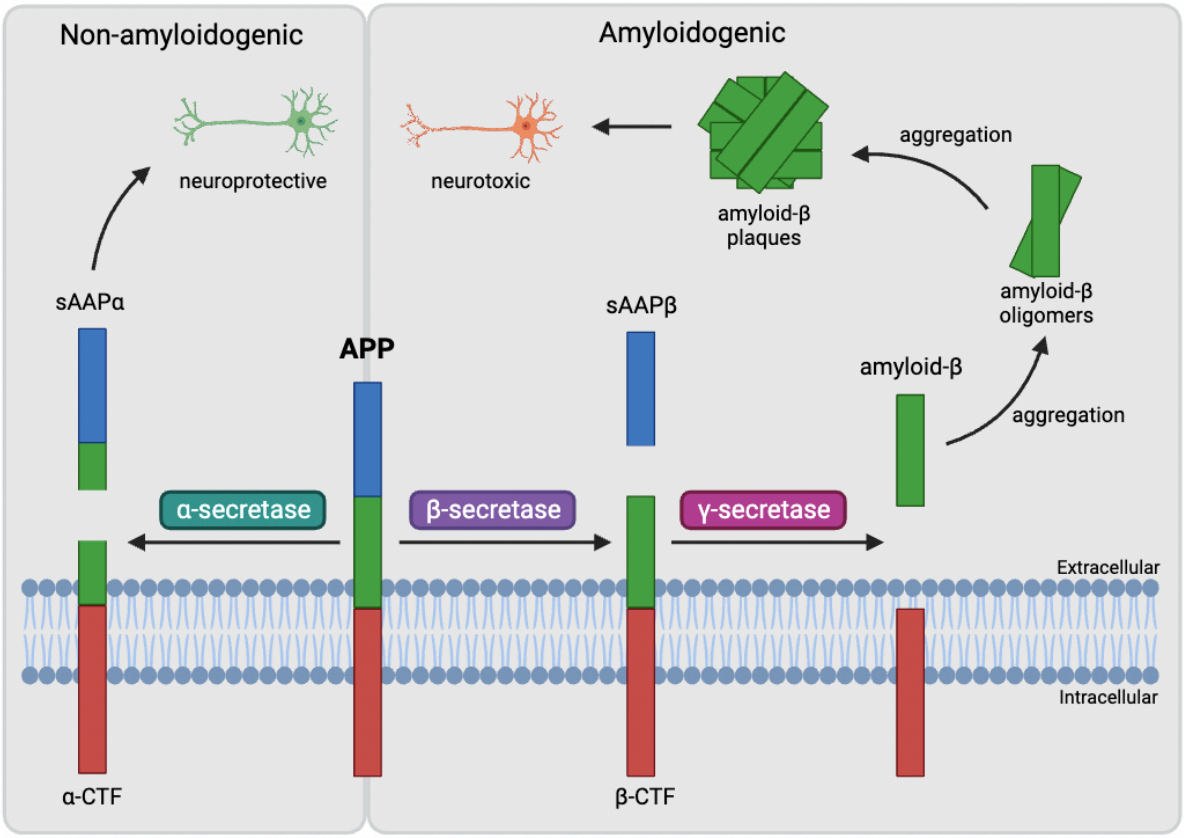

Figure 1.2. Human amyloid precursor protein (APP) non-amyloidogenic and amyloidogenic pathways. The non-amyloidogenic pathway involves APP processing by α-secretase, which cleaves within the amyloid-β domain to generate the membrane tethered α-C terminal fragment (α-CTF) and the neuroprotective N-terminal soluble APP alpha (sAPPα) fragment. The amyloidogenic pathway involves sequential processing of APP by β- and γ-secretases. β-secretase first cleaves APP into membrane tethered β-C terminal fragment (β-CTF) and the N-terminal soluble APP beta (sAPPβ) fragment. β-CTF is then further processed by γ-secretases to produce extracellular amyloid-β peptides, which aggregate to form neurotoxic amyloid-β oligomers and plaques. Figure created in Biorender.com.

Microglia are implicated in clearing amyloid-β to prevent its accumulation and aggregation. Early studies found microglia closely associated with amyloid plaques exhibited a pro-inflammatory phenotype in the brains of AD patients and murine disease models (Perlmutter, Barron and Chui, 1990; Frautschy *et al.*, 1998). Microglial membrane receptors that bind amyloid-β have since been identified and implicated in microglial activation, phagocytosis, and clearance of amyloid-β. These receptors include TLR2 and TLR4 (Tahara *et al.*, 2006).

TLRs can sense amyloid-β aggregates triggering an immune response similar to that seen during an infection. However, in the context of chronic exposure and impaired clearance mechanisms, phagocytosis can be overwhelmed, and chronic activation can lead to

sustained release of pro-inflammatory cytokines and ROS that can cause collateral damage and exacerbate neuronal injury. Indeed, TLR4-mediated microglial activation is implicated in neurotoxicity where TLR4 loss-of-function mutation inhibits microglial activation by amyloid-β and significantly reduces production of IL-6, TNFα and nitric oxide (Walter *et al.*, 2007). Further treatment of primary murine neuronal cells with supernatant from amyloid-β-stimulated microglia showed that TLR4 contributes to amyloid peptide-induced microglial neurotoxicity (Walter *et al.*, 2007).

Another hallmark of AD are intracellular tau neurofibrillary tangles. Tau is a microtubule-stabilising protein found in neurons that is hyperphosphorylated in AD (Brion *et al.*, 1985; Grundke-Iqbal *et al.*, 1986; Naseri *et al.*, 2019). Abnormal phosphorylation of tau leads to its detachment from microtubules, causing it to aggregate into neurofibrillary tangles (Grundke-Iqbal et al., 1986). These tangles disrupt neuronal transport systems, impairing cellular function and ultimately contribute to neuronal death. Tau pathology has been shown to spread between brain regions, and is thought to do this via prion-like propagation (Braak and Braak, 1991; Frost and Diamond, 2010; Mudher *et al.*, 2017; Kaufman *et al.*, 2018).

Microglia have been found in close proximity to neurofibrillary tangles in AD brains and hyperphosphorylated tau can activate microglia (Sheffield, Marquis and Berman, 2000; Bellucci *et al.*, 2004). Hence, microglia are implicated in tau pathology. In fact, activated microglia have been linked to tau propagation; whereby depleting microglia dramatically suppresses tau spreading (Asai *et al.*, 2015). Asai *et al.*, (2015) also found that tau-containing exosomes secreted by activated microglia, are internalised by neurons in culture and *in vivo* and, that this can be prevented by inhibition of microglial activation or exosome synthesis, further supporting the idea that microglia may be involved in the propagation of tau pathology.

Amyloid-β and tau pathologies are not thought to be independent. The amyloid hypothesis suggests that amyloid-β has the potential to induce tau pathology and several studies support this theory. Treating rodent neurons in culture with soluble amyloid-β oligomers causes tau hyperphosphorylation and neurotoxicity, but no dystrophy ensues if tau is knocked down (Jin *et al.*, 2011). Furthermore, amyloid-β has been found to cause tau-dependent microtubule disassembly (King *et al.*, 2006), further supporting the idea that amyloid-β may precede tau pathology, although this is the subject of ongoing debate.

The roles of amyloid-β and tau in neurodegeneration, highlight the significance of these proteins in neuroinflammation and ultimately neuronal loss in AD. While the amyloid hypothesis has spurred research and therapeutic efforts, the complex nature of AD warrants a comprehensive understanding of the intricate mechanisms and receptors involved in order to identify points of therapeutic intervention.

1.4.3. α-Synuclein and microglia in Parkinson's disease

PD is the second most common neurodegenerative disorder, manifesting symptoms relating to motor dysfunction and cognitive decline (Poewe *et al.*, 2017). PD is characterised by the accumulation of intracellular aggregates known as Lewy bodies (LBs) and progressive degeneration of dopaminergic (DA) neurons within the substantia nigra pars compacta. The central component of LBs is α-synuclein which, like amyloid-β in AD, aggregates to form proteinaceous amyloid fibrils and, like tau, has been suggested to spread in a prion-like manner (Masuda-Suzukake *et al.*, 2013).

Microglial roles in PD are not fully understood but microglial activation is proposed to play a significant part of the disease process. Positron emission tomography (PET) has shown that microgliosis occurs early in PD and then remains relatively static, possibly driving the disease via cytokine release (Gerhard et al., 2006). Reactive microglia have also been detected in toxin-induced and transgenic mouse models of PD (Członkowska et al., 1996; Sanchez-Guajardo et al., 2010; Hoenen et al., 2016). Furthermore, induction of microgliosis by LPS, results in loss of DA neurons in the substantia nigra and is often used to model PD *in vitro* and *in vivo* (Dutta, Zhang and Liu, 2008). Thus, microglial activation appears to correlate with PD progression and result in death of DA neurons.

Microglial TLRs have emerged as central players in α-synuclein pathogenesis; α-synuclein activates microglial TLR1/2 across diverse experimental models including BV-2 microglia, primary mouse microglia, and human microglia (Béraud *et al.*, 2013; Kim *et al.*, 2013; Daniele *et al.*, 2015). Media from SH-SY5Y cells overexpressing α-synuclein has been shown to activate microglia in a TLR2-dependent manner (Kim *et al.*, 2013, 2016). Furthermore, TLR1/2 activation by α-synuclein causes microglial release of pro-inflammatory cytokines, TNFα and IL-1β, further supporting a role for microglia in α-synuclein pathology whereby TLR1/2 mediated microglial activation may contribute to PD progression by releasing cytokines that modulate neuroinflammation. TLR4 has been implicated in α-synuclein-mediated microglial activation and phagocytic uptake of α-synuclein (Stefanova *et al.*, 2011; Fellner *et al.*, 2013). In fact, microglial phagocytosis of α-synuclein is impaired in the absence of TLR4 (Stefanova *et al.*, 2011) and, treatment with TLR4 agonist, monophosphoryl lipid A, increases survival of transgenic mice overexpressing α-synuclein (Venezia *et al.*, 2017). Thus, TLR4 appears to promote microglial clearance of α-synuclein, potentially having a beneficial role in controlling α-synuclein spread and PD progression. Hence, the role of microglia in PD is complex. While their ability to phagocytose α-synuclein may be beneficial, their potential to cause inflammation and subsequent neuronal damage can be detrimental. Balancing microglial activation and immune responses is a key challenge in developing effective therapeutic strategies for PD and further investigations are needed to fully map the complex pathways involved in PD with regards to microglial involvement.

1.4.4. Microglia as a target for the modulation of neuroinflammation and phagocytosis

Microglia play a pivotal role in both protective and detrimental immune responses. As such they pose a promising target for the modulation of neuroinflammation and neurodegeneration. Strategies that aim to modulate microglial function could involve altering their activation state, promoting their anti-inflammatory phenotype, or enhancing their clearance of toxic protein aggregates.

One approach for modulating inflammation involves targeting microglial receptors, including TLRs and purinergic receptors. By manipulating these receptors using agonists or antagonists, there is potential to steer microglial responses toward a more balanced inflammatory state. Notably, studies have demonstrated that the blockade of TLR2 and TLR4 effectively curbs the microglial pro-inflammatory response upon exposure to LPS administration (Weber *et al.*, 2013). Additionally, research has shown that TLR4 inhibition using TAK-242 in a transgenic mouse model of AD significantly reduces pro-inflammatory markers like TNFα, and increases M2 phenotypic markers; this TLR4-specific inhibition not only ameliorates the inflammatory profile but resulted in improved neurological function (Cui *et al.*, 2020). Furthermore, inhibition of ATP-gated ion channel and purinergic receptor, P2X7R, using antagonist JNJ-54175446 suppressed cytokine release following LPS stimulation *ex vivo* and has shown possible positive outcomes on mood in phase II clinical trials (Recourt *et al.*, 2020).

The clearance of protein aggregates, a hallmark of many neurodegenerative disorders, is another area of focus. Microglia play a key role in engulfing and clearing toxic protein aggregates like amyloid-β and tau. Strategies that enhance microglial phagocytosis could help alleviate the burden of protein accumulation, potentially slowing down disease progression. Indeed, small-molecule GW5074, an inhibitor of serine/threonine kinase c-RAF, has been shown to significantly increase phagocytic clearance of amyloid-β 1-42 by human microglia-like cells (Connor *et al.*, 2022).

Despite its promise, the idea of microglial modulation faces several challenges. The diversity of microglial functions means that modulating their responses must be carefully balanced to avoid disrupting beneficial functions. The intricate interactions between microglia and other cell types in the CNS further complicate therapeutic strategies. Hence, although microglia represent a compelling target for the modulation of neuroinflammation and CNS disorders, a more comprehensive understanding of their function in health in disease is necessary to harness potential therapeutic applications.

1.5. Extracellular chaperones: Implications for neurodegeneration

The occurrence of protein misfolding and aggregation is closely linked to specific neurotoxic events that underlie several fatal and currently incurable neurodegenerative disorders.

Notable among these are AD, featuring tau and β-amyloid aggregation, and PD, involving accumulation of α-synuclein. The accumulation of misfolded and aggregated proteins imposes a significant burden on cells, necessitating effective management. Protein chaperones play pivotal roles in preventing the aggregation of unfolded polypeptide chains, promoting their folding or refolding, and disassembling protein aggregates (Weibezahn *et al.*, 2005; Hartl, Bracher and Hayer-Hartl, 2011). A mounting body of evidence supports the involvement of chaperone proteins in the pathogenesis of neurodegenerative conditions characterised by aberrant protein aggregation.

In the context of neurodegeneration, protein aggregates often adopt a structured β-sheet-rich arrangement termed amyloid (Chiti and Dobson, 2017). The process of amyloid fibril formation is a multistep pathway involving the conversion of native proteins to aggregation-prone states, subsequent oligomerisation and fibrillisation (Morris, Watzky and Finke, 2009; Knowles, Vendruscolo and Dobson, 2014). Fragmentation and secondary nucleation further amplify the aggregation process by facilitating the formation of additional fibrillar seeds that serve as templates for further rapid amyloid formation (Knowles *et al.*, 2009; Cohen *et al.*, 2013). While amyloid structures are energetically favourable, they coexist in equilibrium with monomeric and oligomeric species (Carulla *et al.*, 2005; Baldwin *et al.*, 2011).

As aberrant protein accumulation occurs, the capacity of chaperones and proteostasis elements to cope with non-native proteins may become overwhelmed. This decline in proteostasis mechanisms is evident in the ageing intracellular context and is presumed to extend to extracellular processes (Gidalevitz, Kikis and Morimoto, 2010), potentially contributing to age-related onset of amyloidosis and related disorders. Despite the significant implication of extracellular protein misfolding and aggregation in various diseases, our understanding of the proteostasis mechanisms in the extracellular environment remains incomplete. Exposed hydrophobic regions on misfolded proteins drive the formation of toxic aggregates and are linked to the aberrant inflammation often observed alongside protein deposition *in vivo* (Seong and Matzinger, 2004; Salminen *et al.*, 2009; Bolognesi *et al.*, 2010). Hence, the identification of extracellular chaperones that preferentially interact with misfolded proteins and reduce their toxicity, is important. Understanding the interplay between these molecules in maintaining proteostasis could offer insights into the origins of amyloidoses and inform strategies for their treatment. Notably, secreted glycoproteins like clusterin and α2-macroglobulin have been identified as potential modulators of protein aggregation (Humphreys *et al.*, 1999; French, Yerbury and Wilson, 2008); these extracellular chaperones exert influence on amyloid formation *in vitro* and exhibit co-localisation with amyloid deposits *in vivo* (Powers *et al.*, 1981; Choi-Miura *et al.*, 1992; Strauss *et al.*, 1992; Van Gool *et al.*, 1993; Kida, Choi-Miura and Wisniewski, 1995; Yerbury *et al.*, 2007, Yerbury *et* al., 2009).

Clusterin is a glycosylated heterodimeric protein that is found ubiquitously in extracellular fluids and can bind to a diverse set of peptides and proteins (Wyatt *et al.*, 2012). Notably, it becomes upregulated in response to injury and in AD (May *et al.*, 1990; Troakes *et al.*, 2017;

Foster *et al.*, 2019). Clusterin has been reported to function in an ATP-independent manner and bind to hydrophobic regions of amyloid-β *in vitro*; this is thought to suppress extracellular aggregation of amyloid-β and reduce its toxicity (Humphreys *et al.*, 1999; Poon *et al.*, 2000; Kumita *et al.*, 2007; Yerbury *et al.*, 2007; Wyatt, Yerbury and Wilson, 2009; Narayan *et al.*, 2012). In transgenic mice, the collaborative action of apolipoprotein E and clusterin is vital for synergistically inhibiting the deposition of fibrillar amyloid-β *in vivo*, as double knockout of apolipoprotein E and clusterin results in a marked increase in levels of amyloid-β, amyloid deposition and early disease onset (DeMattos *et al.*, 2004). Despite the elevated presence of clusterin in the brains of AD patients and the association of clusterin variants with AD in multiple GWAS, highlighting the importance of clusterin in AD (Harold *et al.*, 2009; Lambert *et al.*, 2009), the exact role of clusterin in the progression of AD warrants further exploration. α2-Macroglobulin has also been shown to inhibit protein aggregation *in vitro* (French, Yerbury and Wilson, 2008; Yerbury *et al.*, 2009). The chaperone function of α2-macroglobulin is of particular interest as it is found to be substantially more concentrated in human CSF from AD patients than clusterin (Garton *et al.*, 1991; Wang *et al.*, 2020). Further to its ability to prevent aggregation, α2-macroglobulin retains its ability to sequester proteases, promoting clearance of amyloid-β *in vitro* (Wyatt, Const, *et al.*, 2013). More recently, neuroserpin has been identified as a potential extracellular chaperone for amyloid-β. It is a secreted, monomeric protein, expressed throughout the nervous system and secreted into CSF (Miranda and Lomas, 2006; Nielsen *et al.*, 2007). Furthermore, neuroserpin colocalises with amyloid-β plaques *in vivo,* and can alter amyloid-β aggregation *in vitro* thereby protecting cultured neuronal cells from the amyloid-β-induced toxicity (Kinghorn *et al.*, 2006; West *et al.*, 2021).

Taken together, various chaperones found extracellularly, and in association with amyloid-β *in vivo,* influence amyloid-β aggregation *in vitro*. As AD is associated with an imbalance between amyloid-β production and clearance, and subsequent amyloid deposition and inflammation, future therapeutic approaches for AD may exploit proteostasis machinery, especially extracellular chaperones, to reduce amyloid load and neuroinflammation.

1.5.1. Calreticulin: Functions beyond the endoplasmic-reticulum

Calreticulin is a highly conserved and ubiquitously expressed chaperone that has vital roles within the endoplasmic reticulum (ER) (Coppolino and Dedhar, 1998). Calreticulin consists of three distinct domains (Figure 1.3); 1) the N-terminal domain is highly conserved between species and plays a role in chaperone activity; 2) the P-domain is proline rich and acts as a structural backbone that also takes part in chaperone activity, and; 3) the C-terminal domain is responsible for calcium buffering activity and terminates with a KDEL endoplasmic retrieval sequence (Sönnichsen *et al.*, 1994; Krause and Michalak, 1997; Nakamura *et al.*, 2001). Its primary function within the ER is in the calreticulin/calnexin cycle, where it interacts with

calnexin and ER protein of 57-kDa (ERp57) to ensure the proper folding and glycosylation of nascent glycoproteins before they are secreted (Hammond, Braakman and Helenius, 1994; Michalak et al., 2009). Another crucial role of calreticulin, from which it derives its name, is to maintain calcium homeostasis. Calreticulin serves as a calcium-binding protein, actively participating in intracellular calcium regulation; it sequesters calcium ions in the ER, ensuring proper protein folding and stability and, the binding of calcium to calreticulin acts as a signal that communicates ER stress and modulates downstream signalling pathways (Roderick *et al.*, 1998; Wang, Groenendyk and Michalak, 2012; Venkatesan, Satin and Raghavan, 2021).

Aside from its ER-resident functions, growing evidence supports numerous biological functions for calreticulin on both the cell surface and extracellularly. Among some of these functions, calreticulin is implicated in the uptake of cancer cells by dendritic cells (Obeid *et al.*, 2007) and phagocytosis of apoptotic cells (Gardai *et al.*, 2005), as well as cell adhesion, migration and cellular proliferation (Coppolino *et al.*, 1997; Gold *et al.*, 2010; Huang *et al.*, 2016). Cell surface calreticulin also serves as a cell surface receptor for C1q (Ogden *et al.*, 2001; Duus *et al.*, 2010; Verneret *et al.*, 2014). For apoptotic cell removal, the most widely studied surface signal is phosphatidylserine, however calreticulin may also play a critical role, acting as an “eat-me” signal by binding to and activating LRP1 on macrophages (Gardai *et al.*, 2005). The addition of calreticulin, or increasing cell surface expression of calreticulin, promotes immunogenic cell death of cancer cells through phagocytosis by dendritic cells (Chaput *et al.*, 2007; Obeid *et al.*, 2007). Furthermore, anthracycline-induced immunogenic cell death is prevented when cell surface expression of calreticulin is inhibited (Obeid *et al.*, 2007). Hence, calreticulin is able to signal to phagocytic cells to induce uptake of targets for removal.

The mechanisms by which calreticulin actively translocates to the cell surface and is released remain elusive. It is known that calreticulin can be released from damaged or necrotic cells (Basu *et al.*, 2000; Reid *et al.*, 2022). Yet how calreticulin may be actively released from cells is less well understood and is the subject of continued investigation. Several theories have been proposed (Gold *et al.*, 2010) (Figure 1.3). As calreticulin has a C-terminal KDEL sequence, which ensures the protein is retained in the ER, one theory is that the KDEL sequence is removed or masked under certain conditions, possibly due to interactions with other proteins or altered pH and/or calcium levels. Indeed a number of truncated forms, lacking the KDEL sequence have been identified that may enable calreticulin to exit the ER (Højrup, Roepstorff and Houen, 2001; Klampfl *et al.*, 2013). Another theory proposes that calreticulin retro-translocates to the cytoplasm following ER stress and then exits the cell via exosomes, similar to the secretion of heat shock protein (Hsp) 90α from keratinocytes (Afshar, Black and Paschal, 2005; Cheng *et al.*, 2008; Tucher *et al.*, 2018). A third, suggests that interaction with phosphatidylserine, which is externalised on apoptotic cells by a membrane flip-flop mechanism, might facilitate transport of calreticulin from inside the cell to the outer membrane (Tarr *et al.*, 2010). In addition, calreticulin has been found in the Golgi apparatus and might get to the cell surface and released by remaining bound to secretory

proteins, or proteins that it chaperones, like α-integrins or the major histocompatibility complex I (MHC I) (Howe *et al.*, 2009; Michalak *et al.*, 2009; Liu *et al.*, 2016).

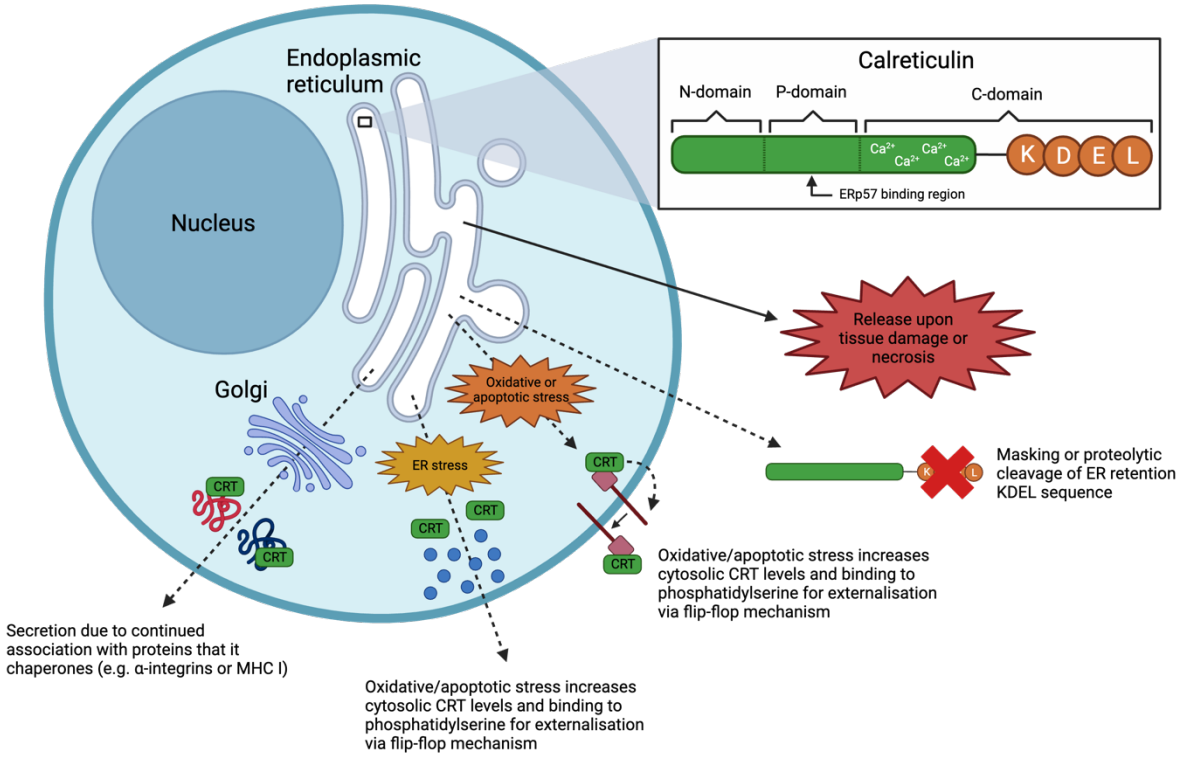

Figure 1.3. Schematic representation of human calreticulin and proposed modes of release to the cytosol, cell surface and extracellular space. Calreticulin (CRT) structure is depicted in the magnified box. Calreticulin has three domains: the N-domain and P-domain are associated with its chaperone functions and, the P-domain harbours the binding region for ERp57; the C-domain, which includes the ER retention sequence (KDEL), is linked to its role in calcium homeostasis. Potential exit routes for calreticulin from the ER to the cytoplasm, cell surface, and extracellular space are depicted. The solid arrow represents known routes for CRT release, through non-specific release following tissue damage or necrosis. Dashed arrows depict potential routes for calreticulin exit from the ER suggested in the literature. Figure created in Biorender.com.

In the brain, C1q has been implicated in generating ROS that induce neuronal cell death in AD (Yasojima *et al.*, 1999; Matsuoka *et al.*, 2001). Further investigations report that calreticulin serves as a trigger for oxidative stress in C1q-mediated ROS generation and so, calreticulin has been associated with exacerbating AD pathology (Stuart *et al.*, 1996; Luo *et al.*, 2003). However, in contradictory reports, a potential beneficial role of calreticulin in AD is described. Calcium imbalance often coincides with AD pathology, where detrimental effects such as cytoskeleton damage, mitochondrial dysfunction, and activation of calcium-dependent degradation enzymes in neurons have been observed (Camacho and Lechleiter, 1995; Liu *et al.*, 1998; Marambaud, Dreses-Werringloer and Vingtdeux, 2009). As calreticulin levels are negatively associated with AD, this might explain the calcium imbalance seen in AD pathology and so elevating calreticulin levels might promote calcium homeostasis (Taguchi *et al.*, 2000; Lin, Cao and Gao, 2014).

Aside from its calcium-buffering properties, calreticulin also plays a role in APP processing (Johnson *et al.*, 2001). Abnormal calreticulin levels and inhibiting calreticulin binding to APP, increases APP interactions with γ-secretase components, which is known to increase aberrant proteolysis subsequently contributing to the amyloidogenic amyloid-β production associated with AD (Stemmer *et al.*, 2013). Calreticulin and ERp57 were found to bind to a substantial portion of soluble amyloid-β in the brain, suggesting that these proteins maintain amyloid-β in a soluble state within the CSF (Erickson *et al.*, 2005). Erickson *et al.,* (2005) also suggest that the failure of post-translational APP processing by calreticulin, along with the absence of amyloid-β-calreticulin complex formation in AD patients, correlates with the worsening of AD. An intriguing idea is that calreticulin, being soluble, can be secreted into the extracellular space. Notably, inflamed macrophages have been found to release calreticulin into the extracellular milieu, where it aids in the recognition and engulfment of target cells by phagocytes (Feng *et al.*, 2018; Cockram, Puigdellívol and Brown, 2019). As calreticulin is released extracellularly, it could potentially function as an extracellular chaperone, like clusterin or neuroserpin, to keep amyloid-β in solution or facilitate its degradation. This has the potential to impact amyloid-β aggregation within the context of AD pathology.

In summary, calreticulin's multifaceted functions encompass its roles in protein folding, calcium homeostasis, immune regulation, and inflammatory modulation. Recent discoveries have expanded its impact beyond the ER, onto the cell surface and into the extracellular space, shedding light on its potential as a therapeutic target in various contexts.

1.6. Glycosylation in the brain

Glycosylation involves the covalent attachment of glycans, which are diverse sugar chains, to proteins, lipids and other molecules (Iqbal *et al.*, 2018). The glycosylation of proteins (which can be N- or O-linked) and lipids is intricately linked to critical functions in the CNS, including brain development, cell adhesion, signal transduction, and cellular differentiation (Ohtsubo and Marth, 2006). Whereas aberrant glycosylation is associated with a range of neurodegenerative disorders (Ohtsubo and Marth, 2006; Iqbal *et al.*, 2018).

Glycoproteins are abundant and play crucial roles in various brain functions. Examples of glycoproteins in the brain include, but are not limited to: 1) neural cell adhesion molecule (NCAM), which is involved in cell adhesion, axon guidance, and neural development, with a role in forming and maintaining synaptic connections in the brain (Weledji and Assob, 2014); 2) neurotransmitter receptors including the N-methyl-D-aspartate receptors which are essential for neuronal signalling and synaptic transmission (Hanson *et al.*, 2023), and; 3) cell surface receptors, including TREM2 and MERTK, which, as mentioned previously, play crucial roles in signal transduction and cellular responses (Takahashi, Rochford and Neumann, 2005; Nomura *et al.*, 2017). In AD, dysregulated N-glycosylation leads to altered

pathways in human AD brains, contributing to synaptic dysfunction, lysosomal dysfunction, neuroinflammation, disrupted cell adhesion, and aberrant cell signalling (Zhang *et al.*, 2020).

Additionally, glycosylation of lipids is important in the CNS. Glycolipids are integral structural components of cell membranes. They are abundant in neurons and glial cells and significantly influence their functions, whereas dysregulated glycolipids resulting from altered glycosylation can underlie several neurodegenerative diseases, including PD and AD (Derry and Wolfe, 1967; Sipione *et al.*, 2020). While glycan-based therapy for neurological diseases remains relatively uncharted territory, ongoing developments in glycobiology and understanding the processes that mediate cellular glycosylation could uncover more effective therapeutic avenues for the future.

1.6.1. Gangliosides synthesis, structure, and functions

Gangliosides are sialic-acid containing glycosphingolipids, first isolated from the human brain by Ernst Klenk in the 1930s (Klenk, 1935). Since their discovery, gangliosides have garnered widespread attention due to their abundance in the nervous system and their various roles in signalling and regulatory mechanisms. Localised primarily to the external side of plasma membrane, gangliosides are glycosphingolipids comprised of a hydrophobic ceramide tail which inserts into the lipid bilayer and a hydrophilic oligosaccharide headgroup that extends into the extracellular space (Yu *et al.*, 2011; Breiden and Sandhoff, 2018). Gangliosides are present in most cell types, but are especially abundant in neurons; they comprise up to 10 – 12 % of the total lipid content in the CNS and are especially enriched in presynaptic membranes (Ledeen and Yu, 1982; Ledeen *et al.*, 1993; D'Azzo, Tessitore and Sano, 2006).

Ganglioside metabolism is carried out by various enzymes, which can add or remove sugar moieties including sialic acid and galactose to form the various ganglioside derivatives (Figure 1.4). Ganglioside biosynthesis begins in the ER where the ceramide portion is generated then transported to the Golgi where sugar residues are added sequentially by a set of membrane-associated glycosyltransferase enzymes. The gangliosides are then transported to the plasma membrane via vesicular exocytotic membrane flow and inserted in the outer leaflet of the plasma membrane (Breiden and Sandhoff, 2018). Conversely, gangliosides are degraded and recycled through the action of several lysosomal glycohydrolase enzymes including neuraminidase 1 (Neu1) and β-galactosidase (Kolter and Sandhoff, 2006).

A-series gangliosides are comprised of GM1 and GD1a, the most abundant ganglioside species in the mammalian CNS, as well as the less abundant derivatives GM2, and GM3 (Tettamanti *et al.*, 1973; Sipione *et al.*, 2020) (Figure 1.4). Every ganglioside species is thought to exhibit important functions within the cell and a homeostatic distribution of gangliosides is maintained within healthy organisms (Yamamoto *et al.*, 1996; Yu, Nakatani and Yanagisawa, 2009; Sekigawa *et al.*, 2011). Conversely, abnormal accumulation or

changes in ganglioside distribution are associated with various diseases including several lysosomal storage disorders (collectively known as gangliosidoses) (Maertens and Dyken, 2007), as well as AD and PD (Kracun *et al.*, 1992; Molander-Melin *et al.*, 2005; Barrier *et al.*, 2007; Caughlin *et al.*, 2018; Seyfried *et al.*, 2018; Huebecker *et al.*, 2019).

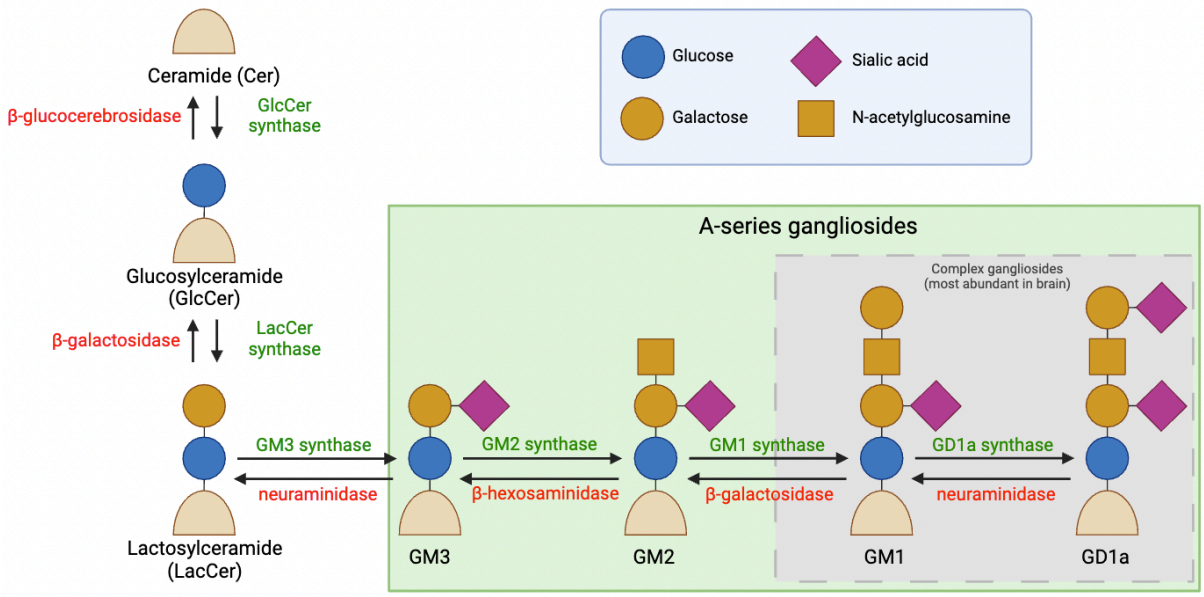

Figure 1.4. Scheme of ganglioside metabolic pathways. Simplified scheme of the metabolic pathways of a-series gangliosides. Biosynthetic glycotransferase enzymes are shown in green. Conversely, catabolic glycohydrolase enzymes are indicated in red. The most abundant brain ganglioside species indicated in dashed grey box. Figure created in Biorender.com.

GM1 is a major component of membrane signalling domains and is the most well studied of the gangliosides. It plays an important role in a variety of processes and has been found to be neuroprotective or disease modifying in PD (Schneider *et al.*, 1992, 2013; Chowdhury and Ledeen, 2022; Galleguillos *et al.*, 2022). GD1a, is almost structurally identical to GM1, except for an additional terminal sialic acid residue which is removed by neuraminidase 3 (Neu3), a sialidase enzyme found in proximity to GD1a in cell membranes (Miyagi *et al.*, 1999; Monti *et al.*, 2000; Wang *et al.*, 2009; Ledeen and Wu, 2018). Hence, one of the primary functions of GD1a is thought to be as a reserve pool for GM1. Understanding of GM2 functions in the adult mammalian brain remains limited, but abnormal accumulation of GM2 in neurons, leads to a wide range of neurological disorders (Demir *et al.*, 2020; Toro, Zainab and Tifft, 2021). Additionally, research has shown increased GM2 expression in mouse brain following traumatic brain injury or stroke (Whitehead *et al.*, 2011; Woods *et al.*, 2013), highlighting the importance of ganglioside homeostasis in the brain. Alterations in ganglioside composition have also been observed with age, specifically a decrease in GD1a levels and an increase in GM2 and GM3 levels (Svennerholm *et al.*, 1994). Moreover, accumulation of GM2 has been observed in AD (Kracun *et al.*, 1992), suggesting an imbalance in ganglioside homeostasis with age may be relevant to age-related neurodegeneration.

The diversity of ganglioside structures suggests they have distinct regulatory functions, but a comprehensive understanding of the underlying molecular mechanisms remains incomplete. Gangliosides are implicated in various biological processes, including cellular recognition, adhesion, signal transduction, growth regulation, and differentiation (Blackburn, Swank-Hill and Schnaar, 1986; Suzuki *et al.*, 1986; Saqr *et al.*, 1995; Stojiljković *et al.*, 1996; Lang *et al.*, 2001; Ledeen and Wu, 2002; Kwak *et al.*, 2006; Lopez and Schnaar, 2009). Additionally, under stress conditions, they are known to initiate apoptosis (Tessitore *et al.*, 2004; Sano *et al.*, 2009). Central to many of these processes is the idea that gangliosides can mediate calcium homeostasis and signalling. Complex gangliosides, like GM1, carry a negative charge, due to their sialic acid content, and are reported to regulate surface ion-channel functions through interactions with proximal cations, including Ca^{2+} (Tanaka *et al.*, 1997). Neu3-mediated conversion of GD1a to GM1 on neuronal membranes has been shown to trigger Ca^{2+} influx via T-type calcium channels and induce neuritogenesis (Wu and Ledeen, 1994). Notably, mice lacking complex gangliosides due to knock-out of specific glycotransferase enzymes exhibit impaired Ca^{2+} regulation (Wu *et al.*, 2001). Furthermore, in a murine model of GM2 gangliosidosis, the aberrant accumulation of GM2 leads to reduced calcium translocation from the cytosol to the sarcoplasmic reticulum, ER stress, neurite atrophy, and apoptosis (Pelled *et al.*, 2003; Virgolini *et al.*, 2019).

1.6.2. Glycohydrolase enzymes in the lysosome: the lysosomal multienzyme complex

As mentioned above, ganglioside degradation is generally performed in the lysosome by various glycohydrolase enzymes. It is known that two of these enzymes, neuraminidase 1 (Neu1) and β-galactosidase are found in a complex, called the lysosomal multienzyme complex, with protective protein cathepsin A (PPCA) (D'Azzo *et al.*, 1982; Verheijen, Brossmer and Galjaard, 1982; Verheijen *et al.*, 1985). Subsequent research recognised PPCA as the scaffold protein, without which the complex is not formed (Galjart *et al.*, 1988; Bonten *et al.*, 1995). Furthermore, PPCA has been found to protect both β-galactosidase and neuraminidase from intra-lysosomal proteolysis (Verheijen *et al.*, 1985). Neu1 and β-galactosidase are the first two enzymes in the sequential removal of sialic acid and galactose from the terminal, non-reducing end of gangliosides and other glycoconjugates. Further catabolism requires other glycohydrolase enzymes including β-hexosaminidase and β-glucocerebrosidase. However, there is no experimental evidence associating these enzymes with the lysosomal multienzyme complex.

The catalytic activation of Neu1 is dependent on its oligomerisation, a process that is mediated by PPCA (Bonten and d'Azzo, 2000; Bonten *et al.*, 2009), and it has been found that Neu1 is only active when associated with PPCA and β-galactosidase (Van Der Spoel, Bonten and D'Azzo, 1998, 2000). Following biosynthesis in the ER, PPCA binds to Neu1 to form a heterodimeric complex that prevents the premature oligomerisation and activation of Neu1; this heterodimer is then transported to the lysosome via the mannose-6-phospate receptor pathway (Bonten *et al.*, 2009). Similarly, β-galactosidase is directed to the

lysosomes in complex with PPCA (Van Der Spoel, Bonten and D'Azzo, 2000). Once in lysosomes, PPCA promotes the catalytic activation of Neu1 and the proper assembly of the multienzyme complex (Bonten, Annunziata and D'Azzo, 2014).

1.6.3. Neuraminidase 1 at the plasma membrane

The initial understanding that glycotransferase and glycohydrolase enzymes are exclusively located in the Golgi and lysosomes, respectively, has been challenged recently, with gangliosides reportedly remodelled by glycotransferases (Vilcaes *et al.*, 2020) and glycohydrolases (Saito and Yu, 1992; Rodriguez *et al.*, 2001) at the plasma membrane. However, the processes and factors governing the relocation of ganglioside-modifying enzymes to the membrane, as well as the resulting implications of this redistribution, remain to be fully elucidated.

Neu1 has been found associated with the plasma membrane as the result of lysosomal fusion mechanisms linked to plasma membrane repair processes (Reddy, Caler and Andrews, 2001) and lysosomal exocytosis due to an excess of intracellular lysosomes (Samarani *et al.*, 2018). Our laboratory recently reported that when microglia are activated, Neu1 translocates to the cell surface and is released into the extracellular space, possibly by lysosomal exocytosis (Allendorf and Brown, 2022). Moreover, the introduction of extracellular neuraminidase or the over-expression of Neu1 resulted in increased microglial phagocytic activity and, the activation of microglia led to the desialylation of microglial phagocytic receptors, TREM2 and MERTK, and enhanced TREM2 binding to galectin-3 (Allendorf, Puigdellívol and Brown, 2020; Allendorf and Brown, 2022). Notably, the culture media derived from activated microglia contained Neu1, and when applied to neurons, induced desialylation while increasing neuronal susceptibility to low levels of glutamate, subsequently intensifying glutamate-induced neuronal cell death (Allendorf and Brown, 2022). Together, these findings suggest that activated microglia have the capacity to release active Neu1, potentially through lysosomal exocytosis, which can augment microglial phagocytosis and sensitise neurons to glutamate. In this study, Allendorf and Brown (2022) suggest that the translocation of Neu1 from the lysosome to the plasma membrane and extracellular space is detrimental to neuronal health, highlighting a consequence of glycohydrolase localisation at the plasma membrane in inflammatory conditions.

1.6.4. β-Galactosidase in the lysosome and at the plasma membrane

Human β-galactosidase is encoded by the *GLB1* gene which is localised to chromosome 3p21.33 and encodes a 677 amino-acid protein (Oshima *et al.*, 1988; Yamamoto *et al.*, 1990). The β-galactosidase protein is synthesised as an 85 kDa precursor, then transported to lysosomes where it is processed to the 64 kDa mature enzyme (Hoogeveen, Verheijen and Galjaard, 1983; Hoogeveen *et al.*, 1984; Zhang *et al.*, 1994; Van Der Spoel, Bonten and D'Azzo, 2000). More than 130 mutations have been identified in the *GLB1* gene, which are associated with a group of rare genetic disorders, with the most well-known and severe form

being GM1 gangliosidosis (Brunetti-Pierri and Scaglia, 2008; Hofer *et al.*, 2009, 2010). In the lysosome, β-galactosidase catabolises glycoproteins, sphingolipids (including ganglioside GM1), and keratan sulphate (Alpers, 1969; Asp and Dahlqvist, 1972; Distler and Jourdian, 1973). In cases where β-galactosidase function is compromised, the degradation of glycoproteins and glycolipids becomes disrupted; this disruption leads to the accumulation of undegraded compounds within lysosomes, which subsequently triggers cellular dysfunction and tissue damage (Ferreira and Gahl, 2017). GM1 gangliosidosis is linked to impairment of the CNS, manifesting symptoms like developmental delay and early onset neurodegeneration, and can be fatal (Sandhoff and Sandhoff, 2018).

Outside of the lysosome, β-galactosidase activity has been found associated with the cell surface in rat cerebellar granule cells during neuronal differentiation and ageing (Aureli, Loberto, Chigorno, *et al.*, 2011) and, increased plasma membrane-associated β-galactosidase activity was also reported during neural stem cell differentiation (Aureli *et al.*, 2012). Moreover, increased β-galactosidase is associated with neuronal membranes in AD (Magini *et al.*, 2015) and, β-galactosidase is increased in the CSF of PD patients, indicating an increase in extracellular β-galactosidase in the brain (van Dijk *et al.*, 2013). The nervous system is especially vulnerable to disturbances in the balance of glycosphingolipid metabolic pathways due to its rich concentration of complex lipids, including gangliosides. An increase in glycohydrolase enzymes such as β-galactosidase could potentially disrupt glycosylation homeostasis, leading to dysfunction in neuronal processes. Hence, understanding the role of increased plasma membrane-associated and extracellular β-galactosidase activity in neurodegeneration is the focus of continued investigation.

1.6.5. β-Galactosidase and senescence

β-Galactosidase activity is the most extensively used biomarker for the detection of cellular senescence. Referred to as senescence-associated (SA)-β-galactosidase, this β-galactosidase becomes detectable at pH 6.0 using detection reagent, X-gal, and occurs as a result of the overexpression of lysosomal β-galactosidase in senescent cells (Dimri *et al.*, 1995; Lee *et al.*, 2006). Briefly, senescence occurs when cells adopt a state of long-term cell cycle arrest; proliferation ceases yet cells remain metabolically active (Narita *et al.*, 2003). Cellular senescence is controlled by one of two tumour suppressor pathways, p53 and pRb (Campisi, 2005). Upon DNA damage, a p53-dependent DNA damage response is triggered (Itahana, Dimri and Campisi, 2001), and transcription of p53-dependent genes (such as p21) induces cell cycle arrest. Inactivation or loss of p53 function can reverse or delay senescence-like growth arrest (Itahana, Dimri and Campisi, 2001). Alternatively, in response to oncogene expression or other cellular stress, the phosphorylated retinoblastoma protein (pRb) pathway is activated (Lowe and Sherr, 2003). Expression of cell cycle inhibitor p16INK4a (hereafter referred to as p16) is induced, and p16 activates pRb which promotes

repressive chromatin reorganisation. Many repressed genes are targets of E2F transcription factors, encoding positive cell cycle regulators, which ultimately prevents cell cycle progression (Lowe and Sherr, 2003). Consequently, senescent cells may be characterised by elevated levels of p16, p53, and p21. Other hallmarks of senescence include, but are not limited to: altered morphology (Noren Hooten and Evans, 2017) and the acquisition of a senescence-associated secretory phenotype (SASP) - a secretory profile of pro-inflammatory cytokines and signalling molecules (eg. IL-1β, IL-6 and IL-8) (Coppé *et al.*, 2008). Although senescence represents a crucial anticancer mechanism, senescent cells may contribute to ageing and certain age-related diseases. Indeed, senescent cells accumulate with ageing and have been found in AD and PD (Baker and Petersen, 2018; Hu *et al.*, 2021; Sikora *et al.*, 2021). However, a link between increased β-galactosidase activity, senescence and neurodegeneration has not been elucidated.

1.6.6. Microglial functions affected by gangliosides and implicated in neurodegeneration

A number of studies indicate that gangliosides activate microglia, which could have serious implications for chronic neuroinflammation and subsequent neurodegeneration. However, the signalling mechanisms involved in ganglioside-mediated microglial activation are yet to be fully understood. It has been reported that gangliosides activate microglia via protein kinase C and NADPH oxidase, which regulate activation of NF-κB and subsequent production of TNFα, IL-1β and inducible nitric oxide synthase (iNOS) in rat brain microglia and BV-2 murine microglia (Min et al., 2004). Furthermore, gangliosides have been shown to activate the complement system *in vitro* (Oshima, Soma and Mizuno, 1993), which is intricately linked to microglial functions as outlined above. Contradictory reports have shown that ganglioside deficiency can cause inflammation and neurodegeneration via the activation of complement system in the spinal cord (Ohmi *et al.*, 2014). The disparity in these results may be attributed to the specific gangliosides used in each study and the potentially divergent roles of complex versus simple gangliosides (Sipione *et al.*, 2020). The dysregulated shedding of gangliosides from neurons may also be responsible for glial activation and it has been reported that TLR4 may mediate ganglioside-triggered inflammation in glia (Jou *et al.*, 2006). Ganglioside GM1 has been shown to be anti-inflammatory in microglia and neuroprotective, both in culture and *in vivo* (van Dijk *et al.*, 2013; Galleguillos *et al.*, 2022). A decrease in ganglioside levels as well as changes in the relative abundance of specific gangliosides has been observed in ageing (Kracun *et al.*, 1991; Svennerholm *et al.*, 1994) and in common neurodegenerative conditions, including PD (Wu *et al.*, 2012; Schneider, 2018) and AD (Davidsson *et al.*, 1991; Kracun *et al.*, 1992). Together, this evidence suggests that changes in brain ganglioside composition might impact microglial functions, contributing to the aberrant activation and subsequent chronic neuroinflammation associated with neurodegeneration. With regard to PD, GM1 levels are deficient across various tissues. Furthermore, extracellular α-synuclein is reported to be internalised by microglia through a GM1-mediated mechanism,

which could have implications for the removal and/or spreading of α-synuclein associated with PD (Park *et al.*, 2009). As mentioned previously, GM1 has been shown to be beneficial in the context of PD and to have disease modifying effects (Schneider *et al.*, 1992, 2013; Chowdhury and Ledeen, 2022; Galleguillos *et al.*, 2022). Recent reports have suggested that reduced GM1 in the CNS may result from an increase in its degradation, rather than a decrease in biosynthesis. It is reported that abnormal GM1 levels coincide with decreased glucosylceramide (GlcCer) levels in PD (Niimi *et al.*, 2021). Furthermore, increased β-galactosidase activity has been found in blood serum from PD patients compared to normal controls; as β-galactosidase is central to the degradation of GM1, it could be that upregulation of β-galactosidase explains the decrease in GM1 seen in PD patients (Niimi *et al.*, 2019).

2 RESEARCH AIMS

In this work, I aimed to establish the effects of calreticulin and β-galactosidase on microglial functions and neuronal loss *in vitro*. I investigated this using a combination of murine and human microglial cell lines, primary microglia and neurons from rat and fixed brain sections from mice. Listed below are the key aims of this work.

1) Aggregated amyloid-β is a hallmark of AD. Amyloid-β can be directly neurotoxic and induce pro-inflammatory activation of microglia, which may contribute to neurodegeneration in AD. Molecular chaperone proteins interact with proteins to prevent their aggregation and facilitate proper folding. The endoplasmic reticulum-resident chaperone protein, calreticulin, can be released from microglia and has been found to bind amyloid-β. I investigated whether exogenous calreticulin can act as an extracellular chaperone to affect amyloid-β aggregation and amyloid-β induced neurotoxicity in vitro. I did this by assessing changes in amyloid-β fibrillisation and oligomerisation using thioflavin T assays and transmission electron microscopy in the presence and absence of recombinant human calreticulin. Then I investigated whether exogenous calreticulin was protective in the context of amyloid-β-induced neuronal loss in primary mixed neuronal-glial cultures from rat. The aim was to test whether calreticulin can act as an extracellular chaperone, and protect neurons against amyloid-β.

2) Glycohydrolase enzymes, including β-galactosidase, play a fundamental role in the degradation of glycoproteins and glycolipids, to maintain homeostasis. Previously it was thought that these enzymes were exclusively located in lysosomes, but recent evidence has found glycohydrolase enzymes associated with the plasma membrane. I investigated whether activated microglia have increased extracellular β-galactosidase activity. To do this, I inflammatory activated BV-2 microglia and primary rat microglia using various stimuli including LPS, PMA and ATP and assessed the presence of extracellular β-galactosidase by

ELISA and β-galactosidase activity using a fluorescence-based *in vitro* assay. I further assessed whether β-galactosidase levels are increased on the surface of microglia in aged brains as this might be implicated in the contribution of ageing and senescence to neurodegeneration. I did this by immunostaining for β-galactosidase in fixed coronal brain sections from 4- and 17-month-old mice. The aim was to determine whether β-galactosidase can be released by microglia in culture or *in vivo*.

3) Extracellular β-galactosidase might remove galactose residues from the surface of microglia and neurons, potentially disrupting homeostasis. I assessed whether addition of β-galactosidase affected microglial activation and neuronal loss in primary mixed neuronal-glial cultures from rats. I further assessed whether inhibition of β-galactosidase in LPS-stimulated cultures affected LPS-induced neuronal loss and microglial activation in these cultures. The aim was to test whether extracellular β-galactosidase can be inflammatory, neurotoxic or neuroprotective, and whether inhibiting extracellular β-galactosidase is neuroprotective.

3 MATERIALS AND METHODS

3.1. Cell culture

3.1.1. Cell lines

Table 3.1. Overview of cell lines used throughout this work.

NAME	SPECIES	DESCRIPTION	SOURCE
BV-2	Mouse	Adherent, microglia-like cell line, immortalised using a v-raf/v-myc carrying retrovirus (Blasi *et al.*, 1990).	A gift from Professor Jennifer Pocock, Department of Neuroinflammation, University College London, UK.
CHME-3	Human	Adherent, human microglial cell line derived from foetal primary microglia, immortalised using SV40 (Janabi *et al.*, 1995). Also known as HCM-3.	A gift from Dr. Svetlana Khoronenkova, Department of Biochemistry, University of Cambridge, UK.
HEK293-T	Human	Adherent, derived from foetal kidney cells, transformed with sheared Adenovirus 5 DNA and immortalised with SV40 (Lin *et al.*, 2014).	A gift from Professor Sarah Lummis, Department of Biochemistry, University of Cambridge, UK.

3.1.2. Cell line culture

All cell-lines (Table 3.1) were kept in a humidified incubator set to 37 °C and 5 % CO_2. BV-2 and CHME-3 microglia cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) (ThermoFisher, 41965039) supplemented with 10 % (v/v) heat inactivated foetal bovine serum (FBS) (ThermoFisher, 10500064) and 1 % (v/v) penicillin/streptomycin (Sigma, P4333) (hereafter referred to as hiDMEM). HEK cells were cultured in DMEM: F12 (ThermoFisher, 31331028), supplemented with 10 % (v/v) FBS and 1 % (v/v) penicillin/streptomycin. Cell lines were passaged, when 80 % confluent, by trypsinisation (ThermoFisher, 15400054). For this, cells were washed with phosphate buffer saline (PBS) then detached with 1X trypsin.

Trypsin was quenched with hiDMEM, and cell suspensions were centrifuged at 150 RCF for 5 min. The resulting cell pellet was resuspended in hiDMEM and seeded in a new flask. For experiments, live cell density was manually counted using a haemocytometer and trypan blue viability stain. Cells were seeded, unless otherwise stated, in low serum DMEM (loDMEM) (supplemented with 0.5 % (v/v) FBS and 1 % (v/v) penicillin/streptomycin).

3.2. Primary cell culture

All experiments were performed in accordance with the U.K. Animals (Scientific Procedures) Act (1986) and approved by the Cambridge University local ethical committee. Mixed glial cultures and mixed neuronal-glial co-cultures (cerebellar granule cells, CGCs) were prepared from the cortex and cerebellum, respectively, of neonatal Wistar rats aged between 3 - 5 days (Figure 3.1). Following decapitation, the cerebellum and cortex were obtained by dissection and placed in ice cold 1X Hanks' Balanced Salt Solution (HBSS) (ThermoFisher, 14185045). Meninges were removed under a microscope and then cortex was digested in 1X trypsin diluted in HBSS for 15 min at 37 °C and cerebellum was digested in 1X versene (ThermoFisher, 15040066) for 7 min at 37 °C. Digests were quenched using supplemented media (Glia media: DMEM supplemented with 10 % (v/v) performance plus heat inactivated FBS (ThermoFisher, 10082147) and 1 % (v/v) penicillin/streptomycin; CGC media: DMEM supplemented with 5 % (v/v) heat inactivated horse serum, 5 % (v/v) performance plus FBS, 5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (Melford Laboratories, H75030), 20 mM KCl (Sigma, 31246), 2 mM L-glutamine (Sigma, G8530), 13 mM glucose (Sigma, 16325) and 1 % (v/v) penicillin/streptomycin). Tissue was then dissociated manually by trituration with a pipette. Versene and trypsin were quenched with media and removed by centrifugation at 300 RCF, 7 min with slow start/stop. The resulting cell pellet was resuspended in the appropriate media. Cortex derived cells were passed through 100 μm and 40 μm cell strainers and seeded, unless otherwise stated, in poly-L-lysine coated T-75 flasks. Cerebellum derived cells were passed through a 40 μm cell strainer and seeded at 1 x 10^5 cells/well in poly-L-lysine coated 96-well plates. After 24 h, cellular debris was removed by gentle agitation and aspiration of the media which was then replaced completely with fresh media. After 7 DIV, primary rat microglia were isolated from mixed glial cultures by rigorous shake-off then collected by centrifugation (300 RCF, 7 min, slow stop/start) and conditioned media retained. The resulting pellet of microglia was resuspended in 1 : 2 ratio of conditioned and fresh glia media. Live microglia were counted on a haemocytometer, using trypan blue viability stain, and seeded onto poly-L-lysine treated plates, at 1 x 10^5 cells/well. Microglia were incubated at 37 °C, 5 % CO_2 overnight, after which they were ready for use. Mixed neuronal-glial co-cultures were treated 7 DIV with various agents as indicated below in section 3.3. and in results.

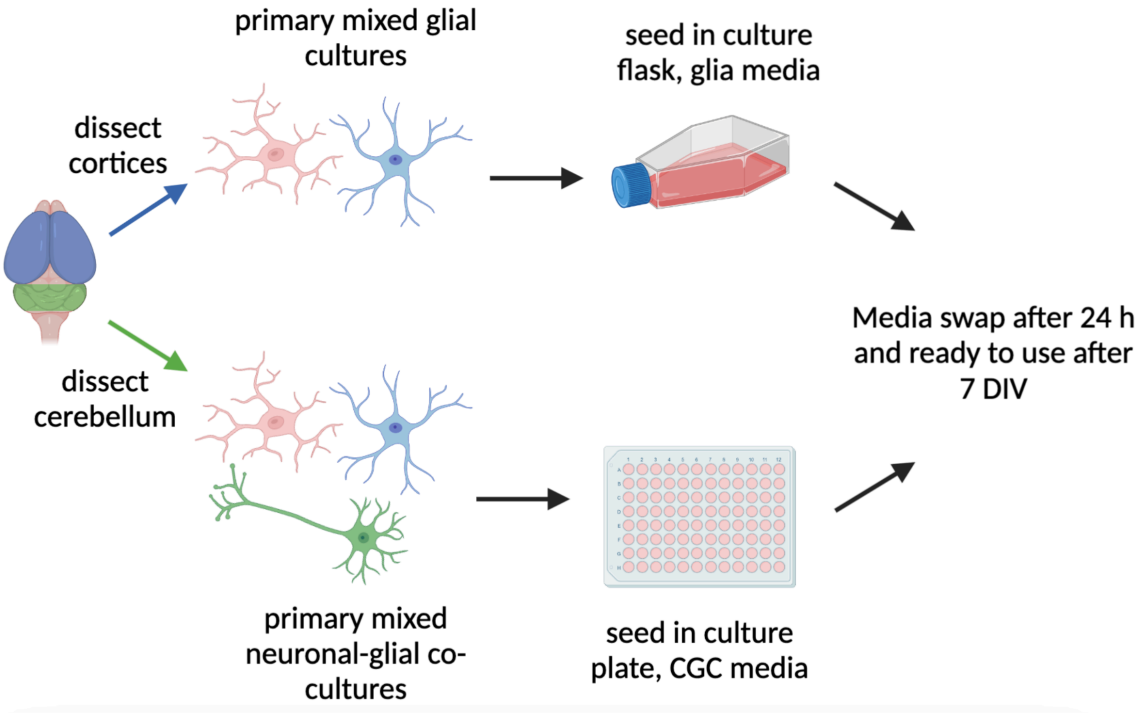

Figure 3.1. Preparation of primary cells. Briefly, brains from neonatal Wistar rats aged between 3 - 5 days were extracted and placed in ice-cold HBSS. Cortices (blue) and cerebellum (green) were dissected, and meninges removed. Cortex derived mixed glial cultures were mechanically dissociated, filtered and, unless otherwise stated, seeded into T-75 tissue culture flasks containing glia media. Cerebellar derived mixed neuronal-glial co-cultures were mechanically dissociated, filtered, and seeded into tissue culture plates containing CGC media. Cultures were incubated at 37 °C, 5 % CO2. After 24 h, a full media swap was performed and after 7 DIV, cultures were ready to use for experiments. Figure made using Biorender.com.

3.3. Cell treatments

In amyloid-β induced neuronal loss experiments, CGCs were treated, at 7 DIV, with 250 nM monomeric synthetic amyloid-β (1-42) (prepared as described in section 3.4.) and 50 nM recombinant human calreticulin (CRT, Abcam, ab91577) for 72 h.

BV-2 cells were seeded in loDMEM at 1 x 10^5 cells/well in 96 well plate and left to adhere overnight. BV-2 cells were then treated, at 37 °C, for 18 h with lipopolysaccharide (LPS) from *Escherichia coli* (Sigma, L4391) at 100 ng/mL. Phorbol 12-myristate 13-acetate (PMA) was used at 100 nM for 18 h. In acute treatments, ATP was used at 1 mM and A23187 was used at 10 μM. For lysis, cells were incubated in 50 μL lysis buffer (150 mM NaCl, 1 % Triton X-100, 50 mM Tris (pH 8.0)) for 15 minutes. Exogenous β-galactosidase from bovine liver (Sigma, G1875) was used at various concentrations and incubation periods at 37 °C, detailed in the results. In some experiments, β-galactosidase was heat-inactivated at 70 °C for 5 min (following optimisation of heat-inactivation conditions using the β-galactosidase activity assay described in section 3.8., Supplementary Figure A 9.1).

For depletion experiments, mixed neuronal-glial co-cultures were treated 3 DIV with 5 μM PLX-3397 (PLX, gift from Aviva Tolkovsky), a colony-stimulating factor-1 receptor (CSF-1R) inhibitor.

β-Galactosidase inhibitors, D-Galactono-1,4-lactone (DGal, BioSynth, MG01323) and 1-Deoxygalactonojirimycin (hydrochloride) (DGJ, Cambridge Bioscience, CAY17179) were used at indicated concentrations in results, generally, 1 mM or 10 mM and with or without LPS for 72 h.

To induce senescence, cells were treated with DNA-damaging reagents: doxorubicin (DOX, Sigma, D1515) and bleomycin sulphate (Bleo, Stratech, A8331). Stock solutions of DOX (1 mM) and Bleo (10 mg/mL) were diluted in hiDMEM (for BV-2 microglia) or conditioned medium (for primary glia) to final concentrations indicated in results. Treatments were done as described previously (Marques, Johnson and Stolzing, 2020; Brelstaff *et al.*, 2021). For DOX experiments, CHME-3 cells were seeded at 5 x 10^4 cells/well in 6 well plate and BV-2 cells were seeded at 2,000 cells/well in 96 well plate. After 48 h DOX treatment, cells were washed 3X with PBS and cultured for a further 6 days in fresh media, with complete media change every 72 h. For bleomycin experiments, BV-2 cells and isolated primary microglia were seeded at 1 x 10^4 cells/well in 96 well plate. Cells were treated with bleomycin for 18 h. After treatment, cells were fixed and stained for SA-β-galactosidase activity as outlined in section 3.13 and imaged (EVOS M5000, Invitrogen). Where indicated, gene expression of p16 and p21 were assessed by qPCR.

Treatments were compared to a vehicle control group. The most appropriate vehicle was chosen for each experiment. Typically, DMSO for depletion experiments, PBS for inhibitor and protein/enzyme experiments or scrambled siRNA for siRNA knockdowns. Where possible a positive control was included, for example, LPS was used as a positive control to induce inflammatory cytokine release.

3.4. Amyloid-β preparation

Peptide synthesised human amyloid-β (1-42) (Anaspec, AS-20276) was prepared as previously described (Neniskyte, Neher and Brown, 2011). Briefly, amyloid-β was dissolved in 1,1,1,3,3,3-hexafluoroisopropanol (HFIP, Sigma) and dried under a stream of nitrogen. Prior to the aggregation assay, amyloid-β was resolubilised in DMSO (Sigma), resuspended in aggregation medium (DMEM, high glucose, HEPES, no phenol red, 21063029, ThermoFisher) and sonicated for 10 min.

3.5. *In vitro* amyloid-β aggregation assay

Amyloid-β aggregation was measured by thioflavin T fluorescence as previously described (Naiki *et al.*, 1989). Briefly, thioflavin T (Sigma, T3516) and amyloid-β (10 μM final concentration for both) were added to aggregation medium ± calreticulin at 0.1 μM or 1 μM. Amyloid-β was incubated at 31.5 °C, with orbital shaking for 10 s, every 10 min and fluorescence (440 nm absorbance, 480 nm emission) was measured in 10 min intervals by a spectrophotometer (FLUOstar OPTIMA, BMG Labtech). Aggregates in wells were imaged with fluorescence microscope (Leica, DMI6000 CS) using HCX PL Fluotar 20x/0.40 dry objective lens and 480/40 nm excitation and 527/30 nm emission filters to detect ThT fluorescence. Acquisition software LAS X v3.52.18963 (Leica Microsystems GmBH, Germany).

3.6. Transmission electron microscopy of amyloid-β oligomers

To generate oligomers, monomeric amyloid-β (10 μM) was incubated with 1 μM calreticulin for 18 h at 4 °C using a thermocycler (ThermoFisher). Oligomeric samples (10 μL) were applied to carbonate coated grids for 1 min and negatively stained with 1 % uranyl acetate for 1 min. Micrographs were obtained on a Tecnai G2 transmission electron microscope. Image post-processing was done in FIJI using a macro that was optimised for the detection of the amyloid-β oligomers. First, noise reduction was done by applying gaussian blur and background subtraction. Identification of oligomeric structures was achieved by thresholding until the segmentation appeared appropriate. Finally, objects were converted to masks and area measurements obtained. Once this was optimised, the same macro was applied to all the images.

3.7. Cell density and morphology analysis

For quantification of cells in Chapter 4, cultures were stained with nuclear marker Hoechst 33342 (HS, blue, ThermoFisher, 62249), microglial marker Alexa Fluor-488 isolectin-B4 (IB4, green, Invitrogen, I21411) and necrosis marker, propidium iodide (PI, red, Sigma, P4170). Cells were imaged on an integrated digital inverted microscope, EVOS M5000 (Invitrogen, LED light source, transmitted light, autofocus mechanism and monochrome CMOS camera (2048 x 1536) with 3.45 μm pixel resolution) using 20x objective (EVOS, fluorite, LWD, phase-contrast, 0.45 NA/ 6.12 WD) and light cubes: DAPI (357/447 nm), GFP (470/525 nm) and Texas Red (585/624 nm). Images from each channel were composited and prepared in (FIJI is Just) ImageJ (FIJI, version 2.9.0) using a macro written by Tim Birkle (https://github.com/timjyb). Cells were then quantified. Microglia were identified by IB4-positive staining. Neurons were distinguished from astrocytes and microglia by their nuclear

morphology and lack of IB4 staining. Apoptotic neurons were identified by chromatin-condensed nuclear morphology and necrotic cells were positively stained with PI. Apoptotic and necrotic cells accounted for <1 % of cells (data not shown). For each treatment condition, four 20x fields were analysed per technical repeat, with three technical repeats per biological repeat and a minimum of three biological repeats performed.

For quantification and morphological analysis of cells in Chapter 6, cultures were stained with Hoechst 33342, neuronal stain Alexa Fluor-488 NeuO (NeuO, green, Stemcell Technologies, 01801) and microglial stain Alexa Fluor-594 IB4 (red, Invitrogen, I21413) and imaged on EVOS M5000 (Invitrogen, LED light source, transmitted light, autofocus mechanism and monochrome CMOS camera (2048 x 1536) with 3.45 μm pixel resolution) using 10x objective (EVOS, fluorite, LWD, phase-contrast, 0.30 NA/ 7.13 WD) and light cubes: DAPI (357/447 nm), GFP (470/525 nm) and Texas Red (585/624 nm)(Figure 3.2A). Four 10x fields were analysed per technical repeat, with three technical repeats per biological repeat and a minimum of three biological repeats performed. Images were then analysed using a pipeline designed by Tim Birkle in CellProfiler (https://github.com/timjyb). Briefly, this pipeline performs background subtraction, nuclei segmentation, intensity normalisation, feature measurement and cell type classification using a trained Random Trees classifier developed in CellProfiler Analyst (Figure 3.2A). From this, cells were segmented and classified to identify neurons, microglia, astrocytes, debris, condensed cells, other and total cell number. CellProfiler was also used for the morphological analysis of microglia in the mixed neuronal-glial co-cultures, providing data on microglial area (Figure 3.2B).

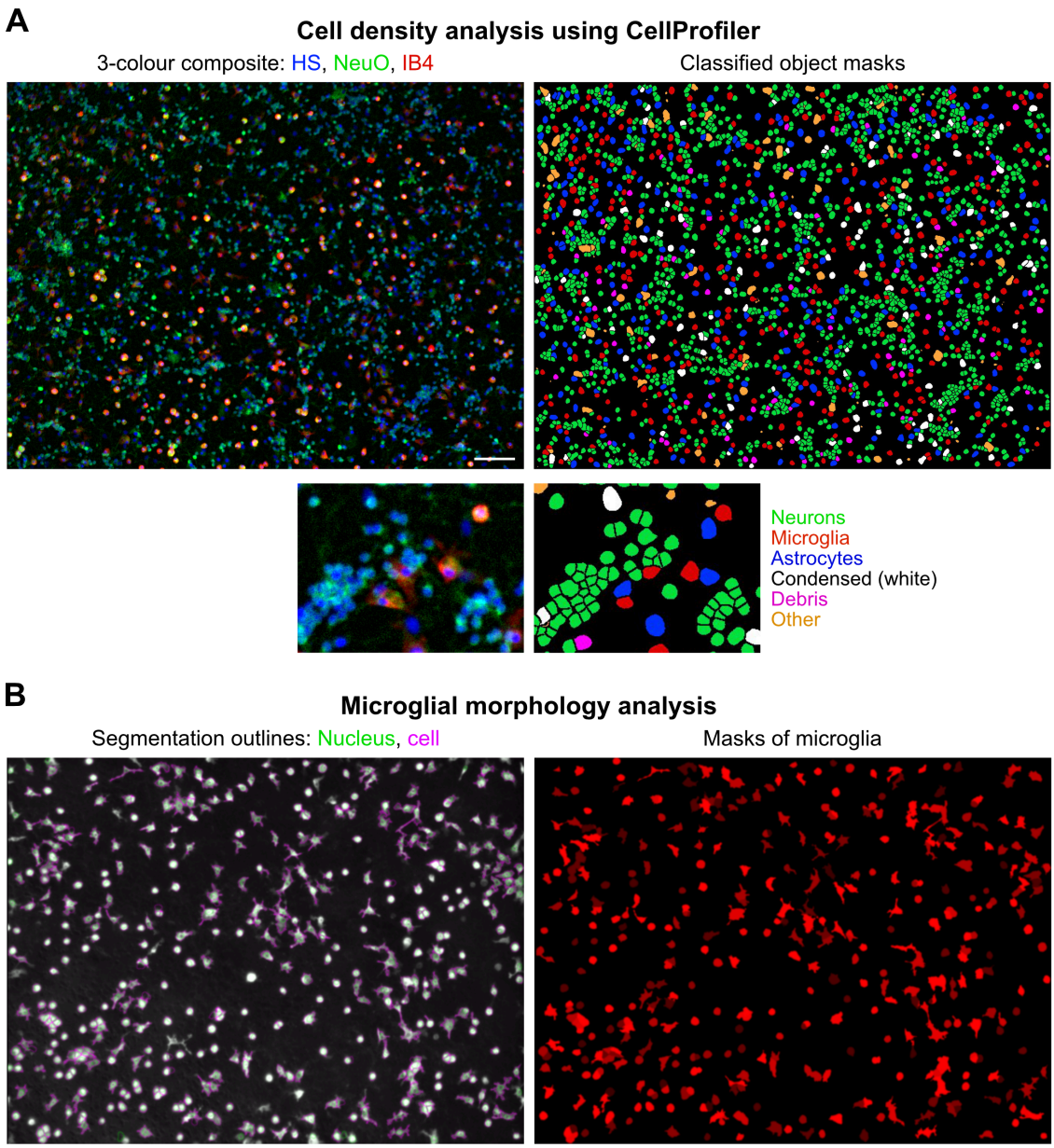

Figure 3.2. Cell density and morphological analysis in CellProfiler. (A) Mixed neuronal-glial cultures were stained with Hoechst 33342 (blue), neuronal stain Alexa Fluor-488 NeuO (green) and microglial stain Alexa Fluor-594 IB4 (red) and imaged on EVOS M5000. Images were then analysed using a pipeline designed by Tim Birkle in CellProfiler. From this, cells were segmented and classified to identify neurons (green), microglia (red), astrocytes (blue), debris (pink), condensed cells (white), other (orange) and total cell number. (B) CellProfiler was also used for the morphological analysis of microglia in the mixed neuronal-glial co-cultures. The Hoechst 33342 (blue) and IB4 (red) channels from (A) were used to segment microglial nuclei (green circles) and outline microglial cells individually (magenta outlines). From this, microglial masks were generated to provide data on microglial area.

Where BV-2 and isolated primary microglial viability and proliferation were assessed, cells were stained with Hoechst 33342, PI and Alexa Fluor-488 IB4 then imaged using EVOS M5000. Four 10x fields were analysed per technical repeat, with three technical repeats per biological repeat and a minimum of three biological repeats performed. Images from each channel were composited and prepared in FIJI using a macro written by Tim Birkle (https://github.com/timjyb). Composited images were uploaded to the imaging software

QuPath which was used to identify nuclei using the Hoechst 33342 staining for total cell counts and PI-positive nuclei, from which % PI-positive cells were determined.

3.8. β-Galactosidase activity assays

BV-2 microglia were seeded at 1 x 10^5 cells/well in black, clear-bottom 96-well plates (Greiner) in loDMEM and treated as in section 3.3. β-Galactosidase activity on live cells was assessed in assay buffer adjusted to pH 7.0, or pH 4.0 for supernatant activity assays. The supernatant activity was assayed at final pH 4.7 as a result of diluting DMEM (which uses a sodium bicarbonate buffer system) with pH 4.0 assay buffer at a 1 : 1 ratio. For the assay, media was removed from live cells and immediately replaced with 50 μL PBS. For supernatants, debris was removed by centrifugation 150 RCF, 5 min and 50 μL used for the assay. The assay was initiated by the addition of 50 μL of 2X assay buffer. Assay buffer (1X) contained 100 mM sodium phosphate (pH 7.0) or 100 mM citric acid/ 200 mM sodium phosphate buffer (pH 4.0), 1 mM $MgCl_2$, 50 μM β-mercaptoethanol and 0.5 mg/mL 4-methylumbelliferyl-β-D-galactopyranoside (MUG, GlycoSynth) in distilled water. 0.2 mU/mL β-galactosidase from bovine liver (Sigma) was used as a positive control. Kinetic experiments were performed using a plate reader (FlexStation 3, Molecular Devices), pre-heated to 37 °C, with fluorescence determinations beginning immediately upon the addition of assay buffer and taken every minute for 2 h, using 360/40 nm excitation and 460/40 nm emission filters. A standard curve was produced using 4-methylumbelliferone (MUB) which was used to convert arbitrary fluorescent units and calculate concentration of MUB produced in μM.

For assessing inhibitors of β-galactosidase, D-galactono-1,4-lactone and DGJ were diluted to 2X the indicated concentrations in PBS containing 2X the final concentration (0.2 mU/mL) of β-galactosidase from bovine liver, then the assay was initiated as above.

3.9. Generation of stable p16 CHME-3 cell line

3.9.1. Cloning of lentiviral plasmid

Lentiviral pWPI plasmid containing the EF1α-EMCV-IRES-EGFP cassette was from Addgene (plasmid #12254). Previously, David Allendorf modified the Pme1 blunt-end restriction site by insertion of a multiple cloning site (5' -AAACTTCCATTGGATCCTACGTATTCCACTAGTCCCATATGTGTTT- 3') which was designed to contain BamH1 and Spe1 restriction sites for sticky-end ligation. Human p16-FLAG cDNA was amplified from pcDNA3.1-p16-FLAG (Genscript) using BamH1-p16 forward (5' -TAAGGATCCATGGAGCCGGCGGCGGGG- 3') and Spe1-FLAG reverse (5' -CAAGGATGACGACGATAAGTGAACTAGTTAT- 3') primers. The PCR product was cloned

into the modified pWPI vector. pWPI-p16-FLAG plasmid was transformed into NEB 10-beta competent *E. coli* (NEB, C3019). Bacteria were plated on ampicillin agar and colonies were selected and expanded. Plasmid DNA was purified using Monarch Plasmid Miniprep kit (NEB, T1010) following manufacturer's instructions (briefly detailed in section 3.11). Plasmid was sent to the Department of Biochemistry Sanger Sequencing lab to ensure sequence was correct before any experiments were conducted.

3.9.2. Transfection of HEK-293T cells

Lentiviral vectors were generated by the transient co-transfection of HEK-293T cells as previously described (Balcaitis *et al.*, 2005). Briefly, the lentiviral transfer vector plasmid (either pWPI empty vector or pWPI-p16-FLAG, 1.64 pmol), packaging plasmid (psPAX2, 1.3 pmol) and envelope plasmid (pMD2.G, 0.72 pmol) DNA were mixed and made up to final volume, 500 μL, in serum-free Opti-MEM (Invitrogen, 51985). 75 μL polyethylenimine (PEI) (1 mg/mL) was mixed with 425 μL serum-free Opti-MEM, then added to the DNA mix. DNA/PEI mixtures were incubated at room temperature (RT) for 15 min. 9 mL of fresh supplemented DMEM was added to a 10 cm plate of ~70 % confluent HEK-293T cells before DNA/PEI mixture was added. Cells were incubated at 37 °C in a 5 % CO_2 humidified incubator. 48 h post-transfection, viral supernatants of empty vector or p16- vector transfected cells were briefly centrifuged to remove cell debris and then filtered through 0.22 μm filters. The virus medium was used for transduction immediately or stored at -80 °C.

3.9.3. Transduction of lentiviral particles into CHME-3 microglia

CHME-3 cells were seeded in T-75 flasks to be ~40 % confluent at point of transduction. After 24 h, all the culture media was removed and replaced with virus medium containing 8 μg/mL polybrene (Sigma, TR-1003). Medium was changed after 24 h to reduce the stress of cells. Transduction efficiency was monitored by fluorescence microscopy of GFP expression 48 h post-transduction.

3.10. Immunofluorescence

Control (pWPI-) and p16-CHME-3 cells were seeded in 24 well plate at 25,000 cells/well in loDMEM and left to adhere overnight. Cells were washed with PBS and fixed with 4 % paraformaldehyde (PFA) for 10 min, RT. Cells were washed with PBS and permeabilised using PBS containing 0.3 % Triton-X for 10 min. Cells were washed again and incubated with blocking solution (PBS with 10 % goat serum) for 1 h, RT. Cells were washed again and incubated with blocking solution containing primary antibodies anti-FLAG (1:500, Sigma, F1804) or anti-Ki67 (1:100, Millipore, AB9260), overnight at 4 °C. After 18 h, cells were washed with PBS and incubated with blocking solution containing the secondary Alexa Fluor-568 anti-mouse IgG (1:200, Invitrogen, A11004) for 2 h in dark, RT. Hoechst was added to wells, incubated for a further 15 min. Cells were washed in PBS and imaged on fluorescent

microscope (Leica, DMI6000 CS) using HCX PL Fluotar 20x/0.40 dry objective lens and 360/40 nm excitation and 470/40 nm emission filters to detect Hoechst and 545/40 nm excitation and 610/75 nm emission for Alexa Fluor-568. Acquisition software LAS X v3.52.18963 (Leica Microsystems GmBH, Germany).

3.11. RNA extraction and reverse transcription

Whole RNA was extracted using the Monarch total RNA Miniprep kit and associated protocol (NEB, T2010). Briefly, cells were harvested by trypsinisation and pelleted, then lysed and spun through columns to remove genomic DNA. RNA was eluted from columns using nuclease-free water. RNA concentrations and purity were assessed spectroscopically using a nanodrop (ThermoFisher). cDNA was generated using SuperScript II Reverse Transcriptase kit (ThermoFisher, 18064014) following the manufacturer's instructions. Briefly, 1 μg whole RNA, 1 μL of 10 mM deoxyribose nucleotide triphosphates (dNTPs) (ThermoFisher, R0192) and 1 μL of 20X random hexamer primers (ThermoFisher, SO142) were combined. Samples were made up to 13 μL with nuclease-free water and incubated at 65 °C for 5 min before being placed on ice. After 1 min, 2 μL of 100 mM 1,4-dithiothreitol (DTT) and 4 μL 5X first strand buffer (250 mM tris(hydroxymethyl)aminomethane (Tris)-HCl (pH 8.3), 375 mM KCl, 15 mM MgCl2) were added per sample. Samples were incubated at RT for 3 min before adding 1 μL (200 units) of Superscript II Transcriptase (Invitrogen, 18064022). The final reaction mixture was placed in a thermocycler (Bio-Rad) and cDNA generated using the following parameters: 25 °C for 10 min, 42 °C for 50 min, 72 °C for 15 min, 4 °C hold.

3.12. Quantitative PCR (qPCR)

For qPCR, SYBR Green JumpStart Taq ReadyMix (Sigma, S4438) was used. Briefly, a master mix was made for each gene (per cDNA sample, in triplicate): 12.5 μL of SYBR Green, 2 μL of 10 μM primers (Table 3.2) and 9.5 μL of nuclease-free water. Of that master mix, 90 μL was aliquoted out for each cDNA sample. 3.75 μL of cDNA was added to the master mix and vortexed. From this, samples were loaded into 0.1 mL PCR strip tubes, in triplicate, for amplification. Amplification was performed using Rotor-Gene Q (Qiagen) system and 'three-step melt' was used under the following conditions: 95 °C for 10 min, 40 cycles of 95 °C for 10 s, 60 °C for 15 s, 72 °C for 20 s, followed by 65 °C melt. Data was analysed with the Rotor-Gene Q software. A comparative quantitation approach was used which calculates comparative concentrations of a target and a calibrator sample according to their take-off point. Comparative concentrations were normalised to housekeeping gene, GAPDH, to correct for different efficiencies of reverse transcription.

Table 3.2. Summary of qPCR primers.

GENE	SPECIES	SEQUENCES	PRODUCT LENGTH	EXON BOUNDARY CROSSED
CDKN2A (p16)	Human	Forward: 5'-CCAACGCACCGAATAGTTAC-3' Reverse: 5'-CACGGGTCGGGTGAGAGT-3'	134	Yes
CDKN1A (p21)	Human	Forward: 5'-TGGAGACTCTCAGGGTCGAAA-3' Reverse: 5'-GGCGTTTGGAGTGGTAGAAATC-3'	164	Yes
GAPDH	Human	Forward: 5'-GGATTTGGTCGTATTGGG-3' Reverse: 5'-GGAAGATGGTGATGGGATT-3'	151	Yes
Cdkn1a (p21)	Mouse	Forward: 5'-GCAGATCCACAGCGATATCCA-3' Reverse: 5'-AACAGGTCGGACATCACCAG-3'	73	Yes
Glb1	Mouse	Forward: 5'-GGTAAACCCCATTCCACGGT-3' Reverse: 5'-GTGGGGCGTCGTAGTCATAG-3'	178	Yes
Gapdh	Mouse	Forward: 5'-GTTGTCTCCTGCGACTTCA-3' Reverse: 5'-GGTGGTCCAGGGTTTCTTA-3'	184	Yes

3.13. Staining for senescence-associated (SA)-β-galactosidase activity

SA-β-galactosidase staining was performed as described (Dimri *et al.*, 1995). Briefly, cells were fixed in 4% paraformaldehyde and washed 3X with PBS then incubated with SA-β-galactosidase staining solution (1 mg/mL 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) (Merck, 11680293001) in dimethylformamide, 40 mM citric acid/sodium phosphate (pH 6.0), 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 150 mM NaCl, 2 mM $MgCl_2$). Staining of cells reached peak levels after 16 - 18 h. To detect lysosomal β-galactosidase, as a positive control, the citric acid/sodium phosphate solution was used at a pH of 4.0. To selectively detect senescent cells, the citric acid/sodium phosphate solution was used at a pH of 6.0. This resulted in only cells with

residual lysosomal activity being stained blue. Cells were imaged on EVOS M5000 (Invitrogen).

3.14. Immunohistochemistry and imaging of free-floating brain slices

Transcardial perfusion was done previously by Mar Puigdellivol and David Allendorf and, after perfusion, tissue sectioning was done by Mar Puigdellivol and Jacob Dundee as described (Dundee *et al.*, 2023). Briefly, 25 μm serial coronal sections were prepared using a sliding microtome and stored in PBS containing 0.025% sodium azide as free-floating sections.

Staining, image acquisition and analysis were performed as previously described (Dundee *et al.*, 2023). Staining was done at RT, with shaking, and between each step slices were washed thoroughly with PBS. For each of five wild-type mice, aged 4- and 17-months-old, three sections were used, taken 300 μm apart per mouse. Sections were incubated with 50 mM ammonium chloride in PBS for 30 min to quench free aldehyde from the fixation process and then, to minimise autofluorescence, sections were incubated in 70 % ethanol containing 0.1 % Sudan Black B for 20 min. To facilitate antibody penetration, sections were permeabilised using 1 % Triton X-100 in PBS for 30 min, and then blocked for 1 h with blocking solution (2 % BSA, 3 % goat serum, and 0.03 % Triton X-100 in PBS). Sections were incubated with mouse anti-Iba1 (1:200, Sigma, MABN92), rabbit anti-β-galactosidase (1:200, ThermoFisher, PA5-102503), and rat anti-CD68 (1:200, Thermo Fisher) antibodies in blocking solution for 2 h at 37 °C (Xiao *et al.*, 2017), then washed with PBS and incubated for 2 h at 37 °C with secondary antibodies, Alexa Fluor-488 goat anti-mouse (1:200, Thermo Fisher, A11001), Alexa Fluor-568 goat anti-rabbit (1:200, Thermo Fisher, A11011) and Alexa Fluor-647 goat anti-rat (1:200, Thermo Fisher). Sections were washed with PBS and mounted on poly-L-lysine treated glass slides in DAPI-containing Vectashield mounting medium (Vector Laboratories, H1500) and imaged on a confocal microscope (Nikon C2si, 63x, 1.35 NA, oil immersion objective lens, LU-N4 laser unit, C2-DU3 detector unit, spectral detection for Channel 1 (405 nm laser, solid state, DAPI) was set between 426-450 nm, Channel 2 (488 nm laser, argon ion, Alexa Fluor-488) was set between 573-613 and Channel 3 (561 nm laser, solid state, Alexa Fluor-568) was set between 650-1000 nm)). Acquisition software NIS-Elements C (Nikon, version 4.51.01). For imaging, Z-stacks (0.5 μm step intervals) were taken of the somatosensory cortex. Fifteen microglia were analysed across the three sections from each mouse. Image analysis was done in FIJI and Imaris (version 9.1.2). Briefly, background subtraction and intensity normalisation were done, then microglial surface rendering (using Iba1 staining) and β-galactosidase staining intensity within Iba1+ structures was analysed in Imaris. The results for the surface-rendered objects were represented as volume (μm^3) and β-galactosidase intensity as mean fluorescence intensity (MFI).

3.15. Enzyme-linked immunosorbent assays (ELISA)

Quantification of cytokines (IL-6 and TNFα) and GLB1 protein was performed using ELISA kits (Table 3.3) according to the manufacturer instructions. Briefly, 96-well plates (MaxiSorb, Nunc) were coated with capture antibody overnight at 4 °C. Plates were then washed 4X with PBS, supplemented with 0.05 % (v/v) Tween-20 (Sigma) and dried by blotting on absorbent paper. Washing was carried out similarly between each step. Plates were blocked to reduce non-specific binding with assay diluent (or 1 % bovine serum albumin (BSA) in PBS) for 1 h. Then 100 μL of standards or samples were added to the plate for 2 h. All samples were suitably diluted with assay diluent to sit within the detectable range of the kit. Subsequently, detection antibody was added for 1 h. Then Avidin-HRP solution was added for 30 min. Plates were washed 5X, incubating wash buffer for 1 min per wash. Colorimetric assessment of cytokine levels was carried out using conversion of tetramethylbenzidine substrate in a peroxidase catalysed reaction. The reaction was stopped after 10 - 25 min by stop solution (or 2N H_2SO_4), causing a visible colour change from blue to yellow. Absorbance was measured at 450 nm on a FLUOstar OPTIMA microplate reader (BMG Labtech). For analysis, optical density for blank was subtracted from each reading and sample cytokine concentrations were interpolated from a standard curve. The dilution of the original samples was accounted for, and final concentrations shown as pg/mL or mU/mL.

Table 3.3. List of ELISA kits used with category number and supplier.

PROTEIN	SPECIES	CAT. NUMBER	SUPPLIER
IL-6	Mouse	431301	BioLegend
IL-6	Rat	437107	BioLegend
TNFα	Mouse	430904	BioLegend
TNFα	Rat	438204	BioLegend
GLB1	Mouse	254126	Abbexa
GLB1	Rat	255673	Abbexa

3.16. RNAi knockdown of *Glb1* in BV-2 microglia

siRNA mediated gene silencing was performed to study the effect of Glb1 deficiency on BV-2 microglia. BV-2s were seeded at 3 x 10^5 cells/well in 6 well plate and after 24 h were incubated in a lipid : siRNA mix containing 3 % (v/v) Lipofectamine 3000 (Invitrogen, L3000001) and 60 pmol of either Glb1-targeting (Silencer™ Select pre-designed siRNA, ID s62969) or non-targeting (Silencer™ Select negative control No. 1 siRNA, 4390843) siRNA in serum-free Opti-MEM. Transfection medium was removed after 3 h incubation at 37 °C and replaced with hiDMEM. 24 h post transfection, cells were detached by trypsinisation, counted, and seeded in loDMEM. RNA extraction was performed 48 h post transfection.

3.17. Statistical analysis

Statistical analysis was performed using GraphPad Prism (version 10.0) and data was collected from a minimum of 3 independent experiments. Shapiro–Wilk test of normality was performed. No outlier tests were performed. Statistical significance was assessed by repeated measures one-way ANOVA, with sphericity assumed, followed by Tukey's, Šídák's or Dunnett's post-hoc test, unless stated otherwise (see figure legends). P-values of $p \leq 0.05$ are considered significant.

4 CALRETICULIN INHIBITS AMYLOID-β AGGREGATION AND PREVENTS NEUROTOXICITY

All data presented in this chapter is my own work and some content has been published in: Reid KM*, Kitchener EJA*, Butler CA, Cockram TOJ and Brown GC. 'Brain cells release calreticulin that attracts and activates microglia, and inhibits beta amyloid aggregation and neurotoxicity' (2022). *Frontiers in Immunology*. DOI:10.3389/fimmu.2022.859686. (*co-first authors).

4.1. Introduction

Several human disorders are associated with the accumulation and deposition of aberrant proteins in various organs and tissues. Indeed, the aggregation of amyloid-β to form toxic oligomers and insoluble amyloid plaques in the brain is a hallmark of AD and has been implicated in the death of neurons that characterise the disease.

Molecular chaperone proteins are commonly found intracellularly, where they interact with proteins to prevent their aggregation and facilitate proper folding. However, an increasing number of extracellular molecular chaperones have been identified, including: clusterin (Humphreys *et al.*, 1999), haptoglobin (Yerbury *et al.*, 2005), α2-macroglobulin (French, Yerbury and Wilson, 2008), neuroserpin and transthyretin (West *et al.*, 2021). These act extracellularly to fold other proteins into a functional or non-pathogenic form (Wyatt, Yerbury, *et al.*, 2013). This could be important in the context of AD, where extracellular chaperones may prevent the aberrant accumulation of amyloid-β into extracellular plaques (Wyatt *et al.*, 2012).

Calreticulin is a highly conserved and ubiquitously expressed lectin, initially recognised for its essential role within the ER (Krause and Michalak, 1997). Intracellularly, calreticulin acts as a molecular chaperone by binding to exposed glucose residues on nascent glycan chains, to facilitate protein glycosylation and folding (Hammond, Braakman and Helenius, 1994). Under certain conditions, such as apoptosis or ER stress, calreticulin can be translocated to the cell surface and act as an "eat-me" signal, promoting phagocytosis of target cells by macrophages, through activation of the LRP1 receptor (Ogden *et al.*, 2001; Gardai *et al.*, 2005; Obeid *et al.*, 2007). Calreticulin is a soluble protein and once at the cell-surface is capable of being released into the extracellular space. Notably, inflammatory-activated macrophages have been found to secrete calreticulin into the extracellular milieu and opsonise target cells, facilitating their recognition and engulfment by phagocytes (Feng *et al.*, 2018; Cockram, Puigdellívol and Brown, 2019).

Calreticulin can directly bind amyloid-β via the polypeptide binding site of calreticulin and the hydrophobic C-terminus of amyloid-β (Houen, Duus and Hansen, 2008), and a significant fraction of amyloid-β in human CSF is bound to calreticulin (Erickson *et al.*, 2005), suggesting that extracellular calreticulin may impact amyloid-β aggregation in the human brain. Hence, it is possible that calreticulin acts as an extracellular chaperone to keep amyloid-β in solution or promote its degradation.

In this chapter, I assessed the potential extracellular chaperone function of calreticulin on amyloid-β aggregation and subsequently investigated whether calreticulin modulates amyloid-β-induced neuronal loss *in vitro*.

4.2. Results

4.2.1. Amyloid-β fibrillises with standard lag-phase kinetics in thioflavin T assay

In vitro assays that track the fibrillisation of amyloid-β are commonly used to understand the aggregation process and to identify modulators, which could be of potential therapeutic interest. Amyloid-β aggregation is highly heterogeneous, giving rise to a number of aggregation states (Figure 4.1A). Thioflavin T (ThT) fluoresces upon binding to β-sheet rich assemblies and is considered the gold standard for monitoring the phases of amyloid-β aggregation, generating a sigmoidal curve (Figure 4.1B). During the initial lag phase, peptide monomers self-assemble to generate disordered, small oligomers. These then rapidly polymerise into large oligomers and fibrils in the growth phase, which contain more structured β-sheet elements. The plateau phase is reached when fibrils have formed, and monomeric peptide is depleted. Using these *in vitro* assays, it is often difficult to control the aggregation and obtain reproducible results in the test tube (Faller and Hureau, 2021). Hence, it is important to optimise assay conditions with each new batch of amyloid-β. In this work, I validated conditions used previously in our lab to track the fibrillisation of synthetic amyloid-β (1-42) peptide using ThT. Aggregation of 10 μM of monomeric amyloid-β, incubated at 31.5 °C in aggregation media over 12 h with 10 μM thioflavin T (ThT), produced a typical sigmoidal curve, with standard lag-phase kinetics (Figure 4.1C).

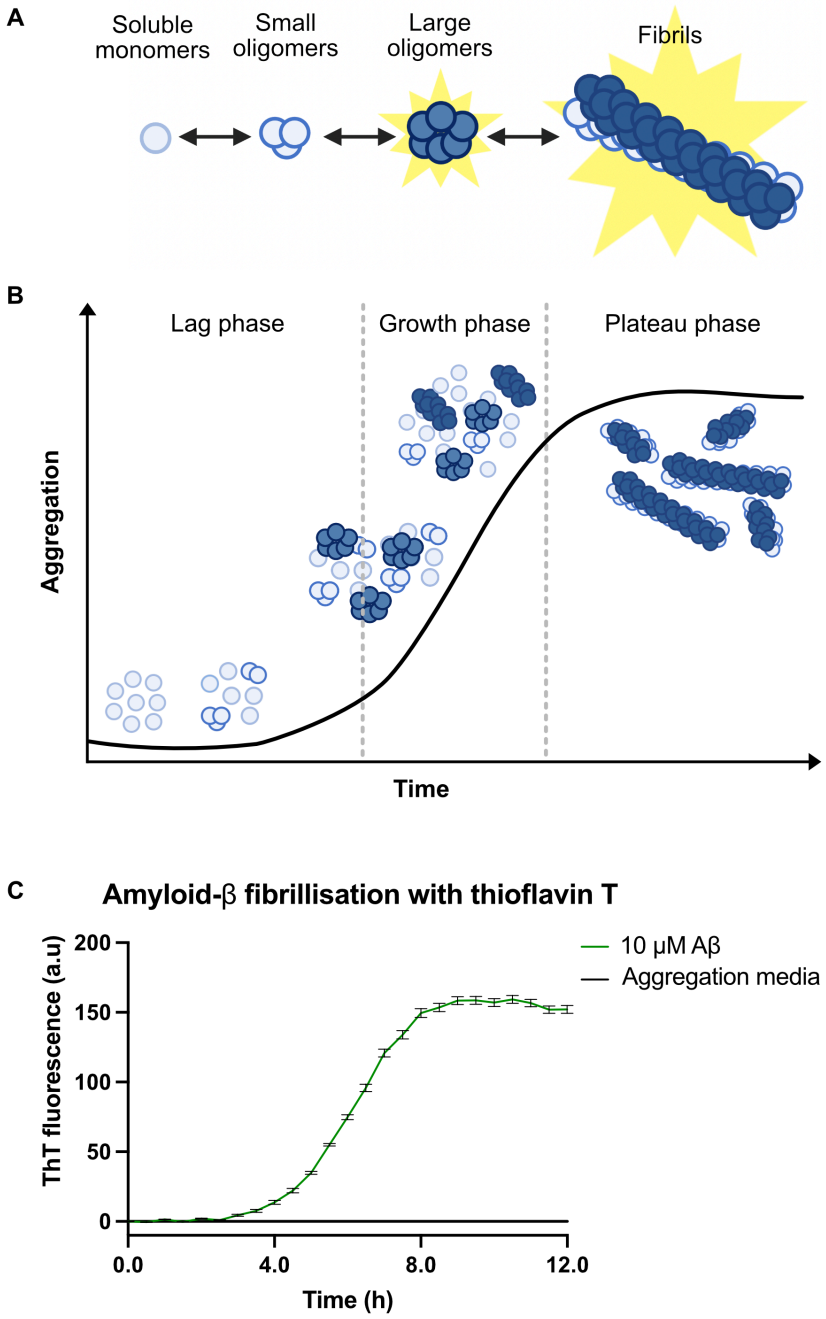

Figure 4.1. Synthetic amyloid-β fibrillisation kinetics can be assessed by thioflavin T fluorescence. (A) A simplified schematic of the main protein species formed during the amyloid fibril formation process: soluble monomers, small oligomers, large oligomers, and fibrils. Thioflavin T (ThT) fluorescence can be used to monitor the self-assembly of amyloid-β from soluble monomers and oligomers into fibrils. (B) Schematic representation of amyloid aggregation assay. The kinetics of amyloid aggregation over time generates a sigmoidal curve in the presence of ThT. Initially peptide monomers self-assemble in the lag phase to generate disordered, small oligomers, which then rapidly form large oligomers in the growth phase. The plateau phase is reached when fibrils have formed, and monomeric peptide is depleted. (C) Aggregation of synthetic amyloid-β (1-42) produced a typical sigmoidal kinetic curve. 10 μM of monomeric synthetic Aβ (1-42) was incubated at 31.5 °C in aggregation media (DMEM) with 10 μM thioflavin T (ThT) and the fluorescence recorded over time. Mean ± SEM of 3 independent experiments are shown.

4.2.2. Calreticulin prevents fibrillisation of amyloid-β

Calreticulin is an intracellular chaperone, which can bind to amyloid-β (Houen, Duus and Hansen, 2008), but it is not known whether calreticulin can act as an extracellular chaperone for amyloid-β, which would be particularly relevant for neurodegeneration. Using the validated *in vitro* ThT fibrillisation assay conditions above, I investigated whether recombinant human calreticulin modulates synthetic amyloid-β aggregation. In the absence of calreticulin, amyloid-β (10 μM) fibrillised with standard lag-phase kinetics at 31.5 °C (Figure 4.2A). In the presence of 1 μM calreticulin (i.e., a molar ratio of calreticulin : amyloid-β = 1 : 10), fibrillisation of amyloid-β was almost completely prevented (Figure 4.2A). In the presence of 0.1 μM calreticulin, the level of ThT fluorescence at the endpoint was significantly reduced by around 30% (Figure 4.2B), however, the kinetics of amyloid-β fibrillisation were little affected, as indicated by the shape of the fibrillisation curve in Figure 4.2A. This suggests that calreticulin may help fold amyloid-β into forms with less β-sheet structure, consistent with calreticulin acting as a chaperone for amyloid-β. Imaging the amyloid-β aggregates at the end of the fibrillisation assay confirmed that calreticulin reduced the ThT fluorescence of the amyloid-β aggregates (Figure 4.2C). This data suggests that calreticulin inhibits amyloid-β fibrillisation, or folds amyloid-β into aggregates with less β-sheet structure.

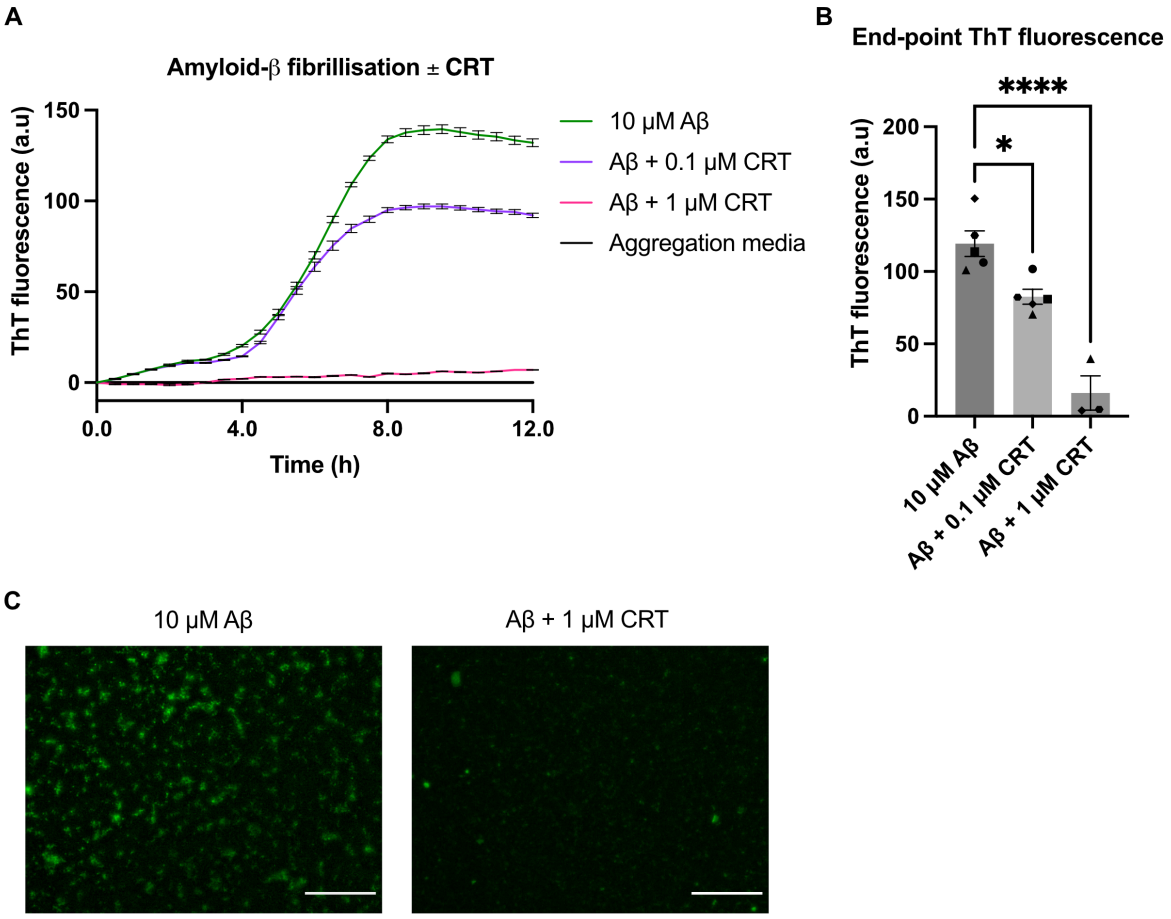

Figure 4.2. Calreticulin prevents fibrillisation of amyloid-β. (A) 10 μM of monomeric Aβ was incubated at 31.5 °C in aggregation media (DMEM) with 10 μM thioflavin T (ThT) ± 0.1 μM or 1 μM calreticulin (CRT), and the fluorescence recorded over time. Mean ± SEM of at least 3 independent experiments are shown. (B) End-point ThT fluorescence readings from (A). Statistical comparisons were made by mixed effects one-way ANOVA followed by Šídák's multiple comparisons test. Asterisk (*) indicate significance (*: $p<0.05$, ****: $p<0.0001$) of end-point fluorescence compared to 10 μM Aβ condition. (C) At the end of the assay, the bottom of the well was imaged using a fluorescence microscope 20x objective. Scale bar = 50 μm.

4.2.3. Calreticulin promotes the formation of large amyloid-β oligomers

As oligomeric amyloid-β is considered to be more neurotoxic than fibrillar amyloid-β (Amin and Harris, 2021), I next assessed the effect of calreticulin on amyloid-β oligomer formation. To do this, I incubated 10 μM monomeric amyloid-β ± 1 μM calreticulin for 18 h at 4 °C, conditions known to produce amyloid-β oligomers (Stine *et al.*, 2011). The resulting amyloid-β oligomers were imaged by transmission electron microscopy (TEM) and oligomeric structures analysed in FIJI. Amyloid-β oligomerised in the presence of 1 μM calreticulin resulted in an increased number of amyloid-β structures ≥ 60 nm^2 compared to amyloid-β

oligomers formed in the absence of calreticulin (Figure 4.3A & B). Hence, calreticulin promotes the formation of larger oligomeric structures.

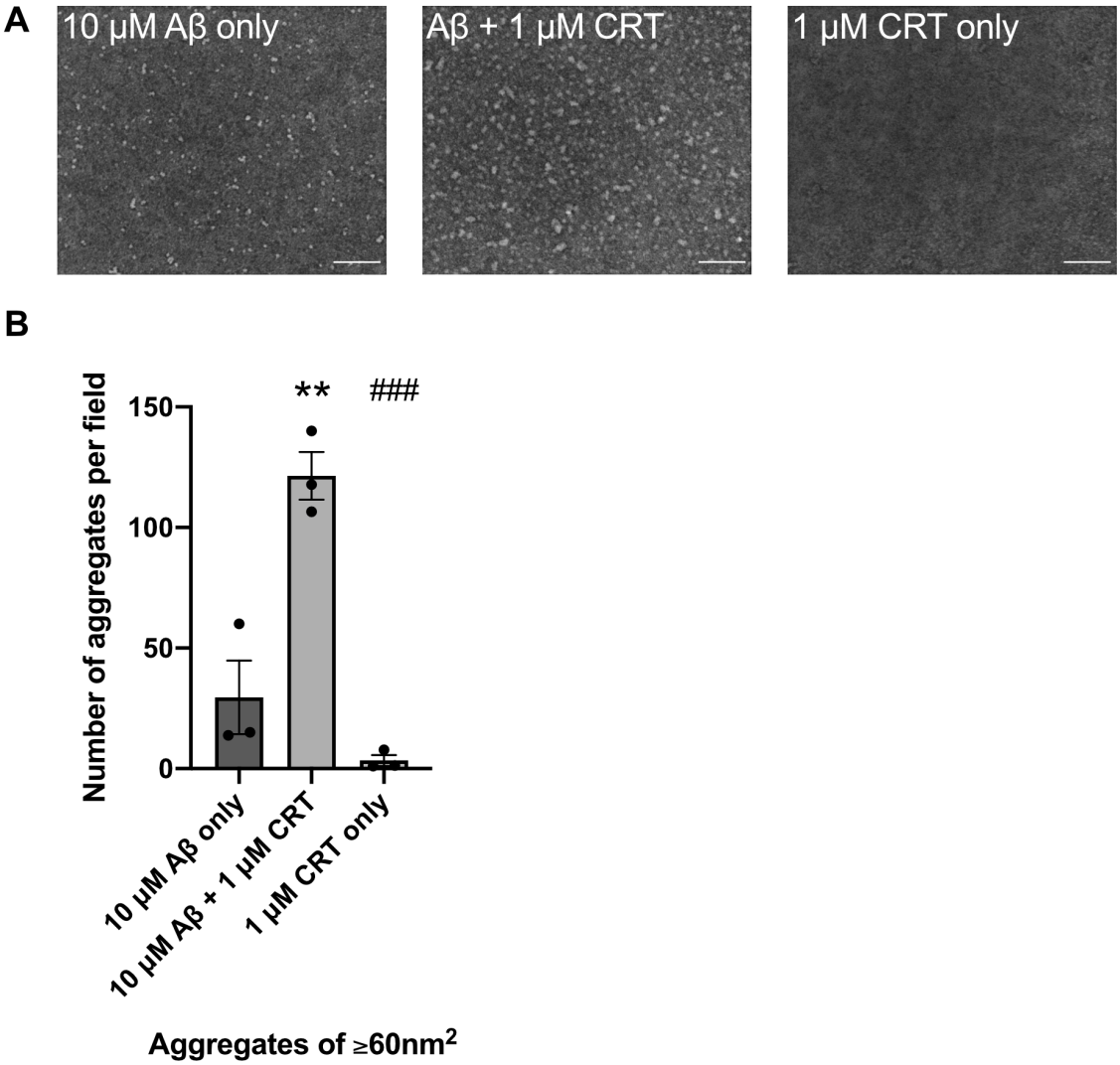

Figure 4.3. Calreticulin promotes formation of large oligomeric structures of amyloid-β. (A) 10 µM of monomeric amyloid-β was incubated for 18 h at 4 °C ± 1 µM CRT. TEM images revealed morphological changes to Aβ oligomers in the presence of CRT compared to Aβ only control. Scale bar = 100 nm. (B) Quantification of aggregates ≥60 nm² quantified from (A). Means ± SEM of 3 independent experiments are shown. Statistical comparisons were made by one-way ANOVA followed by Tukey's multiple comparisons test. Asterisk (*) indicate significance (**: $p<0.01$) to 10 µM Aβ only. Hash (#) indicate significance (###: $p<0.001$) to 10 µM Aβ + 1 µM CRT.

4.2.4. Calreticulin protects against amyloid-β induced neuronal loss

As calreticulin affected fibrillisation and oligomerisation of amyloid-β, I wanted to investigate whether calreticulin affected neurotoxicity induced by amyloid-β. To test this, I prepared mixed neuronal-glial cultures from the cerebellum of neonatal rats. Our lab has previously shown that nanomolar concentrations of amyloid-β induce neuronal loss mediated by

microglia in these cultures (Neniskyte, Neher and Brown, 2011) and that nanomolar concentrations of calreticulin do not cause cell death (Reid *et al.*, 2022). After 72 h treatment ± 250 nM amyloid-β ± 50 nM calreticulin, cultures were stained with nuclear marker Hoechst 33342 (blue), microglial marker IB4 (green) and necrosis marker, propidium iodide (PI, red). Cultures were imaged and cells were identified and quantified. Microglia were identified by IB4-positive staining. Neurons were distinguished from astrocytes by their nuclear morphology. Apoptotic neurons were recognised by chromatin-condensed nuclear morphology and necrotic cells were positively stained with PI (Figure 4.4).

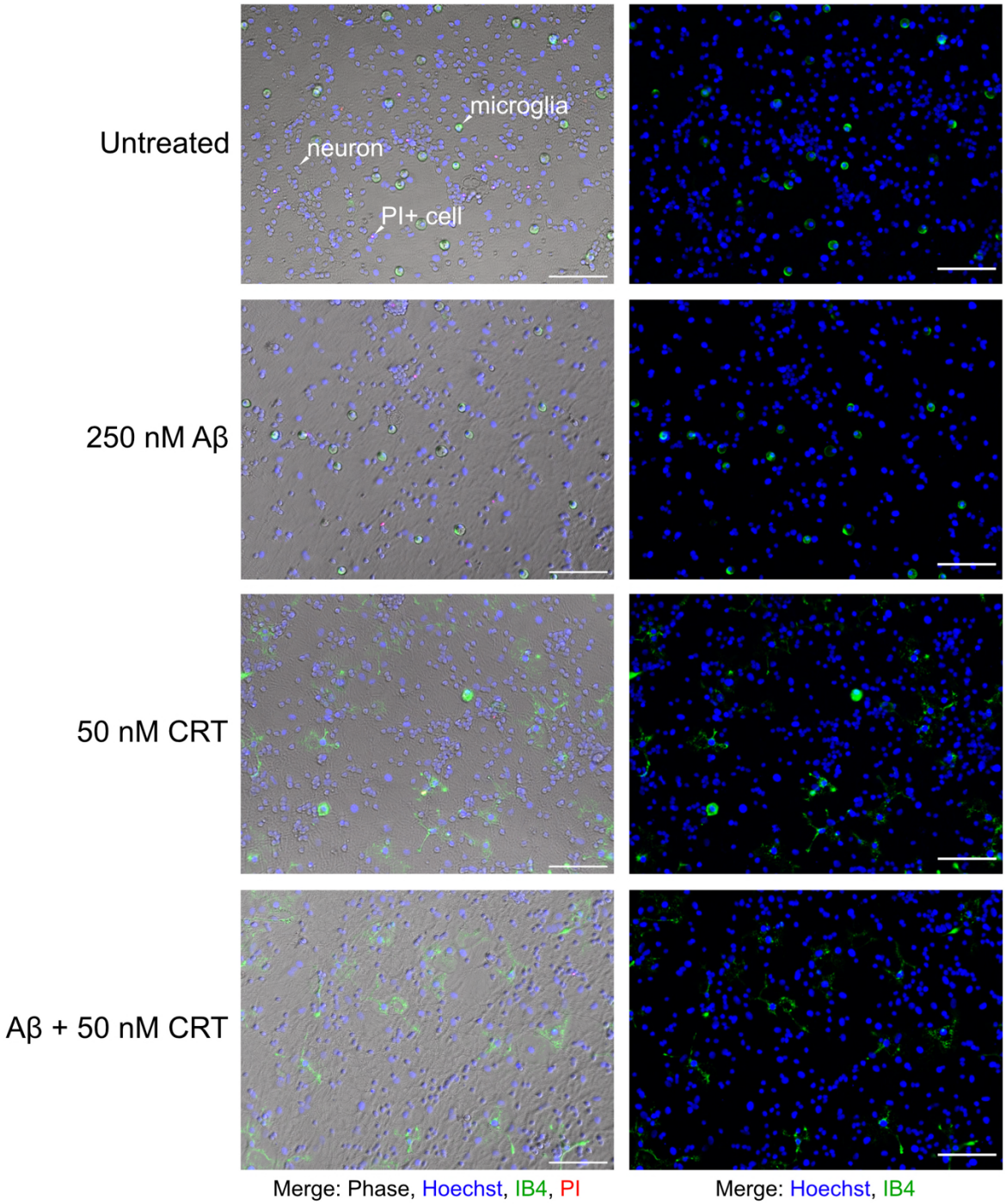

Figure 4.4. Representative microscopy images of primary neuronal-glial cultures 72 h after treatment ± 250 nM amyloid-β ± 50 nM calreticulin. Primary neuronal-glial cultures were treated ± 250 nM monomeric synthetic amyloid-β ± 50 nM recombinant human calreticulin (CRT). After 72 h, cultures were stained with nuclear marker Hoechst 33342 (blue), microglial marker IB4 (green) and necrosis marker, propidium iodide (PI, red). Cultures were imaged and cells quantified (four 20x fields were analysed per condition with three technical repeats per independent experiment). Microglia were identified by IB4-positive staining. Neurons were distinguished from astrocytes through their nuclear morphology. Apoptotic neurons were recognised by chromatin-condensed nuclear morphology and necrotic cells were positively stained with PI. Scale bar = 100 μm.

I found that, 250 nM amyloid-β induced around 35 % loss of neurons (Figure 4.5). Addition of 2.5 nM calreticulin did not reduce neuronal loss, however, 10 nM calreticulin significantly inhibited amyloid-β induced neuronal loss, and 50 nM calreticulin almost completely prevented the amyloid-β induced loss of neurons (Figure 4.5). Addition of 50 nM calreticulin alone (in the absence of amyloid-β) had no effect on neuronal counts. Thus, extracellular calreticulin has neuroprotective effects.

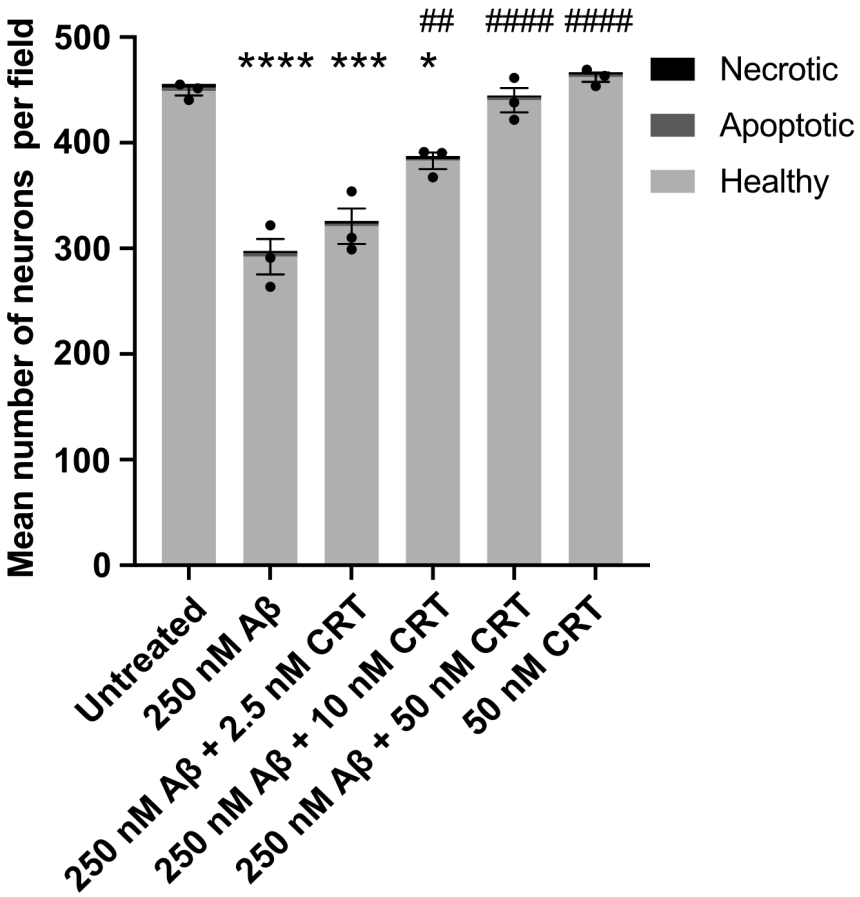

Figure 4.5. Calreticulin protects against amyloid-β induced neuronal loss. Mixed neuronal-glial cultures were treated ± 250 nM monomeric synthetic Aβ ± 2.5 nM, 10 nM, or 50 nM recombinant human CRT for 72 h at 37 °C. Neuronal counts were quantified from cultures stained with Hoechst 33342 (to identify nuclei and apoptotic cells), IB4 (to distinguish microglia) and PI (to identify necrotic

cells). Means ± SEM of 3 independent experiments are shown. Asterisk (*) indicate significance compared to untreated control (*: $p<0.05$, ***: $p<0.001$, ****: $p<0.0001$). Hash (#) indicate significance compared to Aβ only (##: $p<0.01$, ####: $p<0.0001$). Statistical comparisons were made by repeated measures one-way ANOVA followed by Šídák's multiple comparisons test.

4.2.5. Calreticulin promotes microglial proliferation and morphology changes

From the imaging data (Figure 4.4 & Figure 4.5A), I observed that calreticulin affected the number of microglia and dramatically altered the morphology of the microglia in the mixed neuronal-glial cultures, both factors which could indicate microglial activation. Quantification of these observations in Figure 4.5B & C, revealed that treatment with 50 nM calreticulin alone (in the absence of amyloid-β) had no effect on microglial number, but did affect microglial morphology, as microglia became flatter with increased area. There was an increase in both the number of microglia and microglial area when cultures were treated with calreticulin (10 nM or 50 nM) in the presence of 250 nM amyloid-β. Together with the neuroprotective effects described above, these results suggest that calreticulin activates microglia in a way that is not detrimental to neurons.

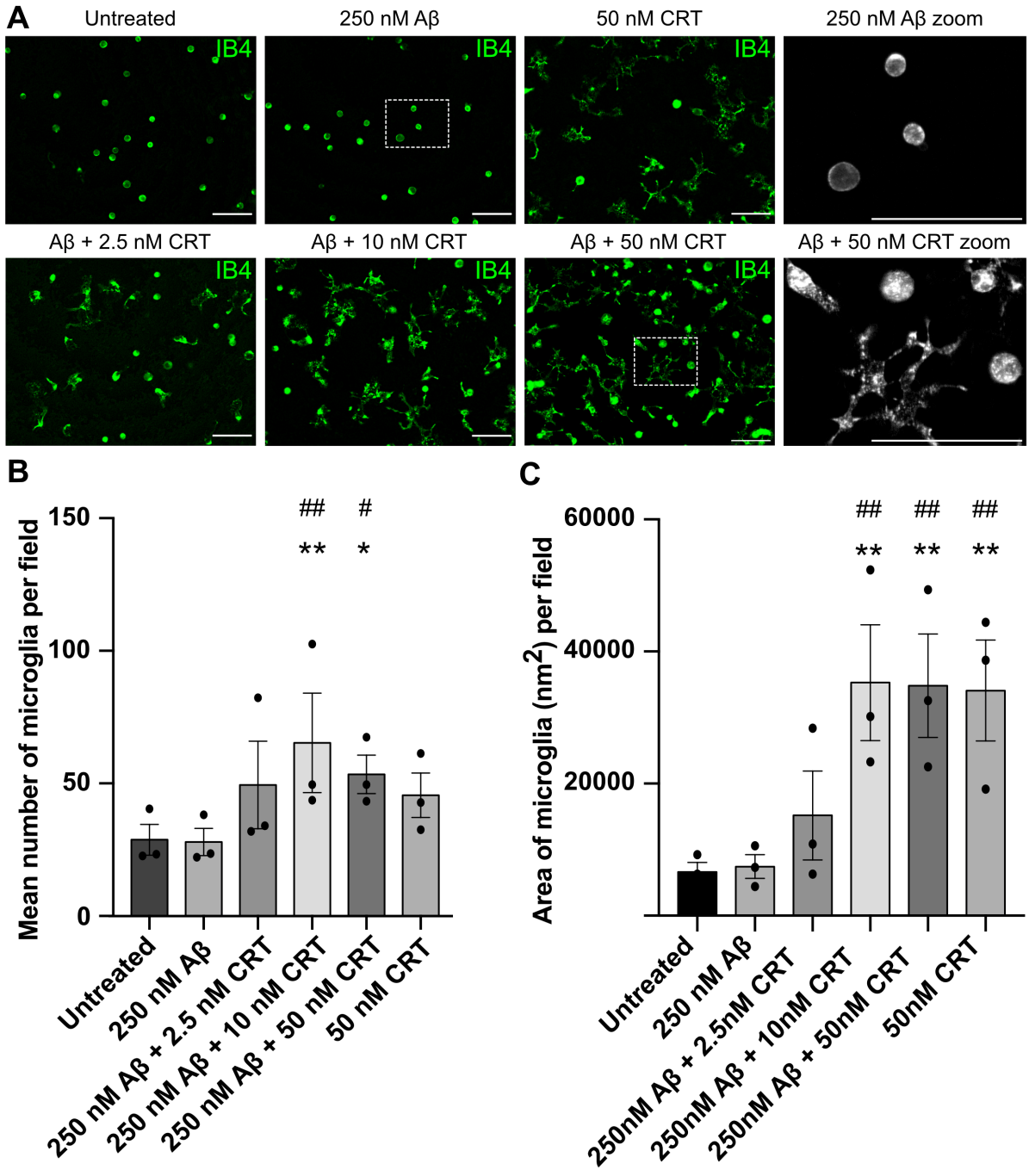

Figure 4.6. Calreticulin promotes microglial proliferation and morphology changes. (A) Mixed neuronal-glial co-cultures were treated ± monomeric 250 nM Aβ and ± 2.5 nM, 10 nM, or 50 nM calreticulin (CRT) for 72 h at 37 °C. Cultures were stained with IB4 (green, to identify microglia), PI (to identify necrotic cells (not shown)), Hoechst 33342 (to identify nuclei and apoptotic cells (not shown)) and imaged, representative fields shown. White boxes correspond to zoomed greyscale images for 250 nM Aβ and Aβ + 50 nM CRT. Scale bars = 100 µm. (B) Microglial counts quantified from (A). (C) Area of field stained with IB4 (i.e., microglial area), quantified from (A). (B & C) Means ± SEM of 3 independent experiments are shown. Asterisk (*) indicate significance compared to untreated control (*: $p<0.05$, **: $p<0.01$). Hash (#) indicate significance compared to Aβ only (#: $p<0.05$, ##: $p<0.01$). Statistical comparisons were made by repeated measures one-way ANOVA followed by Šídák's multiple comparisons test.

4.3. Discussion

Amyloid-β is implicated in the pathology of AD through multiple mechanisms, including direct neurotoxicity and microglial activation. Microglia phagocytose and digest amyloid-β resulting in its clearance (Lai and McLaurin, 2012) however, microglial activation by amyloid-β may contribute to tissue damage (Akiyama *et al.*, 2000). It is now widely accepted that soluble amyloid-β oligomers play a crucial role in pathology, rather than the fibril plaques that are observed post-mortem in AD-patient brains (Zhu, Lin and Lal, 2000). In fact, oligomeric species elicit toxicity via different mechanisms, dependent on their size, with smaller aggregates affecting membrane permeability and larger aggregates inducing toxicity via an increased inflammatory response (De *et al.*, 2019). Hence, selective inhibition of amyloid-β oligomer formation is a potential therapeutic target.

The majority of amyloid-β is chaperone-bound in the CSF of non-AD brains (Erickson *et al.*, 2005). As an ER-resident chaperone that has been found bound to extracellular amyloid-β (Erickson *et al.*, 2005), it is possible that calreticulin acts as an extracellular chaperone to keep amyloid-β in solution or promote its degradation. In this chapter, I assessed the potential extracellular chaperone function of calreticulin on amyloid-β aggregation and subsequently investigated whether calreticulin modulates amyloid-β-induced neuronal loss *in vitro*.

Amyloid-β aggregation is believed to occur via a nucleated polymerisation mechanism. Monomers, soluble oligomers, and large oligomers are relatively unstructured, but upon fibril formation, conformational changes occur to form structured β-sheet elements (Chen *et al.*, 2017). The β-sheet-rich structures react strongly with the ThT dye to yield a bright fluorescence. Hence. I investigated whether calreticulin modulates amyloid-β aggregation by measuring changes in ThT fluorescence over time. Consistent with previous findings (Kitauchi and Sakono, 2016), calreticulin affected amyloid-β fibrillisation in a concentration-dependent manner. Expanding on the observations made by Kitauchi and Sakono, I found that calreticulin folded amyloid-β into aggregates with less β-sheet structure and reduced overall fibrillisation, suggesting that calreticulin might act a chaperone to prevent fibril formation.

As oligomeric amyloid-β is widely considered to be more neurotoxic than amyloid-β fibrils (Amin and Harris, 2021), I assessed the effect of calreticulin on amyloid-β oligomer formation by TEM analysis and found that calreticulin increased the formation of larger oligomeric structures. These effects on fibrillisation and oligomerisation are consistent with calreticulin acting as an extracellular chaperone for amyloid-β. Others have shown that several chaperones, including: clusterin, haptoglobin and α2-macroglobulin block amyloid-β

induced neurotoxicity by binding to amyloid-β oligomers and promoting their assembly into larger oligomeric species with consequent shielding of the reactive surfaces and reduced fibrillisation (Mannini *et al.*, 2012; Narayan *et al.*, 2012). My findings suggest calreticulin might function in a similar way, supporting the idea that calreticulin acts as an extracellular chaperone for amyloid-β. Once amyloid fibrils are formed, molecular chaperones may act to reverse protein aggregation. Indeed, disaggregating chaperones for amyloidogenic proteins have been identified, including lipocalin-type prostaglandin D synthase (Low *et al.*, 2023). It would be interesting to evaluate whether calreticulin interacts with fibrillar amyloid-β and, if it has a reversal effect on fibrillisation.

Nanomolar levels of calreticulin are released from mechanically stressed primary neurons, apoptotic microglia and microglia under ER- or LPS-induced stress (Cockram, Puigdellívol and Brown, 2019; Reid *et al.*, 2022). It can then chemoattract and activate microglia to release pro-inflammatory cytokines and potentially restore homeostasis of damaged tissues (Reid *et al.*, 2022) and, has been shown to opsonise target cells, facilitating their recognition and phagocytosis by microglia (Cockram, Puigdellívol and Brown, 2019). Furthermore, nanomolar concentrations of amyloid-β induce neuronal loss mediated by microglia in mixed neuronal-glial cultures (Neniskyte, Neher and Brown, 2011). As calreticulin can activate microglia and alter the aggregation state of amyloid-β, I tested whether calreticulin affected amyloid-β induced neurotoxicity in mixed neuronal-glial cultures and found that nanomolar levels of calreticulin can prevent neuronal loss.

I did not investigate the mechanism by which calreticulin was neuroprotective, however, it could be that calreticulin interferes with amyloid-β aggregation or microglial activation. Neuroprotection could be attributed to calreticulin: 1) folding amyloid-β into a less neurotoxic form, protecting against direct amyloid-β induced neuronal loss, 2) binding and opsonising amyloid-β to promote its uptake by microglia, thereby reducing its neurotoxic effects, 3) inflammatory activating microglia to promote uptake and degradation of amyloid-β, 4) directly blocking microglial phagocytosis of neurons, or indeed a mixture of these suggested mechanisms.

Microglial morphology can change dramatically in response to stimuli. Generally, in healthy brains, microglia have extended, motile processes that allow them to monitor the brain parenchyma. In response to pathogens, stress and injury, microglia become more amoeboid as they retract their processes, promoting infiltration of the site of damage or infection (Torres-Platas *et al.*, 2014; Okajima and Tsuruta, 2018). My results show that calreticulin affected the morphology of microglia in mixed neuronal-glial cultures, with microglia transitioning from a spheroidal to a ramified shape, with significantly increased area. This is contrary to the morphological shift often described *in vivo,* but not uncommon *in vitro*. Our group and others have observed that, in the absence of a stimulus, cultured BV-2 and primary microglia are

more amoeboid, becoming ramified and attached to the culture plate in response to stimuli including: LPS, ATP, TNFα and IFNγ (Wollmer *et al.*, 2001; Lively and Schlichter, 2018; Goshi *et al.*, 2020). My observations suggest that calreticulin may activate microglia, causing them to assume a more ramified shape *in vitro.* However, microglia can be activated into a variety of different states (Paolicelli *et al.*, 2022). The morphology of microglia in the presence of calreticulin is unusual, and accompanied by neuroprotection, rather than neurotoxicity. Thus, it is possible that calreticulin activates microglia into a neuroprotective state, or blocks activation of microglia into a neurotoxic state. This would require further investigation.

Microglial proliferation is often regarded as a sign of activation and in a murine model for amyloidosis, microglial proliferation is significantly increased (Füger *et al.*, 2017). I found that calreticulin addition in the absence of amyloid-β had no significant effect on microglial number in mixed neuron-glial cultures, but co-treatment with calreticulin and amyloid-β increased microglial number. Therefore, the microglial proliferation observed is likely a result of the amyloid-β treatment rather than addition of calreticulin in these mixed neuronal-glial cultures. Indeed, calreticulin has been found to inhibit the proliferation of a variety of cell types (Pike *et al.*, 1999; Alur *et al.*, 2009; Weng *et al.*, 2015). It is widely debated whether microglial proliferation is beneficial or detrimental and this may depend on several factors including the type of insult (i.e., disease state, type of injury or infection). Inhibiting proliferation has been shown to slow neuronal damage and disease progression, and improve cognitive function after injury (Gómez-Nicola *et al.*, 2013; Poulen *et al.*, 2021). Yet, in cases of coronavirus infection, microglial depletion delays infection resolution and prevents remyelination (Wheeler *et al.*, 2018; Sariol *et al.*, 2020). It is known that there are multiple forms of microglial activation (Town, Nikolic and Tan, 2005; Dubbelaar *et al.*, 2018), some neurotoxic and others neuroprotective (Chen and Trapp, 2016; Bellver-Landete *et al.*, 2019). Hence, it is not straightforward to assume that activation coincides with proliferation, or that they are inevitably neurotoxic. In fact, my results suggest calreticulin activates microglia without being neurotoxic. A mechanism to explain how calreticulin activates microglia remains unknown. Other work in our lab has demonstrated that calreticulin-induced activation is not mediated by LRP1, as inhibition of LRP1 by LRPAP1 (LRP-associated protein 1) was not sufficient to prevent microglial activation (Reid *et al.*, 2022). Alternatively, calreticulin can induce the release of a soluble form of LRP1 (sLRP1), which can induce cytokine release (Brifault *et al.*, 2017). It could be that sLRP1 release and subsequent cytokine production results in microglial activation. Furthermore, research has shown that a recombinant fragment of calreticulin can promote the release of cytokines from human myeloid cells via TLR4/CD14 (Li *et al.*, 2015), so calreticulin may activate microglia in a TLR4/CD14-dependent manner. Nevertheless. a mechanism by which calreticulin activates microglia is yet to be elucidated and is the subject of ongoing work in the lab.

In summary, neurons and microglia can release calreticulin in response to multiple stressors, resulting in nanomolar levels of extracellular calreticulin. Nanomolar calreticulin can induce microglial activation, and interfere with amyloid-β aggregation and associated neurotoxicity

(Figure 4.7). It may be that calreticulin acts as an extracellular chaperone and neuroprotectant by beneficially activating microglia. In mice, a reduction in calreticulin expression in ageing neurons and glial cells has been associated with cognitive decline (Pawlowski *et al.*, 2009) and increased accumulation of misfolded proteins (Yang *et al.*, 2008). Moreover, the level of calreticulin found in the CSF from AD patients is significantly lower than that in healthy individuals (Lin, Cao and Gao, 2014). Hence, increasing extracellular calreticulin levels in the brain might be beneficial in brain pathologies, such as AD. While further work is required to understand the underlying mechanisms of neuroprotection by calreticulin, it would be interesting to assess whether the effect on neuronal loss I have seen *in vitro* can be recapitulated *in vivo*.

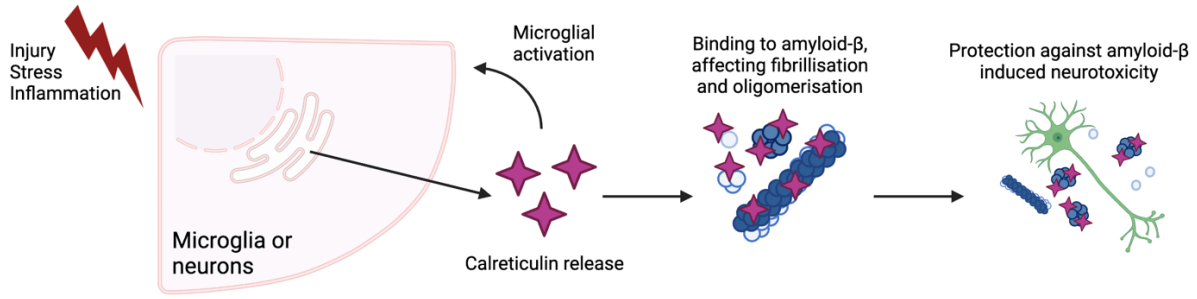

Figure 4.7. Summary of Chapter 4. In response to stress, injury and inflammation, neurons and microglia can release calreticulin, resulting in nanomolar levels of extracellular calreticulin. Nanomolar calreticulin can induce microglial activation, and bind to and alter amyloid-β aggregation and associated neurotoxicity. Figure created in Biorender.com.

5 BETA-GALACTOSIDASE IS RELEASED FROM INFLAMMATORY ACTIVATED MICROGLIA

All data presented in this chapter is my own work or the result of collaborative experiments performed by myself and Jacob Dundee, another PhD student in the Brown lab. Data obtained from collaborative work is shown in Supplementary Figure A9.3. Some content is currently in review for publication in: Kitchener EJA, Dundee JM and Brown GC. 'Activated microglia release β-galactosidase that promotes inflammatory neurodegeneration'.

5.1 Introduction

Gangliosides are sialic-acid containing glycosphingolipids that regulate multiple cellular processes, including proliferation, differentiation and inflammation (Mutoh *et al.*, 1995; Fukumoto *et al.*, 2000; Kim *et al.*, 2002; Jou *et al.*, 2006). Gangliosides are particularly abundant in neuronal cell membranes where they are implicated in brain physiology and pathology (Derry and Wolfe, 1967; Sipione *et al.*, 2020). The ganglioside composition at the plasma membrane is dynamic due to the constant action of biosynthesis, catabolism and, complex trafficking mechanisms; disruption of any of these pathways can affect glycosphingolipid content and subsequently affect neuronal differentiation and cause neurodegeneration (Aureli *et al.*, 2023). The initial understanding that glycosphingolipid biosynthetic enzymes (glycotransferases) and catabolic enzymes (glycohydrolases) are exclusively located in the Golgi apparatus and lysosomes, respectively, has been challenged by recent evidence that various glycohydrolase and glycotransferase enzymes are found at the plasma membrane where they may remodel membrane composition (Aureli, Loberto, Chigorno, *et al.*, 2011).

Glycohydrolase enzymes include sialidases (e.g., Neu1), β-glucocerebrosidase, β-hexosaminidase and β-galactosidase. Sialidase enzymes are some of the most well characterised glycohydrolase enzymes. Neu1 is described to hydrolyse terminal sialic acid residues from glycolipids and glycoproteins, to reveal terminal galactose residues, which are then hydrolysed by β-galactosidase (Alpers, 1969; Asp and Dahlqvist, 1972; Distler and Jourdian, 1973; Sandhoff and Sandhoff, 2018). The majority of human Neu1 and β-galactosidase are located within the lysosomes, where they form a complex with protective protein cathepsin A (PPCA), which protects both β-galactosidase and neuraminidase from intra-lysosomal proteolysis (Verheijen *et al.*, 1985). However, it has been shown that the localisation of certain glycohydrolase enzymes varies according to different stimuli. Neu1 has been found associated with the plasma membrane as the result of lysosomal fusion mechanisms linked to plasma membrane repair processes (Reddy, Caler and Andrews, 2001) and lysosomal exocytosis due to an excess of intracellular lysosomes (Samarani *et al.*, 2018).

Our lab recently reported that activation of microglia, the resident macrophages in the brain, resulted in the translocation of Neu1 to the cell surface and extracellular space by lysosomal exocytosis (Allendorf and Brown, 2022). As Neu1 can be structurally and functionally coupled to β-galactosidase (Verheijen *et al.*, 1985), it may be that β-galactosidase is also released by activated microglia. Chronic activation of microglia can be damaging to neurons and is implicated in many pathologies, including AD and PD (Thameem Dheen, Kaur and Ling, 2007). Furthermore, with ageing, microglia may become dystrophic or senescent (Streit *et al.*, 2004). Senescent cells are often characterised by increased β-galactosidase activity and lysosomal mass (Dimri *et al.*, 1995; Kurz *et al.*, 2000). The accumulation of senescent cells in neurodegenerative diseases, including AD and PD (Baker and Petersen, 2018; Hu et al., 2021) suggests a contribution of senescence to the pathophysiology of these disorders, yet little is known about the role of senescent cells in actively driving neurodegenerative processes. Hence, glycohydrolase enzymes, such as β-galactosidase, might be released by microglia under inflammatory conditions or as a result of ageing and, if so, might affect neurons or neurodegeneration. In this chapter, I sought to investigate whether, and in what conditions, microglia could release β-galactosidase to the cell surface or extracellularly.

5.2 Results

5.2.1 β-Galactosidase activity can be measured using 4-methylumbelliferyl-β-D-galactopyranoside

Human β-galactosidase catalyses the hydrolysis of terminal β-galactose residues from glycoproteins, sphingolipids, and keratan sulphate (Alpers, 1969; Asp and Dahlqvist, 1972; Distler and Jourdian, 1973). A number of synthetic compounds, each with a β-D-galactopyranoside moiety, have been developed which when hydrolysed by active β-galactosidase produce a measurable fluorescent product that can be used to trace β-galactosidase activity in fluorescence-based assays. One such compound is 4-methylumbelliferyl-β-D-galactopyranoside (MUG) (Figure 5.1A). Here, I validated the use of MUG, and determined the activity of an exogenous source of β-galactosidase from bovine liver, by monitoring the fluorescence output when a range of enzyme concentrations were incubated with 0.5 mg/mL MUG at 37 °C (Figure 5.1B). The resulting fluorescent output was dependent on the concentration of β-galactosidase, confirming the exogenous enzyme was active and suitable for use in future experiments.

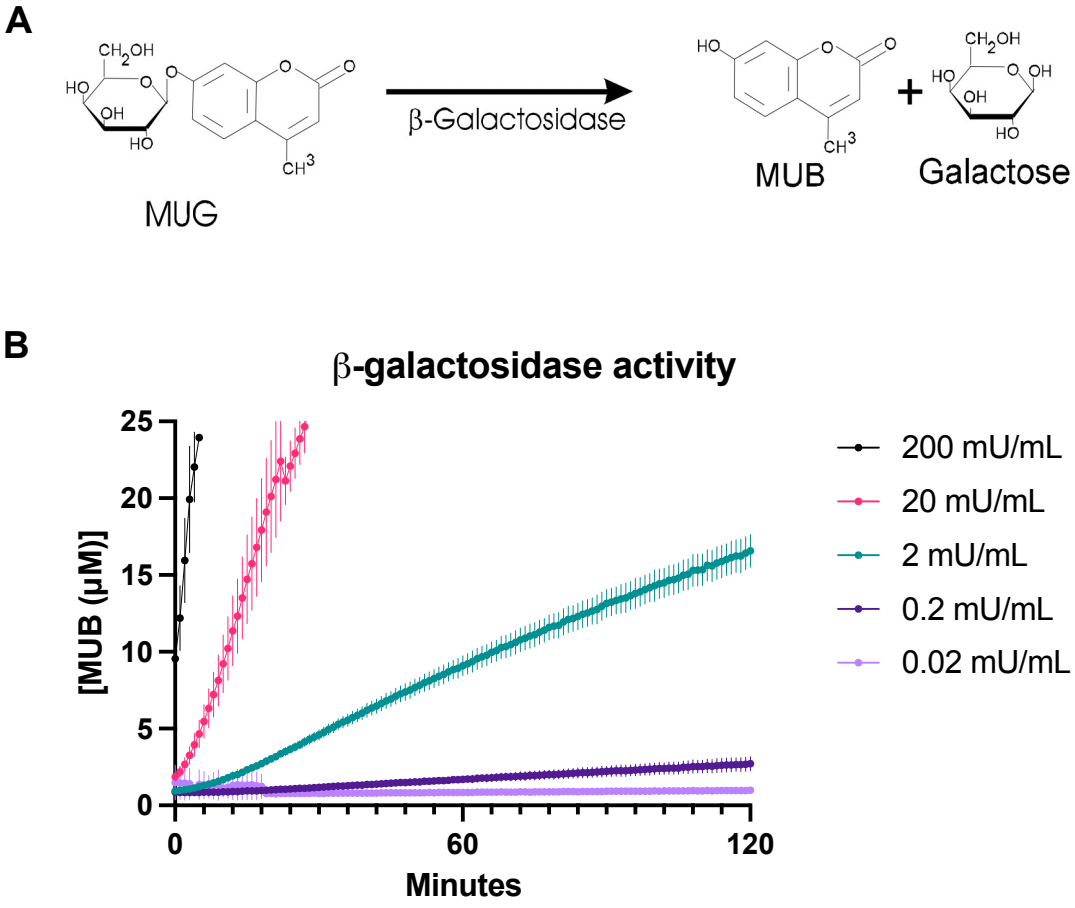

Figure 5.1. β-Galactosidase activity can be measured using 4-methylumbelliferyl-β-D-galactopyranoside. (A) β-Galactosidase activity was monitored using a fluorescence-based assay, whereby the fluorogenic substrate 4-methylumbelliferyl-β-D-galactopyranoside (MUG) is hydrolysed by active β-galactosidase to produce a fluorescent product 4-methylumbelliferone (MUB) and galactose. (B) Exogenous β-galactosidase from bovine liver at different concentrations was incubated with 0.5 mg/mL MUG at 37 °C and conversion to fluorescent product (MUB) was recorded over time. Mean ± SD of 3 technical repeats from one biological experiment are shown.

5.2.2 Chronic activation of microglia induces β-galactosidase activity and release

MUG is thought to have no significant cell permeability, and therefore when added extracellularly can be used to measure cell surface β-galactosidase activity (Aureli *et al.*, 2009). Thus, surface β-galactosidase activity was determined using the rate at which MUG was converted to 4-methylumbelliferone (MUB), when added to a monolayer of cultured cells and recorded over time (Figure 5.2A). Lysis of the cell, by disrupting membrane integrity, resulted in a dramatic increase in measured β-galactosidase activity, as shown by a steep incline of MUB production over time - much higher than from intact, vehicle-treated cells (Supplementary Figure A 9.2). This finding supports the theory that the MUG assay is measuring extracellular rather than intracellular β-galactosidase activity, and the assay can be used to measure β-galactosidase release.

β-Galactosidase is found in a lysosomal multienzyme complex with Neu1 and protective protein cathepsin A (PPCA) (Verheijen *et al.*, 1985). Previous work has shown that Neu1 is released from activated microglia (Allendorf and Brown, 2022). To assess whether active β-galactosidase is also released from inflammatory activated cells, I treated BV-2 microglia for 18 h with LPS or PMA. LPS is known to cause inflammatory activation of microglia via TLR4 (Hines *et al.*, 2013), while PMA causes activation and senescence of microglia via protein kinase C (Cao *et al.*, 2020). LPS and PMA both induced a significant increase in β-galactosidase activity on the cell surface compared to vehicle control cells (Figure 5.2A & B).

In order to measure β-galactosidase release into the extracellular medium, β-galactosidase activity in supernatant was assessed by MUG to MUB conversion (Figure 5.2C & D). Again, both LPS and PMA treatment caused a significant increase in β-galactosidase activity in the supernatant compared to vehicle control cells.

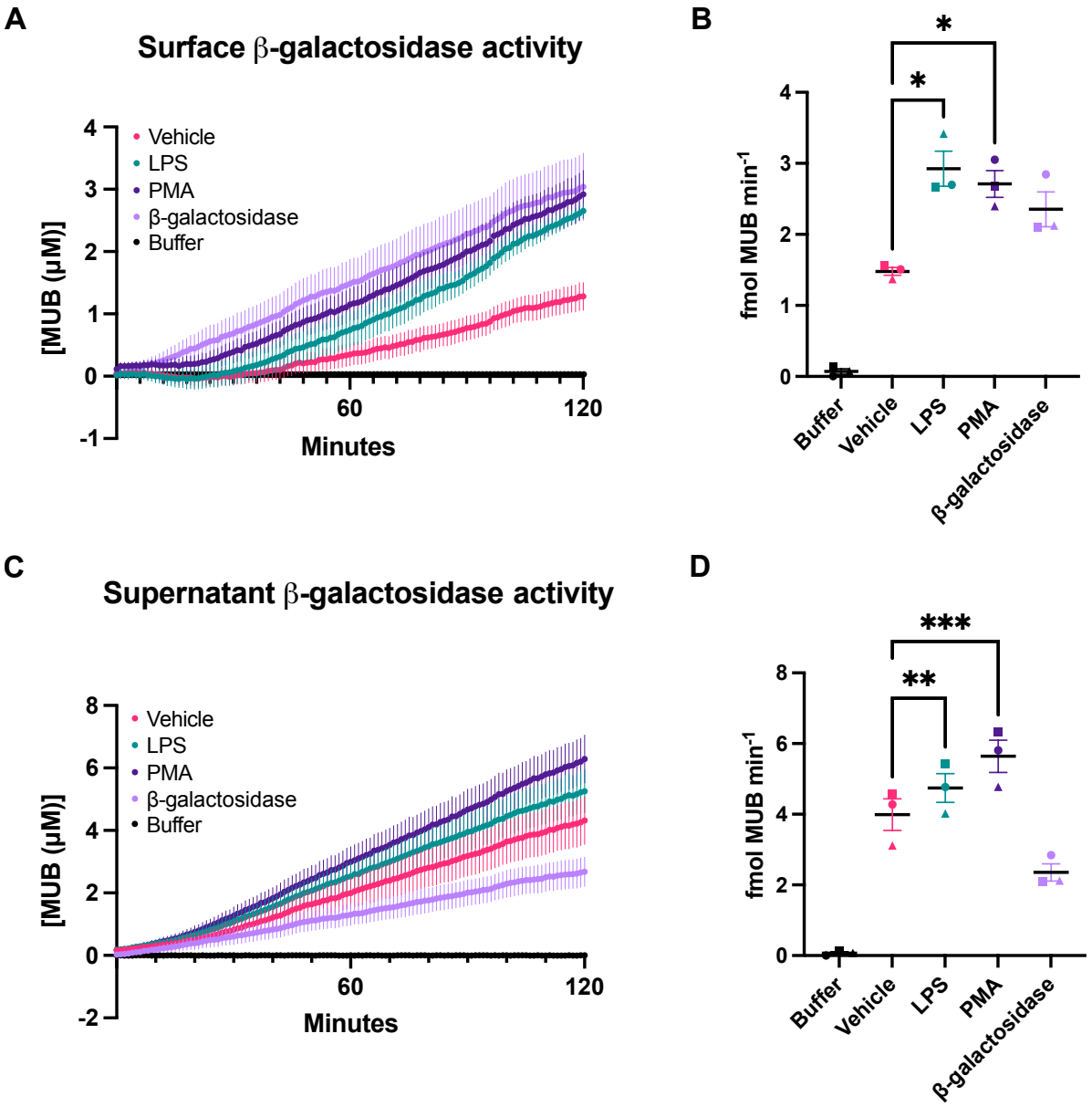

Figure 5.2. Chronic activation of BV-2 microglia induces β-galactosidase release. β-Galactosidase activity on the surface of BV-2 cells and released into the supernatant was determined using the rate at which MUG was converted to the fluorogenic product, MUB, when added to a monolayer of cells (at pH 7.0) or to the cleared supernatant (at pH 4.0) from these cells. Buffer was used as a negative control and denotes assay buffer at pH 7.0 in the absence of cells and β-galactosidase (0.2 mU/mL) was used as a positive control for the assay. (A) BV-2 cells were treated with vehicle (PBS), LPS (100 ng/mL) or PMA (100 nM) for 18 h, then supernatant was removed, and β-galactosidase activity was assayed on the cell surface. (B) The rate at which MUG was converted to MUB was determined from (A). (C) The supernatants of LPS and PMA treated cells from (A) were cleared and assayed for β-galactosidase activity and rate determined in (D). Data represents mean values ± SEM of at least 3 independent experiments. Statistical comparisons were made by repeated measures one-way ANOVA with Dunnett's post-hoc comparisons test. Asterisk (*) indicate significance compared to untreated control (*: $p<0.05$, **: $p<0.01$, ***: $p<0.001$).

To validate the MUG assay results and confirm that β-galactosidase protein is released from the cells into the medium, I assessed β-galactosidase protein concentration in the supernatants of LPS and PMA treated cells by ELISA. Indeed, LPS and PMA promoted a

large and significant increase in β-galactosidase concentration in the supernatants of treated cells compared to the vehicle control (Figure 5.3A), without inducing cell death (Figure 5.3B).

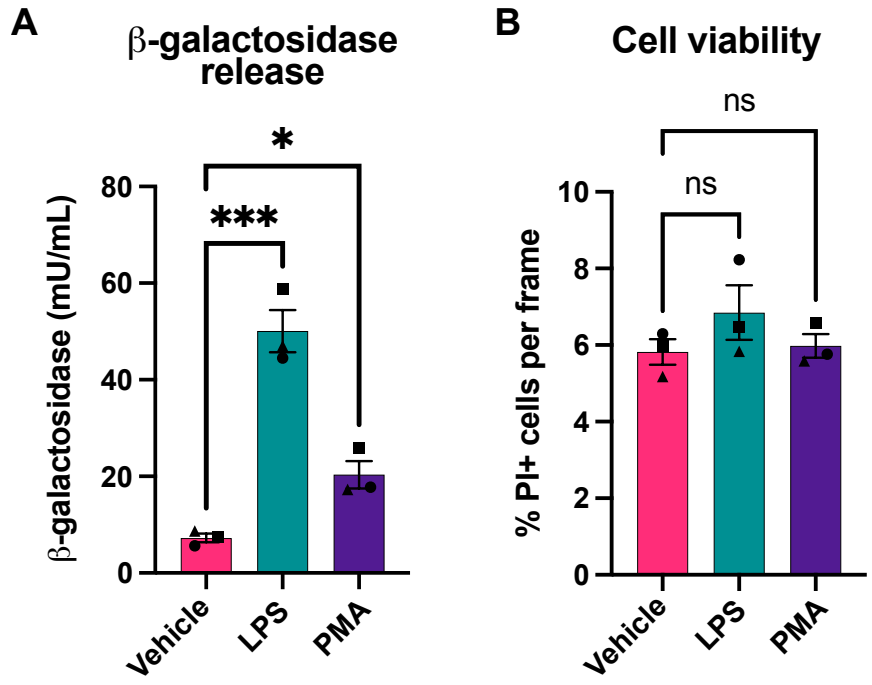

Figure 5.3. LPS and PMA promote β-galactosidase release from BV-2 microglia without affecting cell death. (A) BV-2 cells were treated with LPS (100 ng/mL) or PMA (100 nM) for 18 h, then supernatant was collected, and β-galactosidase concentration was determined by ELISA. (B) Cell viability of BV-2 microglia, post-treatment, was assessed by propidium iodide uptake and presented as % of total cells (determined by Hoechst staining). Data represents mean values ± SEM of 3 independent experiments. Statistical comparisons were made by repeated measures one-way ANOVA with Dunnett's post-hoc comparisons test. Asterisk (*) indicate significance compared to untreated control (ns: $p \geq 0.05$, *: $p < 0.05$, ***: $p < 0.001$).

5.2.3 Acute activation of microglia induces β-galactosidase activity

One potential cause of β-galactosidase release from lysosomes is lysosomal exocytosis, which can be induced by a rise in intracellular calcium. To induce a rise in intracellular calcium, ATP (a P2X7 agonist) or A23187 (a Ca^{2+} ionophore) were added to cells during the assay (i.e., 1 h after the assay had been initiated). β-Galactosidase activity released by cells was measured using MUG to MUB conversion, as above. Both treatment of BV-2 microglia with ATP or A23187, promoted an acute increase in β-galactosidase activity (Figure 5.4A & B), suggesting that lysosomal exocytosis may cause β-galactosidase release. Thus, activated microglia can release active β-galactosidase to their surface and into their extracellular environment.

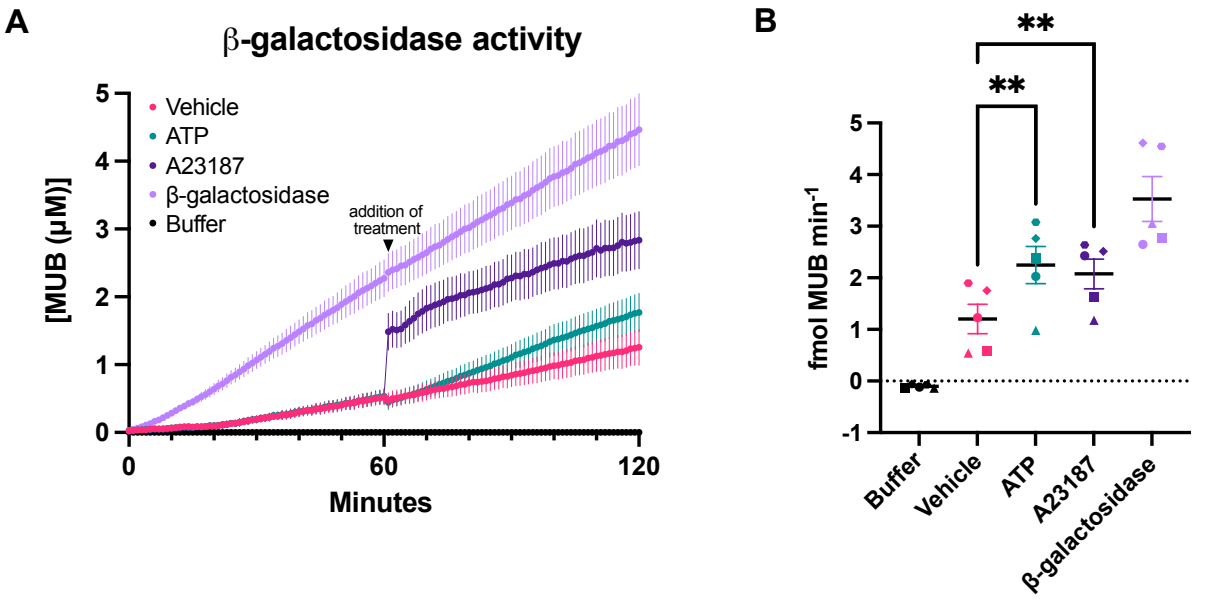

Figure 5.4. Acute activation of BV-2 microglia induces β-galactosidase release. β-Galactosidase activity on the surface of BV-2 cells and released into the supernatant was determined using the rate at which MUG was converted to the fluorogenic product, MUB, when added to a monolayer of cells (at pH 7.0). Buffer was used as a negative control and denotes assay buffer at pH 7.0 in the absence of cells and β-galactosidase (0.2 mU/mL) was used as a positive control for the assay. (A) Cells were acutely treated with ATP (1 mM) or A23187 (10 μM) 1 h after the initiation of the MUG assay and the rate of MUG-MUB conversion after treatment addition was calculated in (B). Data represents mean values ± SEM of at least 3 independent experiments. Statistical comparisons were made by repeated measures one-way ANOVA with Dunnett's post-hoc comparisons test. Asterisk (*) indicate significance compared to untreated control (**: $p<0.01$).

5.2.4 Activation of primary mixed neuronal-glial cultures and isolated primary microglia induces β-galactosidase protein release

As BV-2 microglia may diverge from primary cells as a result of transformation and/or mutation, we also tested whether activation by LPS or PMA could induce β-galactosidase release in mixed neuronal-glial cultures and primary microglia isolated from rat brain. As a major component of the cell wall of Gram-negative bacteria, LPS is commonly used in studies of immune cell activation and function (Paludan, 2000). PMA is a protein kinase C activator that can cause microglial activation through the up-regulation of inducible nitric oxide synthase (iNOS) expression and the posttranslational activation of nicotinamide adenine dinucleotide phosphate (NAPDH) oxidase (Czapiga and Colton, 1999). LPS and PMA induced a significant increase in β-galactosidase protein levels in the extracellular medium of mixed neuronal-glial cultures, measured after 72 h (Figure 5.5A). In isolated primary microglia PMA, but not LPS, induced a significant increase in β-galactosidase protein release (Figure 5.5B), measured after 24 h, without inducing cell death (Figure 5.5C).

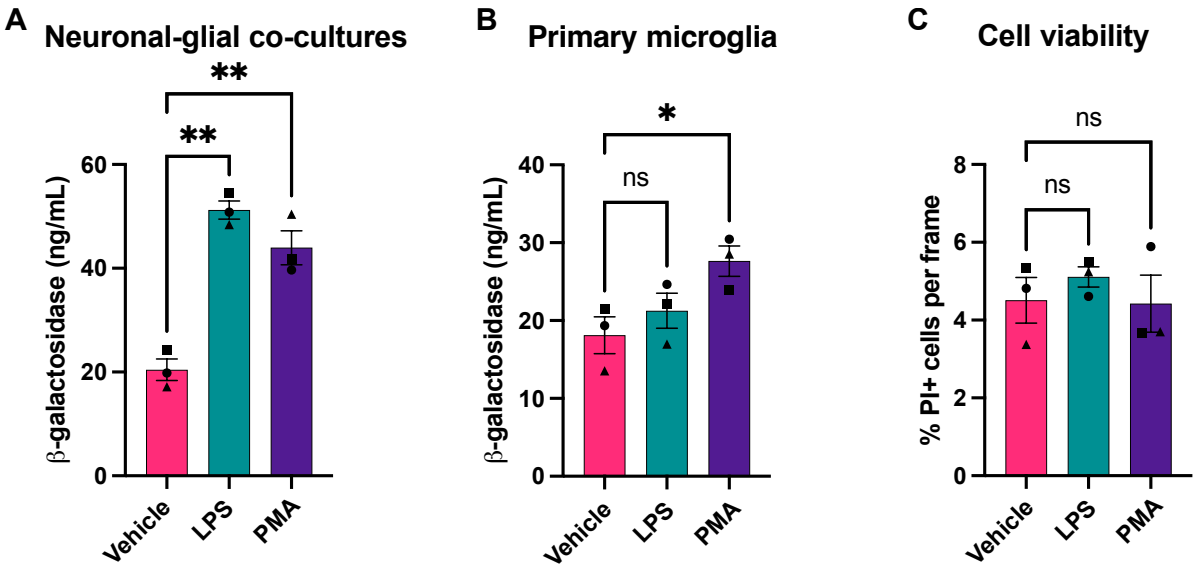

Figure 5.5. Activation of primary mixed neuronal-glial cultures and isolated primary microglia induces β-galactosidase protein release. (A) Mixed neuronal-glial cultures were treated with LPS (100 ng/mL) or PMA (100 nM) for 72 h and supernatants were assessed for β-galactosidase levels by ELISA. (B) Isolated primary microglia were treated with LPS (100 ng/mL) or PMA (100 nM) for 18 h and β-galactosidase levels assessed by ELISA. (C) Viability of primary microglia, post-treatment, was assessed by propidium iodide uptake and presented as % of total cells (determined by Hoechst staining). Data represents mean values ± SEM of 3 independent experiments. Statistical comparisons were made by repeated measures one-way ANOVA with Dunnett's post-hoc comparisons test. Asterisk (*) indicate significance compared to untreated control (ns: $p \geq 0.05$, *: $p < 0.05$, **: $p < 0.01$).

5.2.5 Overexpression of p16 in CHME-3 microglia does not prevent proliferation

The accumulation of senescent cells in neurodegenerative diseases, including AD and PD, suggests the contribution of senescence in the pathophysiology of these disorders. Yet the mechanisms by which senescent cells contribute to disease are not understood. A characteristic marker of senescent cells is an increased number of lysosomes and activity of lysosomal β-galactosidase, often referred to as SA-β-galactosidase (Dimri *et al.*, 1995). It has been shown that lysosomal glycohydrolase β-glucocerebrosidase levels are increased in the culture medium of senescent melanoma cells (Rovira *et al.*, 2022). Hence, I wanted to assess whether β-galactosidase was released from senescent microglia. To generate an *in vitro* model of senescent microglia, I set out to induce cell cycle arrest in CHME-3 microglia cells by overexpressing p16, a key regulator of the pRb pathway. Using lentiviral transduction, p16 with a C-terminal FLAG-tag was expressed in CHME-3 microglia and, negative control cells were generated using an empty pWPI vector. The lentiviral transfer vector pWPI was used as it has a mammalian promotor sequence (elongation factor 1, EF1) and co-expresses GFP with the protein of interest. Successful transduction of CHME-3 cells was confirmed visually by expression of eGFP (Figure 5.6).

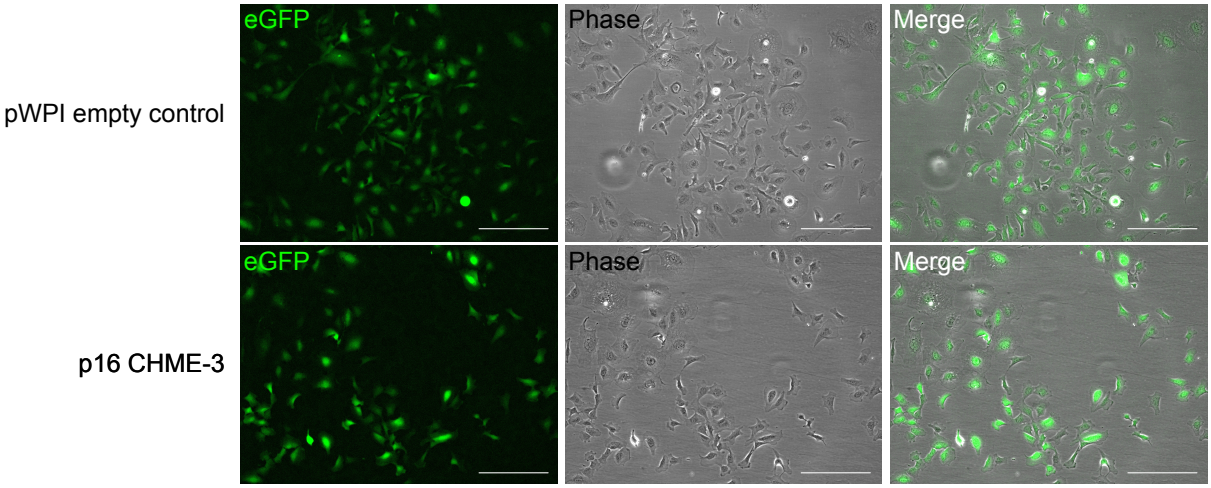

Figure 5.6. p16 expression in CHME-3 cells. Stable CHME-3 microglia cell-line was created using lentiviral transduction with a pWPI plasmid containing the gene of interest and eGFP. Fluorescence microscopy images of transduced CHME-3 microglia expressing eGFP confirm successful transduction. Scale bar = 100 μm.

CHME-3 microglia transduced with p16 clearly showed expression of the FLAG-tag (Figure 5.7A) compared to negative control cells (Figure 5.7B). Transfection efficiency was estimated to be ~95 %, by dividing the number of p16-FLAG-expressing cells by total cells as indicated by Hoechst nuclei staining (data not shown). Furthermore, p16 gene expression was determined by qPCR, with approximately 25-fold increase on endogenous levels, compared to empty-pWPI control cells (Figure 5.7C). Despite highly efficient overexpression of p16, proliferation of these cells was unaffected. Cells were left to reach confluency before being passaged several times. Finally, immunostaining for proliferation marker, Ki67, confirmed that p16 overexpression did not prevent proliferation of CHME-3 cells (Figure 5.7D & E), suggesting that inducing senescence in CHME-3 cells, by p16 overexpression, was not viable.

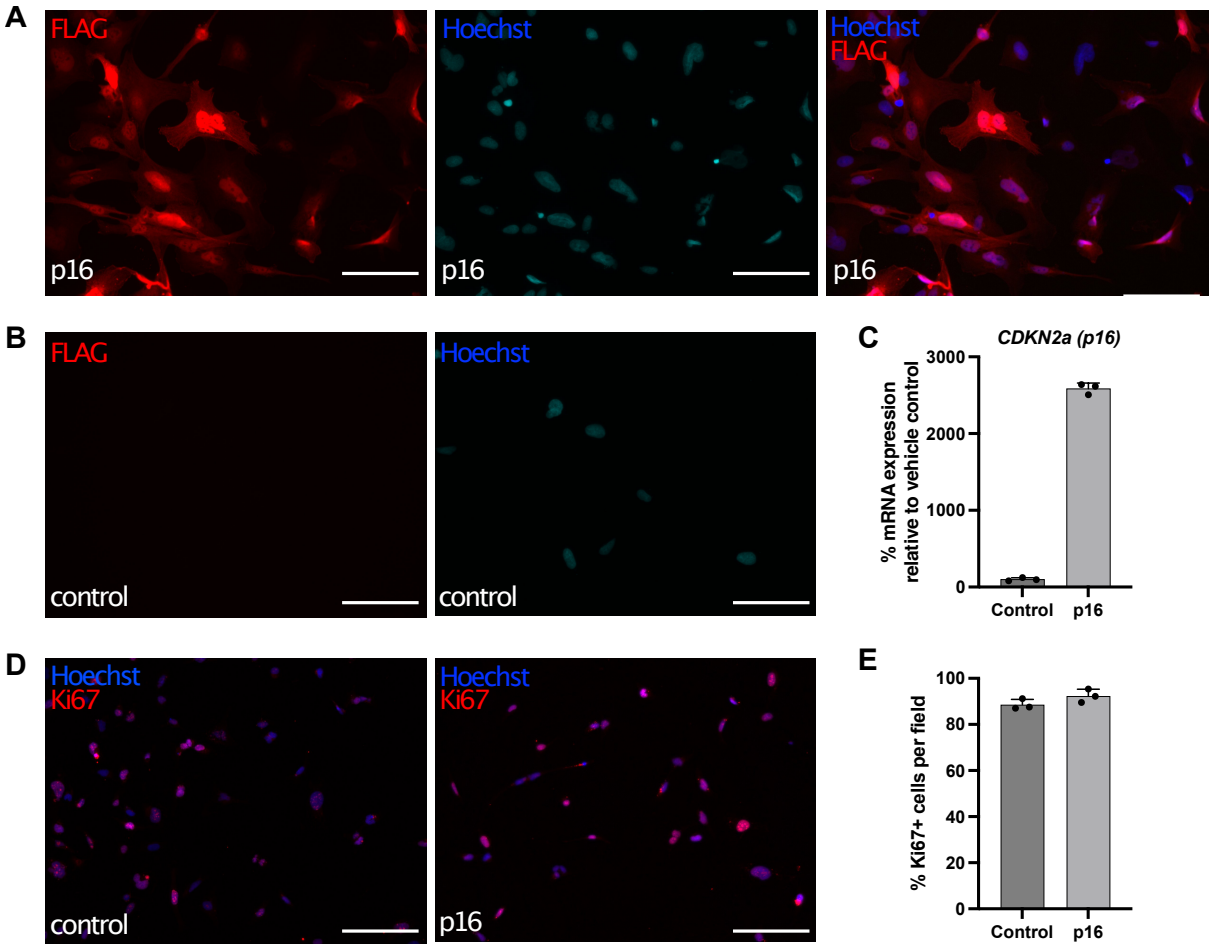

Figure 5.7. p16 expression in CHME-3 microglia does not prevent proliferation. CHME-3 microglia transduced with lentiviral plasmids for (A) p16 or (B) control (empty), were stained with anti-FLAG antibody (red) and Hoechst 33342 (blue). (C) Percentage change of p16 expression in transduced cells determined by qPCR. (D) Immunofluorescence of control- and p16-expressing cells stained for proliferation marker Ki67 (red) and Hoechst (blue), scale bar = 100 μm. (E) Quantification of Ki67 staining in transduced cells from (D). Data from one biological repeat, with triplicate technical repeats shown.

5.2.6 DNA-damage reagents fail to induce senescence in microglia

Next, I decided to try an alternative approach to induce senescence, by causing DNA-damage using chemical agents. Doxorubicin is thought to cause DNA damage by intercalating into DNA and by generating DNA-damaging free radicals (Kim *et al.*, 2006; Anders *et al.*, 2013). It has been shown to induce senescent characteristics in rat microglial cell line, CHME-5 (Marques, Johnson and Stolzing, 2020). Here, the ability of doxorubicin treatment to induce senescence in human CHME-3 microglia was investigated. Based on data from Marques *et al.*, I trialled 50 nM and 100 nM concentrations. Cells were treated for 48 h ± doxorubicin then further cultured in fresh media for 6 days (with a media change every 72 h). Imaging data showed that untreated cells continued to proliferate, however with doxorubicin treatment the number of cells observed after 8 days in culture was reduced and cell morphology was altered; doxorubicin treated cells were markedly larger, flatter and irregular in shape with granulated nuclei than control cells (Figure 5.8A). As major markers

for ageing and senescence, *CDNK2A (p16)* and *CDKN1A (p21)* expression were determined by qPCR. p16 expression levels were reduced in a dose-dependent manner, by ~25 % with 50 nM doxorubicin, and ~50 % with 100 nM treatment (Figure 5.8B). Conversely, p21 endogenous levels were almost 2-fold higher with both 50 nM and 100 nM doxorubicin treatment compared to control cells (Figure 5.8C).

SA-β-galactosidase activity has been considered the gold standard for identification of senescent cells since 1995 (Dimri *et al.*, 1995), so doxorubicin treated and untreated control cells were stained for SA-β-galactosidase using X-gal. β-Galactosidase activity should be detectable in senescent cells at pH 6.0 and cells stained at pH 4.0 act as a positive control, where eukaryotic lysosomal β-galactosidase is active in all cells (Dimri *et al.*, 1995). Doxorubicin treatment repeatedly failed to induce SA-β-galactosidase activity (indicated by blue staining) compared to the untreated control at pH 6.0 (Figure 5.8D). Notably, positive X-gal staining was not observed at pH 4.0., suggesting that staining with X-gal was unsuccessful and further optimisation was required.

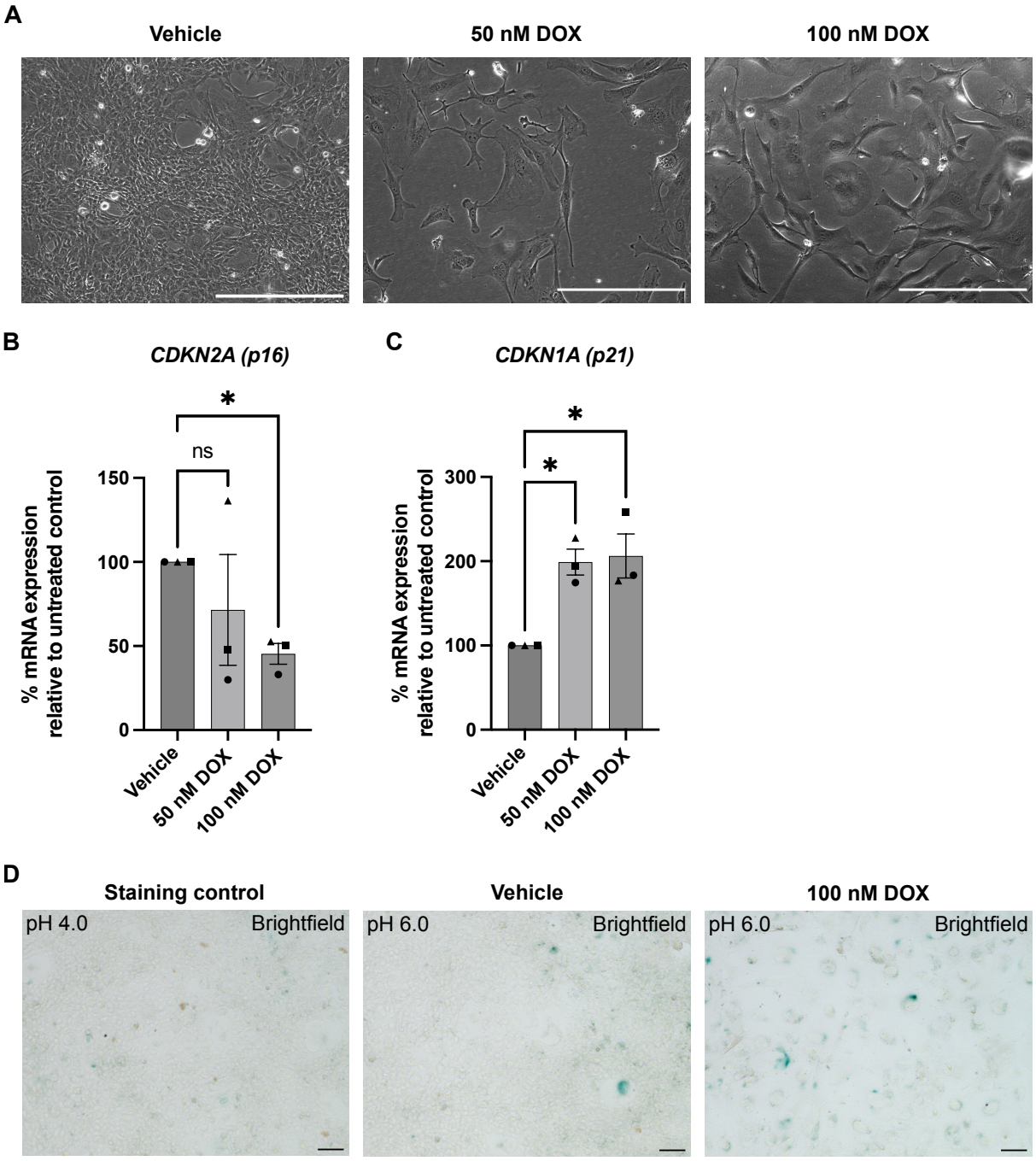

Figure 5.8. Doxorubicin treatment to induce senescence in CHME-3 microglia. CHME-3 microglia were treated with 50nM or 100 nM doxorubicin (DOX) for 48 h then cultured for a further 6 days in fresh DMEM. (A) Representative phase images of CHME-3 cells ± 50nM and 100 nM DOX following 48 h treatment and 6 days in culture. Scale bar = 100 μm. (B) Percentage change of p16 gene expression following DOX treatment, determined by qPCR. (C) Percentage change of p21 gene expression following DOX treatment, determined by qPCR. (D) Representative images of untreated and DOX treated cells stained for SA-β-galactosidase using X-gal at pH 4.0 (positive control) and pH 6.0, scale bar = 100 μm. Data in B & C represent mean values ± SEM of 3 independent experiments. Statistical comparisons were made by repeated measures one-way ANOVA with Dunnett's post-hoc comparisons test. Asterisk (*) indicate significance compared to untreated control (*: $p<0.05$).

Further experiments to optimise X-gal staining in CHME-3 microglia revealed that the absence of staining in the positive control (at pH 4.0) might be cell type specific as X-gal staining in murine

microglial cell line, BV-2, was successful at pH 4.0 (Figure 5.9). X-gal staining in BV-2 cells was clearly visible, with potent blue staining within cells observed at positive control pH 4.0 and relatively low staining at pH 6.0.

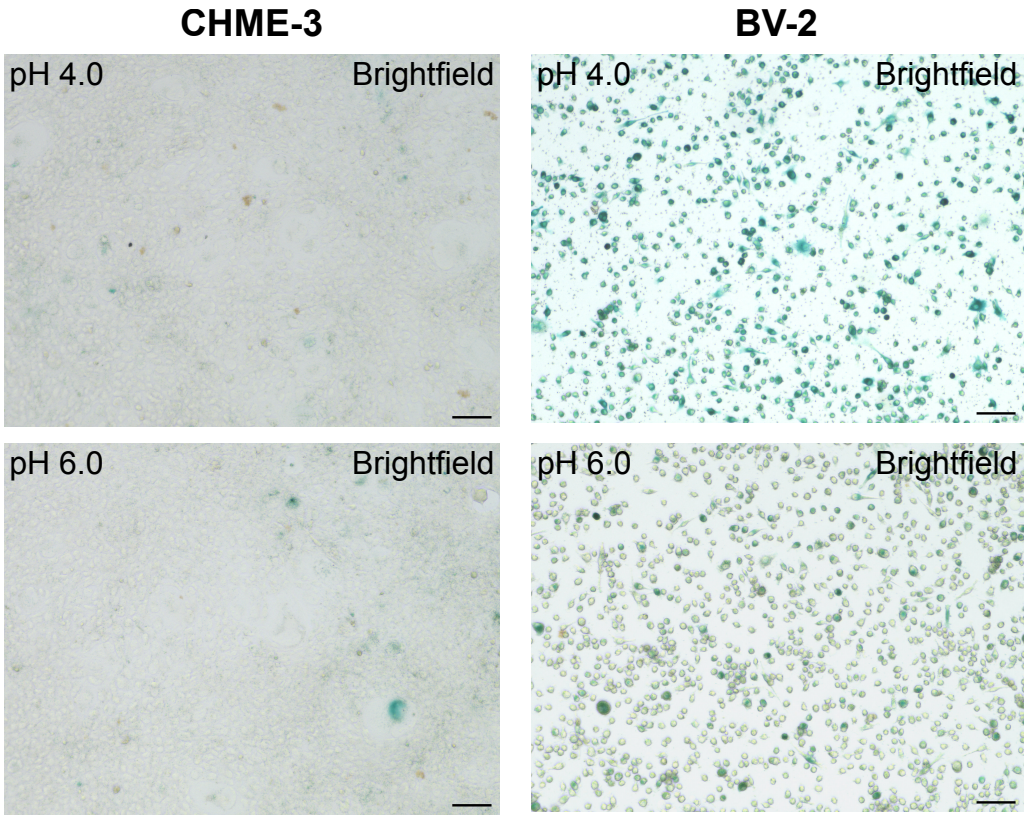

Figure 5.9. CHME-3 microglia stain have less X-gal staining than BV-2 microglia. Representative images of untreated CHME-3 and BV-2 cells stained for SA-β-galactosidase using X-gal at pH 4.0 (positive control for staining protocol) and pH 6.0, scale bar = 100 μm.

As SA-β-galactosidase staining by X-gal could be detected in BV-2 cells, I used BV-2s as an alternative microglial cell line for doxorubicin treatments. Our lab has often observed that BV-2 microglia are more sensitive to treatments than CHME-3 cells, therefore BV-2 cells were treated with 0 nM, 25 nM, and 50 nM doxorubicin to establish optimal treatment conditions (Figure 5.10A). 25 nM doxorubicin did not affect cell count after the 48 h treatment and a further 6 day recovery period compared to untreated control, suggesting that 25 nM doxorubicin treatment was not sufficient to prevent proliferation. 50 nM doxorubicin treatment reduced cell number compared to the untreated condition although this was not significant and high variability in the data made it difficult to draw a firm conclusion. Cell viability was also assessed (as measured by PI staining) and there was no significant difference in necrosis with increasing doxorubicin treatment. As multiple factors are often necessary to characterise senescent cells, *Cdkn1a (p21)* expression was determined by qPCR, but expression levels were not affected by doxorubicin treatment (Figure 5.10B). Cells were also assessed for SA-β-galactosidase activity, but doxorubicin treatment repeatedly failed to induce SA-β-galactosidase activity (indicated by blue staining) compared to the untreated

control at pH 6.0 (Figure 5.10C). Together, these results suggest that doxorubicin, at the concentrations tested, was unable to induce a senescence-like state and so an alternative approach, using bleomycin was trialled in BV-2 cells.

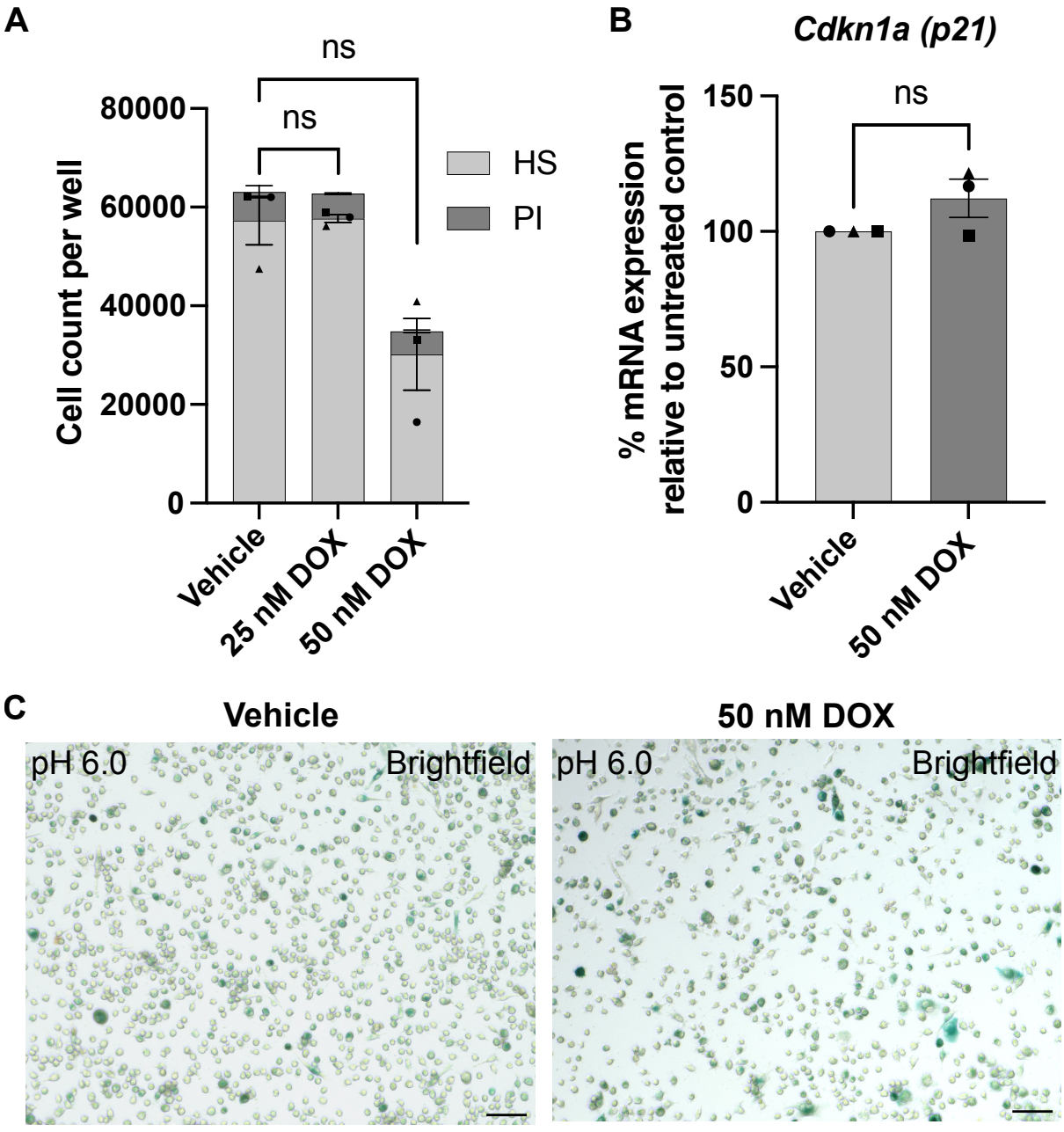

Figure 5.10. Doxorubicin treatment to induce senescence in BV-2 microglia. BV-2 microglia were treated with 0 nM, 25 nM or 50nM doxorubicin (DOX) for 48 h then cultured for a further 6 days (recovery period) in fresh DMEM. (A) After the treatment and recovery period, cells were stained with Hoechst (to identify nuclei) and propidium iodide (to identify necrotic cells), whole well images were taken, and cells counts quantified. (B) Percentage change of *Cdnk1a (p21)* gene expression following DOX treatment, determined by qPCR. (C) Representative images of untreated and DOX treated cells stained for SA-β-galactosidase using X-gal at pH 6.0, scale bar = 100 μm. Data in B & C represent mean values ± SEM of 3 independent experiments. Statistical comparisons were made on Hoechst

cell counts by repeated measures one-way ANOVA with Dunnett's post-hoc comparisons test. Asterisk (*) indicate significance compared to untreated control (*: $p<0.05$).

Bleomycin has been shown to induce senescence in primary microglia (Brelstaff *et al.*, 2021). Here, BV-2 cells were treated ± 50 μg/mL or 100 μg/mL bleomycin for 24 h. Cell viability after the treatment period was assessed by % PI uptake (Figure 5.11A). 100 μg/mL induced a significant amount of cell death, so was not used in further investigations. Although 50 μg/mL did increase % cell death, it was not significant. However, after fixation and staining for SA-β-galactosidase activity, no increase in SA-β-galactosidase was observed compared to untreated control (Figure 5.11B & C). Together, these results suggest that bleomycin, at the concentrations tested, did not induce senescence-like characteristics in BV-2 cells.

These results suggest that inducing senescence with DNA-damaging agents in CHME-3 and BV-2 cell lines is not sufficient to generate a reliable senescence model, especially for downstream experiments that would look to assess β-galactosidase activity and release. As bleomycin was used as a positive control to induce senescence in primary microglia by Brelstaff *et al.*, replicating the conditions described, I treated isolated primary microglia from neonatal rat brains with 50 μg/mL bleomycin for 12 h. However, this repeatedly failed to induce a sufficient increase in SA-β-galactosidase staining *in vitro* (data not shown).

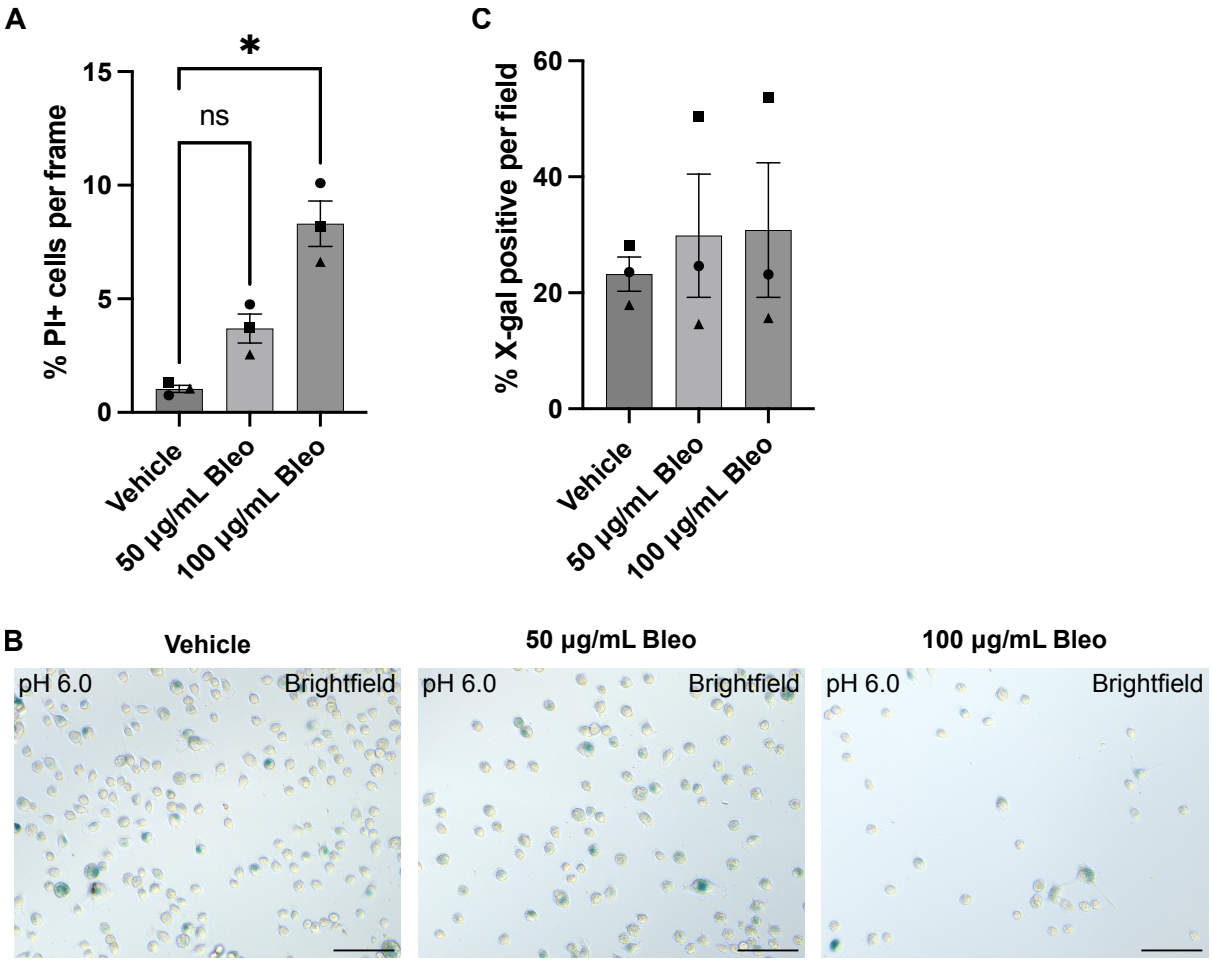

Figure 5.11. Bleomycin treatment to induce senescence in BV-2 microglia. BV-2 microglia were treated ± 50 µg/mL or 100 µg/mL bleomycin for 24 h. (A) After treatment, cells were stained with Hoechst (to identify nuclei) and propidium iodide (to identify necrotic cells) and cells counts quantified. (B) Representative images of untreated and bleomycin treated cells stained for SA-β-galactosidase using X-gal at pH 6.0, scale bar = 100 µm. quantified in (C). Data in A & C represent mean values ± SEM of 3 independent experiments. Statistical comparisons were made by repeated measures one-way ANOVA with Dunnett's post-hoc comparisons test. Asterisk (*) indicate significance compared to untreated control (*: $p<0.05$).

5.2.7 Young and aged brain sections do not have differential SA-β-galactosidase activity

As inducing senescence in cell lines and neonatal primary cells proved inconclusive, I assessed β-galactosidase activity in aged brain sections, previously prepared by other members of our lab. For this, I used fixed coronal brain sections from the brains of wild-type mice at 4- and 17- months-old and assessed SA-β-galactosidase activity by X-gal staining the sections. At pH 6.0 there was minimal SA-β-galactosidase activity (Figure 5.12A) and there was no difference observed between 4- and 17-month-old brains (Figure 5.12B).

It should be noted that these sections were prepared several months prior to the X-gal staining, and it may have been that the enzymatic activity of β-galactosidase was not

preserved, due to loss or degradation of the enzyme, potentially leading to false-negative results.

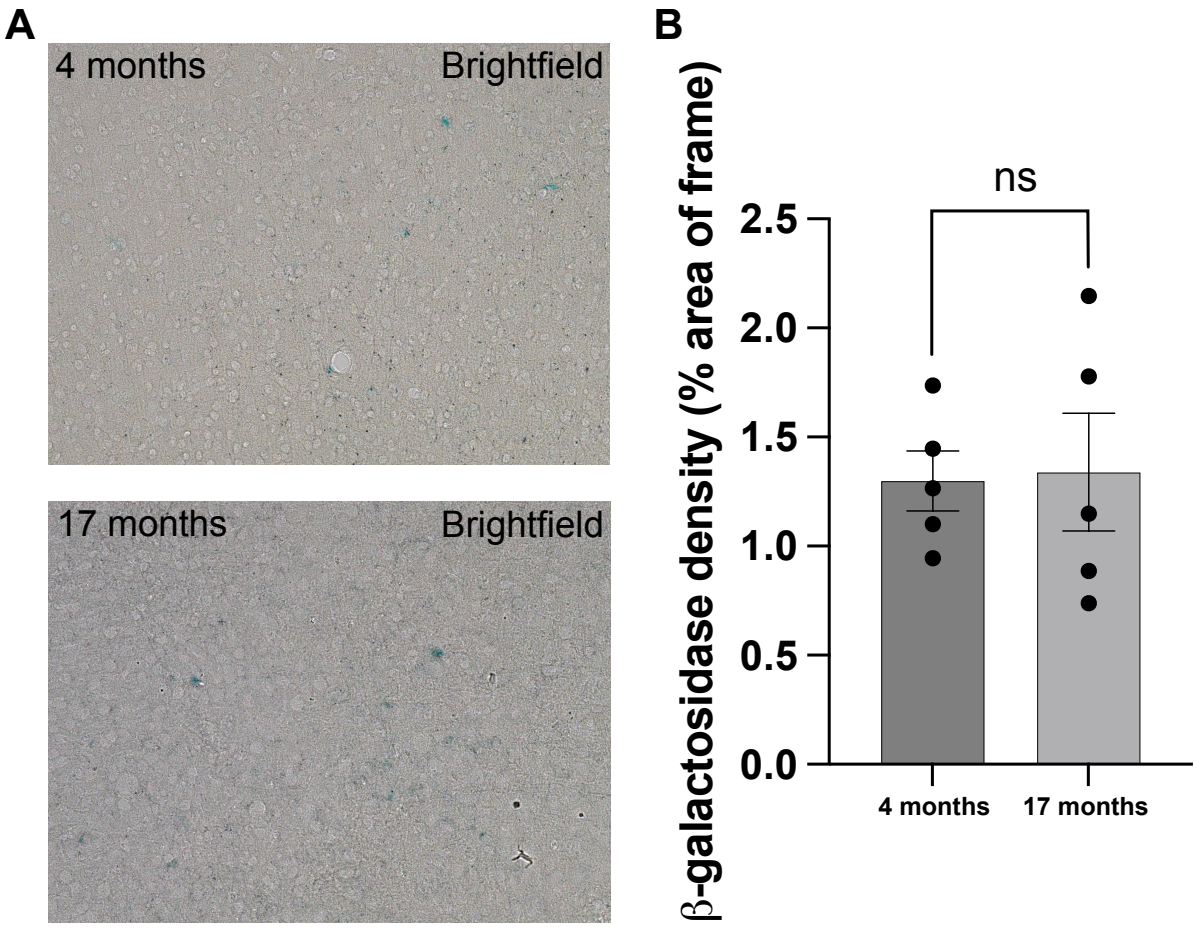

Figure 5.12. Brain sections show no difference in active β-galactosidase levels. (A) Representative images of brain sections from 4- and 17-month-old mice stained for active β-galactosidase activity with X-gal at pH 6.0 (as indicated by blue staining) and quantified as percentage (%) of total area of frame in (B). Each point represents the average % of blue staining from one animal, where three images were taken per section, across three equidistant sections per animal. Error bars represent SEM and statistical comparisons were made via unpaired t-test (ns: $p \geq 0.05$).

5.2.8 Aged brains have increased levels of β-galactosidase

To further investigate total microglial β-galactosidase levels, rather than active enzyme alone (as was the case with the X-gal staining above), I immunostained the sections using antibodies to β-galactosidase and Iba1 (microglial marker) (Figure 5.13A). I analysed the staining intensity of β-galactosidase within Iba1-positive cells in the somatosensory cortex using confocal microscopy and further image analysis of rendered microglial structures using Imaris software (Figure 5.13B). I found that there was no significant difference associated with age in microglial Iba1 volume, indicating no effect of age on microglial size (Figure 5.13C). The intensity of β-galactosidase within Iba1-positive structures was significantly increased in old (17-month-old) compared with young wild-type mice (4-month-old) (Figure

5.13D), indicating a significant increase in β-galactosidase levels with age. In addition, in aged mice brains, the β-galactosidase staining appeared to be on the surface of the microglia, rather than confined to lysosomes (Figure 5.13A & B), consistent with translocation to the cell surface, although this was not possible to quantify. This was further supported by staining of slices with Iba1 and the lysosomal marker, CD68 (staining done by Jacob Dundee, Supplementary Figure A 9.3). This confirmed that although lysosomes appear larger in aged brains, the lysosomes are still largely confined to the perinuclear cell body, and their distribution is distinct from that of β-galactosidase in aged brains.

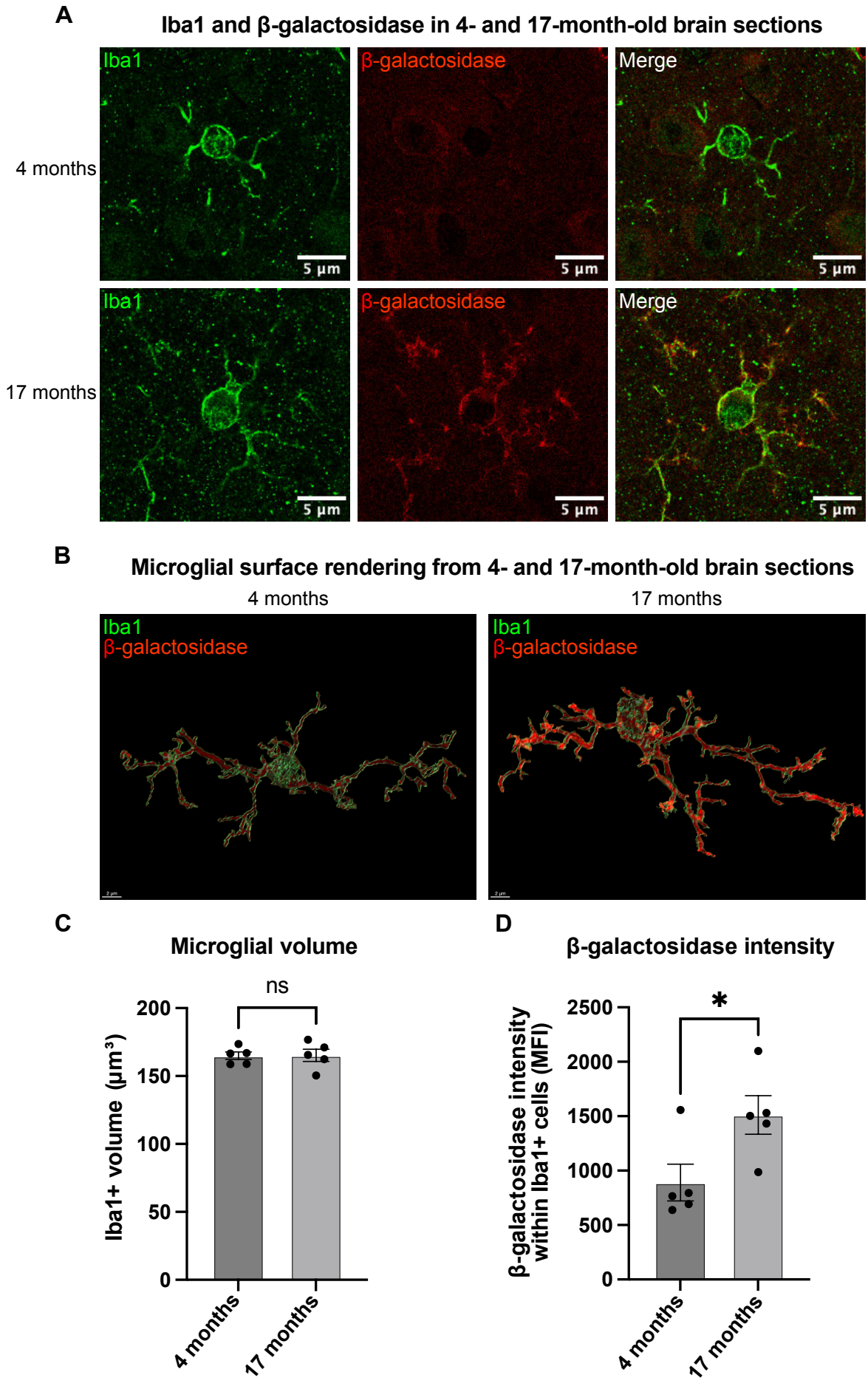
A
Iba1 and β-galactosidase in 4- and 17-month-old brain sections
Iba1
β-galactosidase
Merge
4 months
17 months
5 µm
B
Microglial surface rendering from 4- and 17-month-old brain sections
4 months
17 months
Iba1
β-galactosidase
C
Microglial volume
ns
Iba1+ volume (µm³)
200
150
100
50
0
4 months
17 months
D
β-galactosidase intensity
β-galactosidase intensity within Iba1+ cells (MFI)
*
2500
2000
1500
1000
500
0
4 months
17 months

Figure 5.13. Microglia in aged brains have increased levels of β-galactosidase. (A) Representative confocal microscopy images of microglia from 4- and 17-month-old mice stained for Iba1 (green, microglial marker) and β-galactosidase (red) in the somatosensory cortex. Scale bar = 5 μm. (B) Representative renders of microglia from confocal images generated in Imaris. Scale bar = 2 μm. (C) Microglial volume as measured by area (μm^3) of Iba1 staining. (D) Mean fluorescence intensity (MFI) of β-galactosidase within Iba1-positive microglia. Each point represents one animal comprised of 15 microglia analysed across three equidistant sections. Error bars represent SEM and statistical comparisons were made via unpaired t-test. Asterisk (*) indicate significance (ns: $p \geq 0.05$, *: $p < 0.05$).

5.3 Discussion

Glycohydrolase enzymes including Neu1 and β-galactosidase, play a fundamental role in the degradation of glycoproteins and glycolipids, to maintain cellular constituent turnover and glycosylation homeostasis. Neu1 hydrolyses terminal sialic residues to expose galactose residues, which can then be hydrolysed by β-galactosidase (Sandhoff and Sandhoff, 2018). Previously it was thought that Neu1 and β-galactosidase were exclusively located in the lysosomes where they form part of a multienzyme complex with PPCA (Verheijen *et al.*, 1985), but recent evidence has shown glycohydrolase activity at the plasma membrane (Aureli, Loberto, Chigorno, *et al.*, 2011).

It was previously reported that LPS stimulated BV-2 microglia release a sialidase activity that desialylated co-cultured cells (Nomura *et al.*, 2017). Our lab has since shown that Neu1 can be translocated to the cell surface upon microglial activation (Allendorf and Brown, 2022). It is unknown whether intact microglia can release β-galactosidase, but as Neu1 activity has been associated with the plasma membrane and can be structurally and functionally coupled to β-galactosidase in a lysosomal multienzyme complex, I investigated here whether BV-2 and primary rat microglia can release β-galactosidase activity when activated by a variety of inflammatory stimuli, including: LPS, PMA, ATP and, calcium ionophore A23187.

Various synthetic compounds have been developed to quantify β-galactosidase enzyme activity. In this work I first validated the use of one of these compounds, MUG, as a tool for quantifying β-galactosidase activity, using isolated β-galactosidase from bovine liver. Active β-galactosidase hydrolyses MUG to a fluorescent product, MUB, and galactose. MUG is cell impermeant, so when added to intact cells, the assay only measures the cell surface or extracellular activity and has been used previously to measure β-galactosidase activity on the surface of fibroblasts (Aureli *et al.*, 2009).

First, to test whether β-galactosidase is released from activated microglia, I stimulated BV-2 microglia with LPS which is commonly used in studies of immune cell activation and function (Paludan, 2000). Here, LPS induced a significant increase in β-galactosidase activity on the surface of the cells, compared to cells not treated with LPS. PMA is a protein kinase C activator that can cause microglial activation (Czapiga and Colton, 1999) and has also been reported to induce senescence of microglia, characterised by an increase in intracellular SA-β-galactosidase (Cao *et al.*, 2020). I tested whether PMA treatment of microglia could induce β-galactosidase release, and found that BV-2 microglia cultured with PMA had significantly more β-galactosidase activity on the cell surface.

Stimulation by LPS and PMA also caused a significant increase in β-galactosidase activity measured in the culture medium compared to vehicle treated controls. Further analysis, by

ELISA, of supernatants from BV-2 microglia confirmed that β-galactosidase protein levels were significantly increased after treatment with LPS and PMA. As BV-2 microglia are a cell line, which may diverge from primary cells as a result of transformation and/or mutations (Henn *et al.*, 2009), I also tested whether inflammatory stimulation by LPS and PMA could induce β-galactosidase release from primary cultures from rat brain. LPS and PMA treatment of both mixed neuronal-glial cultures and isolated primary microglia resulted in a significant increase in β-galactosidase protein release as measured by ELISA, consistent with LPS and PMA-induced release of β-galactosidase from microglia. I did not investigate the mechanism by which LPS induces β-galactosidase release. However, Neu1 has been found associated with the plasma membrane as the result of lysosomal fusion mechanisms linked to plasma membrane repair processes (Reddy, Caler and Andrews, 2001) and lysosomal exocytosis due to an excess of intracellular lysosomes (Samarani *et al.*, 2018). Furthermore, our lab has shown that LPS induces release of Neu1 by lysosomal exocytosis (Allendorf and Brown, 2022), so it is possible that β-galactosidase may be translocated in a similar way. Indeed, the transcription factor EB (TFEB) regulates lysosomal secretion, inducing docking and fusion of lysosomes with the plasma membrane and promotes the translocation of β-galactosidase to the cell surface (Magini *et al.*, 2013).

Lysosomal exocytosis can be induced by a rise in intracellular calcium (Reddy, Caler and Andrews, 2001). To investigate if lysosomal exocytosis promotes β-galactosidase release, I treated BV-2 microglia with agents that induce a rise in intracellular calcium: A23187 and ATP. A23187, also known as calcimycin, is a calcium ionophore that promotes rapid influx of calcium and subsequently the fusion of lysosomes with the plasma membrane (Jaiswal, Andrews and Simon, 2002). In addition to an immediate, artifactual, increase in fluorescence, A23187 induced a genuine increase in β-galactosidase activity above that of the vehicle treated control. ATP is a P2X7 agonist which has been shown to stimulate calcium influx (Kaya, Tanaka and Koike, 2002) and subsequent microvesicle exocytosis in human monocytes (MacKenzie *et al.*, 2001) and murine microglial cells (Bianco *et al.*, 2005). I found that ATP induced an increase in β-galactosidase activity compared to vehicle control. These results are consistent with the notion that lysosomal exocytosis may cause β-galactosidase release. However, to confirm that release occurs by lysosomal exocytosis would require further investigation.

The accumulation of senescent cells in AD and PD, suggests the contribution of senescence in the pathophysiology of these disorders. However, there is relatively little evidence for the contribution of senescent microglia in neurodegeneration. A characteristic marker of senescent cells is an increased number of lysosomes and activity of SA-β-galactosidase (Dimri *et al.*, 1995). It has been shown that lysosomal glycohydrolase β-glucocerebrosidase levels are increased in the culture medium of senescent melanoma cells (Rovira *et al.*, 2022). Hence, I wanted to assess whether β-galactosidase was released from senescent microglia.

To do this, I looked to generate an *in vitro* model of senescent microglia which could then be used to assess β-galactosidase release.

Cellular senescence occurs when cells reach their replicative capacity and adopt a state of long-term cell cycle arrest (Hayflick, 1965). Hallmarks of senescence are used to identify senescent cells and include, but are not limited to: increased SA-β-galactosidase activity - which occurs as a result of the overexpression of lysosomal β-galactosidase (Dimri *et al.*, 1995); altered morphology - where cultured senescent cells become flat, large, multi-nucleated, and vacuolated (Denoyelle *et al.*, 2006); long-term exit from the cell cycle - therefore lacking markers of cell proliferation (e.g., Ki67) (Rhinn, Ritschka and Keyes, 2019) and; the acquisition of a senescence-associated secretory phenotype (SASP) - a secretory profile of pro-inflammatory cytokines and signalling molecules (eg. interleukin (IL)-1β, IL-6 and IL-8) (Coppé *et al.*, 2010). Replicative senescence is triggered by telomere attrition (Harley, Futcher and Greider, 1990), but senescence can also be induced by activating oncogenes and DNA damage (Petrova *et al.*, 2016). Senescent cell cycle arrest is controlled by the activation of tumour suppressor pathways, p53/p21WAF1/CIP1 and/or p16INK4A/pRB (Kumari and Jat, 2021), which are reported to be interlinked (Zhang *et al.*, 2006; Yamakoshi *et al.*, 2009). The cyclin-dependent kinase inhibitors, p21WAF1/CIP1 (p21) and p16INK4A (p16) act as negative regulators of cell cycle progression with p21 acting downstream of p53 and p16 acting upstream of pRB. Increased expression of p16 and/or p21 are also used as senescence-identifying markers and, retroviral overexpression of p21 or p16 is sufficient to induce senescence in human diploid fibroblasts (McConnell *et al.*, 1998).

In this work, I investigated whether overexpression of p16 could induce senescence in human CHME-3 microglia by causing an irreversible arrest of cell proliferation, a characteristic feature of senescence. I found that p16 overexpression in human CHME-3 microglia did not prevent proliferation, as demonstrated by sustained expression of proliferation marker, Ki67. DNA tumour virus oncoproteins, including SV40 large T antigen, that bind to and interfere with Rb and p53 function are used to overcome replicative senescence and to generate immortalised cells (Shay, Pereira-Smith and Wright, 1991). CHME-3 microglia were established in this way using human microglial cells (Janabi *et al.*, 1995). As p16 is upstream of pRb, p16 overexpression may have little to no effect on CHME-3 cell fate and may explain why reduction in proliferative capacity of the CHME-3 cells was not observed. Furthermore, inducing senescence by activation of either the p16INK4A/pRB or p53/p21WAF1/CIP1 pathways may be cell-type specific, considering the complexity of the two tumour suppressor pathways and the fact that there can be cross-talk between the two. In fact, it may be that overexpression of p16 alone was not sufficient to prevent proliferation. As p16 overexpression in CHME-3 microglia did not prevent proliferation and optimisation would likely have been complex and resource intensive, I decided to investigate a different approach to generate a robust senescent microglial model.

Senescence can be induced by DNA damage. The anti-cancer drug doxorubicin generates DNA damage via several mechanisms, including, but not limited to: DNA intercalation, free radical generation, DNA binding and alkylation and DNA cross-linking (Gewirtz, 1999). Doxorubicin has been used to induce senescence in various non-microglial cell types (Sliwinska *et al.*, 2009; Yang *et al.*, 2012; Piegari *et al.*, 2013; Bielak-Zmijewska *et al.*, 2014). In rat microglial cell line, CHME-5, doxorubicin treated cells presented characteristics of senescence, including: an enlarged cell body, elevated levels of SA-β-galactosidase, p16 and inflammatory cytokines (Marques, Johnson and Stolzing, 2020). I therefore assessed the use of doxorubicin to induce senescence in CHME-3 microglia and found that doxorubicin affected CHME-3 proliferation and morphology, with treated cells becoming markedly larger, flatter and irregular in shape with granulated nuclei, indicative of cells taking on a senescent-like phenotype. Furthermore, changes in p16 and p21 expression were observed; p16 levels decreased and p21 increased with doxorubicin treatment. As mentioned above, both act as negative regulators of cell cycle progression and their increased expression is often used to characterise senescence. However, in human fibroblasts, differential roles for p16 and p21 have been described (Stein *et al.*, 1999). p21 may be involved in achieving senescent cell-cycle arrest, with increased levels in the early-stages of senescence and, p16 may then be crucial for maintaining cell-cycle arrest (Stein *et al.*, 1999). This could explain why we see a difference in the expression of p16 and p21 after doxorubicin treatment.

Finally, the most widely used marker for senescence is SA-β-galactosidase activity, detected by histochemical staining of cells using the artificial substrate X-gal, which is cleaved to a blue precipitate by active β-galactosidase. SA-β-galactosidase activity was originally thought to result from a β-galactosidase enzyme unique to senescent cells, but has since been shown to be normal lysosomal β-galactosidase overexpressed in senescent cells (Lee *et al.*, 2006). Due to increased lysosomal β-galactosidase levels in senescence, β-galactosidase activity should be detectable at pH 6.0 in senescent cells. At pH 4.0, eukaryotic lysosomal β-galactosidase is active in all cells, so staining at pH 4.0 is used as a positive control for the staining protocol (Dimri *et al.*, 1995). In vehicle and doxorubicin treated CHME-3 cells, staining at pH 6.0 was minimal. At pH 4.0, staining levels were also very low, suggesting that the X-gal staining procedure was unsuccessful and required further optimisation. Possible reasons for poor staining included: 1) improper fixation of the tissue or cells to preserve the enzymatic activity of β-galactosidase; 2) endogenously low β-galactosidase activity and/ or 3) expired or degraded X-gal reagents. Previous work with CHME-3 cells suggests that the fixation conditions used here were suitable for the cell type (Fernandes *et al.*, 2018), so I looked to ascertain whether the reagent quality was suitable. To do so, I prepared fresh staining solutions using new reagents and stained CHME-3 and BV-2 microglia at pH 4.0 and pH 6.0. CHME-3 cells repeatedly showed negligible levels of detectable stain in both conditions, but BV-2 microglia demonstrated intense staining at pH 4.0, indicating that reagent quality was not an impacting factor and suggesting that CHME-3 cells may only

produce low endogenous levels of β-galactosidase, although to confirm, would require further investigation. For the purpose of generating a suitable *in vitro* model of senescence, I decided to continue investigations with BV-2 microglia. However, doxorubicin treatment in BV-2 cells, at the concentrations tested, was unable to induce a senescence-like state, as treatment did not significantly alter several markers of senescence including, proliferation, p21 expression and SA-β-galactosidase activity.

Numerous small molecule compounds that induce cellular senescence have been identified (Petrova *et al.*, 2016). Bleomycin, is another DNA-damaging agent that has been used to induce senescence in primary microglia (Brelstaff *et al.*, 2021). However, I found that bleomycin treatment of BV-2 microglia was unable to induce cellular senescence suitable for subsequent use as a model system. Ultimately, trying to induce senescence in immortalised cell lines may be difficult, as by their very nature immortalised cells are modified to proliferate indefinitely. As cell line work was inconclusive, I attempted to induce senescence in isolated primary microglia using bleomycin. However, this repeatedly failed to induce a sufficient increase in SA-β-galactosidase staining *in vitro.* My attempts to generate a suitable senescent model using CHME-3, BV-2 and primary microglia were unsuccessful. This is complicated further by a well acknowledged lack of universal markers of senescence (Rossi and Abdelmohsen, 2021). The literature suggests that although many markers for senescence have been identified, they are each limited by their specificity. Notably, detecting SA-β-galactosidase using the X-gal staining assay is not completely specific to senescent cells. In fact, researchers have shown that β-galactosidase activity at pH 6.0 might occur in autophagic cells due to the associated biogenesis of lysosomes (Young *et al.*, 2009). β-Galactosidase activity was also detected in immortalised cultured cells and macrophage-like cells at pH 6.0 (Yegorov *et al.*, 1998), and in confluent fibroblast cultures (Severino *et al.*, 2000). Furthermore, β-galactosidase activity at pH 6.0 has also been detected in differentiated neurons (Piechota *et al.*, 2016), and at early stages of development (de Mera-Rodríguez *et al.*, 2019). Together, these reports suggest that SA-β-galactosidase activity is unreliable as a marker of senescence, since the enzyme is not always specific for senescent cells. Hence, most accounts of characterising and identifying senescent cells use more than one marker. In my experiments, where there was some indication that senescence might have been induced, when looking at multiple markers, the results were difficult to conclude. The combination of senescence-inducing agents and cell types used were not suitable to generate a robust model for senescence in microglia that could then be used to ascertain whether β-galactosidase activity on the surface or released by cells was affected by a senescent phenotype.

Microglia are known to become activated and senesce with age in mice (Matsudaira *et al.*, 2023). So, I examined β-galactosidase levels in sections from the brains of mice at 4- and 17-months-old. The sections were prepared previously in the lab for alternative end-point

analysis, not relevant to my project. However, as producing a senescent model in cell culture to assess the β-galactosidase levels on the surface of senescent microglia was unsuccessful, I decided to use these sections to assess β-galactosidase levels potentially on the surface or released with age *in vivo*. First, to see if there was an increase in SA-β-galactosidase in these sections, I X-gal stained the sections. Histochemical analysis of the sections showed no sign of increased SA-β-galactosidase activity with ageing. However, the tissue was prepared several months prior to the X-gal staining and, the fixation and storage conditions used may have been inappropriate for this end-point analysis. Indeed, it has been reported that tissue should be frozen in liquid nitrogen immediately after surgical isolation (Debacq-Chainiaux *et al.*, 2009), so it may be that the enzymatic activity of β-galactosidase was not preserved, potentially leading to false-negative results. Instead, to investigate total microglial β-galactosidase levels, rather than only active enzyme, immunohistochemistry was performed, and sections were stained for Iba1 and β-galactosidase. Iba1 is a calcium binding protein, that is widely used to identify microglial/macrophage populations (Imai *et al.*, 1996). Here, I saw no effect of age on microglial volume, according to analysis of Iba1-positive structures, yet β-galactosidase intensity within microglial cells was increased with age. Notably, much of the β-galactosidase staining appeared to localise with the microglial surface, rather than within the lysosomes, consistent with translocation to the cell surface. Although lysosomes appeared larger in aged brains, the lysosomes were still largely confined to the perinuclear cell body and their distribution was distinct from that of β-galactosidase in aged brains. Although Iba1 is used here to identify microglia, it is not a microglial surface marker, so staining with such a marker (e.g., TMEM119) may provide more conclusive evidence that β-galactosidase is on the surface of the cells. However, Iba1 is cytoplasmic so it may be inferred that, as β-galactosidase staining in aged brains was observed at high intensity at the periphery of the Iba1 stain and not solely in lysosomal structures, enzyme localisation is increased at the cell surface with ageing.

In summary, it was not known if activated, senescent or aged microglia release β-galactosidase. Senescent cells, which are characterised by increased β-galactosidase activity and expansion of their lysosomal content, are implicated in neurodegeneration (Baker and Petersen, 2018). However, a true causal relationship between senescence and neurodegeneration has not been established. My work indicates that better, more robust models and characterisation of the senescent phenotype are needed to achieve this. However, in aged brains, β-galactosidase appeared to be translocated from the lysosomal compartment to the cell surface. Furthermore, inflammatory activation of microglia *in vitro* resulted in increased β-galactosidase activity levels at the cell surface and release of β-galactosidase activity and protein into the culture media. Thus, activated microglia can release β-galactosidase (Figure 5.14).

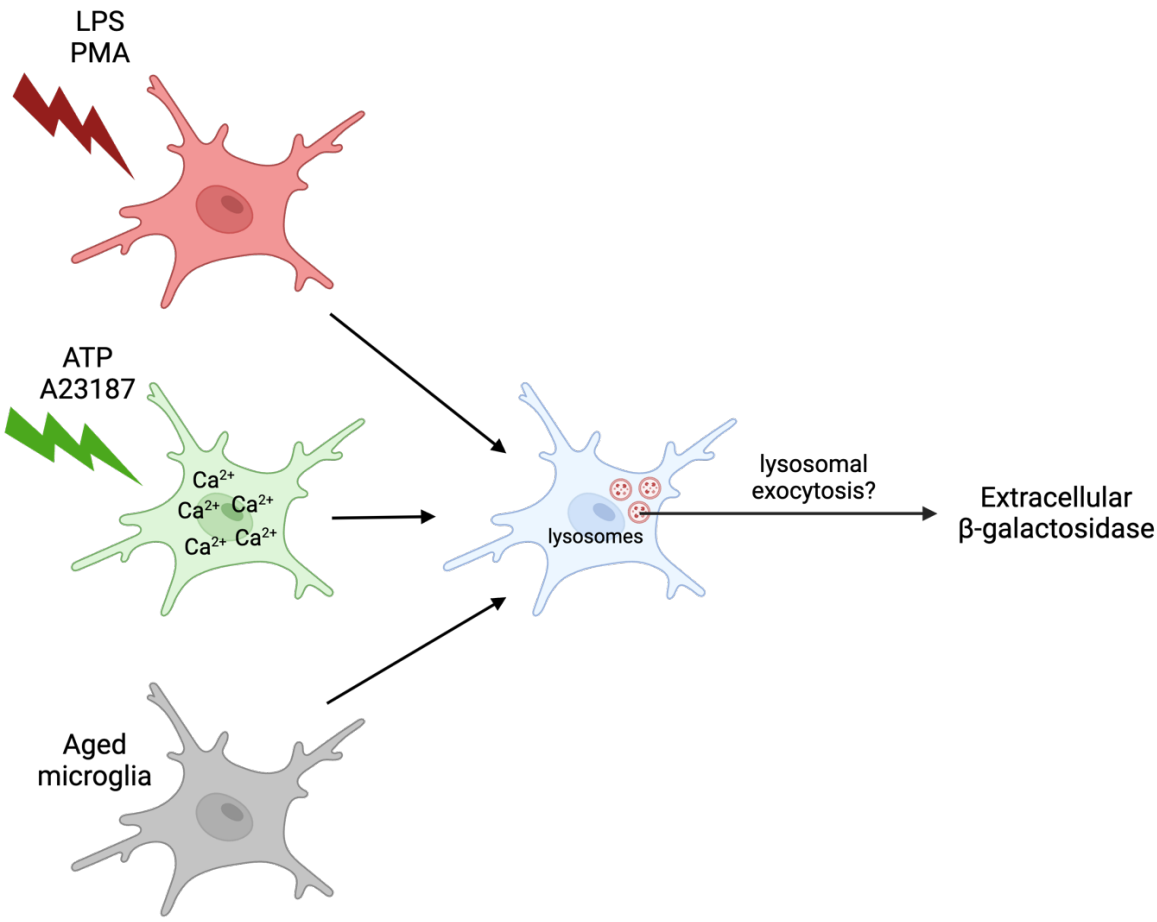

Figure 5.14. Summary of Chapter 5. β-Galactosidase is generally considered a lysosomal enzyme, but β-galactosidase can be released by microglia under certain conditions, including: i) exposure to inflammatory stimuli like LPS and PMA; ii) in response to agents that stimulate a calcium rise and potentiate lysosomal exocytosis, including ATP and calcium ionophore A23187; and iii) as a result of ageing. This release might result from lysosomal exocytosis. However, to confirm that release occurs by lysosomal exocytosis would require further investigation.

6 THE EFFECTS OF BETA-GALACTOSIDASE ON MICROGLIA AND NEURONAL LOSS

All data presented in this chapter is my own work. Some content is currently in review for publication in: Kitchener EJA, Dundee JM and Brown GC. 'Activated microglia release β-galactosidase that promotes inflammatory neurodegeneration'.

6.1 Introduction

Increasing evidence suggests that several lysosomal glycohydrolase enzymes are found associated with the plasma membrane in their active form, challenging the previous notion that these glycohydrolases are located only in lysosomes (Aureli *et al.*, 2023). β-Galactosidase activity has been found associated with the surface of several cell types. In human fibroblasts, a β-galactosidase activity has been found to act in *trans* on neighbouring cells (Aureli *et al.*, 2009). In rat cerebellar granule cell cultures, researchers found an increased β-galactosidase activity associated with the cell surface during neuronal differentiation and ageing (Aureli, Loberto, Lanteri, *et al.*, 2011) and, increased plasma membrane-associated β-galactosidase activity was also seen during neural stem cell differentiation (Aureli *et al.*, 2012). In chapter 5, I found that under inflammatory conditions, β-galactosidase is found on the surface of microglia and released into the culture medium *in vitro.* Furthermore, I found that microglia in aged mice have increased levels of β-galactosidase on their surface *in vivo*. However, the functional role of plasma membrane-associated β-galactosidase remains largely unknown.

Gangliosides, like GM1, are sialic-acid containing glycosphingolipids, particularly abundant in neuronal cell membranes where they are implicated in brain physiology and pathology (Derry and Wolfe, 1967; Sipione *et al.*, 2020). β-Galactosidase catalyses the hydrolysis of the terminal galactose from ganglioside GM1 (Li and Li, 1999). In humans, β-galactosidase deficiency has been associated with GM1 gangliosidosis, a lysosomal storage disorder, characterised by the accumulation of the ganglioside GM1 (Sandhoff and Sandhoff, 2018). GM1 gangliosidosis is linked to impairment of the CNS, manifesting symptoms like developmental delay and early onset neurodegeneration, and can be fatal (Sandhoff and Sandhoff, 2018). Conversely, increased β-galactosidase levels have been associated with neurodegenerative diseases including AD and PD. β-galactosidase associated with the neuronal membrane is increased in AD (Magini *et al.*, 2015) and, β-galactosidase is increased in the CSF of PD patients, indicating an increase in extracellular β-galactosidase in the brain (van Dijk *et al.*, 2013). Extracellular and cell surface β-galactosidase can potentially degrade cell surface ganglioside GM1, which is otherwise neuroprotective and

systemically deficient in PD tissues (van Dijk *et al.*, 2013; Chowdhury and Ledeen, 2022). GM1 also potently inhibits microglial activation (Galleguillos *et al.*, 2022). The balance between glycosylation and de-glycosylation at the plasma membrane is likely important in maintaining healthy physiology. So, increased extracellular and plasma membrane-associated β-galactosidase could result in a loss of GM1 and glycosylation homeostasis. Hence, understanding the role of increased plasma membrane-associated β-galactosidase activity in neurodegeneration is the focus of continued investigation.

Having found in chapter 5 that activated and aged microglia release higher levels of β-galactosidase over baseline or young controls, I sought to investigate whether β-galactosidase promoted inflammatory neurodegeneration and if inhibiting β-galactosidase could prevent this.

6.2 Results

6.2.1 Exogenous β-galactosidase promotes neuronal loss and increases microglial number

As I have shown that activated microglia release β-galactosidase, I next wanted to assess whether this released β-galactosidase could contribute to neurodegeneration. In chapter 5, I found that addition of LPS (100 ng/mL) or PMA (100 nM) induced a significant increase in β-galactosidase protein in the culture medium from mixed neuronal-glial cultures. As LPS and PMA induced an increase in extracellular β-galactosidase, I tested whether addition of isolated β-galactosidase from bovine liver affected neuronal viability and microglial activation in mixed neuronal-glial cultures, by adding 20 mU/mL β-galactosidase to these cultures for 72 h. For comparison, 100 ng/mL LPS was added to the cultures as a positive control. Addition of β-galactosidase induced a loss of about 25 % of the neurons over 72 h (Figure 6.1A), and doubled the number of microglia in the cultures, compared to the vehicle-treated control (Figure 6.1B).

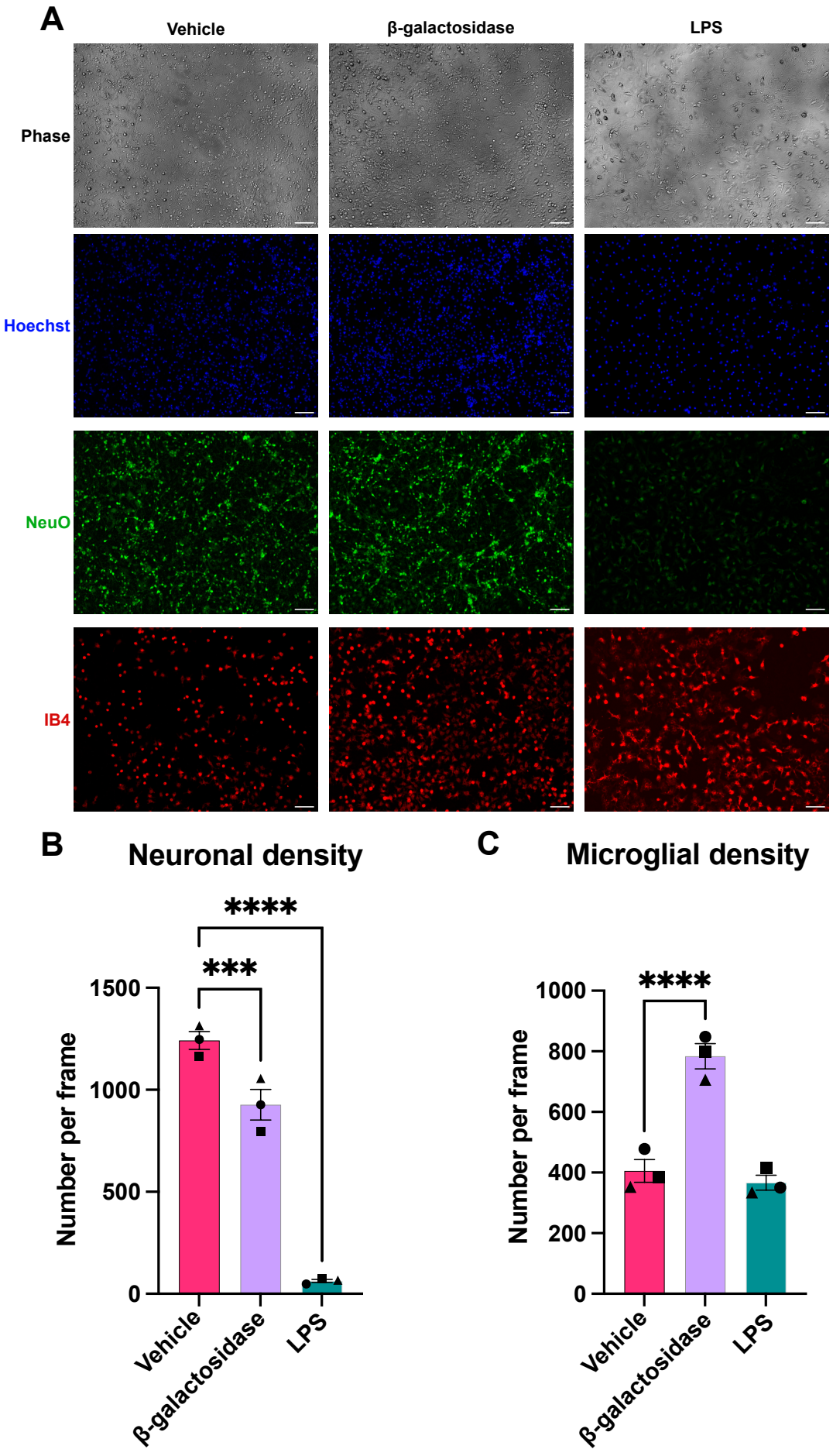
A
Vehicle
β-galactosidase
LPS
Phase
Hoechst
NeuO
IB4
B
Neuronal density
****
***
1500
1000
500
0
Number per frame
Vehicle
β-galactosidase
LPS
C
Microglial density
****
1000
800
600
400
200
0
Number per frame
Vehicle
β-galactosidase
LPS

Figure 6.1. β-Galactosidase promotes neuronal loss and microglial proliferation. (A) Primary neuronal-glial cultures were treated ± PBS (vehicle), β-galactosidase (20 mU/mL) or LPS (100 ng/mL). After 72 hours, cultures were stained with Hoechst 33342 (blue, to identify nuclei), NeuO (green, to identify live neurons) and isolectin B4 (red, to identify microglia). Cultures were then imaged (four, 10x images per technical repeat and three technical repeats per biological repeat). *In vitro*, primary microglia become more ramified after treatment with β-galactosidase and LPS. The number of live neurons are almost completely lost with LPS treatment. Scale bar = 100 μm. (B) Live neuronal cell numbers were quantified using CellProfiler. (C) Microglial cell numbers quantified by fluorescent microscopy using CellProfiler. Data represents mean values ± SEM of 3 independent experiments. Statistical comparisons were made by repeated measures one-way ANOVA with Dunnett's post-hoc comparisons test. Asterisk (*) indicate significance compared to untreated control (***: $p<0.001$, ****: $p<0.0001$).

6.2.2 β-Galactosidase promotes changes in microglial morphology

One measure of microglial activation is changes in microglial morphology. The imaging data showed that β-galactosidase affected microglial morphology (Figure 6.2A) and quantification of microglial area revealed that this was similar to, although to a lesser degree than, treatment with LPS (Figure 6.2B).

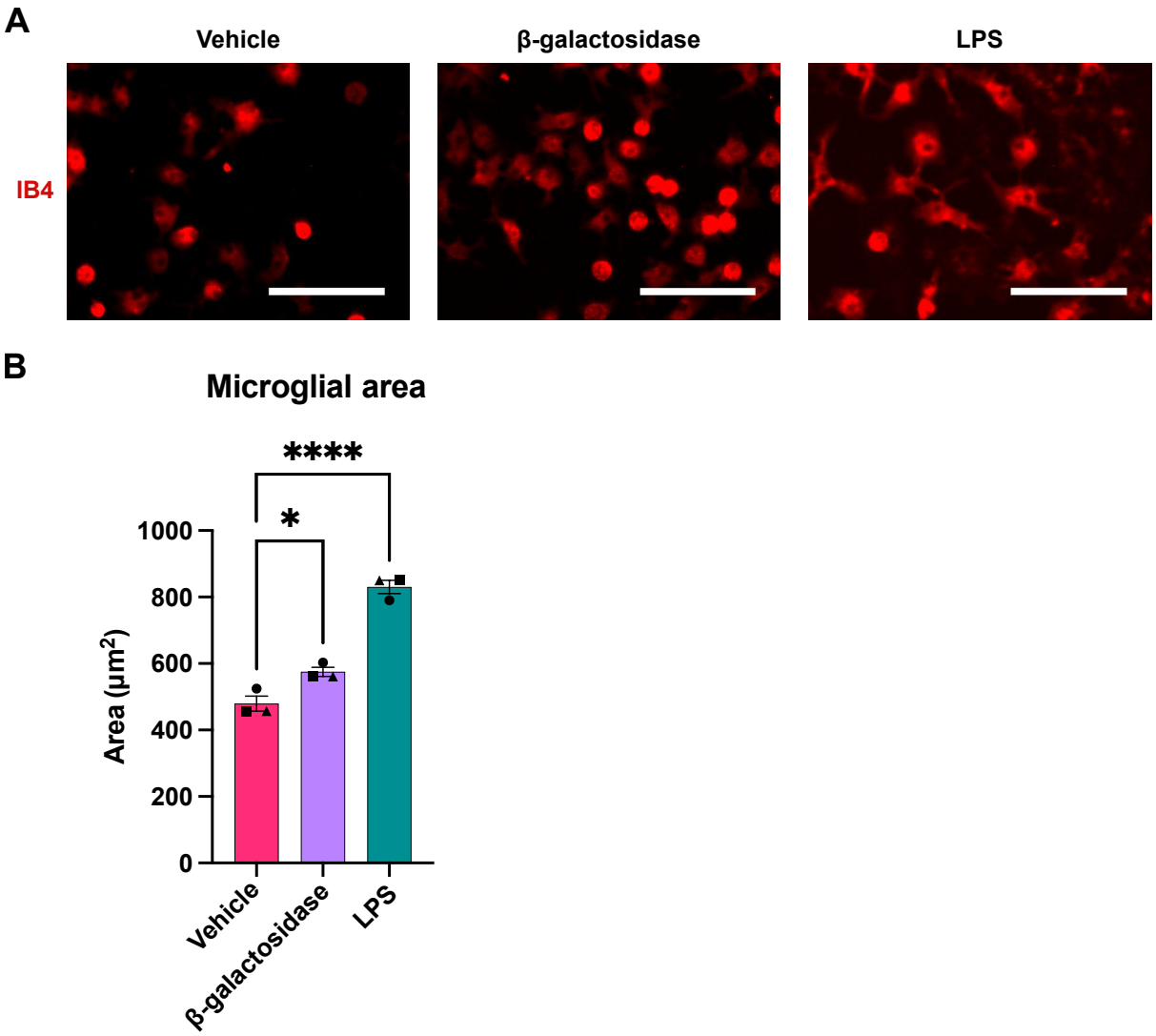

Figure 6.2. β-Galactosidase promotes changes in microglial morphology. (A) As in Figure 6.1A, primary neuronal-glial cultures were treated ± PBS (vehicle), β-galactosidase (20 mU/mL) or LPS (100 ng/mL). After 72 hours, cultures were stained with Hoechst 33342, NeuO and IB4. Here, zoomed in panels are to show microglia in greater detail. Scale bar = 100 µm. (B) Cell area was quantified in CellProfiler. Data represents mean values ± SEM of 3 independent experiments. Statistical comparisons were made by repeated measures one-way ANOVA with Dunnett's post-hoc comparisons test. Asterisk (*) indicate significance compared to untreated control (*: $p<0.05$, ****: $p<0.0001$).

6.2.3 Addition of β-galactosidase to primary rat neuronal-glial cultures promotes TNFα release

Microglia are known to release pro-inflammatory cytokine, TNFα, upon activation with LPS *in vitro* (Nakamura, Si and Kataoka, 1999). Having found that microglia proliferate and undergo morphological changes with β-galactosidase addition, both indicators of activation, I tested whether β-galactosidase could induce release of pro-inflammatory cytokine release in mixed neuronal-glial cultures by measuring the amount of extracellular TNFα by ELISA. Vehicle-treated cultures had no detectable TNFα, while addition of 20 mU/mL β-galactosidase

caused a significant and substantial release of TNFα, similar to that induced by LPS (Figure 6.3).

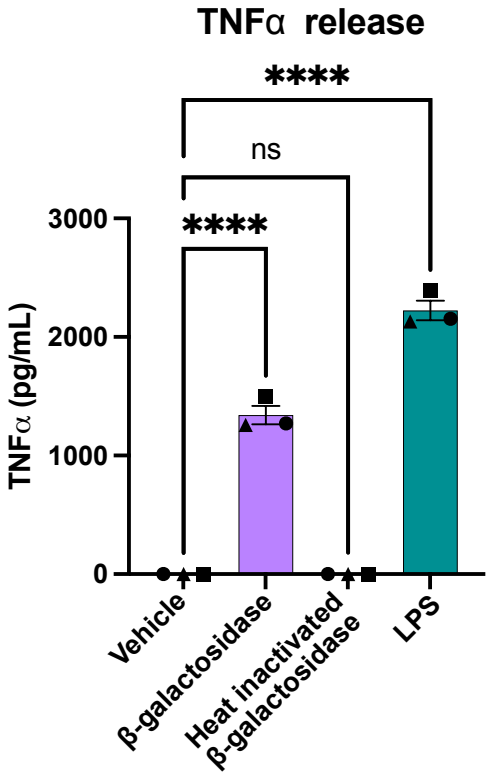

Figure 6.3. β-Galactosidase promotes TNFα release in mixed neuronal-glial cultures. Mixed neuronal-glial cultures were treated ± PBS (vehicle), 20 mU/mL β-galactosidase, 20 mU/mL heat inactivated β-galactosidase and LPS (100 ng/mL) for 72 h. Supernatants were assessed for TNFα levels by ELISA. Data represents mean values ± SEM of 3 independent experiments. Statistical comparisons were made by repeated measures one-way ANOVA with Dunnett's post-hoc comparisons test. Asterisk (*) indicate significance compared to untreated control (ns: $p \geq 0.05$, ****: $p < 0.0001$).

6.2.4 Heat inactivated β-galactosidase does not affect neuronal loss or microglial activation

To test whether the effects of added β-galactosidase were due to the β-galactosidase activity, rather than the protein or potential contaminants, I added 20 mU/mL heat inactivated β-galactosidase to mixed neuronal-glial cultures for 72 h and found that heat inactivated β-galactosidase had no effect on TNFα release (Figure 6.3). I also assessed whether heat inactivated β-galactosidase had an effect on neuronal number, microglial number or microglial morphology and found that the effects induced by active β-galactosidase were prevented by heat inactivation; neuronal loss (Figure 6.4A), microglial proliferation (Figure 6.4B) and microglial morphology changes (Figure 6.4C) were all prevented and not significantly different to the vehicle-treated control.

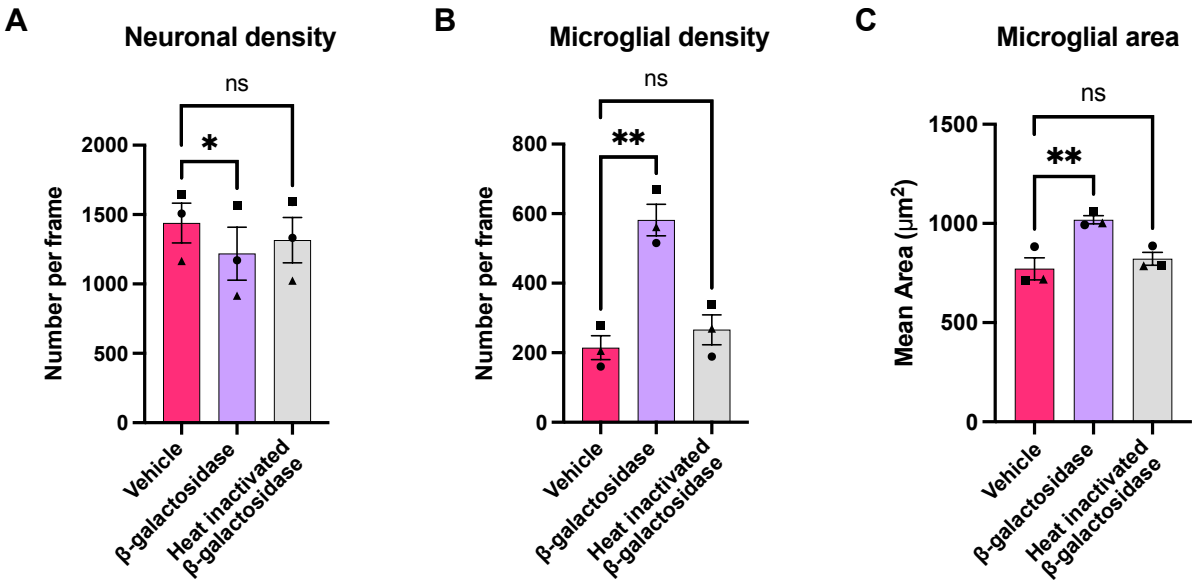

Figure 6.4. Heat inactivated β-galactosidase does not promote neuronal loss or microglial activation. Mixed neuronal-glial cultures were treated with 20 mU/mL or 20 mU/mL heat-inactivated exogenous β-galactosidase for 72 h then cultures were stained with Hoechst 33342 (to identify nuclei), isolectin B4 (to identify microglia), NeuO (to identify live neurons). (A) Live neuronal cell numbers, and (B) microglial cell numbers were quantified by fluorescent microscopy using CellProfiler. (C) Microglial area was quantified in CellProfiler. Data represents mean values ± SEM of 3 independent experiments. Statistical comparisons were made by repeated measures one-way ANOVA with Dunnett's post-hoc comparisons test. Asterisk (*) indicate significance compared to untreated control (ns: $p \geq 0.05$, *: $p < 0.05$, **: $p < 0.01$).

6.2.5 β-Galactosidase promotes TNFα release from isolated primary microglia

I also tested whether β-galactosidase could induce cytokine release from isolated microglial cultures by measuring the amount of extracellular TNFα by ELISA, 24 h after treatment with β-galactosidase. Vehicle-treated cultures had no detectable TNFα, LPS-treated cultures had a large amount of TNFα (~7,500 pg/mL), while β-galactosidase-treated cultures contained an intermediate level of TNFα (~3,000 pg/mL) (Figure 6.5). Again, heat inactivated enzyme did not affect TNFα release compared to the vehicle control. Hence, exogenous β-galactosidase activity promotes neuronal loss and activates microglia to a pro-inflammatory state.

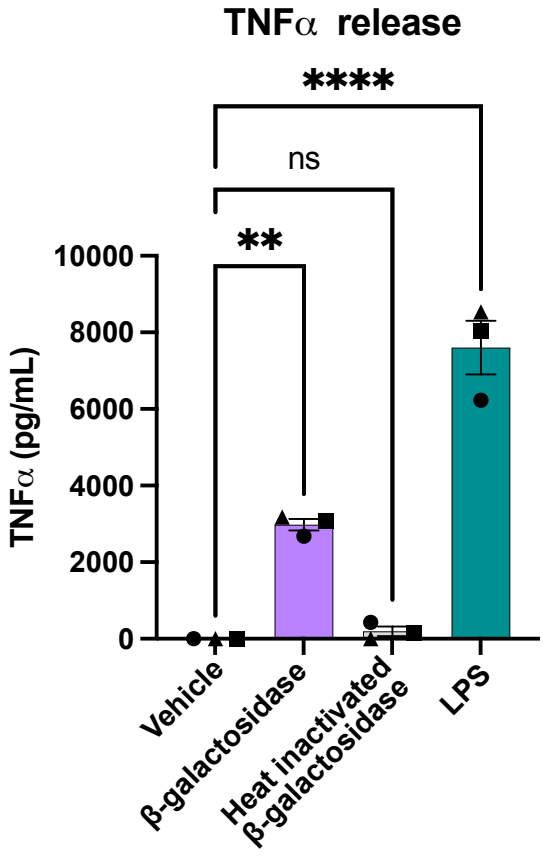

Figure 6.5. β-Galactosidase promotes TNFα release in isolated primary microglial cultures. Isolated primary microglia were seeded at 1 x 10^5 cells/well in a 96 well plate and were treated with 20 mU/mL β-galactosidase, 20 mU/mL heat inactivated β-galactosidase and LPS (100 ng/mL) for 72 h. Supernatants were assessed for TNFα levels by ELISA. Data represents mean values ± SEM of 3 independent experiments. Statistical comparisons were made by repeated measures one-way ANOVA with Dunnett's post-hoc comparisons test. Asterisk (*) indicate significance compared to untreated control (ns: $p \geq 0.05$, **: $p < 0.01$, ****: $p < 0.0001$).

6.2.6 Microglial depletion prevents β-galactosidase induced neuronal loss and inflammation

To investigate whether the effects of β-galactosidase on neurotoxicity and inflammation require microglia, I depleted microglia in the mixed neuronal-glial cultures using PLX-3394, a colony stimulating factor 1 receptor (CSF1R) inhibitor, prior to β-galactosidase treatment. PLX-3394 has been used previously to deplete microglia from mixed glial cultures (Van Zeller, Sebastião and Valente, 2022). Here, PLX treatment depleted the microglia in mixed neuronal-glial cultures by at least 50 % both in the absence and presence of exogenous β-galactosidase treatment (Figure 6.6A). PLX treatment completely prevented the increase in TNFα levels induced by addition of β-galactosidase (Figure 6.6B). Furthermore, depletion of microglia prevented the loss of neurons induced by addition of β-galactosidase (Figure 6.6C). Together, these results suggest β-galactosidase is not directly neurotoxic, but that β-galactosidase induced neuronal loss is mediated by the activation of microglia and subsequent inflammation.

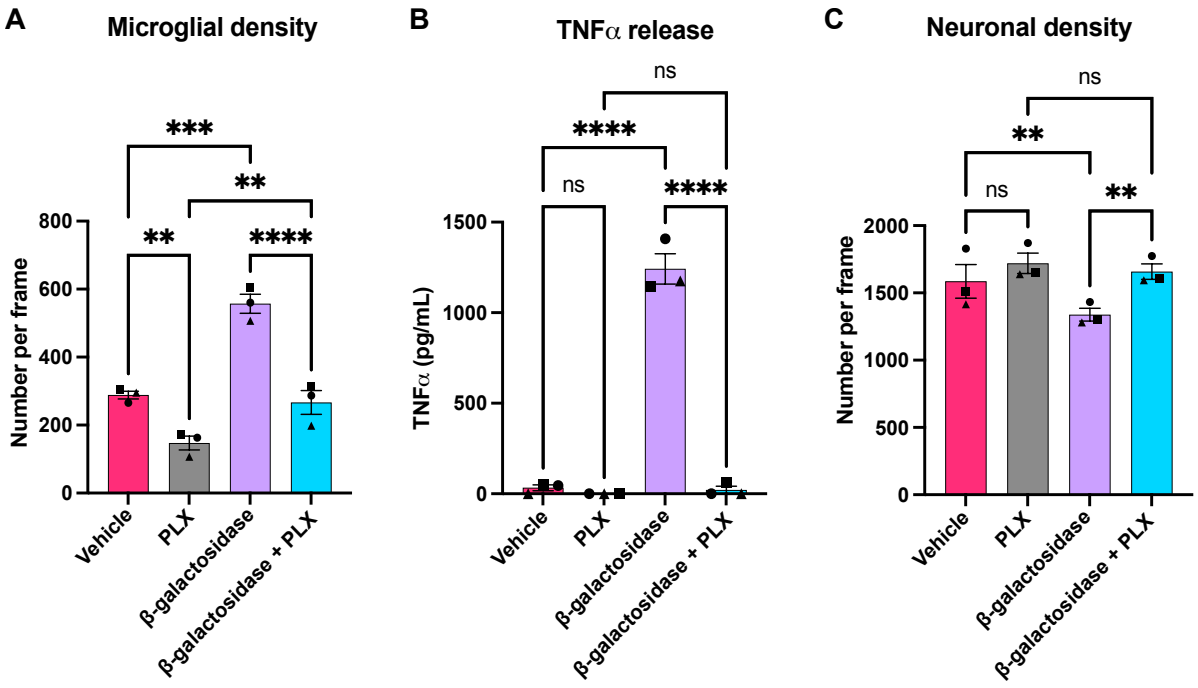

Figure 6.6. Microglial depletion protects against β-galactosidase induced neuronal loss and microglial activation. Mixed neuronal-glial cultures were treated with PLX-3397 (5 μM) 3 DIV, then ± 20 mU/mL exogenous β-galactosidase 7 DIV for 72 h. Cultures were then stained with Hoechst 33342 (to identify nuclei), isolectin B4 (to identify microglia), NeuO (to identify live neurons). (A) Microglial cell numbers were quantified by fluorescent microscopy using CellProfiler. (B) Supernatants were assessed for TNFα levels by ELISA. (C) Live neuronal cell numbers were quantified by fluorescent microscopy using CellProfiler. Data represents mean values ± SEM of 3 independent experiments. Statistical comparisons were made by repeated measures two-way ANOVA with Šídák's post-hoc comparisons test. Asterisk (*) indicate significance compared to untreated control (ns: $p \geq 0.05$, **: $p < 0.01$, ***: $p < 0.001$, ****: $p < 0.0001$).

6.2.7 Lipofectamine 3000 mediated RNAi knock down of mouse GLB1 in BV-2 cells was unsuccessful

As LPS induced β-galactosidase release in primary cultures, and addition of isolated β-galactosidase induced microglial activation and neuronal loss, I wanted to understand the role of β-galactosidase in neuronal loss and microglial activation. To do this, I attempted to modulate the expression of β-galactosidase using lipofectamine mediated RNA interference (RNAi) knock down of endogenous GLB1 expression. Knock down by this method has been done previously in our lab (Allendorf, Franssen and Brown, 2020) and here, I followed a similar RNAi-lipofectamine protocol. However, surface β-galactosidase activity of non-targeting (siNT) or Glb1-targeting (siGlb1) siRNA-transfected BV-2 microglia was reduced but this was not significant (Figure 6.7A & B) and β-galactosidase release from these cells into the culture medium was also not affected significantly (Figure 6.7C & D). I decided to check whether there was a significant difference in Glb1 mRNA expression with the knock down. 24 h post-transfection, there was a reduction in Glb1 mRNA levels by about 35 %, but

this reduction was variable and was not significant (Figure 6.7E). Further analysis of the supernatants from the transfected cells confirmed that knock down was not sufficient to significantly reduce the release of β-galactosidase enzyme, as quantified by ELISA (Figure 6.7F).

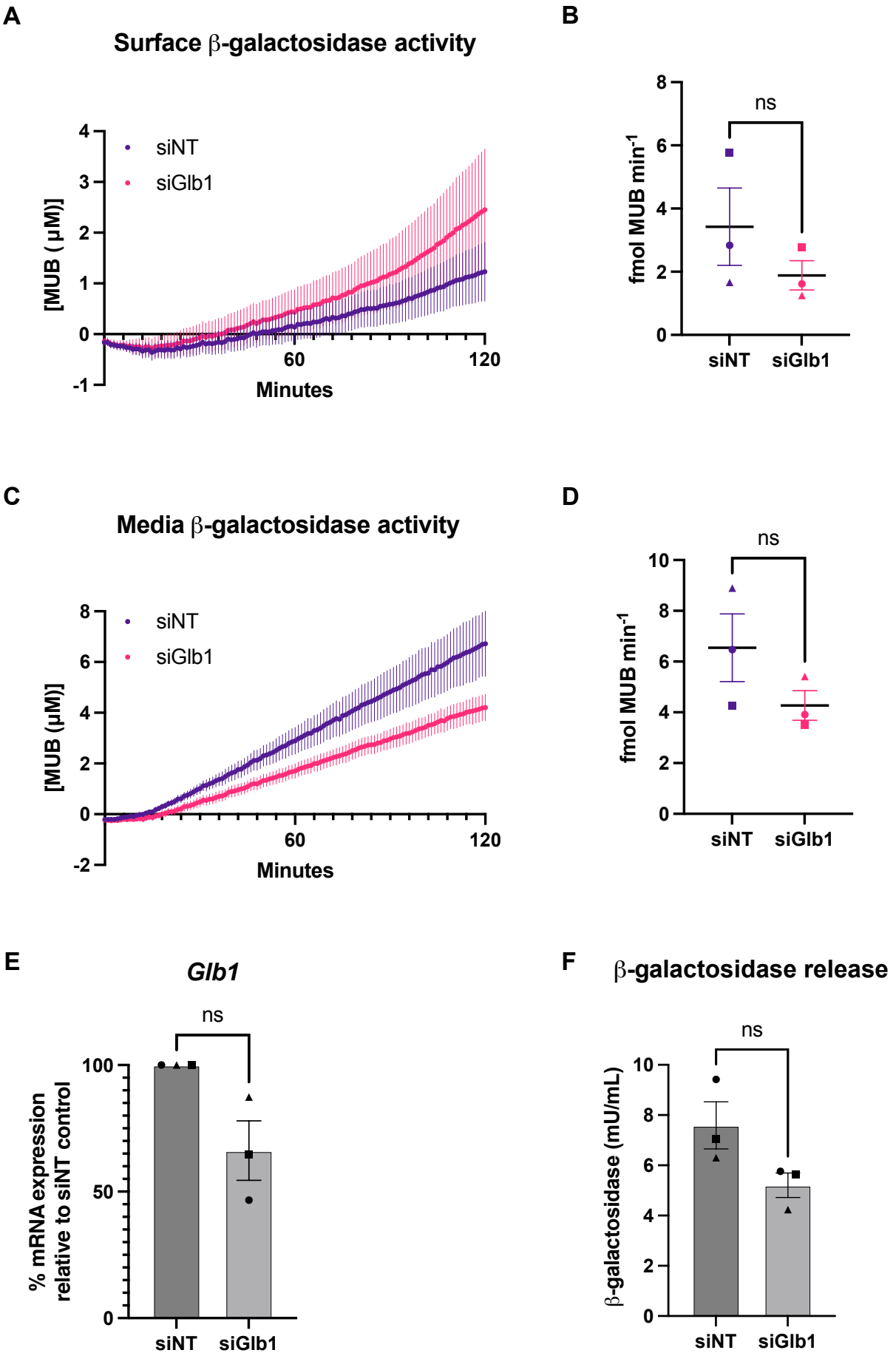
A
Surface β-galactosidase activity
siNT
siGlb1
[MUB (μM)]
Minutes
B
ns
fmol MUB min-1
siNT
siGlb1
C
Media β-galactosidase activity
siNT
siGlb1
[MUB (μM)]
Minutes
D
ns
fmol MUB min-1
siNT
siGlb1
E
Glb1
ns
% mRNA expression relative to siNT control
siNT
siGlb1
F
β-galactosidase release
ns
β-galactosidase (mU/mL)
siNT
siGlb1

Figure 6.7. Lipofectamine 3000 mediated RNAi knock down of GLB1 in BV-2 cells was unsuccessful. β-Galactosidase activity on the surface of non-targeting (siNT) or siGlb1 siRNA-transfected BV-2 cells and released into the supernatant of these cells was determined using the rate at which 4-Methylumbelliferyl-β-D-galactopyranoside (MUG) was converted to the fluorogenic product, 4-Methylumbelliferone (MUB) when added to a monolayer of cells (at pH 7.0) or to the cleared supernatant (at pH 4.0) from these cells. (A) β-Galactosidase activity was assayed on the cell surface of siNT and siGlb1-transfected cells. (B) The rate at which MUG was converted to MUB was determined from (A). (C) The supernatants of siNT and siGlb1-transfected cells from (A) were cleared and assayed for β-galactosidase activity and rate determined in (D). (E) qPCR analysis of mRNA level of mouse Glb1 compared to control (non-target) 24 h post transfection. (F) Supernatant was collected from cells in (A) and β-galactosidase concentration was determined by ELISA. Data represents mean values ± SEM of 3 independent knock down experiments. Statistical comparisons were made by paired t-test (ns: $p \geq 0.05$).

6.2.8 Identifying suitable β-galactosidase inhibitors

As knock down was unsuccessful, I wanted to test whether inhibiting β-galactosidase activity would affect LPS-induced microglial activation and neuronal loss. To do this, I first looked to identify inhibitors of β-galactosidase. I found two structurally unrelated inhibitors, D-galactono-1,4-lactone (DGL) (Figure 6.8A) and 1-deoxygalactonojirimycin (hydrochloride) (DGJ) Figure 6.8D), which are reported to inhibit β-galactosidase and are commercially available. I tested these and found that D-galactono-1,4-lactone and DGJ significantly reduced the activity of 0.2 mU/mL isolated β-galactosidase when added at 10 mM final concentration (Figure 6.8B, C, E & F).

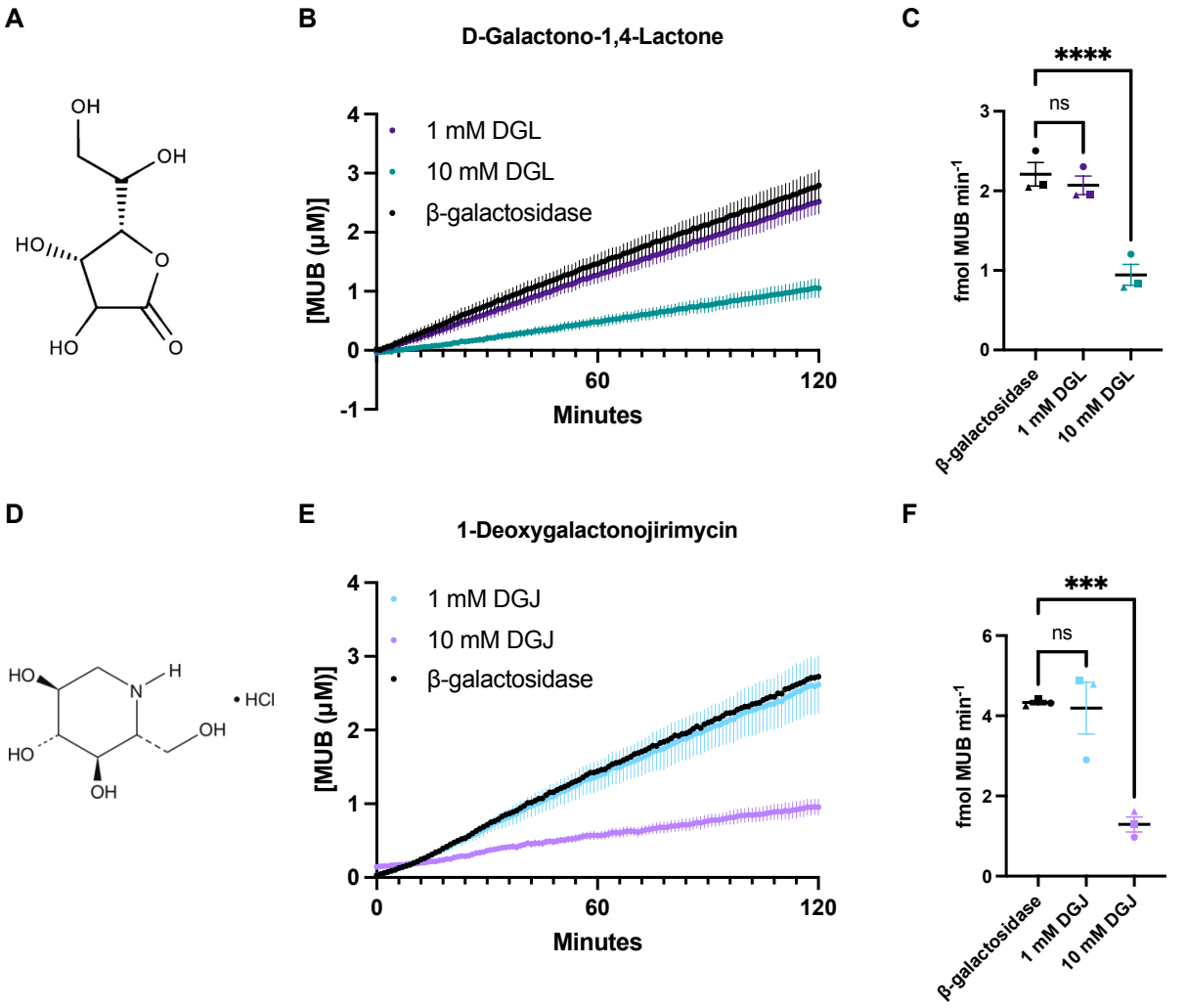

Figure 6.8. D-galactono,1,4-lactone and 1-deoxygalactonojirimycin inhibit β-galactosidase activity. Inhibition of β-galactosidase (from bovine liver) activity by D-galactono,1,4-lactone (DGL) and 1-deoxygalactonojirimycin (DGJ) was determined using the rate at which 4-Methylumbelliferyl-β-D-galactopyranoside (MUG) was converted to the fluorogenic product, 4-Methylumbelliferone (MUB). (A) Chemical structure of DGL. (B) 0.2 mU/mL β-galactosidase was incubated ± 1 mM or 10 mM DGL for 2 h and fluorescence recorded over time. (C) The rate at which MUG was converted to MUB was determined from (B). (D) Chemical structure of DGJ. (E) 0.2 mU/mL β-galactosidase was incubated ± 1 mM or 10 mM DGJ for 2 h and fluorescence recorded over time. (F) The rate at which MUG was converted to MUB was determined from (E). Data represents mean values ± SEM of at least 3 independent experiments. Statistical comparisons were made by repeated measures one-way ANOVA with Dunnett's post-hoc comparisons test. Asterisk (*) indicate significance compared to 0.2 mU/mL β-galactosidase control (ns: $p \geq 0.05$, ***: $p < 0.001$, ****: $p < 0.0001$).

6.2.9 D-Galactono-1,4-lactone (DGL) protects against LPS-induced neuronal loss

I then tested whether these inhibitors affected the neuronal loss induced by LPS. Addition of LPS to mixed neuronal-glial cultures for 72 h resulted in significant neuronal loss as expected, but co-treatment with 10 mM D-galactono-1,4-lactone substantially protected against this loss (Figure 6.9A). D-galactono-1,4-lactone did not completely negate LPS-induced neuronal loss, indicating that inhibiting β-galactosidase alone is not sufficient to completely protect against the effects of LPS on neuronal loss. D-galactono-1,4-lactone did not affect microglial

numbers here, however, it should be noted that the expected increase in microglial number with LPS treatment was not seen (Figure 6.9B), making it difficult to conclude from microglial counts alone whether inhibiting β-galactosidase by D-galactono-1,4-lactone in these cultures is sufficient to prevent LPS-induced microglial activation.

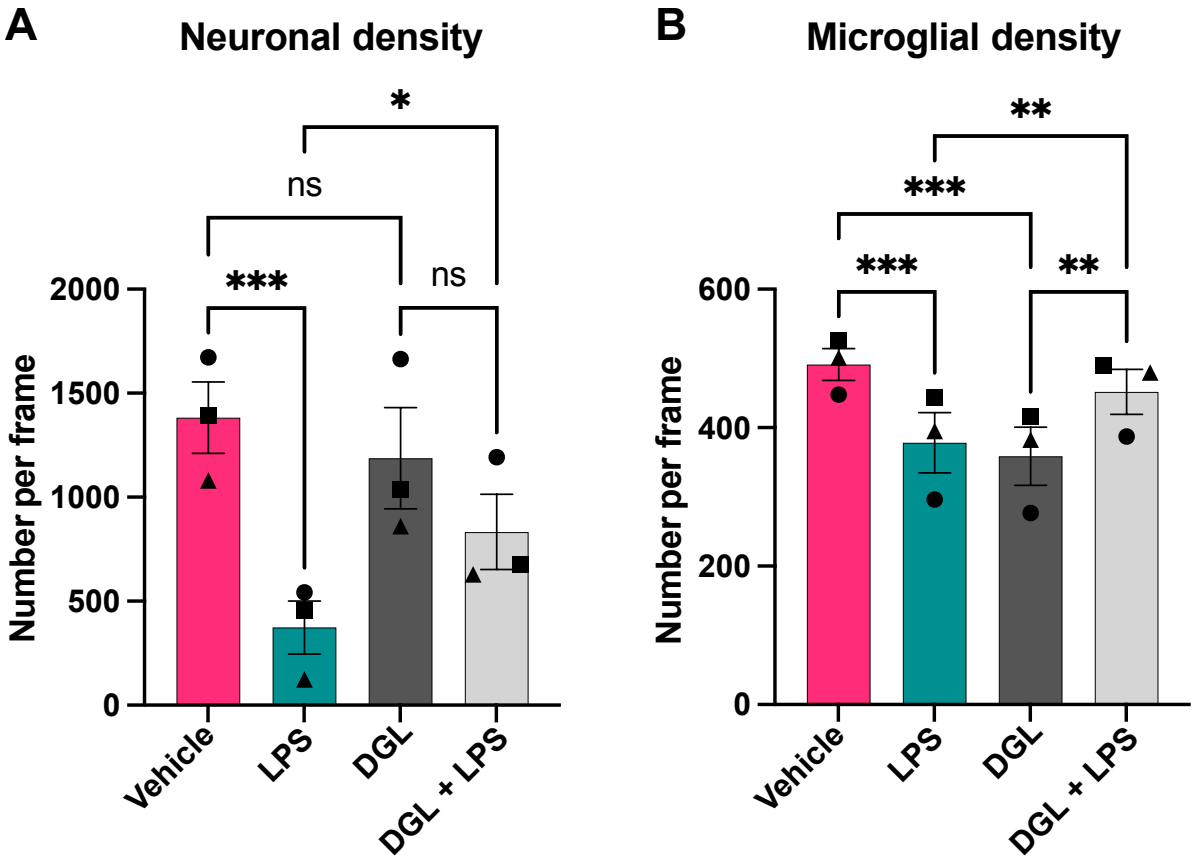

Figure 6.9. D-Galactono-1,4-lactone protects against neuronal loss. Mixed neuronal-glial cultures were treated with LPS (100 ng/mL) ± 10 mM D-galactono-1,4-lactone (DGL) for 72 h then cultures were stained with Hoechst 33342 (to identify nuclei), isolectin B4 (to identify microglia), NeuO (to identify live neurons). (A) Live neuronal cell numbers and, (B) microglial cell numbers were quantified by fluorescent microscopy using CellProfiler. Data represents mean values ± SEM of 3 independent experiments. Statistical comparisons were made by repeated measures two-way ANOVA with Šídák's post-hoc comparisons test. Asterisk (*) indicate significance compared to untreated control (ns: $p \geq 0.05$, *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$).

6.2.10 D-Galactono-1,4-lactone does not affect LPS-induced microglial morphology changes

To further assess the effects of β-galactosidase inhibition by D-galactono-1,4-lactone on microglial activation, I assessed the effect of D-galactono-1,4-lactone on LPS-induced morphological changes. I found that D-galactono-1,4-lactone did not affect changes in microglial area (Figure 6.10) induced by LPS. Hence, D-galactono-1,4-lactone inhibition of β-galactosidase appears to protect against neuronal loss without affecting microglial morphology.

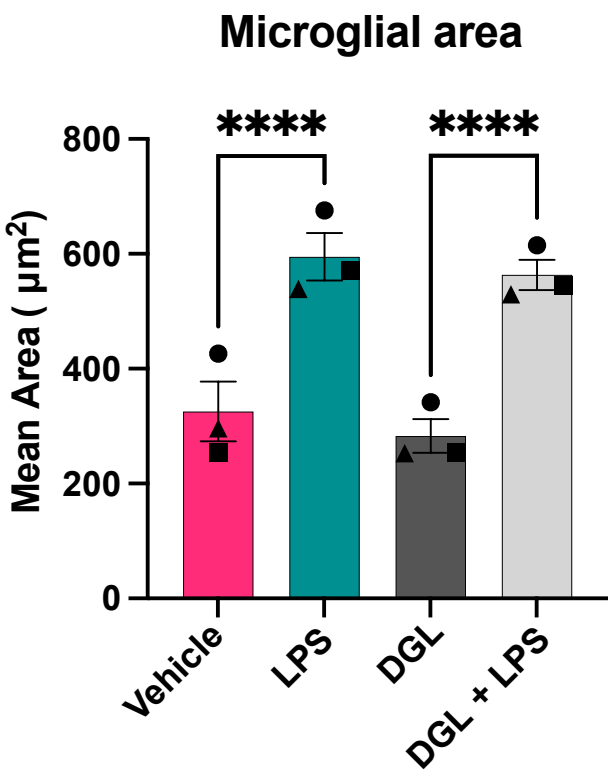

Figure 6.10. D-Galactono-1,4-lactone does not affect microglial morphology. Mixed neuronal-glial cultures were treated with LPS (100 ng/mL) ± 10 mM D-galactono-1,4-lactone (DGL) for 72 h then cultures were stained with Hoechst 33342 (to identify nuclei), isolectin B4 (to identify microglia), NeuO (to identify live neurons). (A) Microglial area was quantified by fluorescent microscopy using CellProfiler. Data represents mean values ± SEM of 3 independent experiments. Statistical comparisons were made by repeated measures two-way ANOVA with Šídák's post-hoc comparisons test. Asterisk (*) indicate significance compared to untreated control (****: $p<0.0001$).

6.2.11 D-Galactono-1,4-lactone modulates LPS-induced release of IL-6 and TNFα

Finally, to assess the effect of β-galactosidase inhibition by D-galactono-1,4-lactone on LPS-induced cytokine release, TNFα and IL-6 levels were assessed after treatment of mixed neuronal-glial cultures with LPS ± D-galactono-1,4-lactone for 72 h. Here, inhibition of β-galactosidase by D-galactono-1,4-lactone significantly reduced LPS-induced release of both TNFα (Figure 6.11A) and IL-6 (Figure 6.11B). Together, these results suggest that inhibition of β-galactosidase by D-galactono-1,4-lactone protects against LPS-induced neuronal loss and LPS-induced cytokine release without preventing LPS-induced microglial morphological changes. Hence, D-galactono-1,4-lactone may protect neurons by inhibiting some aspects of the LPS-induced activation of microglia, potentially by inhibiting the increased levels of β-galactosidase activity after LPS treatment.

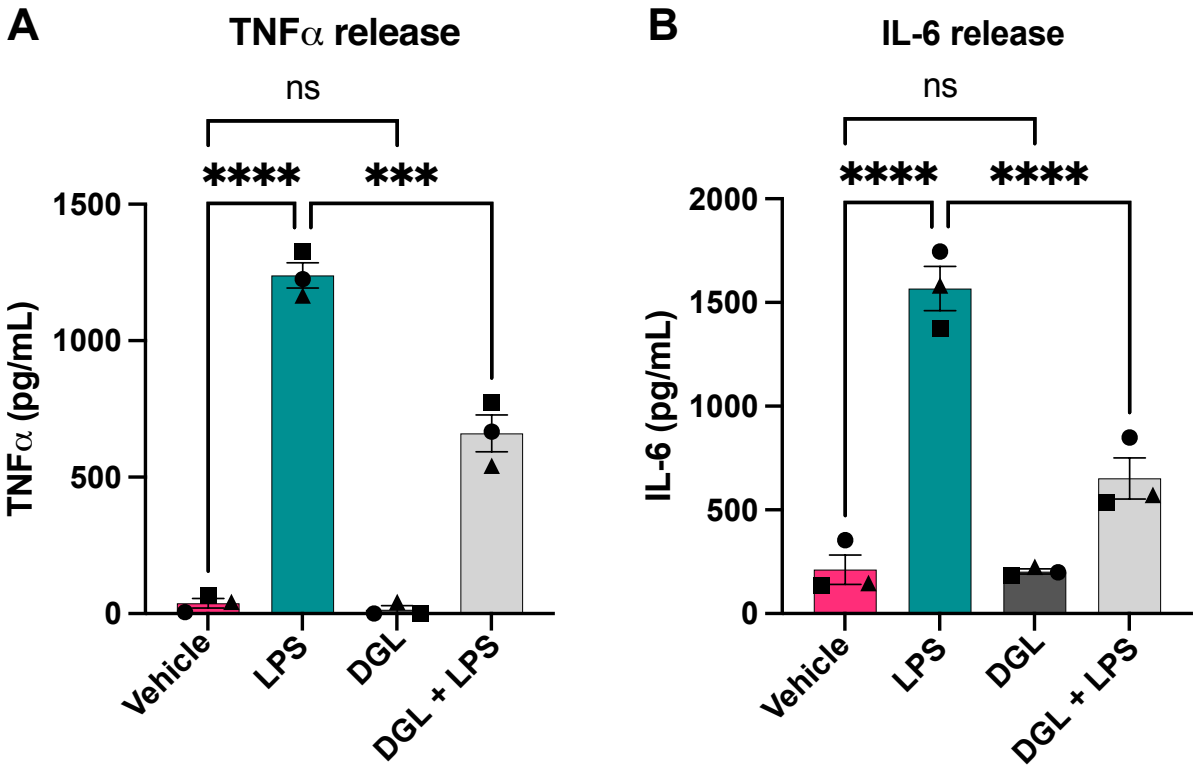

Figure 6.11. D-Galactono-1,4-lactone prevents microglial release of pro-inflammatory cytokines. Mixed neuronal-glial cultures were treated with LPS (100 ng/mL) ± 10 mM D-galactono-1,4-lactone (DGL) for 72 h. Supernatants were assessed for (A) TNFα and (B) IL-6 levels by ELISA. Data represents mean values ± SEM of 3 independent experiments. Statistical comparisons were made by repeated measures two-way ANOVA with Šídák's post-hoc comparisons test. Asterisk (*) indicate significance compared to untreated control (ns: $p \geq 0.05$, ***: $p < 0.001$, ****: $p < 0.0001$).

6.2.12 1-Deoxygalactonojirimycin (DGJ) protects neurons against LPS-induced loss

I then tested whether the other identified β-galactosidase inhibitor, DGJ, affected the neuronal loss induced by LPS. As expected, addition of LPS to mixed neuronal-glial cultures resulted in significant neuronal loss, but co-treatment with 10 mM DGJ significantly protected against this loss (Figure 6.12A). DGJ almost completely prevented LPS-induced neuronal loss, suggesting the possibility that DGJ may be more effective at inhibiting β-galactosidase in these conditions than D-galactono-1,4-lactone.

LPS induced some increase in microglial density, and DGJ had no effect on this LPS-induced increase, but none of these changes were significant (Figure 6.12B). So, it is unclear from these results whether DGJ affects LPS-induced microglial proliferation.

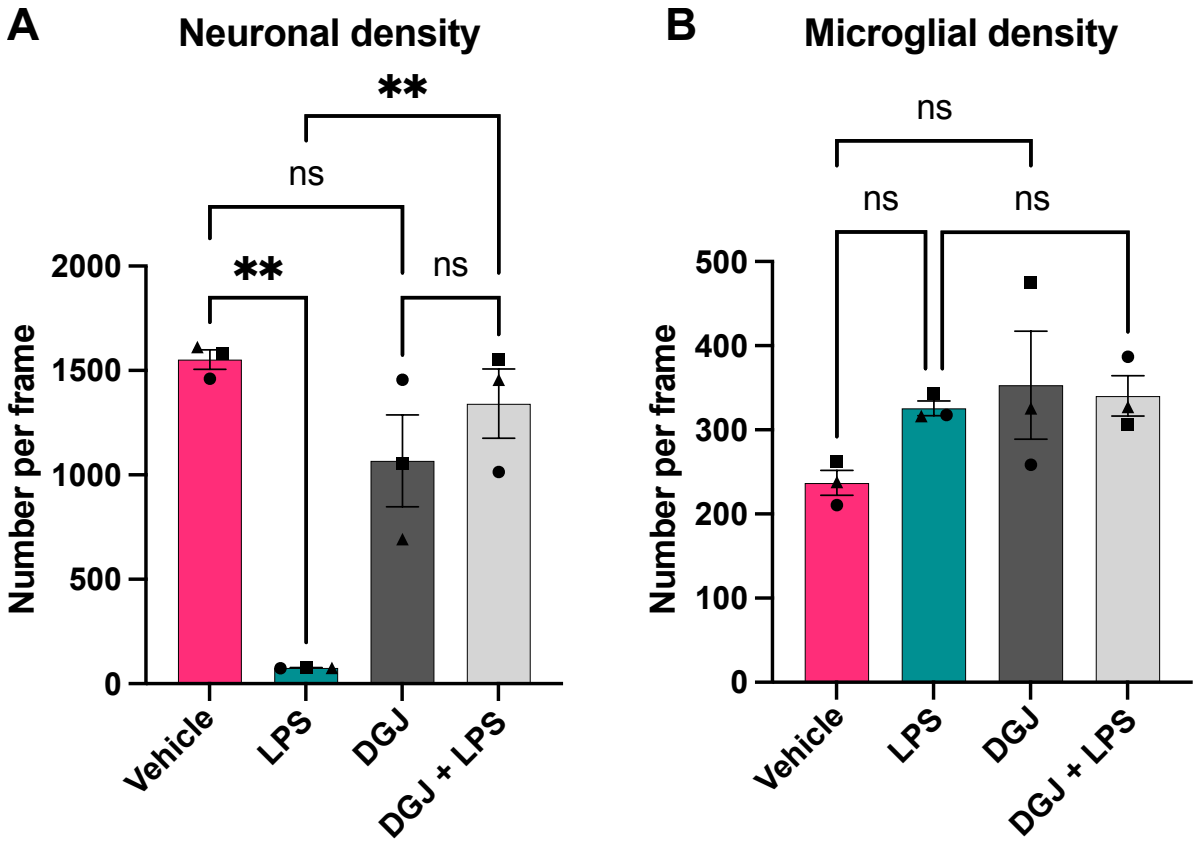

Figure 6.12. 1-Deoxygalactonojirimycin protects against neuronal loss. Mixed neuronal-glial cultures were treated with LPS (100 ng/mL) ± 10 mM 1-Deoxygalactonojirimycin (DGJ) for 72 h then cultures were stained with Hoechst 33342 (to identify nuclei), isolectin B4 (to identify microglia), NeuO (to identify live neurons). (A) Live neuronal cell numbers and, (B) microglial cell numbers were quantified by fluorescent microscopy using CellProfiler. Data represents mean values ± SEM of 3 independent experiments. Statistical comparisons were made by repeated measures two-way ANOVA with Šídák's post-hoc comparisons test. Asterisk (*) indicate significance compared to untreated control (ns: $p \geq 0.05$, **: $p < 0.01$).

6.2.13 1-Deoxygalactonojirimycin does not affect LPS-induced microglial morphology changes

To further assess the effects of β-galactosidase inhibition by DGJ on microglia, I examined the effect of DGJ on the LPS-induced morphological changes of microglia. I found that DGJ did not affect the LPS-induced increase in microglial area (Figure 6.13), however none of these changes were significant. Hence, it is not possible to conclude whether DGJ affects the LPS-induced changes in microglial morphology.

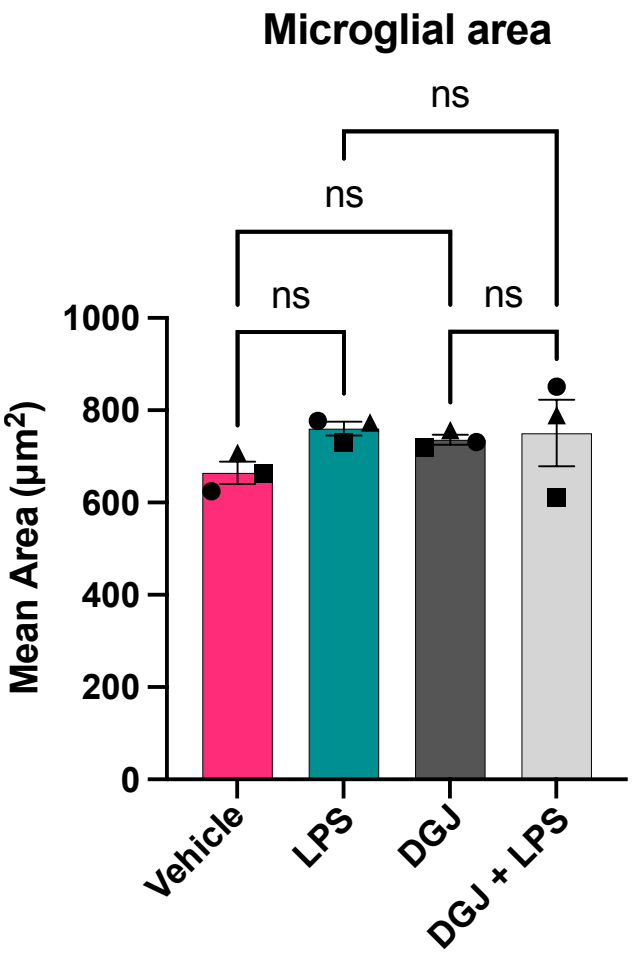

Figure 6.13. 1-Deoxygalactonojirimycin does not affect microglial morphology. Mixed neuronal-glial cultures were treated with LPS (100 ng/mL) ± 10 mM 1-Deoxygalactonojirimycin (DGJ) for 72 h then cultures were stained with Hoechst 33342 (to identify nuclei), isolectin B4 (to identify microglia), NeuO (to identify live neurons). (A) Microglial area and, (B) microglial formfactor were quantified by fluorescent microscopy using CellProfiler. Data represents mean values ± SEM of 3 independent experiments. Statistical comparisons were made by repeated measures two-way ANOVA with Šídák's post-hoc comparisons test (ns: $p \geq 0.05$).

6.2.14 1-Deoxygalactonojirimycin modulates LPS-induced TNFα and IL-6 release

To determine the effect of inhibiting β-galactosidase by DGJ on LPS-induced cytokine release, I assessed the levels of pro-inflammatory cytokines, TNFα and IL-6, in the supernatants of mixed neuronal-glial cultures after treatment with LPS ± DGJ for 72 h. Here, inhibition of β-galactosidase by DGJ significantly reduced LPS-induced release of both TNFα (Figure 6.14A) and IL-6 (Figure 6.14B). Together, these results suggest that inhibition of β-galactosidase by DGJ protects neurons against LPS-induced neuronal loss and prevents the microglial release of pro-inflammatory cytokines. Like D-galactono-1,4-lactone, DGJ may protect neurons by inhibiting the pro-inflammatory activation of microglia induced by increased levels of β-galactosidase activity after LPS treatment.

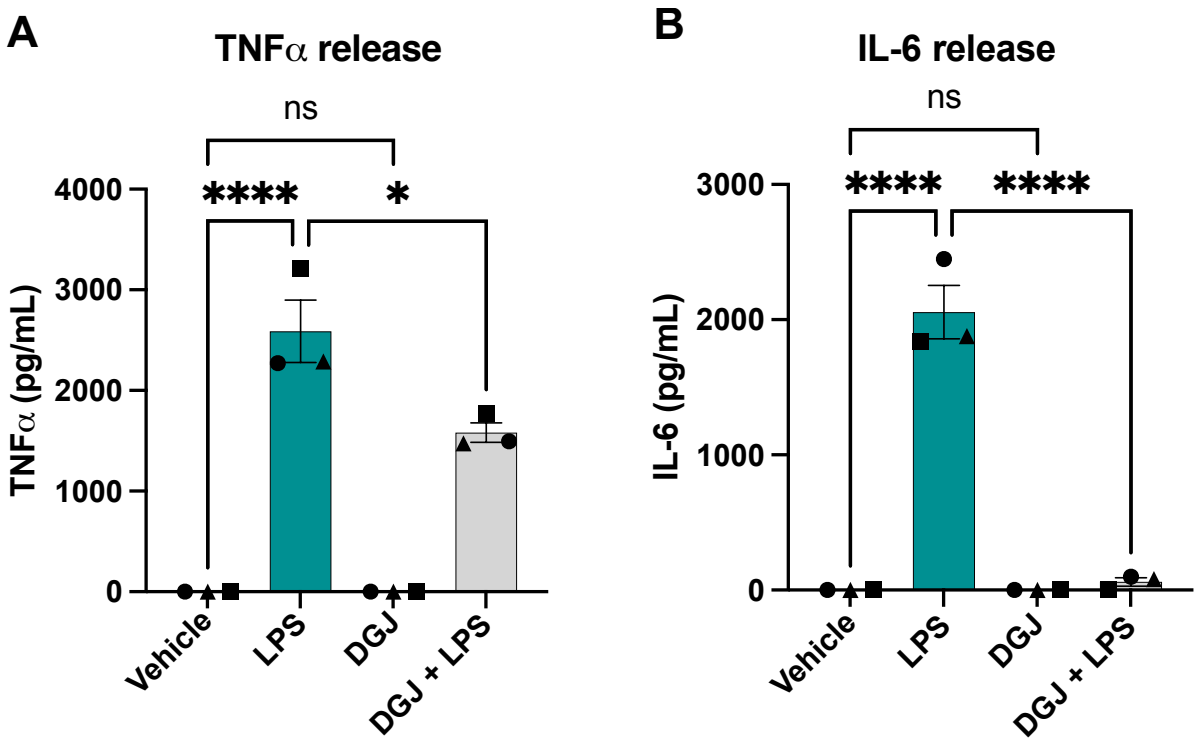

Figure 6.14. 1-Deoxygalactonojirimycin prevents microglial release of pro-inflammatory cytokines. Mixed neuronal-glial cultures were treated with LPS (100 ng/mL) ± 10 mM 1-Deoxygalactonojirimycin (DGJ) for 72 h. Supernatants were assessed for (A) TNFα and (B) IL-6 levels by ELISA. Data represents mean values ± SEM of 3 independent experiments. Statistical comparisons were made by repeated measures two-way ANOVA with Šídák's post-hoc comparisons test. Asterisk (*) indicate significance compared to untreated control (ns: $p \geq 0.05$, *: $p < 0.05$, ****: $p < 0.0001$).

6.3 Discussion

Neuronal loss is implicated in neurodegenerative diseases like AD and PD, and correlates with cognitive impairment and memory deficits (Ehringer and Hornykiewicz, 1960; Olanow, Stern and Sethi, 2009; Andrade-Moraes *et al.*, 2013). So, it could be that neuronal loss causes these dysfunctions, and preventing loss might halt disease progression. However, our current understanding of the mechanisms underlying neuronal loss in AD and PD is limited. As mentioned previously, gangliosides, like GM1, are glycosphingolipids that are particularly abundant in neuronal cell membranes where they are implicated in brain physiology and pathology. Active β-galactosidase at the plasma membrane might affect GM1 or glycosylation homeostasis at the cell surface, disrupting healthy neuronal physiology. Having found in chapter 5 that activated and aged microglia release increased levels of β-galactosidase compared to controls, the focus of this chapter was to investigate the effects of β-galactosidase on microglial function and neuronal loss.

I found that addition of β-galactosidase to primary mixed neuronal-glial cultures caused a significant loss of neurons and promoted an increase in the number of microglia, suggesting that β-galactosidase may activate microglia in a way that promotes neuronal loss. Addition of β-galactosidase also altered microglial morphology, as observed by fluorescent microscopy, and these changes were similar to, although to a lesser degree, than those seen in LPS-stimulated cultures. To further interrogate the effects of β-galactosidase on microglial activation, I assessed the levels of pro-inflammatory cytokine release in these cultures. β-Galactosidase promoted a significant release of TNFα compared to the vehicle-treated control, further suggesting that β-galactosidase promotes microglial activation. TNFα release, neuronal loss, microglial proliferation, and changes to microglial morphology all reverted to base-line levels by heat-inactivating β-galactosidase. Hence, the effects of β-galactosidase may be attributed to the active enzyme rather than addition of the protein, generally, or potential contamination.

Although microglia have been implicated as the major source of TNFα in neurodegenerative disorders and CNS insult, studies have shown that astrocytes and neurons may also produce this pro-inflammatory cytokine (Lee *et al.*, 1993; Breder *et al.*, 1994). I isolated primary microglia from mixed glial cultures and confirmed that β-galactosidase promotes TNFα release from microglia, although this does not rule out the possibility that astrocytes and neurons may contribute to β-galactosidase-mediated TNFα production. Furthermore, the effects on neuronal loss and TNFα release in mixed neuronal-glial cultures caused by isolated β-galactosidase were prevented by depleting microglia from these cultures, suggesting β-galactosidase is not directly neurotoxic, but that β-galactosidase induced neuronal loss is mediated by microglial activation and possibly cytokine release.

To investigate the involvement of endogenous β-galactosidase rather than exogenous addition of isolated β-galactosidase in neuronal loss and microglial activation, BV-2 microglia were subjected to siRNA-mediated knockdown of Glb1. This approach was not pursued as the β-galactosidase activity measured on the cell surface and released into the medium was not significantly down-regulated. Although Glb1 mRNA expression and protein levels were reduced compared to controls, transfection efficiency was variable and this reduction was not significant.

Alternatively, to explore the effects of endogenous enzyme activity, I identified two different β-galactosidase inhibitors. Previous work has identified sugar lactones as inhibitors of several mammalian glycosidases (Leaback, 1968; Levvy and Snaith, 1972) and D-galactono-1,4-lactone has been described as a competitive inhibitor against β-galactosidase isolated from rabbit brain (Jungalwala and Robins, 1968). Furthermore, 1-Deoxygalactonojirimycin (DGJ) is an iminosugar compound that has been investigated for its potential as an inhibitor; DGJ can mimic the structure of galactose and competitively inhibit the active site of β-galactosidase (Fantur *et al.*, 2010). In this work, I found that both D-galactono-1,4-lactone and DGJ inhibited enzyme activity of isolated bovine β-galactosidase, albeit at high concentrations.

LPS is a major structural glycolipid component of the outer membrane of Gram-negative bacteria. It is highly immunogenic and a strong pro-inflammatory agent that is widely used as a model of inflammation both *in vitro* and *in vivo*. I found previously that LPS induced β-galactosidase release in primary neuronal-glial cultures. Here, both β-galactosidase inhibitors reduced LPS-induced neuronal loss in these cultures and reduced the LPS-induced release of pro-inflammatory cytokines, IL-6 and TNFα. Neither inhibitor was capable of preventing LPS-induced neuronal loss completely, but the inhibition was substantial. Hence, my data suggest that microglia are activated by cell surface and/or extracellular β-galactosidase, and this contributes to the LPS-induced neuronal loss in mixed neuronal-glial cultures.

Together, my data suggests that β-galactosidase, in particular extracellular β-galactosidase, may be a good target to prevent inflammatory neurodegeneration. Unfortunately, these two inhibitors, D-galactono-1,4-lactone and DGJ, had to be used at millimolar concentrations to elicit an inhibitory effect in culture, reflecting the high concentrations necessary to inhibit activity of the isolated β-galactosidase enzyme. In this work, I did not identify any commercially-available inhibitors of mammalian β-galactosidase with higher potency. To further test and translate the role of β-galactosidase, the development of more potent inhibitors or other methods may be required.

I did not investigate further the mechanism by which β-galactosidase induced microglial activation or neuronal loss in these cultures, but it might be due to: i) mediation by pro-inflammatory cytokines (e.g., TNFα and IL-6), which are sufficient to induce neuronal loss (Conroy *et al.*, 2004; De Lella Ezcurra *et al.*, 2010; Smith *et al.*, 2012; Neniskyte, Vilalta and Brown, 2014); ii) loss of galactose residues and exposure of N-acetylglucosamine residues on the cell surface, which may induce microglial activation and microglial phagocytosis of neurons by opsonisation or complement activation (Neher *et al.*, 2011; Cockram *et al.*, 2021); and/or, iii) loss of GM1, which may protect neurons.

Mammalian β-galactosidase functions to remove terminal galactose residues from glycolipids and glycoproteins, including the ganglioside GM1 (Li and Li, 1999). GM1 has been shown to be anti-inflammatory in microglia and neuroprotective, both in culture and *in vivo* (van Dijk *et al.*, 2013; Galleguillos *et al.*, 2022). As aberrant and chronic microglial activation and neuroinflammation are implicated in many neurodegenerative disorders (Perry, Nicoll and Holmes, 2010), treatment with GM1 may ameliorate disease progression. In PD patients, GM1 deficiency has been reported and GM1 replacement has been proposed as a potential therapy (Wu *et al.*, 2012). Indeed, GM1 has been shown to ameliorate parkinsonism in primates (Schneider *et al.*, 1992) and, in humans, GM1 treatment may provide some clinical benefit in PD patients (Schneider *et al.*, 2013). Moreover, β-galactosidase activity is increased in the CSF of PD patients (van Dijk *et al.*, 2013). Thus, it is possible that cell surface β-galactosidase degrades protective GM1, resulting in microglial activation and neuronal loss. In theory, targeting β-galactosidase might pose an alternative or adjuvant treatment strategy. However, testing this would require further investigation.

Furthermore, the dysregulated shedding of gangliosides from neurons may be responsible for glial activation and it has been reported that TLR4 may mediate the ganglioside-triggered inflammation in glia (Jou *et al.*, 2006). If, under certain conditions, β-galactosidase activity is elevated in the brain, it could potentially lead to the degradation of GM1 ganglioside at a faster rate than usual, resulting in accumulation of ganglioside GM2. Elevated levels of GM2 in the brain is characteristic of GM2 gangliosidosis, including Tay Sachs disease, where GM2 build-up disrupts normal neuronal cellular processes and can be fatal (Demir *et al.*, 2020). Accumulation of GM2 has also been observed in AD (Kracun *et al.*, 1992), but the relationship between GM2 and neurodegeneration remains to be elucidated. It has been shown that GM2 promotes T-cell apoptosis and leads to immune dysfunction (Biswas *et al.*, 2009) and, GM2 might act as a target recognition structure for human natural killer cells (Ando *et al.*, 1987). Hence, accumulated GM2 may affect immune cell function and related inflammatory and immune processes. Interestingly, GM2 has been shown to markedly enhance TNFα expression in monocyte-derived cells and might elicit further inflammation (Mizutani *et al.*, 1999). So, it could be that activated microglia release β-galactosidase causing aberrant accumulation of GM2, then subsequent microglial interaction with GM2 could perpetuate chronic inflammation and microglia-mediated neurodegeneration.

In addition, extracellular β-galactosidase may remove terminal galactose residues from other receptors and/or cell surface glycoproteins to trigger an immune response via opsonisation and/or activation of the complement system which may alter microglial behaviour and affect neuronal loss. Indeed, it has been reported that gangliosides activate the complement system *in vitro* (Oshima, Soma and Mizuno, 1993). Ficolins and MBL are pattern recognition receptors of the innate immune system, involved in the activation of the lectin pathway of the complement system and subsequent, activation of inflammation, opsonisation, and immune clearance. MBL recognises and binds certain sugar residues, including N-acetylglucosamine and mannose, that are common on the surface of many microorganisms (Holmskov *et al.*, 1994). MBL is generally described to discriminate self from non-self; under normal physiological conditions most self-components are protected from MBL-binding by sialic acid or galactose residues (Petersen, Thiel and Jensenius, 2001). However, in principle, MBL might eliminate self-components that have lost these protective terminal residues through the action of neuraminidase and β-galactosidase enzymes. Much like MBL, ficolins have binding specificity towards N-acetylglucosamine and may opsonise microorganisms to facilitate their clearance by phagocytosis (Matsushita and Fujita, 2002). Furthermore, ficolins can activate the lectin complement pathway through interaction with MASPs (Matsushita, Endo and Fujita, 2000; Matsushita *et al.*, 2002). Ficolin-1 has been shown to be secreted from human monocytes/macrophages and found in human plasma and serum (Honoré *et al.*, 2008). Ficolin-2 can opsonise necrotic cells, activate complement and promote uptake of the necrotic cells by macrophages (Jensen *et al.*, 2007). Thus, it could be that removal of galactose residues by β-galactosidase exposes N-acetylglucosamine residues that are recognised by lectins (e.g., ficolins and/or MBL) which in turn promote their phagocytic uptake or removal via activation of the complement system. Further work is needed to assess this as a possible mechanism.

In sum, although β-galactosidase is generally considered a lysosomal enzyme, active β-galactosidase can be released by microglia under certain conditions, including: i) exposure to inflammatory stimuli like LPS and PMA; ii) in response to agents that stimulate a calcium rise and potentiate lysosomal exocytosis; and iii) as a result of ageing. Extracellular β-galactosidase can then mediate further activation of microglia and subsequent neuronal loss which can be reduced by inhibiting the enzyme (Figure 6.15). Hence, β-galactosidase induced neuronal loss is mediated by the activation of microglia and subsequent inflammation. Further work is required to establish the underlying mechanism for β-galactosidase-mediated microglial activation and neuronal loss, and it would be interesting to assess whether the effects of β-galactosidase that I have seen *in vitro* can be recapitulated *in vivo*. Due to the ubiquitous nature of β-galactosidase, targeting all β-galactosidase may be toxic long-term but it might, in principle, be possible and beneficial to target extracellular β-galactosidase, over lysosomal β-galactosidase.

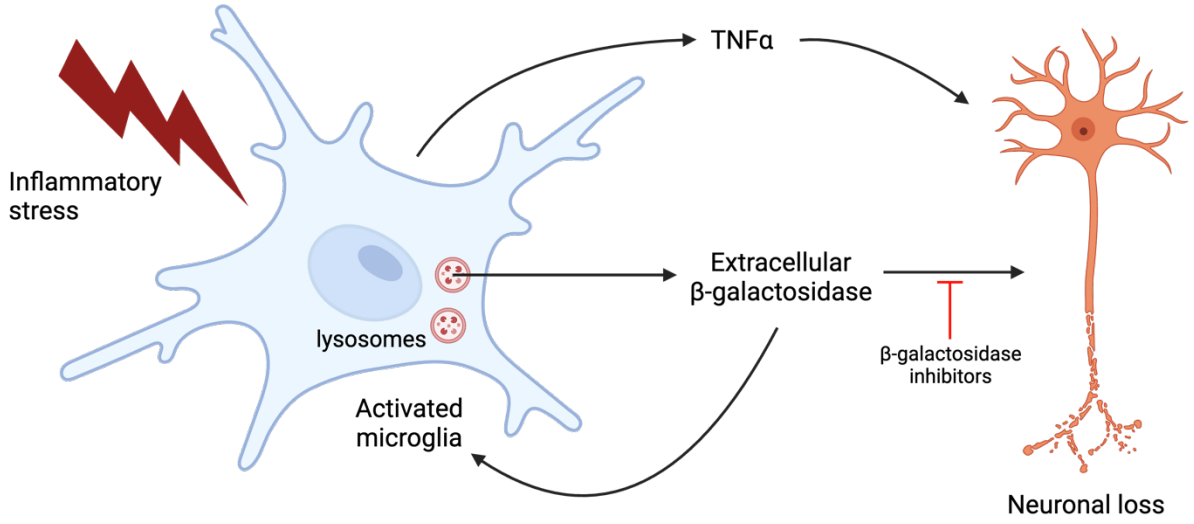

Figure 6.15. Summary of Chapter 6. Upon exposure to inflammatory stimuli like LPS, active β-galactosidase can be released by microglia. Extracellular β-galactosidase can then mediate further activation of microglia, increase TNFα release, and cause subsequent neuronal loss which can be reduced by inhibiting the enzyme.

7 DISCUSSION, LIMITATIONS AND FUTURE PERSPECTIVES

Microglia are the primary immune cells of the CNS; they have dynamic roles in brain development, maintaining homeostasis, and responding to insult and injury, thereby contributing to CNS health. However, increasing evidence highlights a role for microglial dysfunction in many brain pathologies. As ageing is the greatest risk factor for neurodegenerative disorders like AD, understanding the processes that microglia undergo in the ageing brain may hold the key to developing novel therapeutic approaches for many age-related brain diseases. In this dissertation, I present novel findings pertaining to the roles of calreticulin and β-galactosidase - two proteins associated with ageing, chronic inflammation, and neurodegeneration - on microglial functions and neuronal loss.

Traditionally, research has focussed on understanding calreticulin and β-galactosidase within the ER and lysosomes, respectively. Here, I present work that considers the release and extracellular functions of calreticulin and β-galactosidase. By uncovering previously unexplored functions and locations of these otherwise intracellular proteins, research may provide insights into various biological processes and potential therapeutic targets. For instance, understanding how chaperones, like calreticulin, assist in the folding of extracellular proteins may have implications for diseases associated with misfolded proteins, such as neurodegenerative disorders. Additionally, the extracellular release of enzymes like β-galactosidase could be relevant in contexts including intercellular signalling and immune responses. Such unconventional roles challenge the established dogma and offer exciting opportunities for advancing our understanding of various physiological and pathological processes.

The aggregation of amyloid-β to form oligomers and insoluble amyloid plaques in the brain is a hallmark of AD. Amyloid-β can be directly neurotoxic and induce pro-inflammatory activation of microglia, which may contribute to neurodegeneration. Over the last twenty years, researchers have extensively investigated the harmful effects of oligomeric amyloid-β species as a key pathophysiological process in AD, although the precise mechanisms of toxicity remain to be fully elucidated. As part of the cellular quality control machinery, molecular chaperones interact with other proteins to prevent their aggregation and facilitate proper folding; they can intervene at different stages of the aggregation process to inhibit or reverse aberrant protein aggregation or counteract the toxicity associated with amyloid species. An increasing number of extracellular chaperone proteins have been identified for amyloid-β, including: clusterin (Humphreys *et al.*, 1999) and neuroserpin (West *et al.*, 2021).

The ER-resident chaperone protein, calreticulin, can be released from microglia and has been found to bind amyloid-β (Erickson *et al.*, 2005; Cockram, Puigdellívol and Brown, 2019;

Reid *et al.*, 2022). I investigated whether exogenous calreticulin affects amyloid-β aggregation and amyloid-β-induced neurotoxicity. I present data that suggests calreticulin can act as an extracellular chaperone and neuroprotectant by beneficially activating microglia. *In vitro* assays revealed inhibition of amyloid-β fibrillisation by calreticulin, as measured by a reduction in ThT fluorescence. This supported previous reports that amyloid-β fibrillisation is affected, in a concentration-dependent manner, by calreticulin (Kitauchi and Sakono, 2016). Further examination by TEM showed that calreticulin promoted the formation of larger amyloid-β oligomers. De *et al.*, (2019) suggested that large amyloid-β oligomeric species induce neurotoxicity via an increased inflammatory response. As microglia are thought to be the main mediators of inflammation in the brain, I assessed the effects of exogenous calreticulin on amyloid-β-induced neuronal loss in primary mixed neuronal-glial cultures. I found that nanomolar concentrations of calreticulin were neuroprotective in these cultures, yet microglia showed signs indicative of activation, with changes to microglial number and morphology. Microglia can be activated into a variety of different states, but how and when microglia are beneficial, detrimental or both, is still under investigation. In this context, calreticulin may promote activation of microglia into a neuroprotective state, but further work is required to understand the underlying mechanism and whether this neuroprotection can be replicated *in vivo.* To do this, calreticulin and amyloid-β could be co-injected into mice or, calreticulin could be injected into transgenic mice that model amyloid pathology and subsequent levels of amyloid-β deposition or the effect on cognitive function assessed. This approach has been used previously to assess the effects of clusterin in the 5XFAD murine model of amyloid pathology (Qi *et al.*, 2018) and the Bri2 BRICHOS chaperone domain in *App* knock-in mouse models (Manchanda *et al.*, 2023). Alternatively, calreticulin could be overexpressed in these mouse models to assess effects on amyloid pathology; and by introducing a modified form of calreticulin that is targeted for secretion outside the cell, increased levels of extracellular calreticulin could be interrogated more closely. Furthermore, acute injection of calreticulin into wild-type mice might inform whether calreticulin activates microglia *in vivo* and in what way.

Proteolytic cleavage of APP by β- and γ-secretases results in the production of amyloid-β peptides from 38 to 43 amino acids long (Chow *et al.*, 2010). Of these isoforms, the most abundantly generated are amyloid-β 1-40 and 1-42 (Murphy and Levine, 2010). Amyloid-β 1-42 has a higher tendency to self-aggregate and this has been attributed to the two additional hydrophobic amino acids at the C-terminus (Nirmalraj *et al.*, 2020). Furthermore, longer peptide forms, like amyloid-β 1-42, are implicated in AD pathology as mutations resulting in increased production of this isoform are associated with early onset and accelerated pathology (Haass and Selkoe, 2022). Previous work suggests that calreticulin binds to the hydrophobic C-terminus of amyloid-β; although amyloid-β 1-40 showed some binding, strongest binding was to the 1-42 peptide (Houen, Duus and Hansen, 2008). In this

work, I used synthetic amyloid-β 1-42 exclusively, hence the implications of my data are limited to this form of amyloid-β. Interactions of calreticulin with other amyloid-β peptides, especially those lacking residues 41 and 42, may differ and would need further investigation.

Calreticulin levels in the CSF of AD patients are significantly lower than in healthy individuals (Lin, Cao and Gao, 2014) and reduced calreticulin expression in murine neurons and glia has been associated with cognitive decline (Pawlowski *et al.*, 2009). Hence, my data suggests that increasing extracellular calreticulin in the brain might be beneficial for AD. For example, viral vectors expressing a secreted form of calreticulin might be targeted to the brain. However, it is important to note that the direct therapeutic use of calreticulin in AD requires further research, with several challenges to consider. 1) Delivering calreticulin to the brain in a therapeutically effective manner may be challenging, especially considering its size and potential immunogenicity. 2) Modulating calreticulin's activity may have unintended consequences on cellular function, given its multifaceted roles in protein folding and calcium regulation. Thus, any therapeutic approach would need to carefully balance potential benefits and risks. 3) While calreticulin's roles in protein quality control and calcium homeostasis may indirectly relate to AD pathology, its therapeutic potential in AD remains largely unexplored. Future research may uncover ways to leverage these mechanisms or develop calreticulin-related therapies, but it remains an emerging area of study, and other therapeutic approaches for AD continue to be the primary focus of research and drug development. Ultimately, identifying chaperone proteins associated with amyloidogenic species, and understanding their role in modulating protein aggregation and influencing cellular outcomes, has the potential to uncover innovative therapeutic targets for neurodegenerative diseases.

Glycohydrolase enzymes, including Neu1 and β-galactosidase, play a fundamental role in the degradation of glycoproteins and glycolipids, to maintain cellular constituent turnover and glycosylation homeostasis. Neu1 hydrolyses terminal sialic residues to expose galactose residues, which can then be hydrolysed by β-galactosidase. Previously it was thought that these enzymes were exclusively located in lysosomes, but recent evidence has found Neu1 activity also associated with the external surface of the plasma membrane. As Neu1 can be structurally and functionally coupled to β-galactosidase in a lysosomal multienzyme complex, I investigated whether BV-2 microglia and primary rat microglia have increased extracellular β-galactosidase activity when activated by a variety of stimuli. I found that inflammatory activation of BV-2 microglia by LPS and PMA increased β-galactosidase activity at the cell surface and increased β-galactosidase protein levels extracellularly.

One potential cause of β-galactosidase release from lysosomes is lysosomal exocytosis, which can be induced by a rise in intracellular calcium. To induce a rise in intracellular calcium, I stimulated BV-2 cells acutely with ATP and calcium ionophore A23187. Both agents induced an immediate increase in surface β-galactosidase activity, supporting the

idea that lysosomal exocytosis may result in β-galactosidase release. It has been reported that LPS-stimulation of BV-2 microglia increases lysosomal membrane protein, lysosome-associated protein-1 (LAMP-1), levels at the plasma membrane, suggesting lysosomal exocytosis is induced upon inflammatory activation of microglia (Allendorf and Brown, 2022). However, to confirm that β-galactosidase release occurs via lysosomal exocytosis would require further investigation. One approach could be to inhibit lysosomal exocytosis. Vacuolin-1 blocks the calcium-dependent fusion of lysosomes with the plasma membrane and the release of lysosomal contents (Cerny *et al.*, 2004). A reduction in extracellular neuraminidase activity in BV-2 and primary rat microglia has previously been observed when cells are pre-treated with vacuolin-1 (Allendorf and Brown, 2022), indicating that lysosomal exocytosis may be involved in the release of neuraminidase. A similar approach could be applied to investigate the mechanism for β-galactosidase release.

β-Galactosidase, Neu1 and PPCA are generally described as soluble proteins, raising the question, how can they be retained at the plasma membrane? Recent evidence suggests that Neu1 may also have a cell membrane-associated form (Lukong *et al.*, 2001) and another study demonstrated that mature Neu1 protein has two hydrophobic regions that may form putative transmembrane domains (Maurice *et al.*, 2016). Hence, Neu1 may exist in both membrane and soluble forms, which can translocate to the plasma membrane or be released, respectively. It is therefore conceivable that β-galactosidase, in complex with Neu1, translocates to the plasma membrane or into the extracellular milieu. Moreover, PPCA which is found in complex with Neu1 and β-galactosidase in the lysosome, has been detected in BV-2 supernatants (Allendorf and Brown, 2022). Thus, it may be that Neu1, β-galactosidase and PPCA are released in complex. This hypothesis could be tested by co-immunoprecipitation experiments to assess β-galactosidase binding to Neu1 or PPCA in culture supernatants. Additionally, lysosomal β-galactosidase is reported to have a pH optimum of pH 4.0 – 4.5 (Butor *et al.*, 1995; Gary and Kindell, 2005; Ohto *et al.*, 2012), but it is not known how much of this activity is retained at the cell surface where the pH may be higher. It has been shown that, although 5 – 10 fold lower, β-galactosidase does remain active at pH 7.0 (Butor *et al.*, 1995; Gary and Kindell, 2005). It is possible that β-galactosidase interacts with other proteins at the cell surface, and this alters its pH sensitivity. Furthermore, several groups have reported that mature β-galactosidase isolated from mammalian tissues can form a monomer (Norden, Tennant and O'Brien, 1974), dimer (Hubbes, D'Agrosa and Callahan, 1992) or tetramer (Van Der Spoel, Bonten and D'Azzo, 2000). It has been suggested that these structural variations result from differing experimental procedures, particularly influenced by pH conditions (Ohto *et al.*, 2012). For example, porcine β-galactosidase demonstrates pH-dependent oligomerisation, adopting a monomeric form at pH 7.0 and reversibly associating to a dimer in acidic conditions (Yamamoto, Fujie and Nishimura, 1982). Therefore, it may be that β-galactosidase is more active as a dimer, but

retains some enzymatic activity as a monomer at neutral pH. In addition, the local pH of the cell surface microenvironment may be lower than the bulk extracellular pH because the cell surface has a net negative charge. Overall, understanding how β-galactosidase gets to the cell surface, or is released, and remains active outside the lysosome is unclear and requires further investigation.

Senescent cells, which are characterised by increased β-galactosidase activity and expansion of their lysosomal content, are implicated in neurodegeneration (Baker and Petersen, 2018). Yet, a causal relationship between senescence and neurodegeneration has not been established. As senescent cells are characterised by an increased number of lysosomes and SA-β-galactosidase activity (Dimri *et al.*, 1995), I wanted to assess whether β-galactosidase was released from senescent microglia. In this work, I was unable to produce a robust model for senescent microglia, highlighting fundamental limitations of current available models and the methods used to characterise senescence. Instead, as microglia are known to become activated and senesce with age in mice (Matsudaira *et al.*, 2023), I examined β-galactosidase levels in sections from the brains of mice at 4- and 17-months-old. I found that, in aged brains, there were increased levels of β-galactosidase, which may be translocated from the lysosomal compartment to the cell surface. Therefore, it may be that microglia in aged brains have elevated levels of surface β-galactosidase. This might lead to microglial activation and harmful consequences for neurons, potentially contributing to the neurodegeneration linked to ageing. However, substantiating this hypothesis would require further investigation, in particular that β-galactosidase is on the surface of aged microglia and that this activates microglia and damages neurons.

Having found that activated and, potentially, aged microglia release β-galactosidase, I sought to investigate whether β-galactosidase promoted inflammatory neurodegeneration. Extracellular β-galactosidase might remove galactose residues from the surface of microglia and neurons, potentially disrupting homeostasis. I found that addition of β-galactosidase to primary mixed neuronal-glial cultures caused a significant loss of neurons and promoted microglial activation, indicated by a significant increase in TNFα release. Neuronal loss and TNFα release were both prevented by microglial depletion. Moreover, inhibition of β-galactosidase in LPS-stimulated cultures reduced LPS-induced neuronal loss and microglial activation, suggesting that β-galactosidase may activate microglia in a way that promotes neuronal loss. To investigate whether neuronal loss is due to neuronal phagocytosis by microglia, neurons could be labelled with a pH-sensitive dye (e.g., pHrodo dyes) which are nonfluorescent at neutral pH and fluoresce under acidic conditions. Consequently these dyes can be used as highly specific sensors of phagocytic internalisation and lysosomal sequestration in live cells. Together, my data suggests that β-galactosidase, in particular extracellular β-galactosidase, may be a good target to prevent inflammatory

neurodegeneration. However, due to the essential roles of β-galactosidase in lysosomes, targeting all β-galactosidase may be toxic long-term. It might be possible to target extracellular β-galactosidase relative to lysosomal β-galactosidase, based on its location or pH. Delineating the mechanism by which β-galactosidase activates microglia and promotes neuronal loss in these cultures warrants further investigation, to better understand how β-galactosidase might be targeted therapeutically.

Commercial sources of mammalian, especially human β-galactosidase, are limited and expensive. Throughout this work, exogenous β-galactosidase from bovine liver was used. This enzyme caused significant neuronal loss and a substantial release of the pro-inflammatory cytokine TNFα, which was prevented by inhibiting or heat-inactivating the enzyme. However, addition of high enzyme concentrations were necessary to see these effects which can lead to the formation of non-physiological complexes or reactions that do not occur under normal physiological conditions. Furthermore, high concentrations of added enzyme can lead to non-specific effects on the experimental system and the enzyme may interact with other molecules in ways that are not related to its intended catalytic activity. To further assess the link between released β-galactosidase and its neurotoxic effects, it would be better to block endogenous extracellular β-galactosidase using more potent inhibitors or function blocking antibodies to reduce potential off-target effects. A potent inhibitor of mammalian β-galactosidase, DLHex-DGJ, which is a derivative of 1-deoxygalactonojirimycin, has been reported (Fantur *et al.*, 2010). DLHex-DGJ was not available for this study, but might be worth resynthesizing to test for neuroprotection and potential translational studies. It is also worth further investigating other means of β-galactosidase knockdown as the siRNA-transfection approach used here was insufficient for further analysis. Future work to assess the effects of extracellular β-galactosidase *in vivo* would also be important. β-Galactosidase could be introduced into the mouse brain through several methods, including direct injection or by expressing it as an extracellular form. Alternatively, in conditions where extracellular β-galactosidase levels might be elevated, like with LPS stimulation or ageing, targeting extracellular β-galactosidase could involve the use of function blocking antibodies or targeted inhibitors.

There are several general limitations to the work presented in this dissertation. The use of different model systems to understand the role of microglia in neurodegeneration is important but can generate conflicting evidence. Firstly, I assessed the effects of human or bovine proteins in rodent cell systems. Although, this is common in research, minimising mixed systems where possible is preferable. I used commercially sourced calreticulin and β-galactosidase which could potentially be contaminated with endotoxin, thereby affecting microglial activation. To control for endotoxin, calreticulin was assessed previously in the lab using an LAL kit. However, due to the expense and sustainability of LAL tests, this was not

done for β-galactosidase. Instead, heat-inactivated enzyme was used as an alternative. Furthermore, this work is carried out exclusively using *in vitro* models. Results obtained from experiments conducted in monoculture do not account for the diverse interactions attributed to the composite architecture of the brain, including the complex biology of other brain cell types such as astrocytes or oligodendrocytes. Hence, where possible, work was complemented with data using mixed primary neuronal-glial cultures. Although immortalised cell lines, like BV-2 microglia, represent an easy to use and relatively cheap system in which to test the effects of proteins on microglial functions, the use of immortalised cell lines is controversial in the field. Immortalised by retroviral introduction of v-*myc*/v-*raf* genes into primary mouse microglia, BV-2 cells present typical immune cell functions in response to LPS on a transcriptional and function level (Blasi *et al.*, 1990). However, in the absence of a stimulus, compared to microglia *in vivo*, cultured BV-2 cells appear more amoeboid and secrete higher levels of cytokines indicating a higher baseline activation. Therefore, it is important to be cautious when extrapolating the physiological relevance of data obtain in cell lines. Although their use should not be ignored, as they provide a crucial starting point for future research. Alternatively, iPSC-derived microglia are gaining popularity in research. When co-cultured with neurons, iPSC-derived microglia express key microglia-specific markers, display dynamic ramifications, and have phagocytic capacity (Haenseler *et al.*, 2017). Although iPSCs require more complex maintenance and differentiation protocols, when using patient derived cells, they represent a robust, more disease-relevant model. As microglia in mice and humans show differences in their biology, in addition to some of the *in vivo* experiments suggested above, it would be interesting to use human iPSCs to assess the effects of calreticulin and β-galactosidase.

In addition to using immortalised cell lines, isolating microglia from new-born rodent pups is common practice and here I used primary cells from neonatal rat brains to supplement cell line work. Primary cells represent a more physiologically relevant system, but are time consuming to isolate and maintain. Moreover, they have limited replicative capacity so only a finite number of cells can be obtained per animal and as these cells are derived from neonates, they might not recapitulate the diverse properties of mature microglia. It should also be noted that microglia *in vitro* and *in vivo* have different responses to amyloid-β, with murine primary microglia displaying substantial transcriptional changes, while *in vivo* microglia do not, suggesting that primary microglia poorly reflect *in vivo* conditions (McFarland *et al.*, 2021). Moreover, the regional heterogeneity of mature microglia within the adult brain may not be recapitulated in immortalised or primary cultures. An *ex vivo* system, like organotypic slice cultures, might represent an alternative model that retains elements of brain tissue architecture. However, all of the aforementioned models are maintained in serum-supplemented media. In my experiments, FBS was used to supplement media, which can itself activate microglia. *In vivo*, microglial exposure to serum components is minimal, so this might account for the different microglial responses to stimuli observed *in vitro*. Arguably, the most physiologically relevant models available are healthy and disease animal models, used for *in vivo* testing. However, they have their own drawbacks as the diseases they are

used to simulate are complex and, any one model cannot fully reflect the changes occurring in humans. Ultimately, it is important to work with multiple systems, to generate a broader understanding of the cellular and molecular mechanisms underlying neurodegeneration and disease, and to better design targeted therapeutics.

Investigation into early disease-stage biomarkers is also key to improving therapeutic outcomes. Brain-imaging using positron emission tomography (PET) scans and magnetic resonance imaging (MRI) devices have enabled better diagnosis. Moreover, molecular biomarkers, such as amyloid-β and phosphorylated tau in the CSF can be used to determine severity of disease. However, these expensive and invasive procedures are often employed to confirm diagnosis once a patient is symptomatic. Given that pathophysiological processes likely commence years prior to symptom manifestation, there is a need to adjust our approach to diagnosis. The identification of early biomarkers in available biofluids, such as blood, urine, and saliva, might enable relatively non-invasive, rapid, and early detection of disease status. Early diagnosis should allow mechanism-targeting therapies to benefit patients more effectively by preventing or slowing down the disease in the early stages. In concert with more relevant translational disease models, early biomarkers will help to bridge the gap between basic research and clinical outcomes. Calreticulin has been described as a negative biomarker for AD (Lin, Cao and Gao, 2014) and evidence has been found that calreticulin levels increase in the urine of patients with bladder cancer, where it has been proposed as a possible biomarker (Kageyama *et al.*, 2004). Further work is necessary to determine whether calreticulin could have a diagnostic role in AD. β-Galactosidase activity has long been used as a biomarker for the detection of cellular senescence, but how this might link to neurodegeneration and translate to diagnosis is yet to be ascertained. Measuring CSF levels of calreticulin and β-galactosidase in neurodegenerative disease patients and age-matched controls would be a useful starting point for such research.

Understanding the non-canonical, extracellular roles of proteins, like calreticulin and β-galactosidase, holds promise for the future with the potential to significantly advance our understanding of brain function, neurodegenerative diseases, and therapeutic interventions. The concept of extracellular chaperones is relatively new; these proteins may play a role in maintaining protein homeostasis outside cells and could have implications for protein aggregation diseases, making them relevant targets for therapeutic intervention. Furthermore, understanding how extracellular chaperones participate in protein quality control mechanisms and interact with misfolded proteins can shed light on the underlying mechanisms of neurodegeneration. Moreover, the future of research on extracellular glycohydrolase enzymes and cell surface glycan patterns in the brain holds significant promise. Cell surface glycosylation patterns may impact immune responses and neuroinflammation and, further research in this area may uncover new insights into the role of the immune system in brain health and disease. Furthermore, glycohydrolases may be involved in the remodelling of the extracellular matrix in the brain. Future research to understand the glycosylation patterns of neurons, and how they are influenced by

extracellular glycohydrolases, could shed light on neural development, function, plasticity, and disease. Advances in technology, including proteomics, glycomics, and imaging techniques, are making it increasingly feasible to study extracellular chaperones and cell surface glycosylation in the brain.

Finally, with our increasing understanding of the diverse mechanisms at play in neurodegeneration, addressing neurodegenerative diseases such as AD requires a multifaceted approach involving multiple therapeutic targets. Due to the complex and multifactorial nature of these disorders, focusing on a single target may not provide comprehensive solutions. A diversified strategy that targets various aspects, such as protein aggregation, inflammation, and neuroprotection, holds the potential to yield more effective treatments and better outcomes for patients. Hence, continued efforts to understand the complex biology of neurodegenerative processes and identify novel therapeutic targets remain the focus of ongoing research efforts.

7.1. Conclusion

In this work, I used microglial cell lines and primary rodent neuronal-glial cultures to investigate the effects of calreticulin and β-galactosidase on microglial functions and neuronal loss *in vitro*. My findings indicate that the normally ER-resident protein, calreticulin, may act as a potential extracellular chaperone for amyloid-β when released from cells under stress conditions and this has neuroprotective effects in the model used. I also present evidence that β-galactosidase can be released from inflammatory activated microglia and assess the subsequent effects of extracellular β-galactosidase on microglial functions and neuronal loss. I show that β-galactosidase can contribute to inflammatory activation of microglia and subsequent microglial-mediated neuronal loss. Together, these data expand our understanding of how calreticulin and β-galactosidase may be involved in neurodegenerative processes and support the idea that these proteins may be targeted for therapeutic intervention.

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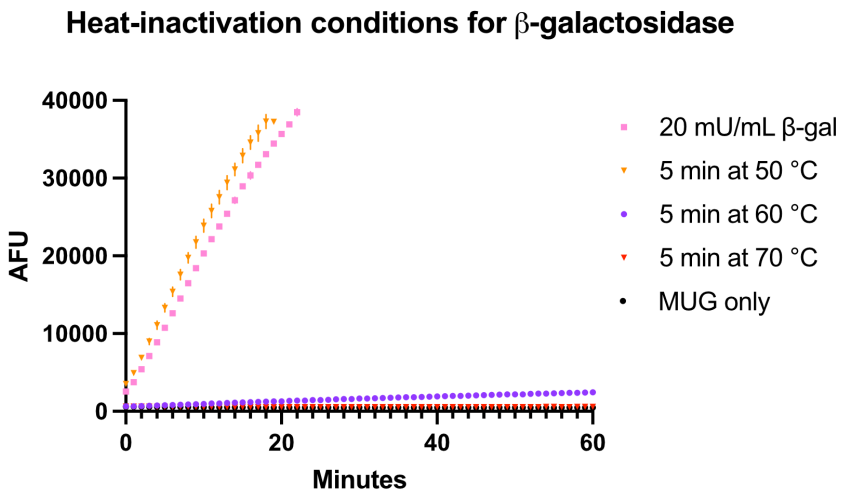
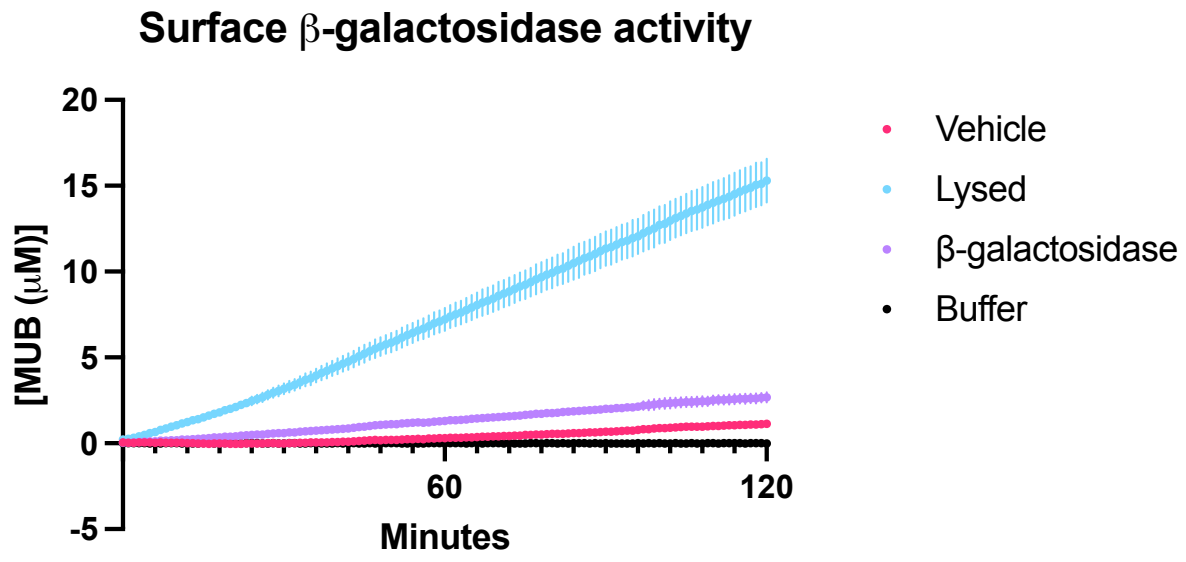
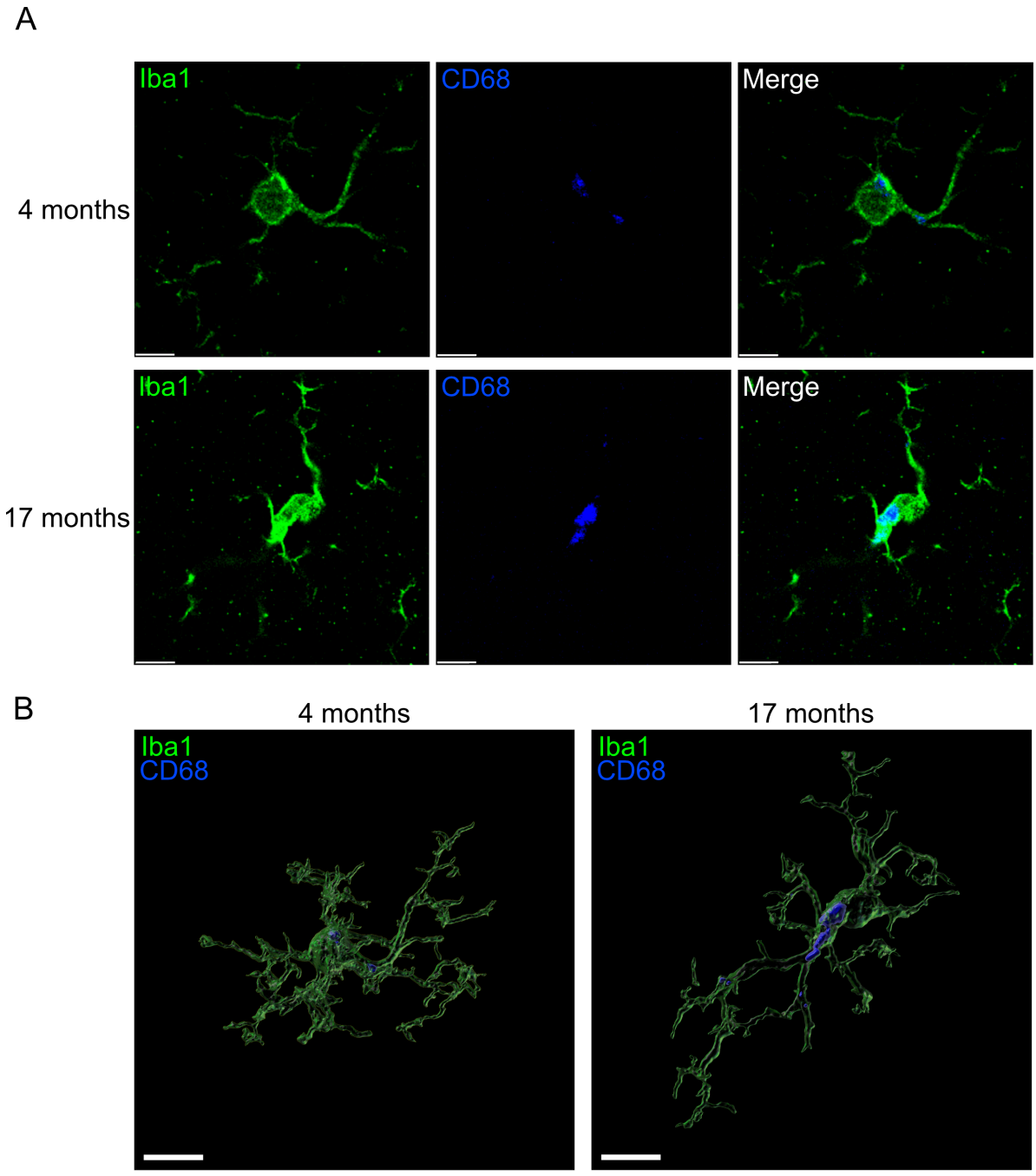
9 APPENDIX



Supplementary Figure A 9.1 Heat-inactivation conditions for β-galactosidase. 20 mU/mL β-galactosidase from bovine liver was incubated for 5 min at 50 °C, 60 °C and 70 °C, then activity was monitored by conversion of 4-Methylumbelliferyl-β-D-galactopyranoside (MUG) to fluorescent product, 4-Methylumbelliferone (MUB) for 1 h and activity compared to non-heat denatured control. Means ± SD from 3 technical repeats of one biological repeat.



Supplementary Figure A 9.2. Cell lysis causes a dramatic increase in β-galactosidase activity. β-galactosidase activity on the surface of BV-2 cells was determined using the rate at which 4-Methylumbelliferyl-β-D-galactopyranoside (MUG) was converted to the fluorogenic product, 4-Methylumbelliferone (MUB) when added to a monolayer of cells (at pH 7.0). Buffer was used as a negative control and denotes 1X assay buffer at pH 7.0 in the absence of cells and β-galactosidase (0.2 mU/mL) was used as a positive control for the assay. BV-2 culture medium was removed from cells and immediately replaced with 50 μL PBS (vehicle control) or lysis buffer, which was then incubated on cells for 15 minutes before the assay was initiated with MUG containing assay buffer. Data represents mean values ± SEM of at least 3 independent experiments.



Supplementary Figure A 9.3. Microglial and lysosomal staining in 4- and 17-month-old brain sections. (A) Representative confocal microscopy images of microglia from 4- and 17-month-old mice stained for Iba1 (green, microglial marker) and CD68 (blue, lysosomal marker) in the somatosensory cortex. Scale bar = 3 μm. (B) Representative renders of microglia from confocal images generated in Imaris. Scale bar = 5 μm. Imaging data and renders produced by Jacob Dundee.